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SYNERGISTIC EFFECT OF (*S*)-*CIS*-VERBENOL WITH THE ANTIBIOTICS AMOXICILLIN AND GENTAMICIN AGAINST SENSITIVE AND RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS

D. Bobadilla [1], M. E. Flores-Giubi [2] and N. Alvarenga [*1]

Department of Phytochemistry [1], Department Biological Chemistry [2], Faculty of Chemical Sciences, National University of Asunción, San Lorenzo, Paraguay.

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Correspondence to Author:
Prof. Nelson Alvarenga

Department of Phytochemistry, Faculty of Chemical Sciences, National University of Asunción, Dr. Jose A. Decoud c/ Escuela Agricola Mcal. Lopez, Campus Universitario de San Lorenzo Km 9.5, San Lorenzo, Paraguay.

E-mail: nelson@qui.una.py

ABSTRACT: The synergistic activity among the monoterpene (*S*)-*cis*-Verbenol and the antibiotics amoxicillin and gentamicin were evaluated against a sensitive and a resistant strain of *Staphylococcus aureus*, being the last one also resistant to methicillin (MRSA). The combinations were evaluated by the checkerboard method. The combinations of both antibiotics with the terpene showed synergic effects for the MRSA strain, whereas for the sensitive strain one combination showed synergy with amoxicillin and additionally one combination showed the additive effect with gentamicin. To confirm the observed results, the combination that had the lowest concentration of amoxicillin and the monoterpene were assayed by the time-kill curve method. An even lower concentration of the terpene that did not show cytotoxicity against human cells was also assayed. The results obtained confirmed those of the checkerboard method and synergy was also observed for the non-cytotoxic concentration of (*S*)-*cis*-Verbenol analyzed. This opens interesting possibilities about the use of purely natural products in combination with antibiotics to treat infections produced by sensitive and resistant bacteria.

INTRODUCTION: Resistant bacteria has become a serious threat to public health in recent times. Especially important are those that are resistant to two or more antimicrobial agents that belong to different types. Among those, *S. aureus* has become relevant because it is responsible for many intrahospitalary and community infections and is associated with an increment of morbidity and mortality in intensive care units [1, 2]. Methicillin-resistant *S. aureus* (MRSA) is becoming isolate with increased frequency since 1961, and it has developed resistance to non-beta-lactam antibiotics as well, like vancomycin [3, 4].

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The substances commonly employed for treatment of infections produced by these bacteria are *β*-lactam antibiotics (alone or combined with aminoglycosides) against methicillin-sensitive *S. aureus* (MSSA) whereas glycopeptides and daptomycin are used against MRSA [6, 7]. One of the alternatives to a fight the bacterial resistance is the search for new antimicrobial drugs from natural products. Medicinal plants represent a vast source of molecules that can be candidates or lead compounds to the development of new substances that can be used as antimicrobials.

The literature offers examples of natural products isolated from plants and microorganisms that possess antibacterial activity and may be useful in helping stop the spread of resistant strains [8]. Among these substances, essential oils have become increasingly important in the last years, due to its antimicrobial properties that include antibacterial, antiprotozoal and antiviral as some of

the most relevant. Essential oils are complex mixtures of terpenes, mainly mono and sesquiterpenes, which are compounds of 10 or 15 carbon atoms that are hydrocarbons or mono-oxygenated alcohols or ketones [9].

Although the antimicrobial properties of the essential oils are known from many years ago, only recently the individual compounds that constitute them have started being tested. Among examples of this, thymol and carvacrol showed antibacterial activity against MRSA and *S. epidermidis* and *Klebsiella pneumonia* [10]. The (+) isomer of *α*-pinene has shown antibacterial activity against *Staphylococcus aureus,* and *Escherichia coli* strains [11]. In a recent study, 65 monoterpenoids and 3 phenylpropanes, that are components of essential oils, were assayed against 24 pathogenic bacteria and the yeast *Candida albicans*. The results showed that oxygenated monoterpenes have higher activity than those belonging to the hydrocarbon type, with minimal inhibitory concentration (MIC) values ranging from 0.03 to 16 mg/mL [9].

