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Green tea (*Camellia sinensis*) leaf extract inhibits the activity of β-lactamases

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Abstract

The most emerging resistance mechanism against β-lactam antibiotics present in bacteria is the production of β-lactamases. The aim of this study was to explore the phytocompounds of *Camellia sinensis* (green tea) that can inhibit the activity of β-lactamases. Moreover, the antibacterial effect of its extract with a combination of antibiotics against resistant bacterial strains was also appraised. *In silico* docking was carried out against the resistance causing enzymes such as AmpC and SHV-1. Antimicrobial susceptibility testing against bacterial strains, i.e., *Klebsiella pneumoniae* and *Escherichia coli*, was performed. Then, for exploring the synergistic effects, both antibiotics and green tea extract were applied in combination. The docking studies revealed that the inhibitors like epigallocatechin gallate with AmpC, and myricetin with SHV-1 enzyme displayed high binding affinities of -8.2 kJ/mol, and -7.5 kJ/mol, respectively. The *in vitro* combination of *C. sinensis* extract with ampicillin and penicillin also potentiated the antibacterial activity of these antibiotics. Thus, the study elucidated that the phytochemicals of *C. sinensis* could inhibit β-lactamases produced by the pathogens. Also, it has enhanced antimicrobial effects when combined with antibiotics.

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Introduction

The growing rate of antimicrobial resistance in bacteria is a major health concern these days. Out of all resistance mechanisms adopted by bacteria, the most prominent one is the inactivation of antibiotics through β-lactamases (βLs). These are the enzymes involved in the hydrolysis of various β-lactam antibiotics such as penicillin, carbapenems, and cephalosporin, etc. (Kapoor et al., 2017; Narendrakumar et al., 2023). Among *Enterobacteriaceae* members, different pathogens like *Escherichia coli* and *Klebsiella pneumoniae* are becoming resistant to β-lactam antibiotics as they are all able to produce βLs to inactivate them. The most emerging βLs are the extended-spectrum beta-lactamases (ESBLs) such as TEM, SHV and CTX-Ms etc. that are the leading cause of antibiotic ineffectiveness in *K. pneumoniae* and *E. coli* (Freitas et al., 2013; Husna et al., 2023). Hundreds of ESBLs have been recognized so far in different *Enterobacteriaceae* members. All the pathogens producing ESBLs being frequently responsible for diarrhea and urinary tract infections are difficult to treat with various β-lactams (Raut et al., 2015).

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Resistance in *K. pneumoniae* is becoming a global issue because many of the broad-spectrum antibiotics, including various β-lactams, are being considered ineffective for treating its infections (Ferreira et al., 2019; Sharma et al., 2023). Half of nosocomial and 90% of community-acquired UTIs are caused by *E. coli* (Farshad et al., 2012; Zhou et al., 2023). However, the range and incidence of antimicrobial-resistant UTIs have increased in recent years (Zhou et al., 2023). Due to various intrinsic and extrinsic factors, these infectious bacterial pathogens have become resistant to most of the antibiotics, like aminoglycosides, fluoroquinolones, and β-lactams (Raeispour and Ranjbar, 2018). Therefore, to combat these pathogens, antimicrobial compounds derived from various natural sources such as plants have become potential sources for such vital compounds (Radji et al., 2013; Vaou et al., 2021; Karnwal and Malik, 2024). Medicinal plants have been proved effective against resistant pathogens and have also various advantages over synthetic drugs. Plant-derived compounds have good antibacterial effects, greater therapeutic benefits and less side-effects, so they can be used to develop better drugs (Parvez et al., 2019; Zhou et al., 2023).

Various secondary metabolites derived from plants contain drug-like characteristics. For the identification of such metabolites, different advanced bioinformatics tools are available nowadays instead of other time-consuming approaches (Parida et al., 2021; Javid and Ahmed, 2023). *In silico* molecular docking is the best method for screening different natural compounds that are considered as potential drugs against pathogens (Romano and Tatonetti, 2019; Vistoli et al., 2023; Zhang and Li, 2024). Thus, this study aimed to identify the phytochemicals present in *Camellia sinensis* leaves that can effectively inhibit βLs.

