Full Length Research Article

Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

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In Vitro Synergistic Activity of Ceftazidime-Avibactam and Aztreonam against New Delhi Metallo-β-Lactamase-Producing Clinical Enterobacterales Isolates

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Abstract

Background: Carbapenemase-producing-Enterobacterales (CPE) infections are on the rise and associated with increased morbidity and mortality, due to a limited number of therapeutic options. The goal of this study was to assess the synergistic activity of ceftazidime-avibactam (CZA) and aztreonam (ATM) combination against phenotypically and genetically characterized bla_{MBL}-producing Enterobacterales.

Methods: In this study, forty (n=40) non-repeat, CPE clinical isolates, including: n=35 *Klebsiella pneumoniae*, n=2 each of *Escherichia coli* and *Klebsiella oxytoca*, and n=1 *Enterobacter cloacae* isolates were identified and susceptibilities were assessed using the Vitek-II compact (bioMérieux, Inc., France) and Microscan Walkaway (Beckman Coulter) systems. Genotypic analysis of clinically relevant carbapenemases was performed by using Xpert-Carba-R assay on GeneXpert (Cepheid, USA). The minimum inhibitory concentrations (MICs) and synergy testing of CZA and ATM, were tested by the Etest fixed ratio method (bioMérieux, Inc., France). The fractional inhibitory concentration index (FICI) was calculated for each antibiotic.

Results: Our results showed that 97.5% of bla_{MBL}-producing Enterobacterales isolates were susceptible to an in-vitro CZA + ATM combination regimen. The fractional inhibitory concentration index (FICI) ranged from 0.001 to 1.001. Among the tested CPE isolates, *Synergy* was observed in 36/40 (90%), an *additive effect* was observed in 5% (n=2)' while two (5%) isolates showed indifference. There was *no antagonism* observed in our study.

Conclusion: Our study exhibited a potent activity of CZA and ATM combination synergy against clinical CPE metallo- β-lactamase producers. More extensive studies involving a variety of Gram-negative pathogens with different resistance mechanisms are required to determine the efficacy of this combination regimen.

Introduction

Carbapenem-resistant Enterobacterales (CRE) infections are rising globally [1, 2]. Many countries report rising CRE cases [3]. High resistance and a lack of new antimicrobials limit therapeutic options and increase risk of morbidity and mortality [4]. *Klebsiella pneumoniae* and bla_{KPC} dominate CRE [5]. A bla_{KPC} epidemiology review includes global reports [6]. CREs have also been reported from Saudi Arabia, in contrast to other reports, bla_{NDM} and bla_{OXA48} are the most common genotypes nationwide. A Majority of the CRE studies are reported from Riyadh, Saudi Arabia [3]. Alarmingly, the presence of CRE has also been documented in municipal wastewater from Jeddah [7]. T The mortality rate associated with CRE cases is 26-46% [3].

The hydrolytic mechanism at the active site divides carbapenemases into serine- and metallo-carbapenemases [8]. Several Enterobacterales genes confer carbapenem resistance including bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{VIM}, and bla_{IMP}. Metallo-beta lactamases (MBL) include bla_{NDM}, bla_{VIM}, and bla_{IMP}, while bla_{KPC} and bla_{OXA48} are serine carbapenemases [9]. Beta-lactamase inhibitors (BLIs) were developed to combat different beta-lactamases. First-line BLIs included clavulanic acid, tazobactam, and sulbactam. Their narrow spectrum of activity makes them ineffective against carbapenemases. New BLI like avibactam, relebactam, zidebactam, and nacubactam are active against serine carbapenemases but not metallo-carbapenemases [10]. The newly introduced beta-lactam/BLI combination CZA has successfully treated CRE infections. CZA is inactive against MBL producers. Aztreonam is resistant to MBL hydrolytic activity, but other hydrolytic enzymes produced in MBL-positive strains like ESBL, serine carbapenemases like bla_{OXA48}, bla_{KPC}, and other cephalosporinases inactivate ATM, making it generally unsuitable for MBL producing CRE infections [11].

