

Antibacterial Activity of Extract, Fractions and Four Compounds Extracted from *Piper solmsianum* C. DC. var. *solmsianum* (Piperaceae)

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Piper solmsianum C. DC. var. *solmsianum* (Piperaceae) is a shrub commonly found in areas with wet tropical soils. Other *Piper* species have been used in folk medicine as antitumoral and antiseptic agents. We studied the crude methanolic extract, some organic fractions and compounds isolated from this plant for possible antimicrobial activity against Gram-positive and Gram-negative bacteria. The bioautographic assays disclosed three inhibition zones. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined showing excellent activity, particularly against the Gram-positive bacteria (*Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus saprophyticus* and *Streptococcus agalactiae*). It appears that the antimicrobial activity of *Piper solmsianum* is related mainly to the presence of conocarpan and eupomatenoid-5 (neolignans). However another, as yet unidentified, active compound could also be extracted from the plant.

Key words: *Piper solmsianum*, Antimicrobial Activity, Conocarpan, Eupomatenoid

Introduction

The Piperaceae is a large family of plants, which have been used in a variety of medicinal and pest control applications (Chauret, 1996). The family comprises about 10 genera and approx. 2000 species. These are plants with a mainly tropical distribution and most of them are herbaceous (Evans, 1991). The two larger genera of the family, *Piper* and *Peperomia*, are well-represented in Brazilian flora (Joly, 1998).

The plants belonging to the genus *Piper* are widely used in folk medicine for the treatment of rheumatism, toothache, epilepsy and stomach ache (Hou *et al.* 1989), anxiety disorders (Singh and Singh, 2002; Tonks, 2003), as anti-inflammatories (Sosa *et al.*, 2002), and antioxidants (Choudhary and Kale, 2002). Besides these uses, it has been reported that extracts and/or essential oils of many species of this genus are potential antimicrobial sources (Bruneton, 1991; Costa, 1994; Costantin *et al.*, 2001; Dorman and Deans, 2000; Holetz *et al.*, 2002; Lentz *et al.*, 1998; Lopez *et al.*, 2001; Orjala *et al.*, 1993; Perez and Anesini, 1994; Shitut *et al.*, 1999; Tirillini *et al.*, 1996).

These biological properties can generally be attributed to the presence of lignans and/or amides, such as alkylic or olefinic isobutylamides (Chauvet, 1996; Freixa *et al.*, 2001; Maxwell *et al.*, 1999).

Piper solmsianum (syn. *P. leucathum* or *P. santosanum*) is a shrub, known popularly as “pariparoba” in Brazil. The biological properties of this plant have not yet been completely investigated. Phytochemical studies have indicated the presence of aliphatic hydrocarbons, monoterpenes, sesquiterpenes, arylpropanoids, neolignans (Martins *et al.*, 2000; Moreira *et al.*, 1995, 2001) and flavonoids (Campos *et al.*, 2005).

In this study, we investigated the antibacterial activity of *Piper solmsianum* by using minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) methods.

Material and Methods

Plant material

Piper solmsianum C. DC. var. *solmsianum* (Piperaceae) was collected (May, 2001) in Ponta

Grossa, in the state of Paraná, Brazil. A voucher specimen is deposited in the “Barbosa Rodrigues Herbarium” under the number HBR 52537.

Phytochemical analysis

The plant *P. solmsianum* was extracted, fractionated and purified as previously described by Campos and co-workers (2005).

The leaves were macerated at room temperature for one week in methanol. The crude methanolic extract (CME) was concentrated in an evaporator under reduced pressure. The residue was then suspended in water and successively partitioned with *n*-hexane, dichloromethane (DCM) and ethyl acetate (EtOAc), affording respective fractions (Hexane Fr., DCM Fr. and EtOAc Fr.).

The hexane fraction was fractionated using silica gel column chromatography with the following elutents: a gradient of *n*-hexane, *n*-hexane/ethyl acetate, ethyl acetate/methanol with increasing polarity and methanol. Sub-fractions were later re-chromatographed as in previous cases and eluted with *n*-hexane, *n*-hexane/ethyl acetate gradient and ethyl acetate, yielding eupomatenoid-3 (**1**) and eupomatenoid-5 (**2**).

