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ORIGINAL PAPER

Validation of models to estimate the fumigant and larvicidal activity of *Eucalyptus* essential oils against *Aedes aegypti* (Diptera: Culicidae)

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Abstract The aim of this work is to validate the pre-existing models that relate the larvicidal and adulticidal activities of the *Eucalyptus* essential oils on *Aedes aegypti*. Previous works at our laboratory described that the larvicidal activity of *Eucalyptus* essential oils can be estimated from the relative concentration of two main components (*p*-cymene and 1,8-cineole) and that the adulticidal effectiveness can be explained, to a great extent, by the presence of large amounts of the component 1,8-cineole in it. In general, the results show that the higher adulticidal effect of essential oils the lower their larvicidal activity. Fresh leaves was harvested and distilled. Once the essential oil was obtained, the chemical composition was analysed, evaluating the biological activity of 15 species of the genus *Eucalyptus* (*Eucalyptus badjensis* Beuzev and Welch, *Eucalyptus badjensis*×*nitens*, *Eucalyptus benthamii* var *Benthamii* Maiden and Cambage, *Eucalyptus benthamii* var *dorrigoensis* Maiden and Cambage, *Eucalyptus botryoides* Smith, *Eucalyptus dalrympleana* Maiden, *Eucalyptus fastigata* Deane and Maiden, *Eucalyptus nobilis* L.A. S. Johnson and K.D.Hill, *Eucalyptus polybractea* R. Baker, *Eucalyptus radiata ssp radiata* Sieber ex Spreng, *Eucalyptus resinifera* Smith, *Eucalyptus robertsonii* Blakely, *Eucalyptus robusta* Smith, *Eucalyptus rubida* Deane and Maiden, *Eucalyptus smithii* R. Baker). Essential oils of these plant species were used for the validation of equations from preexistent models, in which observed and estimated values of the biological activity were compared. The regression analysis showed a strong validation of the models, re-stating the trends previously observed. The models were expressed as follows: A, fumigant activity [$KT_{50(min)}$=10.65–0.076×1,8-cineole (%)]($p<0.01$; F, 397; R^2, 0.79); B, larval mortality $(\%)_{(40\ ppm)}$=103.85+0.482×*p*-cymene (%)−0.363×α-pinene (%)−1.07×1,8-cineole (%) ($p<0.01$; F, 300; R^2, 0.90). These results confirmed the importance of the mayor components in the biological activity of *Eucalyptus* essential oils on *A. aegypti*. However, it is worth mentioning that two or three species differ in the data estimated by the models, and these biological activity results coincide with the presence of minor differential components in the essential oils. According to what was previously mentioned, it can be inferred that the model is able to estimate very closely the biological activity of essential oils of *Eucalyptus* on *A. aegypti*.

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Introduction

Aedes aegypti (L.) (Diptera: Culicidae) is an urban mosquito that feeds almost exclusively on humans (Christophers 1960). It is one of the most important species in the world because it is the main vector of the yellow fever virus and the primary vector of dengue viruses (Eldridge 2005).

The insecticides usually used in the control of this mosquito were temephos for larvicidal treatment in water containers (focal treatment) and pyrethroids as adulticidal ultra low volume formulations (spatial treatment) in the event of an outbreak (Chavasse and Yap 1997).

Synthetic pyrethroid formulations are used because of their good insecticidal activity of low application rates, their short persistence in the environment and their drop effect (PAHO 1994). However, there is a great need to develop effective insecticides with activity against larvae and with adulticide effect to improve control strategies.

Several substances of plant origin have been identified as having toxic, repellent, antifeedant and/or growth and development inhibiting-potential on arthropod pests (Coats 1994).

Essential oils are particular plant products made up of volatile substances found in a variety of species (Weinzieri et al. 1994; Weinzieri 2000). The active components are isoterpenoid compounds, mainly mono- and sesquiterpenes, and are carriers of the odor of aromatic plants (Franzios et al. 1997).

The main characteristics of the essential oils are that they are easily extractable, ecofriendly, biodegradable, possess low or no toxicity against mammals and are very effective against wide spectra of insect pests (Tisserand and Balacs 1995; Isman 2000; Shaaya and Rafaeli 2007; Abdel-Ghaffar and Semmler 2007; Amer and Mehlhorn 2006a, b; Bagvan et al. 2008).

The toxic action mechanisms of terpenoid components of essential oils have not been discovered and are still obscure; however, the onset of toxic signs is usually rapid (Enan 2001), and this rapid effect on some pests is indicative of a neurotoxic mode of action (Isman 2006). For example, Grundy and Still (1985) and Ryan and Byrne (1988) reported that these oils were competitive inhibitors of acetylcholinesterase; others suggested that another possible target for essential oil activity is the octopaminergic system of insects (Enan 2001; Kostyukovsky et al. 2002) and Priestley et al. (2003) showed interference with GABA-gated chloride channels.

The potential of essential oils to act as fumigants has been demonstrated for stored-product pests (Klingauf et al. 1983; El-Nahal et al. 1989; Singh et al. 1989; Shaaya et al. 1991; Rice and Coats 1994; Sarac and Tunc 1995) as well as for parasitic mites of honey bees (Delaplane 1992; Calderone and Spivak 1995). Their volatility has potential benefits as it brings the pesticide vapour into close contact with the pest while at the same time does not leave residues that might cause adverse effects to the protected objects, either crops or food products.

The genus *Eucalyptus* has leaves that contain aromatic oils with a characteristic odour whose recovery by steam distillation produces essential oils (Denny 2002).

There are several reports showing the insecticidal activity of *Eucalyptus* essential oils and their main components in different species (Lee et al. 2001; Papachristos and Stamopoulos 2002; Lucia et al. 2007; Batish et al. 2008), including head lice (Yang et al. 2004; Toloza et al. 2006, 2008). Previous works of our laboratory showed significant relationships between component concentration of *Eucalyptus* essential oils and their insecticide effect on insect pests.

Toloza et al. (2008) determined the fumigant activity of essential oils from *Eucalyptus* and its main components on *Pediculus humanus capitis* De Geer, and a simple regression analysis revealed a significant correlation between KT_{50} data and percentage of 1,8-cineole in these essential oils. Similar correlation between KT_{50} data produced by *Eucalyptus* essential oils and the percentage of 1,8-cineole was found by Juan et al. (2011) in *Haematobia irritans* and Alzogaray et al. (2011) in *Blattella germanica*.

