


## ORIGINAL ARTICLE

# Synergistic antimicrobial activities of epigallocatechin gallate, myricetin, daidzein, gallic acid, epicatechin, 3-hydroxy-6-methoxyflavone and genistein combined with antibiotics against ESKAPE pathogens

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**Abstract**

**Aim:** To verify synergistic effects, we investigated the antimicrobial activity of seven phenolic phytochemicals (gallic acid; epicatechin; epigallocatechin gallate; daidzein; genistein; myricetin; 3-hydroxy-6-methoxyflavone) in combination with six antibiotics against multidrug-resistant isolates from the ESKAPE group.

**Methods and Results:** To investigate single phytochemicals and combinations, initial microdilution and checkerboard assays were used, followed by time-kill assays to evaluate the obtained results. The research revealed that phenolic compounds on their own resulted in little or no inhibitory effects. During preliminary tests, most of the combinations resulted in indifference (134 [71.3%]). In all, 30 combinations led to antagonism (15.9%); however, 24 showed synergistic effects (12.8%). The main tests resulted in nine synergistic combinations for the treatment of four different bacteria strains, including two substances (3-hydroxy-6-methoxyflavone, genistein) never tested before in such setup. Time-kill curves for combinations with possible synergistic effects confirmed the results against *Acinetobacter baumannii* as the one with the greatest need for research.

**Conclusions:** The results highlight the potential use of antibiotic–phytochemical combinations for combating infections with multi-resistant pathogens. Synergistic combinations could downregulate the resistance mechanisms of bacteria.

**Significance and Impact of the Study:** The aim of this study is to demonstrate the potential use of phenolic natural compounds in combination with conventional antibiotics against multidrug-resistant bacteria of the ESKAPE group. Due to synergistic effects of natural phenolic compounds combined with antibiotics, pathogens that are already resistant to antibiotics could be resensitized as we were able to reduce their MICs back to sensitive. In addition, combination therapies could prevent the development of resistance by reducing the dose of antibiotics. This approach opens

David Buchmann and Nadin Schultze shared first authorship

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up the basis for future development of antimicrobial therapy strategies, which are so urgently needed in the age of multidrug-resistant pathogens.

#### KEYWORDS

checkerboard, combination with antibiotic, ESKAPE strain, natural products

## INTRODUCTION

Bacterial infectious diseases are generally treated with antibiotics, but their over- and misuse have promoted a frightening situation of antibiotic resistance around the world. Therefore, and due to a lack of development of new antibiotics, therapeutic options are constantly getting fewer. The U.S. Food and Drug Administration (FDA) approved only six new antibiotics in 2017 (FDA, 2018), four in 2018 (FDA, 2019) and also four in 2019 (FDA, 2020) not one of these belonged to a new class. Linezolid and daptomycin are representatives of the last discovered classes since the 1980s (Durand et al., 2019). In addition to the 41 antibiotic compounds belonging to known classes, currently there is only one compound in the development pipeline that belongs to a new class, namely darobactin (Imai et al., 2019). Although a large proportion (44%) of these substances have the potential to treat infections caused by ESKAPE pathogens, it must be taken into account that only 60% of the substances on phase-III studies will be approved and enter the market (Kim, 2020).

Owed to this predicament, a growing number of infections—such as pneumonia, urinary tract infections, gonorrhoea and salmonellosis—are becoming more difficult to treat as the antibiotics used loose effectiveness. Therefore, the World Health Organization (WHO) declared antibiotic resistance as ‘one of the biggest threats to global health’ (WHO, 2018). Moreover, the medical costs to treat infections caused by resistant bacteria are higher due to longer hospital stays, longer duration of sickness and the use of more expensive drugs (Zhen et al., 2019).

For these reasons, research in the field of drug resistance mechanisms and drug development requires actions across all states and societies around the world. For combating the economic burden of antibiotic resistance, the WHO published in 2017 an overviewed global priority list of antibiotic resistant bacteria to guide research, discovery and development of new antibiotics (Tacconelli, 2017). In this list, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* as well as Enterobacteriaceae are classified as critical; *Enterococcus* spp. and *Staphylococcus aureus* are listed as high priority. These collectively named ESKAPE group are increasingly involved in infectious diseases and are the leading cause of nosocomial infections all over the world (Santajit & Indrawattana, 2016). The difficulty to treat is based on their capability to escape biocidal action of well-known and commonly used antibiotics (Pendleton et al., 2013). Deeper studies on these

pathogenic organisms are urgently necessary. Hence, five species from the ESKAPE group were selected for this study.

The study of the activity of natural products is a promising approach since more than 75% of all antimicrobials currently used for the treatment of bacterial infections are natural products or their derivatives (Durand et al., 2019). While most of them were isolated from micro-organisms such as *Penicillium notatum* or *Streptomyces* spp. (Barbieri et al., 2017; Clardy et al., 2006; Patridge et al., 2016), many plants have been used to treat human bacterial infections since ancient times due to secondary metabolites they compromise (Barbieri et al., 2017; Rakholiya et al., 2013). This heterogeneous group of chemical compounds has been developed over a long period of time to defend plants from different environmental factors, including bacteria. In addition to alkaloids, sulphur-containing phytochemicals, terpenoids and polyphenols also display antimicrobial activity and have become a focus of research. Phytochemicals often have significantly higher minimal inhibitory concentrations (MICs) compared to the commonly used antibiotics. However, numerous studies demonstrated increasing effectiveness of phyto-compounds as well as synergic activity when combining antibiotics with phytochemicals (Amin et al., 2015; Ayaz et al., 2019; Cho et al., 2011; Cui et al., 2012; Dey et al., 2016; Hemaiswarya et al., 2008; Hu et al., 2001; Lin et al., 2005; Rakholiya et al., 2013; Regueira et al., 2017; Rondevaldova et al., 2018). With the aim to find new promising effective combinations against multidrug-resistant ESKAPE species and to confirm the applicability of phenolic compounds in combined therapies, five antibiotics from different classes were chosen, for combinatorial testing with seven phenolic phytocompounds (epigallocatechin gallate, myricetin, daidzein, genistein, epicatechin, gallic acid and 3-hydroxy-6-methoxyflavone). Starting with preliminary testing, the most promising combinations with a potential partial or synergistic effect were selected for further investigation by checkerboard and time-kill assays.

