# In Vitro Potentiation of Carbapenems with Tannic Acid Against Carbapenemase Producing Enterobacteriaceae: Exploring Natural Products as Potential Carbapenemase Inhibitors

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Running Title: Tannic acid, a potential carbapenemase inhibitor.

**Abstract**

**Aims:** We hypothesised and confirmed that tannic acid (TA) reverses carbapenem resistance by inhibiting carbapenemases in class A and B carbapenemase-producing Enterobacteriaceae.

**Methods and Results:** MICs of carbapenems in the presence and absence of TA and other efflux pump inhibitors, TA-carbapenemases inhibition assays and computational studies showed that TA had the greatest effect on metallo-β-lactamases (MBLs) followed by class A serine-β-lactamases (SBLs). TA completely reversed the MICs of MBL producers from between 32— ≥512mg/L to susceptible values (< 4mg/L) whilst substantially reducing the MICs of SBLs from between 16— > 512mg/L to <4—16mg/L. Tolerable cytotoxic effect was observed for the concentrations tested (8 – 1024 mg/L). TA inhibited enzymes with a marked difference of ≈50% inhibition ($IC_{50}$) for NDM-1 (270 μM) and KPC-2 (15 μM).

**Conclusion:** TA inhibited both MBLs and SBLs by targeting their hydrophobic sites. Moreover, TA had a stronger binding affinity for MBLs than SBLs as the MBLs, specifically VIM-1 (-43.7220±0.4513 kcal/mol) and NDM-1(-44.2329±0.3806 kcal/mol), interact with a larger number of their catalytic active-site residues than that of OXA-48 (-22.5275±0.1300 kcal/mol) and KPC-2 (-22.1164±0.0111 kcal/mol).

**Significance and impact of study:** Tannic acid or its analogues could be developed into carbapenemase-inhibiting adjuvants to restore carbapenem activity in CRE infections, save many lives and reduce healthcare associated costs.

**Keywords:** Tannic acid; carbapenem; carbapenemase; *Enterobacteriaceae*; efflux pump; efflux pump inhibitors; enzymatic inhibition assay; MBL inhibitors.

## 1. Introduction

Antibiotic-resistant infections increase morbidities and mortalities worldwide (Powledge, 2004; Osei Sekyere and Asante, 2018). Bacterial $\beta$-lactamases are members of an enzyme family capable of destroying the efficacy of $\beta$-lactam antibiotics by hydrolyzing their $\beta$-lactam rings. There are two globally accepted classification schemes for $\beta$-lactamases. The first scheme is based on the amino acid sequence of the enzyme and comprises of four classes i.e., classes A, B, C, and D. The second scheme is based on the enzyme's functionality or substrate and includes three main groups: group 1, cephalosporinases (class C); group 2, serine $\beta$-lactamases (SBLs) (classes A and D); and group 3, metallo-lactamases (MBLs) (class B) (Somboro *et al.*, 2018).

The exponential rise in both prevalence and dissemination of carbapenem-resistant and carbapenemase-producing *Enterobacteriaceae* (CRE and CPE, respectively) globally in communities, hospitals, and the environment are of grave concern as carbapenems are considered last-resort antibiotics for difficult-to-treat multi-drug resistant infections (Osei Sekyere *et al.*, 2015; 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 antibiotic-resistance enzymes are gaining widespread interest (Ayhan *et al.*, 2016; Osei Sekyere and Amoako, 2017). Chusri *et al.* (Chusri *et al.*, 2009) and Tintino *et al.* (Tintino *et al.*, 2016) showed that tannic acid (TA), a polyphenolic biomolecule,

acts as an efflux-pump inhibitor (EPI) and potentiates the effects of several non-$\beta$-lactam antibiotics in *Acinetobacter baumannii* and *Staphylococcus aureus*, respectively. Payne *et al.* (2002) also characterized a series of tricyclic natural product derivatives as potent broad-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 and Amoako, 2017; Osei Sekyere and 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, and Essack, 2016; Osei Sekyere and Amoako, 2017; Osei Sekyere and 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 72 *Enterobacteriaceae* isolates included *Klebsiella pneumoniae* (n=25)*, Enterobacter spp.* (*E. cloacae, E. kobei, E. asburiae, E. cloacae complex "Hoffman cluster II"* and *E. cloacae complex "Hoffman cluster IIV"*) (n=18)*, Serratia marcescens* (n=16)*, Escherichia coli* (n=8)*, Klebsiella michiganensis* (n=1)*,* and *Providentia rettgeri* (n=1). *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 (Osei Sekyere and 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 strains to determine their innate toxicity to the isolates using the broth microdilution method (BMD) following the Clinical and Laboratory Standards Institute (CLSI) guidelines(Clinical and Laboratory Standards Institute., 2017; Osei Sekyere and Amoako, 2017). Briefly, twofold dilutions of each compound's solution were prepared in a microtiter plate and a 0.5 McFarland-standardized bacterial inoculum was used to inoculate the microtiter wells. The plates were then incubated at 37◦C for 18 to 20 h. The MIC was determined as the lowest concentration at which no visible bacterial growth was observed. These MICs were used as a guide in choosing the sub-MICs (i.e. ½ MIC) of these compounds for further testing with MEM and IPM: sub-MICs used were 10mg/L (CCCP), 32mg/L (TZ), 16mg/L (CPZ), 256mg/L (VRP), 256mg/L (RES). These sub-MICs were 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 the above-described MIC method and CLSI guidelines (Clinical and Laboratory Standards Institute., 2017; Osei Sekyere and 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 *et al.*, 2014). Briefly, increasing concentrations (64mg/L, 128mg/L, 256mg/L, and 512mg/L) of TA were added to the MICs of imipenem (IPM) and meropenem (MEM) (0.5 to 512mg/L) for each isolate to determine the MICs of IPM and MEM in the presence of TA per isolate. The antibacterial assays (MICs) were performed in triplicate.