Regression analysis of component concentrations of different *Eucalyptus* species with toxicity parameters obtained in *A. aegypti* larvae and adults showed significant correlations. Relationships were revealed between larvae mortality and the concentration of 1,8-cineole and *p*-cymene. This indicated that *Eucalyptus* species with higher 1,8-cineole concentration and lower *p*-cymene concentration have less effect on *Ae. aegypti* larvae (Lucia et al. 2008). Furthermore, a significant correlation was observed between the content of 1,8-cineole in *Eucalyptus* essential oils and the corresponding KT_{50} data in *A. aegypti* adults (Lucia et al. 2009).

In this study, we present the chemical composition of *Eucalyptus* essential oils and their corresponding toxic effects on *A. aegypti* larvae and adults. On the other hand, the data of larvicide and adulticide effects presented in this work were used to validate and adjust the regression model to predict *A. aegypti* toxicity produced by *Eucalyptus* essential oils with their respective compositions.

Materials and Methods

Insects

An insecticide-susceptible strain of *A. aegypti* (L.) (CIPEIN strain), reared according to previous reports (Lucia et al. 2007), was used. The laboratory colony has been kept in the laboratory since 1996, free of exposure to pathogens, insecticides, or repellents, at 25–30°C, 80–90% RH, and L_{12}/D_{12} photoperiod.

Chemicals

Essential oils were extracted from the following species of *Eucalyptus*: *E. badjensis* Beuzev and Welch, *E. badjensis*×

nitens, *E. benthamii* var *Benthamii* Maiden and Cambage, *E. benthamii* var *dorrigoensis* Maiden and Cambage, *E. botryoides* Smith, *E. dalrympleana* Maiden, *E. fastigata* Deane and Maiden, *E. nobilis* L.A.S. Johnson and K.D. Hill, *E. polybractea* R. Baker, *E. radiata* ssp. *radiata* Sieber ex Spreng, *E. resinifera* Smith, *E. robertsonii* Blakely, *E. robusta* Smith, *E. rubida* Deane and Maiden and *E. smithii* R. Baker. Essential oils were extracted on a laboratory scale for 70 min using the hydrodistillation method in a modified Clevenger-type apparatus (Bandoni 2002). The chemical composition of each oil was already determined by our laboratory in previous studies (Juan et al. 2011) (Table 1).

Larvicidal bioassay

The larvicidal bioassay was performed according to a protocol established during a meeting of the Latin American Network for Vector Control held in Iguazú (Misiones, Argentina) in December 2004 (Bisset et al. 2005), with some minor modifications (Lucia et al. 2008). Essential oils were tested at a final concentration of 40 ppm in 250 ml water. Seven replicates were made for each treatment.

Bioassay for fumigant activity

The bioassay was conducted following the method of Lucia et al. (2009). Fumigant tests developed in the laboratory were conducted in an enclosed specifically designed chamber that allowed concentration of the test vapours. Experimental units consisted of transparent acrylic tubes, 11.9 cm long, with an inner diameter of 4.4 cm and 164.5 ml capacity (Bio Quip, USA) (Fig. 1).

The acrylic cylinder was placed over the Petri dish with the end covered by the metallic mesh facing downwards and thermostabilised for 10 min at 26–28°C. Batches of 13–15 adult mosquitoes were collected with a mouth aspirator and introduced to the tube through the neoprene disc located on the upper side. This way, the insects were exposed to the essential oil vapours but had no direct contact with the source. The tube was closed and carefully examined to ensure that the transferred mosquitoes were in good conditions.

The number of knocked-down mosquitoes was recorded every minute for approximately 20–30 min. Knockdown was considered as the inability of adult mosquitoes to fly. Seven replicates were made for each tested substance. The total number of mosquitoes in each tube was counted after each test. Fumigant activity [$KT_{50\ (min)}$] values were calculated.

Models to estimate the fumigant and larvicidal activity

Models to estimate the fumigant activity

In a previous study in our laboratory, a regression analysis evidenced a statistically significant relationship between fumigant activity [$KT_{50\ (min)}$] of *Eucalyptus* essential oil against *A. aegypti* and their inverse function of 1,8-cineole concentration [1/1,8-cineole (%); $p<0.01$], according to the following equation: $KT_{50\ (min)}=2.94\pm0.22+143.66\pm6.67\times$ 1/1,8-cineole- (%) (R^2, 91.70; F, 446.13; DF, 51) (Lucia et al. 2009). This equation was used as model to obtain estimated values of the fumigant activity of new *Eucalyptus* essential oils, starting from their 1,8-cineole concentration. The 95% of confidence intervals for coefficient estimate

Table 1 Chemical composition of essential oils from 15 species of *Eucalyptus*, expressed as a relative percentage of the total area of the chromatogram

Relative abundance (%) of the majority components of *Eucalyptus* essential oils used for the validation of the biological activity predictive models against *Aedes aegypti* (data from Juan et al. 2011)

Essential oils	α-Pinene	*p*-Cymene	1,8-Cineole
Eucalyptus badjensis	5.09	2.36	71.73
Eucalyptus badjensis×*nitens*	4.51	1.24	82.75
Eucalyptus benthamii var *Benthamii*	73.15	0.43	0.00
Eucalyptus botryoides	4.42	19.91	13.26
Eucalyptus dalrympleana	2.36	5.61	80.31
Eucalyptus benthamii var dorrigoensis	2.67	1.50	74.73
Eucalyptus fastigata	0.68	37.56	14.69
Eucalyptus nobilis	12.89	18.21	30.43
Eucalyptus polybractea	0.20	4.12	85.01
Eucalyptus radiata ssp. *radiata*	2.80	0.70	68.36
Eucalyptus resinifera	10.02	11.95	58.60
Eucalyptus robertsonii	1.64	2.77	62.01
Eucalyptus robusta	41.69	8.50	0.64
Eucalyptus rubida	1.41	0.00	82.53
Eucalyptus smithii	4.61	0.00	78.49

