NEW ANTIMICROBIAL COMBINATIONS: SUBSTITUTED CHALCONES- OXACILLIN AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

Juan Manuel Talia*, Nora Beatriz Debattista, Nora Beatriz Pappano

Laboratory of Physical Chemistry, Department of Chemistry, San Luis National University, Lavalle 1155. 5700 San Luis, Argentina.

ABSTRACT

Staphylococcus aureus, the most virulent *Staphylococcus* species, is also the prevalent pathogen isolated from hospitalized patients and the second most common from patients in outpatient settings. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents. Related studies of antimicrobial activity indicate that crude extracts containing flavonoids, triterpenes and steroids have showed significative activity against several *Staphylococcus aureus* strains. Combination effects between flavonoids and antibiotics also have been reported. The aim of the present work was to investigate *in vitro* synergism between several chalcones substituted in combination with oxacillin, an antibiotic used conventionally against *S. aureus* ATCC 43 300 that is resistant to meticillin, using the kinetic turbidimetric method developed earlier. The results were satisfactory for all assayed combinations and in accordance with the mechanism of bacteriostatic inhibition previously proposed, except for 2´,4´-dihydroxy-3´-methoxychalcone – oxacillin. The best combination was 2´,3´-dihydroxychalcone - oxacillin (MIC: 11.2 µg/mL). Further investigations are needed to characterize the interaction mechanism with antibiotics. Thus, chalcones - oxacillin combination could lead to the development of new antibiotics against methicillin resistant *S. aureus* infection.

Key words: substituted chalcones, oxacillin, combinations, antimicrobial, *S. aureus*

INTRODUCTION

Staphylococcus aureus, the most virulent *Staphylococcus* species, is also the prevalent pathogen isolated from hospitalized patients and the second most common from patients in outpatient settings (14). Humans are a natural reservoir of *S. aureus*. The emergence of a pathogen community depends on its ability to survive in different environments and to interact successfully with the host. These microorganisms cause a wide range of syndromes, from skin and soft tissue minor infection up to life-threatening pneumonia and toxemy such as toxic shock syndrome. Both methicillin-sensitive and methicillin-resistant strains isolates are persistent colonizers. Rates of staphylococcal colonization

---
*Corresponding Author. Mailing address: Laboratory of Physical Chemistry, Department of Chemistry, San Luis National University, Lavalle 1155. 5700 San Luis, Argentina.; Tel.: 54 02652 426780 Int. 122 Fax: 54 02652 430224.; E-mail: jmtalia@unsl.edu.ar

are high among patients with diabetes, intravenous drug users, surgical patients and patients with the acquired immunodeficiency syndrome (2, 6).

Even though in the last three decades pharmacological industries have produced a number of new antibiotics, the resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs which are utilized as therapeutic agents. New infections can occur in hospitals resulting in high mortality. Such a fact is cause for concern, because of the number of patients in hospitals who have suppressed immunity, and due to new bacterial strains which are multi-resistant (13).

Methicillin-resistant *Staphylococcus aureus* (MRSA), identified in the early 1960s coincident with the introduction of methicillin, is now one of the most common causes of bacterial nosocomial infections, mainly in intensive care units. Methicillin resistance results from the production of an altered penicillin binding protein which has decreased affinity for most beta-lactam antibiotics (7, 24).

Flavonoids are phenolic substances widely distributed in all vascular plants and increasingly are becoming the subject of medical research. They have been reported to possess many properties as anti-inflammatory (4), oestrogenic (11), enzyme inhibition (9), antimicrobial (10), antiallergic (8), antioxidant (23), vascular (22) and cytotoxic antitumor (3) activities, between others. Most of the beneficial health effects of flavonoids are attributed to their ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving reactive oxygen species (12). Related studies of antimicrobial activity indicate that crude extracts containing flavonoids, triterpenes and steroids have showed significative activity against various strains of *S. aureus* (21). The combination of antibiotics with different modes of action against bacterial cells is useful for treatment of bacterial infections. Combination effects between flavonoids and antibiotics have also been reported. Thus, Shibata et al. (20) found that flavone and its derivatives have synergistic action

with the ß-lactams, intensified the susceptibility in MRSA and MSSA. Furthermore, the flavonoid synergistic activity against antibiotic resistant bacteria, under low concentration minimizing the possible toxic effects, was reported (1, 18, 19).

