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Inhibition of acetylcholinesterase by Tea Tree oil

Clive Mills, Brian J. Cleary, John F. Gilmer and John J. Walsh

Abstract

Pediculosis is a widespread condition reported in schoolchildren. Treatment most commonly involves the physical removal of nits using fine-toothcombs and the chemical treatment of adult lice and eggs with topical preparations. The active constituents of these preparations frequently exert their effects through inhibition of acetylcholinesterase (AChE, EC 3.1.1.7). Increasing resistance to many preparations has led to the search for more effective treatments. Tea Tree oil, otherwise known as Melaleuca oil, has been added to several preparations as an alternative treatment of head lice infestations. In this study two major constituents of Tea Tree oil, 1,8-cineole and terpinen-4-ol, were shown to inhibit acetylcholinesterase at IC50 values (inhibitor concentrations required to give 50% inhibition) of 0.04 and 10.30 mM, respectively. Four samples of Tea Tree oil tested (Tisserand, Body Treats, Main Camp and Irish Health Culture Association Pure Undiluted) showed anticholinesterase activity at IC50 values of 0.05, 0.10, 0.08 and 0.11 μL mL^{-1}, respectively. The results supported the hypothesis that the insecticidal activity of Tea Tree oil was attributable, in part, to the anticholinesterase activity of Tea Tree oil.

Introduction

Infestation with head lice (*Pediculus humanus capitis*), pediculosis capitis or pediculosis, has been recorded for generations (Parish 1985). It is a common condition in schoolchildren and is usually detected when children are seen scratching their heads. Infestation is confirmed by the presence of live adult lice, viable eggs and nits. The recognized management of head lice infestations involves combing hair with purpose made fine-toothcombs to remove eggs and nits, and the application of topical insecticidal and ovicidal preparations. Typically, these preparations are shampoos, lotions or crèmes which contain pyrethrines, synthetic pyrethroids (e.g. permethrin and phenothrin), organophosphates (e.g. malathion) or carbamates (e.g. carbaryl) (Vander Stichele et al 1995; Elgart 1999; Mazurek & Lee 2000; Downs et al 2002; Frankowski & Weiner 2002; Meinking et al 2002; Nash 2003). Organochlorides such as dicophane (DDT) and lindane have been used to good effect in topical preparations (Vander Stichele et al 1995; Elgart 1999; Mazurek & Lee 2000; Meinking et al 2001, 2002; Frankowski & Weiner 2002; Orion et al 2002; Jones & English 2003). Although there have been many reported instances of resistance to these preparations, treatment failures are often the result of non-compliance with directions, improper use of the product, use of outdated preparations, re-exposure to lice or incomplete removal of viable eggs (Witkowski & Parish 2002). However, genuine resistance to commonly used pediculoside treatments including pyrethrum and the synthetic pyrethroids, lindane, malathion and carbaryl have been reported (Goldsmid 1990; Dodd 2001a; Downs et al 2002; Witkowski & Parish 2002 and references therein). In addition, multiresistance to commonly used preparations have been reported (Rasmussen 1986; Downs et al 1999; Picollo et al 2000; Dodd 2001b). As a result, an increase in the number of head lice infestations has been recorded. Importantly, there have been reported cases of toxicity relating to the use of such preparations (Mortensen 1986). A more recent approach for persistent infestations has been to use oral drugs such as ivermectin (Burkhart & Burkhart 1999; Elgart 1999; Mazurek & Lee 2000; Frankowski & Weiner 2002), cotrimoxazole (Morsy et al 1996; Burkhart et al 1998) or drug combinations (Hipolito et al 2001; Pollack 2001). Alternative forms of topical therapy include preparations containing essential oils such as ylang ylang (*Cananga odorata*)

Department of Pharmacognosy, School of Pharmacy, Trinity College Dublin, College Street, Dublin 2, Ireland

Clive Mills, Brian V. Cleary, John J. Walsh

Department of Pharmaceutical Chemistry, School of Pharmacy, Trinity College Dublin

John F. Gilmer

Correspondence: J. J. Walsh, Department of Pharmacognosy, School of Pharmacy, Trinity College Dublin, College Street, Dublin 2, Ireland. E-mail: jjwalsh@tcd.ie