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

Sub-MICs of RSP (256mg/L), VRP (256mg/L), TZ (32mg/L), CPZ (16mg/L), and CCCP (10mg/L) were added to increasing concentrations (from 0.5mg/L to 512mg/L) of MEM and IMP in wells containing 0.5 McFarland's standard concentrations of the isolates' cultures. Afterwards, the microtiter plates were incubated at 37◦C for 18 to 20 h. The new MEM and IPM MICs were recorded and deducted from the original MEM and IPM MICs to determine the effects of the EPIs on the MICs of MEM and IMP (Osei Sekyere and Amoako, 2017; Osei Sekyere and Amoako, 2017). This was done to determine whether efflux pumps are involved in TA's reduction of IMP and MEM MICs.

### 2.4. $IC_{50}$ enzyme inhibition assay:

To confirm whether TA has the ability to inhibit the activity of MBLs and SBLs, representative enzymes i.e., NDM-1 and KPC-2, were respectively used to conduct an *in vitro* enzyme inhibition assay using purified enzymes 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 $7.3 \pm 0.3$, were mixed with 30 µM nitrocefin after 10 to 15 minutes pre-incubation with TA at 30°C. The enzymes were supplemented with 100 µM 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 of NDM-1, VIM-2 and KPC-2 (PDB ID: 3QX6, 3RXW, 5ACU and 5FAS) were retrieved from the RSCB Protein Data Bank (https://www.rcsb.org/pdb/). These three enzymes were chosen to represent MBLs (NDM-1 and VIM-2) and SBLs (KPC-2). The missing residues i.e., missing amino acid components of the enzyme and ligand, which can be added through the chimera tool, 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 and Rarey, 2010). System preparation, molecular docking and molecular dynamic simulations were carried out to ascertain the interactions of the enzymes (NDM-1, VIM-2, and KPC-2) with the ligand (TA). Geister partial charges were assigned and the AutoDock atom types were defined using the AutoDock graphical user interface supplied by MGL tools (Sanner, 1999). The docked conformations were generated using the Lamarckian genetic algorithm (LGA), which is considered to be one of the best docking methods available (Huey *et al.*, 2007). The reports for each calculation were in (Kcal/mol). This technique has been validated in previous studies (Kumalo and Soliman, 2016).

### 2.5.1. Molecular dynamics simulation

Due to the lack of parameters needed for the ligand in the Cornell et al. force field (Cornell *et al.*, 1995), the missing parameters were created. Optimization of the ligands were first performed at the HF/6-31G* level with the Gaussian 03 package (Frisch et al., 2004). The restrained electrostatic potential (RESP) procedure (Cieplak *et al.*, 1995) was used to calculate the partial atomic charges. GAFF (Wang *et al.*, 2004) force field parameters and RESP partial charges were assigned using the ANTECHAMBER module in the Amber14 package. Hydrogen atoms of the proteins were added using the Leap module in Amber12. The standard AMBER force field for bioorganic systems (ff03) was used to define the enzyme parameters. Counter ions were added to neutralize the charge enzyme. The system was enveloped in a box of equilibrated TIP3P water molecules with 8 Å distance around the enzyme. Cubic periodic boundary conditions were imposed and the long-range electrostatic interactions were treated with the particle-mesh Ewald method (Essmann *et al.*, 1995) implemented in Amber12 with a non-bonding cut-off distance of 10 Å.

