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A Method for Screening of Volatile Antimicrobial Compounds

Raman Preet Singh

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Abstract Presently available methods for determining antimicrobial activity include broth dilution and disc diffusion. However, these methods can not be employed for study of vapor phase antimicrobial activity. The present study describes a new method and a new apparatus for determination of vapor phase antimicrobial activity of volatile substances against bacteria. The method can be used for assessing effect of new and existing compounds on environmental microflora.

Keywords Antimicrobial · Phenol · Volatile compounds · Vapor phase

Microbial infections constitute an important health burden globally. The control of bacteria is usually achieved by disinfection (usually liquid disinfectants) rather than sterilization as the latter is difficult to achieve when dealing with large amounts of material or when human health may be at risk due to stringent sterilizing procedures. However, the liquid disinfectants suffer from the disadvantage of a local antibacterial effect at the place of application (e.g. liquid phenol disinfectants). This is of concern in conditions requiring aseptic environments like operation theaters in hospitals, microbiology laboratories and microbiological quality control laboratories in food and pharmaceutical industries. Thus, volatile compounds and gases are used in such cases to achieve disinfection/sterilization. Additionally, these agents lack specificity and may affect useful, nonpathogenic microbes as well.

The presently available methods for evaluation of disinfectants include broth dilution and disc diffusion (Holley and Patel 2005). The fundamental limitation in these methods is that only solid and liquid compounds can be tested for disinfectant activity. Also, the potential of compounds to exhibit disinfectant activity in volatile form can not be determined. Further, some compounds may even show better antimicrobial activity in vapor-phase than in liquid phase (Lopez et al. 2005, 2007; Becerril et al. 2007; Tullio et al. 2007). Another limitation while using volatile compounds is that the compounds may be insoluble in water leading to their precipitation in the aqueous culture medium. Such hydrophobic compounds are dispersed in water with the aid of an organic solvent but the compound may itself partition into the organic solvent and desirable concentrations may not be achieved in the aqueous medium.

The most commonly used method for screening of volatile/gaseous antibacterial agents utilizes the killing of *Bacillus subtilis* spores (Kozo et al. 1986; Demitrius et al. 1993; Kida et al. 2007). An inherent disadvantage associated with this method is that it utilizes killing of spores as an endpoint. It is known that not all bacteria form spores and many of them exist only in their vegetative forms. Also, the susceptibility of spores and vegetative forms are different: spores are more resistant to all kinds of antibacterial treatments. Thus, the extrapolation of results from spores to vegetative form will be different. Also, this method can miss the volatile compounds with low activity against spores but which may be highly active against

R. P. Singh (✉)
Department of Pharmacology, Akal College of Pharmacy and Technical Education, Mastuana Sahib, Sangrur, Punjab 148 001, India
e-mail: ramanps@hotmail.com

Present Address:
R. P. Singh
Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar, Punjab, India

vegetative forms of bacteria. Further, this method requires exposure to large quantities of the volatile compounds and specialized equipments for exposure and determination of activity against spores. Recently, a laboratory-scale screening method for determining vapor phase antimicrobial activity of volatile compounds against microbes has been reported that can be easily adopted in laboratory conditions (Lopez et al. 2005, 2007; Becerril et al. 2007; Tullio et al. 2007; Nedorostova et al. 2009). Sato et al. reported the evaluation of volatile compounds for antimicrobial activity against air-borne microorganisms (Sato et al. 2006). Aufderheide reported a system for determining genotoxic potential of cigarette smoke (Aufderheide and Mohr 2004; Aufderheide and Gressmann 2007).

However, the earlier methods allow loss of volatile compounds in surroundings leading to a reduction of vapor concentration exposed to the bacteria (Lopez et al. 2005, 2007; Becerril et al. 2007; Nedorostova et al. 2009). The CULTEX system is costly equipment (Aufderheide and Mohr 2004; Aufderheide and Gressmann 2007). Here, we report a method for determination of antimicrobial activity of volatile compounds against vegetative forms of bacteria. The method involves exposure of bacteria to vapors of the test compound followed by culturing in liquid medium to determine the death/survival of the exposed bacteria.

Materials and Methods

The essential oils (EOs) from cinnamon (*Cinnamon zeylanicum*), clove (*Syzygium aromaticum*) and ginger (*Zingiber officinalis*) were extracted by Clevenger apparatus and were a generous gift from Gurpreet Kaur. These three spices for chosen because of their reported antimicrobial activity (Lopez et al. 2005, 2007; Becerril et al. 2007). For the extraction of EOs, the spices were exhaustively extracted using Clevemger apparatus during March–April, 2006. The EOs were stored in air tight, amber colored bottles and used within 1 week of extraction. All chemicals used were of analytical grade.