Few studies have shown that CZA and ATM work synergistically to treat CRE infections [12, 13]. CZA and ATM combinational therapy treated successfully a CRE bloodstream infection in neutropenic patients, caused by *K. pneumoniae*. The synergistic testing resulted in a substantial decrease in MIC of aztreonam from >256 to 0.094 ug/mL [14]. CZA and ATM were successfully used as prophylactic therapy in a cancer patient undergoing a solid organ transplant with a previous bla_{NDM}-producing *K. pneumoniae* infection [15]. Pragasam and colleagues tested CZA and ATM's synergistic activity against 12 *K. pneumoniae* isolates with bla_{NDM}, bla_{OXA48}-like, and co-producers of bla_{NDM} + bla_{OXA48}. In vitro CZA MIC ranged from 0.5 to >8 μg/ml, while ATM MIC was 64 to 2048 μg/ml. A >8-fold MIC difference (synergistic effects) was observed when CZA MIC was combined with ATM at 4 μg/ml using the checkerboard method [16]. Another study found that CZA alone or in combination with ATM synergistically killed twenty-eight (28) bla_{NDM} and two (02) bla_{NDM}/bla_{KPC}-producing *K. pneumoniae* isolates in vitro. Notably, 90% (27/30) strains showed synergism, while 3.3% (1/30) showed additive effects. Synergy was stronger against the tested isolates [17]. ATM and other β-lactamase inhibitors have shown synergistic activity against Enterobacterales isolates in vitro. The studies found 86%, 50%, and 20% of clinical isolates susceptible to CZA, amoxicillin-clavulanic acid, and ceftolozane-tazobactam [18]. To the best of our knowledge, no Saudi Arabian study examined CZA+ATM combination therapy for such cases. However, CZA and ATM have treated CRE infections in other countries. CZA + ATM combination therapy successfully treated bla_{NDM} in the US [13]. French researchers also reported two successful CZA + ATM combinations in two isolates with bla_{NDM} and bla_{OXA} genotypes in the same year [12].

To find efficacious drug combinations that specifically combat high-level resistance due to New Delhi metallo-beta-lactamase-producing clinical Enterobacterales, we conducted an in-vitro study to evaluate the synergistic effects of CZA and ATM against Enterobacterales harboring bla_{NDM}.

Methods

Microorganisms, Identifications, and Susceptibilities:

A total of forty (n=40) bla_{NDM}-positive Enterobacterales clinical isolates from inpatients, were isolated from a tertiary care hospital, King Khalid University Hospital in Riyadh, Saudi Arabia, between January 2017 and December 2019. The institutional review board of the hospital granted approval for the study. Carbapenem-resistant isolates were subjected to full identification and susceptibilities testing by using the VITEK®2 system (bioMérieux, Inc., France) and Microscan Walkaway system (Beckman Coulter, USA). All study isolates were stored at -80˚C in skimmed milk with 10% glycerol (SPML, Saudi Arabia). Before the experiment, isolates were revived by subculturing on sheep blood agar (SPML, Saudi Arabia). Antimicrobial testing was conducted using Mueller-Hinton agar plates (SPML, Saudi Arabia).

Institutional Review Board Statement:

The Institutional Review Board of the Faculty of Medicine, King Khalid University Hospital, King Saud University Medical City, Riyadh, Saudi Arabia, approved the study by the Declaration of Helsinki guidelines (approval number: Ref #: 20/0118/IRB).

Genotypic Analysis:

The genotypic analysis of carbapenem-resistant Enterobacterales isolates was conducted using the

Xpert-Carba-R assay on the GeneXpert system (Cepheid, USA).

Antimicrobial Agents and MIC Determination:

We used ATM and CZA Etest strips from bioMérieux, Inc. in France. *Klebsiella pneumoniae* ATCC 70063 and *Escherichia coli* ATCC 25922 were used for quality control testing. Using gradient diffusion Etest strips, the MICs for CZA and ATM were found (bioMérieux, Inc., France). For these calculations, the manufacturer's recommendations were adhered to. For CZA and ATM, the range of antibiotic concentrations on an Etest strip was 0.016 to 256 µg/ml. The results were interpreted according to the breakpoints in CLSI 2020 [19]. The tested antibiotics' resistance thresholds were as follows: ATM = 16 µg/ml; CZA = 16 µg/ml. Minimum inhibitory concentration 50 (MIC_{50}) and minimum inhibitory concentration 90 (MIC_{90}) were defined as antibiotic concentrations at which 50% and 90% of the tested isolates were inhibited.

