In Vitro Potentiation of Carbapenems with Tannic Acid Against Carbapenemase Producing Enterobacteriaceae:

Exploring Natural Products as Potential Carbapenemase Inhibitors

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Abstract

Resistance to antibiotics is increasing worldwide, necessitating urgent action to sustain the efficacy of existing antibiotics in clinical use. We show that tannic acid (TA) in combination with carbapenems can reduce and/or reverse the minimum inhibitory concentrations (MICs) of carbapenems to susceptible values in Enterobacteriaceae that express class A and B carbapenemases. MICs of carbapenems in the presence and absence of TA and other efflux pump inhibitors, TA-carbapenemase inhibition assays and computational studies were undertaken to determine the effect of TA on carbapenem susceptibility in *Enterobacteriaceae*. TA had the greatest effect on metallo- β -lactamases (MBLs) followed by class A serine- β -lactamases (SBLs). Antibiotic susceptibility testing showed that TA reversed the MICs of MBLs to susceptible values whilst substantially reducing the MICs of SBLs (class A). Tolerable cytotoxicity effect was observed for the concentrations tested. TA inhibited enzymes with a marked difference between $\approx 50\%$ inhibition (IC_{50}) for NDM-1 and KPC-2. Computational studies including molecular docking, molecular dynamics simulations and binding free energy calculations showed that TA interact with both MBLs and SBLs hydrophobic sites. Moreover, TA had a stronger binding affinity for MBLs than SBLs as the MBLs, specifically VIM-1 and NDM-1, interact with a larger number of their catalytic active-site residues than that of OXA-48 and KPC-2. These *in vitro* evaluations together with computational simulation explain the potentiating effect of TA toward carbapenems against carbapenem-resistance enterobacteriaceae. This study proposes TA as a promising adjuvant for MBLs and SBLs.

Keywords: Tannic acid; carbapenem; carbapenemase; *Enterobacteriaceae*; efflux pump, MBL inhibitors

1. Introduction

Antibiotic-resistant infections are increasing morbidities and mortalities worldwide (Powledge 2004). The exponential rise in the prevalence and global dissemination of carbapenem-resistant and carbapenemase-producing *Enterobacteriaceae* (CRE and CPE, respectively) in communities, hospitals, and the environment is of grave concern as carbapenems are considered “last-resort antibiotics” for difficult-to-treat multi-drug resistant infections (Sekyere et al. 2016; Osei Sekyere, Govinden, Bester, et al. 2016; Yang et al. 2016). The World Health Organization (WHO) recently listed CRE/CPE as “critical pathogens” which are currently the biggest threat to healthcare due to their multidrug resistance to all known classes of antibiotics. Therefore, there is the need to develop novel and efficient strategies to overcome the WHO global priority pathogen list (PPL) (World Health Organization 2017).

Several intervention strategies have thus far been proposed to overcome antibiotic resistance, including the inhibition of mutations, sequential treatment, and use of antisense oligomers to prevent expression of the *acrA* gene that forms part of the *acrAB* efflux pump, which mediates multidrug resistance (Ayhan et al. 2016). As novel antibiotics are bound to also become ineffective over time, it is imperative to sustain the efficacy of existing antibiotics. In this context, the use of adjuvants that inhibit efflux and hydrolysing enzymes of antibiotics are gaining widespread interest (Ayhan et al. 2016; Sekyere & Amoako 2017). For instance, Chusri et al. (Chusri et al. 2009) and Tintino et al. (Tintino et al. 2016) showed that tannic acid (TA) a type of polyphenolic biomolecule which acts as an efflux-pump inhibitor (EPI) and potentiates the effects of several non- β -lactam antibiotics in *Acinetobacter baumannii* and *Staphylococcus aureus*, respectively. Payne et al. also characterized series of tricyclic natural product-derived as potent large-spectrum MBL inhibitors (Payne et al. 2002). Whilst plant-based agents or extracts are perceived as safe, none are currently

approved for clinical use. This study herein characterized TA, a plant-derived polyphenol compound for its property to protect carbapenems from MBLs and SBLs hydrolysis.

Using a large international collection of clinical CPEs of diverse species that express known class A, B, and D carbapenemases (Nordmann et al. 2012; Osei Sekyere & Amoako 2017; Sekyere & Amoako 2017), we show herein for the first time, to our knowledge, that TA reduces class A and B carbapenemase-mediated carbapenem resistance. We show that this reduction in MICs is due to inhibition of carbapenemase activity in the CPE isolates, which was supported by enzymatic assay and computational simulation. This study also showed that TA does not act as an efflux pump inhibitor (EPI) in Enterobacteriaceae contrary to what was reported in *A. baumannii* and *S. aureus* (Chusri et al. 2009; Tintino et al. 2016).

2. Materials and Methods

2.1. Bacterial strains, efflux pump inhibitors (EPIs), and tannic acid (TA).

Seventy-two CRE isolates comprising 46 clinical isolates obtained from private hospitals in South Africa (Osei Sekyere, Govinden, & Essack 2016; Osei Sekyere & Amoako 2017; Sekyere & Amoako 2017) and 26 reference isolates of international origin obtained from Institut National de la Santé et de la Recherche Médicale (U914), Paris, France, (Nordmann et al. 2012) were used in this study. The species breakdown of the 72 *Enterobacteriaceae* isolates are shown in Tables 1-3; Tables S1-S2 and S4. *E. coli* ATCC 25922 was used as control in all experiments. Certified pure products of meropenem (MEM), imipenem (IPM), tannic acid (TA), carbonyl cyanide m-hydrophenylhydrazine (CCCP), reserpine (RSP), verapamil (VRP), thioridazine (TZ), and chlorpromazine (CPZ) as well as cation-adjusted Mueller-Hinton broth and agar were purchased from Sigma Aldrich (St. Louis, MO, USA). VRP, RSP, TZ, and CCCP's ability to inhibit efflux activity have been already described (Sekyere & Amoako 2017).

2.2. MICs of CCCP, EPIs and TA. Checkerboard assay of TA.

The MICs of RSP, VRP, TZ, CPZ, and CCCP were determined for all the 72 isolates and the control (Tables S1-S4) to determine their innate toxicity to the isolates using protocols already described by the CLSI (Clinical and Laboratory Standards Institute. 2017; Sekyere & Amoako 2017). These MICs were used as a guide in choosing the sub-MICs (i.e. $\frac{1}{2}$ MIC) of these compounds for further testing with MEM and IPM. A sub-MIC was chosen to limit the possibility of the inhibitors killing the cells and interfering with the actual value of the antibiotic-inhibitor combination.