The dichloromethane fraction was similarly chromatographed and eluted with increasing amounts of *n*-hexane in ethyl acetate and ethyl acetate/methanol, yielding conocarpan (**3**).

Also, the ethyl acetate fraction was fractionated on a silica gel column eluted with a chloroform/methanol gradient giving several sub-fractions, some of which exhibited a positive test for flavonoids with FeCl_3 , and with a mixture of ethyl acetate (25 mL)/acetone (8 mL)/water (2 mL) yielding the flavone orientin (**4**) together with two flavonoids not yet identified.

The identification of the isolated compounds was performed by analyses of melting points, IR spectra, ^1H and ^{13}C NMR spectra as well as comparison of the physical data with those reported in the literature.

Thin layer chromatography (TLC)

Silica gel 60 F_{254} aluminium sheets (Merck) were used for TLC. Dilutions corresponding to 10 μg of the reference compounds and 20 μg of the plant extract and fractions were applied. The TLC plates were developed with four different solvent systems: *n*-hexane/ethyl acetate (85:15), *n*-hexane/ethyl acetate (9:1), chloroform/methanol (9:1)

and chloroform/methanol/water (65:35:5). The chromatograms were dried using a hair dryer in order to completely remove the solvents. All the TLC plates were run in duplicate; one of them was used as the reference chromatogram. UV-active spots were detected at 254 and 366 nm.

Microorganisms

To determine antibacterial activity, the following microorganisms were used:

Bacillus cereus (ATCC 14579), *Enterobacter cloacae* (ATCC 35030), *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 6538P), *Staphylococcus saprophyticus* (ATCC 35552) and *Streptococcus agalactiae* (ATCC 13813). These were purchased from the tropical culture collection of “André Tosello Technology and Research Tropical Foundation”, Campinas, State of São Paulo, Brazil.

Bioautography

A suspension of *Staphylococcus aureus* at a final concentration of 10^6 cells/mL in Mueller-Hinton agar (Merck) (inoculum), maintained at 37 °C, was applied to the developed plate. Chromatograms were placed on a hot plate maintained at 37 °C. Approx. 10 mL of the inoculum was rapidly distributed over the TLC plate (10 × 10 cm) using a sterile pipette. After solidification of the medium, the TLC plates were incubated overnight at 37 °C in polyethylene boxes lined with moist chromatography paper. The bioautograms were sprayed with an aqueous solution (2.5 mg/mL) of 2,3,5-triphenyltetrazolium chloride and incubated for 4 h at 37 °C. Clear inhibition zones were observed against a dark background.

Quantitative antimicrobial evaluation

The minimal inhibitory concentration (MIC) of the extract, fractions and compounds of *Piper solmsianum* was determined for Gram-positive and Gram-negative bacteria, using the two-fold serial agar dilution assay in concentrations ranging from 1 $\mu\text{g}/\text{mL}$ to 1000 $\mu\text{g}/\text{mL}$. The tested extracts were added to sterile Mueller-Hinton agar medium dissolved in dimethylsulfoxide (DMSO)/water (1:1). Solvent blanks were included.

The MIC values were taken as the lowest concentration of extract, fractions, or compounds that inhibited the growth of the organism after 24 h of

incubation at 37 °C and the minimal bactericidal concentration (MBC) was determined by subculture of the tube with inhibition in an appropriate agar plate. When the microorganism did not grow, the product was considered as bactericide.

Results and Discussion

The phytochemical investigation of *Piper solmsianum* led to the isolation and identification of four pure compounds: eupomatenoid-3 (**1**), eupomatenoid-5 (**2**), conocarpan (**3**) and orientin (**4**). Their structural elucidation was based on the comparison of the physical and spectral data (m.p., IR, ¹H and ¹³C NMR) with those reported in the literature. The compounds eupomatenoid-3 (**1**) (Maxwell *et al.*, 1999; Pessini *et al.*, 2003), eupomatenoid-5 (**2**) (Chauret *et al.*, 1996; Freixa *et al.*, 2001; Maxwell *et al.*, 1999; Pessini *et al.*, 2003), and conoconarpan (**3**) (Benevides *et al.*, 1999; Chauret *et al.*, 1996; Freixa *et al.*, 2001; Maxwell *et al.*, 1999; Pessini *et al.*, 2003) have been found in other species of *Piper*. However, we were the first to demonstrate the presence of the flavonoid orientin (**4**) in this genus (Campos *et al.*, 2005).

