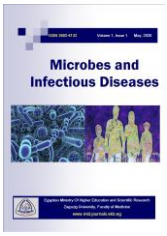
Microbes and Infectious Diseases 2024; 5(4): 1479-1493



Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

The effect of tigecycline-usnic acid combination on tigecycline-non-susceptible *Acinetobacter baumannii* clinical isolates and the role of usnic acid as an adjuvant efflux pump inhibitor

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ARTICLE INFO

Article history:
Received 26 March 2024
Received in revised form 16 April 2024
Accepted 21 April 2024

Keywords:
Antimicrobial resistance
Plant extracts
Resistance genes
Acinetobacter baumannii
Usnic acid

ABSTRACT

Background: Concerns are arising about *Acinetobacter baumannii* (*A. baumannii)* gaining resistance to tigecycline (TGC). *AdeB* efflux pump gene has been strongly associated with TGC resistance and usnic acid (UA) has been postulated to act as an efflux pump inhibitor. This study aimed at determining the antibacterial effect of UA and its effect on TGC susceptibility and on *adeB* gene expression in TGC non susceptible multidrug resistant (MDR) *A. baumannii* clinical isolates. **Methods:** Fifty multidrug resistant *Acinetobacter baumannii* isolates were identified as TGC resistant using disc diffusion method (DD). Minimal inhibitory concentration (MIC) of TGC and UA were determined using microbroth dilution (MBD) test. Checkerboard synergy test was conducted on TGC non susceptible isolates to determine UA effect on MIC of TGC. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to measure *adeB* gene expression before treatment, after treatment with TGC alone and with TGC/UA combination. **Results:** Tigecycline non susceptibility determined by MIC values occurred in 44/50 (88%) isolates. UA had antibacterial activity against 44/44(100%) of the isolates. Reduction in MIC of TGC in response to UA was detected in 10/44 (22.3 %) isolates in which high *adeB* gene expression was detected. In addition, 9/10 (90%) of them revealed highly significant reduction in *adeB* gene expression after treatment with TGC/UA combination. **Conclusion:** UA has an in vitro antibacterial action against TGC non susceptible *A. baumannii* with the potential ability of TGC and UA to synergistically act against them. Such synergism can be significantly attributed to UA efflux pump inhibitory action.

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Introduction

Acinetobacter baumannii (*A. baumannii*) is listed as one of the most clinically significant ESKAPE organisms that comprise *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*. They are mostly health care-associated, clinically significant and potentially highly resistant to antibiotics [1]. It has been estimated that *A. baumannii* causes mortality in critically ill individuals that ranges from 26% to 55.7% [2].

Multidrug resistant (MDR) *A. baumannii* has evolved resistance to the majority of current antibiotics, including carbapenems [3]. Alternative therapies for carbapenem-resistant *Acinetobacter*

DOI: 10.21608/MID.2024.279500.1866
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isolates include TGC and colistin [4]. However, TGC resistance rates have reached 74.2% [5].

Tigecycline is a tetracycline antibiotic that binds to the 30S ribosomal subunit, preventing translation and thereby inhibiting protein synthesis [6]. Tetracycline antibiotic resistance is conducted through an ATP-dependent efflux pump, enzyme inactivation, and ribosomal protective proteins [7].

Bacterial efflux systems are membrane-spanning, tripartite systems with broad substrate specificity that expel potentially harmful chemicals from the periplasm into the extracellular environment [8]. Tetracycline resistance in *A. baumannii* is mediated by two types of efflux pumps: resistance-nodulation-cell division (RND) pumps and *A. baumannii* tetracycline major facilitator superfamily (MFS) efflux pumps [9]. RND family-type pumps are constitutive, chromosomally encoded non-specific pumps derived from the *adeA, adeB,* and *adeC* genes. They elevate MIC of TGC, minocycline, and tetracycline [10]. MFS efflux pumps include pumps encoded by *TetA* and *TetB* genes [9].

Inhibition of efflux pump could be achieved in a variety of ways, including down regulating the expression of efflux pump genes by interfering with genetic regulation, modifying antibiotics so that they become no longer recognized as substrates, competitive binding to the active site preventing drug binding and breaking down the energy system responsible for energizing these pumps [11].

Some compounds have demonstrated anti-efflux pump activity but they were reported to have poor solubility, toxicity and challenges with cell permeability which render them inapplicable in clinical field [11].These findings focused attention to naturally occurring, harmless plant-derived compounds. UA, a secondary metabolite derived from lichens, has been reported to have a broad spectrum of antibacterial activity against many MDR organisms including Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas* and *Mycobacterium tuberculosis* [12–15].

The mechanisms of UA interactions are still not yet clear, however, UA was reported as an inhibitor of RNA and DNA synthesis and bacterial biofilm formation and it could be involved in interfering with the pathogens' cell membrane functions by inhibiting Ca+ influx ,and can also act as an efflux pump inhibitor [12].

The combination of UA and TGC was studied previously in one TGC resistant *A. baumannii* isolate. This combination was found to produce reduction in TGC MIC and decrease in the expression of *adeB* efflux pump gene [16]. To our knowledge, no other wide scaled studies worked to prove such finding.