The MIC of TA alone was also determined for all isolates and the control to determine its activity to the isolates according to previously described method and CLSI guideline (Clinical and Laboratory Standards Institute. 2017; Sekyere & Amoako 2017). The checkerboard method was used to evaluate the effect of different concentrations of TA on the carbapenems as described previously (Tascini et al. 2013; King, Sarah A Reid-Yu, et al. 2014). Concentrations of 64, 128, 256, and 512mg/L of TA were added to increasing concentrations of IPM and MEM to determine the MICs of IPM and MEM in the presence of TA (Tables 1-2). The antibacterial assays (MICs) were performed in triplicate.

2.3. Role of efflux pumps in carbapenem resistance and in TA's activity.

A sub-MIC of RSP, VRP, TZ, CPZ, and CCCP (Table S3-S4) was used in addition to increasing concentrations of MEM and IMP to evaluate the effects of the former on the MICs of MEM and IMP; a detailed description of this protocol is already described (Osei Sekyere & Amoako 2017; Sekyere & Amoako 2017). This was done to determine whether efflux pumps are involved in TA's reduction of IMP and MEM MICs.

2.4. IC₅₀ enzyme inhibition assay:

To confirm whether TA has the ability to inhibit the activity of NDM-1 and KPC-2, an *in vitro* enzyme inhibition assay was conducted using purified

enzymes that was purchased from Raybiotech (Norcross, GA 30092, USA) and the colorimetric β -lactamase substrate nitrocefin, as previously described (Siemann et al. 2002). Briefly, NDM-1 and KPC-2 enzymes (4nM and 5nM respectively) in HEPES with a pH adjusted to 7.3 ± 0.3 were mixed with 30 μ M nitrocefin after 10 to 15 minutes pre-incubation with TA at 30°C. The enzymes were supplemented with 100 μ M of $ZnCl_2$ and 0.1mg/L bovine serum albumin (BSA). The use of BSA was to minimize the denaturation of the enzymes during experiments. Assays were read in 96 well microtiter plate at 490 nm using a plate reader (SPECTROstar^{Nano} BMG Labtech, Germany) at 25°C. Enzyme assays were conducted in triplicate.

2.5. Molecular modelling

The crystal structures (PDB ID: 3QX6, 3RXW, 5ACU and 5FAS) were retrieved from the RCSB Protein Data Bank (<https://www.rcsb.org/pdb/>). The missing residues were added using a graphical user interface of Chimera, a molecular modelling tool (Pettersen et al. 2004). A ligand interaction map was generated using the web version of PoseView (Stierand & Rarey 2010). System preparation, molecular docking and molecular dynamic simulations were carried out to ascertain the interactions of the enzymes (NDM-1, VIM-2, KPC-2) with the ligand (TA). Details of the procedure used has been stated in the supplementary document.

2.6. Cytotoxicity Assay

HepG2 cells were maintained at 37°C in 10% complete culture medium (CCM; Eagles minimum essential medium supplemented with foetal bovine serum, antibiotics, and L-glutamine) until confluent. Following trypsinization, 15000 cells/well (300 μ l) were allowed to adhere to a 96-well plate overnight. The cells were treated with seven dilutions of TA (16 - 1024mg/L) prepared in CCM; untreated cells (CCM only) served as the control.

After 24 hours the treatment was replaced with 20 μ L MTT solution (5mg/mL MTT in PBS) and 100 μ L CCM for 4 hours. The resulting formazan product was solubilized in 100 μ L DMSO (1hour) and the absorbance at 570nm/690nm was determined (BioTek μ Quant plate reader; BioTek Instruments Inc., USA). The average absorbance values performed in triplicate were used to calculate cell viability.

2.7. Phylogenetic analysis

Fifteen carbapenemase genes were found in all the 72 isolates included in this study. Reference amino acid sequences of these 15 carbapenemase genes viz., NDM-1, NDM-4, NDM-5, IMP-1, IMP-8, VIM-1, VIM-19, KPC-2, GES-5, IMI-1, SME-1, SME-2, OXA-48, OXA-181, and OXA-232 were downloaded from GenBank and aligned with MAFFT online server (<http://mafft.cbrc.jp/alignment/server/>). The phylogenetic relationship between the carbapenemases was determined using the MAFFT phylogeny server (<http://mafft.cbrc.jp/alignment/server/phylogeny.html>). The phylogenetic tree was viewed online and downloaded with Newick internal labels (Fig. 2). This was carried out to offer possible insights into the potentiating differences of TA on the different carbapenemase enzymes.

2.8. Data and statistical analysis

The frequency of the carbapenemases per Enterobacteriaceae species was determined from Tables 1 and 2 by calculating the number of isolates expressing a particular enzyme (Table 3). The MIC fold change (Δ), defined as the ratio of the MIC of MEM or IMP alone to that of TA plus MEM or IMP, was calculated for each isolate and shown in a square bracket in Tables 1 and 2. A fold change (Δ) of ≥ 4 was adopted as significant and all Δ below 4 are coloured green in Tables 1 and 2. A mean fold change per antibiotic was calculated for every enzyme (Table 3) using the following equation:

109 $Sum (\Delta \text{ of all isolates expressing } x) / \text{Total number of isolates expressing } x$

110 Where x is any of the carbapenemases found in this study's isolates. The mean fold changes per antibiotic and carbapenemase was translated into
111 a bar graph using Microsoft Excel to show the relative effect of TA (512 mg/L) on each carbapenemase (Fig. 1).

112 GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used to determine the IC_{50} , the significance of the Δ and mean Δ per
113 antibiotics per isolate/species and per expressed carbapenemase in the presence of the efflux pump inhibitors and TA (Tables S5-S6). MIC fold
114 changes (Δ) with a P-value of <0.01 were considered statistically significant.

115 3. Results

116 3.1. TA significantly reduces imipenem (IPM) and meropenem (MEM) MICs in classes A and B carbapenemase-producing isolates.