For the direct bioautographic assay, those solvent systems were selected that presented the best resolutions in the chromatograms; these were *n*-hexane/ethyl acetate (85:15) and chloroform/methanol (9:1).

A recent study carried out in our laboratories revealed that *Piper solmsianum* possesses potent

antifungal activity (Campos *et al.*, 2005). Also, in a preliminary study, it was verified that the plant extract was active against Gram-positive bacteria and therefore a bioautographic assay was performed with *Staphylococcus aureus* where inhibition zones were observed against microorganism, particularly in the chromatogram zones corresponding to the non-polar chemical constituents giving positive reactions for the neolignans.

We found three different inhibition zones. One corresponds to compound **2**, and the other is due to compound **3**, while the third inhibition zone consists of an unidentified compound. In the direct bioautographic assay, the activity found in the CME and in the hexane and DCM fractions indicated that compounds **2** and **3** and one other compound not yet identified must be present.

When the extract, fractions and pure compounds showed MIC values $\leq 1000 \mu\text{g/mL}$, they were considered active. Table I shows the results obtained by the *in vitro* agar dilution method. The starting material (CME) of *Piper solmsianum* inhibited the growth of the *B. cereus*, *S. aureus*, *S. saprophyticus* and *S. agalactiae* strains with MIC values of 10, 10, 30 and 6 $\mu\text{g/mL}$, respectively. This activity also was verified for the hexane and DCM fractions, however, the EtOAc fraction was practically inactive against the microorganisms tested, except for *B. cereus* and *S. agalactiae* with MIC values of 800 and 300 $\mu\text{g/mL}$, respectively.

Regarding the minimal bactericidal concentration, the profile of antimicrobial activity was, in

Table I. Antimicrobial activity of *Piper solmsianum* extract, fractions and compounds against Gram-positive and Gram-negative bacteria expressed as minimal inhibitory concentration (MIC).

Material tested	MIC [$\mu\text{g/mL}$]							
	Gram-positive bacteria				Gram-negative bacteria			
	<i>B.c.</i>	<i>S.a.</i>	<i>S.s.</i>	<i>S.ag.</i>	<i>E.cl.</i>	<i>E.c.</i>	<i>P.a.</i>	<i>S.t.</i>
CME	10	10	30	6	> 1000	> 1000	> 1000	> 1000
Hexane Fr.	20	20	100	10	> 1000	> 1000	> 1000	> 1000
DCM Fr.	20	9	30	6	> 1000	> 1000	> 1000	> 1000
EtOAc Fr.	800	> 1000	> 1000	300	> 1000	> 1000	> 1000	> 1000
1	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
2	6	3	> 1000	2	> 1000	> 1000	> 1000	> 1000
3	5	4	7	4	> 1000	> 1000	> 1000	> 1000
4	> 1000	200	600	1000	> 1000	> 1000	> 1000	> 1000
Amoxicillin	nt	nt	nt	nt	4	6	2	1
Vancomycin	0.7	2	2	0.8	nt	nt	nt	nt

CME, crude methanolic extract; Hexane Fr., hexane fraction; DCM Fr., dichloromethane fraction; EtOAc Fr., ethyl acetate fraction; **1**, eupomatenoid-3; **2**, eupomatenoid-5; **3**, conoconarpan; **4**, orientin; nt, not tested; *B.c.*, *Bacillus cereus*; *S.a.*, *Staphylococcus aureus*; *S.s.*, *Staphylococcus saprophyticus*; *S.ag.*, *Streptococcus agalactiae*; *E.cl.*, *Enterobacter cloacae*; *E.c.*, *Escherichia coli*; *P.a.*, *Pseudomonas aeruginosa*; *S.t.*, *Salmonella typhimurium*.