Initial energy minimization, with a restraint potential of 2 kcal/mol $Å^2$ applied to the solute, was carried out using the steepest descent method in Amber12 for 1000 iterations followed by conjugate gradient protocol for 2000 steps. The entire system was then freely minimized for 1000 iterations. Harmonic restraints with

force constants 5-kcal/mol Å$^2$ were applied to all solute atoms during the heating phase. A canonical ensemble (NVT) MD was carried out for 50 ps, during which the system was gradually annealed from 0 to 300 K using a Langevin thermostat with a coupling coefficient of 1/ps. Subsequently, the system was equilibrated at 300 K with a 2 fs time step for 100 ps whilst maintaining the force constants on the restrained solute. The SHAKE algorithm (Ryckaert *et al.*, 1977) was employed on all atoms covalently bonded to a hydrogen atom during equilibration and production runs. With no restraints imposed, a production run was performed for 2 ns in an isothermal isobaric (NPT) ensemble using a Berendsen barostat (Berendsen *et al.*, 1984) with a target pressure of 1 bar and a pressure coupling constant of 2 ps. The coordinate file was saved every 1 ps and the trajectory was analyzed every 1 ps using the Ptraj module implemented in Amber14.

### 2.6. Cytotoxicity Assay

HepG2 cells were maintained at 37$^o$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 (300µl) 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 20µL MTT solution (5mg/mL MTT in PBS) and 100µL CCM for 4 hours. The resulting formazan product was solubilized in 100µL DMSO (1hour) and the absorbance at 570nm/690nm was determined (BioTek µQuant plate reader; BioTek Instruments Inc., USA). The average absorbance values performed in triplicate were used to calculate cell viability.

### 2.7. Phylogenetic analysis

The phylogenetic relationship between the carbapenemases was determined using the MAFFT phylogeny server (http://mafft.cbrc.jp/alignment/server/phylogeny.html) to offer insights into the potentiating differences of TA on the different carbapenemase enzymes based on their amino acid sequences. The phylogenetic tree was viewed online and downloaded with Newick internal labels.

### 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 manually calculated for each isolate by dividing MEM or IPM MIC by that of TA-MEM or TA-IPM; the Δ are showed 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 (Osei Sekyere and Amoako, 2017; Osei Sekyere and Amoako, 2017). A mean fold change per antibiotic was calculated for every enzyme (Table 3) using the following equation:

*Sum (Δ of all isolates expressing x)/Total number of isolates expressing x*

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

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 antibiotics per isolate/species and per expressed carbapenemase in the presence of the efflux pump inhibitors and TA (data not shown). MIC fold changes (Δ) with a P-value of <0.01 were considered statistically significant.

**Table 1: Minimum inhibitory concentrations (MIC) of meropenem (MEM), imipenem (IPM) and Tannic acid (TA) as well as of MEM and IPM 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 | | |
| ***Klebsiella pneumoniae*** | | | | | | | |
| **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] | | |
| **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 |

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

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 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] | | |
| **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] | | |
| | MEM-256 | 64 [4] | 16 [16] | 8 [32] | 4 [64] | >512 | NDM-1 |

[2] No known carbapenemase present
[3] Enterobacter asburiae
[4] Enterobacter sp.
[5] Enterobacter cloacae complex "Hoffman cluster III"

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **16_S9**[6] | 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 |
| | IPM-128 | 16 [8] | 8 [16] | 4 [32] | 2 [64] | | |
| ***Klebsiella* michiganensis** | | | | | | | |
| **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] | | |

[6] Enterobacter kobei
[7] Enterobacter cloacae complex "Hoffman cluster IIV"

**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** | | | | | | |
| *E. coli* 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** | | | | | | |
| E. coli KPC-2 | MEM- 16 | 8 [2] | 8 [2] | 8 [2] | 4 [4] | >512 |
| | IMP- 64 | 8 [8] | 8 [8] | 8 [8] | 8 [8] | |
| E. cloacae KPC-2 | MEM- 32 | 16 [2] | 16 [2] | 16 [2] | 16 [2] | >512 |
| | IMP- 128 | 64 [2] | 64 [2] | 64 [2] | 32 [4] | |
| C. freundii KPC-2 | MEM- 16 | 4 [4] | 4 [4] | 4 [4] | 4 [4] | >512 |
| | IMP- 64 | 16 [4] | 16 [4] | 16 [4] | 16 [4] | |
| S. marcescens KPC-2 | MEM- >512 | 512 [≥1] | 512 [≥1] | 512 [≥1] | 512 [≥1] | >512 |
| | IMP- 512 | 512 [≥1] | 512 [1] | 256 [2] | 256 [2] | |
| **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 |

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

| | | | | | | |
|---|---|---|---|---|---|---|
| | 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] | |

**Table 3:** MM/GBSA binding free energy profile of NDM-1, VIM-2, KPC-2 and OXA-48 systems.

| Systems* | Evdw | Eelec | ΔGsolv | ΔGgas | ΔGbind |
|---|---|---|---|---|---|
| **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 |
| ΔEvdw, van der Waals energy; ΔEelec, electrostatic energy; ΔGsolv, polar solvation energy; ΔGgas, nonpolar solvation energy; ΔGbind, total binding free energy; *Enzyme-ligand complex. All energy terms are presented in kcal/mol. | | | | | |

