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Synergistic interaction of extracts of garlic (*Allium sativum*) and propolis against methicillin-resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a public health problem, being a cause of severe diseases in hospitals and communities in general. To confront this contingency at present, the effectiveness, in combination with diverse natural products is being studied in order to inhibit this microorganism. The objective of the present work was to evaluate the combined inhibitory effects of ethanolic extracts of garlic (*Allium sativum*) and propolis (Propolis –ppl-) against MRSA strains. We tested two types of extracts: at 20 and 30% for each. Microbial resistance assays were evaluated by the macrodilution method and the combinations were assessed by isobolographic studies. The study strains were divided into two groups based on their resistance to garlic as sensitive (36.8 ± 7.4 mg/mL) and resistant (67.2 ± 8.9 mg/mL). The results show that for strains catalogued as sensitive, the combinations in both concentrations suggest a synergistic effect; on the other hand, for strains catalogued as resistant, the combinations no longer presented a synergic effect.

Key words: Synergism, isobolographic analysis, methicillin-resistant *Staphylococcus aureus* (MRSA), garlic, propolis.

INTRODUCTION

Staphylococcus aureus is a medically important microorganism. For several years, it has been recognized as the main pathogenic agent of its genus for infections of community as well as hospital origin. *S. aureus* forms parts of the Micrococcaceae family, genus *Staphylococcus*, which comprises more than 30 different species, many of

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which are natural inhabitants of human skin and mucous membranes. (Bustos et al., 2006; Camarena and Sánchez, 1997). It is a versatile pathogen that can cause diverse affectations in humans, among which the following are included: superficial lesions, such as skin abscesses and wound infections; systemic infections such as bacteremia or toxemic syndromes such as food poisoning (Espinoza and Gómez, 2007). In addition to its participation in multiple infectious processes, the staphylococci possess great clinical importance due to the progressive increase of mutations that have conferred resistance to multiple antibiotics (Jarraud et al., 2002; Gil, 2000).

The *S. aureus* strains that present resistance to beta-lactams have been denominated methicillin-resistant or MRSA (Hernández et al., 2003). In Mexico, the Hospital Network of Epidemiological Surveillance (RHOVE in spanish) has notified that the percentage of mortality among patients infected with *S. aureus* ranges between 5 and 70% and that percentages of attributable mortality can be high (up to 50%). With data derived from general, pediatric, university, and specialty hospitals, the RHOVE Network has reported that during a period of 1997-2003, S*. aureus* occupied third place in morbility and fourth in mortality (Velazquez, 2005). The great majority of MRSA are not only resistant to beta-lactams, but also to multiple antibiotics, including vancomycin, oxacillin, erythromycin, rifamycin and ciprofloxacin (Camarena and Sánchez, 1997; Filimon et al., 2009)

Due to the great problem caused by MRSA strains in humans, diverse and varied components and substances of plant origin are studied in order to inhibit and control the growth of the microorganism; thus, *Allium sativum* (garlic), a monocotyledon species belonging to the Liliacea family of Asiatic origin, whose medicinal properties have been known since ancient times, has been studied for its anti-microbial effects, which are attributed mainly to the allicin, which possesses potent inhibitory effects on certain enzymes, such as the cysteine proteinases and the alcohol-dehydrogenases, which play important roles in fungal, bacterial and virus infections (Tejerina, 2001; Bptosta, 2005; Duran et al., 2010)

Another natural substance that has also been extensively studied is propolis. Propolis (ppl) is defined as a combination of diverse substances that contain balsamic, ether oils, pollen, vitamins, among others, and that confer antifungal, antibacterial and anti-inflammatory properties, elaborated by bees (*Apis mellifera*) for protection of the beehive (Bracho et al., 2009; Marcen, 2002; Boyanova et al., 2003). Although it has been reported that the antibiotic effects vary by region, the ppl gathering season, and the solvent utilized for extraction, ppl is already being currently taken as a potent anti-microbial, especially against *S. aureus*, whose mechanisms of action include cell-wall debilitation, inhibition of protein synthesis, and inhibition of the process of replication and genetic expression (Palomino et al., 2009; Bustos et al., 2006)