## MATERIALS AND METHODS

### Chemicals

Investigated phytochemicals were obtained from Carbosynth (Compton), Acros Organics, Alfa Aesar,

ChemFaces and Cayman Chemical. Antibiotics were purchased from VWR, Cayman Chemical, Alfa Aesar, Sigma Aldrich and Carl Roth as shown in Table 1. Stock solutions of antibiotics or phenolic compounds were prepared taking into account the salt concentrations with water, concentrated ethanol (chloramphenicol) or DMSO (phenolic compounds) up to the following concentrations ( $\text{mg ml}^{-1}$ ): ampicillin (12.8), cefotaxime (6.4), chloramphenicol (12.8), ciprofloxacin (0.4), gentamicin (12.8), tetracycline (12.8), phytochemicals (10.0) and stored at  $-20^{\circ}\text{C}$ .

## Bacterial strains

The different bacterial strains comprise reference strains of major ESKAPE pathogens (*A. baumannii* ATCC 19606, DSM 9308; *E. coli* ATCC 25922; *S. aureus* ATCC 29213; *Klebsiella pneumoniae* ATCC 700603; *P. aeruginosa* ATCC 27853) and clinical isolates (MRSA PBIO483; *A. baumannii* PBIO721; *E. coli* PBIO730, PBIO1442; *K. pneumoniae* PBIO1455, PBIO1990; *P. aeruginosa* PBIO712 and PBIO2208). Their detailed resistance pattern is summarized in Table 2. All strains were stored in cryovials, containing 20% glycerol, at  $-80^{\circ}\text{C}$ . Before use, fresh bacterial suspension was prepared from an overnight culture on LB-agar-plates in cation adjusted Müller-Hinton-II medium (MH-II-medium, Carl Roth). The inoculum of one colony in 5 ml medium was incubated at  $37^{\circ}\text{C}$  under shaking conditions (200 rpm) overnight.

## Antimicrobial sensitivity testing

Preparation and dilution of all solutions were carried out according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012) and the recommendations of manufacturers.

The MICs were determined by a broth dilution method carried out for 11 natural phenolic compounds and six antibiotics using cation adjusted MH-II-medium. Each experiment was performed in three replicates.

Starting with the antibiotic stock solutions, a serial twofold dilution was performed from row A to row G of the microtiter plates (96 wells, sterile F, Carl Roth) with the following final concentrations ( $\mu\text{g ml}^{-1}$ ): ampicillin (256–4), cefotaxime (128–4), chloramphenicol (256–4), ciprofloxacin (8–0.125), gentamicin (256–4) and tetracycline (256–4). Row H remained without antibiotic as growth control. Ten microliters of inoculum adjusted to an OD 0.05 at 600 nm (biochrom Ultraspec 10) were added to the 90  $\mu\text{l}$  of compound solutions. Tests were conducted in duplicates in columns 2–11, columns 1 and 12 remained

without any additions (substances or bacteria). The same procedure was performed for the phenolic compounds starting with  $400 \mu\text{g ml}^{-1}$ . The final concentration of solvent was less than 2% (v/v) and did not affect the bacterial growth. The MIC value was visually assessed and recorded after incubation at  $37^{\circ}\text{C}$  for 24 h. The minimum concentration of compound at which no visible growth occurred was taken as the MIC value.

## Initial testing

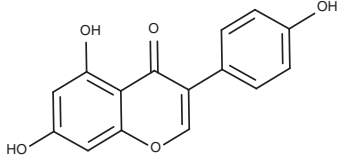
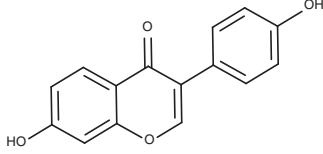
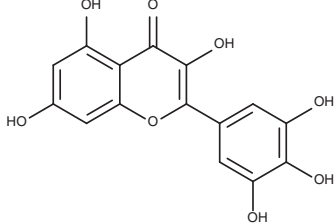
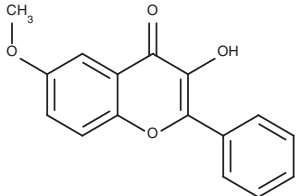
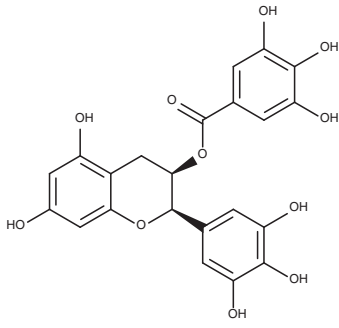
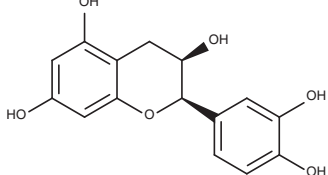
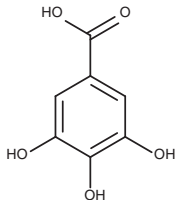
For a preliminary combination testing, the plates contained diluted antibiotics in final concentrations as described above in a total volume of 45  $\mu\text{l}$ . Each well was mixed with 45  $\mu\text{l}$  of a phytocompound solution ( $889 \mu\text{g ml}^{-1}$  in MH-II-medium). An overnight bacterial suspension was set to an optical density (OD) of 0.05, measured by photometer (biochrom Ultraspec 10) at 600 nm. Ten microliters of diluted suspension were filled into the prepared microtiter plates resulting in approx.  $10^5$  CFU per ml, thus yielded a final volume of 100  $\mu\text{l}$ . In summary, the test wells comprised  $400 \mu\text{g ml}^{-1}$  of the phenolic substances combined with twofold decreasing antibiotic concentrations. The last well without turbidity after incubation at  $37^{\circ}\text{C}$  for approximately 24 h (depending on species according to the CLSI guidelines) was visually detected as the lowest inhibitory concentration.