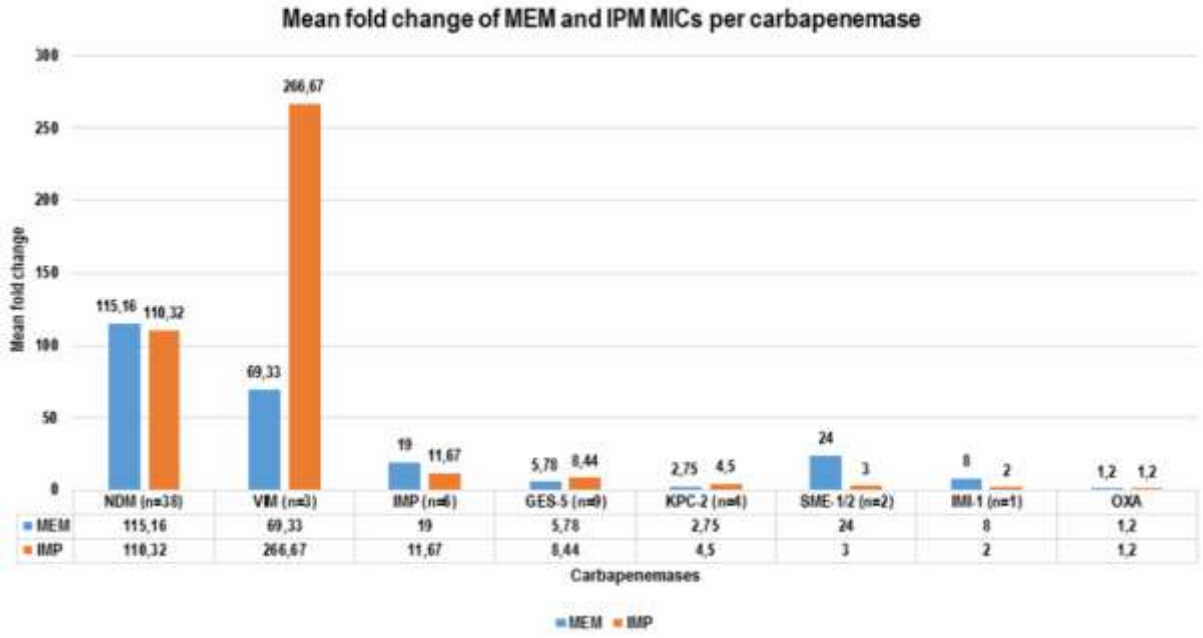


**Fig. 1: MEM and IPM MIC fold change of isolates per carbapenemase upon addition of tannic acid (TA).** The effect of tannic acid (512mg/L) in reducing and/or reversing meropenem (MEM) and imipenem (IPM) resistance among Enterobacteriaceae is shown above as a mean fold change(Δ). The mean Δ is measure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

## 3. Results

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

The checkerboard method was used for this assay. Increasing concentrations of TA resulted in decreasing IPM and MEM 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) (Table 1-2). TA reversed resistance to IPM and MEM in all MBL-positive isolates (p-value < 0.0001-0.01) except in one *Serratia marcescens* $bla_{IMP-1}$-positive strain (Fig.

1). On the contrary, TA significantly (p-value < 0.001-0.01) reduced the MEM MICs of most isolates expressing IMI and SME carbapenemases whilst it reversed resistance to only a few KPC-2- and GES-5-positive isolates (Tables 1-2 and Fig. 1). The effect of TA on OXA-48/-181/-232-producing isolates was insignificant (p-value > 0.01) although it halved the MIC of one *Klebsiella pneumoniae* OXA-48-positive strain (Tables 1-2 and Fig. 1). TA's 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 (Tables 1-2 and Fig. 1). 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 (Tables 1-2); 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 (Tables 1-2). 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).

### 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 (data not shown). All the EPIs and CCCP failed to significantly reduce or reverse resistance to MEM and IMP in all but one *Serratia marcescens* isolate, B (UNN38 _S2) (data not shown).