The apparatus design is as described in Fig. 1. The apparatus consists of a 5 mL glass vial (JSGW, India) (1) containing the test compound (2) at the bottom of vial. The mouth of vial is covered with a filter paper impregnated with bacterial suspension (3) and a lid (4). The Whatman No. 1 filter paper discs were were cut to the size of rim of the vial, soaked overnight in distilled water and autoclaved thereafter. The overnight washing with distilled water was carried out to ensure removal of antimicrobial compounds present in the filter paper disc, if any. The test material was placed at the bottom of an autoclaved vial and closed with an autoclaved lid. The vial was left undisturbed for 3 h at 37°C to allow saturation of air inside the vial with vapors

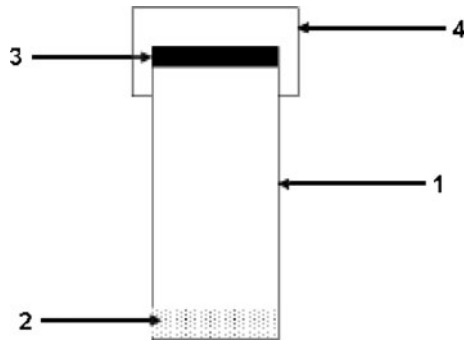

Fig. 1 Design of apparatus

of the test material. The autoclaved filter paper strips were impregnated with 0.01 mL of the microbial suspension of a Gram-positive (*Staphylococcus aureus*) or a gram-negative bacteria (*Escherichia coli*) containing 1×10^9 bacteria. mL^{-1}. The filter paper strips were placed on the rim of vial using sterile forceps and the lid was closed. The filter paper strip was inserted quickly to minimize vapor loss from vial. The total time involved in opening the lid, loading the filter paper strip and closing the lid was less than 60 s. The filter paper strips were removed at various time intervals and incubated (37°C) overnight in nutrient broth (HiMedia, India). All procedures were performed aseptically. The liquid medium was observed after overnight incubation for bacterial growth as described elsewhere (Aufderheide and Mohr 2004; Kaur and Singh 2008).

In parallel experiments, the vapor phase antimicrobial activity was also determined by sealed petri plate method as described earlier (Lopez et al. 2005, 2007; Becerril et al. 2007; Tullio et al. 2007) and compared by One way ANOVA.

Results and Discussion

There is a need for development of methods/systems for determination of vapor phase antimicrobial activity of volatile compounds. This is because the diffusion-based methods (disc diffusion and cylinder plate methods) may not be suitable for identification and development of new antimicrobial agents active in the vapor phase. An obvious reason for the unsuitability of diffusion-based assays is that in these methods the test compound interacts with the microbe(s) in liquid state while in its natural environment the compound may be active in vapor phase as in case of essential oils, environmental pollutants and gaseous/volatile disinfectants. Further, the compounds may show differences in antimicrobial activity in liquid state and in vapor phase (Lopez et al. 2005, 2007; Becerril et al. 2007; Tullio et al. 2007).

In the present study, the antibacterial activity of solid (cinnamon, clove and ginger) and liquid (EOs, ethanol, glutaraldehyde, hydrochloric acid and phenol) samples containing volatile compounds was determined. The antibacterial activity of vapors was a function of the nature of compound and time of exposure. The apparatus was validated by including EOs with reported antimicrobial activities in vapor-phase (Lopez et al. 2005, 2007; Becerril et al. 2007; Tullio et al. 2007). Ethanol, glutaraldehyde and hydrochloric acid were also found to possess antibacterial activity (Table 1). In parallel experiments, the commonly used sealed petri plate method was also used (Lopez et al. 2005, 2007; Becerril et al. 2007; Tullio et al. 2007). The extent of killing was similar in both methods (Table 1).

Phenolic disinfectants are the most extensively used class of disinfectants. In order to identify whether phenol exhibits antimicrobial activity, phenol was also included. Surprisingly, phenol was also found to exhibit vapor phase antimicrobial activity (Table 1). Further, the time-dependent antimicrobial activity of phenol was also determined. Phenol showed a time-dependent killing of bacteria (Table 2).

The method was validated in terms of accuracy, reproducibility, precision and sensitivity. The method was found to be sensitive, reproducible, precise and accurate with intra-assay R.S.D. $< 5\%$ and $R2 > 0.99$. The inter-assay R.S.D. was found to be $<10\%$.

The results of the present study suggest that the method and apparatus described herein is a cheap, sensitive and reproducible method for determining vapor-phase antibacterial activity of volatile compounds. The advantages of the apparatus are numerous: (1) it requires very small amount of test compound which is of particular interest when screening costly compounds or natural products present in minute quantities, (2) the solvent minimally interferes with the estimation of antimicrobial activity, (3) the simple design of apparatus can be easily adopted in laboratories, (4) the apparatus is a closed system and can be used for screening of antimicrobial activity against pathogenic microbes, (5) the apparatus allows screening of compounds without sterilization. Thus, pharmaceutical formulations, toiletries, solids and even plant parts can be screened for antimicrobial activity. The size of the test vessel could be further reduced to enable high throughput screening of volatile test compounds.

