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Bioactivity of selected essential oils and some components on listeria monocytogenes biofilms.

Bioactivity of selected essential oils and some components on *Listeria monocytogenes* biofilms

C.M. Leonard [a],*, S. Virijevic [b], T. Regnier [b], S. Combrinck [b]

[a] *Department of Pharmaceutical Sciences, Tshwane University of Technology, Private Bag X680, Pretoria 0001, South Africa*
[b] *Department of Chemistry, Tshwane University of Technology, PO Box 56208, Arcadia, Pretoria 0001, South Africa*

Abstract

Listeria monocytogenes is a Gram-positive bacterium, able to survive and grow in water, soil, agricultural products, various foods and the food-processing environment. The ubiquitous nature of the organism, coupled with its ability to colonise food-processing surfaces by forming biofilms, causes it to be of a major concern to the industry. Increased foodborne pathogen resistance and negative consumer perceptions regarding the use of synthetic bacteriocides, has resulted in natural antimicrobials being sourced from the plant kingdom. The listerial antibiofilm activities of *Syzygium aromaticum* (clove), *Mentha spicata* (spearmint), *Lippia rehmannii* and *Cymbopogon citratus* (lemongrass) essential oils and their major components were evaluated using the crystal violet assay and confocal scanning laser microscopy. Listerial biofilms treated with *S. aromaticum* or *M. spicata* essential oils, or the pure compounds nerol and citral, exhibited a similar biofilm biomass to the positive control. However, the essential oils of lemongrass, *L. rehmannii*, eugenol and R-carvone caused biofilm enhancement, rather than inhibition. *L. rehmannii* and lemongrass essential oils did not display any antibiofilm properties. Results obtained were confirmed by microscopic observations indicating either a reduction (inhibition) or an increase (enhancement) in biofilm biomass when exposed to the essential oils or pure compounds. The present study revealed that *M. spicata* and *S. aromaticum* essential oils as well as pure citral and nerol are good candidates for further development of ecofriendly disinfectants.
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1. Introduction

Listeria monocytogenes, a Gram-positive bacterium commonly found in soil, water, plants, sewage and foods, such as soft cheeses (Farber and Peterkin, 1991; Gandhi and Chikindas, 2007), is the causal agent of listeriosis, a rare but lethal food-borne infection. The ubiquitous nature of the microorganism is of a major concern to the food industry. Its ability to grow at refrigeration temperatures, and tolerate salt and low pH (Ryser and Marth, 2007), allows it to multiply in food-processing facilities. In the past decade, several outbreaks of infections caused by *L. monocytogenes* have been reported from all over the world (Salamina et al., 2000), with many cases occurring in Europe (European Food Safety Authority (EFSA), 2008) and in the United States (USDA–FSIS, 2009). The ability of *L. mono-*

cytogenes to form a biofilm has been established (Jeayasekaran and Karunasagar, 2000; Sandasi et al., 2008). This structured community of pathogens, encapsulated within a self-developed polymeric matrix, is well protected against bacterial agents and renders the organism difficult to control within the food chain. Up to now, strict food safety regulations, as well as good handling and processing methods, were adequate to restrict the lethal incidence of the disease. However, the emergence of resistant bacterial strains and consumer concerns regarding the use of chemical sanitisers, have placed pressure on the food industry to find natural antimicrobial substitutes for synthetic agents. Alternative bacteriocides must be safe for consumers, but also harmless to the environment (Davidson, 1997). Such antibacterial agents are often sourced from the plant kingdom (Burt, 2004; Bakkali et al., 2008). Numerous studies have been conducted on the antimicrobial potential of plant extracts and essential oils (Smith-Palmer et al., 1998) and it is well established that many essential oils have the ability to control *Listeria in vitro* (Aureli et al., 1992; Mourey and

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* Corresponding author. Tel.: +27 12 3826392.
E-mail address: leonardcm@tut.ac.za (C.M. Leonard).

