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In vitro efficacy of over-the-counter botanical pediculicides against the head louse *Pediculus humanus* var *capitis* based on a stringent standard for mortality assessment

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Abstract. Infestation of the head louse *Pediculus humanus* var *capitis* DeGeer (Phthiraptera: Pediculidae) is an important public health problem in Australia, with up to a third of children infested in some primary schools. Insecticide resistance and inadequate attention to the application instructions of topical pediculicides are common reasons for treatment failure. This study evaluated six popular Australian over-the-counter products against head lice, primarily comprised of different botanical extracts, and compared them with permethrin 1% (Quellada®) and a non-treatment control in order to assess their *in vitro* efficacy. We also assessed commonly used criteria for evaluating pediculicide efficacy *in vitro.* All tested products failed to demonstrate high levels of efficacy with the exception of Tea Tree Gel®, which outperformed 1% permethrin. Permethrin had a high level of efficacy, but using stringent criteria 18% of lice were not dead at 3 h, indicating some resistance to Quellada®. Commonly used less stringent criteria were shown to overestimate mortality of head lice as a result of the protective phenomenon of stasis or sham death observed in exposed lice that may recover after some time. Using two different levels of stringency resulted in different rankings of efficacy for most products, with the exception of the first ranked product, Tea Tree Gel®. Rankings of efficacy also varied over time, even within the different assessment criteria. Government regulatory agencies should require standard *in vitro* tests using stringent mortality criteria, with an observation period of $\geq$ 6 h, to determine the efficacy of new pediculicides, and only products that cause a minimum mortality rate (e.g. 80%) in head lice collected from the target population should be licensed for sale.

Key words. *Pediculus capitis*, botanical pesticides, head lice treatment, *in vitro* testing, pediculicides, resistance, Australia.

Introduction

Hundreds of millions of human infestations with head lice are thought to occur annually (Taplin & Meinking, 1987). Numbers have increased worldwide since the mid-1960s (Gratz, 1997) and an estimated 6-12 million people, mainly children, undergo some sort of treatment annually.

Current topical head lice treatments include organochlorines (lindane), organophosphates (malathion), carbamates (carbaryl), pyrethrins and pyrethroids (permethrin, D-phenothrin, bioallethrin). Surprisingly, this arsenal of pediculicides has failed to attain adequate lice control (Mumcuoglu, 2006). However, topical insecticides, such as those including permethrin or malathion, are still the first choice of consumers in most cases,

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despite the availability of many alternative plant-based compounds (Heukelbach *et al.*, 2007).

Unfortunately, only a few *in vitro* experiments and clinical studies have evaluated the effectiveness of these alternative compounds and the bulk of them remain to be scientifically tested (Heukelbach *et al.*, 2007). In addition, criteria for defining 'mortality' of head lice *in vitro* are not standardized. To prevent overestimates of mortality, we recently used more stringent criteria to assess the *in vitro* efficacy of compounds against head lice (Heukelbach *et al.*, 2006; Oliveira *et al.*, 2007), as suggested by Burkhart & Burkhart (2006a, 2006b).

Table 1 lists all the 49 papers in the literature of which we are aware, describing *in vitro* studies of the efficacy of pediculicides. In 59% of these, a state of reduced activity (rather than organic death) was used to define mortality. The criteria used varied in stringency and included the inability of lice to walk in a progressive fashion (Mougabure Cueto *et al.*, 2002), loss of righting reflex when rolled over (Hunter & Barker, 2003), lack of response when mechanically stimulated (Pollack *et al.*, 1999), and the presence of only minor vital signs such as antenna and gut movements (Downs *et al.* 2000; Oliveira *et al.*, 2007). Only 14% of papers used 'no vital signs', including complete cessation of gut peristalsis, as the criterion for mortality. In 27% of the papers the criteria were not clear or were not provided.

In Australia, head lice infestation is an important public health problem and up to a third of students are infested in some schools (Speare & Buettner, 1999). Control measures have been hampered in the past not only by incorrect use of topical insecticides, but also by the increasing resistance of lice, mainly against permethrin, the most common topical pediculicide used in Australia (Bailey & Prociv, 2000; Hunter & Barker, 2003).

