Journal of Medical Microbiology (2004), **53**, 1023–1027 DOI 10.1099/jmm.0.45567-0

Influence of subinhibitory concentrations of plant essential oils on the production of enterotoxins A and B and α-toxin by *Staphylococcus aureus*

A. Smith-Palmer,[1] J. Stewart[2] and L. Fyfe[1]

[1]Department of Dietetics, Nutrition and Biological Sciences, Queen Margaret University College, Clerwood Terrace, Edinburgh EH12 8TS, UK

[2]Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AC, UK

Correspondence
L. Fyfe
LFyfe@qmuc.ac.uk

Received 18 December 2003
Accepted 5 June 2004

The data presented show the ability of subinhibitory concentrations of plant essential oils to influence the production of enterotoxins A and B and α-toxin by *Staphylococcus aureus*. Subinhibitory concentrations of the oils of bay, clove, cinnamon, nutmeg and thyme had no significant effect on the overall quantity of extracellular protein produced. Haemolysis due to α-toxin was significantly reduced after culture with all five plant essential oils. This reduction was greatest with the oils of bay, cinnamon and clove. These three oils also significantly decreased the production of enterotoxin A; the oils of clove and cinnamon also significantly decreased the production of enterotoxin B.

INTRODUCTION

Staphylococcus aureus is an important medical pathogen, with different strains being responsible for a number of disease states including toxic shock syndrome, scalded skin syndrome and foodborne illness (Le Loir *et al.*, 2003). The ability to cause disease is, in part, dependent upon the production of a range of extracellular proteins, including catalase, fibrinolysin, superoxide dismutase, hyaluronidase, haemolysins (α, β, γ and δ), epidermolytic toxins and enterotoxins. The enterotoxins are a group of serologically distinct proteins (A, B, C_{1-3}, D, E and F) that are the causative agents of staphylococcal food poisoning. Although their exact mode of action has yet to be fully elucidated they are believed to stimulate an enteric-vagus nerve reflex triggering the vomiting centres of the brain (Sears & Kaper, 1996; Arbuthnott *et al.*, 1990). The enterotoxins can also act as superantigens, stimulating T lymphocytes to release cytokines and T-cell proliferation (Balaban & Rasooly, 2000; Krakauer, 1999). Considerable research at present is also aimed at investigating the properties of newly identified enterotoxins, including enterotoxins K, L and U (Orwin *et al.*, 2001, 2003; Letertre *et al.*, 2003).

One of the most extensively studied of the extracellular proteins is α-haemolysin (α-toxin). This is secreted as a soluble protein of 33 kDa. Penetration of host cell membranes by α-toxin results in the formation of a hexameric transmembrane pore that causes the leakage of ions and low molecular mass compounds. These pores also trigger secondary cellular reactions such as eicosanoid production, cytokine release and apoptosis.

In both the food and pharmaceutical industries there is a continuing need to find new and improved antimicrobial agents, especially in view of the increasing incidence of antibiotic resistance. One of the areas which is subject to considerable interest is plant extracts and in particular their essential oils. The antimicrobial properties of plant essential oils against a wide range of micro-organisms are well established (Vardar-Unlu *et al.*, 2003; Valero & Salmeron, 2003; Burt & Reinders, 2003; Smith-Palmer *et al.*, 1998, 2002; Friedman *et al.*, 2002; Delaquis *et al.*, 2002; Elgayyar *et al.*, 2001; Hammer *et al.*, 1999; Deans & Ritchie, 1987). However, in comparison to many other conventional antimicrobial agents, relatively little is known about the influence of plant essential oils on microbial physiology and pathogenicity. The data presented here show the influence of subinhibitory concentrations of five plant essential oils on the production of α-toxin and enterotoxins A and B.

METHODS

Micro-organism. *Staphylococcus aureus* NCTC 10657 was maintained on tryptone soya agar (TSA) slopes (Oxoid) at 4 °C.

Preparation of cultures and culture supernatants. Tryptone soya broth (TSB) (10 ml), containing subinhibitory concentrations of plant essential oils bay, clove, cinnamon and thyme at 0·01 % (v/v) and oil of nutmeg at 1 % (v/v) (kindly supplied by F. D. Copeland and Sons, London, UK), was inoculated with 10 µl of an overnight culture of *S. aureus*. The antimicrobial properties of these five plant essential oils had previously been reported (Smith-Palmer *et al.*, 1998). These subinhibitory concentrations were selected as they ensured that the bacterial concentration after 24 h in the experimental sample was the

same as in the control (data not shown). Supernatants from 24 h cultures were obtained by centrifugation (1600 ***g***) for 10 min.

