

The Antimicrobial Effects of the Organosulfa-Compounds (OSC), Allicin and S-allylcysteine (SAC) found in *Allium sativum* against *Staphylococcus aureus* (SA).

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ABSTRACT

Background: The increasing frequency of Methicillin resistant SA (MRSA) and Vancomycin Intermediate SA (VISA) are causing care providers to consider alternative forms of treatment. Consequently, the study of botanicals for antimicrobial properties is becoming important. One such botanical, *Allium sativum* (Garlic) contains OSC, which are antimicrobially active. These compounds are allin, allicin, and SAC. The purpose of this work was to investigate the antimicrobial effect of these compounds against SA.

Methods: Antimicrobial susceptibility testing was performed on MRSA and MSSA using pure garlic oil, odorless garlic oil (w/o allicin), garlic capsules with allin, allicin, and allinase, Kyolic with SAC, and penicillin as a comparator in minimal inhibitory concentration (MIC) trays. Time kill was performed to test the rate of bactericidal activity of the garlic oil and its components against MRSA and MSSA. Checkerboard Synergy MIC trays containing penicillin and the OSC were tested against MRSA and MSSA.

Results: The MIC results for MRSA and MSSA respectively were: Garlic Oil 8000µg/ml and 400µg/ml, Capsules 500µg/ml and 500µg/ml, Odorless oil 16000µg/ml and 16000µg/ml and Kyolic >16% sol.

The time kill studies were performed at 1X, 2X and 4X the MIC of MRSA and MSSA to the OSC and penicillin. The Garlic oil at 1X killed the MRSA at 2 hrs. but did not completely kill the MSSA. The capsules were not effective against the MRSA but killed the MSSA in 2 hrs. However, regrowth did occur after 24 hrs. The odorless oil killed at 64000µg/ml against the MSSA at 4 hrs and MRSA at 6 hrs. The penicillin killed the MRSA and MSSA at 4X from 4 to 6 hrs, however regrowth was apparent at 24 hrs. Synergistic results were observed combining penicillin with the garlic oil, odorless oil, and Kyolic against the MSSA and MRSA.

Conclusions: This study suggests that all of the OSC within *Allium sativum* are necessary in order for it to be most effective against MRSA.

INTRODUCTION

Does the botanical garlic work as effectively as the antibiotic penicillin against two strains of the bacteria *Staphylococcus aureus*? These two strains are Methicillin Resistant *Staphylococcus aureus* (MRSA) and Methicillin Susceptible *Staphylococcus aureus* (MSSA). Does the *Allium sativum* (garlic) kill the bacteria at the same rate as the penicillin? Will the garlic oil and penicillin work synergistically or antagonistically together when combined?

Vancomycin is the drug currently used to treat MRSA infections, however, resistance to this drug is beginning to occur. Through this research, it will be determined if there is an alternative treatment of MRSA to help decrease the use of vancomycin. Previous research has shown garlic oil to be effective against MRSA through in vitro testing. This study analyzes several separate components of garlic to determine if they are as effective, more effective, or less effective against MRSA and MSSA than the garlic oil as a whole. Using the garlic oil and penicillin as controls in this experiment, the organosulfa-compounds that will be tested include S-allylcysteine (SAC) through Kyolic, allicin through the use of odorless garlic without the allicin, and the combination of allin, allicin, and allinase through the use of garlic capsules.

PROCEDURES

- Grow MRSA and the MSSA on a blood agar plate (BAP) in an incubator at 35°C overnight

Next day:

Test the *Staphylococcus* with the Murex Staphaurex rapid latex test kit (Remel Inc., Lenexa, Kansas) for the identification of *Staphylococcus aureus* following the manufactures instructions.

- Inoculum preparation:

- Take 2-3 colonies from your BAP and using a sterile cotton swab transfer into a 5ml of demineralized water adjusting to a 0.5 McFarland Standard (1.5 x 10⁸ cfu/ml) using a calibrated nephelometer.
- Add 10µl of the standard to 10ml of Mueller Hinton Broth (MHB).

- Inoculate a Sensititre plate (TREK Diagnostic Systems, Cleveland, Ohio) containing Penicillin, Oxacillin and Vancomycin with the MRSA and MSSA following the manufactures instructions.

- Seal each panel and place them within a 35°C incubator overnight.

Next day:

- Read the results:

- Read the positive growth control well first, using a magnifying mirror reader, to insure valid results.
- Then, read each well for growth. The MIC of the antibiotic is the first well that inhibits the growth of the organism.
- Record the results of each well.

Stock Solutions:

- Garlic oil, odorless garlic oil, and Kyolic stock preparation:
 - Prepare a 10ml stock solution that contains 100,000µg/ml garlic oil using ethanol as a solvent. (1ml garlic oil, odorless garlic oil, or Kyolic to 9ml ethanol).