The versatility of apparatus makes it compatible with sophisticated analytical techniques. The air inside the vial can be sampled and analyzed by chromatographic techniques (GC, GC–MS, etc.) for identification of active volatile chemical principles. Genetically engineered microbes expressing reporter genes like luciferase, green fluorescent protein, etc. may be employed for rapid determination of bacterial counts. The viable bacteria can also be analyzed by ATP production, carbon dioxide production, metabolite production, oxygen consumption, substrate consumption, DNA concentration, radionucleotide incorporation or other

Table 2 Time-dependent effect of phenol (50%) on bacterial survival

Time (min)	Mean bacterial survival (%)*	
	S. aureus	*E. coli*
10	69	73
20	25	40
45	13	10
60	0	0

* Data is mean of 3 experiments with n = 5/experiment

Table 1 Effect of various test materials on bacterial survival

Test compound (Concentration in %)	Quantity added	Mean bacterial survival (%)*			
		S. aureus		*E. coli*	
		Vial method	Sealed petri plate	Vial method	Sealed petri plate
Glutaraldehyde (25)	0.1 mL	0	0	0	0
Phenol (50)	0.1 mL	0	0	0	0
Ethanol (95)	0.1 mL	10	6	5	4
Hydrochloric acid (33)	0.1 mL	25	27	40	42
Cinnamon EO (100)	0.1 mL	0	0	0	0
Clove EO (100)	0.1 mL	0	0	0	0
Ginger EO (100)	0.1 mL	0	0	0	0
Cinnamon	100 mg	0	0	0	0
Clove	100 mg	0	0	0	0
Ginger	100 mg	0	0	0	0
Water	0.1 mL	100	100	100	100

* Data is mean of 3 experiments with n = 5/experiment. The difference in results from the vial method described in this study and the previously reported sealed petri plate method were statistically insignificant ($p > 0.05$)

fast methods like optical density, conductance and impedance measurements. Thus, the adaptability of the method allows further modifications for screening of volatile antimicrobial compounds and rapid assessment of bacterial survival.

In conclusion, the method and apparatus described is a versatile system and can be easily adapted in laboratories for determination of antibacterial activity. The application of the apparatus can be extended to determine antimicrobial activity against fungi and other types of microbes. Further, the method can be employed for screening of solids, liquids, gases, aerosols, etc. for antimicrobial activity.

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References

Aufderheide M, Gressmann H (2007) A modified Ames assay reveals the mutagenicity of native cigarette mainstream smoke and its gas vapour phase. Exp Toxicol Pathol 58:383–392

Aufderheide M, Mohr U (2004) A modified CULTEX system for the direct exposure of bacteria to inhalable substances. Exp Toxicol Pathol 55:451–454

Becerril R, Gómez-Lus R, Goñi P, López P, Nerín C (2007) Combination of analytical and microbiological techniques to study the antimicrobial activity of a new active food packaging containing cinnamon or oregano against E coli and S. aureus. Anal Bioanal Chem 388:1003–1011

Demitrius CA, Duran AP, Chamberlain VC, Hitchins VM (1993) Comparison of European and US biological indicators for ethylene oxide sterilization. J Ind Microbiol Biotech 12:399–402

Holley RA, Patel D (2005) Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. Food Microbiol 22:273–292

Kaur G, Singh RP (2008) Antibacterial and membrane damaging activity of Livistona chinensis fruit extract. Food Chem Toxicol 46:2429–2434

Kida N, Mochizuki Y, Taguchi F (2007) Sterilization of a biological safety cabinet by vaporized KMT reagent. Biocontrol Sci 12:65–69

Kozo I, Nariko S, Hidetoshi M (1986) Inactivation of *Bacillus* spores by gaseous ozone. J Appl Microbiol 60:67–72

Lopez P, Sanchez C, Batlle R, Nerin C (2005) Solid- and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. J Agric Food Chem 53:6939–6946

Lopez P, Sanchez C, Batlle R, Nerin C (2007) Vapor-phase activities of cinnamon, thyme, and oregano essential oils and key constituents against foodborne microorganisms. J Agric Food Chem 55:4348–4356

Nedorostova L, Kloucek P, Kokoska L, Stolcova M, Pulkrabek J (2009) Antimicrobial properties of selected essential oils in vapour phase against foodborne bacteria. Food Control 20:157–160

Sato K, Krist S, Buchbauer G (2006) Antimicrobial effect of trans-cinnamaldehyde, (−)-perillaldehyde, (−)-citronellal, citral, eugenol and carvacrol on airborne microbes using an airwasher. Biol Pharm Bull 29:2292–2294

Tullio V, Nostro A, Mandras N, Dugo P, Banche G, Cannatelli MA, Cuffini AM, Alonzo V, Carlone NA (2007) Antifungal activity of essential oils against filamentous fungi determined by broth microdilution and vapour contact methods. J Appl Microbiol 102:1544–1550