

44/80

Inhibition of *Clostridium botulinum* by Spice Extracts and Aliphatic Alcohols

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ABSTRACT

Alcoholic extracts of spices were prepared and tested for inhibition of *Clostridium botulinum* in culture media. Mace (the outer covering of the seed of *Myristica fragrans*) and achiote (annato, *Bixa orellana*) were the most inhibitory of 33 spices studied. Also quite active were bay leaf (*Laurus nobilis*), white and black pepper (*Piper nigrum*) and nutmeg (the seed of *M. fragrans*). Less active were rosemary (*Rosmarinus officinalis*), clove (*Eugenia caryophyllata*), oregano (*Oreganum vulgare*), turmeric (*Curcuma longa*), thyme (*Thymus vulgaris*), and paprika (*Capsicum annum*). Of the series C₁ to C₁₈, aliphatic straight chain alcohols of C₁₄ or C₁₆ chain-lengths were the most inhibitory against *C. botulinum* with a minimum inhibitory concentration (MIC) of 0.6 ppm. A plot of alcohol chain length versus MIC showed a highly significant (P < 0.01) cubic function.

Spices are inhibitory to a number of species of aerobic bacteria. Dold and Knapp (3) showed garlic and onion to be active against all microorganisms tested. Nutmeg and mustard inhibited all organisms tested except *Bacillus subtilis*. Beuchat (1) found oregano and thyme were inhibitory to *Vibrio parahaemolyticus*. Pepper was active against *Escherichia coli* in sausage according to Salzer et al. (12), but they found the curing organisms, the micrococci and lactobacilli, to be unaffected by relatively high concentrations. Farbood et al. (6) reported that a rosemary spice extract was active against *Staphylococcus aureus* in culture media; gram negative bacteria were unaffected.

Fatty acids have been shown by several workers to possess antibacterial activity (5,7,11). Borick et al. (2) showed fatty acid salts to be capable of inhibiting a variety of bacteria. Huhtanen and Micich (9) showed aliphatic amines to be active in inhibiting *Clostridium botulinum*.

The literature on spice-inhibition of bacteria contains no references to studies with *C. botulinum* nor has the effect of aliphatic alcohols been reported. The studies reported here were undertaken to determine the effect of spices and a series of straight chain aliphatic alcohols, from C₁ to C₁₈, against *C. botulinum*.

MATERIALS AND METHODS

The assay method and medium were those of Huhtanen (8). Spices were obtained from local retail outlets. Extracts were made by steeping 10 g of a spice in 90 ml of ethanol for 48 h at room temperature with

¹Agricultural Research, Science and Education Administration, U.S. Department of Agriculture.

occasional stirring. A 0.1-ml portion of the undiluted, particle-free supernatant liquid of an extract was added to each of two tubes each containing 5 ml of sterile assay medium. Serial dilutions of each extract were made with sterile assay medium to contain 1/4, 1/16, 1/256 and 1/1024 the original concentration of extractives per unit volume, and 0.1 ml of each dilution was added to each of two tubes each containing 5 ml of assay medium. Extraction efficiency was not determined, but the six levels of each spice extract added to the assay tubes represented the extract equivalents of about 2000, 500, 125, 31, 8 and 2 ppm of the original spice in terms of the final contents of the assay tubes.

The C₁ to C₁₂ alcohols were added in amounts of 0.2, 0.1 and 0.05 ml to 5-ml quantities of assay medium in tubes. The final concentrations of alcohols (v/v) were 40,000, 20,000, and 10,000 ppm. The alcohols were then diluted by adding 0.5 ml to 3.5 ml of assay medium. This 1:8 dilution was added to assay tubes in 0.2, 0.1 and 0.05-ml amounts, giving final concentrations of 5,000, 2,500 and 1,250 ppm. The 1:8 dilutions and additions were repeated, giving a series of log₂ dilutions.

The insoluble alcohols, C₁₃, C₁₄, C₁₆ and C₁₈, were made up in 10 mg/ml concentrations in ethyl alcohol, and 0.1 and 0.05 ml were added to tubes of autoclaved assay medium. The alcohols were diluted 1:8 in assay medium as above, and, though these alcohols formed suspensions, they were fine and uniform enough so they could be diluted and pipetted as with the C₁ to C₁₂ alcohols.

Determination of MIC was made visually by observing turbidity. When the organism grew, turbidity was usually readily apparent, especially when viewed through transmitted light. In cases where a slight amount of growth was apparent in one tube, for example at 40,000 ppm, and maximum growth in the next dilution, 20,000 ppm, the MIC was estimated to be between the two or, in this example, 30,000 ppm. In cases where insoluble alcohols formed turbidity, verification of growth or no growth was made by observing gas production when a hot loop was plunged into tubes warmed to 50 C. In cases of questionable gas production by the hot loop method, further evaluation of growth was made by examining gram stains of the cultures.

RESULTS AND DISCUSSION

The most inhibitory spices (Table 1) were mace and achiote with a MIC of 31 ppm. Nutmeg, bay leaf and white and black pepper were also quite active with a MIC of 125 ppm. Lesser activities, with MICs of 500 ppm, were exhibited by the extracts of paprika, rosemary, cloves, oregano, turmeric and thyme. The rest of the spices showed little or no inhibition at 2,000 ppm.

