Original Article

Carvacrol Conjugated Zinc Oxide Nanoparticles as Prospective Agents against Clinically Isolated Carbapenem Resistant Acinetobacter Species

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ABSTRACT

Objective: To evaluate the antibacterial activity of Carvacrol conjugated zinc oxide nanoparticles against Carbapenem resistant Acinetobacter isolates and to assess its combination with meropenem.

Study Design: Experimental Study

Place and Duration of Study: This study was conducted at the Dr Ziauddin Hospital, North Campus, Karachi from February, 2021 to January, 2022.

Materials and Methods: The study was conducted on 50 Carbapenem resistant Acinetobacter isolates. The study was approved by ethics review committee (ERC) reference code: 2971220SHPHA. Minimum inhibitory concentration of Carvacrol zinc oxide nanoparticles was determined using broth macrodilution method on 5 isolates and the concentration which inhibited bacterial growth was noted. Agar well diffusion method was then performed on 50 isolates to observe zone of inhibition of the compound at observed MIC. Colistin was used as a standard drug. Checker board assay was done to explore type of interaction between Carvacrol zinc oxide nanoparticles and meropenem. Inter-group comparison was done using Kruskal wallis ANOVA and p-value of less than 0.05 was considered statistically significant.

Results: The minimum concentration of Carvacrol zinc oxide nanoparticles which inhibited bacterial growth was found to be 0.04mg/ml against all the isolates. Zone of inhibition on agar well diffusion was found to be in the range of 20-30mm as compared to colistin (0-14mm) and meropenem (0mm) with p-value being 0.00. Additive interaction was found between carvacrol zinc oxide nanoparticles and meropenem with FICI of 1.5.

Conclusion: This study brings forth anti- bacterial activity of a new compound that in future can be helpful for new antibiotic development.

Key Words: Acinetobacter, Meropenem, Carvacrol

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INTRODUCTION

Acinetobacter is the gram negative, aerobic, non-motile, catalase positive, oxidase negative, non-lactose fermenting coccobacilli[1].

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These are the source of multitude of nosocomial infections such as Ventilator associated pneumonia (VAP), septicemia, meningitis, wound infections and others .They are notorious for their remarkable antimicrobial resistance as they withstand antibacterial action of nearly all conventional antibiotics. Particularly, Carbapenem resistant Acinetobacter (CRA) is a major health concern recognized by WHO as it severely limits the therapeutic options and instigate the scientist to seek new compounds having the potential to combat such resistant organisms [2 3].

Nanoparticles usually defined as those having their size in the range of 1-100nm, are currently an interesting area of scientific research[4]. Owing to the characteristics of small size, large surface area to volume ratio, high reactivity and advantageous delivery kinetics, metallic nanoparticles have also shown the potential to inhibit growth of drug resistant organisms [5]. Amongst others, the zinc oxide nanoparticles have shown tremendous antimicrobial activities against both Gram positive and Gram negative organisms[6]. Zinc oxide nanoparticles

have been reported as relatively nontoxic to human cells and are being used as a part of sunscreens and ointments which prompts these particles to be as future antibiotics[7]. Carvacrol is a mono terpenoid phenol found in the essential oils of plants such as oregano & thyme[8]. It is categorized as Generally Recognized As Safe (GRAS) by FDA and its feed additives are also being employed in commercial markets. It also has reported antibacterial activity against several organisms such as Aspergillus, E.coli, Salmonella typhimurium, Proteus vulgaris etc[9].

Hence, this study was designed to investigate the antibacterial activity of zinc oxide synthesized using conjugation with Carvacrol in contending the exceptionally drug resistant Acinetobacter species. Moreover, we also investigated the potential of these nanoparticles in restoring the activity of carbapenems which might help in reviving once highly effective drugs against Acinetobacter.