Table II. Antimicrobial activity of *Piper solmsianum* extract, fractions and compounds against Gram-positive and Gram-negative bacteria expressed as minimal bactericidal concentration (MBC).

Material tested	MBC [$\mu\text{g/mL}$]							
	Gram-positive bacteria				Gram-negative bacteria			
	<i>B.c.</i>	<i>S.a.</i>	<i>S.s.</i>	<i>S.ag.</i>	<i>E.cl.</i>	<i>E.c.</i>	<i>P.a.</i>	<i>S.t.</i>
CME	20	20	50	50	> 1000	> 1000	> 1000	> 1000
Hexane Fr.	30	30	> 1000	80	> 1000	> 1000	> 1000	> 1000
DCM Fr.	20	10	60	20	> 1000	> 1000	> 1000	> 1000
EtOAc Fr.	900	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
1	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
2	5	5	> 1000	6	> 1000	> 1000	> 1000	> 1000
3	6	6	10	8	> 1000	> 1000	> 1000	> 1000
4	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000

CME, crude methanolic extract; Hexane Fr., hexane fraction; DCM Fr., dichloromethane fraction; EtOAc Fr., ethyl acetate fraction; **1**, eupomatenoid-3; **2**, eupomatenoid-5; **3**, conocarpan; **4**, orientin; *B.c.*, *Bacillus cereus*; *S.a.*, *Staphylococcus aureus*; *S.s.*, *Staphylococcus saprophyticus*; *S.ag.*, *Streptococcus agalactiae*; *E.cl.*, *Enterobacter cloacae*; *E.c.*, *Escherichia coli*; *P.a.*, *Pseudomonas aeruginosa*; *S.t.*, *Salmonella typhimurium*.

general, similar to the MIC values, as expected (Table II).

No activity was observed against Gram-negative bacteria. This can be explained because the outer membrane of Gram-negative bacteria is known to present a barrier to the penetration of numerous antibiotic molecules, and the periplasmic space contains enzymes which are able of breaking down foreign molecules introduced from the outside (Duffy and Power, 2001; Poole, 1994; Schaechter *et al.*, 1999).

Many Gram-negative organisms exhibit intrinsic high-level resistance to a range of antibiotics, which supports the role of the outer membrane and active efflux as a barrier to antibiotics (Hancock and Bell, 1988; Köhler *et al.*, 1999; Nikaido, 1989, 1994; Van Bembeke *et al.*, 2003).

Antimicrobial activity in species of the Piperaceae family has been found for amides, essential oils, lignans, alkaloids, phenylpropanoids, neolignans and chromene (Benevides *et al.*, 1999; Constantin *et al.*, 2001; Dorman and Deans, 2000; Masuda *et al.*, 1991). Compounds **2** and **3** exhibited the widest activity. The Gram-positive bacteria were more sensitive to conocarpan, which showed MBC values of 6 $\mu\text{g/mL}$ against *B. cereus* and *S. aureus*, 10 $\mu\text{g/mL}$ against *S. saprophyticus* and 8 $\mu\text{g/mL}$ against *S. agalactiae*. Compound **2** showed a MBC value of 5 $\mu\text{g/mL}$ against *B. cereus* and *S. aureus* and 6 $\mu\text{g/mL}$ against *S. agalactiae*. In addition, compounds **2** and **3** presented excellent activity against Gram-positive bacteria, with a potency similar to the antibiotics used in antimicro-

bial therapeutics, as can be observed in Tables I and II.

Compound **4** (flavonoid) was obtained from the ethyl acetate fraction and the MIC value was 200 $\mu\text{g/mL}$ against *S. aureus*, 600 $\mu\text{g/mL}$ against *S. saprophyticus* and 1000 $\mu\text{g/mL}$ against *S. agalactiae*; however, the MBC values were very high.

The neolignans present in *Piper solmsianum* may be the main factor responsible for the antimicrobial activity of this plant, however, no activity was observed for compound **1** against the microorganisms tested.