117 The checkerboard method was used for this assay. Increasing concentrations of TA resulted in decreasing imipenem (IPM) and meropenem (MEM)
118 MICs for particularly NDM-, VIM-, IMP-, GES-5-, SME-1/-2-, IMI-1-, and KPC-2-producing isolates in a descending order of magnitude (Δ) (Tables 1-
119 2; Fig. 1). TA reversed resistance to IPM and MEM in all MBL-positive isolates (p-value $< 0.0001-0.01$) except in one *Serratia marcescens* bla_{IMP-1} -
120 positive strain. On the contrary, TA significantly (p-value $< 0.001-0.01$) reduced the MEM MICs of most isolates expressing IMI and SME
121 carbapenemases whilst it reversed resistance to only a few KPC-2- and GES-5-positive isolates. The effect of TA on OXA-48/-181/-232-producing
122 isolates was insignificant (p-value > 0.01) although it halved the MIC of one *Klebsiella pneumoniae* OXA-48-positive strain (Tables 1-2; Fig. 1). TA's
123 resistance-modulating effect was most potent against producers of MBLs such as NDM, VIM, and IMP than against producers of serine- β -

lactamases such as GES, KPC, SME, and IMI. The effect of TA on the tested isolates was greatest at a concentration of 512mg/L for all the carbapenemases and was thus used in calculating the mean Δ (Fig. 1); unless otherwise stated, the effects of TA on the CRE in this manuscript are discussed with the premise that a concentration of 512mg/L was used. TA on its own had no effect or activity on the isolates even at >512mg/L, whilst CCCP and the other EPIs exhibited inhibition to all the tested organisms (Tables 1-2, and Tables S1-S2).

3.2. Efflux pump inhibitors and CCCP fail to reduce MEM and IMP MICs significantly

In order to investigate whether TA reduces MICs of MEM and IMP through efflux pumps inhibition, EPIs and CCCP were employed to elucidate this hypothesis. As shown in Tables S1-S2, all the EPIs and CCCP, which is known to indirectly block efflux activity through reduction in both adenosine triphosphate (ATP) production and the proton motive force (PMF), (Sekyere & Amoako 2017) failed to significantly reduce or reverse resistance to MEM and IMP in all but one *Serratia marcescens* isolate, B (UNN38 _S2) (Table S1). This indicated that TA did not reduce the MICs of MEM and IMP through inhibition of efflux pumps activity as reported in methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Myint et al. 2013).

3.3. Enzymatic assay and molecular modelling and docking

Inhibition of efflux pumps not being involved in the protection of MEM and IMP, we therefore investigated the interaction between carbapenemase enzymes and TA to evaluate the potentiating effect of TA toward MEM and IMP. Half-maximum inhibitory concentration (IC_{50}) values of TA were determined using nitrocefin for NDM-1 (MBL) and KPC-2 (SBL) to determine the difference in inhibition between the two classes. TA respectively inhibited $\approx$ 50% of NDM-1 and KPC-2 at 15 μ M and 270 μ M indicating that TA possess an effect on both classes of carbapenemase enzymes.

Furthermore, computational molecular dynamic analyses were employed to ascertain that TA interacts with class A and class B carbapenemase enzymes as proof of concept. Thus, root-mean-square deviation (RMSD) analysis verified that all four tested systems (ligand-enzymes complexes: TA-NDM-1, TA-VIM-2, TA-KPC-2, TA-OXA-48) were quite stable over the molecular docking simulations and that, the interactions with the ligand site were strong enough to maintain the latter bonding (Fig. 2A, and Table S7). The radius of gyration (Rg) of the four systems were also steady, highlighting the moderate conformational changes occurring during the simulation (Fig. 2B). These results demonstrated that TA interact with each enzyme by reducing their hydrolysis activities at different levels, and also showed a stable compact structure. However, OXA-48 and KPC-2 (serine- β -lactamases) exhibited a significant overall high radius of gyration in comparison to the metallo- β -lactamases (VIM-2 and NDM-1) (Fig. 2B). The difference(s) in the interactions between the catalytic active site residue(s) and TA amplified the conformational flexibility of the complex and ultimately affected the receptor grip on the inhibitor, resulting in a higher radius of gyration of the SBLs. Further analysis of the binding free energy showed that the MBLs (VIM-2 and NDM-1) exhibited a higher binding affinity when compared to the SBLs. These findings corroborate the results from the Rg and the *in vitro* experiments. Analysis of ligand-enzymes interaction (Fig. 3) showed that the MBLs interact with a larger number of their active site residues as opposed to that of OXA-48 and KPC-2. TA also forms electrostatic interactions with zinc.

3.4. Cytotoxicity assay

The MTT assay was used to measure TA cytotoxicity (0 – 1024mg/L) in HepG2 cells after 24 hours exposure. An increase in cell viability was associated with TA concentrations; 16mg/L (118.98 $\pm$ 6.95%) and 32mg/L (108.52 $\pm$ 7.28%) (Fig S2). However, for the 64mg/L, 128 mg/L and 256 mg/L the cell viability decreased to 89.26 $\pm$ 8.37%, 63.08 $\pm$ 4.82% and 63.40 $\pm$ 4.73% respectively. The metabolic activity of TA was increased again at 512mg/L (93.50 $\pm$ 4.86%) and 1024mg/L (93.86 $\pm$ 4.43%) (Fig S2). TA was having tolerable cytotoxic effect for all the concentrations tested.

3.5. Phylogenetic tree of the carbapenemases

A phylogenetic tree of all the carbapenemases (n=15) was drawn to ascertain offer plausible insights into the potentiating effects on the various carbapenemases. The tree (Fig. 4) shows that all the members of the same carbapenemase class are clustered closer together (or share closer sequence identity) and separated from members of other classes that were found on different branches. MBLs (NDM, VIM and IMP) were clustered together and farther from the other subclasses. GES-5 was more distant from KPC-2, IMI-1 and SME whilst IMI-1 and SME were very closely related and distant from KPC-2. All the three OXA-type carbapenemases had a closer sequence similarity as shown by their close clustering on the same branch (Fig. 1 and 4).