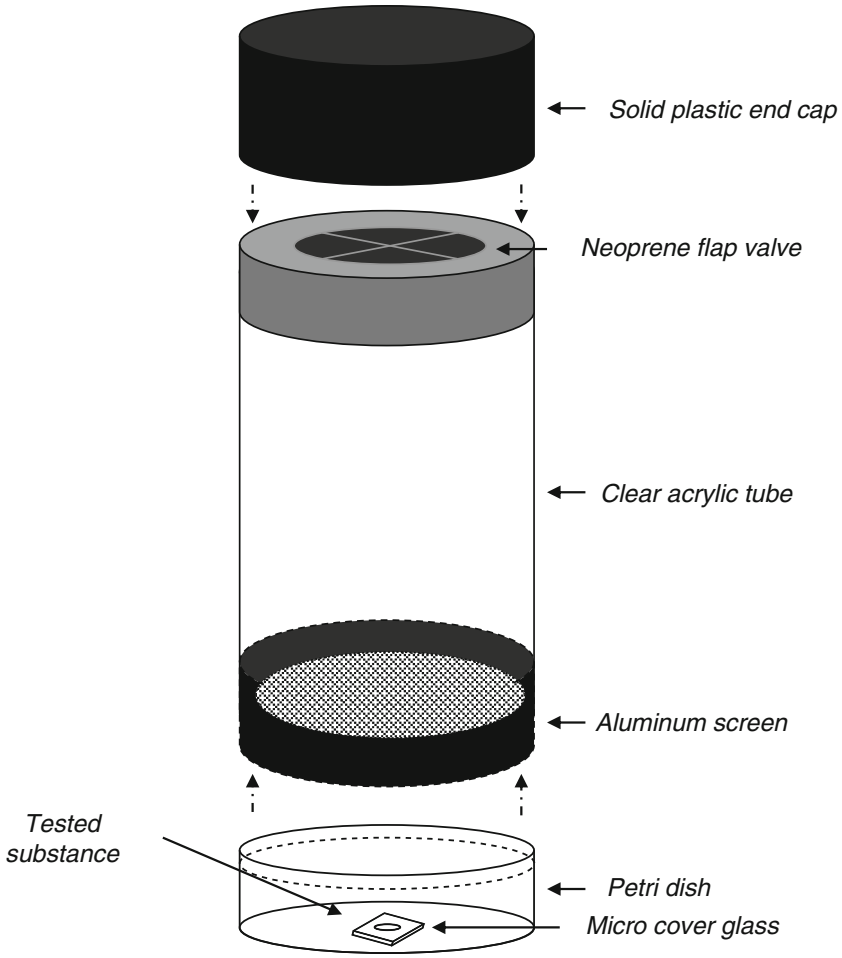

Fig. 1 Test device used to evaluate the fumigant activity of essential oils against *Aedes aegypti* (Lucia et al. 2009)

standard was used for the predicted $KT_{50\ (min)}$ values (constant: lower limit, 2.527/upper limit, 3.419; 1,8-cineole: lower limit, 127.642/upper limit, 154.47).

This model has shown a good fitness to predict the knockdown effect of *Eucalyptus* essential oils with percentages of 1,8-cineole ranging from 18.0 to 91.2. For this reason, *Eucalyptus* essential oils within this range of concentration were used to validate the model of fumigant activity against *A. aegypti*.

Models to estimate the larvicidal activity

Former results of the larvicidal activity (per cent mortality) against larvae *A. aegypti* also showed a statistically significant relationship with two components of essential oils from 12 *Eucalyptus* species. The fitted regression model was as follows: larval mortality (%)=94.46±11.69+ 1.24±0.55×*p*-cymene−1.13±0.15×1,8-cineole (%) (R^2, 97.20; *F*, 833.00; DF, 48; Lucia et al. 2008). As regards the standard for coefficient estimates, the 95% of confidence intervals was used for the predicted larval mortality values (constant: lower limit, 83.066/upper limit, 103.489; 1,8-cineole: lower limit, −1.250/upper limit, −0.992; *p*-cymene: lower limit, 0.813/upper limit, 1.769).

The addition of *E. grandis* essential oil to the initial equation resulted in a second equation considering the α-pinene concentration in the regression model (see results).

However, these equations alone described the toxicity of *Eucalyptus* essential oils against *A. aegypti* larvae; therefore, in order to modify the predictive model, it was necessary to apply logistic regression.

Statistical analysis

The value of larval mortality (%) was determined after 24 h exposure to a final concentration of 40 ppm and expressed as such by means of seven replicates±standard deviation. These values of larval mortality for each essential oil were transformed, analysed by variance analysis (ANOVA) and compared a posteriori by Tukey's honestly significant difference (HSD) mean multiple comparison test using Software Statgraphics Plus for Windows Version 4.0-Corp. SGWIN P®. A $p<0.05$ was considered statistically significant.

The 95% of confidence intervals for the larval mortality value was obtained using the computer software Instat V. 3.01 for Windows (Graphpad Software, San Diego, CA, USA). These confidence intervals were used for comparing observed versus predicted values and were considered significantly different when the 95% confident limits (CL) did not overlap.

The larval mortality (%) predictive models were determined using linear multiple regression and logistic regression (logit model), where P_x=probability of occurrence of an event [larval mortality (%)] and Q_x is the probability of no occurrence of an event. Furthermore, odds=P/Q, where $Q=1-P$; logit (P)=ln (odds)=ln (P/Q).

Knockdown data for each essential oil were subjected to probit analysis (Litchfield and Wilcoxon 1949) and observed $KT_{50\ (min)}$ values of the *Eucalyptus* essential oils were obtained using POLO PC 2.0 software (LeOra Software 2002). $KT_{50\ (min)}$ values were considered significantly different when the 95% confident limits (CL) did not overlap.

The fumigant toxicity ($KT_{50\ (min)}$) predictive model was determined using simple linear regression analysis.

The regression analysis was performed using Statistical Graphics SGWIN® software (Statgraphics Plus 4.0; Statistical Graphics Corporation, 1994–1999, Herndon, VA, USA).

