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A Qualitative Investigation of Volatile Organic Components of Antimicrobial Oil Smoke Vapors

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Abstract

A petroleum middle distillate, known as fog oil (FO), has been used in the United military battlefield to create obscurant smoke screens. During studies on the feasibility of replacing FO with relatively environmentally benign natural oil esters, with similar flow properties, such as methyl soyate (MS), it was observed that FO and MS aerosols and vapors were lethal to *Salmonella typhimurium* strains (Ames strains used to test for mutagenic activity in the Modified Ames Assay) even after very short exposures. It was further shown that vapors produced from the vegetable oil esters under certain conditions exhibited antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria. In this study, we examined the antimicrobial properties of volatile organic compounds detected in vegetable oil ester vapors. The experiments involved introduction of a known amount of specific compounds present in oil smoke vaors, individually and in combination, into an exposure chamber containing nutrient agar petri dishes inoculated with *Salmonella typhimurium*. Petri dishes were removed from the chamber after varied exposure periods to determine survival of the bacteria. The results of the experiments showed that individual compounds exhibited antimicrobial activity but lower than the vapors produced during thermal aerosol generation process suggesting the antimicrobial activity of the vapors is likely a synergistic activity of multiple components of the vapors.

Keywords

Antimicrobials, Disinfectants, Vapors, Fog Oil, Methyl Soyate, Bacteria

1. Introduction

In recent years, there has been a growing awareness of the need for the develop-

ment of new and safe antimicrobial agents against contamination by various microorganisms in different areas like medical devices, food industry, feed supplies and storage spaces which has led to an increased use of disinfectants and antiseptics by the general public [1] [2] [3]. Disinfectants are antimicrobial agents that are applied to non-living objects to destroy microorganisms, in a process which is known as disinfection [4]. Disinfectants should generally be distinguished from antibiotics that kill or inhibit microorganisms within the body, and from antiseptics, which destroy microorganisms on living tissue. Sanitizers are high level disinfectants that kill over 99.9% of a target microorganism in applicable situations [5] [6]. Very few disinfectants and sanitizers can sterilize (complete elimination of all living microorganisms). Those treatments that can sterilize depend entirely on their mode of action. Disinfectants are abundantly used in hospitals, laboratories and other health care facilities to treat different surfaces [7] [8].

There are different types of disinfectants from various sources. Perfume oils (mixture of natural essential oils and odoriferous organic chemicals) were active in destroying microorganisms either by direct contact with the oils or their vapors [9] [10] [11] [12]. In addition, volatile compounds like trans-2-hexenal, 2,4-hexadienal, furfural, β-ionone, and 1-nonanal found to occur naturally in corn ears inhibit the growth of *Aspergillus flavus* [13]. *In vitro* studies of phytochemical oils possess great antifungal activity in fungal cultures [14]-[21]. Isolation of nonisoprenoid alkyl side chain phenolic compounds, such as anacardic acids, cardols, methylcardols and cardanols from cashew *Anacardium occidentale* (Anacardiaceae) apple, were found to have antimicrobial activity on *Bacillus subtilis*, *Staphylococcus*, *Streptococcus mutans* and *Pseudomonas aeruginosa* [22].

A petroleum middle distillate, known as fog oil (FO), has been used in the military battlefield to create obscurant smoke screens [23]. Traditional, petroleum-based, FO has numerous potential health risks to humans and animals and limits to exposure have been developed [23]. Our research group has been investigating the use of biogenic oils (primarily methyl soyate (MS) as a renewable, potantially less toxic, and more environmentally benign altenative to petroleum based-FO. To determine the potential toxicity of biogenic oil esters for generating obscurant smoke, we explored the mutagenic potential of the oil smoke vapors generated using FO and MS using the modified Ames test [24]. During these studies it was observed that FO and MS aerosols and vapors were lethal to the *Salmonella typhimurium* strains used for the Modified Ames Assay) even after very short exposures. Further studies showed that vapors produced from the vegetable oil esters under certain conditions exhibited high antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria [25]-[32]. We previously showed that aerosols/vapors obtained from mineral oil or vegetable oil ester under proper conditions can serve as an excellent antibacterial disinfectant [33]. The study demonstrated the potential use of volatile biogenic oils as novel antimicrobials and disinfectants for future investigation and clinical ap-