Etest Fixed Ratio Synergy Method:

The individual MICs and synergistic activity of CZA and ATM against CRE isolates were determined by inoculating Mueller-Hinton agar (MHA) plates with 0.5 McFarland suspension of test isolates. When testing synergy, (combination setups), the antibiotics under study were applied to the bacterial lawn one after the other using Etest strips. After five minutes of incubation at room temperature, the first Etest strip (ATM) was taken out and preserved as a scale for determining the minimum inhibitory concentration (MIC). The impression of the first Etest strip, ATM, was immediately covered by the second Etest strip (CZA). To make it easier to read the ATM MIC when CZA is present, the ATM strip was then positioned on top of CZA. The plates were kept for 18 hours at 36°C. Each Mueller-Hinton agar plate contained two Etest strips. The point at which all growth was completely inhibited was observed to determine the MIC of individual drugs and drug combinations. The Etest strip's inhibition zone intersection with the scale was used to calculate the minimum inhibitory concentration (MIC) [12].

Data Storage and Analysis:

IBM SPSS software version 20.0 was used to conduct the statistical analysis (IBM Corp.; Armonk, NY). The qualitative data were described using numbers and percentages. The findings were considered significant at a level of 5% ($p < 0.05$). The chi-square test was used to compare categorical groups, and Fisher's exact was used to adjust the chi-square test when more than 20% of the cells had an expected value of less than 5. The following formula was used to determine the fractional inhibitory concentration index (FICI) for each antibiotic in the combination: FICA + FICB equals FICI. FICA represents the MIC of ATM in combination divided by the MIC of ATM alone, and FICB represents the MIC of CZA in combination divided by the MIC of CZA alone. The FICIs have been interpreted as follows: Synergy = FICI ≤0.5, Additivity = FICI >0.5 to ≤1, No interaction (indifference) = FICI >1 to ≤4, and Antagonism = FICI >4.

Results

Using the agar-based Etest fixed ratio method, the recent study assessed the in vitro synergy between CZA and ATM against 40 Enterobacterales isolates, *bla*$_{NDM}$. *K. pneumoniae* (n=35, 87.5%), *E. coli,* and *K. oxytoca* (n=2, 5%) each (Total= 10%), respectively, and 2.5% (n=1) *E. cloacae* were the main isolates (data not shown here). Table 1 displays the MIC distributions by Etest for CZA, ATM, and CZA+ATM, besides this, fractional inhibitory concentration index values for each tested isolate are shown along with interpretation Test results for all 40 isolates showed CZA resistance (100%) when tested alone, while 17.5% of isolates exhibited aztreonam resistance alone (n=7). In 90% of the tested isolates (n=36/40), the combination of CZA and ATM demonstrated synergistic effects. The decrease in MIC after CZA and ATM synergy is displayed in Table 1, the lowest and highest MIC decreases were observed to be 3 and 13 doubling dilutions, respectively (Table 1).

Figure 1: Depicting CRE-2, *Klebsiella pneumoniae* isolate resistant to ceftazidime-avibactam (CZA) aztreonam (ATM) **(a)** and synergy noticed for combination of ceftazidime-avibactam (CZA) plus aztreonam (ATM), **(b)**. The minimum inhibitory concentrations (MICs) of CZA, AT, and CZA+AT were >256, >256 **(a)**, and 0.38 (µg/ml) **(b)**, respectively. Fractional Inhibitory concentration (FIC) for CZA and ATM was 0.0015 and 0.0015, respectively. This isolate's Etest MIC was 0.38µg/ml on combination testing (CZA+ATM), and the fractional Inhibitory concentration index was 0.003 (ΣFIC: 0.003, Synergy).

K. pneumoniae (n=35), MIC range, MIC50, MIC90 against CZA+ATM: 0.032-24, 0.25, and 0.75, respectively. Out of the 35 tested *K. pneumoniae* isolates, 97.1% of isolates were susceptible to the combination Etest method. Overall, all tested isolates exhibited very satisfactory results against CZA+ATM, as shown by the MIC range, MIC50, and MIC90 of 0.032-24, 0.25, and 0.5, respectively (Table 2). Figure 1 shows susceptibility testing of CRE-2, *K. pneumoniae* isolate showing resistance to CZA and ATM, however, synergy is noticed for the combination of CZA+ATM.