The aim of the present work was to investigate *in vitro* synergism between several chalcones substituted in combination with oxacillin, antibiotic used conventionally against *S. aureus* 43 300 (MRSA) which is resistant to it. Kinetic turbidimetric method developed earlier was employed (15).

MATERIALS AND METHODS

Compounds

Oxacillin, sodium salt monohydrate (Sigma-Aldrich); 2´,3-dihydroxychalcone (1), 2´,4-dihydroxychalcone (2), 2´,4´-dihydroxychalcone (3) and 2´,4´,3-trihydroxychalcone (4) were all synthesized in our laboratory by Claisen-Schmidt condensation and identified by chromatographic and spectroscopic techniques (TLC, UV-Vis, IR, NMR) (5). In addition, 2´,4´-dihydroxy-3´-methoxychalcone (5), a natural compound isolated from *Zuccagnia punctata* Cav., a native plant to San Luis (Argentina) (17) was assayed. Figure 1 illustrates the compound structures. Oxacillin and different chalcones solutions were prepared in absolute ethanol and diluted for antimicrobial assays.

Bacterial strains

Staphylococcus aureus ATCC 43 300 (MRSA) strain (purchased from American Type Culture Collection), maintained by successive subcultures in trypticase soy agar BBL (Becton Dickinson) at 4ºC and by lyophilization, was used.

Cultura media

Broth and agar nutritive and broth and agar Müller-Hinton (Oxoid) were used.

Kinetic-turbidimetric assays

In order to determine quantitatively the sensitivity of *S. aureus* to oxacillin and its increase when oxacillin is used in combination with several chalcones, a previously developed kinetic-turbidimetric method was employed (15).

A 24 h culture of *S. aureus* ATCC 43 300 in agar slant was transferred to 30 mL of Müller-Hinton broth and incubated at 35ºC for 18 h, with permanent stirring, in order to be used as inoculum. Kinetic experiments of microbial growth were performed in Erlenmeyer flasks containing 100 mL of Müller-Hinton broth with addition of increasing concentrations of antibiotic oxacillin and 2 mL of previously prepared inoculum. Subsequently, Erlenmeyer flasks were incubated in a Rosi 1000 culture chamber (35ºC, 180 rpm). Aliquots were extracted at 20 min intervals for 5 h and the transmittances were read at 720 nm. A flask without antibiotic was used as control. This first experiment enabled us to choose the optimal oxacillin concentration to be used in the next trials (6 μg.mL^{-1}).

For synergism determination, similar experiments in presence of chalcone in increasing concentrations, alone or in combination with oxacillin, were performed.

In the following equation:

$$\ln N_t = 27.4 - 10.3 \cdot T \tag{1}$$

the transmittance (T) values at 720 nm were related to the number CFU/mL (N_t) (15).

All trials were conducted in triplicate and standard deviations of the specific growth rate, were in all cases lower than 0.001.
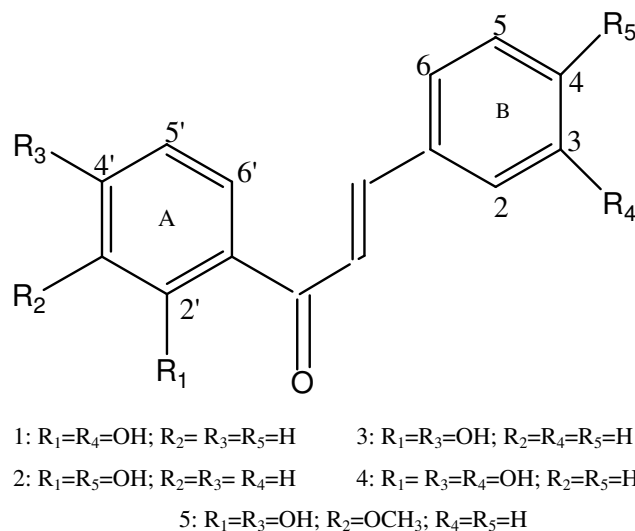

1: $R_1=R_4=OH$; $R_2=R_3=R_5=H$ 3: $R_1=R_3=OH$; $R_2=R_4=R_5=H$

2: $R_1=R_5=OH$; $R_2=R_3=R_4=H$ 4: $R_1=R_3=R_4=OH$; $R_2=R_5=H$

5: $R_1=R_3=OH$; $R_2=OCH_3$; $R_4=R_5=H$

Figure 1. Structure of compounds: 1: 2´,3- dihydroxychalcone; 2: 2´,4- dihydroxychalcone; 3: 2´,4´- dihydroxychalcone; 4: 2´,4´,3- trihydroxychalcone; 5: 2´,4´-dihydroxy-3´-methoxychalcone.