plication. Subsequent analaysis by our lab also characterized the volatile and semivolatile organics with Gas Chromatography-Mass Spectroscpy (GC-MS) and identified several candidate compounds responsible for the antibacterial activity [34]. Using the defined chemical components found in FOG oils by GC-MS analysis, we examined specific volatile organic compounds in vegetable oil ester vapors to determine their antimicrobial activity in isolation or in combination with each other.

Results—Exposure of Salmonella typhimurium *to different concentrations of neat compounds*.

2. Effect of Varying Concentrations of Hexanal on Bacterial Survival

Trypticase Soy agar plates pre-inoculated with *S. typhimurium* were placed inside the exposure chamber. Either 1 microliter or 10 microliters pure hexanal was taken up in a gas chromatography syringe and sprayed into the exposure chamber through the vacuum inlet. The samples were exposed for time intervals of 1, 2, 5, and 10 minutes and later incubated for 24 hrs at 37˚C. The number of colonies were counted after the incubation period. The results are provided in Table 1. No antimicrobial activity was detected at either time intervals or concentration. Therefore, the same sampling procedure was followed using 50 µL hexanal, and the exposure time interval was increased in order to detect the antimicrobial activity of hexanal. The results are provided in Table 2.

Table 1. *S. typhimurium* colony count after exposure to 1 µL and 10 µL Hexanal at different time intervals.

Concentration	1 min	2 min	5 min	10 min
Control	112	113	117	112
1 µl	105	105	95	112
10 µl	101	107	107	118

Table 2. *S. typhimurium* colony counts after exposure to 50 µL Hexanal for different time intervals.

Exposure time in minutes	Volume in µL	Number of Colonies after exposure	Control
30	50	TMTC	TMTC
45	50	300	TMTC
60	50	200	TMTC
75	50	45	TMTC
90	50	No growth	TMTC
120	50	No growth	TMTC

TMTC = too many to count (colony count > 500).

3. Effect of Varying Concentrations of Heptanal on the Bacterial Strains

As with hexanal, 10 µL pure heptanal was taken in a GC syringe and sprayed into the exposure chamber through the vacuum inlet to expose trypticase soy agar plates pre-inoculated with *S. typhimurium*. In addition to varying exposure time, different cell concentration were also employed. The samples were exposed to different time intervals of 1, 2, and 5 minutes followed by incubation for 24 hrs at 37˚C. The number of colonies were counted after the incubating period. The results are provided in Table 3. Different sets of experiments were performed by increasing heptanal concentration and exposure time. The results are presented below in Figure 1 and Table 4. In these experiments, 50 µL heptanal were used and the trypticase soy agar plates were exposed for 75, 90 and 120 minutes.

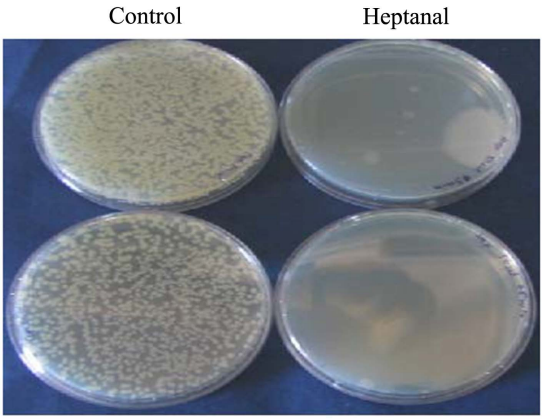

Figure 1. A photograph of representative trypticase soy agar plates incubated with *S. typhimurium* after exposure to 50 µL heptanal. The left plate was the unexposed and on the right plate was 75 minutes exposure time.