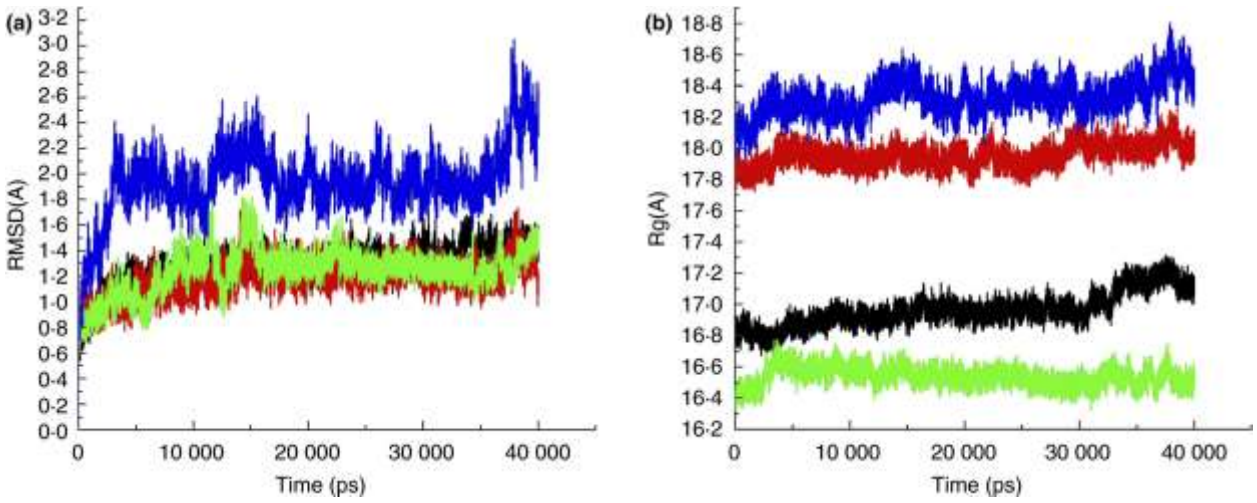


**Fig. 2: The Root-mean-square deviation (RMSD) and Radius of gyration (Rg) diagrams of the four complexes**: A) RMSD showing the stability of the systems (enzyme-ligand complex); B) Rg illustrating the compactness of the systems.

### 3.3. Enzymatic assay, molecular modelling and docking

As efflux pumps' inhibition was not involved in the reduction of MEM and IMP MICs by TA, we investigated the interaction between carbapenemase enzymes and TA to explain the potentiating effect of TA on MEM and IMP (Figures 1-2). 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 TA inhibition between the two carbapenemase classes. TA respectively inhibited ≈50% of NDM-1 and KPC-2 at 15 µM and 270 µM indicating, that TA possess an effect on both classes of carbapenemase enzymes (Figures 2-3).