Canillac, 2002*)*. The range of essential oils associated with the *in vitro* inhibition of *Listeria* species include *Cymbopogon citratus* and *Syzygium aromaticum* (Lis-Balchin and Deans, 1997). According to these researchers, the antilisterial activity can often be correlated with a high percentage of specific monoterpenes, such as citral, present in the oils.

In this study, the antimicrobial activity of *S. aromaticum* (clove), *Mentha spicata* (spearmint), *Lippia rehmannii* and *C. citratus* (lemongrass) essential oils against preformed *L. monocytogenes* biofilms were tested and compared. Furthermore, confocal scanning laser microscopy was used to visually confirm the results of the crystal violet assay.

2. Materials and methods

2.1. Essential oils and pure compounds

Aerial parts of seven individual plants of *L. rehmannii* were collected from Zandfontein cemetery (S 25.41′28.3″, E 28.04′ 04.8″) in the environs of Pretoria, South Africa in March 2007. A voucher specimen "Bosman & Combrinck 21" was deposited in the herbarium of the South African National Biodiversity Institute in Pretoria. The essential oil of *L. rehmannii* was obtained by steam distillation as described by Linde et al. (2010).

All commercial samples of *C. citratus* , *S. aromaticum* and *M. spicata* were purchased from Holistic Emporium (Johannesburg, South Africa) and stored in the dark at room temperature until required. Pure R-(-)-carvone, eugenol, citral and nerol were obtained from Sigma Aldrich (Johannesburg, South Africa). All essential oils and pure compounds were diluted to 1 mg/mL using methanol (AR grade, Merck Saarchem, Johannesburg) according to the method described by Sandasi et al. (2008).

2.2. Gas chromatography

The four essential oils were prepared as described by Linde et al. (2010). Each solution (1 µL) was analysed with GC-MS (Agilent 6890 N GC system coupled simultaneously to a 5973 MS and a flame ionization detector), equipped with an autosampler. The FID chromatograms were used to obtain the relative percentage peak areas of the individual components. Relative retention indices (RRI) were determined for individual components using *n*-alkanes as reference points. For identification of the compounds, mass spectra and retention indices were compared using NIST®, Mass Finder®, Flavour® data libraries and the Başer Library of Essential Oil Constituents.

2.3. Preparation of a standardized culture

L. monocytogenes (ATCC 19111) was reactivated from a glycerol stock culture kept at −80 °C. The isolate was sub-cultured on Tryptone Soy Agar (TSA) and incubated for 24 h at 37 °C. A pure colony was then inoculated into Tryptone Soy Broth (TSB) and incubated for 24 h at 37 °C at 150 rpm. Following incubation, *L. monocytogenes* was standardized to an optical density of 0.02 $(1.0 \times 10^6 \text{ CFU/mL})$ using a spectrophotometer (Helios alpha, Thermoelectron Corporation) at 590 nm.

2.4. Crystal violet assay

One hundred microlitres of the standardised culture was transferred into each of the wells of a 96-well microtitre plate and incubated at 37 °C for 6 h to allow the formation of a preformed listerial biofilm. Thereafter, 100 µl of the individual essential oils or pure compounds (diluted in methanol to a final concentration of 1 mg/mL) were added to the wells and the plates were then incubated for a further 18 h. Crystal violet staining was carried out using the method described by Djordjevic et al. (2002) and modified by Sandasi et al. (2008). Ciprofloxacin (0.00125 mg/ mL) was used as a positive control while the negative controls were water and biofilm or methanol and biofilm (Sandasi et al., 2008).

2.5. Confocal scanning laser microscopy

For the confocal scanning laser microscopy, the culture was standardised as previously described. The standardised culture $(1 \times 10^6 \text{ CFU/mL})$ was placed in sterile beakers containing sterile microscope slides and incubated at 37 °C for 6 h to allow for the formation of listerial biofilms. Following this, the preformed biofilms were exposed to 100 µL of the essential oils and pure compounds (1 mg/mL) and further incubated for 18 h. The slides were removed from the cultures and stained with BacLight® (Biorad, Johannesburg, South Africa). Prior to staining, the dye was diluted 12.5 times with sterile distilled water. A volume of 25 µl of the diluted dye was placed onto the slide and allowed to adsorb to the cells for 10 min. A cover slip was then placed and sealed onto the slide, whereafter the biofilm was viewed on a microscope (Carl Zeiss 510 META), using SYTO 9 and propidium iodide stains and excitation radiation at 488 nm and 560 nm, respectively. Twenty fields per slide were evaluated of which two representative fields were photographed.