Table 3. *S. typhimurium* colony count present after exposed to 10 µL Heptanal at different time intervals.

Culture	1 min	2 min	5 min
105 cells/ml	TMTC	TMTC	TMTC
Control	TMTC	TMTC	TMTC
104 cells/ml	400	300	200
Control	400	400	400

TMTC = too many to count (colony count > 500).

Table 4. *S. typhimurium* colony count of bacterial strains after exposed to 50 µL of heptanal.

Volume	75 min	90 min	120 min
50 µL	No growth	No growth	No Growth
Control	TMTC	TMTC	TMTC

TMTC = too many to count (colony count > 500).

4. Effect of Varying Concentrations of Pentanal on the Bacterial Strains

50 µL pure pentanal was sprayed into the exposure chamber through the vacuum inlet to expose trypticase soy agar plates pre-inoculated with *S. typhimurium*. The samples were exposed to time intervals of 75, 90 and 120 minutes followed by incubation for 24 hrs at 37˚C. The numbers of colonies were counted after the incubation period. The results are provided in Table 5.

5. Effect of Varying Concentrations of Propanal on Bacterial Strains

Trypticase Soy agar plates pre-inoculated with *S. typhimurium* were placed inside the exposure chamber. 50 µL of pure propanal was taken up in a gas chromatography syringe and sprayed into the exposure chamber through the vacuum inlet. The samples were exposed to time intervals of 75, 90 and 120 minutes and later incubated for 24 hrs at 37˚C. The number of colonies were counted after the incubation period. The results are provided in Figure 2 and Table 6.

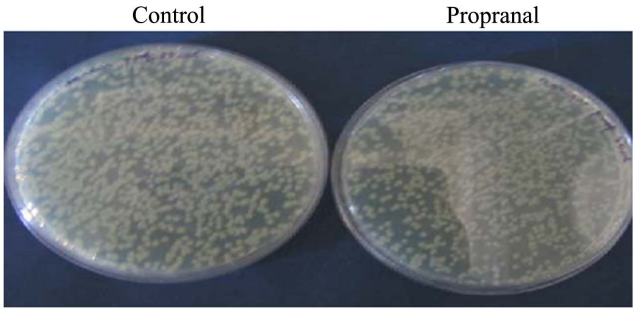

Figure 2. A Photograph of incubated with *S. typhimurium* trypticase soy agar plates after exposure to 50 µL of propanal for 120 minutes.

Table 5. *S. typhimurium* colony count of bacterial strains present after exposure to 50 µL pentanal.

Volume	75 min	90 min	120 min
50 µL pentanal	175	210	255
Control	TMTC	TMTC	TMTC

TMTC = too many to count (colony count > 500).

Table 6. *S. typhimurium* colony count of bacterial strains after exposed to 50 µL Propanal.

Volume	75 min	90 min	120 min
50 µL Propanal	400	350	300
Control	TMTC	TMTC	TMTC

TMTC = too many to count (colony count > 500).

The results of the tests presented above clearly illustrate that volatile organic compounds found in aerosol released during rapid volatilization of methyl soyate (hexanal, heptanal, pentanal and propanal) kills *S. typhimurium.* However, the concentrations at which the pure samples of the compounds possess antimicrobial activity were very high relative to the concentrations detected in analysis of methyl soyate vapors and the exposure time required is much longer. A summary of the analytical report of methyl soyate aerosol chemical composition and concentration used in our analysis is listed in Table 7. Antimicrobial activities of the compounds presented in the tests are also time dependent. In Table 2, it is shown that increased exposure time from 30 to 120 minutes led to an increase in antimicrobial activity. A comparative summary of the data was provided in Table 8 and Table 9.

Table 7. List of volatile compounds and their concentration in comparison with analytical report.

Compounds	Concentration(μg/L) found in analytical report	Concentration (μg/L) of 50 μL aldehydes studied for antimicrobial activity
Propanal	108.40 μg/L	9088.8 μg/L
Pentanal	65.21 μg/L	9044.4 μg/L
Hexanal	160.80 μg/L	8866.6 μg/L
Heptanal	79.42 μg/L	9000 μg/L

Bacterial culture was *S. typhimurium*.