(Mumcuoglu et al 2002), lavender (*Lavandula augustifolis*) (Veal 1996), anise (*Pimpinella anisum*) (Mumcuoglu et al 2002), neem (*Azadirachta indica*) (Morsy et al 2000), tea tree (*Melaleuca alternifolia*) (Nash 2003) and others (Veal 1996; Frankowski & Weiner 2002). These essential oils are complex mixtures of compounds, many of which are monoterpenes, and have been shown to possess a wide range of useful properties. Two of the major components of Tea Tree oil, 1,8-cineole and terpinen-4-ol, have been shown to inhibit acetylcholinesterase activity (Greenberg-Levy et al 1993; Keane & Ryan 1999) and terpinen-4-ol has been shown to be lethal to head lice at concentrations of 10% in isopropanol (Downs et al 2000). The insecticidal activity of malathion and carbaryl is attributed to similar activity (Main 1979). However, although Tea Tree oil is used as an active ingredient in topical preparations for the treatment of head lice, no reference to inhibition of acetylcholinesterase by Tea Tree oil could be located in the literature. Therefore, the aim of this study was to determine whether the insecticidal activity of Tea Tree oil was, at least in part, due to inhibition of acetylcholinesterase activity.

Materials and Methods

Materials

Electric eel acetylcholinesterase type III (AChE, EC 3.1.1.7) and acetylthiocholine iodide (ATCI) were purchased from Sigma (Sigma-Aldrich Ireland Ltd, Dublin, Ireland). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and 1,8-cineole were purchased from Aldrich (Sigma-Aldrich Ireland Ltd, Dublin, Ireland). Terpinen-4-ol was obtained by elution of a sample (1.5 mL) of Tisserand Tea Tree Oil from a silica column (45 × 100 mm, Merck Kieselgel 40–60 μm) with hexane. Fractions were pooled according to TLC results (Merck Kieselgel F_{254}/hexane developed by 0.5% anisaldehyde in 5% sulfuric acid spray) and the identity of the compound confirmed by GC/MS fragmentation patterns and NMR spectroscopy. Tea Tree oils were purchased from local pharmacies. Tea Tree oil and monoterpene stock solutions were prepared with methanol (Riedel-de Haën Chromasolv). AChE and DTNB stock solutions were prepared using phosphate buffer (pH 8) and ATCI stock solution was prepared with glass distilled water. The reaction progress was monitored using a Spectronics Genesys 5 spectrophotometer.

Enzyme assay

AChE activity was determined by a modified method of Ellman et al (1961). AChE (50 μL 0.22 U mL^{-1} in phosphate buffer pH 8) and inhibitor solution (20 μL) were added to 2.0 mL phosphate buffer (pH 8) and incubated at room temperature for 5 min. DTNB (20 μL, 10 mM in phosphate buffer pH 8) was added and the reaction initiated by addition of ATCI substrate (20 μL) at concentrations ranging from 0 to 1.5 mM as required. The reaction was monitored at 412 nm for 6 min. Results are presented as the mean ± s.e.m. from a minimum of triplicate readings.

Enzyme inhibition studies

Kinetic data were analysed by GraphPad Prism version 3.03. The values of K_m (Michaelis constant (substrate concn at which $V_o = V_{max}/2$)) and V_{max} (maximal or limiting reaction rate) were calculated by non-linear regression analysis. IC50 values, inhibitor concentrations required to give 50% inhibition of AChE activity, were calculated graphically from the concentration–response curves for each inhibitor. Inhibitor constant values ($K_{i(app)}$) were derived from plots of (V_o/V_i)–1 vs inhibitor concentration (Salvesen & Nagase 1989), where V_o is the initial reaction rate and V_i is the reaction rate in the presence of an inhibitor. The true inhibitor constant, the K_i value, was subsequently calculated from $K_i = K_{i(app)}/(1 + [S]/K_m)$. Where S is the substrate concentration.

GC/MS analysis

The identification and quantitation of 1,8-cineole and terpinen-4-ol in each of the Tea Tree oils evaluated was made by GC/MS analysis on the basis of mass spectral fragmentation and retention time comparison. Tea Tree oil, terpinen-4-ol and 1,8-cineole samples were dissolved in ethanol and analysed on a Varian ChromPak CP300 GC coupled to a Saturn 2000 MS detector. Data were analysed using Varian Saturn GC/MS Workstation software (version 5.52). The monoterpene or Tea Tree oil solutions (1.5 μL) were separated on a Varian DB35MS mid polarity column (30 m × 0.25 mm, 0.1 mm film thickness), with an injection port (split 1:20) temperature of 150 °C using a temperature program from 60 °C (held for 5 min) to 100 °C at 4 °C min^{-1} (held for 5 min), to a final temperature of 180 °C at 8 °C min^{-1}. Relative percentages of each component were determined by area normalization of the total detector response (Russell & Southwell 2003).