3.6. Species, carbapenemases and mean fold change

The study comprised of Enterobacteriaceae isolates from six genera and 12 species expressing eight carbapenemases, namely NDM (n=38), IMP (n=6), VIM (n=3), GES-5 (n=9), KPC-2 (n=4), SME (n=2), IMI-1 (n=1), and OXA-48-types (n=5) (Tables 1-2). Three clinical isolates from South Africa i.e. 1-S1, 65_S32, and 45_S21 were non-CPEs and had tested negative both for the Carba-NP test (Osei Sekyere et al. 2015; Osei Sekyere, Govinden, & Essack 2016) and efflux-mediated carbapenem resistance (as shown in the MIC fold change of 1), but the latter two isolates had very high IPM and MEM MICs, which were drastically reduced by TA (Table 1). Of note, isolate 3_S2 was the only one that expressed OXA-232 with an IPM and MEM MIC of 128mg/L; however, it was Carba-NP negative (Osei Sekyere, Govinden, & Essack 2016).

The mean Δ of IPM and MEM for each carbapenemase was calculated from the individual Δ of all the isolates to serve as an index of the relative effect of TA on each carbapenemase (Fig. 1). Thus, carbapenemases with higher mean Δ were most affected by TA. Disparities were also observed

between the mean Δ of IPM and MEM for all the carbapenemases. The highest mean Δ for IPM and MEM were observed in isolates expressing NDM, VIM and IMP followed by those expressing GES-5, SME, KPC-2, and IMI-1. OXA-48-type-expressing isolates had the least mean Δ for the two antibiotics (Fig. 1 and 4).

4. Discussion

Carbapenems have become the last-resort antibiotic for treating fatal and multidrug-resistant bacterial infections due to increased fluoroquinolone, aminoglycoside and cephalosporin resistance (Osei Sekyere, Govinden, & Essack 2016; Osei Sekyere 2016; Osei Sekyere & Amoako 2017). The increased use of carbapenems to battle these resistant infections have thus led to the selection and dissemination of CPE, which are resistant to almost all known β -lactam antibiotics (Nordmann & Poirel 2014; Sekyere et al. 2016). In this study, we report on a plant-derived agent, TA, which protect carbapenem antibiotics from enzymatic hydrolysis by inhibiting the activity of mostly class B MBLs and class A SBLs, thus, reducing carbapenem MICs and resistance.

The inability of CCCP and the other EPIs to reverse IPM and MEM resistance suggests that TA does not inhibit efflux pumps to reverse IPM and MEM MICs. Moreover, TA alone had no biocidal effect on the isolates at concentrations >512 mg/L, as seen from the MIC of TA (Tables 1-2), ruling out the possibility that TA's mechanism of action is through protein inhibition. As well, TA does not interact negatively with the carbapenems, as such an interaction would not have produced the results seen. Rather, a combination of TA with IPM and MEM resulted in a reduction or modulation of carbapenem resistance in most isolates, thus revealing the potentiating property of TA toward both tested carbapenem antibiotics.

187 Notably, the effect of TA was mainly dependent on the type of carbapenemase enzyme expressed by the isolates rather than by the species or strain
188 type. For instance, irrespective of the species or strain, TA modulated carbapenem resistance in all MBL-producing isolates as well as in most
189 isolates expressing certain types of class A SBLs. However, resistance in the same species expressing the same class D carbapenemase types
190 were barely modulated. Hence, the resistance-modulating effect of TA was mainly based on the type of carbapenemase rather than on the species
191 or strain, indicating that TA interacts with the carbapenemase enzymes to modulate carbapenem resistance, similarly to previous studies that
192 demonstrated inhibition of carbapenemase enzymes to improve carbapenem antibiotics such as aspergillomarasmine A, 1,4,7-triazacyclononane-
193 1,4,7-triacetic acid (NOTA), di-(2-picolyl)amine (DPA) but with broader spectrum of activity (King, Sarah A. Reid-Yu, et al. 2014; Somboro et al.
194 2014; Azumah et al. 2016). To confirm this hypothesis, *in vitro* enzyme inhibition assays and computational molecular analysis were conducted. The
195 enzyme assay showed a wide difference in TA inhibition capacity per carbapenemase. The IC₅₀ values further affirmed that TA inhibits MBLs more
196 prominently than SBLs. Computational studies predicted that TA inhibit both MBLs and SBLs by targeting their hydrophobic sites. Analysis of ligand-
197 enzymes interaction indicated that the MBLs interact with a larger number of their active site residues as opposed to that of OXA-48 and KPC-2
198 (SBL). TA also forms hydrogen bonds with zinc, which is important for MBL activity. This binding decreases the enzyme activity harming or killing the
199 bacteria. Further analysis of the binding free energy showed that the MBLs (VIM-2 and NDM-1) exhibited a stronger and stable binding affinity when
200 compared to the SBLs, which is caused by the differences in the interactions between the catalytic active site residue(s) and TA, amplifying the
201 conformational flexibility of the complex and ultimately affecting the receptor grip on the inhibitor (Table 4). This results in a higher radius of gyration
202 of the SBLs than MBLs. These findings corroborate the results from the *in vitro* experiments and confirmed that TA interacts with the enzymes to

potentiate IMP and MEM efficacy. The per-residue interaction energy decomposition calculations applied in this study will potentially assist medicinal chemists in the design of inhibitors that can interact with these carbapenemase residues in future studies.

Among carbapenemases, class B is known to have little amino acid homology and longer evolutionary distance to classes A and D as seen on the phylogenetic tree, which shows that class A and D have a closer evolutionary distance (Fig. 2). In addition, NDM is most closely related to VIM than to IMP with a sequence identity of 32.4% (Saini 2012). Hence, the closer amino acid sequence homology of VIM to NDM rather than its closer evolutionary distance to IMP might in part explain the observation in which VIM-positive isolates had greater mean MIC fold changes than IMP-positive ones (Fig. 2)

Isolates 65_S32 and 45_S21 both tested negative for the Carba-NP test and no known carbapenemase was found in their genome although they both had very high IPM and MEM MICs (45_S21=32mg/L; 65_S32=256 and 512mg/L respectively) (Osei Sekyere, Govinden, & Essack 2016; Osei Sekyere & Amoako 2017; Sekyere & Amoako 2017). Furthermore, both isolates had no MIC change when tested with carbapenems-EPIs, indicating that their efflux pumps were not possibly responsible for the high MICs recorded. However, TA could reduce/modulate resistance to carbapenems in these isolates. As the OXA-232-producing isolate, 3_S2, was also Carba-NP test negative and could not be affected by TA, but was highly resistant to IPM and MEM, we suspect that an unknown or undiscovered MBL or class A SBL might be mediating the high carbapenem MIC recorded in these two strains (Table 1).