The inhibition of *C. botulinum* by aliphatic alcohols is shown in Table 2. Methanol and ethanol gave a MIC of 30,000 ppm; this decreased to 0.6 ppm for tetradecanol and hexadecanol, increasing again to 25 ppm for octadecanol. The curve of chain length plotted against Ln MIC, shown in Fig. 1, shows a highly significant (p < 0.001) cubic function and a less significant (p < 0.005) quadratic function. The increase in MIC with

TABLE 1. Inhibition of *Clostridium botulinum* by spices.

Common	Botanical	MIC* (µg/ml)
Allspice	<i>Pimenta officinalis</i>	2000
Parsley	<i>Petroselinum crispum</i>	> 2000
Marjoram	<i>Marjoram hortensis</i>	> 500
Mustard	Blend of <i>Brassica hirta</i> and	
	<i>Sinapis alba</i>	> 2000
Garlic	<i>Allium sativum</i>	> 2000
Celery flakes	<i>Apium graveolens</i>	> 2000
Celery seed	<i>Apium graveolens</i>	2000
Chives	<i>Allium schoenoprasum</i>	> 2000
White pepper	<i>Piper nigrum</i> (water soaked)	125
Black pepper	<i>Piper nigrum</i> (dried fruit)	125
Sweet pepper	<i>Capsicum annuum</i>	> 2000
Paprika	<i>Capsicum annuum</i>	500
Anise	<i>Pimpinella anisum</i>	> 2000
Sage	<i>Salvia officinalis</i>	2000
Ginger	<i>Zingiber officinale</i>	> 2000
Caraway	<i>Carum carvi</i>	> 2000
Fennel	<i>Foeniculum vulgare</i>	> 2000
Achiote	<i>Bixa orellana</i>	31
Tarragon	<i>Artemisia dracunculus</i>	> 2000
Dill	<i>Anethum graveolens</i>	> 2000
Rosemary	<i>Rosmarinus officinalis</i>	500
Cinnamon	<i>Cinnamomum zeylanicum</i>	2000
Cloves	<i>Eugenia caryophyllata</i>	500
Red Pepper	<i>Capsicum frutescens</i>	> 500
Bay Leaf	<i>Laurus nobilis</i>	125
Cumin	<i>Cuminum cyminum</i>	> 2000
Oregano	<i>Lippia graveolens</i>	500
	<i>Oreganum vulgare</i>	500
Turmeric	<i>Curcuma longa</i>	> 2000
Onion	<i>Allium cepa</i>	500
Thyme	<i>Thymus vulgaris</i>	125
Nutmeg	<i>Myristica fragrans</i> (seed)	31
Mace	<i>Myristica fragrans</i> (external coat)	> 2000
Coriander seed	<i>Coriandrum sativum</i>	> 2000

*10% ethanol extracts, MIC (minimum inhibitory concentrations) values are on a whole spice basis. Levels assayed were 2000, 500, 125, 31, and 8 µg/ml.

TABLE 2. Inhibition of *Clostridium botulinum* by aliphatic alcohols.

Alcohol	MIC µg/ml
Methanol ^a	30,000
Ethanol	30,000
Propanol	15,000
Butanol	10,000
Pentanol	5,000
Hexanol	10,000
Heptanol	600
Octanol	200
Nonanol	100
Decanol	25
Undecanol	5
Dodecanol	2.5
Tridecanol ^b	1.25
Tetradecanol	0.6
Hexadecanol	0.6
Octadecanol	25

^aMethanol to dodecanol were diluted in assay media.

^bTridecanol to octadecanol were 1% solutions in ethanol with further dilutions in assay medium.

the octadecanol appears to account for the cubic trend of the curve. This curve is similar to that reported for n-alkyl esters of benzoic acid by Dymicky and Huhtanen (4), who found the C₁₁ ester to be most active with decreasing activity to C₁₈; the curve of these results (chain length vs. Ln MIC) was also cubic.

A comparison of C₁₀ to C₁₈ alcohols and amines of the same chain length [data from Huhtanen and Micich (9)]

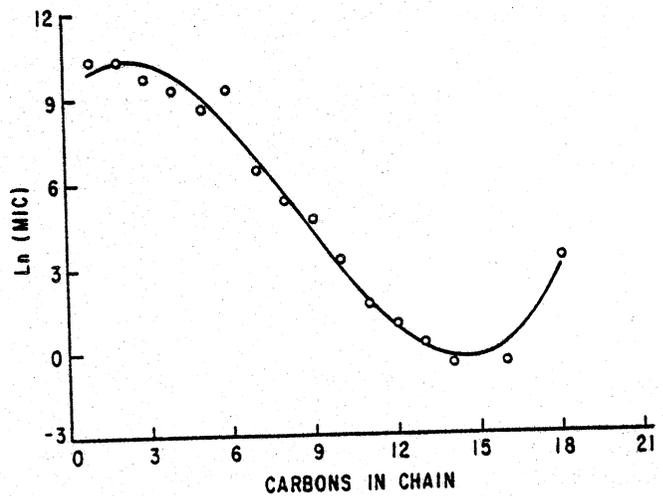


Figure 1. Inhibition of *C. botulinum* as function of Ln (MIC) and length of aliphatic alcohol chain length.