Data analysis

Data were statistically compared using XLstatistics version 5.68 (XLent Works, Australia (Carr 2000)). Differences between treatment groups were statistically examined using either the Kruskal-Wallis test, followed by Nemenyi's post hoc test to identify individual differences, or alternatively the Mann Whitney U-test. A significance value of 5% was selected.

Results

Composition of oils

The two major constituents of interest were 1,8-cineole and terpinen-4-ol. The percentage of these monoterpenes, as determined by GC/MS analysis, in each of the Tea Tree oil samples tested is shown in Table 1.

Concentration-dependent inhibition

Concentration-dependent inhibition of AChE was observed for 1,8-cineole and terpinen-4-ol (Figure 1). The IC50

Table 1 Proportion of 1,8-cineole and terpinen-4-ol in the Tea Tree oil samples tested.

Sample	1,8-Cineole (%)	Terpinen-4-ol (%)
Body Treats	20.4	26.7
Main Camp	7.0	24.8
IHCA Pure Undiluted	14.6	31.7
Tisserand	13.2	30.4

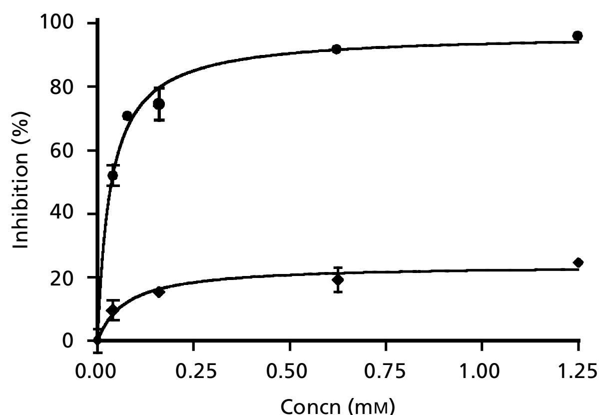

Figure 1 Inhibition of AChE activity by 1,8-cineole (•) and terpinen-4-ol (⁄) (mean ± s.e.m., n = 3).

values obtained for 1,8-cineole and terpinen-4-ol using a substrate concentration of 0.06 mM were 0.04 ± 0.00 and 10.30 ± 0.61 mM, respectively (Table 2). Similarly, a concentration-dependent inhibition was observed for each of the Tea Tree oils used and the IC50 values of each are given in Table 3.

Inhibition kinetics

In the absence of inhibitor, the K_m and V_{max} values were 0.07 ± 0.00 mM and 3.98 ± 0.10 μmol/0.01 U min^{-1}, respectively, as calculated by direct non-linear regression analysis. The kinetic data for the monoterpenes and Tea Tree oil samples are shown in Tables 2 and 3. The monoterpenes showed competitive inhibition of AChE as determined by non-linear regression, where the $K_{m(app)}$ values increased with increasing inhibitor concentration, and by 1/V vs 1/[S] (Lineweaver-Burke) plots of the data. Similarly, the Tisserand sample showed competitive inhibition; however, the remaining samples were shown to be non-competitive inhibitors of AChE. K_i values were derived from Salvesen-Nagase data replots as shown in Figure 2 for 1,8-cineole. K_i values calculated for 1,8-cineole and terpinen-4-ol were 0.03 ± 0.01 and 4.70 ± 0.60 mM, respectively (Table 2). The kinetic data for the oil samples can be seen in Table 3.

Discussion

The incidence of head lice resistance to conventional topical preparations and persistent infestation problems has lead to the search for more effective treatments. The use of preparations containing plant essential oils has been of some benefit (Veal 1996; Frankowski & Weiner 2002). Tea Tree, or Melaleuca, oil has been incorporated into a wide range of products for use in the pharmaceutical, cosmetic, veterinary, industrial and household product industries and the antimicrobial properties of Tea Tree oil are well documented. Several topical preparations that contain Tea Tree oil are available to treat head lice infestations.

Resistance is now widespread to even, arguably, the most effective treatments, those that contain malathion (Abou el-Ela et al 2000; Meinking et al 2001). The target of these treatments is the enzyme AChE. The two main constituents of Melaleuca oil, 1,8-cineole and terpinen-4-ol, are known to inhibit AChE (Greenberg-Levy et al 1993; Keane & Ryan 1999), however no reference to the inhibition of AChE by Tea Tree oil could be located in the literature.

