Journal of Microbiology, Biotechnology and Food Sciences

Betts et al. 2012 : 2 (3) 1068-1078

REGULAR ARTICLE

SYNERGISTIC ANTIBACTERIAL EFFECTS OF THEAFLAVIN IN COMBINATION WITH AMPICILLIN AGAINST HOSPITAL ISOLATES OF *STENOTROPHOMONAS MALTOPHILIA*

Jonathan W. Betts[*1], Christine Murphy[2], Stephen M. Kelly[1] and Stephen J. Haswell[1]*

Addresses: [1]Department of Chemistry, Faculty of Science, University of Hull, Cottingham Road, Hull, HU6 7RX, UK.

[2]Department of Biological Sciences, Faculty of Science, University of Hull, Cottingham Road, Hull, HU6 7RX, UK.

*Corresponding author: Jono.betts@btinternet.com, Phone. No.: +447716141359

ABSTRACT

Stenotrophomonas maltophilia is an important opportunistic nosocomial pathogen that shows intrinsic resistance to many antibiotics. This often limits treatment options and can cause lengthy hospital stays. Combination treatments are often used to combat resistance and using natural compounds such as polyphenols could give increased treatment options and even the reuse of antibiotics to which high levels of resistance have been observed. A checkerboard assay was used to determine if any synergy exists between ampicillin and the polyphenol theaflavin against 9 clinical isolates and one control isolate (NCTC 13014) of *S. maltophilia*. It was discovered that significant synergy ($P \leq 0.05$) does exist between theaflavin and ampicillin, reducing the mean MIC of ampicillin from 12.5-22.9 µg/mL, in liquid culture, to 3.125-6.25 µg/mL. The FIC index was calculated to be 0.22-0.35 confirming synergy. From these results, significant potential for medical applications can be seen and further investigation is recommended.

Keywords: antibacterial, theaflavin, polyphenol, synergy, checkerboard, *Stenotrophomonas maltophilia*

INTRODUCTION

Throughout the world, pathogens are increasingly exhibiting resistance to clinically available antibiotics **(Karchmer, 2004)**. Bacterial resistance to antibiotics has been described as a natural phenomenon resulting from selective pressure due to continued exposure to their presence **(Guillemot, 1999)**. There are five major mechanisms that underlie bacterial resistance: target site modification, enzyme-catalyzed inactivation, metabolic by-pass, efflux or prevention of antibiotic entry into cells **(Alanis, 2005)**. All groups of antibiotics have associated resistance and in some cases manufacturers have been forced to modify their structures in an attempt to reverse this. Despite modifications, resistance to the modified drugs can rapidly arise.

Stenotrophomonas maltophilia is an opportunistic pathogen, which can readily colonise epithelial cells in the human respiratory tract often leading to pneumonia in immune-compromised patients, individuals with cystic fibrosis and those on respirators **(Alonso and Martinez, 1997; Looney, *et al.*, 2009)**. This bacterium possesses intrinsic resistance to important clinical antibiotics **(San Gabriel *et al.*, 2004)** and combination therapy can be required to treat infections.

The use of antibiotic formulations containing two or more active agents has its basis in successful therapy and is particularly valuable in preventing future emergence of antibiotic resistance. However, this would not be the case for resistance genes acquired by bacteria, through horizontal gene transfer. Beyond the traditional antibiotic cocktails it is likely that there are many antibacterial mixtures yet to be identified. Combining clinical antibiotics with non-traditional antibacterial agents could provide treatments that are more effective than using the antibiotic alone **(Cushine and Lamb, 2011)**.

Natural compounds such as the polyphenols found in tea may provide promising additions to the armoury of conventional drugs, in order to meet the urgent requirement for new antimicrobial therapies to help combat the resistance problem. For example, the polyphenol theaflavin, found in black tea has been shown to possess antimicrobial activity against bacteria including *Bacillus cereus* and *Shigella* spp. **(Vijaya *et al.*, 1995; Friedman *et al.*, 2006)**. Both *Acinetobacter baumannii* and *S. maltophilia* have been shown to be susceptible to the green tea polyphenol epigallocatechin gallate **(Osterburg *et al.*, 2009; Gordon and Wareham, 2010)**.