The increasing global prevalence of pediculosis and accompanying increased awareness highlight the need to approach alternatives to head lice control more seriously. There is also a dire need to properly evaluate the plethora of over-the-counter (OTC) head lice repellents and preventatives. To address and remedy this situation nationally, six popular Australian OTC products, primarily comprised of different botanical extracts, were compared with a commercial permethrin product for their *in vitro* efficacy against head lice.

Materials and methods

Head lice used

Head lice, *Pediculus humanus* var *capitis* DeGeer, were collected by dry combing from schoolchildren in Townsville (QLD, Australia) on four occasions over a 3-month period. After collection, lice were held on human hair in 5-cm Petri dishes at 27 °C and 50% relative humidity.

Captured adult female and male lice were blood-fed on the dorsum of the hand by two investigators (JH and FAO) and used within 1 h after feeding. Before testing, all lice were examined for activity and morphological integrity under a dissecting microscope, and only fully active and intact adult lice were used, irrespective of sex. There appears to be no difference in responses to insecticides by sex (Blommers & van Lennep, 1978).

Products tested

Six commercial Australian OTC products for the treatment of head lice were compared *in vitro* with a positive (Quellada® Head Lice Treatment; Glaxo Smith Kline, Ermington, NSW, Australia) and a negative (no treatment) control group. Table 2 shows the products, their active and other ingredients, their presentation and their purchase prices as of October 2005. Only products that contained the most commonly used active ingredients based on plant extracts or essential oils were selected. The selection of specific products and manufacturers was arbitrary.

In vitro *tests and mortality criteria*

For each of the eight experimental groups (six treatment and two control groups), 25 ± 3 lice were tested. Lice clasping hair strands were immersed completely in the product for 1 min and then placed with hairs onto Whatman filter paper in Petri dishes. Pools of the products were wiped from the lice with a jeweller's forceps directed under a dissecting microscope. The lice in the negative control group were placed directly on moistened filter paper without any treatment. They were not dipped in water or wiped with forceps, as these actions have been shown in previous studies to have no effect on lice activity levels (Canyon & Speare, 2007). To prevent lice from desiccation, the filter paper had been previously moistened with 200 μL tap water. To simulate treatment on an infested host, head lice were washed in tap water after 20 min and placed into a new Petri dish with unused filter paper.

Lice on the filter paper were examined under a dissecting microscope by a single observer in all cases to prevent inter-observer variation. The standard criteria for evaluating mortality were compared with a more stringent method to see if standards require revision (Hunter & Barker, 2003; Heukelbach *et al.*, 2006):

Standard criteria for 'mortality'. The inability to walk in a progressive fashion and the absence of a righting reflex when rolled onto the back were defined as 'some vital signs' for the present study. These criteria were originally used by the World Health Organization (WHO) to measure sub-lethal effects as opposed to mortality in basic toxicological studies and to test for insecticide resistance. The criteria were meant to be used to compare the sub-lethal effects of particular products on different populations of insects, over a range of doses, rather than as absolute measures of mortality (World Health Organization, 1981). However, the criteria have been used by many to test the overall 'efficacy' of lice control products.

Stringent criteria. Death of a head louse was defined as the complete absence of any vital signs such as gut movement and movement of antennae or legs, with or without stimulation using forceps. This status was defined as 'no vital signs' for the present study.

Lice were defined as 'active' if no changes in their levels of activity or behaviour were observed post-treatment. An evaluation of these categories was conducted 30, 60 and 180 min post-treatment. All lice were maintained at 27 °C during the tests.

Table 1. Details of published studies of pediculicide efficacy against body lice, head lice and pubic lice. If peristalsis was not looked for as evidence of internal viability, the criteria used by the authors were classed as minor or major vital signs.