Materials and Methods

Molecular docking

Molecular docking was carried out to determine the mode of interaction between bacterial enzymes and phytochemicals (ligands) with the help of MGL-docking tools and AutoDock Vina (Fan et al., 2019). Briefly, from the PDB database (https://www.rcsb.org/), 3D structures of four bacterial enzymes such as AmpC, and SHV-1, with the attached ligands AMP, and 1OG respectively, were obtained. The 3D structures of phytocompounds of *Camellia sinensis* were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) in SDF format and then converted to PDBQT by the AutoDock tools (Trott and Olson, 2010). Also, Avibactam, a synthetic βL inhibitor, was included as a control. All four enzymes were prepared for docking by removing water molecules and adding polar hydrogens, and then converted into the PDBQT file using the AutoDock tools (Guedes et al., 2014; Stanzione et al., 2021). The grid box was adjusted around the ligand attached to enzymes and all the X, Y and Z coordinates were recorded to get active site information.

Subsequently, the configuration text file and PDBQT file of the ligands and the enzymes were pasted into one folder and prepared for docking (Javid and Ahmed, 2023; Muhammed and Aki-Yalcin, 2024). Docking was performed using AutoDock Vina by the vina command ("\vina\vina.exe" --config conf.txt --log log.txt) in command prompt. After the docking was complete, the docked complex was analyzed through PyMOL and their binding affinity values (kJ/mol) were examined.

Extract preparation and phytochemical analysis

Dried leaves of *Camellia sinensis* were collected from a local grocery store and after proper plant identification, the extract was prepared by maceration (Barreira et al., 2021). Then, the crude extract was concentrated to obtain in dried powder form, which was freeze-dried until further use (Gulo et al., 2021). Different biochemical procedures were performed for the qualitative screening of phytochemicals such as phenols, alkaloids, saponins, tannin, triterpenes, and flavonoids present in the *C. sinensis* extract following Adamu et al. (2022).

Bacterial strains

Fully characterized clinical isolates of *K. pneumoniae* and *E. coli* were obtained from the Institute of Microbiology and Molecular Genetics, University of the Punjab. These clinical strains were stored at -80 °C under aseptic conditions. The susceptibility of these strains against a variety of β-lactam antibiotics such as cefotaxime 30 µg (CTX), cefoxitin 30 µg (CXT), penicillin 10 µg (PEN) and ampicillin 10 µg (AMP) was determined by the disc diffusion assay according to CLSI instructions (CLSI, 2012; Cepas and Soto, 2020).

Antibacterial activity of plant extract

The antibacterial activity of different concentrations of the plant extract was determined by the well diffusion assay under CLSI guidelines (CLSI, 2012; Danish et al., 2020). Briefly, Mueller-Hinton (MH) agar

plates were inoculated with freshly prepared bacterial cultures. Then, the wells were made in agar plates under aseptic conditions. Different concentrations of plant extract, i.e., 50 mg/mL, 150 mg/mL, and 200 mg/mL were prepared by resuspending the powdered extract in methanol, and poured in the respective wells. The plates were then incubated at 37 °C, and the zones were measured after 24 h. All the *in vitro* experiments were run in triplicates.

Synergistic activity of plant and antibiotics

The synergistic assay was performed by the D-zone assay to analyze the combined effect of antibiotics with the plant extract as compared with the individual effects of antibiotics, as described by CLSI (Arora et al., 2021; Gadisa and Usman, 2021). For this purpose, the antibiotics discs of cefotaxime 30 µg (CTX), cefoxitin 30 µg (CXT), penicillin 10 µg (PEN) and ampicillin 10 µg (AMP), purchased from Abtek Biological Ltd., were separately soaked each in 200 mg/mL concentration of *C. sinensis* extract in an Eppendorf under aseptic conditions. After the antibiotics discs were got soaked with the plant extract, they were then placed equidistantly onto the already inoculated MH agar. All plates were placed in an incubator at 37 °C for 24 h. The zone of inhibition around each antibiotic was measured.

Time kill assay

As the highest activity was observed in case of combination of penicillin and plant extract, therefore, this combination was subjected to the time-kill assay to further analyze the synergistic interaction (Amaral et al., 2020; Pal and Tripathi, 2020). Briefly, 100 µL MH broth, 10 µL penicillin (10 to 150 mg/mL) and 20 µL plant extract (0.5-4 mg/mL) were added in the wells of the microtiter plate. Then, an aliquot of 10 µL of freshly cultured bacterial strains was added in each well, and the plates were incubated at the room temperature. The absorbance was recorded at 610 nm every 3 h.