Examples have started to appear in the literature, and the results have been good in many cases, validating this as an alternative that deserves more research in order to transform the employment of the essential oils and its components with the antibacterials in an option to treat infections produced by multi-resistant micro-organisms [12, 13, 14, 15]. There are also examples of synergistic effects of individual components of the essential oils and antibiotics against bacteria and the yeast *Candida albicans*, pointing that those compounds can be valid alternatives to revert the resistance of microorganisms against the drug presently used [16, 17]. (*S*)-*cis*-Verbenol **Fig. 1** is a mono-oxygenated monoterpene alcohol, frequently found in many essential oils, but rarely as the main component [18, 19, 20, 21, 22].

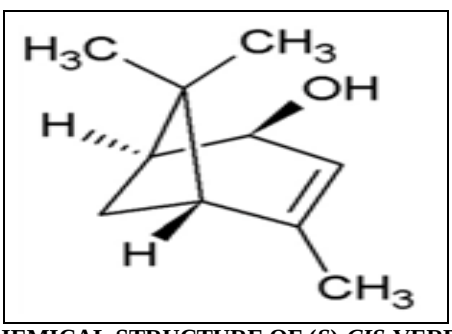

FIG. 1: CHEMICAL STRUCTURE OF (*S*)-*CIS*-VERBENOL

This substance is an important commodity in the food and perfume industry and is a component of pheromone traps [23]. The compound has shown anti-ischemic and anti-inflammatory properties and antiprotozoal activity against species of *Leishmania* and *Trypanosoma cruzi* [24, 25]. The compound has been evaluated for antibacterial activity, showing MIC values between 1 - 2 mg/mL against many Gram (+) and Gram (-) pathogenic bacteria [9]. With these antecedents and the need of finding new alternatives for infectious diseases produced by resistant microorganisms has to lead us to search if the synergistic effect exists between this compound and the drugs amoxicillin and gentamicin against sensitive and resistant strains of *Staphylococcus aureus*. Although both antibiotics are not normally used against MRSA because it is usually resistant, they could be useful if the resistance is reversed by its combination with the terpene.

MATERIALS AND METHODS:

Reagents, Materials, and Chemicals: All the reagents employed were of analytical grade. (*S*)-*c*is-Verbenol and the sodium salt of resazurin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was from Anedra (Research AG, Buenos Aires, Argentine). Mueller-Hinton broth culture media was from Oxoid Ltd., (Basingstoke, Hampshire, England). Baird-Parker agar was from Conda S.A. (Madrid, Spain). Tween 80 was purchased from Hi-Media Laboratories Pvt. Ltd., (Mumbai, India). Amoxicillin and gentamicin were both obtained from local pharmaceutical companies. Sterile 96-well microplates were from Eppendorf AG (Hamburg, Germany). For both antibiotics, a stock solution of 1,000 µg/mL was prepared dissolved in sterile saline solution, and the necessary dilutions were made in Mueller-Hinton broth with 10% DMSO. In the case of (*S*)-*cis*-Verbenol, a stock solution of 100 mg/mL was prepared in Mueller-Hinton broth with 10% DMSO and 10% Tween 80 and dilutions were made to carry on the assays.

Microorganisms: Two strains of *Staphylococcus aureus* were tested. ATCC 6538 and a methicillin-resistant clinical isolate (MRSA). The Microbiology Department of the Central Laboratory, Clinical Hospital of National University of Asuncion, Paraguay, kindly provided the last.

The isolate was obtained in 2016 from a 44-year-old male with cardiac insufficiency in which were detected gram-positive *Cocci* in the blood culture. The identity and sensibility of the microorganism were determined by a Vitek® 2 compact instruments (BioMerieux Inc., Durham, NC) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [26]. The stock microorganisms were maintained freeze at -20 ºC in nutrient broth with 15% glycerol. The initial inoculum was prepared to take aseptically a small portion and put in a screw cap tube with 15 ml of Mueller-Hinton broth. It was then incubated for 24 h at 36 ± 1 ºC. The suspension was then diluted with sterile saline solution to the turbidity equivalent to 0.5 McFarland standard with a turbidimeter (AN 2100, Hach Company, Loveland, CO, USA). This suspension contains 1×10^8 CFU /mL. It was then diluted again with sterile saline solution to yield a suspension that contains 5×10^5 UFC/mL.