Criteria for mortality	Exposure technique	Sex of lice	Species	Reference
1. No vital signs				
No movement of lice and peristalsis of the gut	Kitchen cotton-polyester towels	Mixed adults, nymphs	Head lice	Meinking *et al.* (1986)
No movement of lice or peristalsis of the gut	Filter paper	Mixed adults, nymphs	Head lice	Meinking *et al.* (2001)
Absence of antenna, claw or leg movements and of internal organ movements	Lice covered with shampoo dilutions	Mixed adults, nymphs	Head lice	McCage *et al.* (2002)
Lack of movement of limbs and gut; failure to respond when limbs poked with forceps	Filter paper	Mixed	Body lice	Priestley *et al.* (2006)
Lack of internal movements, movement of antennae, and minimal leg movements even after stimulation with forceps; mortality with reference to major vital signs also presented	Immersion	Mixed	Head lice	Heukelbach *et al.* (2006)
Lack of internal movements, movement of antennae, and minimal leg movements even after stimulation with forceps	Immersion	Mixed	Head lice	Jadhav *et al.* (2007)
No movement or peristalsis for several hours even after stimulation with forceps	Solutions were spread over lice and filter paper	Mixed adults, third-stage nymphs	Head lice	Carpinella *et al.* (2007)
2. Minor or major vital signs				
Not stated, but describes stupefaction and paralysis in results section	Immersion; direct contact; crawl on solids; fumigation	Mixed	Body lice	Aschner & Mager (1945)
Inability to walk after 24 h	Filter paper	Mixed	Head lice	Maunder (1971)
No movement of legs and antennae on stimuli, no comment on peristalsis	Lice stuck to sticky tape on slides; immersion	First-stage nymphs	Head lice	Blommers (1980)
Incapable of co-ordinated movement and sucking blood	Human hair and fishing line impregnated	Mixed	Head lice	Rupes *et al.* (1984)
No movement without stimulation	Lice collected from head after treatment and washed	Mixed	Head lice	Pitman *et al.* (1987)
'Knockdown', no mortality	Patch impregnated	Mixed	Body lice	Sholdt *et al.* (1989)
Motility, but not clearly defined; twitching of limbs discounted as life	Immersion	Mixed	Head lice	Burgess *et al.* (1992)
Not given, but comments that tremors present in dead lice	Immersion	Mixed	Body lice	Burgess *et al.* (1994)
No activity, no antennal movement on stimulation, no comment on peristalsis	Filter paper	Mixed	Pubic lice	Ragheb *et al.* (1995)
Inability to walk properly; motionless but twitching limbs and peristalsis	Immersion	Mixed	Head lice	Veal (1996)
Inability to walk on artificial hair	Simulated hair impregnated	Mixed	Head lice	Rupes *et al.* (1995)
No movement after probing	Filter paper	Mixed	Head lice	Pollack *et al.* (1999)
Absence of all movements or irreversible intoxication (paralysis or continuous tonic-clonic spasms)	Filter paper	Mixed	Body lice	Downs *et al.* (1999)

continued

Table 1. Continued.