The possible synergies between tea-derived compounds and other antibiotics have important implications and potential applications. Investigations into the synergies of

flavanols with other compounds such as ascorbic acid **(Hatano *et al.,* 2008)** and polyphenols **(Betts *et al.,* 2011)** have proven to be successful by enhancing or prolonging the antibacterial action. Previously the green tea polyphenol epicatechin gallate was shown to reverse the resistance of Methicillin-resistant *Staphylococcus aureus* (MRSA) to oxacillin **(Anderson *et al.,* 2005)**. Also, epigallocatechin gallate (EGCG) showed synergy with the macrolide antibiotic clarithromycin **(Yanagawa *et al.,* 2003)**. Flavanoids, including epicatechin from tea, reduced the minimum inhibitory concentrations (MICs) of isoniazid in *Mycobacterium smegmatis* **(Lechner et al., 2008)**. Some conflicting research suggests that a gallate group must be present for any synergy to be shown **(Stapleton *et al.,* 2004)**.

Very little research has been published to provide evidence of synergy between theaflavin and antibiotics. Previous research **(Neyestani et al., 2007)** has shown that black tea extracts cause synergistic and antagonistic effects when applied with various antibiotics against *Streptococcus pyogenes*. However, this research used a crude black tea mixture and did not identify the specific compounds responsible for the observed effects. Previous research **(Tiwari *et al.,* 2005)** has also found synergy between black tea extracts and antibiotics such as chloramphenicol and gentamicin against various pathogens including *Shigella dysenteriae* and *Salmonella enterica* serovar Typhimurium. This work also suggested that the synergy results from the exploitation of dual binding sites on the surface of the bacteria. Although like previous research **(Neyestani et al., 2007)** mixed tea extracts were used and the active chemical components were not purified. The rationale of using polyphenols with antibiotics is the possibility of effectively reusing antibiotics such as Beta-lactams, in the treatment of bacterial infections, where resistance has previously shown them to be ineffective.

The objective of the preliminary research described in this paper was to assess whether any synergistic effects exist between ampicillin with theaflavin against clinical isolates of *Stenotrophomonas maltophilia*.

MATERIAL AND METHODS

Clinical and control isolates

Nine strains of *S. maltophilia* were isolated over three months from sputum samples of respiratory patients at the Hull Royal Infirmary, UK. All isolates were cultured on blood and MacConkey agar (Oxoid, Basingstoke, UK) and identified using Gram staining, BSAC

(British Society for Antimicrobial Chemotherapy) antibiotic susceptibility testing **(Andrews, 2007)** and biochemical profiling with API 20E testing kits (BioMérieux, France). A control strain of *S. maltophilia* (NCTC 13014) was purchased from the Heath Protection Agency Cultures Collections, Porton Down, UK.

Culture media, antibiotics and tea polyphenols

Ampicillin (AMP) powder was purchased as ampicillin sodium salt from Sigma-Aldrich, UK. All media and ampicillin discs were purchased from Oxoid, UK. Ampicillin was selected as previous laboratory testing had shown that 25 µg discs containing ampicillin produced no zones of inhibition against *S. maltophilia* **(Betts *et al.*, 2012)** and therefore resistance was concluded. All media and materials were autoclaved before their use. Theaflavin (TF) with purity ≥ 95 was donated by Unilever, Shanghai, China.

Ampicillin susceptibility testing

All isolates were inoculated onto IsoSensitest agar (Oxoid, UK) using the method standardised by **Moodsdeen, Williams and Secker (1988)**. Ten IsoSensitest agar plates (6 mm in depth) were poured and inoculated with the control strain and each of the 9 clinical isolates. Discs were added containing ampicillin (25 µg). All isolates were incubated at 30°C for 20 h, in accordance with the BSAC methods (BSAC, 2012) of susceptibility testing for *S. Maltophilia* **(Andrews, 2007).** At the end of the incubation period, for each test disc the diameter of the disc plus zone of inhibition was measured (mm) and recorded. Each experimental plate was replicated six times to check for consistency.

Checkerboard assay

Stock solutions of theaflavin and ampicillin were initially made up in 0.5 mL of 100% DMSO based on the method by **Gordon and Wareham (2010),** for ease of solubility. Stock solutions were then diluted into 19.5 mL of ISO-sensitive broth leaving DMSO concentrations of 2.5%. To a standard 96-well round-bottomed microtitre plate, 50 µL of the theaflavin and ampicillin solutions were pipetted into each well, so that each row and column contained a fixed amount of one antimicrobial agent and increasing concentrations of the other. This gave final well

concentrations of 3.125-800 µg/mL of theaflavin and 3.25-100 µg/mL of ampicillin (Figure 1). To inoculate each well with *S. maltophilia*, a 100 µL volume of a 0.5 MacFarland suspension of was pipetted **(Andrews, 2001)**. This procedure was replicated for each isolate tested. All microtitre plates were incubated at 30°C for 24 h. Control wells were also prepared using theaflavin in 2.5% DMSO (3.125-800 µg/mL), ampicillin in 2.5% DMSO (3.25-100 µg/mL) and 2.5% DMSO only.