3. Results and discussion

Wide-spectrum antibacterial activities of essential oils and their components against Gram-positive and Gram-negative bacteria are well documented (Chorianopoulos et al., 2008; De Oliviera et al., 2010; Sandasi et al., 2008; van Vuuren, 2008). Listerial biofilms, treated with *S. aromaticum* or *M. spicata* essential oils, exhibited the same biomass (absorbance 0.09) as that of the positive control (ciprofloxacin). It was therefore assumed that the antilisterial activity of these essential oils could be attributed to the activity of their major chemical constituents, eugenol and carvone, respectively (Tables 1 and 2). However, surprisingly, these compounds alone, caused biofilm enhancement rather than inhibition (Table 1). These results are in agreement with Sandasi et al. (2008) who reported that treatment of listerial biofilms with some pure essential oil components enhanced biofilm growth. These researchers attributed the antibiofilm activity to the synergistic effect of various oil components and not exclusively to the action of a major component. In agreement with our results, Lis-Balchin and Deans (1997) reported a good antilisterial activity for *S. aromaticum* oil, but since these researchers used the planktonic form, rather than the

Table 1
Crystal violet assay at absorbance (590 nm) for essential oils and pure compounds.

Essential oils/pure components	Common name	Crystal violet test	Major components [1]	References [1]
		Absorbance at 590 nm		
Cymbopogon citratus	Lemongrass	0.38±0.06	Geranial (41%), neral (32%)	Tsortzakis and Economakis (2007) Linde et al. (2010)
Lippia rehmannii	–	0.46±0.05	Geranial (35%), neral (22%), nerol (0.5 to 1%)	Burt and Reinders (2002) Linde et al. (2010)
Mentha spicata	Spearmint	0.09±0.01	R-(-)-carvone (between 50 and 80%), Limonene (between 10 and 22%), 1,8-cineol (between 1 and 3%)	Jirovetz et al. (2002) Da Porto and Decorti (2009) Chauhan et al. (2009)
Syzygium aromaticum	Clove	0.09±0.01	Eugenol (79%), isocaryophyllene (5%)	Smith-Palmer et al. (1998) Burt and Reinders (2002)
R-(-)-carvone	–	0.62±0.09		
Citral	–	0.08±0.01		
Eugenol	–	0.36±0.09		
Nerol	–	0.08±0.01		
Ciprofloxacin	–	0.09±0.01		
Water	–	0.10±0.02		

[1] References reported on the major components of each essential oil.

sessile (biofilm) form, their results cannot be directly correlated with ours.

Despite the observed enhancement of *L. rehmannii* and *C. citratus* essential oils on the listerial biofilms, their major compound, citral (a mixture of the *cis/trans*-isomers geranial and neral) exhibited a similar antibiofilm activity to ciprofloxacin (Table 1). These results contradict the findings of Lis-Balchin and Deans (1997) who evaluated the antilisterial activity of *C. citratus* oil against 20 food-borne strains and found it to be an effective inhibitor of the organism. However, results obtained using planktonic cells may not compare well to those using sessile cells.

Results obtained for *C. citratus* essential oils, contradict those of De Oliviera et al. (2010), who reported that *C. citratus* (lemongrass) and *Cymbopogon nardus* (citronella) essential oils displayed very high antimicrobial activities against listerial biofilms. However, the concentrations of essential oils applied to the biofilm were substantially higher than those used in our study. Citral, in contrast to the citral-rich oils *L. rehmannii* and *C. citratus* displayed a significant inhibition of the listerial biofilm (Table 1). Nerol, a minor component of *L. rehmannii* essential oil, gave similar results to citral.