Criteria for mortality	Exposure technique	Sex of lice	Species	Reference
'Knockdown', inability to cling to paper on edge when tapped	Chromatography paper	Mixed	Body lice	Zeichner (1999)
'Knockdown' (immobile, body distorted and shrunken; recovered = began to move and swell; dead = body shrinkage progressed to severe changes)	Filter paper	First-stage nymphs	Head lice	Lee *et al.* (2000)
Inability to walk over filter paper	Filter paper	Mixed adults, third-stage nymphs	Head lice	Picollo *et al.* (2000)
Absence of movements or irreversible intoxication (spasms, paralysis)	Filter paper	Mixed	Head lice	Downs *et al.* (2000)
Inactivity, no response by antennae on stimulation, no comment on peristalsis	Filter paper	Mixed	Head lice	Abou El-Ela *et al.* (2000)
Absence of all internal and external movement on tactile stimuli, spastic paralysis, continuous tonic-clonic movements	Filter paper	Mixed	Head lice	Downs *et al.* (2002)
Inability to walk over filter paper	Immersion	Mixed adults, third-stage nymphs	Head lice and body lice	Mougabure Cueto *et al.* (2002)
Inability to right when rolled onto back	Filter paper	Mixed adults, third-stage nymphs	Head lice	Hunter & Barker (2003)
Inability to walk from centre to border of filter paper (7 cm)	Filter paper, direct application	Mixed	Head lice	Vassena *et al.* (2003)
Inability to migrate down to another piece of cloth	Immersion	Mixed; 17 days old	Body lice	American Society for Testing and Materials (2004)
Lethargic response or no movement	Filter paper; vapour	Female	Head lice	Yang *et al.* (2004a)
Cessation of motility or movement of appendages when touched with a needle, no comment on peristalsis	Direct application	Mixed	Head lice, body lice	Oladimeji *et al.* (2000)
'Dead' or inability to move when disturbed	Direct application	Mixed	Body lice and head lice	Kristensen *et al.* (2006)
Inability to walk from centre to border of filter paper (7 cm) in 15 s	Direct application	Mixed	Head lice	Mougabure Cueto *et al.* (2006)
Not walking, but presence of internal movements, movements of antennae, or leg movements after stimulation with forceps; data using no vital signs also presented, but not mortality criteria	Immersion	Mixed	Head lice	Oliveira *et al.* (2007)
Unable to right when inverted and no movements in legs when probed	Immersion	First-stage nymphs	Head lice	Strycharz *et al.* (2008)
3. Not stated or not clearly stated:				
Not given	Cloth around hair; vapour; hair dipped	Mixed	Head lice	Scobbie (1945)
'Dead' 3 days later in pillboxes worn on leg	Spray test; dip test; dust test	Mixed	Body lice	Busvine (1946)
Not given	Filter paper	Mixed	Head lice	Blommers & van Lennep (1978)
Not given	Filter paper	Mixed	Head lice	Blommers (1979)
Not given	Immersion	Mixed	Head lice	Maunder (1981)
Not given	Filter paper	Mixed	Head lice	Mumcuoglu *et al.* (1995)
Not given	Filter paper	Males	Head lice	Morsy *et al.* (2000)

continued

Table 1. Continued.

Criteria for mortality	Exposure technique	Sex of lice	Species	Reference
No definition; but lack of mobility not criteria	Immersion	Mixed	Head lice	Burkhart & Burkhart (2000)
Not given	Filter paper	Mixed adults, nymphs	Head lice	Meinking *et al.* (2002)
Not given	Filter paper	Female	Head lice	Yang *et al.* (2003)
Not given	Filter paper	Mixed adults and nymphs	Head lice	Yoon *et al.* (2003)
Not given	Filter paper, vapour	Female	Head lice	Yang *et al.* (2004b)
Not given	Filter paper	Female	Head lice	Yang *et al.* (2005)

Statistical analysis

For each experimental group 25 ± 3 lice were analysed. SPSS Version 15 (SPSS Inc., Chicago, IL, U.S.A.) was used to compare differences in relative frequencies between experimental groups using the exact version of the chi-square test. The McNemar-Bowken test was used to compare paired (longitudinal) data within each experimental setting.

Results

The activity levels of lice treated with botanical products, 1% permethrin (Quellada®) and the negative control group over time are shown in Fig. 1. The efficacy of the treatments (i.e. the proportions of treated lice with no vital signs) differed significantly at all three post-treatment observation times (all $P < 0.0001$). After 30 min, Neutralice® and Tea Tree Gel® appeared to have been quite effective. Over time, the efficacy of Lysout® and Neutralice® decreased significantly ($P = 0.001$ for both). Lice treated with Neutralice® recovered after 180 min, resulting in only 16.7% mortality. By contrast, the positive control (Quellada®) gradually increased in efficacy over time ($P = 0.012$), causing 82.1% mortality after 180 min. Overall, only Tea Tree Gel® was highly effective at all time-points, with only a single louse out of 25 showing minor vital signs throughout, resulting in an overall mortality rate for this treatment of 96%.

The effect of the treatment using stringent criteria did not change significantly over time for the negative control ($P = 1.0$), the Lice Blaster® group ($P = 0.5$), the Moov® group ($P = 0.5$), the Praneem® group ($P = 0.3$) or the Tea Tree Gel® group ($P = 1.0$).

Figure 1 shows clearly that the less stringent criteria of 'some vital signs' produced a striking overestimate of the efficacy of some products, even at 30 min post-treatment, and especially at 60 and 180 min post-treatment, compared with our more

Table 2. Details of over-the-counter head lice products evaluated.