The validation of regression models was performed using the new data-collecting technique and confirmation runs. The quality of each regression model was evaluated using the coefficient of determination (R^2), p value and F ratio value (F) and by comparing predicted and observed values. The 95% of confidence intervals for the coefficients in the predictive model was obtained by regression analysis. The observed and predicted $KT_{50\ (min)}$ and larval mortality values were considered significantly different when the 95% confident limits (CL_{95}) did not overlap.

Results and discussion

Validation of Fumigant activity model

Table 2 shows the fumigant activity values of every *Eucalyptus* essential oil evaluated. The essential oil of the *E. polybractea* was the most effective, with a $KT_{50\ (min)}$ value of 3.86, without being significantly different from *E. smithii* and *E. rubida*.

Figure 2 shows predicted versus observed fumigant activity $KT_{50\ (min)}$ values for 11 *Eucalyptus* essential oils.

The estimated values of $KT_{50\ (min)}$ for four essential oils were not obtained (*E. robusta*, *E. benthamii* var *Benthamii*, *E. fastigata* and *E. botryoides*) due to the relative percentages of 1,8-cineole component, under the minimum limits acceptable by the predicting model (0.64%, 0.20%, 14.69% and 13.26%, respectively).

The remaining 11 essential oils drew predicted values that differed from the observed values in <1 U, implying that the model could estimate unbiased approximations (Fig. 2). Significant differences were observed only between the observed and estimated values for the essential oils of *E. radiata* ssp. *radiata* and *E. polybractea*, although these differences were minimal (0.699 and 0.772 respectively).

From the data analysis, it was inferred that the minimum square model used [$KT_{50\ (min)}=2.94\pm0.22+143.66\pm6.67\times$ 1/1,8-cineole (%)] did not predict the observations better than when it was used with the original data. However, the "loss" in R^2 was small for the prediction (91.70% vs 86.33%; $p<0.01$; F, 57; R, 0.92; Fig. 3).

In order to obtain a unique predictive linear model for the dependent variable $KT_{50\ (min)}$ in a higher range of percentage values for the component 1,8-cineole varying between 0.64% and 91.2%, 27 out of 28 evaluated oils were used. Thus, a new predictive model for the knockdown effect was obtained (Fig. 4) as described herein: $KT_{50\ (min)}=10.65–0.076\times$1,8-cineole (%) ($p<0.01$; F, 397; R, −0.88). The correlation coefficient indicated a strong relationship between variables; the statistical R^2 revealed that the model explained the 78.93% of the variability in the knockdown effect.

These results confirmed the importance of the 1,8-cineole component in the knockdown effect of *Eucalyptus* essential oils on *A. aegypti*.

For other species, the fumigant activity of the essential oils from the evaluated *Eucalyptus* showed a strong tendency to increase the knockdown effect as the essential oils became rich in the 1,8-cineole component. Alzogaray et al. (2011) evaluated the fumigant activity of 13 essential oils of first-stage *B. germanica* nymphs and reported that the fumigating activity values of the essential oils could be mildly explained by the presence of the 1,8-cineole component [$TV_{50\ (min)}=49.65+1347.66\times$1/1,8-cineole (%); F, 11; R^2, 51.00; $p<0.01$].

Likewise, previous studies, in which fumigant activity was evaluated on the same 13 essential oils of *H. irritans*, determined that *Eucalyptus* essential oils with more 1,8-cineole content showed a positive correlation with the knockdown effect (Juan et al. 2011).

In turn, Toloza et al. (2008, 2010) determined the knockdown effect of the essential oils from *E. grandis*, *E. camaldulensis*, *E. tereticornis*, *E. grandis*×*E. tereticornis* and *E. grandis*×*E. camaldulensis* and their main components on *P. humanus capitis* De Geer. As seen in previous works, fumigant activity could be explained by the presence of the 1,8-cineole in the essential oil [$TV_{50\ (min)}=33.51–0.26\times$1,8-cineole (%); F, 9; R^2, 68.00; $p<0.05$] and [$TV_{50\ (min)}=86.74–0.74\times$1,8-cineole (%); F, 44; R^2, 91.66; $p<0.01$] and to a greater extent by the addition of the α-pinene to the precedent model [$TV_{50\ (min)}=42.20+0.22\times$α-

Table 2 Fumigant activity of the essential oils vapours from different species of *Eucalyptus* against *Aedes aegypti*

Essential oils	Fumigant activity			
	KT_{50} (min)[a]	Statistics[b]		
	(95%CI)	Slope±SE	χ^2	*df*
E. robusta	10.35 (9.68–10.38)	8.44±0.53	13.45	16
E. benthamii var *Benthamii*	9.45 (9.12–9.79)	9.44±0.65	13.02	16
E. fastigata	9.06 (8.31–9.80)	9.43±0.64	55.61	13
E. botryoides	8.35 (8.03–8.67)	9.07±0.67	2.78	12
E. nobilis	7.70 (7.34–8.06)	7.54±0.61	6.49	8
E. radiata ssp. *radiata*	5.74 (4.95–6.53)	8.41±0.71	26.51	6
E. resinifera	5.25 (4.96–5.52)	7.67±0.61	3.49	9
E. robertsonii	5.20 (4.86–5.52)	9.09±0.77	11.38	9
E. badjensis	5.02 (4.79–5.25)	8.36±0.68	2.40	7
E. badjensis × *nitens*	4.77 (4.54–4.99)	10.26±0.95	3.29	9
E. dalrympleana	4.77 (4.47–5.06)	6.75±0.61	2.96	7
E. benthamii var *dorrigoensis*	4.45 (4.19–4.69)	8.65±0.82	1.74	8
E. smithii	4.28 (3.90–4.66)	7.71±0.82	5.61	5
E. rubida	4.25 (3.97–4.51)	6.71±0.57	3.05	8
E. polybractea	3.86 (3.61–4.10)	6.87±0.60	2.14	7

[a]Time to 50% knockdown with a 95% confidence interval (CI). $KT_{50\ (min)}$ values are the means of seven replicates using 13–15 adults (total 54–60 adults) for each essential oil, determined at 1-min intervals until 100% of the adults were knocked down