is shown in Fig. 2. In this range, the curve for the alcohols exhibited a highly significant binomial trend ($p < 0.01$); the curve for the amines was flatter, though still significantly ($p < 0.05$) quadratic in nature. In both studies, the C₁₄ and C₁₆ aliphatic amines and alcohols were the most inhibitory. Fatty acids in the range of C₇ to C₁₂ (the longest chain-length studied) were found by Karabinos and Ferlin (10) to be highly toxic to *S. aureus*, with undecylenic acid (C₁₁) the most bactericidal. Eisler and von Metz (5) indicated that saturated fatty acids of C₁₂, C₁₄ and C₁₆ carbon atoms were more effective than the lower chain length acids in killing *Pasteurella pestis*. As in our studies with alcohols, the C₁₈ fatty acid was relatively inactive.

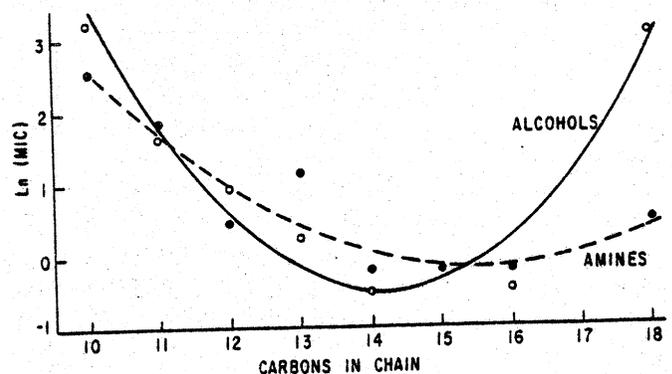


Figure 2. Comparison of aliphatic straight chain amines and alcohols as functions of Ln (MIC) versus chain length.

Although formulations for cured meats and sausage vary widely depending on the type of product and the manufacturer, most contain at least one or more of those spices reported here to be active against *C. botulinum* in the test tube assay. Information on exact quantities of spices used in meat products is not available from manufacturers since this is proprietary information. They are only required to list, qualitatively, the spices in their product. Achiote is not used, however, since it is a suspected carcinogen. The value of these spices as anticlostridial agents in foods has not been demon-

strated. Research now underway in our laboratories will establish the value of these active spices and alcohols in inhibiting *C. botulinum* in experimental meat systems.

REFERENCES

1. Beuchat, L. R. 1976. Sensitivity of *Vibrio parahaemolyticus* to spices and organic acids. *J. Food Sci.* 41:899-902.
2. Borick, P. M., M. Bratt, A. G. Wilson, L. Weintraub, and M. Kuna. 1959. Microbiological activity of certain saturated and unsaturated fatty acid salts of tetradecylamine and related compounds. *Appl. Microbiol.* 7:248-251.
3. Dold, H., and A. Knapp. 1948. The antibacterial activity of spices. *Z. Hyg.* 128:696-706.
4. Dymicky, M., and C. N. Huhtanen. 1979. Inhibition of *Clostridium botulinum* by p-hydroxybenzoic acid n-alkyl esters. *Antimicrob. Agents Chemother.* 15:798-801.
5. Eisler, D. M., and E. K. von Metz. 1968. Anti-*Pasteurella pestis* factor. III. Effect of fatty acids on *Pasteurella pestis*. *J. Bacteriol.* 95:1767-1773.
6. Farbood, M. I., J. H. MacNeil, and K. Ostovar. 1976. Effect of rosemary spice extractive on growth of microorganisms in meats. *J. Milk Food Technol.* 39:675-679.
7. Galbraith, H., T. B. Miller, A. M. Paton, and J. K. Thompson. 1971. Antibacterial activity of long chain fatty acids and the reversal with calcium, magnesium, ergocalciferol and cholesterol. *J. Appl. Bacteriol.* 37:803-813.
8. Huhtanen, C. N. 1975. Some observations on a Perigo-type inhibition of *Clostridium botulinum*. *J. Milk Food Technol.* 38:762-763.
9. Huhtanen, C. N., and T. J. Micich. 1978. Inhibition of *Clostridium botulinum* by aliphatic amines and long chain aliphatic aminodiamides. *J. Amer. Oil Chem. Soc.* 55:854-855.
10. Karabinos, J. V., and H. J. Ferlin. 1954. Bactericidal activity of certain fatty acids. *J. Amer. Oil Chem. Soc.* 31:228-232.
11. Neiman, C. 1954. Influence of trace amounts of fatty acids on the growth of microorganisms. *Bacteriol. Rev.* 18:147-163.
12. Salzer, U.-J., U. Bröker, H-F. Klie, and H-U. Liepe. 1977. Wirkung von Pfeffer und Pfefferinhaltsstoffen auf die Microflora von Wurstwaren. *Die Fleischwirtschaft* 57:1975-1976.