The present study aims at demonstrating the antibacterial effect of UA and its potential synergistic effect on TGC against TGC non susceptible A. *baumannii* clinical isolates and detecting the effect of TGC/UA combination on the level of *adeB* efflux pump gene expression.

Methodology

This exploratory observational cross-sectional study was conducted on non-repetitive 63 clinical multi drug resistant (MDR) *A. baumanii* isolates obtained from clinical samples collected from ICU or general wards and sent to Ain Shams University Hospital's central microbiology labs. The study was approved by the Research Ethical Committee of Faculty of Medicine, Ain Shams University (No. FMASU M D 138/2022).

Data collection and species confirmation using conventional methods

For each isolate, type of specimen and hospital distribution were identified. Isolates were identified up to species level using conventional culture and identification methods [17]. Isolates were preserved in glycerol at – 80 ºC.

Antibiotic susceptibility testing using DD method

Multi drug resistance was confirmed and TGC resistance was identified using DD method according to Tendencia, and following CLSI 2022 guidelines [18,19]. The following antibiotic discs were used: Ampicillin-Sulbactam (10/10µg), Ceftazidime (30µg), Ceftriaxone (30µg), Imipenem (10µg), Meropenem (10µg), Gentamicin (10µg), Tobramycin (10µg), Tetracycline (30µg) and Tigecycline (15µg). The diameter of the zones of inhibition was interpreted according to the standard cut off points provided by Food and drug administration (FDA) for TGC [20], and according to CLSI 2022 guidelines for other antibiotics [19]. Isolates were defined as MDR when they had shown resistance to at least three different classes of antimicrobial agents, mainly extended spectrum Cephalosporins (ceftazidime or ceftriaxone), Carbapenems (imipenem or meropenem), Aminoglycosides (gentamicin and tobramycin),

tetracycline and *β*-lactam/*β*-lactamase inhibitors (ampicillin-sulbactam) [21].

Detection of TGC MIC for TGC resistant isolates using resazurin based MBD test

Minimal inhibitory concentration of TGC was determined by MBD test following CLSI 2022 guidelines for antimicrobial susceptibility testing using TGC serial dilutions ranging from (0.125 µL /mL-128 µL /mL) in a 96 well microtiter plate with bacterial inoculum adjusted at 10^6 CFU/mL. After 24 hr incubation, 30 µL of resazurin was added to each well and the plate was further incubated for 2–4h to observe color change [16,19,22]. Bacterial growth was indicated by oxidation of resazurin from blue to pink. MIC results were interpreted according to the standard cut off points provided by FDA as follows: ≤2 mg/L, susceptible; 4 mg/L, intermediate; ≥8 mg/L, resistant [20]. For isolates showing discrepancy in the sensitivity profile between MBD and DD methods, MIC was retested using VITEK2 system.

Detection of the antibacterial activity of UA on TGC non susceptible isolates using resazurin based MBD test

Isolates that were confirmed to be TGC non susceptible by MBD and VITEK2 were subjected to MBD testing to determine the MIC of UA following CLSI 2022 guidelines in a 96 well microtiter plate using serial dilutions ranging from 2 to 4096 µg /mL of UA 98% (Sigma, Aldrich) dissolved in Di Methyl Sulfoxide (DMSO) [19,23]. Aiming at preventing the antibacterial effect of DMSO from interfering with the results of UA tests ,the subinhibitory concentration of DMSO was first determined using MBD test and was used as a solvent for UA [21].

Testing the effect of UA on TGC susceptibility using checkerboard synergy assay

The effect of UA on TGC MIC was studied on the TGC non susceptible isolates by a two-dimensional MBD test using 96 well microtiter plate following CLSI 2022 guidelines for antimicrobial susceptibility testing [19], Mueller–Hinton broth was added to each well, with increasing concentrations of TGC (0.125 µg/mL – 128 µg/mL) in rows and of UA (64 µg/mL - 4096 µg/mL) in columns [25]. The plate was inoculated with 10^6 CFU/mL bacterial inoculum. Final UA and TGC concentrations in each well are shown in **Figure 1**.

Results were interpreted by measuring the fractional inhibitory concentration index (FICI) using the following formula [26]:

$\sum FIC = FICA + FICB$

(FIC_A = MIC of C_A in combination/MIC of C_A

FIC_B = MIC of C_B in combination/MIC of C_B

A and B are the concentrations of TGC and UA, respectively.

The FICI results were interpreted as follows: FICI ≤ 0.5, synergism; 0.5 < FICI < 1, additive; 1 ≤ FICI < 2, indifference; FICI ≥ 2, antagonism.

***AdeB* efflux pump gene expression analysis**

Based on the results of checkerboard assay the isolates were divided into 2 groups: Group A: isolates showing synergistic or additive effect of UA on TGC and Group B: isolates showing indifferent effect of UA on TGC.

Baseline *adeB* gene expression was determined in both group A and B isolates relative to a TGC sensitive isolate and was compared between both groups. In addition, each of group A isolates was treated once with TGC alone at the subinhibitory concentration corresponding to each isolate and once with TGC/UA combination using TGC subinhibitory concentration combined with the most effective UA concentration based on the checkerboard test results. The effect of each treatment on gene expression was determined and compared to base line gene expression in the tested isolates.

