Antibacterial Activity of (+) Usnic Acid against Multi Drug Resistant *Acinetobacter baumannii* from Clinical Isolates

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Abstract

Acinetobacter baumannii (Ab) is developing resistance to a variety of common antibiotics. and become multidrug resistant, extreme drug resistant, and pan drug resistant pathogens, requiring the identification of new antibiotics as well as the identification of new plant compounds capable of acting against Ab. Recent research has revealed MDR Ab co-infections with COVID-19,, raising alarm bells. Since its isolation, Usnic acid has been investigated for a variety of pharmacological activities, including antioxidant, antitumor, antibacterial, antifungal, antiviral, antiprotozoal, and insecticidal. Many Plant-derived drugs show promising activity as new antimicrobial agents against multidrug resistant (MDR) strains. There is insufficient data to support the antibacterial activity of (+)-UA against MDR Acinetobacter baumannii.. In the present study, we evaluated the antimicrobial activity of naturally occurring compound (+) usnic acid (UA) against MDR Ab. We determined the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC), time-kill assay in twenty multidrug resistant *Acinetobacter baumannii* clinical isolates collected from two different centers. Results revealed promising activity of (+)-UA with MIC concentration of 512–1024 µg/mL and MBC 2048 –4096 µg/mL. The MBC/MIC index indicated that the compound was bactericidal. The time-kill assay revealed a gradual decrease in the log10 value of the bacteria. Since there is a limited research available on the activity of usnic acid against MDR Acinetobacter baumannii, present study fills the gap.

Abbreviations: *Acinetobacter baumannii* = Ab; MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; UA = (+) - usnic acid

Keywords: *Antibacterial activity, multi-drug resistant, Acinetobacter baumannii, (+) usnic acid.*

Introduction

Antimicrobial resistance is a global issue in infectious diseases control. About 700,000 deaths occur globally in a year due to antimicrobial resistant infections. According to the Antimicrobial Resistance report, 10,000,000 deaths may occur annually worldwide by 2050 causing heavy burden on the economy [(1)]. In the present pandemic situation, the antimicrobial resistance is also aggravating. A meta-analysis from five countries showed 3.5% of co-infection and 14.3% of secondary infection with COVID–19 infection [(2)]. According to a recent study from Iran, 19 patients infected with COVID-19, Out of them 17 patients co-infected with MDR A. baumannii all of them died which demonstrating the pathogen's risk [(3)]. Efforts are being undertaken to control antimicrobial resistance by governmental organizations, giving awareness on the effect of overuse of antibiotics and its impact on health. The bacteria survive in the presence of antibiotics by adapting various mechanisms of resistance by

synthesizing proteins and developing new pathways [4]. Among various microorganisms that cause infections, a group of organisms known as ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species) cause concern. These pathogens cause life threatening hospital acquired infections [5].

Multi drug resistant (MDR) *Acinetobacter baumannii* (Ab) is considered to be a hospital acquired infection globally. High mortality and prolonged hospital stay are reported in patients infected with MDR Ab [6, 7]. The bacteria undergo mutations and resistance mechanisms like efflux pump and enzyme degradation. In 2017, the World Health Organization released a list of bacteria and emphasized member countries to promote research and development for new antibiotics. In this list Ab has been categorized as one of the most critical organisms [8]. In carbapenem resistant isolates of Ab, the alternative treatment is tigecycline and colistin [9]. In some instances, resistant to these antibiotics were also reported due to uncontrolled use. Studies have reported, 74.2% and 53.1% resistance to tigecycline and colistin respectively [10, 11]. As multidrug resistance has been observed in these pathogens, there is a demand for new methods and drug treatment. Plant extracts and derivatives are widely evaluated as antimicrobial agents against MDR strains [12]. Hence there is a need to evaluate the efficacy of plant extracts for MDR Ab.