## Checkerboard assay

The possibly existences of synergism or antagonism of the most active combinations reported from the initial testing were examined in more detail using the checkerboard microdilution method (Moody, 2004), according to the Clinical Microbiology Procedures Handbook with slight modifications (Isenberg, 2004). In detail, serial twofold dilutions of the phytochemicals and the antibiotics in combination were processed in 96-well microtiter plates (sterile, F, Carl Roth). Hence, the antibiotic of the combination was diluted vertically from columns 1–9 (resulting concentrations depends on chosen antibiotic) while the phytochemical was pipetted horizontally and diluted from A to F resulting in concentrations between 400 and  $12.5 \mu\text{g ml}^{-1}$ . Ten microliters of bacterial suspension with OD 0.05 were added to the wells containing 90  $\mu\text{l}$  compound combinations, MH-II-medium alone or MH-II-medium with solvent (max. concentration 2% (v/v)).

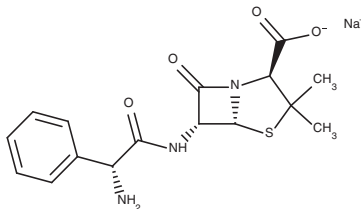
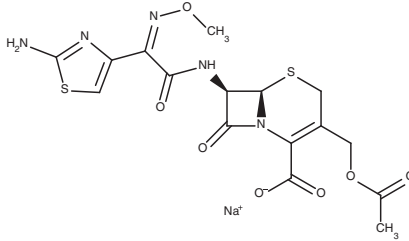
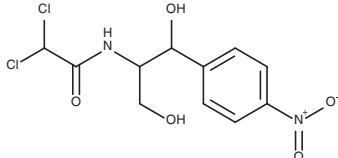
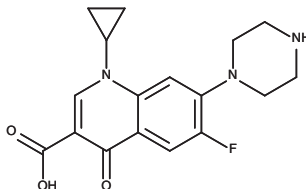
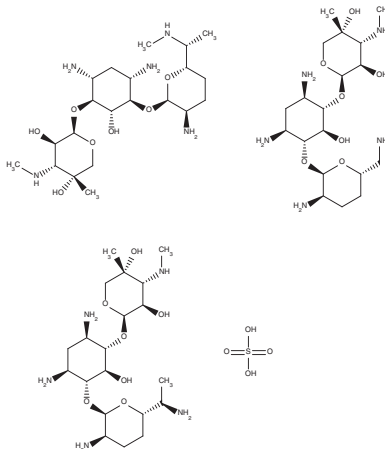
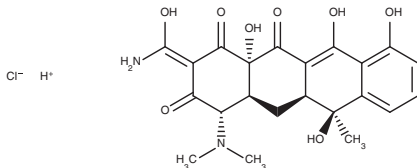
The plates were covered and closed with parafilm to avoid evaporation and then incubated at  $37^{\circ}\text{C}$  for 24 h. The lowest concentration that inhibits visible growth for each combination was determined.

**TABLE 1** List of phytochemicals and antibiotics used in this study

Antimicrobial	Manufacturer	Structure	CAS-number
Genistein (Gin)	Cayman Chemical		446-72-0
Daidzein (Da)	Alfa Aesar		486-66-8
Myricetin (Myr)	Carbosynth		529-44-2
3-hydroxy-6-methoxyflavone (6-MF)	Carbosynth		93176-00-2
(-)-Epigallocatechin gallate (EGCG)	Cayman Chemical		989-51-5
(-)-Epicatechin (EC)	Cayman Chemical		490-46-0
Gallic acid (GA)	Cayman Chemical		149-91-7

(Continues)

TABLE 1 (Continued)

Antimicrobial	Manufacturer	Structure	CAS-number
Ampicillin-Na (Amp)	Sigma Aldrich		69-52-3
Cefotaxime-Na (Cef)	Cayman Chemicals		64485-93-4
Chloramphenicol (Chlor)	VWR		56-75-7
Ciprofloxacin (Cip)	Alfa Aesar		85721-33-1
Gentamicin sulphate (Gent)	Carl Roth		1405-41-0
Tetracycline-HCl (Tet)	VWR		64-75-5

The fractional inhibitory concentration index (FICI) was calculated by the following equation:

$$\frac{A}{MIC A} + \frac{B}{MIC B} = FICI \quad (1)$$

In Formula (1), A stands for the MIC of the antibiotic in combination with the phytochemical compound, MIC A corresponds to MIC of the antibiotic alone and the same for B, the phytochemical, respectively. FICI values of 0.5 or less indicate as synergistic activity; as partial synergism, when

**TABLE 2** Antimicrobial resistances of examined isolates and their origin. \* 3/4MRGN classify Gram-negative bacterial isolates into resistance groups if they are resistant to three or all of the following groups of antibiotics: piperacillin as a penicillin derivative, cephalosporin with an extended spectrum, carbapenems and fluoroquinolones (Kaase 2016)

Strain	Database number	Origin	Resistances/ type strain
<i>Acinetobacter baumannii</i>	ATCC 19606 (PBIO2202)	Human	Intrinsic/type strain
	DSM 9308 (PBIO2212)	Human	Intrinsic/type strain
	PBIO721	Fly	Intrinsic
<i>Escherichia coli</i>	ATCC 25922 (PBIO904)	Human	EUCAST-Ref. strain
	PBIO730	Blackbird	ESBL
	PBIO1442	Human	ESBL/3MRGN*
<i>Klebsiella pneumoniae</i>	ATCC 700603 (PBIO2010)	Human	ESBL/EUCAST-Ref. strain
	PBIO1455	Human	3MRGN*
	PBIO1990	Human	4MRGN*
<i>Pseudomonas aeruginosa</i>	ATCC 27853 (PBIO903)	Human	EUCAST-Ref. strain
	PBIO712	Fly	Intrinsic
	PBIO2208	Human	Intrinsic
<i>Staphylococcus aureus</i>	ATCC 29213 (PBIO901)	Human	EUCAST-Ref. strain
	PBIO483	Human	MRSA

FICIs were between 0.5 and 1.0; FICI values up to 2.0 were named as indifferent, whereas values higher than 2.0 were set as antagonism (Cui et al., 2012; Osterburg et al., 2009). The FICIs represent the mean of three independent experiments, whereas in cases of more than one synergistic, partial synergistic or antagonistic combinations always the lowest FICI (indicates the best combination) was selected.