Available evidence from published studies show that tannins, which includes TA, catechin, ethylgallate, epicatechin gallate, epigallocatechin gallate, methyl gallate, ellagic acid, rosmarinic acid and 1,2,3,4,6-Penta-O-galloyl- β -D-glucopyranose are polyphenolics that are abundant in plants (Leal et al.

2011; Slobodníková et al. 2016) (Fig. S1). Tannin extracts and fractions have been reported to either potentiate or modify the effects of β -lactams, fluoroquinolones, and several other antibiotic classes (Leal et al. 2011; Myint et al. 2013; Slobodníková et al. 2016). Moreover, they have been shown to inhibit biofilm formation, quench quorum sensing, and confer antimicrobial and antioxidant activity against bacteria by binding to bacterial proteins, cell wall and cell membrane structures, resulting in cell lysis and efflux pumps inhibition (Leal et al. 2011; Myint et al. 2013; Slobodníková et al. 2016). However, the reported antibacterial and efflux inhibition effects of tannins were not seen in this study, intimating that other tannins besides TA may be responsible for those effects.

In an earlier study, Leal et al. (Leal et al. 2011) found that the tannin fractions of *Pentaclethra macroloba* was bactericidal to bacteria in a dose-dependent manner by inhibiting protein synthesis, although protein binding is the universally accepted mechanism of tannins' biological and astringent activity (Slobodníková et al. 2016). Notably, the tannin fraction was found to be non-toxic to eukaryotic cells at the effective bactericidal concentration (Leal et al. 2011). As TA alone was not active against the Enterobacteriaceae isolates used in this study it is obvious that it did not inhibit protein synthesis supporting our hypothesis a potential carbapenemase inhibitor.

5. Conclusion

TA did not reverse carbapenem resistance by inhibiting all proteins/enzymes and/or efflux pumps in the cells, but through an inhibition of the carbapenemases by a possible binding/sequestration interaction with their catalytic-active sites, thus freeing the carbapenems to exert their antibiotic effect. Further investigation of TA properties might extend its inhibitory spectrum to cover all carbapenemases and reduce the efficacy of carbapenems in carbapenem-resistant Enterobacteriaceae.

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301 **Table 1: Minimum inhibitory concentrations (MIC) of meropenem (MEM), imipenem (IPM) and Tannic acid (TA) as well as of MEM and IPM**
 302 **in the presence of TA for the clinical South African isolates.**

Isolates	MIC(mg/L) [fold change ¹]					Carbapenemase	
	Antibiotics alone	+ Tannic acid			Tannic acid alone		
		64 mg/L	128 mg/L	256 mg/L			512 mg/L
<i>Klebsiella pneumoniae</i>							
Class B							
C(UNN_S3)	MEM-256	32 [8]	16 [16]	8 [32]	4 [64]	>512	NDM-1
	IPM-128	16 [8]	16 [8]	8 [16]	4 [32]		
D(UNN_S4)	MEM-512	32 [16]	8 [64]	4 [128]	2 [256]	>512	NDM-1
	IPM-128	16 [8]	16 [8]	4 [32]	1 [128]		
I(UNN_S9)	MEM-512	64 [8]	16 [32]	4 [128]	2 [256]	>512	NDM-1
	IPM-256	64 [4]	32 [8]	16 [16]	4 [64]		
J(UNN_S10)	MEM-128	32 [4]	8 [16]	4 [32]	1 [128]	>512	NDM-1
	IPM-256	32 [32]	8 [32]	8 [32]	2 [128]		
12_S5	MEM-64	16 [4]	8 [8]	4 [16]	0.5 [32]	>512	NDM-1
	IPM-128	32 [4]	16 [8]	8 [16]	2 [64]		
13_S6	MEM-128	32 [4]	16 [8]	4 [32]	2 [64]	>512	NDM-1
	IPM-64	16 [4]	16 [4]	4 [16]	1 [64]		
20_S11	MEM-256	32 [8]	16 [16]	4 [64]	4 [64]	>512	NDM-1
	IPM-128	32 [4]	8 [16]	2 [64]	1 [128]		
21_S12	MEM-512	64 [8]	16 [32]	4 [128]	2 [256]	>512	NDM-1
	IPM-128	16 [8]	8 [16]	2 [64]	1 [128]		
29_S13	MEM-512	32 [16]	8 [64]	2 [256]	1 [512]	>512	NDM-1
	IPM-128	16 [8]	8 [16]	8 [16]	4 [32]		

¹ A fold change of ≥ 4 was defined as significant for TA inhibition. Fold changes below 4 are coloured green.

32_S15	MEM-128	16 [8]	4 [32]	2 [64]	0.5 [256]	>512	NDM-1
	IPM-32	8 [4]	4 [8]	1 [32]	0.25 [128]		
53_S27	MEM-256	64 [4]	32 [8]	8 [32]	4 [64]	>512	NDM-1
	IPM-128	16 [8]	8 [16]	4 [32]	1 [128]		
Class A							
15_S8	MEM-16	16 [1]	16 [1]	16 [1]	16 [1]	>512	GES-5
	IPM-128	128 [1]	128 [1]	128 [1]	128 [1]		
18_S10	MEM-512	128 [4]	64 [8]	64 [8]	32 [16]	>512	GES-5
	IPM-512	128 [4]	64 [8]	32 [8]	32 [16]		
30_S14	MEM-256	64 [4]	32 [8]	32 [8]	32 [8]	>512	GES-5
	IPM-128	64 [2]	32 [8]	32 [8]	32 [8]		
34_S15	MEM-512	128 [4]	64 [8]	32 [16]	32 [16]	>512	GES-5
	IPM-512	64 [8]	64 [8]	64 [8]	32 [16]		
35_S17	MEM-512	128 [4]	64 [8]	64 [8]	64 [8]	>512	GES-5
	IPM-512	64 [8]	32 [16]	32 [16]	32 [16]		
36_S18	MEM-128	128 [1]	128 [1]	128 [1]	128 [1]	>512	GES-5
	IPM-128	128 [1]	128 [1]	128 [1]	128 [1]		
38_S19	MEM-16	16 [1]	16 [1]	16 [1]	16 [1]	>512	GES-5
	IPM-32	32 [1]	32 [1]	32 [1]	32 [1]		
52_S26	MEM-512	128 [4]	128 [4]	64 [8]	32 [16]	>512	GES-5
	IPM-256	64 [4]	32 [8]	32 [8]	16 [16]		
Class D							
3_S2	MEM-128	128 [1]	128 [1]	128 [1]	128 [1]	>512	OXA-232
	IPM-128	128 [1]	128 [1]	128 [1]	128 [1]		
Serratia marcescens							
Class B							
B (UNN38 _S2)	MEM->512	64 [>8]	64 [>8]	32 [>16]	8 [>64]	>512	NDM-1
	IPM-512	64 [8]	32 [16]	16 [32]	8 [64]		
E (UNN41 _S5)	MEM-128	16 [8]	8 [16]	4 [32]	4 [32]	>512	NDM-1
	IPM-128	32 [4]	16 [8]	8 [32]	4 [64]		
G (UNN43 _S7)	MEM-16	4 [4]	4 [4]	4 [4]	2 [8]	>512	NDM-1