Ethical Matters: The sample from which the methicillin-resistant *S. aureus* strain was isolated was collected for diagnosing purposes as per medical request. For that case, no consent is required. Authors of this work have no access to information that could permit to identify the patient.

MIC Determination: The minimum inhibitory concentration (MIC) of the (*S*)-*cis*-Verbenol and the antibiotics Amoxicillin and Gentamicin were determined by the method of Sarker *et al*., with slight modifications [27]. For *S. aureus* ATCC 6538 (*S*)-*cis*-Verbenol initial concentration was 10 mg/ mL for the first well of the row. In the case of the antibiotics, the initial concentration was 10 µg/mL for the first well. For MRSA, the initial concentration of (*S*)-*cis*-Verbenol was the same as above, and for the antibiotics, the concentration in the first well of the row was 200 µg/mL. For the first well of every row, 100 µl of either, (*S*)-*cis*-Verbenol, amoxicillin or gentamicin solution was added and 50 µl of Mueller-Hinton broth (MHB) added in the remaining wells. With a multi-channel pipette, 50 µl was taken from the first well and serial dilutions were made in order then all the wells had 50 µl of the solutions of (*S*)-*cis*-Verbenol and the antibiotics in serially descending concentrations. Then 50 µl of the bacterial inoculum was added to all wells, so the final volume in each was 100 µl. The last two columns of the plate were reserved for grown control and sterility control. Broth plus 10% DMSO and the microorganisms were added to the first column and, the same mixture but without microorganisms to the second column (100 µl total volume in each well). After incubation for 24 h at 36 ± 1 ºC, 10 µl of resazurin solution were added to each well, and the plate was incubated again for 24 h. Any coloration change from deep blue to pink was interpreted as positive microorganism growth.

Fractional Inhibitory Concentration Index Determination: The synergism between (*S*)-*cis*-Verbenol and the antibiotics were initially evaluated by determination of the fractional inhibitory concentration index (FICI) by the checkerboard method [12]. The antibiotics and the terpene were a mixture (1: 1 v/v) at concentrations ranging from 2 × MIC to 1/32 MIC and added to the wells (25 µl each, 50 µl total volume). Then 50 µl of the bacterial suspension (5×10^5 CFU/mL) was added, and the plate was incubated for 24 h. After that time, 10 µl of the resazurin solution was added, and the plate was incubated for another 24 h. The growth inhibition was interpreted as above. The FICI was calculated according to the following formula:

$$FICI = \frac{\text{MIC of antibiotic + Verbenol}}{\text{MIC of antibiotic alone}} + \frac{\text{MIC of Verbenol + antibiotic}}{\text{MIC of Verbenol alone}}$$

The results were interpreted as follows: FICI≤0.5 = synergy; 0.5 < FICI ≤ 4 = additive; FICI > 4 antagonism.

Time-Kill Curve Test: The synergistic activity was confirmed by the time-kill curve test. The lowest concentration of the combination between monoterpene and the antibiotics which had shown synergistic activity according to the checkerboard method (FICI≤0.5), was used. In a tube, MHB was added along with the solutions of (*S*)-*cis*-Verbenol and the antibiotic at the lower concentrations obtained by the checkerboard method that showed a synergistic effect. The bacterial inoculums were also added at a concentration of 5.10^5 CFU/mL. Another tube was used for the control, in which MHB broth was added, plus the antibiotics at the concentration obtained by the checkerboard method and the bacterial inoculum at a concentration of

5.10^5 CFU/mL, without the solution of (S)-*cis*-Verbenol. The tubes were incubated at a temperature of 37 ºC for 24 h. Aliquots were taken and serially diluted at 0, 3, 6 and 24 h within the incubation period. Ten microliters of the dilution were spread on plates with Baird-Parker agar and incubated for another 24 h to determine the number of viable cells. With the results obtained, a logarithm curve of the number of viable cells *vs.* time was made. Synergy is defined as a difference greater than 100 times in the number of CFU/mL or the reduction in the number of colonies greater than $2\log_{10}$ at 24 h produced by the combination compared to the most active agent alone (the antibiotic in this case).