The combination of antimicrobials has demonstrated to be a potent form of combating multiresistant strains. The most common way is to combine diverse pharmaceuticals; however, the combination of natural substances has shown to be a field that should be considered with greater detail, due to the good effects that it has exhibited. One of the ways that the interaction among pharmaceuticals can be evaluated is by means of isobolographic analysis. If two drugs are administered together, their effects can be as follows: (a) additive: corresponding to the simple sum of the effects that each of them produces separately; (b) sub-additive: also termed antagonists, which corresponds to a lesser effect than the simple sum of each agent separately, and (c) synergic or supra-additive: a greater effect than the sum of the effects separately of each drug. It is necessary to conduct a qualitative assay to distinguish these cases when they are solely due to the action of simple addition (Vazquez, 2005; Tallarida, 2002)

Although successful combinations of ppl with other compounds have been reported (Lozina et al., 2008; Fernandes et al., 2005), there are no antecedents of the garlic-ppl combination. The aim of present work was to evaluate the synergic effect on combining extracts of ppl and garlic *in vitro*, against MRSA strains for isobolographic studies.

MATERIALS AND METHODS

Reagents and solutions

Mueller-Hinton Broth (MHB) Becton Dickinson BBCTM Lote0340475, Mueller-Hinton Agar (MHA) BD Bioxon Lot 0006404 and Oxacillin discs (Becton, Dickinson and Co., BDBBL Sensi-disc of 1 µg Ref. 231319) were used. For propolis and garlic extracts, we used absolute ethanol (99.304%) CTR Scientific lot 03T14165 (CTR 01160) and, as reference strain, we employed *S. aureus* ATCC 29213 based on recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2010).

Formulation of the extracts

Propolis was collected from the Canatleca apiculture region (Canatlán, Durango state, Mexico); it was cleaned manually from waxes, and from the remains of plants or insects and was macerated. As for the garlic, this was acquired in the market of the locality, it was peeled and cut into short pieces of about 1 mm in diameter. The garlic plant was identified at National Polytechnical Institute-Dgo. Ethanolic extracts at 20 and 30% was prepared as follows: we weighed 20 g of propolis and 20 g of garlic, each separately, and we added 100 mL of ethanol; we allowed these to rest for a period of 8 days with occasional shaking, protected from light with a covering of aluminum foil at room temperature. In this manner, the two extracts were obtained at 20% (propolis PPL20 and garlic 20). In a similar fashion, the extract was prepared at 30% (PPL30 and garlic 30), weighing 30 g of each separately. At the end of the rest period, the extracts were filtered through Whatman filter paper and were aliquoted in 50-mL corning conical tubes. The

extracts were maintained under protection from light and under refrigeration (at 5-7°C) until the time of the study (Bptosta, 2005; Duran et al., 2010; Tallarida, 2002; Fernandes et al., 2005).

Gathering and characterization of strains

25 strains of *S. aureus* were randomly collected in healthy volunteers whose ages ranged from 1 month to 59 years. The strains were characterized as *S. aureus* by biochemical tests according to that established by McFaddin (2003) (coagulase, catalase and mannitol). In order to identify MRSA strains, we employed oxacillin discs according to guidelines established by the CLSI. ATCC29213 strain was utilized as quality control for such identification. The MRSA strains identified were maintained at -20°C in skim-milk medium and were employed later in the present study. The strains were codified X1, X2, X3, etc. A total of 25 strains were collected, and, only SARM was included in the study: X1, X8, X18, X23 and X24.

Inoculum preparation

Each strain was thawed at room temperature and later cultured on plates with MHA by means of crossed striae in four fields and incubated at 37°C for 24 h. Later, two colonies were removed and suspended in 150 mL of MHB, obtaining an initial population of between 1 × 10E5 and 1 × 10E6 colony-forming units (CFU), which constituted the inoculum, according to CLSI recommendations. Additionally, an aliquot of this inoculum was cultured on a plate with the purpose of counting the population of the inoculum expressed as CFU × 10E6. This population was selected as the basis for calculating effective concentrations 50 (EC50), as described later.