	IPM-512	64 [8]	16 [32]	8 [64]	8 [64]		
K (UNN47 _S11)	MEM-128	64 [2]	16 [8]	8 [16]	4 [32]	>512	NDM-1
	IPM-256	32 [8]	32 [8]	16 [16]	8 [32]		
L (UNN38 _S12)	MEM-128	16 [8]	16 [8]	16 [8]	8 [8]	>512	NDM-1
	IPM-128	16 [8]	16 [8]	8 [16]	8 [16]		
7_S3	MEM-64	16 [4]	8 [8]	4 [16]	4 [16]	>512	NDM-1
	IPM-32	16 [2]	8 [4]	8 [4]	8 [4]		
56_S29	MEM-512	64 [8]	32 [16]	16 [32]	8 [64]	>512	NDM-1
	IPM-256	32 [8]	32 [8]	8 [32]	8 [32]		
59_S30	MEM-512	64 [8]	32 [16]	16 [32]	8 [64]	>512	NDM-1
	IPM-256	32 [8]	32 [8]	8 [32]	4 [64]		
67_S33	MEM-256	32 [8]	32 [8]	8 [8]	8 [8]	>512	NDM-1
	IPM-512	64 [8]	32 [16]	16 [32]	8 [64]		
68_S34	MEM-64	16 [4]	8 [8]	4 [16]	4 [16]	>512	NDM-1
	IPM-32	8 [4]	8 [4]	4 [8]	2 [16]		
71_S36	MEM->512	128 [>4]	64 [>8]	32 [>16]	8 [>64]	>512	NDM-1
	IPM-128	32 [4]	16 [8]	8 [16]	4 [32]		
No known carbapenemase gene							
45_S21	MEM-32	16 [2]	8 [4]	8 [4]	4 [8]	>512	NK ²
	IPM-32	16 [2]	8 [4]	4 [8]	4 [8]		
Enterobacter cloacae (unless otherwise stated in the footnote)							
Class B							
A (UNN37 _S1) ³	MEM-64	16 [4]	8 [8]	4 [16]	1 [64]	>512	NDM-1
	IPM-32	8 [4]	4 [8]	2 [16]	2 [16]		
F (UNN42 _S6) ⁴	MEM-512	64 [8]	32 [16]	8 [64]	4 [128]	>512	NDM-1
	IPM-128	32 [4]	8 [16]	4 [64]	2 [64]		

² No known carbapenemase present

³ *Enterobacter asburiae*

⁴ *Enterobacter* sp.

H (UNN44 _S8) ⁵	MEM->512	128 [>4]	64 [>8]	8 [>64]	4 [>128]	>512	NDM-1
	IPM-128	32 [4]	8 [16]	4 [32]	2 [64]		
16_S9 ⁶	MEM-256	64 [4]	16 [16]	8 [32]	4 [64]	>512	NDM-1
	IPM-128	32 [4]	16 [8]	8 [16]	1 [128]		
43_S20 ⁷	MEM->512	64 [>8]	32 [>16]	16 [>32]	4 [>128]	>512	NDM-1
	IPM-64	8 [8]	8 [8]	2 [32]	1 [64]		
49_S24 ³	MEM-512	64 [8]	16 [32]	8 [64]	4 [128]	>512	NDM-1
	IPM-128	32 [4]	8 [16]	8 [16]	4 [32]		
51_S25	MEM->512	128 [>4]	32 [>16]	8 [>64]	4 [>128]	>512	NDM-1
	IPM-128	32 [4]	8 [16]	4 [32]	2 [64]		
55_S28 ⁶	MEM-512	32 [16]	16 [32]	8 [64]	4 [128]	>512	NDM-1
	IPM-256	32 [8]	8 [32]	4 [64]	4 [64]		
63_S31 ³	MEM-512	64 [8]	16 [32]	8 [64]	4 [128]	>512	NDM-1
	IPM-64	16 [4]	8 [8]	4 [16]	1 [64]		
No known carbapenemase gene							
1_S1	MEM-2	2 [1]	2 [1]	2 [1]	2 [1]	>512	-
	IPM-0.5	0.5 [1]	0.5 [1]	0.5 [1]	0.5 [1]		
65_S32	MEM-512	64 [8]	8 [64]	4 [128]	2 [256]	>512	-
	IPM-256	32 [8]	8 [32]	8 [32]	4 [64]		
Escherichia coli							
Class B							
10_S4	MEM->512	32 [>16]	32 [>16]	8 [>64]	4 [>128]	>512	NDM-5
	IPM-512	32 [16]	16 [32]	8 [64]	4 [128]		
Citrobacter freundii							
Class B							
48_S23	MEM-512	32 [16]	32 [16]	8 [64]	4 [128]	>512	NDM-1

⁵ Enterobacter cloacae complex “Hoffman cluster III”

⁶ Enterobacter kobei

⁷ Enterobacter cloacae complex “Hoffman cluster IIV”

	IPM-128	16 [8]	8 [16]	4 [32]	2 [64]		
<i>Klebsiella michiganensis</i>							
Class B							
69_S35	MEM-512	64 [8]	16 [32]	8 [64]	2 [256]	>512	NDM-1
	IPM-128	16 [8]	8 [16]	4 [32]	2 [64]		

Table 2: Minimum inhibitory concentrations (MIC) of meropenem (MEM), imipenem (IPM) and Tannic acid (TA) as well as of MEM and IMP in the presence of TA for the reference isolates