The concentration for the MIC panel are made as follows:

| MIC (µg/ml) | Stock (ml) | MHB (ml) |
|-------------|------------|----------|
| 32000 | 3.2 | 6.8 |
| 16000 | 1.6 | 8.4 |
| 8000 | 0.8 | 9.2 |
| 4000 | 0.4 | 9.6 |
| 2000 | 0.2 | 9.8 |
| 1000 | 0.1 | 9.9 |
| 500 | 0.05 | 9.95 |
| 250 | 0.025 | 9.975 |
| 125 | 0.0125 | 9.9875 |
| 62.5 | 0.006 | 9.994 |
| 31.25 | 0.003 | 9.997 |
| 15.62 | 0.0015 | 9.9985 |
| 7.81 | 0.00075 | 9.99925 |

- Capsule stock solution preparation:

- A solution contains 100,000µg/ml allin-allicin-allinase.
- The solvent needed to bring the gel into solution is DMSO. After the addition of the DMSO, the concentration becomes 50,000µg/ml.
- Follow the above chart starting at 8000µg/ml for the concentration of the MIC panels.

- Inoculate the MIC trays:

- Both the MRSA and MSSA were tested.
- Carefully inoculate 50µl of the stock dilution and 50µl of your inoculum into each well of the MIC tray using a multi-channel pipette.

- Seal each panel and place them within a 35°C incubator overnight.

Next day:

- Read the results as in step 5 in the first procedure.

Time Kill Procedure:

- Prepare the stock solutions of the antimicrobials (as in steps 1-2 in the second procedure)
- The macro tube dilutions will be made at the MIC, 2X the MIC, and 4X the MIC. There will also be a positive growth control tube consisting of only Mueller Hinton Broth with no antibiotic solution in it.
 - The solution of garlic at the MIC for MSSA is 1000µg/ml, 2X the MIC is at 2000µg/ml, and 4X the MIC is at 4000µg/ml.
 - The solution of garlic at the MIC for MRSA is 8000µg/ml, 2X the MIC is at 16000µg/ml, and 4X the MIC is at 32000µg/ml.
 - The solution of penicillin at the MIC for MSSA is 2µg/ml, 2X the MIC is 4µg/ml, and 4X the MIC is 8µg/ml.
 - The solution of penicillin at the MIC for MRSA is 8µg/ml, 2X the MIC is 16µg/ml, and 4X the MIC is 32µg/ml.
 - The solution of the odorless garlic oil at the MIC for MSSA is 16000µg/ml, 2X the MIC is 32000µg/ml, and 4X the MIC is 64000µg/ml.
 - The solution of the odorless garlic oil at the MIC for MRSA is 16000µg/ml, 2X the MIC is 32000µg/ml, and 4X the MIC is 64000µg/ml.
 - The solution of the garlic capsules at the MIC for MSSA is 500µg/ml, 2X the MIC is 1000µg/ml, and 4X the MIC is 2000µg/ml.
 - The solution of the garlic capsules at the MIC for MRSA is 500µg/ml, 2X the MIX is 1000µg/ml, and 4X the MIC is 2000µg/ml.
- Using a pipette, remove 0.1ml for the standardized solutions of the organism and inoculate that into the 9.9ml control, MIC, 2X the MIC, and 4X the MIC. The final volume should equal 10ml.
- From each tube, remove 0.1ml and transfer that into 9.9ml of saline (this equals a 1:100 dilution). Continue with 2 more 1:10 dilutions (0.1ml into 0.9ml).
- Remove 0.1ml from each dilution, including the 1:100, and transfer this into the center of the BAP using a pipette.
- Spread the solution evenly using a sterile plastic puck spreading from the center outward while rotating the BAP.
- Inoculate the BAP in a 35°C incubator overnight.
- This procedure will be performed at 0 hour, then repeated at 2 hours, 4 hours, 6 hours, and 24 hours.
 - Between the procedures, keep the penicillin, garlic oil, odorless oil, and capsule dilution in the 35°C incubator on a shaker.
- Read the results:
 - Count the colonies on the BAPs at each interval tested.
 - Any plate with over 300 colonies will be recorded at "Too Numerous to Count" or TNTC.

Synergy Procedure:

To determine if the garlic oil, the Kyolic, the odorless oil, or the capsules work together with the penicillin synergistically, antagonistically, or have an additive effect on each other.

- Prepare the penicillin stock solution:
 - Prepare an 8ml stock solution that contains 2560µg/ml of penicillin.
 - To make this solution, dissolve the penicillin with deionized water
 - Prepare the garlic oil, odorless garlic oil, and Kyolic stock solution (follow the step 1 in procedure 2)
 - Prepare the garlic capsule stock solution (follow the step 2 in procedure 2)
 - Perform dilutions of stock solutions as in procedure 2.
 - Dispense the dilutions in the MIC trays using an eight-channel pipette.
 - Inoculate the trays using the checkerboard technique (see data charts). The penicillin solution will travel down the MIC trays while the garlic solution will run across the length of the MIC tray.
 - This tests every possible combination of the penicillin and garlic antimicrobial being tested.
 - Inoculate the MIC trays as in procedure 1.
 - Incubate the MIC trays in a 35°C incubator overnight.
- The next day:*
- Read and record the results using a magnifying mirror reader.