Inhibition studies

The studies were carried out in triplicate by the macrodilution method, according to that described by Taroco et al. (2010). Based on previous assays, we established the following work ranges: 25; 12.5; 6; 3; 1.5; 0.7, and 0.3% of extract v/v for garlic 20% and PPL 20%. Likewise, we utilized 25, 12.5, 6, 3, 1.5, 0.7 and 0.3% of extract v/v for garlic 30% and PPL 30%. After the corresponding inoculation, the extracts wad allowed to act for a period of 20 min and then 10 µL of each concentration (and of each series) was seeded on plates with MHA. The plates as well as the tubes were incubated at 37°C for 18-20 h. To discard the possible inhibitory effect of the ethanol used in the extracts, each strain was cultured in the presence of pure alcohol at the same dilutions utilized in the extracts, employing water as diluent.

MIC, MBC and EC50

Minimal inhibitory concentration (MIC) was taken as the concentration in which appreciable turbidity due to growth was not observed. To determine MIC, we measured absorbance by spectrometry at a wavelength of 625 nm. The plates were expressed as the population of colony-forming units (CFU) × 10E6/mL. As minimal bactericidal concentration (MBC), the concentration that did not exhibit growth on the plate was established. Effective concentration 50 (EC50) was calculated in the following manner: a) in each case, mortality was determined as the difference between the initial population (the inoculum) and the population counted on the plate of each antibiotic concentration; b) after, the percentage of effect was determined as a relationship between the average mortality of the three runs in each concentration and the initial population; c) the log was included in a graph of concentrations vs. percentage of effect, obtaining a straight line, and determining the corresponding equation; d) finally, with this equation, the concentration from which 50% of effect was calculated, and EC50 was obtained. The effects of the four groups were compared: garlic 20, garlic 30, propolis 20 and propolis 30.

Statistics and software

The normal distribution of data was calculated by means of the Shapiro-Wilks test. To verify intergroup differences, an analysis of variance (ANOVA, $p \leq 0.05$) was applied. Microsoft Excel and SPSS 19.0 softwares were employed.

Combinations

In order to evaluate the combinations between the extracts of propolis and garlic, we followed the method described by Talladira (2002) for isobolographic studies. This method has demonstrated to be a simple and objective form for evaluating combinations of pharmaceuticals and presents great statistical robustness. The additive line was established based on the average EC50 of the strains studied, as described by the methodology. The strains were grouped based on their resistance to garlic as follows: Group 1 comprised of five strains that presented an average sensitivity to the garlic extracts of 36.8 ± 7.4 mg/mL, and Group 2, made up of three strains that presented an average resistance of 67.2 ± 8.9 mg/mL. It is necessary to clarify that the groups are independent one one form the other and that these are not compared among themselves. Following the methodology, the combinations with ppl are detailed in Tables 1 and 2 for groups 1 and 2, respectively. After making the cultures in broth and seeds which were plated, the EC50 was calculated as has been previously described. The Student t test was applied for comparing average theoretical concentrations (EC50t) against experimental concentrations (EC50e), as indicated in Talladira's methodology. The experimental proportions of each antimicrobial were determined in order to outline the corresponding isobologram as described previously in the methodology and finally, the interactions index was determined.

RESULTS

Nine of the total strains (36%) from healthy carriers were MRSA. For the garlic extract, minimal inhibitory concentrations (MIC) were 46.13 ± 18.51 mg/mL and 67.79 ± 26.7 mg/mL for extracts at 20 and 30%, respectively. With regard to the propolis, MIC were 18.9 ± 6.4 mg/mL and 14.1 ± 10.2 mg/mL for the extracts at 20 and 30%, respectively.

The result obtained in the isobolographic study of group 1 strains for extracts at 20% as well as at 30%, respectively, of garlic and propolis employed for the inhibition of *S. aureus* are depicted in Table 3.

According to the study for Group 1 strains treated with extracts at 20 and at 30%, respectively, a statistically significant difference was found in the inhibitory concentration of antibiotics in the experimental stage (EC50e) in reference to the theoretical concentration (EC50t) against the microorganism ($p < 0.05$).

The proportionality of the concentrations in the Group 1 combinations are depicted in Table 4 and the isobologram in Figure 1.

Table 1. Combinations used in the isobolographic study for extracts at 20% and 30% propolis and garlic for inhibition of Group 1 MRSA strains.