In the absence of any inhibitor, K_m and V_{max} values (0.07 ± 0.00 mM and 3.98 ± 0.10 μmol/0.01 U min^{-1}, respectively) were consistent with literature values for electric eel acetylcholinesterase (e.g. Perry et al 2000). 1,8-Cineole and terpinen-4-ol showed competitive inhibition of AChE, with 1,8-cineole being the more effective inhibitor (IC50 = 0.04 ± 0.00 mM; K_i 0.03 ± 0.01 mM, $P < 0.05$). However, these compounds are considered weak inhibitors of AChE when compared with physostigmine (IC50 = 5×10^{-8} M) or tacrine (IC50 = 2×10^{-8} mM) (Perry et al 2000 and references therein).

Table 2 Kinetic data for 1,8-cineole and terpinen-4-ol.

Inhibitor	[a]K_m (mM)	[a]V_{max} (μmol 0.01 U^{-1} min^{-1})	K_i (mM)	[b]IC50 (mM)
No inhibitor	0.07 ± 0.00	3.98 ± 0.10	—	—
1,8-Cineole	0.25 ± 0.01*	4.12 ± 0.08	0.03 ± 0.01*	0.04 ± 0.00*
Terpinen-4-ol	0.09 ± 0.02	4.19 ± 0.34	4.70 ± 0.60*	10.30 ± 0.61*

Values are mean ± s.e.m., n = 3. [a]Inhibitor concn of 0.04 mM. [b][ATCI] = 0.06 mM. *$P < 0.05$.

Table 3 Kinetic data for Tea Tree oil samples.

Inhibitor	[a]$K_{m(app)}$ (μL mL^{-1})	[a]$V_{max(app)}$ (μmol 0.01 U^{-1} min^{-1})	K_i (μL mL^{-1})	[b]IC50 (μL mL^{-1})
Tisserand	0.18 ± 0.04*	4.44 ± 0.48	0.03 ± 0.00	0.05 ± 0.01*
Main Camp	0.11 ± 0.02*	3.73 ± 0.51	0.03 ± 0.01	0.08 ± 0.02
Body Treats	0.22 ± 0.08*	5.78 ± 1.45	0.04 ± 0.00	0.10 ± 0.02
IHCA Pure Undiluted	0.14 ± 0.03*	4.32 ± 0.58	0.05 ± 0.01	0.11 ± 0.02

Values are mean ± s.e.m., n = 5. [a]Inhibitor concn of 0.02 μL mL^{-1}. [b][ATCI] = 0.05 mM. *P < 0.05.

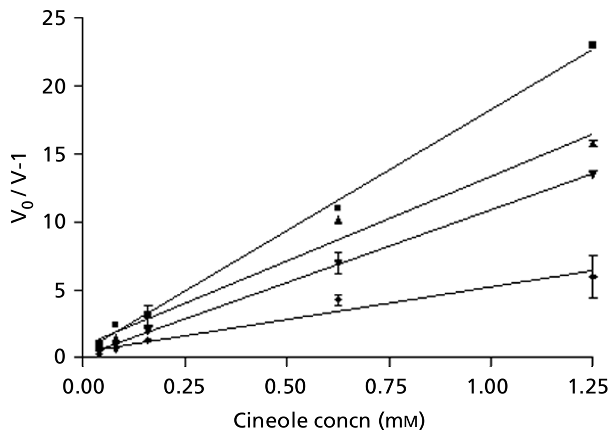

Figure 2 Salvesen & Nagase (1989) replot of AChE activity in the presence of 1,8-cineole used to determine the $K_{i(app)}$ (slope = $1/K_{i(app)}$). [ATCI] = ■ 0.063; ▲ 0.125; ▼ 0.250; ⁄ 0.50 mM.

Three of the Tea Tree oil samples evaluated showed a non-competitive inhibition of AChE. This was in contrast to the competitive inhibition showed by the monoterpenes used in this study. The most active of the oils tested was Tisserand (IC50 = 0.05 ± 0.01 μL mL^{-1}, P < 0.05), which showed competitive inhibition, and the least active was Irish Health Culture Association Pure Undiluted (IHCA) (IC50 = 0.11 ± 0.02 μL mL^{-1}). An increase in the K_i values was accompanied by an increase in the IC50 values for the Tea Tree oils (Table 3).