Isolates	MIC (mg/L) [fold change] ⁸					
	Antibiotics alone	+ Tannic acid				Tannic acid alone
		64 mg/L	128 mg/L	256 mg/L	512 mg/L	
Control						
<i>E. coli</i> ATCC 25922	MEM- 0.0075	0.0075 [1]	0.0075 [1]	0.0075 [1]	0.0075 [1]	>512
	IMP- 0.06	0.06 [1]	0.06 [1]	0.06 [1]	0.06 [1]	
CLASS A						
KPC-2						
<i>E. coli</i> KPC-2	MEM- 16	8 [2]	8 [2]	8 [2]	4 [4]	>512
	IMP- 64	8 [8]	8 [8]	8 [8]	8 [8]	
<i>E. cloacae</i> KPC-2	MEM- 32	16 [2]	16 [2]	16 [2]	16 [2]	>512
	IMP- 128	64 [2]	64 [2]	64 [2]	32 [4]	
<i>C. freundii</i> KPC-2	MEM- 16	4 [4]	4 [4]	4 [4]	4 [4]	>512
	IMP- 64	16 [4]	16 [4]	16 [4]	16 [4]	
<i>S. marcescens</i> KPC-2	MEM- >512	512 [≥1]	512 [≥1]	512 [≥1]	512 [≥1]	>512
	IMP- 512	512 [≥1]	512 [1]	256 [2]	256 [2]	

⁸ A fold change of ≥4 was defined as significant for TA inhibition. Fold changes below 4 are coloured green.

GES-5						
E. cloacae GES-5	MEM- 64	64 [1]	64 [1]	64 [1]	64 [1]	>512
	IMP- 128	128 [1]	128 [1]	128 [1]	128 [1]	
IMI-1						
E. asburiae IMI-1	MEM- 256	128 [2]	32 [8]	32 [8]	32 [8]	>512
	IMP- >512	512 [≥1]	512 [≥1]	256 [≥2]	256 [≥2]	
SME-1						
S. marcescens SME-1	MEM- 512	256 [2]	32 [16]	32 [16]	32 [16]	>512
	IMP- >512	512 [≥1]	256 [≥2]	256 [≥2]	256 [≥2]	
SME-2						
S. marcescens SME-2	MEM- 512	256 [2]	32 [16]	32 [16]	16 [32]	>512
	IMP->512	512 [≥1]	256 [≥2]	256 [≥2]	128 [>4]	
CLASS D						
OXA-48/181						
E. coli OXA-48	MEM- 1	1 [1]	1 [1]	1 [1]	1 [1]	>512
	IMP- 2	2 [1]	2 [1]	2 [1]	2 [1]	
K. pneumoniae OXA-48	MEM- 8	4 [2]	4 [2]	4 [2]	4 [2]	>512
	IMP- 8	4 [2]	4 [2]	4 [2]	4 [2]	
K. pneumoniae OXA-181	MEM- 1	1 [1]	1 [1]	1 [1]	1 [1]	>512
	IMP- 4	4 [1]	4 [1]	4 [1]	4 [1]	
P. rettgeri OXA-181	MEM- 0.5	0.5 [1]	0.5 [1]	0.5 [1]	0.5 [1]	>512
	IMP- 1	1 [1]	1 [1]	1 [1]	1 [1]	
CLASS B						
NDM-1/NDM-4						
E. coli NDM-1	MEM- 64	16 [4]	8 [8]	4 [16]	1 [64]	>512
	IMP- 128	32 [4]	8 [16]	1 [128]	0.5 [512]	
E. cloacae NDM-1	MEM- 16	4 [4]	4 [4]	1 [16]	0.5 [32]	>512
	IMP- 32	8 [4]	4 [8]	0.5 [64]	0.25 [128]	
K. pneumoniae NDM-1	MEM- 512	64 [8]	16 [32]	4 [128]	2 [256]	>512
	IMP- 256	64 [4]	32 [8]	16 [16]	4 [64]	
C. freundii NDM-1	MEM- 16	4 [4]	2 [8]	0.5 [32]	0.5 [32]	>512

	IMP- 8	2 [4]	2 [4]	0.5 [16]	0.25 [32]	
E. coli NDM-4	MEM- 256	32 [8]	8 [32]	4 [64]	2 [128]	>512
	IMP- 128	32 [4]	16 [8]	8 [16]	2 [64]	
VIM-1/19						
E. coli VIM-1	MEM- 64	4 [16]	2 [32]	0.5 [128]	0.5 [128]	>512
	IMP- 128	4 [32]	2 [64]	1 [128]	0.5 [512]	
E. cloacae VIM-1	MEM- 8	2 [4]	2 [4]	0.5 [16]	0.5 [16]	>512
	IMP- 16	4 [4]	2 [8]	1 [16]	0.5 [32]	
K. pneumoniae VIM-19	MEM- 64	16 [4]	8 [8]	2 [32]	1 [64]	>512
	IMP- 512	64 [8]	32 [16]	4 [128]	2 [256]	
IMP-1/IMP-8						
E. cloacae IMP-1	MEM- 64	16 [4]	8 [8]	4 [16]	1 [64]	>512
	IMP- 128	32 [4]	16 [8]	8 [16]	4 [32]	
E. coli IMP-1	MEM- 16	4 [4]	2 [8]	2 [8]	1 [16]	>512
	IMP- 32	8 [4]	4 [8]	4 [8]	2 [16]	
S. marcescens IMP-1	MEM- 128	64 [2]	64 [2]	64 [2]	32 [2]	>512
	IMP- 128	64 [2]	64 [2]	32 [4]	16 [8]	
E. coli IMP-8	MEM- 16	8 [2]	4 [4]	4 [4]	2 [8]	>512
	IMP- 32	16 [2]	16 [2]	16 [2]	8 [2]	
E. cloacae IMP-8	MEM- 8	4 [2]	2 [4]	2 [4]	1 [8]	>512
	IMP- 16	8 [2]	8 [2]	8 [2]	4 [4]	
K. pneumoniae IMP-8	MEM- 16	8 [2]	8 [2]	4 [4]	1 [16]	>512
	IMP- 64	16 [4]	16 [4]	8 [8]	8 [8]	

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Table 3. Frequency of carbapenemases found in each Enterobacteriaceae species used in the study