Results

In silico analysis

The two commonly reported bacterial βLs that are involved in bacterial resistance mechanisms were selected to dock with the phytochemicals of *C. sinensis* to determine how they efficiently bind with the enzymes and result in the inhibition of the bacterial growth. The phytocompounds such as epigallocatechin gallate with AmpC, and myricetin with SHV-1 enzyme produced an efficient binding energy of -8.2 kcal/mol and -7.5 kcal/mol, respectively (**Table 1**). The docked complex of the enzymes with phytochemicals was also analyzed by PyMOL. The avibactam was used as the synthetic inhibitor. Binding energy obtained after docking the avibactam with AmpC and SHV-1 was -5.9 kcal/mol and -5.4 kJ/mol, respectively.

Epigallocatechin gallate, an ester of gallic acid and epicatechin, showed highest affinity for AmpC βL. The interaction of epigallocatechin gallate at the active site of AmpC βL is shown in **Figure 1**. **Figure 2** shows that myricetin, a polyphenolic flavonoid, exhibited highest affinity for SHV-1 βL, i.e., -7.5 kJ/mol.

Table 1. Phytochemicals of *C. sinensis* docked with respective enzyme, along their top affinity scores (kJ/mol)

Phytochemicals	Pubchem ID	Binding score (kJ/mol)	
		SHV-1	AmpC
Epigallocatechin gallate	65064	-5.7	-8.2*
Rutin	5280805	-5.5	-8.1
Theaflavin	169167	-6.1	-7.9
Isoquercitrin	5280804	-6.2	-7.5
Myricetin	5281672	-7.5*	-7.4
Epicatechin	72276	-6.5	-7.3
Epigallocatechin	72277	-6.9	-7.3
Avibactam	9835049	-5.4	-5.9

***** Phytochemicals with highest binding affinities

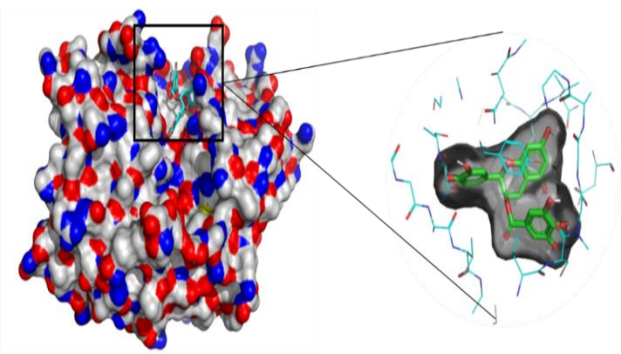

Figure 1. Docking complex and interaction of epigallocatechin gallate within binding site of AmpC βL

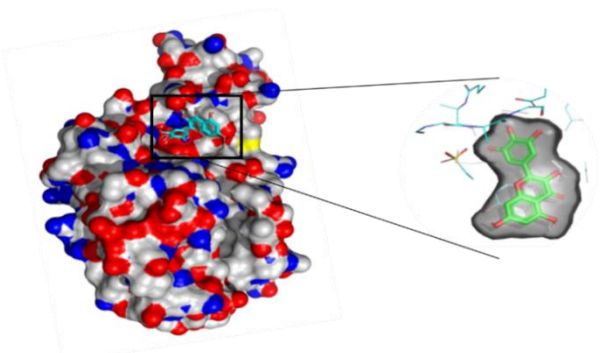

Figure 2. Docking complex and interaction of myricetin within the binding site of SHV-1 βL

Phytochemical screening

The *C. sinensis* extract was also screened for the presence of various phytochemicals by the biochemical testing. The results of the screening indicated the presence of phytochemicals of various types such as phenols, flavonoids, alkaloids, saponins, and tannins in the extract of *C. sinensis*, as shown by color changes in **Figure 3**.

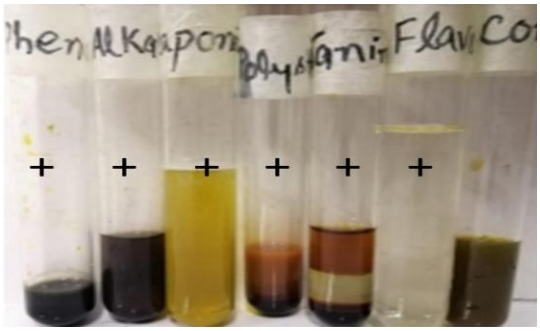

Figure 3. Qualitative testing of phytochemicals of *C. sinensis* extract compared with the control test tube (on left side)

In vitro antibacterial assay

Antibiotic susceptibility was investigated against two strains of *K. pneumoniae* KPSA and KPSB and two of *E. coli,* i.e., ECSA and ECSB, for each of antibiotics such as cefotaxime 30 µg (CTX), cefoxitin 30 µg (CXT), penicillin 10 µg (PEN) and ampicillin 10 µg (AMP). The results from the disc diffusion assay show that no zone of inhibition was produced around any of the antibiotic discs against all clinical strains, so they were considered resistant to each antibiotic (**Table 2**). The antibacterial activity of plant extract determined by the well diffusion assay showed no significant activity for different concentrations tested (**Table 2**).