A purity control via plating of 4 µl from random wells on BD™ CHROMagar™ Orientation Medium was performed for one assay of each strain and day to assure that no cross contamination occurred.

### Time-kill assay

Time-kill assays were performed as described by Jayaraman et al., (2010). Briefly, the best effective combinations obtained in the checkerboard assays were selected and tested against *A. baumannii* (PBIO2202), one of the bacterial species considered by the 'WHO's priority list for research and development of new antibiotics' (Tacconelli, 2017). Tubes filled with MH-II-medium, containing test solutions of single substances with various volumes regarding their test concentrations as well as their solubilities and their combinations, as well as a

growth control only with solvents, were prepared. Based on the MICs, sub-inhibitory antibiotic concentrations (approx. ¼ MIC) were tested. Since the highest tested concentration of the phenolics did not result in a detectable MIC, we used ½ of the highest investigated concentration for this assay.

Bacterial suspension was adjusted to OD 0.5 and diluted 1 : 10 in MH-II-medium. The prepared tubes have been inoculated with 150 µl of diluted bacterial suspension resulting in  $5 \times 10^5$  CFU per ml in a total volume of 15 ml. Samples were collected at 0, 2, 4, 8 and 24 h and plated on LB agar to count the viable colonies after 24 h of incubation at 37°C.

Synergy effects were declared as more than 100-fold decrease (more than two log<sub>10</sub> steps) of CFU per ml by the combinations compared to the most active compound alone. An increase of  $\geq 2$  log<sub>10</sub> CFU per ml of surviving cells is defined as antagonism and a counting between is declared as indifferent (Akinyele et al., 2017). Experiments were performed as independent replicates.

### Statistical analysis

Values are represented as mean ± SD. All analyses were done as three biological independent experiments.

## RESULTS

### Antimicrobial sensitivity test

We investigated the susceptibility of five bacterial strains (*Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*), each with three different isolates, against five classes of antibiotics alone and in combination with seven phytochemicals. In accordance with The European Committee on Antimicrobial Susceptibility Testing (EUCAST), the antibiotic activities based on their MIC were categorized as sensitive, intrinsic resistant and resistant as shown in Table S1. The investigated strains showed different resistance patterns mostly against ampicillin, cefotaxime, chloramphenicol, ciprofloxacin and gentamicin with at least 50% of tested isolates being resistant including intrinsic resistant strains, thereof the most against tetracycline. No bacterial isolate was susceptible to all of the antibiotics investigated.

The phytochemicals alone had no inhibitory effects in tested concentrations (12.5–400  $\mu\text{g ml}^{-1}$ ), except epigallocatechin gallate (EGCG) against *S. aureus* (PBIO483, PBIO901) with a MIC of 200  $\mu\text{g ml}^{-1}$  (data not shown).

### Initial testing

After preliminary testing of one isolate of each strain, it was possible to select interesting combinations with potential partial synergistic effects based on the FICI values. These tests were carried out with a constant concentration of the phenolic compound that was always 400  $\mu\text{g ml}^{-1}$ . According to the equation above, this constant concentration results always in a value of 1 for calculation of summand B (the phytocompound). If you take this into account in Formula 1, additive effects appear in this special test setting when the FICI is between 1 and 1.5 and therefore we regarded these combinations as more interesting for further tests. Combinations of antibiotics and 400  $\mu\text{g ml}^{-1}$  phytocompound, which were declared indifferent due to bacterial growth up to the MIC of the antibiotic, gave values of 1.5–2. Values higher than 2 determine potential antagonisms due to the bacterial growth above the inhibiting antibiotic concentration alone. In total, there were 24 combinations with FICI values between 1.0 and 1.5, meaning a potential synergism, 30 ensembles showed values above 2.0, representing a possible antagonism, and 134 setups resulted in no changes (see Table S2).

The phytochemical with the most combinatorial hits was epigallocatechin gallate with eight hits followed by 3-hydroxy-6-methoxyflavone with six hits, myricetin with

four hits and genistein as well as daidzein with three hits. No potential synergism was observed for epicatechin and gallic acid in any combination nor bacteria tested. Instead, combinations with epicatechin with eight combinatorial hits as well as gallic acid with five hits were mostly antagonistic (see Table S2).

The combination with the broadest spectrum against different bacterial species was epigallocatechin gallate with tetracycline and cefotaxime, respectively, both of which were effective against two different species (*A. baumannii* and *S. aureus* or *P. aeruginosa*). In contrast, chloramphenicol in combination with daidzein showed the most antagonistic effects in different species including *E. coli*, *S. aureus* and *P. aeruginosa*. For *E. coli*, no finding indicated a synergistic combination while nine test setups implied synergisms for *A. baumannii* based on FICI values lower than 1.5 (see Table S2).