Group 1 strains					
Extracts at 20%			Extracts at 30%		
EC50 Garlic (mg/mL)	EC50 Propolis (mg/mL)	Zt (mg/mL)	EC50 Garlic (mg/mL)	EC50 Propolis (mg/mL)	Zt (mg/mL)
18.4			17.3		
9.2	0.26	9.46	8.5	0.165	8.67
4.6	0.13	4.73	4.2	0.08	4.28
2.3	0.06	2.36	2.1	0.04	2.14
	0.53			0.33	

EC50: Effective concentration, 50; Zt: Sum of the fractions of both extracts.

Table 2. Combinations used in the isobolographic study for the extracts at 20 and 30% of propolis and garlic for inhibition of Group 2 methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

Group 2 Strains					
Extracts at 20%			Extracts at 30%		
EC50 Garlic (mg/mL)	EC50 Propolis (mg/mL)	Zt (mg/mL)	EC50 Garlic (mg/mL)	EC50 Propolis (mg/mL)	Zt (mg/mL)
33.6			33		
16.8	0.32	17.12	16.5	0.24	16.74
8.4	0.16	8.56	8.25	0.12	8.37
4.2	0.08	4.28	4.12	0.06	4.18
2.1	0.04	2.14		0.33	
	0.65				

EC50: Effective concentration 50; Zt: Sum of fractions of both extracts.

Table 3. Results of the isobolographic study (Group 1 extracts at 20 and 30%).

MRSA strain	Extracts 20%		Extracts 30%	
	EC50t	EC50e	EC50t	EC50e
X1	9.46	6.70	8.66	6.95
X8	9.46	10.90	8.66	4.70
X18	9.46	4.03	8.66	6.80
X23	9.46	4.75	8.66	4.60
X24	9.46	4.30	8.66	6.70
Average	9.46	6.13	8.66	5.95
Standard deviation (SD)	0	2.86	0	1.19

MRSA = Methicillin-resistant *Staphylococcus aureus*. EC50e = Effective experimental concentration$_{50}$; CE50t = Effective theoretical concentration (n = 5; p <0.05).

Table 4. Proportion of extracts at 20 and 30% to outline the isobologram.

Extract	Proportion of extracts at 20%	Proportion of extracts at 30%
Garlic	5.967 mg/mL	5.938 mg/mL
Propolis	0.169 mg/mL	0.012 mg/mL

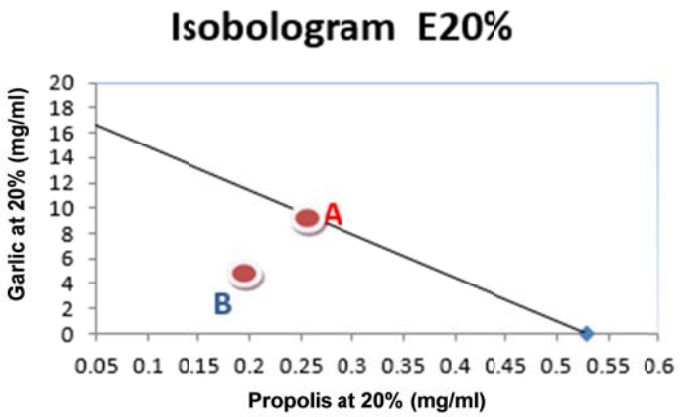

Figure 1. Isobologram of Group 1 extracts at 20%.

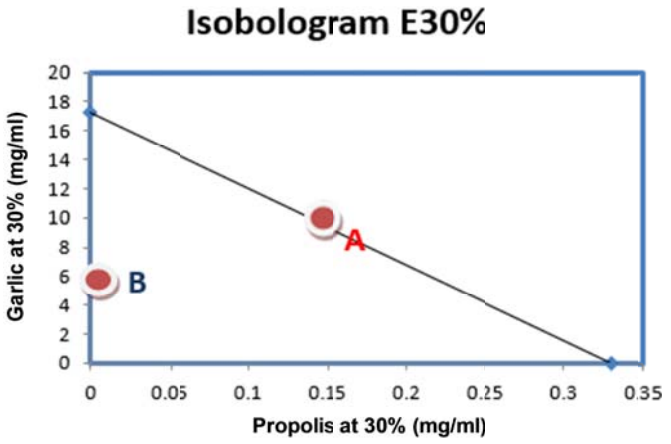

Figure 2. Isobologram of Grupo 1 extracts at 30%.