Isolates (n=72)		NDM (n=38)			VIM (n=3)		IMP (n=6)		KPC-2	GES-5	IMI-1	SME (n=2)		OXA (n=4)		
		-1	-4	-5	-1	-19	-1	-8				-1	-2	-48	-181	-232
		(n=36)	(n=1)	(n=1)	(n=2)	(n=1)	(n=3)	(n=3)	(n=4)	(n=9)	(n=1)	(n=1)	(n=1)	(n=2)	(n=1)	(n=1)
<i>K. pneumoniae</i> (n=25)	Clin ⁹	11	- ¹⁰	-	-	1	-	1	-	8	-	-	-	-	-	1
	Ref ¹¹	1	-	-	-	-	-	-	-	-	-	-	-	1	1	-
<i>S. marcescens</i> (n=16)	Clin	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ref	-	-	-	-	-	1	-	1	-	-	1	1	-	-	-
<i>Enterobacter</i> spp. (n=18)	Clin	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ref	1	-	-	1	-	1	1	1	1	1	-	-	-	-	-
<i>E. coli</i> (n=8)	Clin	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
	Ref	1	1	-	1	-	1	1	1	-	-	-	-	1	-	-
<i>C. freundii</i> (n=3)	Clin	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-

⁹ Clinical South African strains

¹⁰ No known carbapenemase present

¹¹ Reference strains

	Ref	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-
<i>K. michiganensis</i> (n=1)	Clin	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ref	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. rettgeri</i> (n=1)	Clin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ref	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-

Table 4: MM/GBSA binding free energy profile of all four systems . All energy terms are presented in kcal/mol.

Systems*	Evdw	Eelec	ΔG_{solv}	ΔG_{gas}	ΔG_{bind}
MBLs					
NDM-1	-3.889±0.1660	-34.6186±0.3174	-38.5080±0.2285	-5.7249±0.1561	-44.2329±0.3806
VIM-2	-3.90744±0.1014	-31.4537±0.1706	-35.3612±0.1070	-8.3608±0.1485	-43.7220±0.4513
SBLs					
KPC-2	-1.9446 ±0.1870	-17.3093±0.2722	-19.2540±0.1590	-2.8624±0.0752	-22.1164±0.0111
OXA-48	-1.0841±0.1321	-17.9093±0.1887	-18.9935±0.1818	-3.1591±0.1712	-22.5275±0.1300
ΔE_{vdw} , van der Waals energy; ΔE_{elec} , electrostatic energy; ΔG_{solv} , polar solvation energy; ΔG_{gas} , nonpolar solvation energy; ΔG_{bind} , total binding free energy; *Enzyme-ligand complex.					

1 Table legends

2 Table 3: Minimum inhibitory concentrations (MIC) of meropenem (MEM), imipenem (IPM) and Tannic acid
3 (TA) as well as of MEM and IPM in the presence of TA for the clinical South African isolates.

4 Table 2: Minimum inhibitory concentrations (MIC) of meropenem (MEM), imipenem (IPM) and Tannic acid
5 (TA) as well as of MEM and IMP in the presence of TA for the reference isolates.

6 Table 3. Frequency of carbapenemases found in each Enterobacteriaceae species used in the study.

7 Table 4. MM/GBSA binding free energy profile of all four systems. All energy terms are presented in
8 kcal/mol.

9

10 Figure legends

11 **Fig. 1: MEM and IPM MIC fold change of isolates per carbapenemase upon addition of tannic acid**
12 **(TA).** The effect of tannic acid (512mg/L) in reducing and/or reversing meropenem (MEM) and imipenem
13 (IPM) resistance among Enterobacteriaceae is shown above as a mean fold change(Δ). The mean Δ is
14 measure of the relative effect of TA on the MIC of each isolate. TA effect was most potent against bacteria
15 producing metallo- β -lactamases such as NDM (MEM=115, IPM=110), VIM (MEM=69, IPM=266) and IMP
16 (MEM=19, IPM=12) than against those producing serine- β -lactamases such as GES (MEM=6, IPM=8),
17 KPC (MEM=3, IPM=5), SME (MEM=24, IPM=3) and IMI (MEM=8, IPM=2); these effects were statistically
18 significant (p-value < 0.0001-0.01). Effect on producers of OXA-type carbapenemases (MEM=1, IPM=1)
19 was very minimal or null and statistically insignificant (p-value > 0.01). IMP and MRP was thus affected
20 differently by TA per enzyme.

Fig. 2: The Root-mean-square deviation (RMSD) and Radius of gyration (Rg) diagrams of the four complexes: A) RMSD showing the stability of the systems (enzyme-ligand complex); B) Rg illustrating the compactness of the systems.

Fig. 3: A ligplot showing the relative position of Tannic acid inside the active site of: (A) VIM-2, (B) NDM-1 (C) OXA-48, D) KPC-2. TA interact more with MBLs when compared to SBLs at their catalytic active sites.

Fig. 4: Phylogenetic tree of the carbapenemases. The carbapenemases found in the isolates used in this study are clustered into their families of classes A, B, and D, showing the close relationship and amino acid sequence identity between members of the same family. The mean MIC fold change per enzyme and antibiotic (MEM and IMP) are shown besides the enzyme names. The effect of TA differed from carbapenemase to carbapenemase and from carbapenemase class to class, with class B being the most inhibited.

Fig. 1: MEM and IMP MIC fold change of isolates per carbapenemase upon addition of tannic acid (TA). The effect of tannic acid (512mg/L) in reducing and/or reversing meropenem (MEM) and imipenem (IMP) resistance among Enterobacteriaceae is shown above as a mean fold change (Δ). The mean Δ is measure of the relative effect of TA on the MIC of each isolate. TA effect was most potent against bacteria producing metallo- β -lactamases such as NDM (MEM=115, IMP=110), VIM (MEM=69, IMP=266) and IMP (MEM=19, IMP=12) than against those producing serine- β -lactamases such as GES (MEM=6, IMP=8), KPC (MEM=3, IMP=5), SME (MEM=24, IMP=3) and IMI (MEM=8, IMP=2); these effects were statistically significant (p-value < 0.0001-0.01). Effect on producers of OXA-type carbapenemases (MEM=1, IMP=1) was very minimal or null and statistically insignificant (p-value > 0.01). IMP and MRP was thus affected differently by TA per enzyme.

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