Product name (TGA reg. no.)	Producer	Active ingredient(s) and concentration (w/v)*	Selected other ingredients	Quantity	Price (AUD)†
Lice Blaster® (L63221)	Richard Thomson Pty Ltd, Kingsgrove, NSW	*Adhatoda vasica* leaf extract 20%, *Stemona sessifolia* root extract 20%, *Echinacea purpurea* herb 4%, *Melaleuca* (tea tree) oil 2%	Ethanol‡	250 mL	$17.99
Neutralice® spray (L67693)	Key Pharmaceuticals Pty Ltd, Rhodes, NSW	*Melaleuca* (tea tree) oil 10%, *Lavender* oil 1%	Ethanol 24% w/v, benzyl alcohol 0.5% w/v	100 mL	$11.99
Praneem® Repel® natural shampoo (N/A)	NewChem Laboratories Pty Ltd, Sumner Park, QLD	*Azadirachta indica* (neem tree) seed extract‡	Dimethicone copolyol‡	200 mL	$15.45
Moov® head lice treatment	Ego Pharmaceuticals Pty Ltd, Braeside, VIC	*Eucalyptus* oil 10%	Ethanol‡, benzyl alcohol 0.5%	200 mL	$19.99
Tea Tree Head Lice Gel®	Thursday Plantation Laboratories Ltd, Ballina, NSW	*Melaleuca* (tea tree) oil 5% (w/w)	Ethanol 20%	2 x 125 mL	$15.99
Lysout® natural anti-lice foaming gel	National Natural Products, Salisbury, SA	*Stemona sessifolia* 5%, *Echinacea purpurea* 10%, *Canarium luzonicum* gum oil 2%	Diazolidinylurea‡, phenoxyethanol‡, hydroxybenzoates‡	250 mL	$17.59
Quellada® head lice treatment cream	Glaxo Smith Kline, Ermington, NSW	Permethrin 1% w/w	Isopropyl alcohol 20%	40 mL	$10.40

*As stated by the producer.
†At time of purchase (October 2005).
‡Concentration not revealed on the product inlet/description.