MATERIALS AND METHODS

Carvacrol was purchased from Ambeed Inc. USA. Carvacrol conjugated zinc oxide nanoparticles were synthesized in HEJRIC, ICCBS Karachi University. Mueller Hinton Broth (MHB) and Mueller Hinton Agar (MHA) were purchased from Oxoid, UK. The study was approved by ethics review committee (ERC) reference code: 2971220SHPHA. Total of 50 culture plates showing growth of Acinetobacter were included while agar plates showing contamination or double growth were excluded from the study. In order to pick the Carbapenem resistant isolates of Acinetobacter (CRA), antimicrobial susceptibility testing was performed as per CLSI guidelines [10]. A lawn of bacterial inoculum was made on Mueller Hinton agar plate and discs of different antibiotics which include amikacin, ceftriaxone, co-trimoxazole, gentamicin, colistin, ofloxacin, imipenem, tazobactam/piperacillin and meropenem were placed. Plates were incubated at 37c and zone of inhibition around each antibiotic was measured after 24 hours. Isolates in which zone of inhibition of meropenem was less than 14mm were labelled as CRA and were included in our study for the further experiments.

Minimum inhibitory concentration (MIC) of Carvacrol conjugated zinc oxide nanoparticles (CrZnONPs) was determined using broth macro dilution method in accordance with CLSI guidelines. Briefly, bacterial isolates equivalent to 0.5 McFarland turbidity standard were grown overnight in Brain Heart Infusion (BHI). 50ul of this bacterial suspension was then inoculated in sterile tubes containing 5ml of MHB and two fold serial dilutions of CrZnONPs (0.01mg/ml, 0.02mg/ml, 0.04mg/ml, 0.08mg/ml, and 0.16mg/ml). Tubes were incubated aerobically at 37°c for 24hrs and the minimum concentration which inhibited bacterial growth indicated by change in turbidity was considered as the MIC of the nanoparticles. This was further confirmed by plating 100ul of bacterial suspension from each tube on MH agar plates to observe complete inhibition of bacterial colonies at the corresponding MIC. MIC was tested on five different isolates of CRA and each experiment was done in triplicate [11].

Agar well diffusion method was used to further evaluate the antibacterial activity of CrZnONPs. A lawn of bacterial inoculum was made on 150mm MH agar plates with the 100ul of overnight grown bacterial suspension of 0.5McFarland turbidity. After drying for 15 minutes, wells measuring 8mm in diameter were made onto the plates with borer and 10ul of CrZnONPs suspension was dispensed into the wells. Plates were incubated at 37°c for 24hrs and zone of inhibition was measured in millimeters. This experiment was conducted on 50 isolates of CRA[12]. Colistin was used as a standard drug.

Checker board assay, which provides a precise estimation of synergistic, additive, or antagonistic type of drug interaction in vitro, was employed to evaluate the combination of CrZnONPs with meropenem. The experiment was conducted in 12 distinct tubes, labelled 1-12, each containing 15ul of CRA isolate bacterial culture and 1 ml of MHB. Two fold serial dilutions of CrZnONPs ranging 0.01mg/ml, 0.02mg/ml, 0.04mg/ml, 0.08mg/ml was prepared along the x-axis (abscissa) and two fold serial dilutions of meropenem ranging from 0.002mg/ml, 0,004mg/ml and 0.008 mg/ml were prepared along y-axis (ordinate) (Fig 1). A loopful of each tube was smeared over MH agar plates after incubation for 24hrs. The plates were then kept at 37°C for 24 hours to observe growth. The most effective combination in the assay was identified as the earliest one that first suppressed growth of the bacterial cells. Fractional inhibitory concentration index (FICI) was calculated using the following formula:

$FICI = FIC_A + FIC_B$

Where,

FIC_A = MIC of drug A in combination/ MIC of drug A alone

FIC_B = MIC of drug B in combination/ MIC of drug B alone

Interpretation:

FIC Index ≤0.5= Synergy between the compounds

FIC Index >0.5–4.0= Additive or Indifferent interaction between the compounds

FIIC Index >4.0= Antagonism between the compounds.[13]