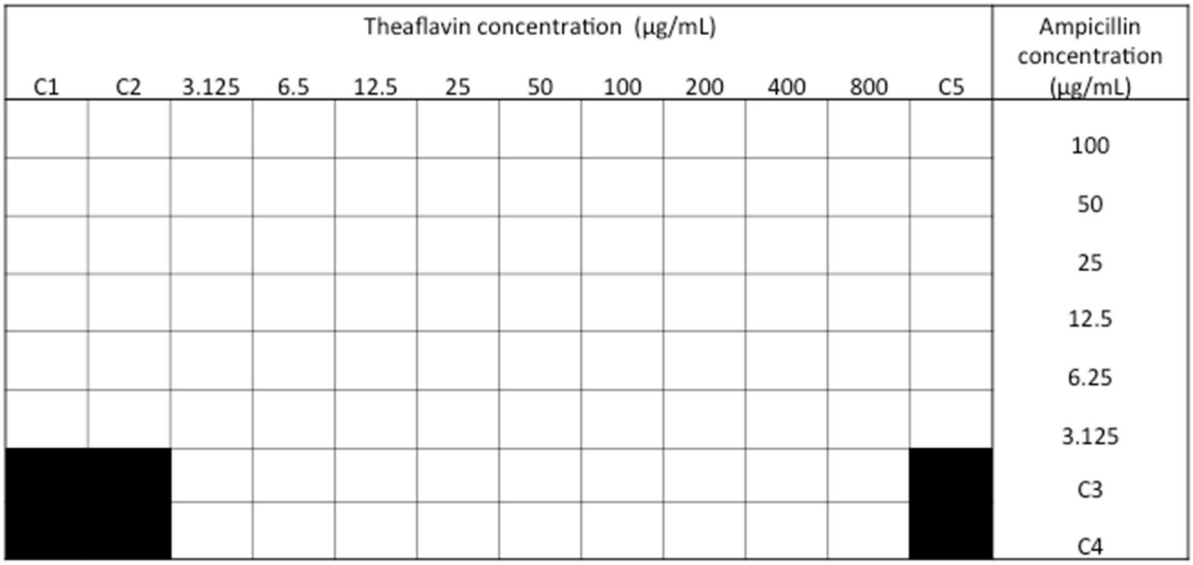

Figure 1 Organisation of the checkerboard assay showing well concentration of theaflavin and ampicillin. C1 = Ampicillin alone (no bacterial inoculant), C2 = Ampicillin (in 2.5% DMSO) + *S. maltophilia*, C3 = DMSO only + *S. maltophilia*, C4 = Theaflavin (in 2.5% DMSO) + *S. maltophilia* and C5 = theaflavin alone (no bacterial inoculant).

At the end of the incubation period, each well was observed for signs of visible turbidity. The lowest concentration not showing visible turbidity was taken as the MIC. The fractional inhibitory concentrations (FIC) were determined based on the method described by **Hall, Middleton and Westmacott (1983)** whereby the FICa = MIC of compound a + compound b/MIC of compound a, the FICb = MIC of compound b + compound a/MIC of compound b and the FICs = FICa + FICb. If the FICs index was equal to 0.5 or less, a synergistic effect was recorded. A value > 0.5 – 4.0 was taken as an additive effect and a value > 4.0 was counted as antagonistic effect between theaflavin and ampicillin. Six-fold replication of all checkerboard assays check for consistency and enabling the results to be provided as mean values. Significant differences between data sets for each combination of

polyphenols were determined using the Wilcoxon Mann–Whitney test, and results showing $P \leq 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Results from the susceptibility testing using 25 µg ampicillin discs, showed that all isolates of *S. maltophilia* presented resistance to ampicillin. However, from the checkerboard assay it was observed that in a liquid culture *S. maltophilia* isolates were susceptible to ampicillin at concentrations of 12.5 µg/mL and above, with mean MICs being 12.5-22.9 µg/mL depending on the isolate (See table 1). The MIC of ampicillin was dramatically reduced when used in combination with theaflavin. With the mean theaflavin additions of 6.25-11.46 µg/mL the mean ampicillin MICs against all *S. maltophilia* were significantly ($P < 0.05$) reduced to 3.125-6.25 µg/mL. The FIC indexes of ampicillin and theaflavin combinations against each *S. maltophilia* isolate was calculated to be between 0.22 and 0.35 confirming that a synergistic, not an additive relationship exists between theaflavin and ampicillin. No antimicrobial effects were produced from DMSO alone and no synergy was observed between DMSO and ampicillin.