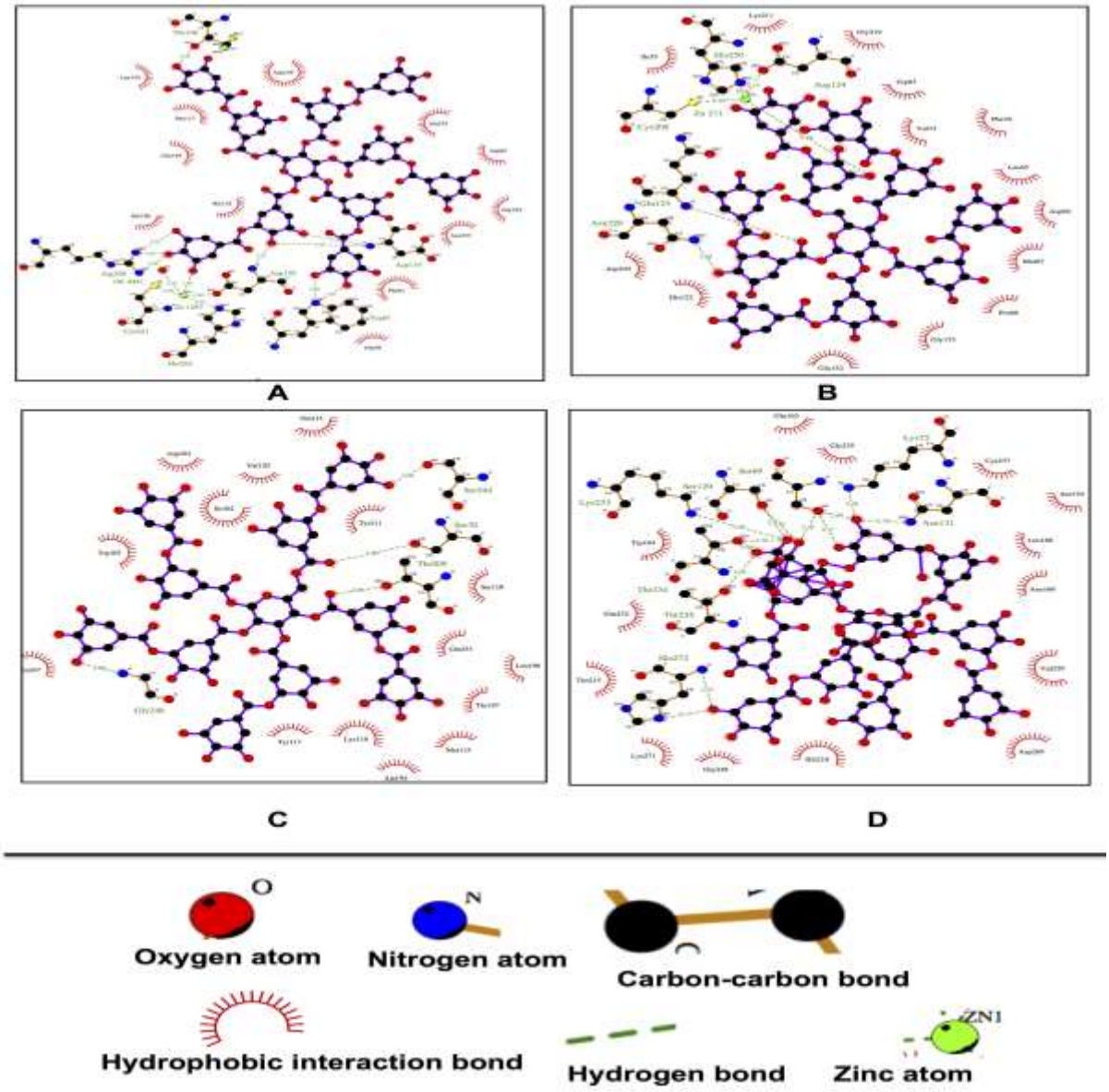


**Fig. 3: A ligplot showing the relative position of Tannic acid inside the active site of:** (A) VIM-2, (B) NDM-1 (C) OXA-48, D) KPC-2. TA interact more with MBLs when compared to SBLs at their catalytic active sites.

Furthermore, computational molecular dynamic analyses were employed to confirm that TA interacts with class A and class B carbapenemase enzymes as proof of concept (Figures 2-3). Thus, root-mean-square deviation (RSMD) 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 (Table 3 and Fig. 2-3). The radius of gyration

(Rg) of the four systems were also steady, highlighting the moderate conformational changes occurring during the simulation (Table 3 and Fig. 2-3). These results demonstrated that TA interact with each enzyme by reducing their hydrolytic activity at different levels, and also showed a stable compact structure (Table). However, OXA-48 and KPC-2 (serine-β-lactamases) exhibited a significant overall higher Rg than the MBLs (VIM-2 and NDM-1) (Fig. 2). 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 than the SBLs (Table 3). 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 than OXA-48 and KPC-2. TA also forms electrostatic interactions with zinc. The closer sequence similarity of OXA-48 and KPC-2 than with the MBLs provides further insight into these observations (Fig. 4).

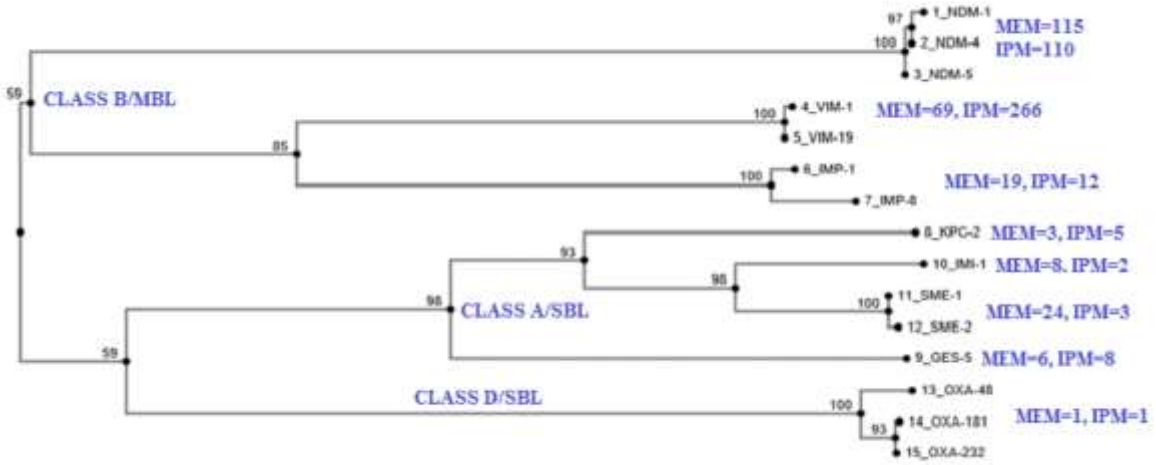


**Fig. 4: Phylogenetic tree of the carbapenemases.** The carbapenemases found in the isolates used in this study are clustered into their families of classes A, B, and D, showing the close relationship and amino acid sequence identity between members of the same family. The mean MIC fold change per enzyme 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.

### 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±6.95%) and 32mg/L (108.52±7.28%) (Fig 5). However, for the 64mg/L, 128 mg/L and 256 mg/L the cell viability decreased to 89.26±8.37%, 63.08±4.82% and 63,40±4.73% respectively. The metabolic activity of TA-treated cells was increased again at 512mg/L (93.50±4.86%) and 1024mg/L (93.86±4.43%) (Fig 5). TA was having tolerable cytotoxic effect for all the concentrations tested.