Quantitative RT-PCR was performed using PureLink™ RNA Mini Kit (Thermo-fisher, USA), GoScript™ Reverse Transcriptase kit (Promega, USA) , DreamTaq Green PCR Master Mix (2X) kit (Thermo-fisher, USA) and 2 pairs of primers specific for *adeB* genes. 16sRNA was used as a housekeeping gene to normalize levels of *adeB* transcripts. The sequences of primers used are described in **Table 1** [16].

Suspensions were cultured overnight at 37 °C then were adjusted at 0.5 MacFarland by adjusting OD between 0.08 and 0.1 at a wavelength of 625 nm before being used for RNA isolation [27]. RNA isolation and RT-PCR steps were done following the instruction of the kits' handbooks and according to the conditions mentioned by Bankan et al [16]. The relative quantitation (RQ) was calculated according to Livak et al [28].

Statistical analysis

Data were collected, revised, coded, and entered into the Statistical Package for Social Science (IBM SPSS) version 27. The quantitative data were presented as median and inter-quartile range (IQR) when data found non-parametric. Also, qualitative variables were presented as number and percentages. The comparison between two independent groups with quantitative data and non-parametric distribution were done by using Mann-Whitney test.

The comparison between two paired groups regarding quantitative data and non-parametric distribution was done by using Wilcoxon Rank test. The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was interpreted as follows: P-value > 0.05: Non-significant (NS), P-value < 0.05: Significant (S), P-value < 0.01: Highly significant (HS).

Results

The study involved 63 non- repeated clinical isolates that were confirmed to be MDR-*A. baumanii*. They were obtained from Ain Shams University Hospital. Most isolates were collected from ICU (53.96% (34/63 isolates)) followed by burn unit (19.05% (12/63 isolates)), general wards (14.29% (9/63 isolates)), outpatient clinics and NICU (4.76% (3/63 isolates)) for each. Minority of isolates were collected from bone marrow transplantation unit and isolation unit (1.59% (1/63 isolate)) for each.

Most of the bacterial isolates were collected from blood (42.86% (27/63 isolates)), followed by wound swabs (23.81% (15/63 isolates)), sputum (15.47% (10/63 isolates)), urine (4.76% (3/63 isolates)), central line and pleural fluid (3.17% (2/63 isolates)) for each. Fewer isolates were collected from CSF, pus sample and drain (1.59% (1/63isolate)) for each.

Antimicrobial susceptibility pattern of isolates as determined by DD

The rate of TGC resistance was found to be 78.1% (50/63 isolates). As shown in **(Figure 2)**, Ampicillin-sulbactam showed the highest rate of resistance (100% (63/63 isolates)), while Imipenem showed the lowest rate of resistance (74.6% (47/63 isolates)).

TGC MIC values for TGC resistant isolates using resazurin based MBD

Out of the 50 isolates showing resistance to TGC by DD, 39 isolates (78%) were resistant to TGC by MBD with MIC ranging between (8- 64 μg/mL), 8 isolates (16%) showed intermediate sensitivity with MIC equal to 4 μg/mL and 3 isolates (6%) were sensitive with MIC ranging between (1-2 μg/mL).

Out of the 11 isolates showing different TGC sensitivity profile by DD and MBD, five isolates were confirmed to be TGC non susceptible using VITEK2 system. The total number of TGC non susceptible isolates that were detected either by MBD or by VITEK2 was 44/50 (88%).

Anti-bacterial activity of UA on TGC non susceptible isolates

UA showed antibacterial effect against all 44/44 (100%) TGC non-susceptible isolates. MIC of UA was found to be 2048 μg/mL in 40/44 (90.9%) isolates and 1024 μg/mL in 4/44 (9.9%) isolates.

Effect of UA on TGC MIC using checkerboard synergy assay (Table 2)

Out of 44 TGC non susceptible isolates, 5 (11.36%) isolates showed synergistic effect of UA on TGC**,** with 4-16-fold reduction in TGC MIC while another 5 (11.36%) isolates showed additive effect, with 2-fold reduction in TGC MIC. The remaining 34 (77.27%) isolates showed indifferent effect.

Based on these results, isolates were divided into 2 groups: Group A: comprising 10 isolates showing synergistic (**Figure 3A)** or additive (**Figure 3B)** effect of UA on TGC MIC and Group B: comprising 10 isolates showing indifferent effect of UA on TGC MIC **(Figure 4)** selected based on exhibiting the highest TGC MIC. **Table 3** provides detailed checkerboard test results of group A isolates.

***AdeB* efflux pump gene expression analysis results**

Baseline expression of *adeB* efflux pump gene was compared between group A and group B isolates. *AdeB* gene expression was found to be high in all 10 (100%) isolates in group A, unlike group B in which only 2/10 (20%) isolates showed high gene expression.

Comparing levels of *adeB* gene expression between group A and B isolates revealed a high

statistically significant difference (p value <0.001) between both groups **(Table 4; Figure 5)**.

A high statistically significant positive correlation was detected between MIC of TGC and baseline a*deB* gene expression **(Table 5; Figure 6)**.