Determination of extracellular protein concentration. The determination of extracellular protein concentration was based on the method of Bradford (1976) using Coomassie Brilliant Blue G dye (Bio-Rad) diluted 1 : 5 and bovine serum albumin (Sigma-Aldrich) as the standard. Five millilitres of dye was added to 1 ml samples of culture supernatants and incubated at room temperature for 30 min. The A_{595} was read in triplicate using a Dynatech MR 500 plate reader. Controls included TSB alone, the value of which was subtracted from the experimental value to give the extracellular protein concentration.

α-Toxin activity in the supernatant after culture with subinhibitory concentrations of plant essential oils. α-Toxin activity was determined by incubating 1 ml samples of culture supernatant with 485 µl PBS (Sigma-Aldrich) to which was added rabbit erythrocytes (Scottish Antibody Production Unit, Law Hospital, Lanarkshire, UK) to achieve a final concentration of 1 %. Tubes were gently mixed by inversion, incubated at 37 °C for 30 min and then centrifuged (1000 ***g***) for 5 min. The A_{550} was read in triplicate using a Dynatech MR 500 plate reader. To determine percentage haemolysis, controls were set up which contained 1 % erythrocytes and deionized water. Experimental results were expressed as the percentage haemolysis compared to the water lysed control, which was taken as 100 % haemolysis. Other controls contained PBS, TSB or TSB with subinhibitory concentrations of plant essential oils. No significant haemolysis was detected in these preparations (Smith-Palmer, 1999).

Direct effect of plant essential oils on the activity of α-toxin. Experiments were conducted to determine whether plant essential oils directly inhibited the activity of α-toxin. Plant essential oils (5 µl) were added to supernatants (10 ml) from *S. aureus* cultured in TSB only (therefore known to contain high levels of α-toxin). All tubes were incubated at 37 °C for 1 h with constant mixing, and the samples were left to settle for 15 min, enabling the plant essential oils to separate out. Samples (1 ml) were removed and haemolytic activity was measured as before (Smith-Palmer, 1999).

Determination of intracellular α-toxin activity after culture with subinhibitory concentrations of plant essential oils. Experiments were conducted to determine whether culture with plant essential oils prevented α-toxin synthesized in the cell from being exported into the culture supernatant. *S. aureus* was cultured with subinhibitory concentrations of plant essential oils for 24 h, the supernatant was discarded, and the cells were washed twice in PBS and resuspended in 10 ml PBS. Cells were lysed on ice by sonication for 5 min and examined via light microscopy to ensure that all the cells were lysed. The lysate was centrifuged (1600 ***g***) for 10 min to remove cell debris and the supernatant was examined for haemolytic activity as above (Smith-Palmer, 1999).

Influence of plant essential oils on enterotoxin production by *S. aureus*. The strain of *S. aureus* used produces enterotoxins A and B. Supernatants from *S. aureus* cultured with plant essential oils were examined for enterotoxin production using a reversed passive latex agglutination kit (Oxoid). The assays were conducted according to the manufacturer's instructions. Controls of TSB and control supernatants with and without plant essential oils showed that the oils did not interfere with the performance of the kit and detection of the enterotoxins.

Reproducibility and statistics. All measurements were conducted in triplicate within each experiment and each experiment was performed on at least three separate occasions. The two-tailed Student's *t*-test was used to analyse the data. Differences were judged to be statistically significant when $P \leqslant 0{\cdot}05$.

RESULTS

Culture with subinhibitory concentrations of plant essential oils had no significant effect on the overall quantity of extracellular protein produced (Table 1).

S. aureus cultured in TSB alone showed 76·5 % haemolysis due to α-toxin (Table 1). This was significantly reduced after culture with all five plant essential oils. The extent of this reduction was very similar for the oils of bay, cinnamon and clove, with values of 19·6–22·9 % haemolysis. The reduction was less after culture with the oils of nutmeg and thyme, with values of 59·6 and 52·2 %, respectively, both of which were still significantly less than the control.

Table 1 shows that the plant essential oils had no significant influence on the activity of α-toxin when added to the supernatant of *S. aureus* cultured in TSB alone and thus shown to contain high levels of haemolytic activity. When *S.*

Table 1. Quantity of extracellular protein, and the haemolytic activity of α-toxin, produced by *S. aureus* cultured with subinhibitory concentrations of plant essential oils

The direct effect of plant essential oils on the activity of α-toxin of *S. aureus* is also shown.