To directly compare Tea Tree oil data with the monoterpenes used in this study, an average molecular weight of 160 g mol^{-1} (Perry et al 2000) was assumed for each of the Tea Tree oils. An average density of 0.89 g mL^{-1} was determined for the Tea Tree oils by weighing known volumes in triplicate of each sample. This showed that the rank order of potency was Tisserand (IC50 = 0.26 mM), Main Camp (IC50 = 0.46 mM), Body Treats (IC50 = 0.58 mM) and IHCA Pure Undiluted (IC50 = 0.61 mM). The 1,8-cineole and terpinen-4-ol content of Tea Tree oils is regulated by Australian Standard AS 2782-1997 and International Standard ISO 4730 to be less than 15% and greater than 30%, respectively. Of the samples tested for activity against AChE, Body Treats had both high 1,8-cineole (20.4%) and low terpinen-4-ol (26.7%) concentrations. Main Camp was shown to have a low terpinen-4-ol concentration (24.8%).

The concentrations of 1,8-cineole and terpinen-4-ol in each of the Tea Tree oils sampled did not account for the total activity of the oil itself. This may have been due to synergistic action of all of the constituents of the oils or the presence of other active components.

Conclusion

This work did not show efficacy of Tea Tree oil in infestation, rather that its known activity might be attributed to AChE inhibition. The low incidence of adverse side effects of Tea Tree oils and the moderate inhibition of AChE activity would suggest that preparations containing Tea Tree oil may be of some benefit as part of a co-ordinated approach to the control of head lice infestation.

References

Abou el-Ela, R. G., Morsy, T. A., Nassar, M. M., Khalaf, S. A. (2000) Evaluation of four pediculicides against the head lice *Pediculus h. capitis*. *J. Egyptian Soc. Parasitol.* **30**: 51–58

Burkhart, C. G., Burkhart, C. N., Burkhart, K. M. (1998) An assessment of topical and oral prescription and over the counter treatments for headlice. *J. Am. Acad. Dermatol.* **38**: 979–982

Burkhart, C. N., Burkhart, C. G. (1999) Another look at ivermectin in the treatment of scabies and head lice. *Int. J. Dermatol.* **38**: 235

Carr, R. (2000) XLStatistics 5.68 XLent Works Australia. URL. http://www.deakin.edu.au/~rodneyc/xlstats.htm (accessed 2003)

Dodd, C. (2001a) Treatment of head lice. Choice of treatment will depend on local patterns of resistance. *Br. Med. J.* **323**: 1084

Dodd, C. S. (2001b) Interventions for treating headlice. Cochrane Database Syst Rev(3): CD001165

Downs, A. M., Stafford, K. A., Harvey, I., Coles, G. C. (1999) Evidence for double resistance to permethrin and malathion in head lice. *Br. J. Dermatol.* **141**: 508–511

Downs, A. M., Stafford, K. A., Coles, G. C. (2000) Monoterpenoids and tetralin as pediculocides. *Acta Derm. Venereol.* **80**: 69–70

Downs, A. M. R., Stafford, K. A., Hunt, L. P., Ravenscroft, J. C., Coles, G. C. (2002) Widespread insecticide resistance in head lice to the over-the-counter pediculocides in England, and the emergence of carbaryl resistance. *Br. J. Dermatol.* **146**: 88–93

Elgart, M. L. (1999) Current treatments for scabies and pediculosis. *Skin Ther. Lett.* **5**: 1–3

Ellman, G. L., Courtney, K. D., Andres, V., Featherstone, R. M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**: 88–95

Frankowski, B. L., Weiner, L. B. (2002) Head lice. *Pediatrics* **110**: 638–643

Goldsmid, J. M. (1990) Head louse treatment: is there an insecticide resistance problem? *Med. J. Aust.* **153**: 233–234

Greenberg-Levy, S. H., Kostjukovsky, M., Ravid, U., Shaaya, E. (1993) Studies to elucidate the effect of monoterpenes on acetylcholinesterase in two stored-product insects. *Acta Horticulturae (ISHS)* **344**: 138–146

Hipolito, R., Mallorca, F., Zuniga-Macaraig, Z. E. A. (2001) Head lice infestation: single drug versus combination therapy with one percent permethrin and trimethoprim/sulphamethoxazole. *Pediatrics* **107**: E30

Jones, K. N., English, J. C. (2003) Review of common therapeutic options in the United States for the treatment of pediculosis capitis. *Clin. Infect. Dis.* **36**: 1355–1361