Further analysis of the effect of TGC alone and of TGC/UA combination on *adeB* gene expression in group A isolates revealed that 8 /10 (80%) isolates showed elevated *adeB* gene expression after treatment with TGC alone and 2 /10 (20%) isolates showed reduction in gene expression. Meanwhile treatment with TGC/UA combination resulted in reduction in *adeb* gene expression in 9/10 (90%) isolates, while only 1/10(10%) isolates showed no change in gene expression levels **(Figure 7)**.

There was a high statistically significant difference between gene expression after treatment with TGC alone and after TGC/UA combination treatment **(Table 6; Figure 8)**.

A statistically significant positive correlation was also detected between gene expression after treatment with TGC alone and gene expression after TGC/UA combination treatment **(Table 7; Figure 9)**.

Table 1. Sequence of primers used in molecular studies

Primer name	Sequence	Reference
AdeB forward	5′-GGATTATGGCGACAGAAGGA-3′	Bankan et al. [16]
AdeB reverse	5′-AATACTGCCGCCAATACCAG-3′	
16sRNA forward	5′-CAGCTCGTGTCGTGAGATGT- 3′	
16sRNA reverse	5′-CGTAAGGGCCATGATGACTT-3′	

Table 2. Checkerboard synergy test results

Effect of UA on TGC	FICI range	N=44	Percent= 100%
Synergistic	0.124-0.375	5	11.36%
Additive	0.53-0.56	5	11.36%
Indifferent	1.03-1.06	34	77.27%
Antagonist	0	0	0%

Table 3. TGC MIC values and fold change in MIC in response to UA treatment in group A isolates

Isolate number	MIC of TGC alone(ug/mL)	MIC of TGC after UA combination(ug/mL)	Fold reduction	FICI	Type of interaction
AB1	16	1	16	0.124	synergistic
AB9	32	2	16	0.312	synergistic
AB37	8	1	8	0.187	synergistic
AB42	8	2	4	0.312	synergistic
AB51	16	4	4	0.312	synergistic
AB11	16	8	2	0.53	additive
AB15	8	4	2	0. 53	additive
AB16	16	8	2	0.53	additive
AB50	16	8	2	0.53	additive
AB60	16	8	2	0.56	additive

Table 4. Comparison between baseline *adeB* efflux pump gene expression in group A and group B isolates relative to the expression in a TGC sensitive *A. baumanii* isolate

	Group A (n=10)	Group B(n=10)	Mann Whitney test		
	Median (IQR)	Median (IQR)	z	*p* value	sig.
Fold change	3.03 (1.8 - 4.89)	0.18 (0.01 - 0.35)	3.176	<0.001	S

Table 5. Correlation between MIC of TGC and baseline *adeB* gene expression

	MIC	
	r	*p*-value
Basic a*deB* gene expression	**0.876****	0.001

Spearman correlation coefficients

Table 6. Comparison between the effect of treatment with TGC alone and with TGC/UA combination on *adeB* gene expression in group A isolates relative to baseline gene expression.

	Gene expression after TGC	**Gene expression after TGC/UA**	**Test value**	**P-value**	**Sig.**
Median (IQR)	5.5 (1.67 - 10.26) 0.18 – 34.77	0.33 (0.18 – 0.46) 0.07 – 1.02	2.599‡	0.009	HS*

*HS: Highly significant

Table 7. Correlation between gene expression after treatment with TGC alone and gene expression after TGC/UA combination treatment

	Gene expression after TGC/UA		
	r	p-value	
Gene expression after TGC	**0.844****	0.002	HS*

Spearman correlation coefficients. *HS: Highly significant

Figure 1. Concentrations of UA and TGC used in checkerboard assay. Black colored blocks represent UA and white colored blocks represent TGC. The arrows represent the direction of dilution of TGC and UA