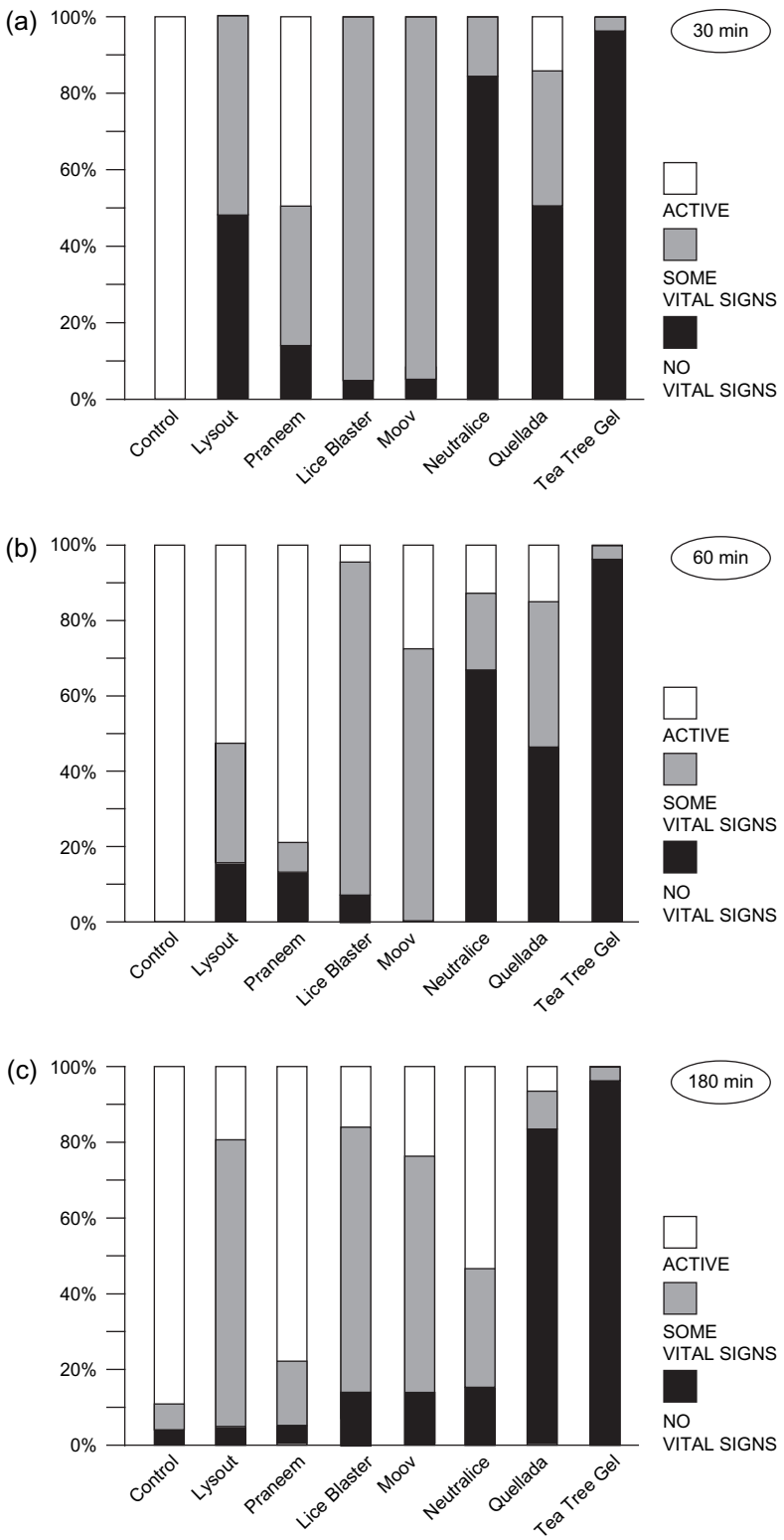

Fig. 1. Post-treatment percentages of lice in the three categories recorded as active, showing weak vital signs, and showing no vital signs, after (a) 30 min, (b) 60 min and (c) 180 min. $n = 25 \pm 3$ lice in each column.

stringent criteria of 'no vital signs'. Six of the seven commercial products were ranked differently for efficacy according to the stringent and less stringent criteria (Table 3). Only Tea Tree Gel® and the control group were consistently ranked first and last, respectively, using both criteria.

Discussion

Our data show that only one botanical preparation performed well *in vitro*: Tea Tree Gel®, which contained 5% tea tree oil. All other botanical preparations were less effective. Quellada®, containing permethrin, the positive control, killed only 82% of lice after 180 min, indicating the possible presence of resistance to permethrin. Hunter & Barker (2003) found a high level of resistance to permethrin in head lice in a Townsville school. (Lice for this study were also collected in Townsville, but in a different school.) In their study, only about 5% of lice were killed at 180 min post-treatment according to the less stringent criteria, compared with our findings of 93% using the same criteria and 82% using our more stringent criteria. There are three possible explanations for this difference. Firstly, there may have been a real difference in levels of permethrin resistance between the two pools of lice tested. This is likely: according to the evidence of Hunter & Barker (2003), the prevalence of resistance in Queensland varies by school. Secondly, the formulation of permethrin may have affected the observed level of resistance. Burgess (2004) has suggested that particular formulations can improve the efficacy of an insecticide even when lice are resistant. Hunter & Barker (2003) tested pure permethrin, whereas we used a commercial product (Quellada®), which comprised 1% w/w permethrin in 20% isopropyl alcohol. Thirdly, the method of testing may affect the level of efficacy of treatments. Hunter & Barker (2003) used an 'arena test' (filter paper), whereas we used an immersion with post-wash. We are unaware of any studies assessing the comparability of results between these two methods of testing pediculicide efficacy.

Tea tree (*Melaleuca alternifolia*) oil has been used in traditional aboriginal medicine for a range of purposes and its efficacy for the treatment of louse infestations has been described repeatedly (Heukelbach *et al.*, 2007). For example, 94% mortality against body lice was observed using a 1% emulsion of tea tree oil in water (Veal, 1996). In preliminary observations, we observed 100% mortality of head lice after 4 h with an emulsion of 4% tea tree oil in 20% ethanol (J. Heukelbach, 2006, unpublished data).