Table 8. Individual compounds (50 μL each) activity at different intervals.

Compounds	75 min	90 min	120 min
Hexanal	−	−	−
Heptanal	−	−	−
Pentanal	+	+	+
Propanal	+	+	+
Formaldehyde	−	−	−
Control	+	+	+

(+) = growth and (−) = no growth of microorganism.

Table 9. Hexanal and Heptanal with two different concentrations individually.

Compounds	75 min	90 min	120 min
Hexanal 20 μl	+	+	+
Hexanal 35 μl	+	+	−
Heptanal 20 μl	+	+	+
Heptanal 35 μl	+	+	PG
Control	+	+	+

(PG) = Partial growth, (+) = growth and (−) = no growth of microorganism.

6. Disinfectant Activity of Mixed Aldehydes

Since the neat compounds did not appear to have the expected antimicrobial activity, it is possible that the strong antimicrobial activity of the oil vapors is due to the synergistic effects of the compounds. Therefore, some of the compounds were tested in combination to look for synergistic effects. Pure hexanal and heptanal in the same amount were taken in equal proportion with two different syringes and injected into the exposure chamber through the vacuum inlet with trypticase soy agar plates pre-inoculated with *S. typhimurium* placed inside the exposure chamber. The samples were exposed for different time intervals of 75, 90 and 120 minutes and later incubated for 24 hrs at 37˚C. The numbers of colonies were counted after the incubation period. The results are provided in Table 10.

7. Disinfectant Activity of Aldehydes and Ketones Mixture

Pure aldehydes and ketones (acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, hexanone, and heptanone) each at 10 times the concentration found in Methyl soyate vapor were mixed to detect the synergistic effects. Antimicrobial activity of the total mixture was tested. The calculated amount of the mixture was taken up in a syringe and sprayed into the exposure chamber through the vacuum inlet of the exposure chamber where trypticase soy agar plates pre-inoculated with *S. typhimurium* were placed inside the exposure chamber. The samples were exposed to different time intervals of 75, 90 and 120 minutes and later incubated for 24 hrs at 37˚C. The results are presented in Table 11.

Table 10. Hexanal and Heptanal mixed in equal proportion.

Compounds	75 min	95 min	120 min
Hexanal (25 µL) Heptanal (25 µL)	+	−	−
Hexanal (15 µL) Heptanal (15 µL)	+	+	+
Hexanal (12.5 µL) Heptanal (12.5 µL)	+	+	+
Control	+	+	+

(+) = growth and (−) = no growth of microorganism.

Table 11. *S. typhimurium* colony count of bacterial strains after exposure to aldehyde and ketones mixtures at different concentrations.

Volume	75 min	90 min	120 min
13 µL	TMTC	TMTC	PG
6.4 µL	TMTC	TMTC	TMTC

TMTC = too many to count (colony count > 500); PG = partial growth.

8. Discussion

Following our initial studies which showed that vapors produced from the vegetable oil esters under certain conditions exhibited high antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria [33]. This revealed the potential use of volatile biogenic oils as novel antimicrobials and disinfectants and worthy of future investigation for clinical application. Vapors generated from heating vegetable oil esters have some limitations due to additional release of residual oil and the complex mixture of organic compoinds. Therefore, subsequent analaysis by our lab characterized the volatile and semi-volatile organics with Gas Chromatography-Mass Spectroscpy (GC-MS) and identified several candidate compounds responsible for the antibacterial activity [34]. In this report, we investigate the defined chemical components to determine their antimicrobial activity in isolation or in combination with each other.