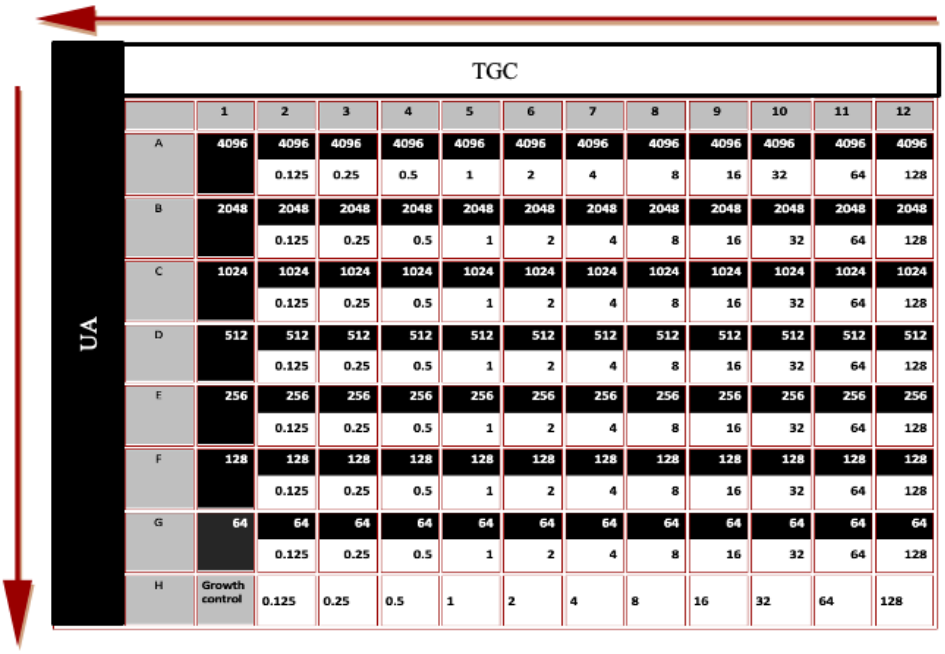


Figure 1. Frequency of antibiotic resistance in *A. baumanii* isolates

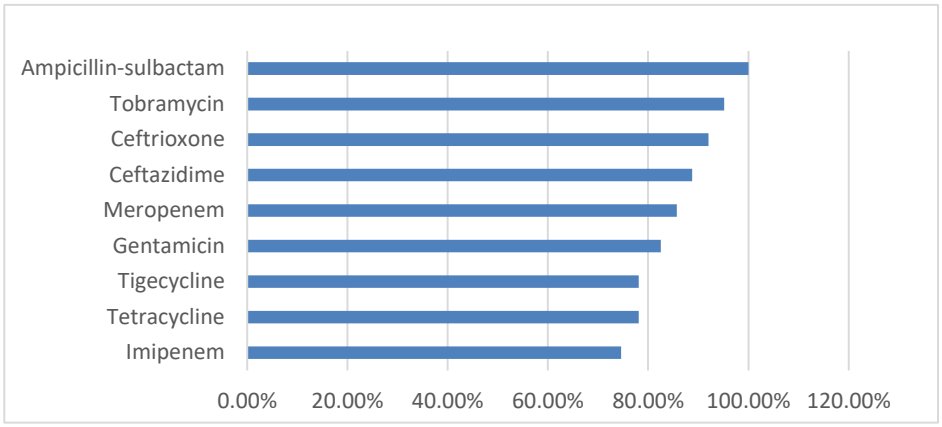


Figure 2. Effect of UA on MIC of TGC in group A isolates. A: Synergistic effect with 16-fold reduction in MIC of TGC from16 µg/mL (well H9) to 1 µg/mL (well F5). B: Additive effect with 2-fold reduction in MIC of TGC from16 µg/mL (well H9) to 8 µg/mL (well F8)

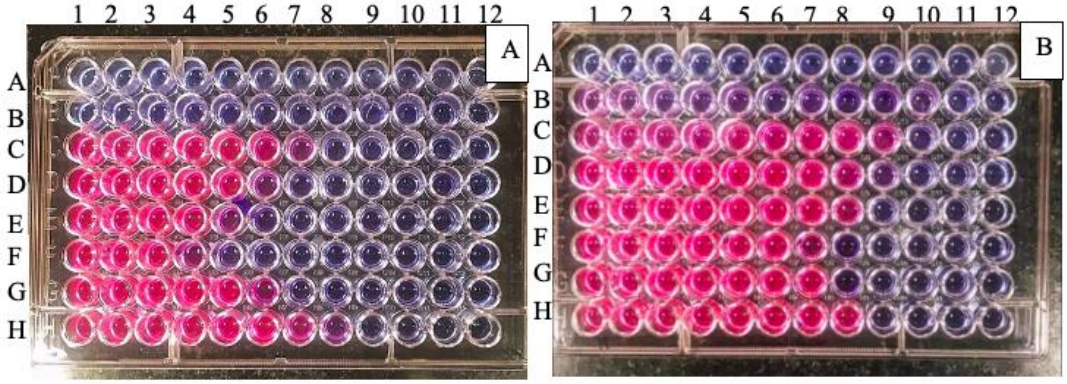


Figure 3. Effect of UA on MIC of TGC in group B isolates. Well H8 represents MIC of TGC alone (16 µg/mL) and well G8 represent MIC of TGC in combination with UA (16 µg/mL). Indifferent effect is determined with no change in MIC of TGC

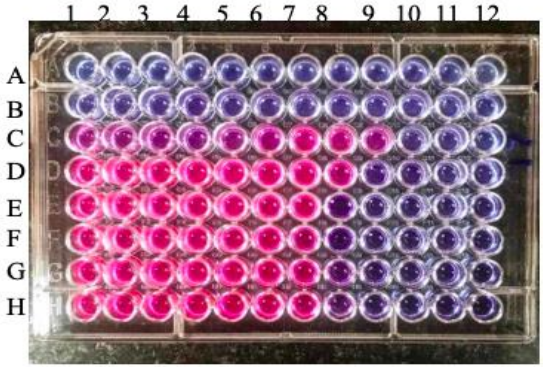


Figure 4. Comparison between baseline *adeB* efflux pump gene expression in group A and group B relative to the expression in a TGC sensitive *A. baumanii* isolate