Many conflicting results exist regarding the *in vitro* inhibition of biofilm and planktonic cells. According to Mourey and Canillac (2002) and Sandasi et al. (2008), the activities of the single components of essential oils often differ from those observed when the compounds are combined.

The micrographs obtained were used to confirm the effects of the *C. citratus* and *S. aromaticum* essential oils, as well as those of nerol and eugenol, on the biofilm biomass. Fig. 1 illustrates the enhanced biofilm formation by *C. citratus* oil (A) and eugenol (B), compared to the inhibition of the listerial biofilm by *S. aromaticum* oil (C) and nerol (D). To our knowledge, this is the first report on the use of confocal scanning laser microscopy to evaluate changes in listerial biofilms biomass after exposure to essential oils or pure compounds.

4. Conclusion

The antibiofilm activities of *S. aromaticum* and *M. spicata* essential oils as well as those of nerol and citral are promising. However, the results for *C. citratus* contradict the findings of de Oliviera et al. (2010). The differences in the reported data could be due to many factors including differences in the strains used,

Table 2
Relative percentage peak areas of the six most abundant compounds above 1% detected in spearmint, lemongrass and *Lippia rhemannii* essential oils by GC-MS and GC-FID.

| Essential oil	Six most abundant compounds (GC-FID) >1%					
	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6
Mentha spicata (spearmint)	R-(-)-carvone (80.8%)	ethylacetoacetate (8.5%)	limonene (2.3%)	dihydrocarvone (1.8%)	dihydrocarveol acetate (1.7%)	dihydrocarveol (1.0%)
Cymbopogon citratus (lemongrass)	geranial (42.5%)	neral (31.7%)	limonene (8.9)	α-terpineol (5.2%)	citronellol (2.8%)	linalool (2.8%)
Lippia rehmannii	geranial 42.3%)	neral (26.8%)	caryophyllene oxide (3.7%)	camphor (3.3%)	isocaryophyllene (3.2%)	β-caryophyllene (2.3%)
Syzygium aromaticum (clove)	eugenol (88.3%)	β-caryophyllene (8.1%)	α-humulene (2.0%)			
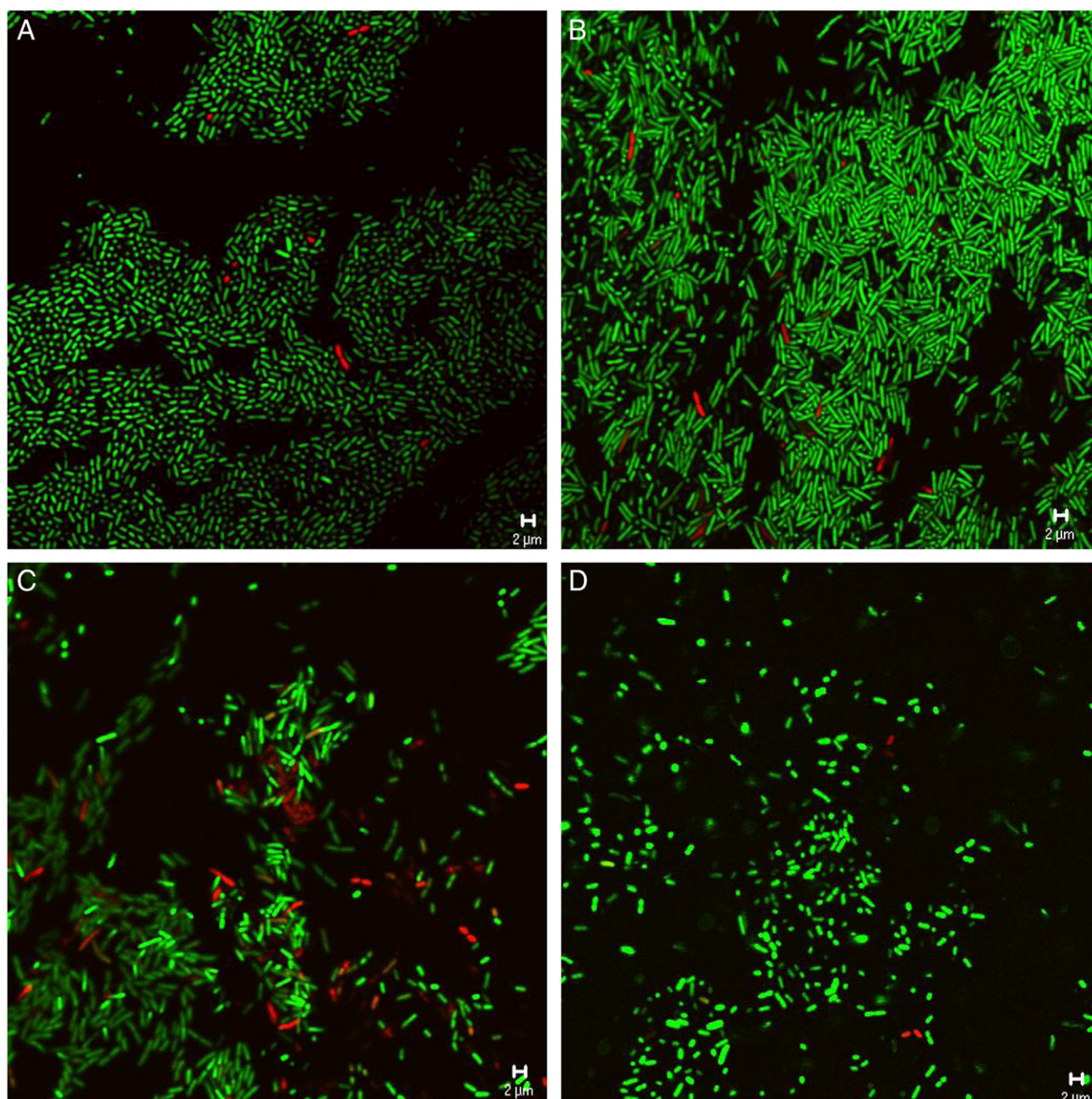
Fig. 1. Confocal scanning laser micrographs of *Listeria.monocytogenes* (ATCC19111) biofilms after treatment with essential oils from *Cymbopogon citratus* (A) and *Syzygium aromaticum* (C), treatment with essential oil components eugenol (B) and nerol (D).