[b]Statistics of the probit analysis of knockdown times

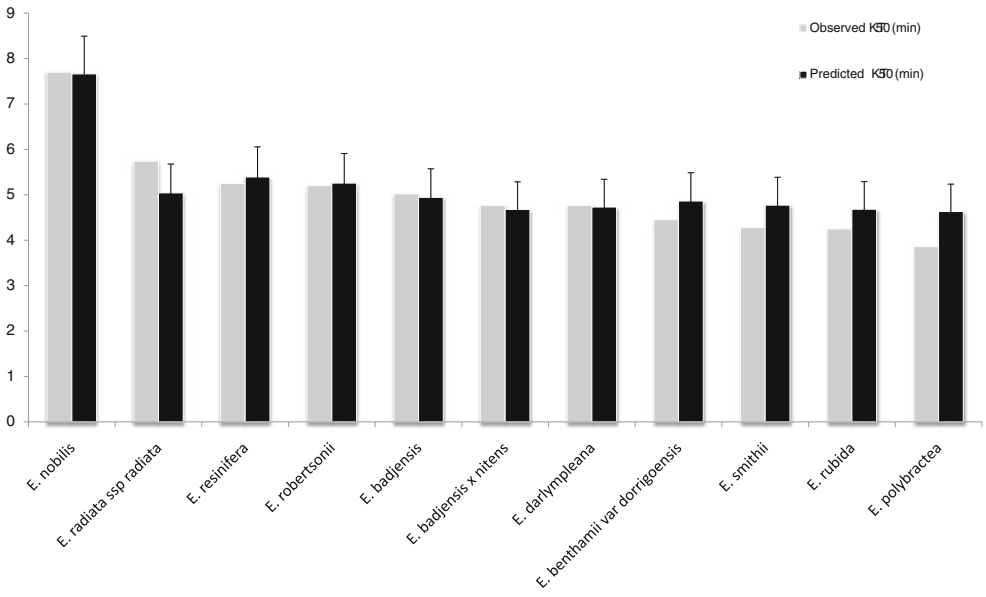

Fig. 2 Plot of predicted versus observed fumigant activity values ($KT_{50\ (min)}$) for 11 *Eucalyptus* essential oils. *Observed* $KT_{50\ (min)}$ values are the means of 7 replicates using 15 adults (total 60 adults) for each essential oil, determined at 1-min intervals until 100% of the adults were knocked down. *Predicted* $KT_{50\ (min)}$ value obtained by means of adjusted least squares: It is the value by means of the equation: $KT_{50\ (min)} = 2.94 \pm 0.22 + 143.66 \pm 6.67 \times 1/1,8\text{-cineole}$ (%), with 95% confidence limits (CL)

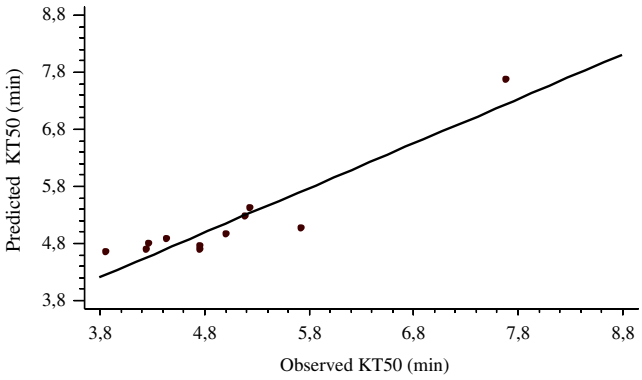

Fig. 3 Lineal simple regression between predicted versus observed fumigant activity values ($KT_{50\ (min)}$)

pinene (%)−0.39×1,8-cineole (%); *F*, 26; R^2, 96.00; $p<0.05$]. These results could be explained by the analysis of previous studies by the same authors (Toloza et al. 2006), in which the fumigant activity of 21 monoterpenes against *P. humanus capitis* was determined, concluding that the most effective components were 1,8-cineole and anisole. Likewise, the α-pinene was rated seventh out of 21 components in order of effectiveness, being the TV_{50} value of 1,8-cineole 3.84 times lower than that of α-pinene.

Similar results have been reported by many authors: Tarelli et al. (2009) studied the knockdown effect of five monoterpenes and five commercial essential oils against *Musca domestica* and arrived at the conclusion that *Eucalyptus* essential oil and 1,8-cineole were the most effective ones.

In another similar study (Sfara et al. 2009), the knockdown effect of five essential oils and seven monoterpenes was evaluated on *Rhodnius prolixus* first-stage nymphs, showing that *Eucalyptus* sp. commercial essential oil and the pure component 1,8-cineole were the most effective.

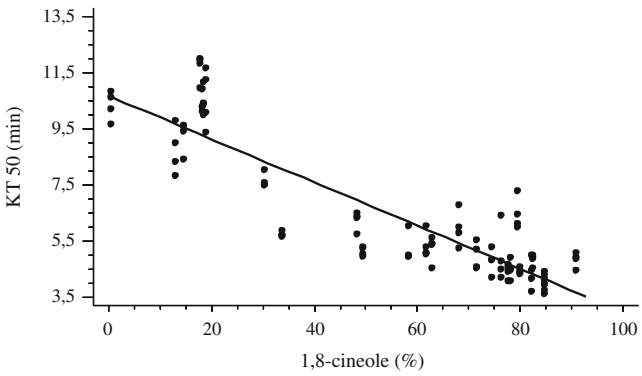

Fig. 4 Relationship between fumigant activity ($KT_{50\ (min)}$) and the percentage of 1,8-cineole in essential oils from different species of *Eucalyptus*. Each point represents the KT_{50} value for each species of *Eucalyptus* and their corresponding 1,8-cineole relative abundance (%). Total points = 112

For Lee et al. (2004a), the 1,8-cineole and the essential oils rich in 1,8-cineole are considered as future commercial products for insect control in stored grains. This is based on the fumigant toxicity of 42 essential oils in species belonging to the *Myrtaceae* family against storage grains pests (*Sitophilus oryzae*, *Tribolium castaneum* and *Rhyzopertha dominica*), assuming that five of the six most effective oils were those rich in 1,8-cineole, corresponding to *Callistemon sieberi*, *E. blakelyi*, *E. nicholii*, *Melaleuca armillaris* and *Melaleuca fulgens* species (58.99%, 56.92%, 82.19%, 42.77% and 77.50% of 1,8-cineole, respectively). However, the most effective species only contains 6% of 1,8-cineole, the main components being piperitone and *p*-cymene (53.31% and 22.78%, respectively). This consideration of Lee et al. (2004a, b) agrees with Negahban and Moharramipour (2007), which respectively evaluate the knockdown effect of *M. fulgens* essential oils, of three *Eucalyptus* species and of 1,8-cineole over stored grains pests, the good effectiveness of the essential oils being attributed to their respective concentration of the 1,8-cineole component, and suggesting that it can be considered as a new fumigant product to use.