Point A represents the theoretic concentration of the combination situated on the additive line. Point B represents the experimental concentration (EC), which situates both antibiotics below the additive line. This suggests that the combination presents greater inhibitory potency than the application isolated from each antibiotic separately, which was confirmed by the determination of the interactions index, the latter less than the unit.

The isobologram was outlined based on the proportionalities of Table 4 for extracts at 30% (Figure 2), in which point A represents the theoretical (t) concentration situated on the additive line and point B, the experimental concentration (EC) found, which was situated under the additive line. This suggests that the extracts at 30% also present greater inhibitory potency than the application isolated from each antibiotic separately. To corroborate the synergistic effect observed in Figure 2, we also determined the interaction index, which also resulted less than the unit. In Group 2 strains, contrariwise, we did not find any synergistic combination, the interactions index greater than the unit in all cases.

DISCUSSION

The mechanism of activity of antimicrobal of garlic is based on the inhibition of the activity of the enzimes alkaline phosfatase, invertase, urease and papain. These enzymes are inhibited by alicine in concentrations of 0.0005 M, specially alkaline phosfatase (Lawson, 1993). On the oder hand, propolis compounds, in special flavonoids, inhibits the alkaline phosfatase too (Gutierrez, 2012). May be, both sustances, intensify the action of alicine over strains. This results in a synergistic effect.

According to the investigations carried out by Waili and colleagues in 2012 on the synergic effects of propolis extracts with ethyl alcohol on *S. aureus*, our results are in agreement that propolis utilizes the effect of another antimicrobial on being used on *S. aureus* isolates, with the difference with Waili and coworkers in 2012 which employed a second active substance: honey; on the other hand, our work highlights the effects of propolis in combination with garlic extracts, obtaining results in an interactions index of $\gamma < 1$. Huang et al. (2011) studied the synergistic combination of chitosan with silver acetate, demonstrating that they work in synergy to inhibit the *in vitro* growth of Gram-positive bacilli, methicillin-resistant *S. aureus* and Gram-negative bacteria, with MRSA strains the most resistant to such a combination. In our work, it is noteworthy that Group 2 strains demonstrated high resistance to the combination in both concentrations of the extracts, as the cited work reports.

On the other hand, Lozano and colleagues in 2014 reported a synergistic effect on applying a combination of the extracts of propolis on oregano MRSA isolates; one part of our work with garlic and propolis extracts on group 1 strains coincides with the work of the Lozano group, because it reports a synergistic effect with an interactions index of $\gamma < 1$; however, Group 2 strains showed greater resistance to the garlic and propolis extracts than to the oregano and propolis extracts.

Due to the absence of reports on combinations of garlic with other products of plant origin, it is difficult to conduct a discussion and in-depth comparison with respect to our results. However, we wish to make it clear that the bases are in order for continued investigation on alternative medicine to combat this type of microorganism. The fact that the same combinations of garlic and propolis were synergistic in the face of a group of strains while they did not present the same effect on confronting another group of these can be explained by the fact that even while they belong to the same species, there are genetic diversities between them. This was reported by Kechrid and Perez-Vazquez (2011) in a study with 236 isolates of *S. aureus*, among which 22% highly marked genetic diversity, thus a different behavior of resistance.

We can search for a possible explanation for this in the work of Krest and Keusgen (1999), in which the authors determined the enzymatic activity of allinase, which is responsible for the production of allicin in the extracts, rapidly decreasing to 42°C, indicating that the optimal

temperature of the enzyme is found between 35 and 37°C, and that inactivation occurs between 42 and 60°C; at temperatures >60°C, enzymatic activity is destroyed. It could be that at temperatures under the optimal range, their activity is also diminished; thus, our extracts were observed to be affected, given that they were worked at room temperature (20-25°C), resulting in some strains exhibiting greater resistance to garlic extracts than others.

Conclusions

Isobolographic analysis of our work suggests a synergistic effect on combining garlic and propolis in extracts at 20 and 30% on Group 1 strains. Contrariwise, possibly due to the genetic diversity of the strains studied or to other factors such as the effect of the temperature on the allinase enzyme, group 2 strains showed resistance to the combinations of the extracts.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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