Synergistic activity

The synergistic effects of antibiotics with *C. sinensis* extract showed that when the extract was combined with penicillin and cefotaxime discs, 10 mm and 11 mm zones of inhibition were observed, respectively, against *Klebsiella* KPSA strain. In case of KPSB strain, inhibition zones of 15 mm, 10 mm and 11 mm were produced when the extract was combined with penicillin, cefotaxime and cefoxitin, respectively. The *E. coli* strain ECSA, showed a 10 mm zone with penicillin, 18 mm zone with ampicillin and 7 mm zone of inhibition with cefotaxime on combining with *C. sinensis* extract. However, the extract exhibited the inhibition zones of 11 mm, 14 mm, and 15 mm diameter on synergism with penicillin, ampicillin and cefotaxime discs, respectively, against the ECSB strain (**Table 2**).

Table 2. *In vitro* antibacterial activity of standard antibiotics, *C. sinensis* extract, and antibiotics plus *C. sinensis* extract against recommended test strains

Strain type	Diameter of zone of inhibition (mm)										
	Standard antibiotics				*C. sinensis* extract			Antibiotics plus extract			
	PEN10	AMP10	CTX30	CXT30	CS50	CS100	CS200	PEN10	AMP10	CTX30	CXT30
KPSA	-	-	-	-	-	-	-	10	-	11	-
KPSB	-	-	-	-	-	-	-	15	-	10	11
ECSA	-	-	-	-	-	-	-	10	18	7	-
ECSB	-	-	-	-	-	-	-	11	14	15	-

PEN 10 = penicillin 10 µg; AMP10 = ampicillin 10 µg; CTX30 = cefotaxime 30 µg; CTX30 = cefoxitin 30 µg; CS50 = *C. sinensis* extract 50 mg/mL; CS100 = *C. sinensis* extract 100 mg/mL; CS200 = *C. sinensis* extract 200 mg/mL;

Time kill assay

In case of *K. pneumoniae*, penicillin when administered with the *C. sinensis* extract showed a significant growth inhibition as compared to when the antibiotic or plant extract was administered individually (**Figure 4**). Although penicillin alone was able to inhibit the *E. coli* growth over the period of 12 h, significantly high growth inhibition was observed in the combination form. The plant extract alone was not able to produce any visible growth inhibition against *E. coli* (**Figure 5**).

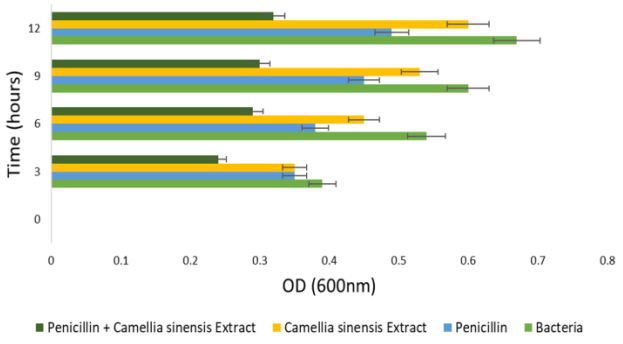

Figure 4. Time kill graph showing growth inhibition of *K. pneumoniae* over the period of 12 h

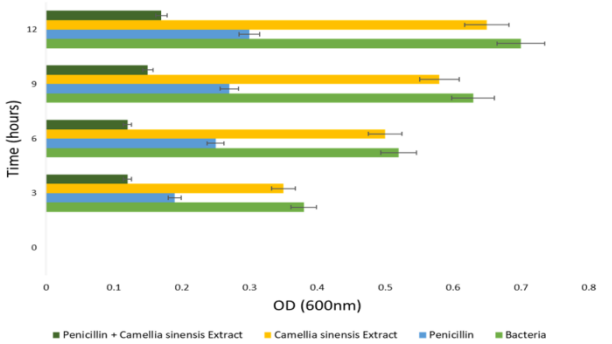

Figure 5. Time kill graph showing growth inhibition of *E. coli* over the period of 12 h

Discussion

The inappropriate and indiscriminate use of antimicrobial agents has become a major cause of antibiotic resistance these days. This situation is becoming deadliest as many of the bacterial strains have adapted different mechanisms of resistance to counter antibiotics, one such mechanism is the antibiotic inactivation through βLs (Dugassa and Shukuri, 2017). In this study, we were mainly concerned with two βLs, AmpC, and SHV-1, that are involved in the inactivation of various β-lactams. Different studies have reported the contributing activity of these enzymes in increasing resistance in *K. pneumoniae*. Moya and Maicas (2020) described the occurrence of different resistant genes in the *K. pneumoniae* encoding various types of Class A βLs.