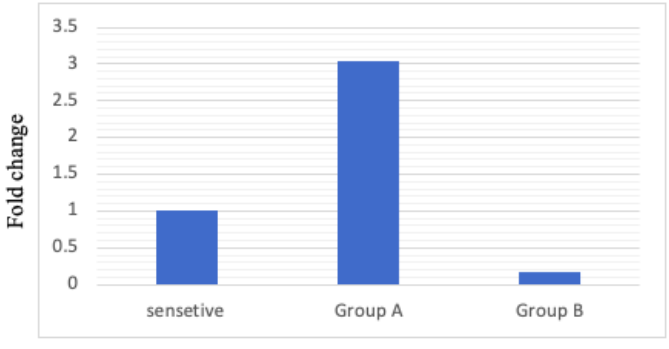


Figure 5. Correlation between MIC of TGC and baseline *adeB* gene expression

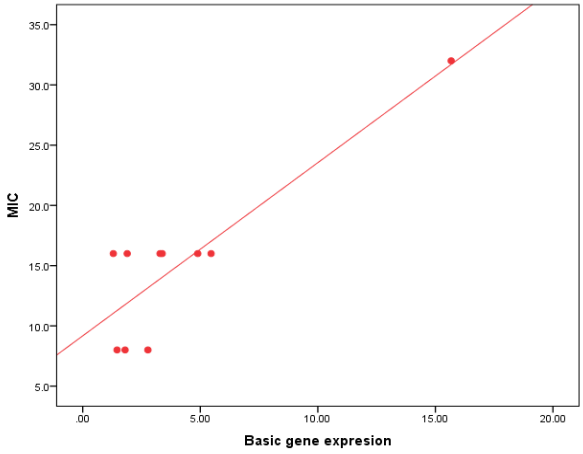


Figure 6. Effect of treatment with TGC alone and with TGC/UA combination on *adeB* gene expression in relation to baseline gene expression in each of group A isolates

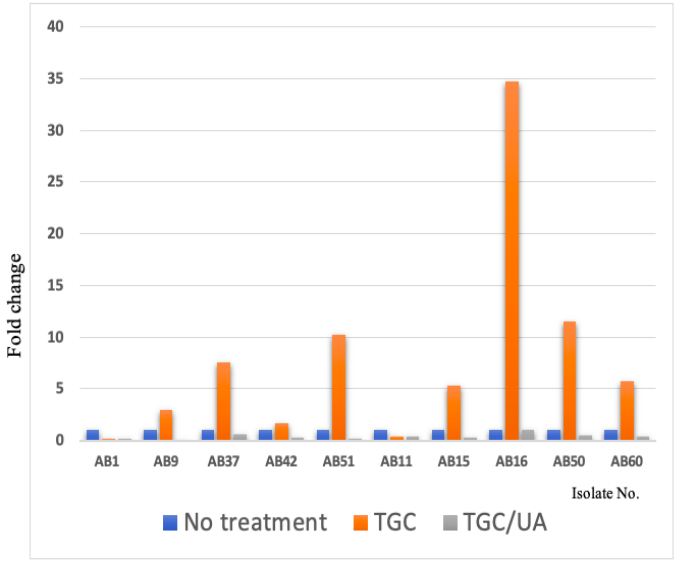


Figure 7. Fold change in *adeB* gene expression after treatment of group A isolates with TGC alone and with TGC/UA combination relative to baseline gene expression

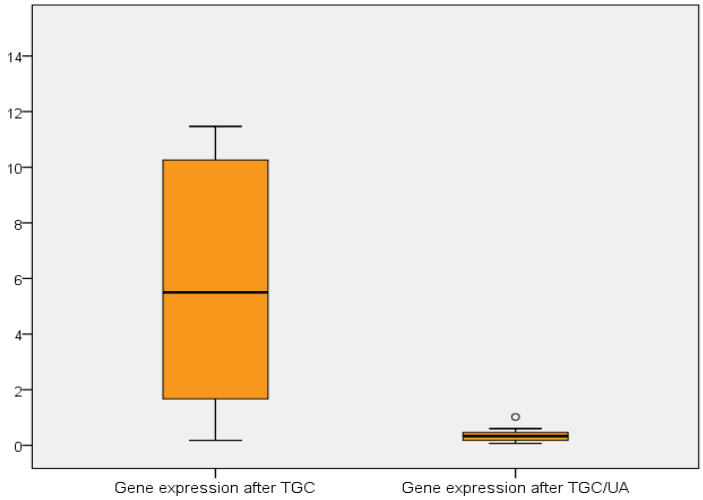
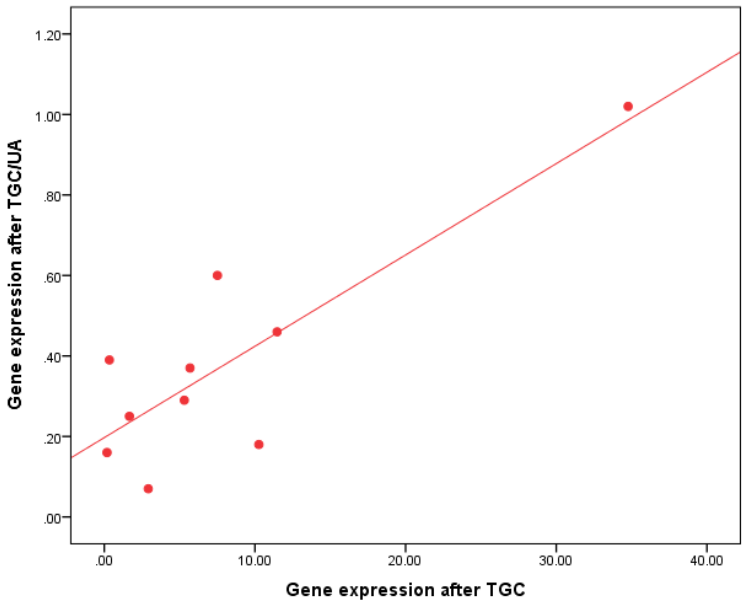