Generally, the insecticide activity of essential oils is species dependent, and the chemical composition may influence their insecticide properties (Rice and Coats 1994; Isman 1999; Enan 2001). However, it is worth noticing the importance of 1,8-cineole as a pure component or as a part of an essential oil in the knockdown effect on at least nine different species of insects (*P. humanus capitis*, *R. prolixus*, *M. domestica*, *S. oryzae*, *T. castaneum. R. dominica*, *H. irritans*, *B. germanica* and *A. aegypti*).

Validation of larvicidal activity model

Table 3 shows the values of the larvicidal activity of the different *Eucalyptus* essential oils evaluated. The essential oil of *E. fastigata* was the most effective, with a larval mortality value of 92.85%, without significant differences from *E. radiata* ssp. *radiata*, *E. nobilis* and *E. robusta*.

The four models used to predict larvicidal acticity of *Eucalyptus* essential oils on *A. aegypti* are described below:

1. Larvicidal activity model published by Lucia et al. 2008, as a result of the analysis of essential oils from 12 *Eucalyptus* species. The fitted regression model was as follows: larval mortality (%)=94.46±11.69+1.24±0.55× *p*-cymene−1.13±0.15×1,8-cineole (%) (R^2, 97.20; *F*, 833; DF, 48) (Lucia et al. 2008). As regards the standard of coefficient estimates, the 95% of coefficient intervals was used for the predicted larval mortality values (constant: lower limit, 83.066/upper limit, 103.489; 1,8-cineole: lower limit, −1.250/upper limit, −0.992; *p*-cymene: lower limit, 0.813/upper limit, 1.769).

Table 3 Larvicidal activity of the essential oils from different species of *Eucalyptus* against *Aedes aegypti*

Essential oils	Larval mortality (%) (95%CI)	Standard deviation
E. rubida	12.14 (9.67–14.61)	2.67h
E. badjensis×*nitens*	17.14 (11.89–22.38)	5.67g,h
E. dalrympleana	17.85 (11.93–23.74)	6.36g,h
E. polybractea	17.85 (3.76–31.95)	15.23g
E. benthamii var *dorrigoensis*	25.00 (16.99–33.01)	8.66g
E.badjensis	25.71 (6.38–45.04)	20.9g
E. robertsonii	44.28 (39.34–49.22)	5.35f
E. resinifera	56.42 (44.57–68.28)	12.81e
E. smithii	67.85 (53.26–82.44)	15.77d
E. botryoides	77.15 (74.67–79.61)	2.67c,d
E. benthamii var *Benthamii*	79.28 (75.12–83.44)	4.49b,c
E. robusta	82.85 (76.97–88.74)	6.36a,b,c
E. nobilis	86.42 (82.93–89.92)	3.78a,b,c
E. radiata ssp. *radiata*	90.00 (78.36–101.64)	12.58a,b
E. fastigata	92.85 (90.38–95.33)	2.67a

Each value of larval mortality (%)±SD at 40 ppm final concentration was obtained from the data from seven independent replicates. The larval mortality values with the same letter are not significantly different (Tukey's HSD mean multiple comparison test, $p>0.05$)

2. Larvicidal activity model as a result of the analysis of essential oils from 12 *Eucalyptus* species used in a previous model, with the incorporation of *E. grandis* essential oil (a species rich in α-pinene) (Lucia et al. 2007). The fitted regression model was as follows: larval mortality (%)=93.66±5.77+1.53±0.26×*p*-cymene−1.00±0.07×1,8-cineole (%)−0.30±0.07×α-pinene (%) (R^2, 95.70; F, 565; DF, 51).

   As regards the standard of coefficient estimates, the 95% confidence interval was used for the predicted larval mortality values (constant: lower limit, 86.653/upper limit, 100.24; 1,8-cineole: lower limit, −1.057/upper limit, 1.063; *p*-cymene: lower limit, 1.245/upper limit, 1.830; α-pinene: lower limit, −0.499/upper limit, −0.102).

3. This model is the result of the application of the logistic regression to the original data of model 1) Larvicidal activity model using logistic regression: LN (P/Q)= 7.00±0.82–0.14±0.010×1,8-cineole (%)−0.077± 0.038×*p*-cymene (%) (R^2, 91.25; F, 235; DF, 47), where P=larval mortality (%); Q=larval survival; and LN=natural logarithm. As regards the standard of coefficient estimates, the 95% of confidence intervals was used (constant: lower limit, 5.342/upper limit, 8.664; 1,8-cineole: lower limit, −0.161/upper limit, −0.119; *p*-cymene: lower limit, −0.155/upper limit, 0.000).
4. This model is the result of the application of the logistic regression to the original data of model 2) Larvicidal activity model using logistic regression: LN (P/Q)= 6.530±1.106–0.132±0.013×1,8-cineole (%)−0.063± 0.049×*p*-cymene−0.027±0.014×α-pinene (%) (R^2, 87.64; F, 113; DF, 51), where P=larval mortality (%); Q=larval survival; and LN=natural logarithm. As regards the standard of coefficient estimates, the 95% of confidence interval was used (constant: lower limit, 4.303/upper limit, 8.755; 1,8-cineole: lower limit, −0.158/upper limit, −0.106; *p*-cymene: lower limit, −0.162/upper limit, 0.036; α-pinene: lower limit, −0.055/upper limit, 0.001).

In addition to this, it is important to point out that all predictive models can predict larvicidal activity values of *Eucalyptus* essential oils between predetermined concentration limits for each component: *p*-cymene (0.10–21.25%), 1,8-cineole (18.00–91.20%) and α-pinene (0.50–52.71%).

A linear regression analysis was performed for trying to find different associations among the observed values of larval activity for each essential oil and the expected values obtained by means of each alternative predictive model. From all the analyses, two influential values were observed in all evaluated models, these being those corresponding to *E. radiata* ssp. *radiata* and *E. smithii* essential oils (Table 4).