RESULTS AND DISCUSSION

The number of CFU/mL at different times was obtained by the turbidimetric kinetic method (Eq. 1). The microbial growth can be expressed by the equation:

$$\ln N_t = \ln N_o + \mu \cdot t \tag{2}$$

where t is time in min, N_o is CFU/mL at t = 0, N_t is CFU/mL at t = t and μ is specific growth rate (in min^{-1}).

Growth rates values in media with increasing chalcone

concentration and their combinations with oxacillin constant concentration were obtained from the exponential phase of ln Nt vs. t plots.

Table 1 exhibits the specific growth rates of *S. aureus* obtained in presence of chalcones isolated and chalcones - oxacillin combinations with the respective MIC values.

Table 1. Specific growth rates and minimal inhibitory concentration for all systems assayed against a methicillin-resistant *Staphylococcus aureus* strain.

Chalcone		Concentration (μg.mL^{-1})					MIC μg.mL^{-1}
		0	5.00	10.0	15.0	20.0	
1	μ (isolated)	0.0465	0.0380	0.0158	0.00130	0	15.8
	μ (comb)	0.0465	0.0231	0.00616	0	0	11.2
2	μ (isolated)	0.0453	0.0389	0.0350	0.0314	0.0312	45.0
	μ (comb)	0.0453	0.0150	0.0115	0.0112	0.00653	22.4
3	μ (isolated)	0.0476	0.0373	0.0247	0.00425	0.000400	20.1
	μ (comb)	0.0476	0.0296	0.00928	0.00343	0	15.3
4	μ (isolated)	0.0459	0.0386	0.0314	0.0254	0.0131	30.8
	μ (comb)	0.0459	0.0279	0.0197	0.0157	0.00719	22.5
5	μ (isolated)	0.0464	0.0391	0.0348	0.0292	0.0244	42.5
	μ (comb)	0.0464	0.00988	0.00866	0.00818	0.00766	∞

1: 2´,3- dihydroxychalcone; 2: 2´,4- dihydroxychalcone; 3: 2´,4´- dihydroxychalcone; 4: 2´,4´,3- trihydroxychalcone; 5: 2´,4´-dihydroxy-3´-methoxychalcone. MIC: minimal inhibitory concentration.

The specific growth rate values decreasing when the experiment was made in presence of oxacillin constant concentration. This fact is observed in Table 1 for all assayed combinations. The results were satisfactory, except for 2´,4´-dihydroxy-3´-methoxychalcone – oxacillin.

They are in accordance with bacteriostatic inhibition mechanism previously proposed (15). In the same, the exponential growth law of microorganisms and the possible adsorption of substances on the cell wall were considered, resulting in the reduction or cancellation of some vital function:

$$\mu_T$$
$$N \rightarrow 2N$$
$$k_I$$
$$C+N \rightarrow C\text{-}N$$
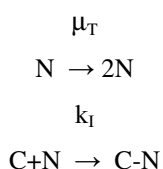
The growth rate of bacteria by this mechanism is expressed as:

$$dN/dt = \mu_T . N - k_I . C . N \qquad o \qquad dN/dt = (\mu_T - k_I.C) N$$

Comparing this relationship with the exponential growth law $dN/dt = \mu.N$, it is inferred that:

$$\mu = \mu_T - k_I . C \qquad\qquad (3)$$

where μ_T: specific growth rate without drug (min^{-1}) (control), k_I: specific inhibition rate (mL.μg^{-1}.min^{-1}) and C: drug concentration (μg.mL^{-1}). The minimal inhibhitory concentration (MIC) was calculated by extrapolation at $\mu = 0$ from the graphical representation of equation 3.

For 2´,4´-dihydroxy-3´-methoxychalcone – oxacillin combination, antagonist is observed, probably due to the strong complex formation between both molecules. This fact provokes a decrease of the chalcone bacteriostatic activity.