Figure 8. Correlation between gene expression after treatment with TGC alone and gene expression after TGC/UA combination treatment



Discussion

Acinetobacter baumannii has recently emerged as a serious pathogen of global significance. The concerns are arising against this organism due to its capacity to develop resistance to practically all antibiotics commonly used and its ability to transmit this resistance rapidly, spanning borders and influencing healthcare settings at many economic levels [29]. Based on WHO in the African area in 2019, seven pathogens were jointly accountable for 821,000 deaths linked to antimicrobial resistance (AMR) of which *A. baumannii* ranked fifth after *Streptococcus pneumoniae, Klebsiella pneumoniae, Escherichia coli*, and *Staphylococcus aureus*, with around 48,000 deaths. In Egypt AMR was linked to 56,600 fatalities of which *A.baumannii* was responsible for 6,200 deaths [30].

The majority of antibiotics available nowadays including carbapenems, the preferred drugs for treating severe *A.baumannii* infections, have been rendered ineffective against MDR *A. baumannii* [3]. TGC demonstrated strong antibacterial action against MDR *A. baumannii*. However, its unregulated use has resulted in a high level of resistance [4,16].

In the present study, 63 confirmed MDR *A. baumannii* isolates from different hospital areas and different specimens were included. For all isolates both hospital and specimen distribution were specified. Antibacterial susceptibility pattern and incidence of TGC resistance were determined using DD method.

Most isolates in this study, (53.69%), were found to be isolated from ICU. This result was close to that reported by Benamrouche et al. and Yadav et al. who stated that the isolation rate of MDR *A. baumannii* isolates from ICU was 51.1% and 49.6% respectively [3,31]. Other studies found higher significant rates of isolation of MDR *A. baumannii* in the ICU (91.3%, 67.7%) in Jordan and Egypt respectively [32,33] .Our findings coinciding with other studies can be conferred to the opportunistic nature of *A. baumannii.* In addition, invasive procedures conducted prior to ICU admission may be substantial risk factors for *A. baumannii* infection.

Most isolates (42.86%) were obtained from blood followed by wound swabs (23.81%) and sputum (15.47%). This result was against many past reports. Abd El-Baky et al. in Minya, Egypt stated that (16, 80%) of isolates were obtained from wound infection [34]. In addition, many previous works declared that respiratory specimens were the major source for *A. baumannii* clinical isolates. In Egypt, Asaad et al. reported that 50% of isolates were from respiratory specimens [33]. A similar outcome was found in Turkey, where 63.1% of the isolates were recovered from respiratory specimens [35]. Numerous studies conducted overseas, including Oman, India, the USA, and Jordan, have similarly revealed the major role of respiratory specimens as

source of *A. baumannii* (44%, 39%, 67.7%, and 93.4%, respectively) [16,32,36,37].

In contrast, this study showed that respiratory specimens ranked third after blood and swab samples. A possible explanation is that the respiratory specimens in this study included only sputum samples collected from patients admitted in wards and outpatient clinics rather than the ICU where *A. baumannii* is identified as major cause of ventilator associated pneumonia. These variations could also be explained by the high incidence of respiratory infections with MDR *A. baumannii* in the period between 2020 till 2022 caused by COVID 19 pandemic that resulted in increased ICU admission and use of mechanical ventilators together with the misuse of antibiotics that resulted in augmentation of the dilemma of antimicrobial resistance. This can be supported by the studies that reported nosocomial outbreaks of multidrug resistant organisms (MDROs) such as carbapenem-resistant *A. baumannii* during the COVID-19 pandemic [38–40].

Regarding antibiotic susceptibility profile, Ampicillin-sulbactam showed the highest rate of resistance (100%). This result was close to that in other studies that detected Ampicillin-sulbactam resistance in 100% of isolates in Minya, Egypt [34] and in 95.7% and 99.4% of isolates involved in two different studies conducted in Turkey [35,41].

The high resistance to Ampicillin-sulbactam in *A. baumannii* strains whethear isolated locally or overseas could be explained by *A. baumannii* intrinsic β-lactamases including class A (TEM-1), class C (ADC-30), class D (OXA), and class B metallo-β-lactamases (MBLs) which are able to degrade both Ampicillin and Sulbactam [42].

Regarding TGC resistance, our study detected that 78.1% of isolates were TGC resistant. Such high rate was also reported in different areas in Egypt including Banha and Tanta (60% and 73.9%) respectively [5,43].

Mrowiec et al., and Konca et al. also reported approximately 74.2% and 73.9% TGC resistance rate in Poland and Turkey respectively [35,44].The increased TGC resistance rate among *Acinetobacter* could be explained by the emergence of MDR resistant strains mostly in health care settings as a result of antibiotic abuse which acquired applying antimicrobial recycling along with the use of restricted antibiotics as TGC.

In contrast to our results, TGC resistance was reported to be very low (3.63%) in Assuit, Egypt [45]. The rate of TGC resistance was also reported to be low in Jordan (7.2%) and Pakistan (27.27%) [32,46]. Such variations in TGC resistance rate could be attributed to the difference in regional distribution of MDR *A. baumannii* strains [47].