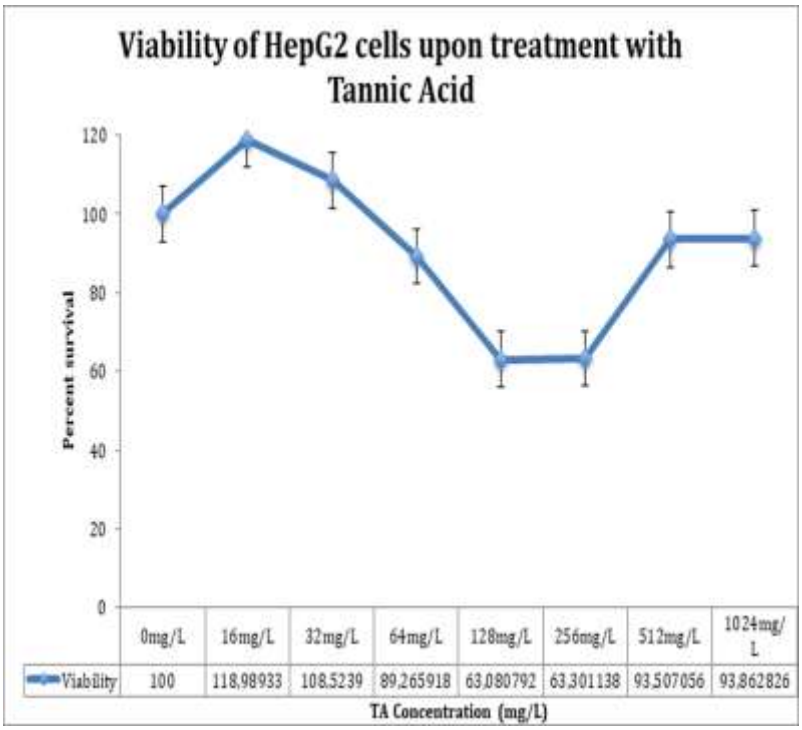


***Fig. 5***: A figure showing HepG2 cell viability following treatment with TA for 24 hours. TA regression analysis yielded a biphasic curve with no significant reduction in cell viability at 512 and 1024mg/L.

### 3.5. 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 and 4). Three clinical isolates from South Africa i.e. 1-S1, 65_S32,

**Table 4. 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 (n=36) | -4 (n=1) | -5 (n=1) | -1 (n=2) | -19 (n=1) | -1 (n=3) | -8 (n=3) | (n=4) | (n=9) | (n=1) | -1 (n=1) | -2 (n=1) | -48 (n=2) | -181 (n=1) | -232 (n=1) |
| ***K. pneumoniae (n=25)*** | Clin[1] | 11 | –[2] | – | – | 1 | – | 1 | – | 8 | – | – | – | – | – | 1 |
| | Ref[3] | 1 | – | – | – | – | – | – | – | – | – | – | – | 1 | 1 | – |
| ***S. marcescens (n=16)*** | Clin | 11 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| | Ref | – | – | – | – | – | 1 | – | 1 | – | – | 1 | 1 | – | – | – |
| ***Enterobacter spp.*** | Clin | 9 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| ***(n=18)*** | Ref | 1 | – | – | 1 | – | 1 | 1 | 1 | 1 | 1 | – | – | – | – | – |
| ***E. coli (n=8)*** | Clin | – | – | 1 | – | – | – | – | – | – | – | – | – | – | – | – |
| | Ref | 1 | 1 | – | 1 | – | 1 | 1 | 1 | – | – | – | – | 1 | – | – |
| ***C. freundii (n=3)*** | Clin | 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| | Ref | 1 | – | – | – | – | – | – | 1 | – | – | – | – | – | – | – |
| ***K. michiganensis (n=1)*** | Clin | 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| | Ref | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| ***P. rettgeri*** | Clin | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| ***(n=1)*** | Ref | – | – | – | – | – | – | – | – | – | – | – | – | – | 1 | – |

[1] Clinical South African strains
[2] No known carbapenemase present
[3] Reference strains

and 45_S21 were non-CPEs and had tested negative both for the Carba-NP test (J. Osei Sekyere *et al.*, 2015; Osei Sekyere, Govinden, and 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, and 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 (Table 4; 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, 2016; Osei Sekyere, Govinden, and Essack, 2016; Osei Sekyere and 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 and Poirel, 2014; Osei Sekyere *et al.*, 2015). 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. Moreover, no significant cytotoxic effect of TA was observed in HepG2 cells.