concentration and chemical composition of essential oils, time of exposure to the essential oils, the growth stage of the biofilm and the nature of the surface to which the organism adheres. The enhancement (increase in biofilm biomass) obtained with *L. rehmannii* essential oil, also containing primarily citral, correlated well with that of *C. citratus*. R-(-) carvone and eugenol also caused an increase in the biofilm biomass. Confocal scanning laser microscopy was found to be a useful tool for confirming the effects of the essential oils and pure compounds on the sessile cells when analysing biofilm growth. This study found that there is a necessity for standardised *in vitro* methods for determining the influence of essential oils and pure compounds on biofilm development.

References

Aureli, P., Costantini, A., Zolea, S., 1992. Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*. Journal of Food Protection 55, 344–348.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils — a review. Food and Chemical Toxicology 46, 446–475.

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in food — a review. International Journal of Food Microbiology 94, 223–253.

Burt, S.A., Reinders, R.D., 2002. Antibacterial activity of selected plant essential oils against *Escherichia coli* O157: H7. Applied Microbiology 36, 162–167.

Chauhan, R.S., Kaula, M.K., Shahia, A.K., Kumara, A., Rama, G., Tawab, A., 2009. Chemical composition of essential oils in *Mentha spicata* L. accession [IIIM(J)26] from North–West Himalayan region, India. Industrial Crops and Products 29, 654–656.