Statistical Analysis: All determinations were in triplicate for the checkerboard method or quadruplicate for the time-kill curve test, on independent days, using means and standard deviations of the values obtained. In the time-kill curve test, to determine if there were statistically significant differences between the assayed compound and the control at 24 h, a paired Student t-test was performed. A significant difference was considered for a P value <0.01.

RESULTS AND DISCUSSION:

MIC Determination: The results of the MIC determination for (*S*)-*cis*-Verbenol and the antibiotics are summarized in **Table 1**.

TABLE 1: MINIMUM INHIBITORY CONCENTRATION (µg/mL) FOR (*S*)-*CIS*-VERBENOL AND THE ANTIBIOTICS GENTAMICIN AND AMOXICILLIN AGAINST *S. AUREUS* ATCC 6538 AND METHICILLIN RESISTANT S. *AUREUS* (MRSA)

	(*S*)-*cis*-Verbenol	Amoxicillin	Gentamicin
Staphylococcus aureus	5300	0.0810	0.0910
MRSA	8600	100.0	107.5

As it can be observed, the MRSA strain is resistant to both antibiotics (MIC >1 µg/mL) according to EUCAST breakpoint tables [28, 29].

FICI Determination: The checkerboard method was used to determine if there was synergy between (*S*)-*cis*-Verbenol and the antibiotics amoxicillin and gentamicin as described previously. A FICI value ≤0.5 was considered to show a synergic interaction between the terpene and the drugs. The results obtained are presented in **Table 2** and **3**. In all the cases, if there were two more combinations that showed a synergic effect, the combinations with the lowest concentration of both substances were selected.

TABLE 2: FRACTIONAL INHIBITORY CONCENTRATION INDEX (FICI) OF THE COMBINATIONS BETWEEN (*S*)-*CIS*-VERBENOL, AMOXICILLIN, AND GENTAMICIN AGAINST *S. AUREUS* ATCC 6538

Staphylococcus aureus ATCC 6538		Individual MIC (µg/mL)	Combined MIC (µg/mL)	FICI	Result
Amoxicillin	+	0.0810	0.0101	0.250	Synergic
(*S*)-*cis*-Verbenol		5300	662.5		
Gentamicin	+	0.0910	0.0455	0.625	Additive
(*S*)-*cis*-Verbenol		5300	662.5		

TABLE 3: FRACTIONAL INHIBITORY CONCENTRATION INDEX (FICI) OF THE COMBINATIONS BETWEEN (*S*)-*CIS*-VERBENOL, AMOXICILLIN, AND GENTAMICIN AGAINST METHICILLIN RESISTANT *S. AUREUS* (MRSA)

MRSA		Individual MIC (µg/mL)	Combined MIC (µg/mL)	FICI	Result
Amoxicillin	+	100.0	25.00	0.313	Synergic
(*S*)-*cis*-Verbenol		8600	537.5		
Gentamicin	+	107.5	26.88	0.375	Synergic
(*S*)-*cis*-Verbenol		8600	1075		

For the sensitive strain of *S. aureus*, the combination of Verbenol with amoxicillin show a FICI of 0.250, indicating a synergic effect, whereas the combination with gentamicin showed no synergic but additive effect. Regarding the MRSA strain, both antibiotics showed a synergic effect with the terpene, with FICI values of 0.313 for amoxicillin and 0.375 for gentamicin. The interesting point here is that the combination lowers the MIC value of the antibiotics 4 times as compared with the antibiotics alone. Although the diminution of the MIC is not enough to revert the

resistance completely, nevertheless a significant improvement was observed, pointing to the positive action of (*S*)-*cis*-Verbenol on the activity of the antibiotics against the microorganism.