### Checkerboard assay

Based on the initial testing, various combinations were picked for further confirming investigations using checkerboard assays. These assays were performed including additional multi-resistant isolates of the tested species. Four of six investigated combinations against *A. baumannii* PBIO2202 yielded FICI below 0.5, which is declared as synergism. The tested combinations against *A. baumannii* isolates PBIO721 and 2212 led to decreased amounts of antibiotics as well, the FICI being between 0.46 and 1.5, 1.63 was obtained once (Table 3). Especially, the concentrations needed for an inhibitory effect of cefotaxime, gentamicin and tetracycline could be reduced in combination with epigallocatechin gallate. For example, on average, the use of cefotaxime could be lowered from 128 to 32  $\mu\text{g ml}^{-1}$  together with 100  $\mu\text{g ml}^{-1}$  epigallocatechin gallate, for gentamicin from 27 to 8  $\mu\text{g ml}^{-1}$  in combination with 50  $\mu\text{g ml}^{-1}$  and for tetracycline the addition reduced the MIC from 8 to 3.3 or 4.7  $\mu\text{g ml}^{-1}$  with epigallocatechin gallate or myricetin. The combination with genistein or daidzein decreased the needed gentamicin on average from 27 to 4 and 8  $\mu\text{g ml}^{-1}$ , respectively (Table 3).

Comparable data were generated with the combinations of ciprofloxacin with epigallocatechin gallate and tetracycline with epigallocatechin gallate or myricetin against *P. aeruginosa*. No explored combination yielded in FICI lower than 0.5, but all test setups resulted in values between 0.52 and 1.56. While they ranged between 0.53 and 0.77 for PBIO903, the values of 1.31–1.56 could be calculated for PBIO712 and PBIO2208 in two of six cases (Table 3). These FICI mean, it was possible to reduce the antibiotic MIC from 0.29 to 0.10  $\mu\text{g ml}^{-1}$  (Cip+EGCG)

**TABLE 3** Checkerboard assay results of examined antibiotics ampicillin (Amp), cefotaxime (Cef), chloramphenicol (Chlor), ciprofloxacin (Cip), gentamicin (Gent) and tetracycline (Tet) combined with phenolic compounds epigallocatechin gallate (EGCG), myricetin (Myr), genistein (Gin), daidzein (Da) and 3-hydroxy-6-methoxyflavone (6-MF) against different isolates of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*

Components	MIC ( $\mu\text{g ml}^{-1}$ )	MIC <sub>C</sub> ( $\mu\text{g ml}^{-1}$ )	FIC index (interpretation)	MIC ( $\mu\text{g ml}^{-1}$ )	MIC <sub>C</sub> ( $\mu\text{g ml}^{-1}$ )	FIC index (interpretation)	MIC ( $\mu\text{g ml}^{-1}$ )	MIC <sub>C</sub> ( $\mu\text{g ml}^{-1}$ )	FIC index (interpretation)	MIC ( $\mu\text{g ml}^{-1}$ )	MIC <sub>C</sub> ( $\mu\text{g ml}^{-1}$ )	FIC index (interpretation)
<i>A. baumannii</i> (PBIO721)												
Tet	3 <sup>‡</sup>	2	1.17 (I)	8 <sup>‡</sup>	3.3	0.48 (S)	7 <sup>‡</sup>	4	0.70 (P)	<i>A. baumannii</i> (PBIO2212)		
EGCG	400	200		400	2.5		400	50				
Tet	3 <sup>‡</sup>	3	1.13 (I)	8 <sup>‡</sup>	4.7	0.84 (P)	7 <sup>‡</sup>	7	1.5 (I)			
Myr	400	50		400	100		400	200				
Gent	3 <sup>#</sup>	1	0.58 (P)	27 <sup>†</sup>	4	0.65 (P)	43 <sup>†</sup>	9	0.46 (S)			
EGCG	400	100		400	200		400	100				
Gent	3 <sup>#</sup>	3	1.25 (I)	27 <sup>†</sup>	4	0.40 (S)	43 <sup>†</sup>	32	0.87 (P)			
Gin	400	100		400	100		400	50				
Gent	3 <sup>#</sup>	2	0.92 (P)	27 <sup>†</sup>	8	0.42 (S)	43 <sup>†</sup>	27	1.63 (I)			
Da	400	100		400	50		400	400				
Cef	16 <sup>‡</sup>	8	0.63 (P)	128 <sup>‡</sup>	32	0.50 (S)	7 <sup>‡</sup>	4.7	0.92 (P)			
EGCG	400	50		400	100		400	100				
<i>Pseudomonas aeruginosa</i> (PBIO712)												
Tet	53 <sup>‡</sup>	27	0.76 (P)	64 <sup>‡</sup>	32	0.53 (P)	48 <sup>‡</sup>	26.7	1.06 (I)	<i>Pseudomonas aeruginosa</i> (PBIO2208)		
EGCG	400	100		400	12.5		400	200				
Tet	53 <sup>‡</sup>	27	1.01 (I)	64 <sup>‡</sup>	32	0.63 (P)	48 <sup>‡</sup>	26.7	1.56 <sup>2</sup> (I)			
Myr	400	200		400	50		400	400				
Cip	0.13 <sup>*</sup>	0.04	1.31 (I)	0.25 <sup>*</sup>	0.13	0.77 (P)	0.5 <sup>†</sup>	0.125	0.75 (P)			
EGCG	400	400		400	100		400	200				
<i>Klebsiella pneumoniae</i> (PBIO1455)												
Tet	11 <sup>†</sup>	4	0.49 <sup>2</sup> (S)	13 <sup>†</sup>	7	0.66 (P)	13 <sup>†</sup>	10.7	1.32 (P)	<i>Klebsiella pneumoniae</i> (PBIO2210)		
EGCG	400	50		400	50		400	200				
Tet	11 <sup>†</sup>	3.3	0.43 (S)	13 <sup>†</sup>	13	2 (I)	13 <sup>†</sup>	8	0.74 (P)			
Myr	400	50		400	400		400	50				
Cip	8 <sup>†</sup>	8	2 (I)	8 <sup>†</sup>	8	2 (I)	0.3 <sup>#</sup>	0.17	0.82 (P)			
EGCG	400	400		400	400		400	100				
Cip	8 <sup>†</sup>	6.7	1.34 (I)	8 <sup>†</sup>	8	2 (I)	0.3 <sup>#</sup>	0.25	0.86 (P)			
Myr	400	200		400	400		400	12.5				

(Continues)



TABLE 3 (Continued)