DRUG B MEROPENEM				
0.008mg/ml	9 (0.01/0.008)	10 (0.02/0.008)	11 (0.04/0.008)	12 (0.08/0.008)
0.004mg/ml	5 (0.01/0.004)	6 (0.02/0.004)	7 (0.04/0.004)	8 (0.08/0.004)
0.002mg/ml	1 (0.01/0.002)	2 (0.02/0.002)	3 (0.04/0.002)	4 (0.08/0.002)
0.00	0.01mg/ml	0.02mg/ml	0.04mg/ml	0.08mg/ml

DRUG A CARVACROL ZINC OXIDE NANOPARTICLES

Figure No.1: Design of Checker Board Assay

Statistical Analysis: Data was analyzed using SPSS software. Normality of the data was checked using Shapiro-Wilk test. Numerical data was expressed as median and interquartile ranges. Kruskal-Wallis ANOVA was applied for comparison between groups. p-value less than 0.05 was considered significant.

RESULTS

MIC of CrZnONPs was tested on five different isolates of CRA and the concentration in each isolate that was able to inhibit growth was found to be 0.04mg/ml (Table 1) (Fig 1).

Table No.1: Minimum Inhibitory Concentration (Mic) of CrZnONPs against Five Different Carbapenem Resistant Acinetobacter Isolates

Serial Dilutions of CrZnOPs	0.01mg/ml	0.02mg/ml	0.04mg/ml	0.08mg/ml	0.16mg/ml
Isolate 1	+	+	-	-	-
Isolate 2	+	+	-	-	-
Isolate 3	+	+	-	-	-
Isolate 4	+	+	-	-	-
Isolate 5	+	+	-	-	-

+ indicates growth, - indicates no growth

Table No.2: Agar Well Diffusion for CrZnONPs on Carbapenem Resistant Acinetobacter Isolates

S.No	Compound	No. of Isolates	Minimum Zone of Inhibition	Maximum Zone of Inhibition	MEDIAN (IQR)	p-value
1	CrZnONPs	50	10	30	20 (6)	0.00
2	Colistin		0	14	14 (0)	
3	Meropenem		0	0	0	

Table No.3: Checker Board Assay between CrZnONPs & Meropenem against CRA Isolate

Bacterial strain	CrZnONPs (Drug A)		FIC_A	MEROPENEM (Drug B)		FIC_B	FICI $=FIC_A+FIC_B$
	MIC alone	MIC in combination		MIC alone	MIC in combination		
Carbapenem resistant Acinetobacter	0.04	0.02	0.5	0.008	0.008	1	1.5

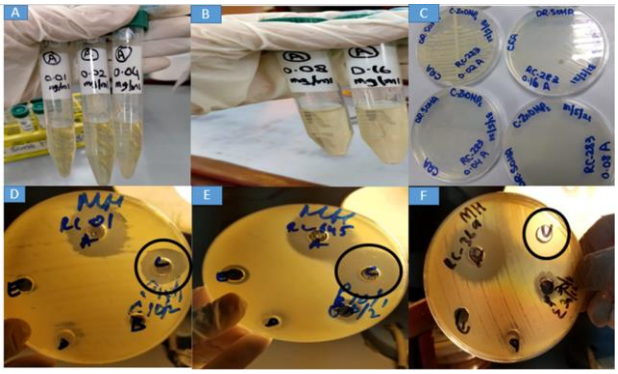

Figure No.2: Mic & Agar Well Diffusion Method

A gar well diffusion was performed to observe the zone of inhibition of CrZNoNPs at its tested MIC. All of the isolates in the study were sensitive to this compound. The data for zone of inhibition for 50 isolates is summarized in (Table 2) (Fig 1).

A & B: Broth macrodilution. C: MIC determined by change in turbidity best observed at 0.04mg/ml as also evident by no bacterial growth on agar plate. D, E & F: MH agar plates showing zone of inhibition of CrZnONPs mentioned as C (encircled) in different CRA isolates.

In the checker board assay tube 10 was the first combination that inhibited bacterial growth, therefore FICI between CrZnONPs and meropenem combination was calculated to be 1.5 which shows that these compounds have an additive interaction between them (Table 3).