Freixa *et al.* (2001) suggested that the absence of a methoxy group at the 3 position of the phenyl-propenyl-benzofuran structure and the saturation of the 2,3 bond plays an important role in the antimicrobial activity. Our results suggest that the presence of a hydroxy group at the 4 position of the phenyl-propenyl-benzofuran structure can play a more important role in the antimicrobial activity. Hence, when this group is absent, no activity was observed.

Interestingly, the more potent inhibitory effects were observed with fractions and isolated compounds of lower polarity, which may have properties enabling them to penetrate the cells and interfere with metabolic processes.

In conclusion, the antibacterial activity of *P. solmsianum* seems to be related mainly to compounds **2** and **3** (neolignans), however, other active compounds can be present. The antimicrobial activity of the neolignans may be related to the

presence of a hydroxy group at the 4 position of the phenyl-propenyl-benzofuran structure.

Further studies are currently in progress to verify the mechanism of antimicrobial action and to identify other active compounds present in this plant.

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Benevides P. J. C., Sartorelli P., and Kato M. J. (1999), Phenylpropanoids and neolignans from *Piper regnellii*. *Phytochemistry* **52**, 339–343.

Bruneton J. (1991), Elementos de fitoquímica y de farmacognosía. Acribia, Zaragoza.

Campos M. P., Cechinel Filho V., Silva R. Z., Yunes R. A., Zacchino S. A., Juarez S., Bella Cruz R. C., and Bella Cruz A. (2005), Evaluation of antifungal activity of *Piper solmsianum* C. DC. var. *solmsianum* (Piperaceae). *Biol. Pharm. Bull.* **28**, 1527–1530.

Chauret D. C., Bernard C. B., Arnason J. T., and Durst T. (1996), Insecticidal neolignans from *Piper decurrens*. *J. Nat. Prod.* **59**, 152–155.

Choudhary D. and Kale R. K. (2002), Antioxidant and non-toxic properties of *Piper betle* leaf extract: *in vitro* and *in vivo* studies. *Phytother. Res.* **16**, 461–466.

Costa A. F. (1994), Farmacognosia. Fundação Calouste Guibenckian, Lisboa.

Costantin M. B., Sartorelli P., Limberger R., Henriques A. T., Steppe M., Ferreira M. J., Ohara M. T., Emerenciano V. P., and Kato M. J. (2001), Essential oils from *Piper cernuum* and *Piper regnellii*: antimicrobial activities and analysis by GC/MS and ¹³C-NMR. *Planta Med.* **67**, 771–773.

Dorman H. J. and Deans S. G. (2000), Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol.* **88**, 308–316.

Duffy C. F. and Power R. F. (2001), Antioxidant and antimicrobial properties of some Chinese plant extracts. *Int. J. Antimicrob. Agents* **17**, 527–529.

Evans W. C. (1991), Farmacognosia. Iberoamericana, México.

Freixa B., Vila R., Ferro E. A., Adzet T., and Cañigueral S. (2001), Antifungal principles from *Piper fulvescens*. *Planta Med.* **67**, 873–875.

Hancock R. E. W. and Bell A. (1988), Antibiotic uptake into Gram-negative bacteria. *Eur. J. Clin. Microbiol. Infect. Dis.* **7**, 713–720.

Holetz F. B., Pessini G. L., Sanches N. R., Cortez A. G., Nakamura C. V., and Dias Filho B. P. (2002), Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. *Mem. Inst. Oswaldo Cruz* **97**, 1027–1031.

Hou C. Y., Zhang J. Q., Zhang Y. M., and Liu Y. L. (1989), Studies on the chemical constituents of *Piper macropodum* C. DC. *Yao Xue Xue Bao* **24**, 789–792.

Joly A. B. (1998), Botânica: Introdução à taxonomia vegetal. Câmara Brasileira do Livro, São Paulo.

Köhler T., Pechère J. C., and Plésiat P. (1999), Bacterial antibiotic efflux systems of medical importance. *Cell Mol. Life Sci.* **56**, 771–778.