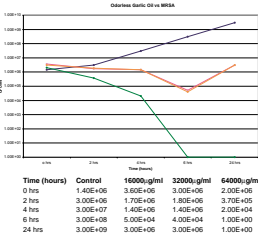
| MIC Results | | | |
|-------------|--------|----------|---------------------|
| Antibiotic | MRSA | MSSA | QC Staph ATCC 29213 |
| Penicillin | 8µg/ml | 1µg/ml | 0.5µg/ml |
| Oxacillin | 2µg/ml | 0.5µg/ml | 0.25µg/ml |
| Vancomycin | 1µg/ml | 1µg/ml | 1µg/ml |

| Antimicrobial | MRSA | MSSA |
|------------------|------------|------------|
| Garlic Oil Lot 1 | 8000µg/ml | 4000µg/ml |
| Garlic Oil Lot 2 | 4000µg/ml | 2000µg/ml |
| Odorless Oil | 16000µg/ml | 16000µg/ml |
| Garlic Capsules | 500µg/ml | 500µg/ml |
| Kyolic | > 16% | > 16% |

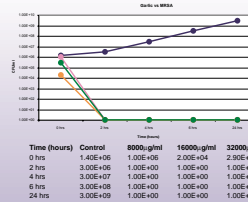
Penicillin vs. MRSA Time Kill Results



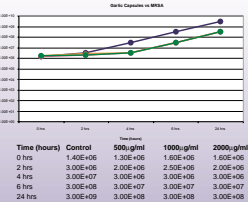
Odorless Garlic Oil vs. MRSA Time Kill Results



Garlic Oil vs. MRSA Time Kill Results



Garlic Capsules vs. MRSA Time Kill Results



MRSA Synergy Plate Format

| | | Garlic Oil | | | | | | | | | | | |
|------------|---|------------------|-------|------|------|------|------|-----|-----|-----|------|-------|----|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Penicillin | A | Positive Control | 16000 | 8000 | 4000 | 2000 | 1000 | 500 | 250 | 125 | 62.5 | 31.25 | 16 |
| | B | 16 | | | | | | | | | | | |
| | C | 8 | | | | | | | | | | | |
| | D | 4 | | | | | | | | | | | |
| | E | 2 | | | | | | | | | | | |
| | F | 1 | | | | | | | | | | | |
| | G | 0.5 | | | | | | | | | | | |
| | H | 0.25 | | | | | | | | | | | |

CONCLUSION

The *Allium sativum* performs more effectively against the MRSA and the MSSA than penicillin, which can be observed through the time kill results.

- The garlic completely killed the MRSA at 2 hours whereas there were still colonies present from the organisms exposed to penicillin for 2 hours. At 24 hours, the MRSA exposed to penicillin began to grow back. However when exposed to garlic for 24 hours there was no re-growth. Indicating *in vitro* that another dose of the penicillin would need to be administered *in vivo*, while one dose of garlic at its highest concentration would still be efficient.
- While exposing the MRSA and the MSSA to the various organosulfa-compounds found in garlic, the results illustrate that these compounds are not as effective against the bacteria, alone as they are when combined in the garlic. Based on the MIC trays and time kill results. With the MIC trays, the garlic capsules (allin, allicin, and allinase) were effective against MSSA and MRSA at 500µg/ml, the odorless oil (allicin free) was effective at 16000µg/ml, and the Kyolic (SAC) was effective at a concentration greater than 16%. The concentration of the odorless oil and the Kyolic are very high when compared to the pure garlic oil, which is effective against MSSA at 4000µg/ml and MRSA at 8000µg/ml.
- The garlic capsules seemed to be extremely effective through the MIC tray results; however, after testing the capsules in the time kill procedure it can be concluded that the capsules were not effective at all against the MRSA. The capsules did immediately kill the MSSA but regrowth was apparent after 24 hours.
- The time kill results for the odorless oil show that it was only effective at 64000µg/ml against the MSSA at 4 hours and against the MRSA at 6 hours. These concentrations are very high when compared to the pure garlic oil, which killed the MRSA at 2 hours at 8000µg/ml.
- The time kill results for penicillin indicate that it did effectively kill the MSSA and MRSA at 8µg/ml from 4 to 6 hours; however, re-growth was apparent.
- The synergy procedure concluded that the garlic and all of the organosulfa-compounds individually work synergistically with the penicillin. Sulfa drugs tend to work synergistically by blocking a bacteria's metabolic pathway at different sites. The compounds in garlic that show antimicrobial activity are the sulfa-compounds; this may be the reason why they work synergistically with the penicillin. The results of the synergy trays show that the garlic and its compounds work synergistically with the penicillin indicating that garlic and penicillin could be more effective against *Staphylococcus aureus* when combined.