Components	MIC ( $\mu\text{g ml}^{-1}$ )	MIC <sub>c</sub> ( $\mu\text{g ml}^{-1}$ )	FIC index (interpretation)	MIC ( $\mu\text{g ml}^{-1}$ )	MIC <sub>c</sub> ( $\mu\text{g ml}^{-1}$ )	FIC index (interpretation)	MIC ( $\mu\text{g ml}^{-1}$ )	MIC <sub>c</sub> ( $\mu\text{g ml}^{-1}$ )	FIC index (interpretation)
<i>S. aureus</i> (PBIO483)									
Amp	256 <sup>†</sup>	17.3	0.32 (S)	32	5.3	0.29 (S)			
EGCG	200	50		200	25				
Amp	256 <sup>†</sup>	53.3	0.46 (S)	32	12	0.50 (S)			
Myr	400	100		400	50				
Amp	256 <sup>†</sup>	64	0.75 (P)	32		n.a.			
Gin	400	200		400					
Chlor	19 <sup>†</sup>	4	0.46 (S)	32 <sup>‡</sup>	8	0.50 (S)			
EGCG	200	50		200	50				
Chlor	19 <sup>†</sup>	4.7	0.75 (P)	32 <sup>‡</sup>	26.7	1.83 (I)			
Myr	400	200		400	400				
Cef	8 <sup>†</sup>	4	0.53* (P)	1	0.5	0.63 (P)			
6-MF	400	12.5		400	50				
Cef	8 <sup>†</sup>	1	0.25 (S)	1	0.5	0.63 <sup>2</sup> (P)			
EGCG	200	25		200	25				

Values are shown as mean of three (except stated differently). Classification according to EUCAST description: † = resistant; ‡ = intrinsic resistant; \* = intermediate; # = susceptible; n.a. = not available; <sup>1</sup> according to CLSI-Guideline. MIC = minimal inhibitory concentration of compounds alone; MIC<sub>c</sub> = minimal inhibitory concentration of components in combination; S = synergism; P = partial synergism; I = indifferent; <sup>2</sup>n = 2.

as well as from 51.7 to 28.6  $\mu\text{g ml}^{-1}$  (Tet+EGCG or Myr) across all tested strains.

*Klebsiella pneumoniae* could also be influenced significantly by the use of antibiotics together with epigallocatechin gallate or myricetin. The FICI ranged between 0.43 and 2, while two combinations led to synergism, five showed FICI between 0.66 and 0.86, five test setups showed neither antagonism nor synergism (Table 3). The composite of tetracycline and epigallocatechin gallate was effective against all isolates and decreased antibiotic MIC, on average from 12.3 to 7.2  $\mu\text{g ml}^{-1}$ . The combinations ciprofloxacin plus epigallocatechin gallate or myricetin were nearly ineffective, especially against the highly resistant isolates PBIO1455 and 1990.

The strongest effects of combinations of antibiotic with phenolic substances could be achieved in *S. aureus*. Altogether, four out of seven tested combinations were synergistic against PBIO483 as well as three out of six against PBIO901 namely ampicillin plus epigallocatechin gallate or myricetin, chloramphenicol plus epigallocatechin gallate as well as cefotaxime plus epigallocatechin gallate (not for PBIO901) (Table 3).

In particular, the concentrations of beta lactams and ciprofloxacin could be reduced by more than 90% and 79%, respectively, in combination with epigallocatechin gallate. Tested combinations of antibiotic and myricetin as well as 3-hydroxy-6-methoxyflavone were also effective but much less than the composites with epigallocatechin gallate.

Overall, the test setups against *E. coli* isolates, not one combination, had a noteworthy effect on the decrease of antibiotic MIC.

## Time-kill curves

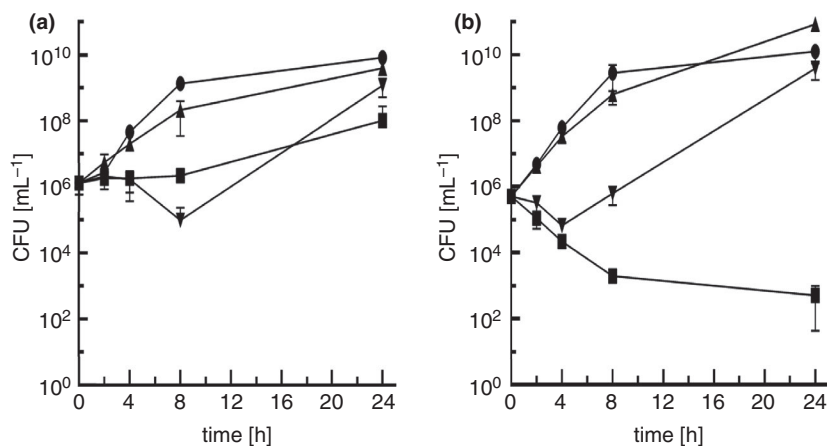
Since *A. baumannii* is a strain with high priority for research and development of antimicrobial drugs around the world, time-kill curves were also performed with combinations that showed positive results in the checkerboard

assay. Nor the antibiotic neither the phenolic substance alone showed any significant reduction of CFU per ml ( $>2 \log_{10}$ ) compared to the control after 24 h incubation. However, there was a decrease in the number of bacteria noticeable during an incubation period of 4 h and 8 h or 8 h and 24 h when *A. baumannii* was treated with antibiotics alone (Figure 1). The combination of cefotaxime and epigallocatechin gallate led to 1–2  $\log_{10}$  reduction of viable cells at 24 h compared to the compounds alone (Figure 1a). The synergistic effects of gentamicin and epigallocatechin gallate were more pronounced. There was a subsequent decrease in CFU per ml over time. After just 8 h, there was a  $>2 \log_{10}$  reduction of the bacterial count, which reached its maximum at 24 h with a 5–6  $\log_{10}$  decrease (Figure 1b).