Chorianopoulos, N.G., Giaouris, E.D., Skandamis, P.M., Haroutounian, S.A., Nychas, G.J.E., 2008. Disinfectant test against monoculture and mixed-culture biofilms composed of technological, spoilage and pathogenic bacteria: bactericidal effect of essential oil and hydrosol of *Satureja thymbra* and comparison with standard acid–base sanitizers. Journal of Applied Microbiology 104, 1586–1696.

Da Porto, C., Decorti, D., 2009. Ultrasound-assisted extraction coupled with under vacuum distillation of flavor compounds from *Mentha spicata* (carvone-rich) plants: comparison with conventional hydrodistillation. Ultrasonic Sonochemistry 16, 795–799.

Davidson, P.M., 1997. Chemical preservatives and natural antimicrobial compounds. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), Food Microbiology Fundamentals and Frontiers. ASM, New York, pp. 520–556.

de Oliviera, M.M.M., Brugnera, D.F., Cardoso, M., das, G., Alves, E., Piccoli, R.H., 2010. Disinfectant action of *Cymbopogon* sp. essential oils in different phases of biofilm formation by *Listeria monocytogenes* on stainless steel surface. Food Control 21, 549–553.

Djordjevic, D., Weidmann, M., McLandsborough, L.A., 2002. Microtiter plate assessment of *Listeria monocytogenes*. Applied and Environmental Microbiology 68, 2950–2958.

European Food Safety Authority, 2008. Zoonoses data collection reports. http://www. efsa.europa.eu/en/science/monitoring_zoonoses/reports.html Retrieved 07.04.10.

Farber, J.M., Peterkin, P.I., 1991. *Listeria monocytogenes*, a food borne pathogen. Microbiological Reviews 55, 476–511.

Gandhi, M., Chikindas, L., 2007. *Listeria*: a food borne pathogen that knows how to survive. International Journal of Food Microbiology 113, 1–15.

Jeayasekaran, G., Karunasagar, I., 2000. Effects of sanitizers on *Listeria* biofilm on contact surfaces. Asian Fisheries Science 13, 209–213.

Jirovetz, L., Buchbauer, G., Shabi, M., Ngassoum, M.B., 2002. Comparative investigation of essential oil and volatiles of *Mentha spicata*. Perfume Flavour 27, 16–22.

Linde, J.H., Combrinck, S., Regnier, T., Virijevic, S., 2010. Chemical composition and antifungal activity of the essential oils of *Lippia rehmannii* from South Africa. South African Journal of Botany 76, 37–42.

Lis-Balchin, M., Deans, S.G., 1997. Bioactivity of selected plant essential oils against *Listeria monocytogenes*. Journal of Applied Microbiology 82, 759–762.

Mourey, A., Canillac, N., 2002. Anti-*Listeria monocytogenes* activity of essential oils components of conifers. Food Control 13, 289–292.

Ryser, E.T., Marth, E.H., 2007. Listeria, listeriosis, and food safety. Marcel Dekker Inc., New York.

Salamina, G., Dalledonne, E., Niccolini, A., Poda, G., Cesaroni, D., Bucci, M., 2000. Lessons from an outbreak of listeriosis related to vacuum-packed gravid and cold-smoked fish. International Journal of Food Microbiology 62, 173–175.

Sandasi, M., Leonard, C.M., Viljoen, A.M., 2008. The effect of five common essential oil components on *Listeria monocytogenes* biofilms. Food Control 19, 1070–1075.

Smith-Palmer, A., Stewart, J., Fyfe, L., 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Journal of Applied Microbiology 26, 118–122.

Tsortzakis, N.G., Economakis, C.D., 2007. Antifungal activity of lemongrass (*Cympopogon citratus* L.) essential oil against key postharvest pathogens. Innovative Food Science and Emerging Technologies 8, 253–258.

United States Department of Agriculture—Food Safety and Inspection Service, 2009. FSIS recalls. Available from http://www.fsis.usda.gov/FSIS_RE-CALLS/. Accessed 08.04.10.

van Vuuren, S.F., 2008. Antimicrobial activity of South African medicinal plants. South African Journal of Botany 119, 462–472.

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