Seq#	Organism	MIC (µg/ml)			MIC Decreased (doubling dilutions)	FICI			Synergy Interpretation
		CZA	ATM	CZA+ATM		FIC-CZA	FIC-ATM	ΣFIC Index$	
CRE-01	Klebsiella pneumoniae	256	256	24	3	0.094	0.094	0.188	Syn.
CRE-02	Klebsiella pneumoniae	256	256	0.38	9	0.001	0.001	0.003	Syn.
CRE-03	Klebsiella oxytoca	256	128	0.125	11	0	0.001	0.001	Syn.
CRE-04	Klebsiella pneumoniae	256	0.19	0.19	10	0.001	1	1.001	Indiff.
CRE-05	Klebsiella pneumoniae	256	256	0.25	10	0.001	0.001	0.002	Syn.
CRE-06	Escherichia coli	256	256	1	8	0.004	0.004	0.008	Syn.
CRE-07	Klebsiella pneumoniae	256	256	0.38	9	0.001	0.001	0.003	Syn.
CRE-08	Klebsiella pneumoniae	256	256	0.38	9	0.001	0.001	0.003	Syn.
CRE-09	Klebsiella pneumoniae	256	1	0.25	10	0.001	0.25	0.251	Syn.
CRE-10	Klebsiella pneumoniae	256	256	0.5	9	0.002	0.002	0.004	Syn.
CRE-11	Escherichia coli	256	256	0.75	8	0.003	0.003	0.006	Syn.
CRE-12	Klebsiella pneumoniae	256	256	0.75	8	0.003	0.003	0.006	Syn.
CRE-13	Klebsiella pneumoniae	256	0.5	0.19	10	0.001	0.38	0.381	Syn.
CRE-14	Klebsiella pneumoniae	256	24	0.032	13	0	0.001	0.001	Syn.
CRE-15	Klebsiella pneumoniae	256	256	0.125	11	0	0	0.001	Syn.
CRE-16	Klebsiella pneumoniae	256	64	0.19	10	0.001	0.003	0.004	Syn.
CRE-17	Klebsiella pneumoniae	256	256	2	7	0.008	0.008	0.016	Syn.
CRE-18	Klebsiella pneumoniae	256	256	0.5	9	0.002	0.002	0.004	Syn.
CRE-19	Klebsiella pneumoniae	256	32	0.094	11	0	0.003	0.003	Syn.
CRE-20	Klebsiella oxytoca	256	32	0.125	11	0	0.004	0.004	Syn.
CRE-21	Klebsiella pneumoniae	256	256	0.25	10	0.001	0.001	0.002	Syn.
CRE-22	Klebsiella pneumoniae	256	0.25	0.19	10	0.001	0.76	0.761	Add.
CRE-23	Enterobacter cloacae	256	32	0.125	11	0	0.004	0.004	Syn.
CRE-24	Klebsiella pneumoniae	256	24	0.125	11	0	0.005	0.006	Syn.
CRE-25	Klebsiella pneumoniae	256	0.25	0.19	10	0.001	0.76	0.761	Add.
CRE-26	Klebsiella pneumoniae	256	256	0.25	10	0.001	0.001	0.002	Syn.
CRE-27	Klebsiella pneumoniae	256	0.19	0.19	10	0.001	1	1.001	Indiff.
CRE-28	Klebsiella pneumoniae	256	0.125	0.064	12	0	0.512	0.512	Syn.
CRE-29	Klebsiella pneumoniae	256	256	0.19	10	0.001	0.001	0.001	Syn.
CRE-30	Klebsiella pneumoniae	256	256	0.25	10	0.001	0.001	0.002	Syn.
CRE-31	Klebsiella pneumoniae	256	256	0.25	10	0.001	0.001	0.002	Syn.
CRE-32	Klebsiella pneumoniae	256	256	0.19	10	0.001	0.001	0.001	Syn.
CRE-33	Klebsiella pneumoniae	256	256	0.25	10	0.001	0.001	0.002	Syn.
CRE-34	Klebsiella pneumoniae	256	256	0.38	9	0.001	0.001	0.003	Syn.
CRE-35	Klebsiella pneumoniae	256	256	0.5	9	0.002	0.002	0.004	Syn.
CRE-36	Klebsiella pneumoniae	256	256	0.38	9	0.001	0.001	0.003	Syn.
CRE-37	Klebsiella pneumoniae	256	256	0.25	10	0.001	0.001	0.002	Syn.
CRE-38	Klebsiella pneumoniae	256	256	0.5	9	0.002	0.002	0.004	Syn.
CRE-39	Klebsiella pneumoniae	256	256	0.75	8	0.003	0.003	0.006	Syn.
CRE-40	Klebsiella pneumoniae	256	256	0.25	10	0.001	0.001	0.002	Syn.