Experiments reported in this study were directed at evaluations of antimicrobial activities of primary chemicals detected in the thermal oxidation products [32]. Analysis of methyl soyate leads to volatile oxygenated species including short chain alcohols, aldehydes, ketones and acids. Antibacterial activities of individual chemicals were assessed by exposing *Salmonella* cultures to vapors of selected chemicals. These experiments also showed that individual aldehydes such as pentanal, hexanal and heptanal exhibit antimicrobial activity but only at very high concentration relative to those observed in the thermal oxidation products. Even the mixture of aldehydes did not exhibit antibacterial activity at concentration 10 times higher than the concentration in the thermal oxidation stream.

The results presented indicate that concentration of several neat compounds required for antibacterial activity was higher than the concentration found in analytical data of vapor. This included additional studies with propanal and pentanal which showed no antimicrobial effect at higher concentration. Based on these results it was concluded that the antimicrobial activity of the methyl soyate vapor is likely due to the synergistic effect of several chemical components in the vapor. Further testing with aldehydes and ketones in a mixture of each at 10 times the concentration found in methyl soyate vapor produced no antimicrobial activity. These results suggest the headspace concentration of the individual aldehydes may not be achieving the desired level for antimicrobial activity.

9. Materials/Methods

Compounds: Methyl soyate vapors were composed of acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, hexanone, and heptanone. High purity Standards (98%) of acetaldehyde, propanal, butanal, pentanal, hexanal, and hexanone were purchased from Sigma-Aldrich, St. Louis, MO. Heptanone was purchased from M. P Biochemical's, Inc Ohio. These compounds were selected based upon mass spectroscopy analysis of MS and FO vapors [32]. All above

listed chemicals were properly stored in order to avoid any contamination and vaporization before experiments.

Nutrient medium for bioassay: Tryptic soy agar (1.5% agar, 3% tryptic soy broth) and tryptic soy broth (3% tryptic soy broth) were used for the anti-microbial assay. The agar was purchased from Fisher Scientific (Fair lawn, NJ). The tryptic soy broth (soybean-casein digest medium) was purchased from Becton, Dickinson and company (Sparks, MD).

Bacterial strain: *Salmonella typhimurium* strain TA97 was acquired from the laboratory of Dr. Bruce Ames at the University of California Berkeley. *S. typhimurium* stock cultures were stored at −80˚C and fresh sub-cultures from frozen stock were prepared on a monthly basis. Active cultures were transferred to fresh media every week.

Inoculations of the bacteria on the nutrient agar plates: An overnight culture (16 hrs) was used for serial dilution. After measuring the optical density (O.D) at 600 nm the culture was serially diluted with trypticase soy broth to final concentration of 10^4 cells/mL. The diluted culture was used for inoculation of tryptic agar plates. From the selected tube 100 µL of the culture was spread using sterile glass beads.

Exposure to neat compounds: After inoculation plates were placed open in the glass exposure chamber. The specified amount of the neat compounds was injected through the outlet in the lid of the exposure chamber. Studies were done with inoculated plates exposed to 5, 10, 15, 30, 45, 75, 90, or 120 minutes. After exposure plates were incubated for 24 hrs at 37˚C on a bench top incubator.

10. Conclusion

The overall conclusion from these initial results is that individual neat compounds identified in the thermal oxidation products exhibit antimicrobial activity. However, the high volumes needed to achieve antimicrobial activity indicate that antimicrobial activity may stem from the synergistic effects of several compounds and/or may be related to unidentified chemical/s in the thermal oxidation product stream. Alternatively, the neat compounds may not achieve the needed concentration due to insufficient vaporization within the chamber. A more comprehensive chemical and antimicrobial analysis of these products is therefore recommended for future studies. Further analysis is also recommended to determine the mechanism of inhibition. The oil vapors are bactericidal and not bacteriostatic and preliminary experiments indicate that the bacteriostatic activity is due to disruption of the cell membrane. However, the sporicidal activity of the vapors also suggests other mechanisms of bactericidal activity.

Author Contributions

Conception and design: A. L, J. K., D. W., and S. K. Manuscript writing: A. L., J. K., D. W., and S. K. Final approval of manuscript: D. W. and S. K. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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