Results of MBD test performed in our study on 50 TGC resistance isolates identified by DD demonstrated that 39/50 (78%) were TGC resistant, while 8/50 (16%) displayed intermediate sensitivity and 3/50 (6%) displayed TGC sensitivity. MIC values ranged between 1-64 µg/mL. Discrepancy in TGC susceptibility results was observed between DD and MBD in 11 isolates and TGC susceptibility for these isolates was retested by VITEK2 system.

The discrepancy in TGC susceptibility results between DD and MBD methods in *A. baumannii* was observed in other researches in Egypt [48] and overseas [49,50] .Such finding could be attributed to the difference in concentration of oxygen and ions (mainly manganese) in the culture media used in the different testing methods that may induce a direct impact on TGC's in vitro activity by affecting the polyphenolic group in its structure [51,52]. Also, it was suggested that breakpoint zone diameter of ≥16/≤12 mm to define susceptibility/resistance, respectively, instead of the FDA approved break points (≥19/≤14 mm), may reduce such discrepancy [53].

The overwhelming crisis of antimicrobial resistance made it evident that new therapeutic approaches must be developed in response to multidrug resistance. The natural and safe UA was reported to have broad spectrum antibacterial activity against MRSA, *M. tuberculosis* and *Pseudomonas* [12–15].

To detect the antimicrobial effect of UA against MDR *A. baumannii*, MBD test was performed on the 44 isolates that were TGC non susceptible.

Usnic acid showed antibacterial activity against 100% of isolates with MIC of 2048 µg/mL detected in 90.9% of isolates and 1024 µg/mL in 9.9% of isolates. Our MIC results were slightly higher compared to those reported by Nagaraju et al. who conducted the test on 20 isolates and revealed that UA had potential efficacy at MIC of 512 in 2 (10%) of isolates and at 1024 µg/mL in 18 (90%) of isolates [23].

Numerous studies demonstrated the potential use of UA as an adjuvant in the treatment of certain MDR pathogens when combined with other antibiotics. Examples include UA/ clarithromycin combination against *Mycobacterium abscessus* [54] ,UA/norfloxacin combination against MRSA [13] and UA/ Polymyxin B combination against *Pseudomonas aeruginosa*[15]. The combination of UA and TGC was reported to cause a 256-fold reduction in MIC of TGC when tested on TGC resistant *A. baumannii* [16].

A checkerboard synergy test was performed on 44 TGC non susceptible isolates. A synergistic interaction between TGC and UA was observed in 11.36% isolates, with a fold reduction in TGC's MIC ranging from 4 to 16 folds, whereas 11.36% of isolates displayed additive interaction with a two-fold reduction in TGC's MIC. The UA concentration that resulted in the highest fold reduction in TGC's MIC in most of the affected isolates was found to be 128 μg/mL.

A previous research suggested that UA influences the replication and synthesis of DNA and RNA in bacteria and can also act as an efflux pump inhibitor [12]. Efflux pump genes including the *adeB* efflux pump gene was found to be strongly connected with TGC resistance in MDR *A. baumannii* [55,56].

We contrasted the basic level of *adeB* gene expression between Group A (isolates showing reduction in TGC MIC in response to UA) and group B (isolates showing no change in TGC MIC in response to UA). The baseline *adeB* efflux pump gene expression was high in 100% of group A isolates, moreover there was a statistically significant correlation between the isolate's TGC MIC and gene expression level. On the other hand, only 2 (20%) of group B isolates showed high gene expression. The low level of gene expression detected in group B isolates may imply that they have adopted an alternative TGC resistant mechanism. Moreover, genotypic resistance profile doesn't always reflect the phenotypic resistance.

Further expression analysis in group A isolates revealed 1.6-10.26-fold increase in *adeB* gene expression after treatment with TGC alone in 80% of isolates and 0.18-0.46 -fold reduction in *adeB* gene expression in 90% of isolates after treatment with TGC/UA combination with a high statistically significant difference between both treatments. Close to our results are those reported by Bankan et al. that stated a 0.65-fold reduction in *adeB* gene expression in response to UA [16].

The capacity of UA to reduce TGC MIC in TGC non susceptible *A. baumannii* isolates and the finding that theses isolates showed high level of *adeB* efflux pump expression, emphasized by the reduction in gene expression after TGC/UA combination treatment highlight the role of UA as an efflux pump inhibitor.

Conclusion:

Usnic acid has an in vitro antibacterial effect against MDR *A. baumannii* with the potential ability of TGC and UA to work synergistically to combat the potentially fatal MDR *A. baumannii*. The synergism between TGC and UA can be strongly contributed to the effect of UA as an efflux pump inhibitor as demonstrated by reduction in expression of *adeB* efflux pump gene in response to UA treatment. Large-scale studies are recommended to determine the optimum effective UA concentration to be used in combination with TGC which could allow using lower concentrations of TGC thus decreasing TGC side effects and resistance rates.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ghada Ayman Fahmy and Rania Alam el-Din Mohamed. The first draft of the manuscript was written by Ghada Ayman Fahmy and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript

Competing interests

The authors have no relevant financial or non-financial interests to disclose

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