Keane, S., Ryan, M. F. (1999) Purification, characterisation, and inhibition by monoterpenes of acetylcholinesterase from the wax moth, *Galleria mellonella* (L.). *Insect Biochem. Mol. Biol.* **29**: 1097–1104

Main, A. R. (1979) Mode of action of anticholinesterases. *Pharmacol. Ther.* **6**: 579–628

Mazurek, C. M., Lee, N. P. (2000) How to manage head lice. *West. J. Med.* **172**: 342–345

Meinking, T. L., Entzel, P., Villar, M. E., Vicaria, M., Lemard, G. A., Porcelain, S. L. (2001) Comparative efficacy of treatments for pediculosis capitis infestations: update 2000. *Arch. Dermatol.* **137**: 287–292

Meinking, T. L., Serrano, L., Hard, B., Entzel, P., Lemard, G., Rivera, E., Villar, M. E. (2002) Comparative in vitro pediculicidal efficacy of treatments in a resistant head lice population in the United States. *Arch. Dermatol.* **138**: 220–224

Morsy, T. A., Ramadan, N. I., Mahmoud, M. S., Lashen, A. H. (1996) On the efficacy of Cotrimoxazole as an oral treatment for pediculosis capitis infestation. *J. Egyptian Soc. Parasitol.* **26**: 73–77

Morsy, T. A., el-Ela, R. G., Nasser, M. M., Khalaf, S. A., Mazyad, S. A. (2000) Evaluation of the in-vitro pediculicidal action of four known insecticides and three medicinal plant extracts. *J. Egyptian Soc. Parasitol.* **30**: 699–708

Mortensen, M. L. (1986) Management of acute childhood poisonings caused by selected insecticides and herbicides. *Pediatr. Clin. North Am.* **33**: 421–445

Mumcuoglu, K. Y., Miller, J., Zamir, C., Zentner, G., Helbin, V., Ingber, A. (2002) The in vivo pediculicidal efficacy of a natural remedy. *Israel Med. Assoc. J.* **4**: 790–793

Nash, B. (2003) Treating head lice. *Br. Med. J.* **326**: 1256–1257

Orion, E., Matz, H., Ruocco, V., Wolf, R. (2002) Parasitic skin infestations II, scabies, pediculosis, spider bites: unapproved treatments. *Clin. Dermatol.* **20**: 618–625

Parish, L. C. (1985) History of pediculosis. Cutaneous infestations and insect bites. In: Orkin, M., Maibach, H. I. (eds) Marcel Dekker, New York, pp 159–162

Perry, N. S. L., Houghton, P. J., Theobold, A., Jenner, P., Perry, E. K. (2000) In-vitro inhibition of human erythrocyte acetylcholinesterase by *Salvia lavandulaefolia* essential oil and constituent terpenes. *J. Pharmaceut. Pharmacol.* **52**: 895–902

Picollo, M. I., Vassena, C. V., Mougabure Cueto, G. A., Vernetti, M., Zerba, E. N. (2000) Resistance to insecticides and effect of synergists on permethrin toxicity in *Pediculus capitis* (Anoplura: Pediculidae) from Buenos Aires. *J. Med. Entomol.* **37**: 721–725

Pollack, R. J. (2001) Head lice infestation: single drug versus combination therapy. *Pediatrics* **108**: 1393

Rasmussen, J. E. (1986) Pediculosis: treatment and resistance. *Adv. Dermatol.* **1**: 109–125

Russell, M. F., Southwell, I. A. (2003) Monoterpenoid accumulation in 1,8-cineole, terpinolene and terpinen-4-ol chemotypes of *Melaleuca alternifolia* seedlings. *Phytochemistry* **62**: 683–689

Salvesen, G., Nagase, H. (1989) Inhibition of proteolytic enzymes. Proteolytic enzymes: a practical approach. In: Beynon, R. J., Bond, J. S. (eds) Oxford University, IRL Press, pp 83–87

Vander Stichele, R. H., Dezeure, E. M., Bogaert, M. G. (1995) Systematic review of clinical efficacy of topical treatments for head lice. *Br. Med. J.* **311**: 604–608

Veal, L. (1996) Headlice. http://www.wingedseed.com/Agora/Lice_page.htm. (accessed 2003)

Witkowski, J. A., Parish, L. C. (2002) Pediculosis and resistance: the perennial problem. *Clin. Dermatol.* **20**: 87–92