## DISCUSSION

Due to the increasing bacterial resistance spreading worldwide and the lack of development of new antibiotic classes or antimicrobial substances, new strategies to combat bacterial diseases are necessary. One option lies in the combination of either antibiotics among themselves or antibiotics with phytochemicals to exploit potential synergistic effects. It is reported that plants and their extracts provide inhibitors against pathogens. Hence, this study focused on phytochemicals seven of which have yet to be investigated, and used in combinations with established antibiotics to be used against highly resistant bacteria, which are known to be responsible for nosocomial or urinary tract infections. These infections are complicated to treat, which results in an urgent need for new treatment strategies (Pendleton et al., 2013; Santajit & Indrawattana, 2016).

Based on this fact, for the investigation, the so-called ESKAPE strains, except *Enterococcus faecalis*, were chosen, each with three resistant isolates and tested against five different classes of antibiotics combined with seven



**FIGURE 1** Time-kill curves from *A. baumannii* (PBIO2202) for synergistic effects of EGCG (200  $\mu\text{g ml}^{-1}$ ) with (a) cefotaxime (32  $\mu\text{g ml}^{-1}$ ); (b) gentamicin (4  $\mu\text{g mL}^{-1}$ ). Colony forming units detected over time for growth control (●), after treatment with EGCG (▲), treatment with antibiotic (▼) or treatment with EGCG and antibiotic in combination (■). Data are shown as mean of three replicates with standard deviation

phytochemicals. After an initial testing, the most interesting combinations were selected. These included six different combinations of substances for *A. baumannii*, four for *K. pneumoniae*, three for *P. aeruginosa* and seven for *S. aureus*. Although there was not one positive initial tested antibiotic–phenolic compound combination for *E. coli*, 12 test setups were examined by checkerboard assays because of particularly relevant treatment needs of *E. coli* infections.

It could be confirmed that combinations of antibiotics with secondary plant compounds, particularly epigallocatechin gallate, are often associated with decreased MIC for the antibiotics in multiple bacterial strains, for example *P. aeruginosa* or *A. baumannii* as well as *K. pneumoniae* or *S. aureus*, as previously described (Jayaraman et al., 2010; Lee et al., 2017; Liu et al., 2019; Sudano Roccaro et al., 2004).

Altogether, strains of two species (*A. baumannii* and *S. aureus*) could be reclassified in our experiments from EUCAST classification ‘Resistant’ to ‘Susceptible’, one strain (*K. pneumoniae*) could be influenced from ‘Susceptible, increased exposure’ to ‘Susceptible’ and *P. aeruginosa* switched from ‘Resistant’ to ‘Susceptible, increased exposure’ by treating with antibiotic–phenolic compound combinations (The European Committee on Antimicrobial Susceptibility Testing., 2020). The potent combinations, as shown in Table 4, were gentamicin plus epigallocatechin gallate, daidzein or genistein, respectively, against *A. baumannii*, chloramphenicol together with epigallocatechin gallate as well as myricetin against *S. aureus* and ciprofloxacin combined with epigallocatechin gallate or myricetin against ESBL-producing *K. pneumoniae* and *P. aeruginosa*. However, this combination did not work for the highly resistant carbapenemase-producing *K. pneumoniae* isolates PBIO1455 and PBIO1990.

The strongest effect on the reduction of used antibiotic could be generated by treatment of *S. aureus*, even though the strain is a multidrug-resistant MRSA. The obtained FICI values reached down to 0.2 and were calculated for four combinations as synergism. Additionally, there was not one combination without any growth inhibition effect. Surprisingly, in this study, no additional effects between ciprofloxacin and pre-tested phytocompounds were detectable, in contrast to Abreu et al., (2017). That might be owed to differences in genetic determinants of the strains. Instead, it was observable that the phytocompounds improved the efficacy of beta lactams as well as the efficacy of chloramphenicol. This may be contributed by increased interruption of cell wall based on inhibition of transpeptidation by beta lactams coupled with potential inhibition of efflux systems such as the efflux pump NorA as described by Braga et al., (2005) for alkaloids as well as downregulation of beta lactamases through phenolic compounds

**TABLE 4** Antimicrobial resistance classification of different bacterial species according to EUCAST of investigated antibiotics that could be changed in combination with a natural compound

Species	Tested antibiotic	EUCAST classification	Antibiotic–natural compound combination	Needed amount of natural compound ( $\mu\text{g ml}^{-1}$ )	Changed EUCAST classification
<i>Acinetobacter baumannii</i>	Gentamicin	Resistant	Gentamicin + EGCG	100–200	Susceptible
			Gentamicin + daidzein	50–100	Susceptible
			Gentamicin + genistein	100	Susceptible
<i>Staphylococcus aureus</i>	Chloramphenicol	Resistant	Chloramphenicol + EGCG	50	Susceptible
			Chloramphenicol + myricetin	200	Susceptible
<i>Klebsiella pneumoniae</i>	Ciprofloxacin	Susceptible, increased exposure	Ciprofloxacin + EGCG	100	Susceptible
			Ciprofloxacin + myricetin	12.5	Susceptible
<i>Pseudomonas aeruginosa</i>	Ciprofloxacin	Resistant	Ciprofloxacin + EGCG	200	Susceptible, increased exposure
			Ciprofloxacin + myricetin	200	Susceptible, increased exposure

(Miklasińska-Majdanik et al., 2018; Zhao et al., 2002). Especially, epigallocatechin gallate enhanced the penetration of small molecules because of disruption of cell wall synthesis (Zhao et al., 2001). The same mechanisms could be assumed for myricetin and genistein because of their structural similarity.

Multiple synergistic inhibition could be observed for *K. pneumoniae* by treatment of tetracycline combined with epigallocatechin gallate or myricetin, noteworthily even against highly resistant isolates. Recent studies reported a synergistic behaviour due to changing the capsule structure of *K. pneumoniae* as well as inhibition of efflux pumps from the resistance-nodulation-cell division (RND)-family (AcrAB) by epigallocatechin gallate (Dey et al., 2016; Mazzariol et al., 2002). Both mechanisms are able to increase bactericidal activity of tetracycline, because of intracellular accumulation and could explain the enhanced susceptibility of different bacterial strains expressing RND-efflux pumps.