Usnic acid (UA) is a lichen derived secondary metabolite with a unique dibenzofuran skeleton and is commonly found in lichenized fungi of the genera *Usnea*, *Ramalina*, and *Cladonia*. The lichens symbiotically coexist with cyanobacteria and produce various secondary metabolites. (+) Usnic acid (UA) is one such compound isolated from various lichens and has been studied for many biological properties including antibacterial activity. The structural characteristics of UA combined with its physiochemical properties are responsible for its pleiotropic biological effects. UA has been used in medicinal products, perfumes, cosmetics. It possesses a broad spectrum of bioactivities, like antimicrobial, analgesic activity, anti-inflammatory antiviral, and anticancer. The antibacterial efficacy of lichen extracts and compounds present in them have been studied for many years. Many researchers patented the antimicrobial effect of UA [13, 14, and 15]. Usnic acid mechanism of action is not completely understood till now. Nonetheless, research indicates that Usnic acid's inhibition of bacterial nucleic acid replication and synthesis results in this action [16]. Hence, in the present study the effect of UA was evaluated for its antibacterial activity against MDR Ab.

Materials and Methods

Chemicals: Mueller Hinton agar, Mueller Hinton broth and brain heart infusion broth were purchased from M/s Himedia (Mumbai, India). Colistin was procured from M/s Cipla (Mumbai, India).

Bacterial Strains: Twenty MDR Ab clinical isolates from S.V.S Medical College and Hospital (Mahabubnagar, Telangana, India) and A.C.S.R Medical College and Hospital (Nellore, Andhra Pradesh, India), collected in 2019 were used for the study. Approval from Institutional Biosafety Committee of Saveetha Medical College and Hospital (001/08/2020/IBSC/SIMATS) and Institutional Ethics Committee of Saveetha Medical College and Hospital (003/09/2020/IEC/SMCH) were obtained for carrying out the present study. All the experiments were conducted in the Biosafety Cabinet (BSL – II) and the used materials were disposed as per the Standard Operating Procedures of the institution..

Isolation of UA: UA was isolated following the reported procedure [17]. About 200 g of shade-dried lichen (*Roccella montagnei* Bel.) was extracted with methanol in cold. After 72 hr the methanolic extract was successively treated with petroleum ether and acetone. The acetone fraction was subjected to column chromatography on silica gel and eluted with solvents of increasing polarity. Elution of the column with benzene afforded yellow shining crystals with melting point of 196 – 198ºC. Based on spectroscopic analysis, the above compound was identified as UA (Yield = 1.0 g). The purities of the above isolated compounds were confirmed by comparison with respective authentic samples by thin layer chromatography mixed and melting point determination and super-imposable infra-red spectroscopy.

Minimum inhibitory concentration (MIC): Identification and estimation of the minimum inhibitory

concentration of all twenty clinical isolates of Ab were done by using Vitek2 system [18]. All the twenty MDR Ab (freshly prepared dilutions from glycerol stock isolates) were tested. Antibacterial activity of the compound was done by microbroth dilution following Clinical and Laboratory Standards Institute (CLSI) guidelines. Stock solution of the compound was prepared in dimethyl sulfoxide (DMSO). In a 96-well microtitre plate, serial dilution of the compound ranging from 2048 - 4 µg/mL was done in Mueller Hinton broth. To this, 50 µL of bacterial inoculum (1.5 x 10^5 CFU/ mL) was added and the microtitre plate was incubated at 37°C for 18 hr. The growth of the organism in each well was visually detected and MIC was noted where the growth was inhibited. The test was performed in triplicates.

Minimum bactericidal concentration (MBC): 10 µL of 1x, 2x, and 4x MIC from the 96 well microtitre plate were sub-cultured on Mueller Hinton agar and incubated for 24 hr at 37°C. After incubation, the highest dilution which yielded no bacterial growth on the plates was recorded as MBC. The test was done in triplicate.

MBC/ MIC Index: The MBC/MIC index was calculated. Equal to or more than 4.0 was taken as bactericidal and less than 4.0 as bacteriostatic [19].

Time kill kinetics assay: Time kill kinetics assay was performed for 1x and 2x MIC following CLSI guidelines by broth macro dilution method [20]. A final inoculum of 1.5 x 10^5 CFU/mL was taken in tubes. The tubes were shaken periodically and incubated at 37°C. At time intervals of 4, 8, 12, 16, 20, and 24 hr, 1 µL of inoculum was collected with a sterile loop from each tube, spread on Mueller Hinton agar, and incubated for 24 hr at 37°C. The colony counts were determined. Colistin was used as positive control and for negative control Ab alone was inoculated in brain heart infusion broth.

Statistical Analysis:. Linear regression analysis was used for time kill kinetics assay. A probability of 0.05 or equal was taken as statistically significant. SigmaPlot 14.5 (Systat Software, USA) was used for statistical analysis and graph plotting.