Table 3. Ranking of efficacy of product groups and control group by 'mortality' using stringent (no vital signs) and less stringent (some vital signs) criteria. Note that possible rankings are from 1 to 8, but rank can be equal if percentage mortality is similar.

	Stringent criteria		Less stringent criteria	
	60 min	180 min	60 min	180 min
Tea Tree Gel®	1	1	1	1
Neutralice®	2	3	3	6
Quellada®	3	2	4	2
Lysout®	4	6	6	4
Praneem®	5	6	7	7
Lice Blaster®	6	4	2	3
Moov®	7	4	5	5
Control	8	8	8	8

In the present study, many lice recovered within 180 min of sham death after exposure to a product. Lice have the ability to physiologically shut down and go into stasis, and then recover from an apparently morbid state (Nuttall, 1917; Canyon & Speare, 2007). In fact, insects are well equipped to metabolize insecticides and recover from insecticide exposure (Bloomquist & Miller, 1986). Thus, in the evaluation of head lice treatments, the end-point observation must be taken after the maximum duration of stasis. This end-point cannot be determined by observations at the gross level, such as the lack of physical response to disturbance with forceps (Pollack *et al.*, 1999), or irreversible toxicity defined as paralysis or continuous tonic-clonic spasm (Downs *et al.*, 1999), because cessation of finer body functions, such as peristalsis, can only be determined by microscopy. Previous researchers (Nuttall, 1917; Meinking *et al.*, 1986; Oliveira *et al.*, 2007) have highlighted the need for an extended period of observation to confirm death, and this was confirmed by this study, where ranking of efficacy varied with time. A previous study indicated that percent mortality remained relatively constant only after 6 h (Oliveira *et al.*, 2007), with a rise at 24 h even in control lice owing to dehydration. Thus, an observation period of $\geq$6 h is recommended in future studies.

Several compounds, such as mayonnaise, petroleum jelly and olive oil, have erroneously been reported to kill head lice, based on the less stringent criteria of efficacy, but in reality these caused only a transient period of stasis (Burkhart & Burkhart, 2001, 2004, 2006a, 2006b). In the present study, we evaluated head lice for 3 h post-treatment. Theoretically, it is possible that the apparent efficacy of Tea Tree Gel® may have been caused by sub-lethal effects or sham death lasting $>$3 h, especially as this product (a rather sticky gel) appeared to be more effective than other products containing tea tree oil. The gel formulation of the product may have caused more persistent stasis than other formulations, leading to effects similar to those caused by mayonnaise or olive oil. We attempted to reduce the probability of misidentifying mortality, however, by using more stringent criteria for the definition of mortality than are commonly used.

A practical method for detecting insecticide resistance in lice on patients, which is based on the assessment of louse movement in combing samples 20 min after treatment and which has been used by some of the authors of the present study (Canyon & Speare, 2005; Speare, 2006), needs further research to determine whether it correlates with level of resistance.

Many producers of lice control products now include a lice comb in the package and instruct consumers to comb out their wet hair several times after applying the product. Thus, consumers cannot determine whether the claimed pediculicidal effects result from the efficacy of the ingredients contained in the product or from their own assiduous combing efforts.

In conclusion, we have shown clearly that the criteria for evaluating pediculicide efficacy used in previous studies usually

overestimate mortality. Furthermore, as rankings of efficacy using stringent and less stringent criteria differ, the more stringent criteria should be chosen as the standard. Stringent criteria (no vital signs including lack of peristalsis) should be used as the criteria for death to avoid the problem of sub-lethal effects which disappear several hours post-exposure. Adequately designed and standardized *in vitro* studies are needed to assess the efficacy of products that are marketed to control head lice infestations. As listed products are not currently bound by government regulatory authorities to meet a defined level of efficacy using rigorous scientific criteria, we recommend two changes. Government regulatory agencies should require standard *in vitro* tests using stringent mortality criteria, with observation periods of ≥ 6 h to determine the efficacy of new pediculicides, and only products that cause a minimum mortality rate (e.g. 80%) in head lice collected from the target population should be licensed for sale.

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