Another noteworthy result should be mentioned is that tetracycline combined with epigallocatechin gallate and myricetin successfully inhibited the growth of intrinsically resistant strains. *Pseudomonas aeruginosa* as only a third of the initially used antibiotic amount was necessary when phenolic compounds were added. By expression of efflux pump systems, namely the AcrAB-TolC as a part of the RND-family, *P. aeruginosa* is naturally resistant against tetracycline treatment (Gibbons, 2008). Since *P. aeruginosa* has efflux pumps from RND-family, it seemed to be that the previously described mechanisms could also apply to this strain. Because of structural similarity to epigallocatechin gallate, the same mode of action could be possible for myricetin.

Comparable observations could be obtained by the treatment of *A. baumannii*, also a highly intrinsic resistant Gram-negative bacterium. A reduction of almost 70% of tetracycline use, as well as 75% of cefotaxime was detectable combined with epigallocatechin gallate. Furthermore, for the aim of investigating combined influences against ESKAPE strains completely, new substances such as daidzein or genistein were found as active additives with antibiotics. Often these substances showed reduced bacterial growth without FICI being declared as synergistic, but partial synergistic ( $0.5 \leq \text{FICI} \leq 1.0$ ).

With this investigation, it could be confirmed that epigallocatechin gallate behaved synergistic together with beta lactams (EGCG + Cef) in accordance with Lee et al (2017). This research as well as others (Nakayama et al., 2013, 2015; Osterburg et al., 2009; Pannek et al., 2006) found synergisms of carbapenems and epigallocatechin gallate against resistant strains by targeting again, a RND-type tripartite efflux pump, named AdeABC. Since daidzein and genistein share structural similarities with epigallocatechin gallate, the obtained results could be explained.

However, the retained ineffectiveness of ampicillin in any combination against *A. baumannii* is not affected by this assumption. Normally, an increased bactericidal effect should apply for ampicillin, as it penetrates through the damaged cell wall just like cefotaxime. Both are inactivated by a beta lactamase, which is the oxacillinase, a class D carbapenemase, in *A. baumannii*. Therefore, it could be suggested that the tested phytochemicals may not affect beta lactam enzymes in *A. baumannii* in the same way.

Additionally, these investigations revealed noticeably more synergistic phytochemical-antibiotic combinations against *A. baumannii* than against the other tested Gram-negative bacterial strains. This could be due to different expression of efflux pumps (Fernández & Hancock, 2012) and their differing inhibition intensities, as well as target affinity of the phytochemicals according to the explained mode of actions above. Pumps, only present in *A. baumannii* are, for example, AbeM (multidrug and toxic compound extrusion) and AdeABC (resistance-nodulation-cell division) which mediates resistances against aminoglycosides, fluoroquinolones and other. Considering that gentamicin showed the most synergistic effects with phytochemicals, it can be speculated that the AbeM and AdeABC pumps have a special role in the synergistic effects. Besides, *A. baumannii* shows a widespread intrinsic resistance against antibiotics due to a low number of influx systems, such as porins, which also do not exist in other Gram-negative bacteria. Due to the difficulty of penetrating antibiotics, the loss of porins leads to resistances, for example against carbapenems via CarO (Limansky et al., 2002). Therefore, future investigations should focus on whether and by which phytochemicals these multidrug resistance mechanisms could be reversed either by inhibition of efflux pumps and, for example, beta lactamases or even by stimulation of specific porins.

The thesis of affecting especially efflux and influx systems by phytochemicals is affirmed by the fact that the substances alone show either no or little bactericidal effects.

Moreover, it was unfortunately impossible to find a combination between antibiotic and phytochemical that inhibits *E. coli*, which is contrary to recent publications. Cui et al. found epigallocatechin gallate and cefotaxime as a synergistic combination. They suggested that exogenous and endogenous reactive oxygen species are responsible for the synergistic effects of epigallocatechin gallate and beta lactams, examined with cefotaxime, because of additive disturbance of the cell wall (Cui et al., 2012). However, the results could be obtained only with non-therapeutically relevant amounts of EGCG ( $1.5 \text{ mg ml}^{-1}$ ).

Current practice that is also used in this investigation is assessing the synergistic or antagonistic effects of substance combinations with calculation of fractional inhibitory concentration values (equation above). Unfortunately, this

procedure is directly dependent on chosen start concentrations for bacterial susceptibility testing. That means, if a higher, mostly clinically negligible, test concentration is chosen, the resulting FICI values decrease substantially, therefore seeming to be significantly synergistic. This could also be one reason, why the results of some combinatorial testing in this study are not in accordance with the findings in the literature.

With this investigation it could be shown that different, but structurally similar phenolic substances in combination with antibiotics can inhibit bacterial growth. However, it was not possible to observe which structural elements are required for bactericidal synergisms with antibiotics in detail. Based on previous publications (Cho et al., 2011; Cui et al., 2012; Hu et al., 2001; Isogai et al., 2001; Lee et al., 2017) and the confirmation in this study epigallocatechin gallate turns out as a strong synergistic phytochemical in combination with different antibiotic classes against many bacterial strains. The achieved data led to the hypothesis that a substituent in position two of the flavonol scaffold is necessary, preferably a gallate residue. That is because positive outcomes could often be yielded for myricetin, whereas for genistein or daidzein (compounds without substitutions in position two) only in some cases. Stronger reductions of structural complexity as presented by epicatechin or gallic acid, the simplest molecules considered in this investigation, is likewise disadvantageous. These substances showed mostly antagonisms. Further examination will have to take into account whether the gallic acid ester is the reason for the stronger effects of epigallocatechin gallate compared to myricetin. Finally, all the reported antagonistic results should also be paid attention to, as they may reveal significant interactions between food and antibiotics. Due to the unknown mechanisms of action, further investigations are also necessary in this regard.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available in the supplementary material to this article.

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### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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