The studentised residuals measure how many standard deviations each observed value deviates from a fitted model using all data, except for that observation. In this case, there were two studentised residuals higher than 3.0 in absolute value. These observations were considered outliers, which should be removed from the model and handled separately.

In the light of these remarks, the regression analysis was performed on each model, with and without these influential values (Table 5).

In Table 5, it is observed that regression model 2 [larval mortality (%)=93.66±5.77+1.53±0.26×*p*-cymene−1.00± 0.07×1,8-cineole (%)−0.30±0.07×α-pinene (%)] predicted the observed data showing the minimal square average predicting error and one of the best R^2.

Table 4 Predicted versus observed larval mortality values for essential oils from different species of *Eucalyptus* against *Aedes aegypti*

	(γ)	Model 1 ($\hat{y}_1$)	Residual ($\gamma-\hat{y}1$)	$(\gamma-\hat{y}_1)^2$	Model 2 ($\hat{y}_2$)	Residual ($\gamma-\hat{y}_2$)	$(\gamma-\hat{y}_2)^2$	Model 3 ($\hat{y}_3$)	Residual ($\gamma-\hat{y}_3$)	$(\gamma-\hat{y}_3)^2$	Model 4 ($\hat{y}_4$)	Residual ($\gamma-\hat{y}_4$)	$(\gamma-\hat{y}_4)^2$
E. nobilis	86.4	82.7	3.8	14.2	87.2	–0.8	0.6	79.2	7.2	52.0	73.5	13.0	168.0
E. radiata ssp *radiata*[a]	90.0	18.1	71.9	5,172.3	25.5	64.5	4,156.3	6.8	83.2	6,929.1	6.8	83.2	6,917.4
E. resinifera	56.4	43.1	13.4	178.5	50.3	6.1	37.2	10.7	45.7	2,092.4	9.7	46.7	2,180.8
E. robertsonii	44.3	27.8	16.5	270.8	35.4	8.9	79.0	13.1	31.2	974.0	13.3	31.0	959.3
E. badjensis	25.7	16.3	9.4	88.0	24.0	1.7	2.9	3.8	21.9	478.8	3.8	21.9	478.9
E. badjensis×*nitens*	17.1	2.5	14.6	214.6	11.4	5.7	32.4	0.9	16.2	263.1	1.0	16.1	260.4
E. darlympleana	17.9	10.7	7.2	51.6	21.2	–3.4	11.4	0.9	16.9	286.5	1.1	16.7	280.2
E. benthamii var *dorrigoensis*	25.0	11.9	13.1	172.3	20.4	4.6	21.0	2.7	22.3	496.5	2.9	22.1	487.2
E. smithii[a]	67.9	5.8	62.1	3,852.8	13.8	54.1	2,922.2	1.8	66.0	4,360.3	1.9	66.0	4,352.3
E. rubida	12.1	1.2	10.9	119.4	10.7	1.4	2.0	1.0	11.1	123.2	1.2	10.9	119.5
E. polybractea	17.9	3.5	14.3	205.7	14.9	3.0	8.7	0.5	17.3	299.7	0.7	17.2	294. .2

γ observed larval mortality; predicted larval mortality: model 1 ($\hat{y}_1$): larval mortality (%)=94.46±11.69+1.24±0.55×*p*-cymene−1.13±0.15×1,8-cineole (%); model 2 ($\hat{y}_2$): larval mortality (%)= 93.66±5.77+1.53±0.26×*p*-cymene−1.00±0.07×1,8-cineole (%)−0.30±0.07×α-pinene (%); model 3 ($\hat{y}_3$): LN (*P*/*Q*)=7.00±0.82 - 0.14±0.010×1,8-cineole (%)−0.077±0.038×*p*-cymene (%); Model 4 ($\hat{y}_4$): LN (*P*/*Q*)=6.530±1.106 - 0.132±0.013×1,8-cineole (%)−0.063±0.049×*p*-cymene−0.027±0.014×α-pinene (%)

[a] Outliers

In Fig. 5, the differences between the observed values and the estimated values can be observed by means of the model 2. The only significant differences between the observed and the estimated values corresponded to *E. radiata* ssp. *radiata, E. robertsonii* and *E. smithii* essential oils, though the difference was minimal in *E. robertsonii* (8.89) and very considerable in *E. radiata* ssp. *radiata* and *E. smithii* (64.47 and 54.06, respectively). The presence of few components in the essential oil may be the cause for the unexpected increment in the larvicidal activity.

In order to obtain a unique linear predictive model for the dependent variable (larval mortality) in a wider range of relative abundance for the three components involved in the multiple regression model (*p*-cymene, 1,8-cineole and α-pinene), it was determined not to add *E. smithii* and *E. radiata* ssp. *radiata* essential oils to the model, which strongly differed in their expected and observed values. The elimination of these influencing data is due to the fact that, with the new larvicidal activity predictive model, we will be able to increase the range of concentrations for the three components (*p*-cymene, 1,8-cineole and α-pinene), in which the same concentration can be used (0.10–37.56; 0.64–91.2 and 0.20–73.15%, respectively).

Moreover, it is important to highlight that the number of observations used for the new equation increased considerably with respect to those used at the beginning (52 vs 104 observations).

For the last case, 26 out of 28 oils evaluated were used, and a new larval mortality predictive model was obtained: larval mortality $(\%)_{(40\ ppm)}=103.85+0.482\times p$-cymene (%)− 0.363×α-pinene (%)−1.07×1,8-cineole (%).

From the ANOVA table reading, it emerges that a statistically significant relationship exists among all the independent variables and their dependent variable (1,8-cineole, $p<0.01$; *p*-cymene, $p<0.05$; and α-pinene, $p<0.01$).

The statistical R^2 reveals that the model explains the 89.99% of the variability in larval mortality, with a statistical *F* value of 300.

From all the results analysed so far, it may be concluded that there are two possible models to estimate the larval mortality value of *Eucalyptus* essential oils on *A. aegypti*.