Lentz D. L., Clark A. M., Hufford C. D., Meurer-Grimes B., Passreiter C. M., Cordero J., Ibrahimi O., and Okunade A. L. (1998), Antimicrobial properties of Honduran medicinal plants. *J. Ethnopharmacol.* **63**, 253–263.

Lopez A., Hudson J. B., and Towers G. H. (2001), Antiviral and antimicrobial activities of Colombian medicinal plants. *J. Ethnopharmacol.* **77**, 189–196.

Martins R. C., Latorre L. R., Sartorelli P., and Kato M. J. (2000), Phenylpropanoids and tetrahydrofuran lignans from *Piper solmsianum*. *Phytochemistry* **55**, 843–846.

Masuda T., Inazumi A., Yamada Y., Padolina W. G., Kikasaki H., and Nakatami N. (1991), Antimicrobial phenylpropanoids from *Piper sarmentosum*. *Phytochemistry* **30**, 3227–3228.

Maxwell A., Dabideen D., Reynolds W. F., and Mclean S. (1999), Neolignans from *Piper aequale*. *Phytochemistry* **50**, 499–504.

Moreira D. L., Kaplan M. A., and Guimarães E. F. (1995), Constituintes químicos de *Piper solmsianum* C. DC. (Piperaceae). *Rev. Bras. Farm.* **76**, 106–109.

Moreira D. L., Souza P. O., Kaplan M. A., Pereira N. A., Cardoso G. L., and Guimarães E. F. (2001), Effect of leaf essential oil from *Piper solmsianum* C. DC. in mice behaviour. *An. Acad. Bras. Ci.* **73**, 33–37.

Nikaido H. (1989), Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrob. Agents Chemother.* **33**, 1831–1836.

Nikaido H. (1994), Prevention of drug access to bacterial targets: permeability barrier and active efflux. *Science* **264**, 382–388.

Orjala J., Erdelmeier C. A., Wright A. D., Rali T., and Sticher O. (1993), Five new prenylated *p*-hydroxybenzoic acid derivatives with antimicrobial and molluscicidal activity from *Piper aduncum* leaves. *Planta Med.* **59**, 546–551.

Perez C. and Anesini C. (1994), Antibacterial activity of alimentary plants against *Staphylococcus aureus* growth. *Am. J. Chin. Med.* **22**, 169–174.

Pessini G. L., Prado Dias Filho B., Nakamura C. V., and Cortez D. A. G. (2003), Antibacterial activity of ex-

tracts and neolignans from *Piper regnellii* (Miq.) C. DC. var. *pallescens* (C. DC.) Yunck. Mem. Inst. Osvaldo Cruz **98**, 1115–1120.

Poole K. (1994), Bacterial multidrug resistance – emphasis on efflux mechanisms and *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* **34**, 453–456.

Schaechter M., Engleberg N. C., Eisenstein B. I., and Medoff G. (1999), Mechanisms of Microbial Disease. Williams and Wilkins, Lippincott.

Shitut S., Pandit V., and Mehta B. K. (1999), The antimicrobial efficiency of *Piper betle* Linn. leaf (stalk) against human pathogenic bacteria and phytopathogenic fungi. *Cent. Eur. J. Public Health* **7**, 137–139.

Singh Y. N. and Singh N. N. (2002), Therapeutic potential of kava in the treatment of anxiety disorders. *CNS Drugs* **16**, 731–743.

Sosa S., Balick M. J., Arvigo R., Esposito R. G., Pizza C., Altinier G., and Tubaro A. (2002), Screening of the topical anti-inflammatory activity of some Central American plants. *J. Ethnopharmacol.* **81**, 211–215.

Tirillini B., Velasquez E. R., and Pellegrino R. (1996), Chemical composition and antimicrobial activity of essential oil of *Piper angustifolium*. *Planta Med.* **62**, 372–373.

Tonks A. (2003), Treating generalised anxiety disorder. *BMJ* **29**, 700–702.

Van Bambeke F., Glupczynski Y., Piésiat P., Pechère J. C., and Tulkens P. M. (2003), Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. *J. Antimicrob. Chemother.* **51**, 1055–1065.