When the threatening situation increases, then plants are considered as the main source of drugs to reverse antibiotic resistance. *Camellia sinensis* contains the phytochemicals that show significant antibacterial, antiviral and anti-inflammatory activities (Barbieri et al., 2017). Thus, this study focused on identifying potential βLs inhibitors from *C. sinensis* by utilizing *in silico* and *in vitro* tools. One of the most influential methods is the molecular docking approach that can screen the abundance of phytochemicals computationally in affordable time. This method describes the affinity of a particular phytochemical against a specific enzyme in terms of binding energy (kJ/mol) (Rampone et al., 2021). Thus, keeping these grounds in mind, *in silico* analysis of docking enzymes and phytochemicals and their *in vitro* analysis against certain resistant bacteria has been carried out in this study. The *in silico* analysis showed that epigallocatechin gallate had the highest affinity of -8.2 kJ/mol against AmpC βL and proved as 40 percent more effective compared to the synthetic inhibitor, avibactam. Kalalo et al. (2021) have also reported the antibacterial activity of epigallocatechin gallate against various infectious pathogens. For SHV-1 βLs, docking with the myricetin showed binding affinity as -7.5 kcal/mol as compared to -5.4 kJ/mol by avibactam. Various other studies also have confirmed the inhibitory activities of myricetin against βLs, hence, proving its ability to protect various β-lactams from hydrolysis (Wang et al., 2020; Javid and Ahmed, 2023). The differences in the results of this study could be attributed to the variable docking procedures used against different β -lactamases in previous studies.

In vitro analysis revealed that the selected strains of *K. pneumoniae* as well as *E. coli* were found to be highly resistant to cefotaxime, cefoxitin, ampicillin and penicillin. Palzkill (2018) also supported the idea that class A βLs hydrolyze penicillin and various newer generation cephalosporins in *K. pneumoniae*. Jameel et al. (2014) reported that AmpC βLs in *E. coli* cause high resistance against cefoxitin and cefotaxime. Liakopoulos et al. (2016) described that various SHV βLs in *Enterobacteriaceae* members have evolved resistance against various β-lactams.

The synergistic activity of *C. sinensis* extract with antibiotics produced the striking difference. Without any synergistic activity with extract, all the strains were resistant to the tested antibiotics, but in contrast, with the combination of *C. sinensis*, a zone of inhibition was observed around the antibiotics. Several studies have reported that the synergistic effect of different polyphenols such as epicatechin, epigallocatechin and epigallocatechin gallate with antibiotics produced the enhanced antibacterial activity against different *Enterobacteriaceae* species. (Haghjoo et al., 2013; Stephen et al., 2014; Manso et al., 2021). The time kill assay also supported that the *C. sinensis* extract contains a variety of phytocompounds that can activate the β-lactam antibiotic, such as penicillin, which alone did not produce any significant results.

The *in silico* and *in vitro* analyses elucidate that how various phytochemicals from green tea can be used to inhibit βLs present in the bacterial species and how we can increase the effectiveness of resistant antibiotics with the combination of green tea extract. Through *in silico* analysis, we can identify all effective phytochemicals that have highest binding affinity with certain βLs. So, *in vitro* analysis shows that when these antibiotics are used synergistically with *C. sinensis* extract they can effectively inhibit the growth of pathogens.

Conclusion

This study suggests that phytochemicals present in green tea are the major sources of β-lactamase inhibitors. Furthermore, the green tea extract can restore the effect of β-lactam antibiotics synergistically and can be used as a potent drug against β-lactamase producing resistant strains of *K. pneumoniae* and *E. coli.*

Author(s), Editor(s) and Publisher's declarations

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This study does not involve human/animal subjects, and thus no ethical approval is needed.

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of the experiment, all materials were properly discarded to minimize/eliminate any types of bio-contamination(s).

Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher. These are available with the corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

Authors' consent
All authors contributed in designing and writing the entire article. All contributors have critically read this manuscript and agreed to publish in IJAaEB.

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