Results

In this present study the antimicrobial activity of Usnic acid to inhibit the bacterial growth of MDR Acinetobacter baumannii were investigated by using MIC, MBC, and time kill assay. The twenty isolates of MDR-Ab used in this study were isolated from various specimens details are given in the table No. 1.

Table No. 1: MDR *Acinetobacter* isolates isolated from various specimens

Sl. No.	Isolate No.	Strain type	Specimen
1	AI 1444	A. baumannii	Endotracheal tube
2	AI 646-2	A. baumannii	Cerebrospinal fluid
3	AI 646-5	A. baumannii	Endotracheal tube
4	AI 4185	A. baumannii	Endotracheal tube
5	AI 829	A. baumannii	Endotracheal tube
6	AI 6142	A. baumannii	Endotracheal tube
7	AI 7783	A. baumannii	Endotracheal tube
8	AI 6553	A. baumannii	Pus
9	AI 5678	A. baumannii	Endotracheal tube
10	AI 6538	A. baumannii	Pus
11	AI 3990	A. baumannii	Endotracheal tube aspirate
12	AI 4888	A. baumannii	Endotracheal tube
13	AI 3074	A. baumannii complex	Pus
14	AI 3927	A. baumannii complex	Sputum
15	AI 899	A. baumannii complex	Pus
16	AI 6428	A. baumannii complex	Pus
17	AI 2760	A. baumannii complex	Endotracheal tube
18	AI 2540	A. baumannii complex	Endotracheal tube
19	AI 7496	A. baumannii	Endotracheal tube
20	AI 2368	A. baumannii	Endotracheal tube

MIC, MBC and MBC/MIC ratio of isolated compound:

In microbroth dilution method the efficacy of (+) - UA showed MIC ranged between 512 – 1024 µg/mL against all the isolates. The MBC of (+) - UA was ranged between 2048 – 4096 µg/mL. The mean of MIC and MBC concentrations of triplicate data are given in the table No. 2. The MIC/ MBC index ratio of twenty isolates were reported at 4, which indicate the bactericidal activity of compound against MDR *Acinetobacter* isolates results are given in table No. 2.

Table No. 2: MIC and MBC of (+) - UA against MDR *Acinetobacter* isolates

Sl. No.	Isolate Number	Strain type	Usnic acid		
			MIC µg/ ml	MBC µg/ ml	MIC / MBC Index
1	AI 1444	A. baumannii	512	2048	4
2	AI 646-2	A. baumannii	1024	4096	4
3	AI 646-5	A. baumannii	1024	4096	4
4	AI 4185	A. baumannii	1024	4096	4
5	AI 829	A. baumannii	1024	4096	4
6	AI 6142	A. baumannii	1024	4096	4
7	AI 7783	A. baumannii	1024	4096	4
8	AI 6553	A. baumannii	1024	4096	4
9	AI 5678	A. baumannii	1024	4096	4
10	AI 6538	A. baumannii	1024	4096	4
11	AI 3990	A. baumannii	1024	4096	4
12	AI 4888	A. baumannii	1024	4096	4
13	AI 3074	A. baumannii complex	1024	4096	4
14	AI 3927	A. baumannii complex	1024	4096	4
15	AI 899	A. baumannii complex	1024	4096	4
16	AI 6428	A. baumannii complex	512	2048	4
17	AI 2760	A. baumannii complex	1024	4096	4
18	AI 2540	A. baumannii complex	1024	4096	4
19	AI 7496	A. baumannii	1024	4096	4
20	AI 2368	A. baumannii	1024	4096	4

Time kill kinetics assay

The time taken to reach the desired reduction for colistin (positive control), Ab alone (negative control), UA is given in Figure 1. The time-kill kinetics assay of the compound against test organism at 1x, 2x MIC concentrations showed a reduction in $\log_{10}$. For 2x MIC's of UA there was 100% bacterial growth inhibition after 8 hr. Statistical correlation between colistin, Ab alone, 1x UA was carried out to find the $\log_{10}$ reduction by linear regression analysis. The linear regression analysis as done in the study [21] was done to see the efficacy of compound when compared with positive and negative control. The correlation coefficient for colistin, Ab alone, and 1x UA were 0.990, 0.943, and 0.959 respectively. The estimated slope for colistin, Ab alone, and UA were 0.129, 0.053, and 0.082 respectively, this shows that UA is having approximately similar effect like positive control.