Culture conditions	Extracellular protein concn [µg protein (ml culture supernatant)$^{-1}$]	Haemolysis (%) [in supernatant after culture of *S. aureus* inTSB and oil(s)]	Haemolysis (%) [after addition of oil(s) to supernatant after culture of *S. aureus* in TSB alone]
Control	37·25 ± 1·59	76·5 ± 3·7	73·6 ± 2·2
Oil of bay	33·02 ± 1·87	22·9 ± 0·9*	71·6 ± 4·5
Oil of cinnamon	29·75 ± 2·25	19·6 ± 1·3*	74·9 ± 5·0
Oil of clove	33·18 ± 1·73	20·4 ± 1·5*	73·1 ± 4·8
Oil of nutmeg	31·75 ± 0·25	59·6 ± 8·6*	78·2 ± 6·2
Oil of thyme	28·25 ± 2·65	52·2 ± 3·7*	80·1 ± 4·9

*$P \leqslant 0{\cdot}05$ compared to the corresponding control.

aureus cultured with plant essential oils was lysed, very little haemolytic activity was detected, with no samples showing greater than 3 % haemolysis (data not shown).

S. aureus cultured in TSB alone secreted 13·1 ng enterotoxin A ml^{-1} (Table 2). When cultured with the oils of bay, cinnamon and clove this was significantly reduced to 3·7, 1·3 and 3·2 ng ml^{-1}, respectively. No significant changes were observed when cultured with the oils of either nutmeg or thyme. A significant decrease in the production of enterotoxin B was also observed following culture with the oils of clove and cinnamon. In the control supernatant, 204·8 ng enterotoxin B ml^{-1} was detected, compared with only 44·8 and 38·4 ng ml^{-1} after culture with the oils of cinnamon and clove, respectively.

DISCUSSION

The data show the ability of plant essential oils, especially the oils of bay, cinnamon and clove, even at subinhibitory concentrations, to cause a significant decrease in the production of key toxins, despite causing no change in the total extracellular protein production (Tables 1 and 2).

It is widely recognized that the production of α-toxin can be modulated by subinhibitory concentrations of antibiotics; for example, Ohlsen *et al.* (1998) reported strong induction of α-toxin by β-lactams and almost complete inhibition of expression by clindamycin. The suppression of α-toxin production has also been reported with other antimicrobials, including glyercol monolaurate (Projan *et al.*, 1994; Schlievert *et al.*, 1992). However, α-toxin has not previously been shown to be modulated by plant essential oils. In contrast, the influence of plant compounds, namely extracts from olives and especially oleuropein (Tassou & Nychas, 1994; Nychas *et al.*, 1990) and garlic (González-Fandos *et al.*, 1994), on the production of enterotoxins has been demonstrated by other researchers. Plant essential oils have, however, recently been shown to influence the production of listeriolysin O and phosphatidylcholine-specific phospholipase C by *Listeria monocytogenes* (Smith-Palmer *et al.*, 2002). Staphylococcal gastroenteritis does not result from the ingestion of *S. aureus* per se, but rather from enterotoxins which are pre-formed within the food (Le Loir *et al.*, 2003). Consequently, the ability of essential oils, in particular clove and cinnamon, to decrease the production of enterotoxins adds impetus to their potential as novel natural food preservatives. This is an area of growing interest, especially as the antimicrobial properties of plant essential oils against a wide range of pathogens is becoming increasingly recognized and their potential application to foods is investigated (Valero & Salmeron, 2003; Smith-Palmer *et al.*, 2001; Hao *et al.*, 1998; Lis-Balchin *et al.*, 1998) as is this potential addition to active packaging and modified atmosphere packaging (Suppakul *et al.*, 2003; Skandamis & Nychas, 2001, 2002; Nielson & Rios, 2000). However, it should be taken into consideration that the results reported here were achieved after culture in TSB. Experiments are needed to establish if a similar reduction can be achieved in food, especially as the structure and properties of the food matrix are likely to influence toxin production.

The inhibition of enterotoxin and α-toxin production by plant essential oils could have occurred at a number of points, including transcription, translation, export from the cell or direct inactivation of the toxin. Direct inactivation seems unlikely as the addition of plant essential oils to supernatants known to contain high levels of α-toxin did not reduce haemolytic activity (Table 1). This is in contrast to the work of Okubo *et al.* (1989), who reported that tea extract was able to inhibit the activity of α-toxin directly. Similarly, the lack of intracellular haemolytic activity detected when the cells were lysed suggests that it was unlikely that there was an intracellular accumulation of α-toxin resulting from the cells' inability to successfully export proteins.