The inability of 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)(Osei Sekyere and Daniel G. Amoako, 2017; Osei Sekyere, 2018), to reverse IPM and MEM resistance suggests that TA does not inhibit efflux pumps to reduce IPM and MEM MICs as reported in methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Myint *et al.*, 2013). 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 carbapenem antibiotics. Moreover, TA alone had no biocidal effect on the isolates at concentrations >512 mg/L, as seen from the MIC of TA, suggesting that TA's mechanism of action is not through universal but specific protein inhibition; specifically, inhibition of β-lactamases. This is because universal protein inhibition would have made TA cytotoxic to the cells, a situation that was not observed.

Moreover, the different responses observed by the isolates upon adding the EPIs and CCCP suggest that the isolates had different efflux expression and activity levels. Equal efflux expression and activity levels would have resulted in same MIC reductions after adding the inhibitors. Further efflux expression analysis tests should be undertaken, particularly for *Serratia marcescens* isolate B (UNN38 _S2), to ascertain the expression levels of efflux pumps and their role in carbapenem resistance.

The reason underlying the cytotoxicity assay results in which TA increased HepG2 cell viability at 16-32mg/L, reduced it at 64-256mg/L and increased it again at 524-1024mg/L is not known to us presently. And further studies are necessary to understand the mechanism(s) underlying this observation. However, we hypothesise that the initial increase in cell viability might be due to TA serving as nutrients for the cells, but became toxic to the cells at 64-256mg/L by reducing nutrient availability and/or inhibiting metabolic enzymes' activity in the cell via its astringent action (https://www.britannica.com/science/tannin) (Leal *et al.*, 2011; Tintino *et al.*, 2016). To overcome this TA cytoxicity at 64-256mg/L, the cell might have used other

mechanisms to either inhibit TA activity or find alternative nutrient acquisition pathways at higher TA concentrations of 512-1024mg/L.

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 type. For instance, irrespective of the species or strain, TA modulated carbapenem resistance in all MBL-producing isolates as well as in most isolates expressing certain types of class A SBLs. However, resistance in the same species expressing the same class D carbapenemase types were barely modulated. Hence, the resistance-modulating effect of TA was mainly influenced by the type of carbapenemase than by the species or strain, indicating that TA interacts with the carbapenemase enzymes to modulate carbapenem resistance. This is similar to previous studies that demonstrated that carbapenemase enzymes (MBLs) were inhibited by aspergillomarasmine A (AMA), 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), and di-(2-picolyl) amine (DPA) to increase the efficacy of carbapenem antibiotics (King *et al.*, 2014; Somboro *et al.*, 2015; Azumah *et al.*, 2016). However, TA exhibited broader spectrum of activity than the above-mentioned three compounds. As reported by other investigators with other molecules (King *et al.*, 2014; Somboro *et al.*, 2015; Azumah *et al.*, 2016), TA was found by enzyme inhibition assays and computational modelling to also directly bind to the carbapenemases, albeit at different Rg and $IC_{50}$ values depending on the carbapenemase class.

While AMA, NOTA, and DPA only inhibited MBLs (King *et al.*, 2014; Somboro *et al.*, 2015; Azumah *et al.*, 2016), TA inhibited both MBLs and SBLs, except OXA-48-like (class D) carbapenemases. This was shown by the computational studies to be due to the targeting of the hydrophobic sites of both MBLs and SBLs by TA, which interacted with a larger number of MBL active site residues than those of OXA-48 and KPC-2 (SBL). Moreover, TA's higher effect on MBLs is due to its formation of hydrogen bonds with zinc, which is important for MBL activity, thus removing the bacterial defence against β-lactams (carbapenems). Further analysis of the binding free energy showed that the MBLs (VIM-2 and NDM-1) exhibited a stronger and stable binding affinity than the SBLs, which is caused by the differences in the interactions between the catalytic

active site residue(s) and TA, amplifying the conformational flexibility of the complex and ultimately affecting the receptor grip on the inhibitor. This results in a higher radius of gyration of the SBLs than MBLs. These explain the broader β-lactamase/carbapenemase inhibition spectrum of TA, which was more potent in inhibiting MBLs than SBLs, than AMA, NOTA and DPA. To our knowledge, this finding has never been reported, making this the first of its kind and providing a promising lead compound (TA) that can be further exploited to engender clinically beneficial MBL and SBL inhibitors to treat CPE infections. 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.

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, and Essack, 2016; Osei Sekyere and Amoako, 2017; Osei Sekyere and 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.

Available evidence from published studies show that tannins, which includes TA, catechin, ethylgallate, epicatechin gallate, epigallocatechin gallate, methyl gallate, ellagic acid, rosmaric acid and 1,2,3,4,6-Penta-O-galloyl-b-D-glucopyranose are polyphenolics that are abundant in plants (Leal *et al.*, 2011; Slobodníková *et al.*, 2016). 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), similar to what was observed herein. As TA alone was not active against the Enterobacteriaceae isolates used in this study, it is obvious that it did not inhibit protein synthesis.

In 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 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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