To facilitate comparison of the inhibitory action between structurally related substances such as chalcones and their combinations with conventional antibiotic oxacillin, should be determined the percentual bacteriostatic efficiency of a drug (PBE) (16), in the form:

$$PBE = 100/MIC \qquad\qquad (4)$$

where MIC: minimum inhibitory concentration (μg.mL^{-1}) and 100: percentage arbitrary numerical factor. Evaluating chalcones PBE tested and comparing with the PBE of these chalcones with oxacillin, can be established that combinations PBE are higher, demonstrating synergism for all of them, except for 2´,4´,dihydroxy-4´-methoxychalcone – oxacillin combination (Table 2).

Table 2. Minimal inhibitory concentration and percentual bacteriostatic efficiency of isolated chalcones and of chalcones-oxacillin combinations against a methicillin-resistant *Staphylococcus aureus* strain.

Chalcone	MIC (comb)	PBE (isolated)		PBE (comb)
1	11.2	6.33	<	8.93
2	22.4	2.22	<<	4.46
3	15.3	4.97	<<	6.53
4	22.5	3.25	<	4.44
5	73.9	2.35	>>>	≈ 0

1: 2´,3- dihydroxychalcone; 2: 2´,4- dihydroxychalcone; 3: 2´,4´- dihydroxychalcone; 4: 2´,4´,3- trihydroxychalcone; 5: 2´,4´-dihydroxy-3´-methoxychalcone. MIC: minimal inhibitory concentration.

Chalcones activity against methicillin-resistant *Staphylococcus aureus* (MRSA) was detected by kinetic-turbidimetric method. This inhibition was observed with the isolated chalcones and with their combinations with oxacillin, an ineffective antibiotic. The chalcones – oxacillin combinations showed an interesting synergic action.

Due to the growing problem of microbial resistant, the outlook for the use of antimicrobial drugs is uncertain. Consequently, the development of researches which seek to increase antibacterial activity and to characterize the interaction mechanism with antibiotics woud be desirable.

Thus, the synergistic effect between chalcones and oxacillin is an important way that could lead to the development of new combination antibiotics against MRSA infections.

REFERENCES

1. Alvarez, M.A.; Debattista, N.B.; Pappano, N.B. (2008). Antimicrobial activity and synergism of some substituted flavonoids. *Folia Microbiol.*, 53 (1), 23-28.

2. Boyle-Vavra, S.; Daum, R.S. (2007). Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton–Valentine leukocidin. *Lab. Investigation.* 87, 3-9.

3. Cárdenas, M.; Marder, M.; Blank, V.C.; Roguin, L.P. (2006). Antitumor activity of some natural flavonoids and synthetic derivatives on various human and murine cancer cell lines. *Bioorg. Med. Chem.* 14, 2966-2971.

4. Cheng, W.; Li, J.; You, T.; Hu, C. (2005). Anti-inflammatory and immunomodulatory activities of the extracts from the inflorescence of *Chrysanthemum indicum* Linné. *J. Ethnopharmacol.* 101, 334-337.

5. Dhar, D.N. (1981). *The Chemistry of chalcones and related compounds.* John Wiley & Sons, New York, p. 5-9.

6. Franklin, D.; Lowy, M.D. (1998). *Staphylococcus aureus* infections. *N. Engl. J. Med.* 339, 520-532

7. Hawkey, P.M.; Jones, A.M. (2009).The changing epidemiology of resistance. *J. Antimicrob. Chemother.* 64, i3-i10.

8. Hirose, E. ; Matsushima, M.; Takagi, K.; Ota, Y.; Ishigami, K.; Hirayama, T.; Hayashi, Y.; Nakamura, T.; Hashimoto, N.; Imaizumi, K.; Baba, K.; Hasegawa, Y.; Kawabe, T. (2009). Involvement of Heme Oxygenase-1 in Kaempferol-Induced Anti-Allergic Actions in RBL-2H3

Cells. *Inflamattion.* 32, 99-108.

9. Loizzo, M. R.; Said, A.; Tundis, R.; Rashed, K.; Statti, G. A.; Hufne, A.; Menichini, F. (2006). Inhibition of angiotensin converting enzyme (ACE) by flavonoids isolated from *Ailanthus excelsa* (Roxb) (Simaroubaceae). *Phytother. Res.* 21, 32-36.