Each of the plant essential oils is a complex mixture of esters, aldehydes, ketones and terpenes (Smith-Palmer *et al.*, 2002), the exact composition depending upon the growth conditions of the plant. Thus it is likely that each plant essential oil will have multiple modes of action and multiple points of disruption to toxin production. This multi-component

Table 2. Production of enterotoxins A and B by *S. aureus* cultured with subinhibitory concentrations of plant essential oils

Enterotoxins A and B were detected using reversed passive latex agglutination.

Culture conditions	Amount of enterotoxin (ng ml^{-1})	
	Enterotoxin A	Enterotoxin B
Control	13·1 ± 3·3	204·8 ± 28·0
Oil of bay	3·7 ± 0·9*	115·2 ± 33·3
Oil of cinnamon	1·3 ± 0·3*	44·8 ± 7·0*
Oil of clove	3·2 ± 0·4*	38·4 ± 5·7*
Oil of nutmeg	12·8 ± 2·3	179·2 ± 28·0
Oil of thyme	14·0 ± 3·0	230·4 ± 22·8

*$P \leqslant 0{\cdot}05$ significant difference from the respective control.

nature of plant essential oils is an advantage over many conventional antimicrobials, which have a single target site, as it is more difficult for bacteria to develop resistance. This is especially relevant at a time of increasing emergence of multi-resistant pathogens. Furthermore, plant essential oils have a more natural image and hence are more readily accepted by consumers than antimicrobial agents they perceive as chemical and artificial.

The data presented here show the ability of selected plant essential oils to significantly reduce the production of key pathogenicity factors by *S. aureus*, namely α-toxin and the enterotoxins A and B. This is potentially of considerable importance in the food and pharmaceutical industries and an exciting area for further development.

ACKNOWLEDGEMENTS

The authors acknowledge F. D. Copeland and Sons, Colanol House, 5 Westfield Street, London, UK, for the generous supply of plant essential oils.

REFERENCES

Arbuthnott, J. P., Coleman, D. C. & De Azavedo, J. S. (1990). Staphylococcal toxin in human disease. *Soc Appl Bacteriol Symp Ser* **69,** 101S–107S.

Balaban, N. & Rasooly, A. (2000). Staphylococcal enterotoxins. *Int J Food Microbiol* **61**, 1–10.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Burt, S. A. & Reinders, R. D. (2003). Antibacterial activity of selected plant essential oils against *Escherichia coli* O157 : H7. *Lett Appl Microbiol* **36**, 162–167.

Deans, S. G. & Ritchie, G. (1987). Antibacterial properties of plant essential oils. *Int J Food Microbiol* **5**, 165–180.

Delaquis, P. J., Stanich, K., Girard, B. & Mazza, G. (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *Int J Food Microbiol* **74**, 101–109.

Elgayyar, M., Draughon, F. A., Golden, D. A. & Mount, J. R. (2001). Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. *J Food Prot* **64**, 1019–1024.

Friedman, M., Henika, P. R. & Mandrell, R. E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli, Listeria monocytogenes*, and *Salmonella enterica*. *J Food Prot* **65**, 1545–1560.

González-Fandos, E., García-López, M. L., Sierra, M. L. & Otero, A. (1994). Staphylococcal growth and enterotoxins (A-D) and thermonucleases synthesis in the presence of dehydrated garlic. *J Appl Bacteriol* **77**, 549–552.

Hammer, K. A., Carson, C. F. & Riley, T. V. (1999). Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol* **86**, 985–990.

Hao, Y. Y., Brackett, R. E. & Doyle, M. P. (1998). Efficacy of plant extracts in inhibiting *Aeromonas hydrophila* and *Listeria monocytogenes* in refrigerated cooked poultry. *Food Microbiol* **15**, 367–378.

Krakauer, T. (1999). Immune response to staphylococcal superantigens. *Immunol Res* **20**, 163–173.

Le Loir, Y., Baron, F. & Gautier, M. (2003). *Staphylococcus aureus* and food poisoning. *Genet Mol Res* **2**, 63–76.

Letertre, C., Perelle, S., Dilasser, F. & Fach, P. (2003). Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus. J Appl Microbiol* **95**, 38–43.