Time-Kill Curve Test: The synergistic activity was confirmed by the time-kill curve test. For this, the concentration of the combination between the monoterpene and the antimicrobial, which had synergistic activity according to the checkerboard method (FICI≤0.5), was used.

The combination of amoxicillin and (S)-*cis*-Verbenol was assayed against *S. aureus* **Fig. 2** and the combination between amoxicillin and the terpene against the MRSA strain **Fig. 3** was also assayed. The combination of gentamicin and the terpene that showed synergism by the checkerboard method was not assayed, because the concentration of (*S*)-cis-Verbenol (1,075 µg/mL) was near the value of the IC_{50} for the terpene against human fibroblasts [25].

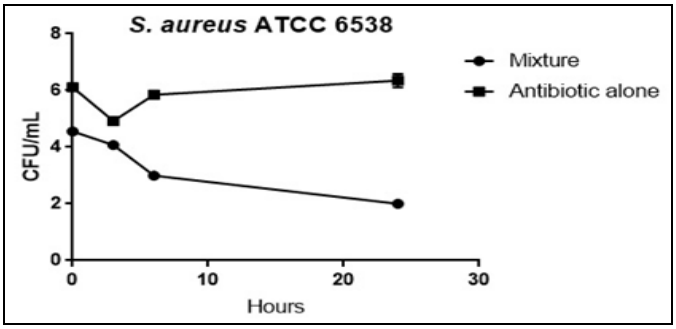

FIG. 2: TIME-KILL CURVE TEST FOR AMOXICILLIN AND (*S*)-*CIS*-VERBENOL AGAINST *S. AUREUS* ATCC 6538. Combination between Amoxicillin (0.01013 µg/mL) and (*S*)-*cis*-Verbenol (662.5 µg/mL) against *S. aureus*. The antibiotic alone was used as control and the combination of the antibiotic and terpene as the assay (P<0.01, Student paired t-test). For some points error bars were omitted for clarity.

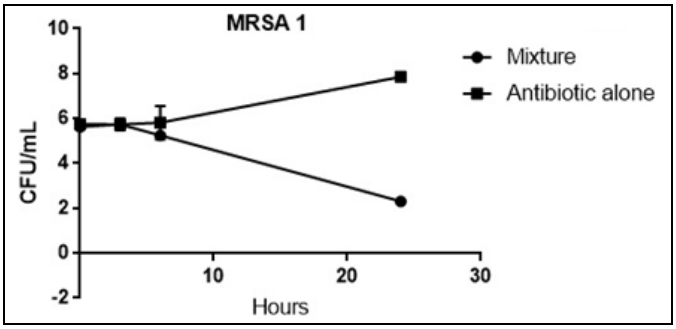

FIG. 3: TIME-KILL CURVE TEST FOR AMOXICILLIN AND (*S*)-*CIS*-VERBENOL AGAINST MRSA. Combination between Amoxicillin (25.0 µg / mL) and (*S*)-*cis*-Verbenol (537.5 µg/mL) against MRSA. The antibiotic alone was used as control and the combination of the antibiotic and terpene as the assay (P<0.01, Student paired t-test). For some points error bars were omitted for clarity.

For the time-kill curve test, a reduction of 2 log_{10} of the CFU (colony forming units) produced by the combination as compared with the control (the antibiotic alone) in 24 h is interpreted as a synergic effect. As can be observed in the graphics and **Table 4**, in both cases the combinations of the terpene with the drugs produce a diminution in the number of CFU superior to 3log_{10} after 24 h concerning the controls indicating a synergistic bactericidal effect [30]. The diminution in the number of CFU was > 4 log_{10} for the ATCC strain and > 5 log_{10} for the MRSA strain.