µg/ml = microgram/milliliter, MIC = minimum inhibitory concentration, CZA+ATM: ceftazidime-avibactam+aztreonam, CZA: ceftazidime-avibactam, ATM: aztreonam, FIC-CZA: Fractional inhibitory concentration of ceftazidime-avibactam, FIC-ATM: Fractional inhibitory concentration of aztreonam, ΣFIC: Index of fractional inhibitory concentration, $ ΣFIC: Summation fractional inhibitory concentration criteria: *synergistic (ΣFIC: ≤0.5), additive (ΣFIC:>0.5 and ≤1), indifferent (ΣFIC: >1 and ≤4), antagonistic (ΣFIC: >4), Syn. = Synergy, Add. = Additive, Indiff. = indifferent.*

Table 1: Ceftazidime-avibactam and aztreonam susceptibility and synergy testing against *blaNDM*-CRE isolates (n=40).

S#	Organism	Antibiotic	MIC Range (µg/ml)	#MIC50 (µg/ml)	##MIC90 (µg/ml)	%-S-by Etest
1	Klebsiella pneumoniae (n=35)	CZA	>256	>256	>256	97.1
		ATM	0.125-256	>256	>256	
		CZA+ATM	**0.032-24**	**0.25**	**0.75**	
2	*Klebsiella oxytoca (n=2)	CZA	>256	>256	>256	100
		ATM	32-128	32	128	
		CZA+ATM	**0.125**	**0.125**	**0.125**	
3	*Escherichia coli (n=2)	CZA	>256	>256	>256	100
		ATM	>256	>256	>256	
		CZA+ATM	**0.75-1**	**0.75**	**1**	
4	*Enterobacter cloacae (n=1)	CZA	>256	>256	>256	100
		ATM	32	32	32	
		CZA+ATM	**0.125**	**0.125**	**0.125**	
5	**Overall, All blaNDM isolates (n=40)**	CZA	>256	>256	>256	97.5
		ATM	0.125-256	>256	>256	
		CZA+ATM	**0.032-24**	**0.25**	**0.5**	

*= Interpret the results with caution as isolates number is not statistically significant, µg/ml = Microgram/Milliliter, MIC = minimum inhibitory concentration, S = susceptible, CZA+ATM: ceftazidime-avibactam + aztreonam, CZA: ceftazidime-avibactam, ATM: aztreonam, #MIC50 = MIC at which 50 percent of isolates were inhibited, ##MIC90 = MIC which inhibits 90 percent of isolates.

Table 2: MIC ranges, MIC50, and MIC90 values of various blaNDM-positive isolates tested against ceftazidime-avibactam, aztreonam, and a combination of ceftazidime-avibactam and aztreonam (n=40).

This isolate's combination Etest value was 0.38μg/ml. The fractional Inhibitory concentration index was 0.003 (ΣFIC: 0.003, Synergy).