Lis-Balchin, M., Buchbauer, G., Hirtenlehner, T. & Resch, M. (1998). Antimicrobial activity of Pelargonium essential oils added to a quiche filling as a model food system. *Lett Appl Microbiol* **27**, 207–210.

Nielson, P. V. & Rios, R. (2000). Inhibition of fungal growth on bread by volatile components from spices and herbs, and the possible application in active packaging, with special emphasis on mustard essential oil. *Int J Food Microbiol* **60**, 219–229.

Nychas, G. J. E., Tassou, S. C. & Board, R. G. (1990). Phenolic extract from olives: inhibition of *Staphylococcus aureus. Lett Appl Microbiol* **42**, 217–220.

Ohlsen, K., Ziebuhr, W., Koller, K.-P., Hell, W., Wichelhaus, T. A. & Hacker, J. (1998). Effects of subinhibitory concentrations of antibiotics on alpha-toxin (*hla)* gene expression of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. *Antimicrob Agents Chemother* **42**, 2817–2823.

Okubo, S., Ikigai, H., Toda, M. & Shimamura, T. (1989). The anti-haemolysin activity of tea and coffee. *Lett Appl Microbiol* **9**, 65–66.

Orwin, P. M., Leung, D. Y., Donahue, H. L., Novick, R. P. & Schlievert, P. M. (2001). Biochemical and biological properties of Staphylococcal enterotoxin K. *Infect Immun* **69**, 360–366.

Orwin, P. M., Fitzgerald, J. R., Leung, D. Y., Gutierrez, J. A., Bohach, G. A. & Schlievert, P. M. (2003). Characterization of *Staphylococcus aureus* enterotoxin L. *Infect Immun* **71**, 2916–2919.

Projan, S. J., Brown-Skrobot, S., Schlievert, P. M., Vandenesch, F. & Novick, R. P. (1994). Glycerol monolaurate inhibits the production of β-lactamase, toxic shock syndrome toxin 1, and other staphylococcal exoproteins by interfering with signal transduction. *J Bacteriol* **176**, 4204–4209.

Schlievert, P. M., Deringer, J. P., Kim, M. H., Projan, S. J. & Novick, R. P. (1992). Effect of glycerol monolaurate on bacterial growth and toxin production. *Antimicrob Agents Chemother* **36**, 626–631.

Sears, C. L. & Kaper, J. B. (1996). Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiol Rev* **60**, 167–215.

Skandamis, P. N. & Nychas, G. J. (2001). Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. *J Appl Microbiol* **91**, 1011–1022.

Skandamis, P. N. & Nychas, G. J. (2002). Preservation of fresh meat with active and modified atmosphere packaging conditions. *Int J Food Microbiol* **79**, 35–45.

Smith-Palmer, A. (1999). *The antimicrobial properties of plant essential oils against foodborne pathogens*. PhD thesis, Queen Margaret University College, Edinburgh, UK.

Smith-Palmer, A., Stewart, J. & Fyfe, L. (1998). Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Lett Appl Microbiol* **26**, 118–122.

Smith-Palmer, A., Stewart, J. & Fyfe, L. (2001). The potential application of plant essential oils as natural food preservatives in soft cheese. *Food Microbiol* **18**, 463–470.

Smith-Palmer, A., Stewart, J. & Fyfe, L. (2002). Inhibition of listeriolysin O and phosphatidylcholine-specific phospholipase C production by *Listeria monocytogenes* by subinhibitory concentrations of plant essential oils. *J Med Microbiol* **51**, 1–8.

Suppakul, P., Miltz, J., Sonneveld, K. & Bigger, S. W. (2003).

Antimicrobial properties of basil and its possible application in food packaging. *J Agric Food Chem* **51**, 3197–3207.

Tassou, S. C. & Nychas, G. J. E. (1994). Inhibition of *Staphylococcus aureus* by olive phenolics in broth and in a model food system. *J Food Prot* **57**, 120–124.

Valero, M. & Salmeron, M. C. (2003). Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. *Int J Food Microbiol* **85**, 73–81.

Vardar-Unlu, G., Candan, F., Sokmen, A., Daferera, D., Polissiou, M., Sokmen, M., Donmez, E. & Tepe, B. (2003). Antimicrobial and antioxidant activity of the essential oil and methanol extracts of *Thymus pectinatus* Fisch. et Mey. Var. *pectinatus* (Lamiaceae). *J Agric Food Chem* **51**, 63–67.