DISCUSSION

The worrisome situation of Carbapenem resistance associated with Acinetobacter infections has endangered human lives due to considerable morbidity and mortality[14]. In this scenario, nanoparticles are now being evaluated for their antibacterial ability specifically against resistant isolates, generating hope for alternative antibiotic for extensively drug resistant organisms in particular such as Acinetobacter species, for which now only limited antibiotics have now been left effective[15]. In our study we used nanoparticles synthesized using natural biomolecule Carvacrol as this form of synthesis is environment friendly, sustainable, economical and above all is free from hazardous chemicals[16].

The minimum concentration of CrZnONPs that inhibited growth of the isolates was found to be 0.04mg/ml in our experiments. There is dearth of studies in literature that specifically reports the antibacterial activity of this compound. However, there are many studies in which the activity of various form of zinc oxide nanoparticles synthesized using different biomolecules or chemicals has been tested against Acinetobacter. In one of such study conducted in Iran reports MIC of 0.5mg/ml of zinc oxide nanoparticles synthesized chemically against the clinical isolates of multi drug resistant (MDR) Acinetobacter baumannii (A.baumannii) [17]. A recent study of China that explored the activity of zinc oxide nanoparticles synthesized using Caryophyllus aromaticus on 10 MDR strains of A.baumannii isolated from pneumonia patients reported much lower MIC in the range of 0.04-0.19ug/ml [18].

In agar well diffusion method CrZnONPs has revealed exceptional results as it did not only effectively clear bacterial colonies but was also able to do this than the standard drug colistin. Amazingly, CrZnONPs has also inhibited growth of an isolate which was resistant to colistin providing remarkable evidence of their exceptional antibacterial activity against resistant bacteria in the current grave situation of emerging pan drug resistant organisms. We found out ZOI for CrZnONPs to be in between 10-30mm. This finding is in line to a study of Egypt on Aspergillus niger synthesized zinc oxide nanoparticles that also revealed ZOI of 21mm for A.baumannii[19]. Also, another study on Eucalyptus globulus synthesized ZnONPs conducted in Algeria against A.baumannii showed that ZnONPs were able to form ZOI in the range of 12.37-17.23mm[20].

In our study we found additive interaction between CrZnONPs and meropenem as the FICI was calculated to be 1.5. This finding is in agreement to a study of Saudi Arabia in which combination of ZnONPs was evaluated along with meropenem, colistin and ciprofloxacin against E.coli & A.baumannii. The study reports combination of ZnONPs with meropenem to be additive or indifferent against both organisms with FICI values of 1.25 and 2 respectively. Also, the combination of ZnONPs with colistin and ciprofloxacin also showed additive/indifferent effects[21].

The foremost reason for the difference in findings of various studies is that in each of the study different plants and chemicals are used for the synthesis of nanoparticles which also impart their own natural properties to these nano formulations. Apart from it, there is also much variation in the physical properties that also determine their antimicrobial potential [22]. Most importantly each of the study is conducted on a different isolate of Acinetobacter from different geographical regions which possess having varied genes for antimicrobial resistance and majority has targeted the most obstinate organism of this genus i.e. A. baumannii while in our study isolates belonging to any species were included.

CONCLUSION

Our study highlights the potential antimicrobial activity of carvacrol conjugated zinc oxide nanoparticles against troublesome Acinetobacter organisms. The results of the checker board assay are too preliminary to correctly estimate the type of interaction between CrZnONPs and meropenem but additive interaction between these two compounds is still an optimistic finding. Future studies should be conducted on larger number of isolates selected from varied geographical regions so that the findings of this study can be generalized by and large.

Author's Contribution:

Concept & Design of Study:	Soha Haque, Kauser Ismail, Raza Shah
Drafting:	Kauser Ismail
Data Analysis:	Ambrina Khatoon, Faisal Afridi, Soha Haque
Revisiting Critically:	Zahida Memon
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Conflict of Interest: The study has no conflict of interest to declare by any author.

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