Taking into account, a recent article where (*S*)-*cis*-Verbenol showed no toxicity against human cells at the concentration of 500 µg/mL [25], that concentration was assayed by the time-kill curve test against the MRSA strain combined the compound with amoxicillin at 25 µg/mL. The results are shown in **Table 4** and in **Fig. 4** (MRSA 2). A reduction of 2 log10 was obtained in the number of CFU, indicating a bacteriostatic synergistic interaction between the terpene and the antibiotic [30]. This behavior is remarkable because it is known that beta-lactam antibiotics are bactericidal, but it could be explained considering that at certain concentration of the terpene with the drug, the effect is bacteriostatic and the increase of the concentration above certain limit produce a bactericidal effect. Also, the molecular target of the terpene could be different from those of the drug, and multiple types of mechanisms for those compounds have been identified [31]. All this could contribute to the observed bacteriostatic effect of the combination.

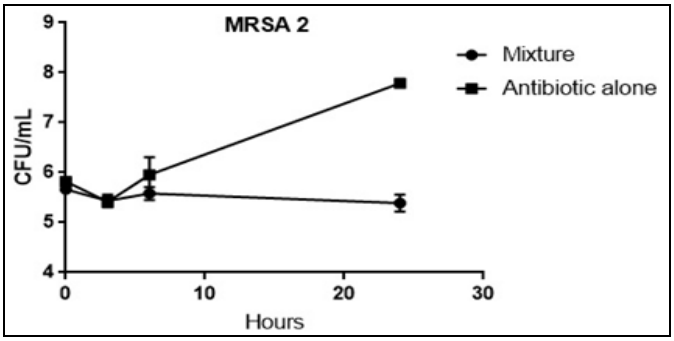

FIG. 4: TIME-KILL CURVE TEST FOR AMOXICILLIN AND (*S*)-*CIS*-VERBENOL AGAINST MRSA. The combination between Amoxicillin (25.0 µg/mL) and (*S*)-*cis*-Verbenol (500.0 µg/mL) against MRSA. The antibiotic alone was used as control and the combination of the antibiotic and terpene as the assay (P<0.01, Student paired t-test).

TABLE 4: Log_{10} VALUES OF CFU AT 24 h FOR THE BACTERIA ASSAYED

		Log_{10} CFU/mL (t= 24 h)	Log_{10} CFU (t24) C – Log_{10} CFU (t24) A
S. aureus	A	2.0 ± 1.2	4.3
	C	6.3 ± 0.24	
MRSA 1	A	2.3 ± 1.3	5.6
	C	7.9 ± 0.041	
MRSA 2	A	5.4 ± 0.17	2.4
	C	7.8 ± 0.025	

MRSA 1: a combination between amoxicillin (25.0 µg/mL) and (*S*)-*cis*-Verbenol (537.5 µg/mL) against methicillin-resistant *S. aureus*. MRSA 2: a combination between amoxicillin (25.0 µg/mL) and (*S*)-*cis*-Verbenol (500.0 µg/mL) against methicillin-resistant *S. aureus*. Assay (A) = Combination Control (C) = Antibiotic alone

Although the resistance was not reverted, as commented above, nevertheless a significant reduction in the concentration of the antibiotics used were observed, indicating that the combination of natural products and clinically used anti-bacterial is a valid alternative to fight microbial resistance. Moreover, in some cases it can improve the safety of the antibiotics, taking into account the toxicity showed by some of them, that could be diminished if lower concentrations are used.

CONCLUSION: The combination of the monoterpene (*S*)-*cis*-Verbenol and the antibiotics amoxicillin and gentamicin showed a synergic effect against a sensitive and a resistant strain of *Staphylococcus aureus*, which is also resistant to methicillin.

The combination of drugs and terpene lower the MIC of the antibiotics 4 times with respect to the antibiotics alone. The synergic activity was also observed for the terpene and amoxicillin by the time-kill curve method, even at a concentration where the terpene showed no cytotoxicity against human cells.

Although, the resistance is not reverted totally, it opens interesting possibilities about the use of purely natural products along with clinically used drugs to overcome bacterial resistance.

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CONFLICT OF INTEREST: Authors have no conflicts of interest.

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