From the results presented, the differences can be seen between the disc diffusion and microtitre assays as methods of showing susceptibility of *S. maltophilia* to ampicillin. This difference confirms previous literature, which mentions the difficulties of accurately determining antibiotic susceptibility of *S. maltophilia* and the unreliability of the disc diffusion method **(Tatman-Otkun *et al.*, 2005)**. It has been shown in this study that the microtitre assay is more accurate and far more easily standardised, in than it does not rely on the slow diffusion of chemical agents into agar, which are highly affected by temperature and pH.

Table 1 Mean (± standard deviation) minimum inhibitory concentrations (MIC) and the fractional inhibitory concentration indexes (FICIs) of ampicillin and theaflavin against clinical isolates of *S. maltophilia*.

Isolate no.	MIC (µg/mL)			
	AMP	TF	AMP + TF	FIC index
1	12.5 (0)	200 (0)	3.125 (0) + 6.25 (0)	0.28
2	14.6 (10.4)	200 (0)	3.125 (0) + 6.25 (0)	0.25
3	12.5 (0)	400 (0)	3.125 (0) + 11.46 (5.2)	0.28
4	12.5 (0)	200 (0)	3.65 (2.6) + 6.25 (0)	0.32
5	22.9 (10.4)	400 (0)	6.25 (0) + 11.46 (5.2)	0.30
6	14.6 (10.4)	200 (0)	4.17 (2.08) + 6.25 (0)	0.32
7	25 (0)	400 (0)	5.21 (2.1) + 12.5 (0)	0.24
8	16.6 (8.3)	200 (0)	3.125 (0) + 6.25 (0)	0.22
9	12.5 (0)	200 (0)	3.65 (2.6) + 11.46 (5.2)	0.35
Control	12.5 (0)	200 (0)	3.125 (0) + 9.38 (3.13)	0.30

Amp= ampicillin, TF = theaflavin, Control = *Stenotrophomonas maltophilia* NCTC 10258.

The results presented confirm previous results regarding the antibacterial activity of theaflavin against *S. maltophilia* **(Betts *et al.,* 2012)**. More importantly, the work demonstrates that significant synergy ($P \leq 0.05$) exists between theaflavin and ampicillin against all isolates of *S. maltophilia* used in this investigation. The research here refutes the findings of other research **(Stapleton *et al.,* 2004)**, which suggest that a gallate group must be present for synergy to occur, and instead supports work which indicates that with some antibiotics an interaction can exist **(Martins *et al.,* 2011)**. The mechanism for this is likely to involve an interaction with β-lactamase, possibly disabling the enzyme and allowing ampicillin to again disrupt peptidoglycan synthesis. This result supports previous polyphenol and antibiotic work **(Zhao *et al.,* 2001)** where the addition of epigallocatechin gallate (EGCG) reversed resistance to penicillin by *Staphylococcus aureus*. Previous studies have proposed that the mechanisms for polyphenol synergy and activity involves modulating the activity of intrinsic β-lactamases **(Zhao *et al.,* 2002)** and also the destabilisation of the bacterial cytoplasmic membrane via by production of hydrogen peroxide **(Wang, Wang and Xie, 2010)**. However, the modes of action require further investigation as other research has suggested that the mechanism behind the antibacterial action of polyphenols is the result of cell aggregation **(Cushnie and Lamb, 2011)**. This mechanism would lead to decreased cell

surface area and result in reduced nutrient uptake and oxygen consumption. As a consequence less energy and materials would be available to produce/maintain elements that they rely on for their defence against antibiotics, such as β-lactamases and efflux pumps. With these resistance mechanisms suppressed, antibiotics such as ampicillin might again become a viable option to treat infections caused by multidrug resistant bacteria.