1. The model resulting from the first analysis, in which 13 essential oils were used to obtain the multiple linear regression model that can predict the larvicidal effect of *Eucalyptus* essential oils, based on the relative quantity of components between predetermined ranges [*p*-cymene (0.10 and 21.25%), 1,8-cineole (18.00 and 91.20%) and α-pinene (0.50 and 52.71%)]. This model explains the 95.70% of the variability in the data, with a statistical *F* value of 357, expressed as follows: larval mortality $(\%)_{(40\ ppm)}=$

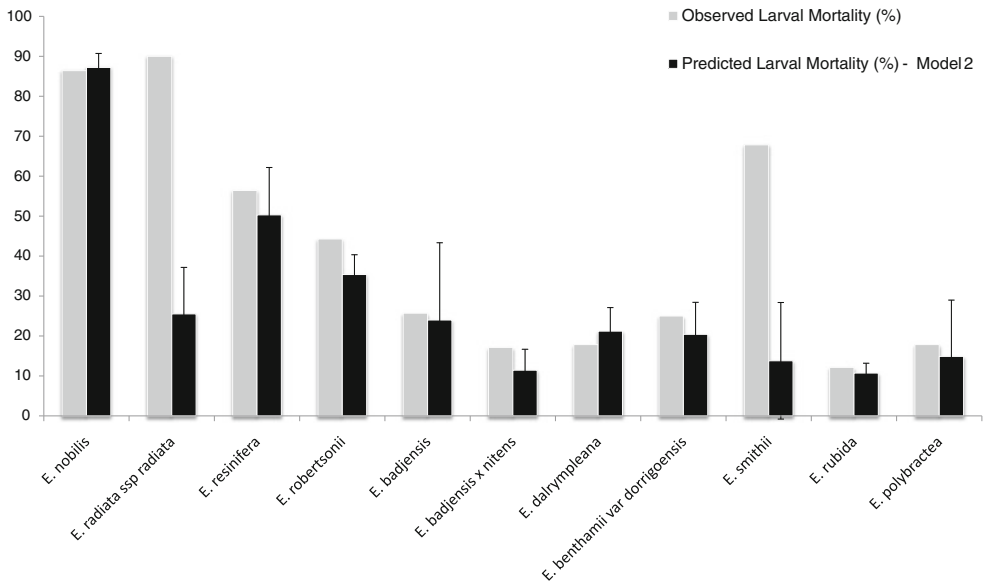

Fig. 5 Plot of predicted versus observed larval mortality values (%) for 11 *Eucalyptus* essential oils. *Observed larval mortality values (%)* values are the means of 7 independent replicates using 20 larvae (total 140 larvae) for each essential oil, determined at 40 ppm final concentration. *Predicted larval mortality values (%)* value obtained by means of adjusted least squares: It is the value by means of the equation: Larval mortality (%) = 93.66 ± 5.77 + 1.53 ± 0.26 × *p*-cymene − 1.00 ± 0.07 × 1,8-cineole (%) − 0.30 ± 0.07 × α-pinene (%), with 95% confidence limits (CL)

$$93.66+1.53\times p\text{-cymene}\ (\%)-0.302\times \alpha\text{-pinene}\ (\%)-1.00\times 1,8\text{-cineole}\ (\%).$$

2. The model resulting from the second analysis, in which 26 essential oils were used to obtain the multiple linear

Table 5 Multiple linear regression between predicted versus observed larval mortality values (%)

	Predicted larval mortality (%), model 1	Predicted larval mortality (%), model 2	Predicted larval mortality (%), model 3	Predicted larval mortality (%), model 4
Analysis of variance (linear regression)	Model with unusual residuals			
Standard error of estímated	23.4	23.9	24.8	24.8
R^2	41.3	38.8	34.2	34.3
F ratio	6.3	5.7	4.7	4.7
p value	0.033	0.041	0.059	0.058
df model	1	1	1	1
df residual	9	9	9	9
Sum of squares (model)	3,465.0	3,253.7	2,865.8	2,873.7
Sum of squares (residual)	4,924.1	5,135.3	5,524.0	5,515.3
Mean squares (model)	3,465.0	3,253.7	2,865.8	2,873.8
Mean squares (residual)	547.1	570.6	613.8	612.8
Error promedio de predicción al cuadrado	10,340.2	7,273.7	16,355.6	16,498.1
Analysis of variance (linear regression)	Model without unusual residuals			
Standard error of estímated	3.7	4.0	11.7	11.7
R^2	98.0	97.7	80.0	79.9
F ratio	348.0	295.3	28.0	27.9
p value	0.000	0.001	0.001	0.001
df model	1	1	1	1
df residual	7	7	7	7
Sum of squares (model)	4,691.6	4,675.2	3,829.4	3,826.3
Sum of squares (residual)	94.36	110.8	956.6	959.7
Mean squares (model)	4,691.6	4,675.2	3,829.4	3,826.3
Mean squares (residual)	13.5	15.8	136.7	137.2
Error promedio de predicción al cuadrado	1,315.0	195.3	5,066.2	5,228.4

regression model that can predict the larvicidal effect of *Eucalyptus* essential oils, based on the relative quantity of components between broader ranges [*p*-cymene (0.10–37.56%), 1,8-cineole (0.64–91.20%) and α-pinene (0.20–73.15%)]. This model explains the 89.99% of the variability in the data, with a statistical *F* value of 300, expressed as follows: larval mortality (%) $_{(40\ ppm)}$=103.85+0.482×*p*-cymene (%)−0.363×α-pinene (%)−1.07×1,8-cineole (%).

Bearing in mind that there are two possible models to estimate the larval activity of *Eucalyptus* essential oils on *A. aegypti*, it is essential to consider in which scenarios each one should be used.

The models previously explained not only intend to predict the insecticide effect of *Eucalyptus* essential oils on *A. aegypti* but also set a strong trend in the involvement of various components. Moreover, these models may provide interesting data by analysing the standard deviation between the observed and estimated data. This is because any larval mortality or fumigant activity value observed for a given essential oil significantly higher than its estimated value is worthy of a deep chemical composition analysis to find the component/s determining the considerable increase in their effectiveness. Therefore, models not only predict with greater or lesser accuracy the larvicidal effectiveness of essential oils but also can give a hint to study that essential oil more thoroughly, when the observed values are significantly higher than expected.

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