Discussion

Carbapenem-resistant Enterobacterales strains pose a significant and widespread risk to public health worldwide. The production of serine and metallo-beta lactamases, such as KPC, NDM, VIM, IPM, and OXA-48-like enzymes, is the primary cause of resistance to carbapenems that are used as a last resort in this species. Metallo-β-lactamases (MBL) production in gram-negative bacteria is a well-known trait, causing deadly infection worldwide, with a potential to disseminate quickly [20, 21]. From 2007 to 2016, the rate of carbapenem resistance among *K. pneumoniae* in Saudi Arabia increased from 0% to 33.3%, making it the most resistant Enterobacterales species [22]**.** *K. pneumoniae* isolates from Saudi Arabia are resistant to carbapenems due to their acquisition of OXA-48-like and NDM carbapenemases, with few recent studies detecting KPC carbapenemases [23, 24]. Our results corroborate previous microbiological studies indicating the higher prevalence of blaMBL in *K. pneumoniae* than any other member of Enterobacterales [25]. Due to the coexistence of numerous carbapenemases and co-resistance to tigecycline, β-lactams, aminoglycosides, quinolones, and colistin, few treatment options are available, which poses a significant therapeutic challenge. Hospitalizations, morbidity, and death rates related to pan-drug-resistant (PDR) *K. pneumoniae* infections that are resistant to traditional treatment have surged worldwide in recent times. [26]. Several studies are reporting the effectiveness of CZA + ATM therapy in comparison with other active antibiotic groups [27]. The Infectious Disease Society of America (IDSA) has recommended the use of CZA with ATM for the management of clinical infections due to MBL producers. Other treatment options for MBL producers include ATM in combination with meropenem-vaborbactam, cefiderocol, polymyxin B (in combination therapy for non-UTI infections), and polymyxin E (colistin) for UTI only. It is imperative to contact the microbiology laboratory for in vitro testing of the synergy between CZA+ ATM [28]. This study investigated ATM and CZA synergy against Enterobacterales that produce blaMBL. All isolates were CZA-resistant due to blaMBL. However, synergy against all isolates was observed when tested in synergy with ATM. Numerous studies [29-31] have documented the synergy between CZA and ATM.

We used the fixed ratio method and found it efficient for calculating synergies between two antimicrobials. Various studies have been performed using the Etest synergy method and researchers have found these methods useful, reliable, and comparable to agar dilution and broth dilution methods. [32, 33]. The interpretation of synergy by fractional inhibitory concentration index showed synergy in 90% of the isolates. Among the remaining, 5% of the isolates showed an additive effect while the other 5% showed no interaction. No antagonism was observed, thus it may be safer to use this antimicrobial regimen, however, further extensive studies are warranted to further understand this aspect and it could be interesting to test these combinations on strains with additional resistance mechanisms, such as blaVIM and bla_{IPM}-producing strains. The addition of ATM to CZA decreased the CZA MIC from 3 folds to 13 folds among various isolates.

Consider these points when interpreting our findings: First, these experiments only used Enterobacterales isolates with *K. pneumoniae* predominance. Second, the study excluded non-fermenter bacteria like *P. aeruginosa*. Given the lack of agents that kill bla_{NDM}-producing Enterobacterales, we wanted to show that CZA and ATM work together and suggest it as a treatment [26]. The results should be interpreted with caution, as all studies of this type need more work to characterize CZA with ATM's effectiveness against bla_{MBL}-producing Gram-negative pathogens. Although this study had many *K. pneumoniae* isolates, only two bla_{NDM}-producing Klebsiella oxytoca, two *E. coli*, and one *E. cloacae* were tested. We suggest further research into bla_{NDM}-producing Enterobacterales isolates and non-Enterobacterales isolates, particularly those with co-occurring β-lactamases. The findings may not apply to Enterobacterales isolates with other bla_{MBL} like bla_{VIM} or bla_{IMP}. The results may not apply to other blaMBL-producing pathogens like *P. aeruginosa* and *A. baumannii*, which have more intrinsic, acquired, and adaptive resistance mechanisms than *K. pneumoniae* and *E. coli*. Finally, these combination regimens need human clinical efficacy and safety testing.

Taken together, bla_{MBL}-producing pathogens remain a major challenge despite recent advancements in the treatment of carbapenem-resistant organisms. ATM can resist the bla_{MBL}-mediated hydrolysis, but co-harbored serine beta-lactamases (KPC, OXA-48, and ESBL) make ATM ineffective, requiring combination therapy. Our study showed that CZA and ATM synergistically inhibited bla_{NDM}-producing Enterobacterales isolates. Similar to all studies of this type, more extensive experiments against a variety of Gram-negative pathogens with different blaMBLs with different hydrolytic capabilities and other resistance mechanisms are needed to determine the efficacy of this combination regimen. Finally, CZA and ATM clinical efficacy and safety must be established in clinical trials, and current use should be based on a risk versus benefit model.

Author Contributions

Absar M, Marie M, and Arshad M conceived the research idea and designed the experiment.

AlAnazi N, Alfuraydi A run the experiment

Absar M, Arshad M collected and analyzed the data, and wrote the manuscript draft.

S Ali and Marie M, Binkhamis K, Arshad M revised and edited the manuscript.

All authors read and approved the final version of the manuscript.

Conflict of Interest

The author declare that there is no conflict of interest regarding the publication of this paper.

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