CONCLUSION

In conclusion this is believed to be the first report providing evidence of synergistic properties of theaflavin when used in combination with ampicillin against clinical isolates of *S. maltophilia*. These results could lead to further work investigating the use of ampicillin in the treatment of *S. maltophilia* infections, an antibiotic normally not used against the bacterium. This work highlights the potential use of natural compounds such as polyphenols in a clinical setting to help combat resistance. The use of conventional antibiotics with polyphenols could increase future treatment options and result in the reuse of many antibiotics to which high levels of resistance have previously been observed. Further work is recommended to investigate the specific mechanisms and underlying interactions between theaflavin and β-lactam antibiotics and its wider effectiveness against resistant bacterial pathogens.

Acknowledgments: We gratefully acknowledge the funding from ESPRC (EP/C53770X/1). We would also like to thank the Northern General Hospital, Sheffield, UK and Hull Royal Infirmary, UK for providing the clinical isolates and to Unilever, China for supplying samples of theaflavin.

REFERENCES

ALANIS, A. J. 2005. Resistance to antibiotics: Are we in the post-antibiotic era? *Archives of Medical Research,* vol. 36, p. 697-705.

ALONSO, A. – MARTINEZ, J. L. 1997. Multiple antibiotic resistance in *Stenotrophomonas maltophilia*. *Antimicrobial Agents and Chemotherapy*, vol. 41, no. 5, p. 1140-1142.

ANDERSON, J. C. – HEADLEY, C. – STAPLETON, P. D. – TAYLOR, P. W. 2005. Synthesis and antibacterial activity of hydrolytically stable (-)-epicatechin gallate analogues

for the modulation of β-lactam resistance in *Staphylococcus aureus*. *Bioorganic and Medicinal Chemistry Letters*, vol. 15, p. 2633-2635.

ANDREWS, A. M. 2001. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, vol. 48, no. 1, p. 5-16.

ANDREWS, A. M. 2007. BSAC standardized disc susceptibility testing method (Version 6). *Journal of Antimicrobial Chemotherapy*, vol. 60, no. 1, p. 20-41.

BETTS, J. W. - MURPHY, C. - KELLY, S. M. - HASWELL, S. J. 2011. Antibacterial effects of theaflavin and its synergy with epicatechin against clinical isolates of *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*. *International Journal of Antimicrobial Agents*, vol. 38, no. 5, p. 421-425.

BETTS, J. – MURPHY, C. – KELLY, S. - HASWELL, S. 2012. Minimum inhibitory and bacterical concentrations of theaflavin and synergistic combinations with epicatechin and quercetin against clinical isolates of *Stenotrophomonas maltophilia*. *Journal of Microbiology Biotechnology and Food Sciences*, vol. 1, no. 5, p. 1250-1258.

BSAC. 2012. BSAC methods for antimicrobial susceptibility testing. Version 11, March 2012.

CUSHINE, T. P. T. – LAMB, A. J. 2011. Recent advances in understanding the antibacterial properties of flavonoids. *International Journal of Antimicrobial Agents*, vol. 38, no. 2, p. 99-107.

FRIEDMAN, M. – HENIKA, P. R. – LEVIN, C. E. – MANDRELL, R. E. – KOZUKE, N. 2006. Antimicrobial activities of tea catechins and theaflavins and tea extracts against *Bacillus cereus*. *Journal of Food Protection*, vol. 69, no. 2, p. 354-361.

GORDON, N. C. - WAREHAM, D. W. 2010. Antimicrobial activity of the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) against clinical isolates of *Stenotrophomonas maltophilia*. *International Journal of Antimicrobial Agents*, vol. 36, no. 2, p. 129-131.

GUILLEMOT, D. 1999. Antibiotic use in humans and bacterial resistance. *Current Opinion in Microbiology*, vol 2, p. 494-498.

HALL, M. J. – MIDDLETON, R. F. – WESTMACOTT, D. 1983. *Journal of Antimicrobial Chemotherapy*, vol. 11, p. 427-433.

HATANO, T. – TSUGAWA, M. - KUSUDA, M. – TANIGUCHI, S. – YOSHIDA, T. – SHIOT, S. *et al.* 2008. Enhancement of antibacterial effects of epigallocatechin gallate using ascorbic acid. *Phytochemistry*, vol. 69, p. 3111-3116.

KARCHMER, A. W. 2004. Increased antibiotic resistance in respiratory tract pathogens: Protekt US – an update. *Clinical Infectious Diseases*, vol. 39, no. 3, p. S142-S150.