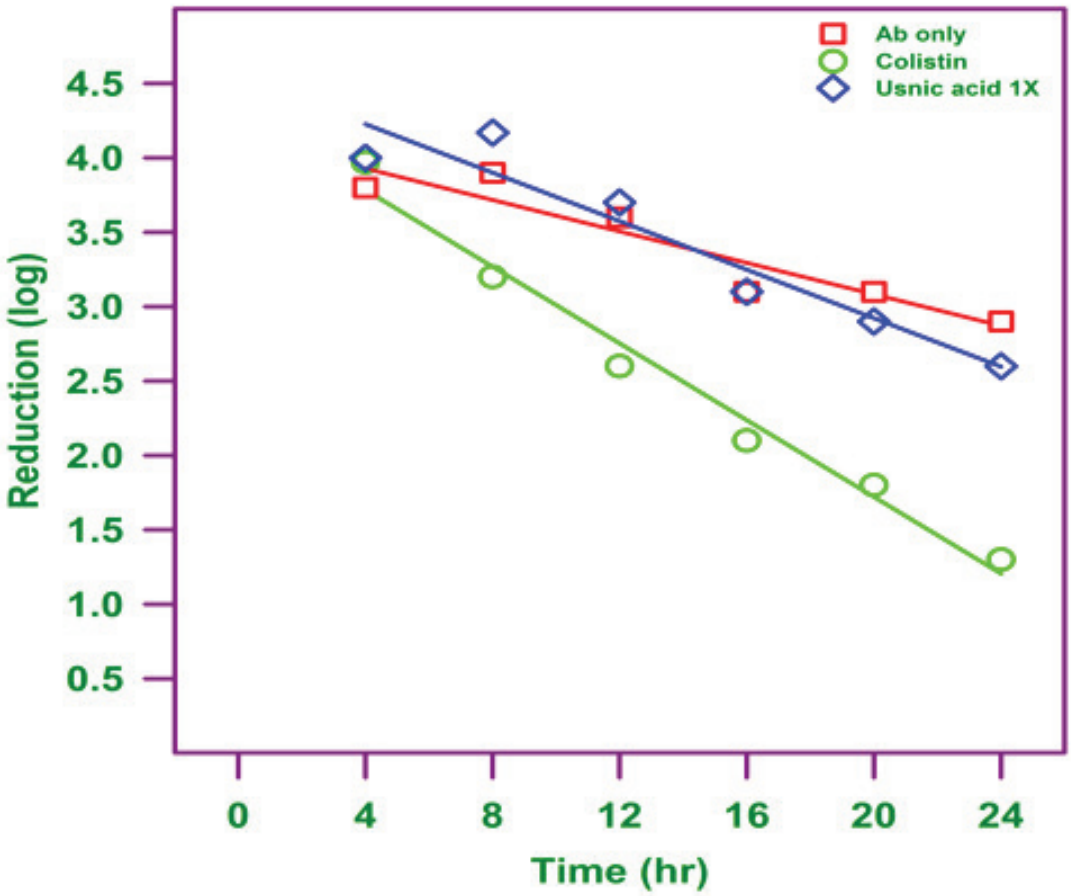

Figure No.1 Time kill kinetics assay of UA

Discussion

Previous studies reported that UA possesses antimicrobial properties against not only Gram-positive but also Gram-negative microorganisms. UA has been shown to be bacteriocidal against Gram-negative bacteria such as *Bacteroides, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia, Proteus vulgaris, Yersinia enterocolitica*, and strong effect on *H. pylori* [15]. Due to the UA's numerous complex pharmacological properties, these findings provided a comprehensive profile of the compound, which has garnered considerable attention in recent years. The present antimicrobial effect

observed here could be linked to the compounds' previously recorded antibacterial effect; UA efficacy for *E. coli* was reported as 1000 µg/mL [22]. The possible mechanism may be due to the inhibitory activity in nucleic acid replication and synthesis of bacteria [16]. However the role of these UA in the anti-bacterial property has to be explored in detail in near feature.

Conclusion

In the present study, the MIC, MBC and time kill assay of UA showed antibacterial efficacy of UA and will be bactericidal for *A. baumannii*. This compound can be used in combination therapy to prevent resistance development to standard antibiotics.

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