10. Mandalari, G.; Bennett, R.N.; Bisignano, G.; Trombetta, D.; Saija, A.; Faulds, C.B.; Gasson, M.J.; Narbad, A. (2007). Antimicrobial activity of flavonoids extracted from bergamot (*Citrus bergamia* Risso) peel, a byproduct of the essential oil industry. *J. Appl. Microbiol.* 103, 2056-2064.

11. Matsumura, A.; Ghosh, A.; Pope, G.S.; Darbre, P.D. (2005). Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells. *J. Steroid Biochem. Mol. Biol.* 94, 383-525.

12. Mellou, F.; Lazari, D.; Skaltsa, H.; Tselepis, A.D.; Kolisis, F.N.; Stamatis, H. (2005). Biocatalytic preparation of acylated derivatives of flavonoid glycosides enhances their antioxidant and antimicrobial activity. *J. Biotechnol.* 116, 295-304.

13. Nascimento, G.G.F.; Locatelli, J.; Freitas, P.C.; Silva, G.L. (2000). Antibacterial Activity of Plant Extracts and Phytochemicals on Antibiotic-Resistant Bacteria. *Braz. J. Microbiol.* 31, 247-256.

14. O'Callaghan, R.J.; McCormick, C.C.; Caballero, A.R.; Marquart, M.E.; Gatlin, H.P.; Fratkin, J.D. (2007). Age-Related Differences in Rabbits during Experimental *Staphylococcus aureus* Keratitis. *Invest. Ophth. Vis. Sci.* 48, 5125-5131.

15. Pappano, N.B.; Centorbi, O.P.; Ferretti, F.H. (1990). Determinación de la concentración mínima inhibitoria a partir de parámetros cinéticos de crecimiento. *Rev. Microbiol.* 21, 183-188.

16. Pappano, N.B.; Centorbi, O.P.; Ferretti, F.H. (1994). Determination of the responsible molecular zone for the chalcones bacteriostatic activity. *Rev. Microbiol.* 25, 168-174.

17. Pederiva, R.; Kavka, J.; D´Arcangelo, A.T. (1985). Chalconas y flavanonas aisladas de *Zuccagnia punctata* Cav. *An. Asoc. Quím. Argent.* 63, 85-90.

18. Sato, M.; Tanaka, H.; Yamaguchi, R.; Kato, K.; Etoh, H. (2004). Synergistic effects of mupirocin and an isoflavanone isolated from *Erythrina variegata* on growth and recovery of methicillin-resistant *Staphylococcus aureus. Int. J. Antimicrob. Ag.* 24, 241-246.

19. Sato, Y.; Shibata, H.; Arai, T.; Yamamoto, A.; Okimura, Y.; Arakaki, N.; Higuti T. (2004). Variation in synergistic activity by flavone and its related compounds on the increased susceptibility of various strains of methicillin-resistant *Staphylococcus aureus* to β-lactam antibiotics. *Int. J. Antimicrob. Ag.* 24, 226-233

20. Shibata, H.; Kondo, K.; Katsuyama, R.; Kawazoe, K.; Sato, Y.; Murakami, K.; Takaishi, Y.; Arakaki, N.; Higuti, T. (2005). Alkyl Gallates, Intensifiers of ß-Lactam Susceptibility in Methicillin-Resistant *Staphylococcus aureus. Antimicrob. Ag. Chemother.* 49, 549-555.

21. Taleb-Contini, S.H.; Salvador, M.J.; Watanabe, E.; Ito, I.Y.; Rodrigues de Oliveira, D.C. (2003).Antimicrobial activity of flavonoids and steroids isolated from two *Chromolaena* species. *Rev. Bras. Cienc. Farm.* 39, 403-408.

22. Xu, Y.C.; Leung, S.W.S.; Yeung, D.K.Y.; Hu, L.H.; Chen, G.H.; Che, C.M.; Man, R.Y.K. (2007). Structure–activity relationships of flavonoids for vascular relaxation in porcine coronary artery. *Phytochemistry.* 68,1179-1188.

23. Yilmaz, Y.; Toledo, R.T. (2004). Major Flavonoids in Grape Seeds and Skins: Antioxidant Capacity of Catechin, Epicatechin, and Gallic Acid. *J. Agric. Food Chem.* 52, 255-260.

24. Zetola, N.; Francis, J. S.; Nuermberger, E. L.; Bishai, W. R. (2005). Community-acquired meticillin-resistant *Staphylococcus aureus*: an emerging threat. *Lancet Infect. Dis.* 5, 275-286.