LECHNER, D. – GIBBONS, S. – BUCAR, F. 2008. Modulation of isoniazid susceptibility by flavanoids in *Mycobacterium*. *Phytochemistry Letters*, vol. 1, p. 71-75.

LOONEY, W. J. – NARITA. M. – MUHLEMANN, K. 2009. *Stenotrophomonas maltophilia*: An emerging opportunist human pathogen. *Lancet Infectious Diseases*, vol 9, p. 312-323.

MARTINS, A. – VASAS, A. – VIVEIROS, M. – MOLNAR, J. – HOHMANN, J. et al. 2011. Antibacterial properties of compounds isolated from *Carpobrotus edulis*. *International Journal of Antimicrobial Agents,* vol. 37, p. 438-444.

MOODSDEEN, F. – WILLIAMS, J. D. – SECKER, A. 1988. Standardization of inoculum size for disc susceptibility testing: a preliminary report of a spectrophotometric method. *Journal of Antimicrobial Chemotherapy*, vol. 21, p. 439-443.

NEYESTANI, T. R. – KHALAJI, N. - GHARAVI, A. 2007. Black and green teas may have selective synergistic or antagonistic effects on certain antibiotics against *Streptococcus pyogenes* in vitro. *Journal of Nutritional and Environmental Medicine*, vol. 16 no. 3-4, p. 258-266.

OSTERBURG, A. – GARDNER, J. – HYON, S. H. – NEELY, A. – BABCOCK, G. 2009. Highly antibiotic-resistant *Acinetobacter baumannii* clinical isolates are killed by the tea polyphenol (-)-epigallocatechin-3-gallate (EGCG). *Clinicial Microbiology and Infection*, vol. 15, no. 4, p. 341-346.

SAN GABRIEL, P. – ZHOU, J. – TABIBI, S. – CHEN, Y. – TRAUZZI, M. *et al.* 2004. Antimicrobial Susceptibility and Synergy Studies of *Stenotrophomonas maltophilia* Isolates from Patients with Cystic Fibrosis. Antimicrobial Agents and Chemotherapy, vol. 48, no. 1, p. 168–171.

STAPLETON, P. D. – SHAH, S. – ANDERSON, J. C. – HARA, Y. - HAMILTON-MILLER J. M. T. *et al*. 2004. Modulation of β-lactam resistance is *Staphylococcus aureus* by catechins and gallates. *International Journal of Antimicrobial Agents*, vol 23, p. 462-467.

TATMAN-OTKUN, M. - GURCAN, S. – OZER, B. – AYDOSLU, B. – BUKAVAZ, S. 2005. The antimicrobial susceptibility of *Stenotrophomonas maltophilia* isolates using three different methods and their genetic relatedness. *BMC Microbiology*, vol. 5, no. 24.

TIWARI, R. P. – BHARTI, S. K. – KAUR, H. D. – DIKSHIT, R. P. – HOONDAL, G. S. 2005. Synergistic antimicrobial activity of tea and antibiotics. Indian Journal of Medical Research, vol. 122, p. 80-84.

VIJAYA, K. – ANANTHAN, S. – NALINI, R. 1995. Antibacterial effect of theaflavin, polyphenol 60 (*Camellia sinensis*) and *Euphorbia hirta* on *Shigella spp*. – a cell culture study. *Journal of Ethnopharmacology*, vol. 49, no. 2, p. 115-118.

WANG, Q. – WANG, H. – XIE, M. 2010. Antibacterial mechanism of soybean isoflavone on *Staphylococcus aureus*. *Archives of Microbiology*, vol. 192, p. 893-898

YANAGAWA, Y. - YAMAMOTO, Y. – HARA, Y. – SHIMAMURA, T. 2003. A combination effect of epigallocatechin, a major compound of green tea catechins, with antibiotics on *Helicobacter pylori* growth in vitro. *Current Microbiology*, vol. 47, p. 244-249.

ZHAO, W. H. – HU, Z. Q. – OKUBO, S. – HARA, Y. – SHIMAMURA, T. 2001. Mechanism of synergy between epigallocatechin gallate and β-lactams against methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, vol. 45, no. 6, p. 1737-1742.

ZHAO, W. H. – HU, Z. Q. – HARA, Y. – SHIMAMURA, T. 2002. Inhibition of penicillinase by epigallocatechin gallate resulting in restoration of antibacterial activity of penicillin against penicillinase-producing *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy, vol. 46, no. 7, p. 2266-2268.