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ORIGINAL PAPER

Insecticidal activity of an essential oil of *Tagetes patula* L. (Asteraceae) on common bed bug *Cimex lectularius* L. and molecular docking of major compounds at the catalytic site of *Cl*AChE1

Flávio Augusto Sanches Politi[1] **· Juliana Damieli Nascimento**[2] **· Alexander Alves da Silva**[1] **· Isabela Jacob Moro**[2] **· Mariana Lopes Garcia**[3] **· Rafael Victório Carvalho Guido**[3] **· Rosemeire Cristina Linhari Rodrigues Pietro**[2] **· Antônio Francisco Godinho**[4] **· Maysa Furlan**[1]

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Abstract Emerging resistance to insecticides has influenced pharmaceutical research and the search for alternatives to control the common bed bug *Cimex lectularius*. In this sense, natural products can play a major role. *Tagetes patula*, popularly known as dwarf marigold, is a plant native to North America with biocide potential. The aim of this work was to evaluate the biological activity of *T. patula* essential oil (EO) against adult common bed bugs via exposure to dry residues by the Impregnated Paper Disk Test (IPDT) using cypermethrin as a positive control. We selected the enzyme acetylcholinesterase as a target for modeling studies, with the intent of investigating the molecular basis of any biological activity of the EO. Chemical analysis of the EO was performed using gas chromatography coupled to mass spectrometry (GC-MS). Additionally, oral and dermal acute toxicity tests were performed according to Organization for Economic Cooperation and Development (OECD) guidelines. The sulforhodamine B assay (SRB) was performed to verify the cytotoxicity of EO to HaCaT cells. The EO eliminated 100 % of the bed bugs at 100 mg mL^{-1} with an LC_{50} value of 15.85 mg mL^{-1}. GC-MS analysis identified α-terpinolene, limonene, piperitenone, and piperitone as major components of the mixture. Molecular modeling studies of these major compounds suggested that they are acetylcholinesterase inhibitors with good steric and electronic complementarity. The in vitro cytotoxicity evaluation revealed a $LC_{50} = 37.06$ μg mL^{-1} and in vivo acute toxicity showed an LC_{50} >4000 mg kg^{-1}, indicating that the EO presents low risk of toxic side effects in humans. The *T. patula* essential oil components provide a promising strategy for controlling bed bug populations with low mammalian toxicity. These findings pave the way for further in vivo studies aimed at developing a safe and effective insecticide.

Highlights • *Tagetes patula* essential oil showed similar insecticidal effects to cypermethrin.
• Major compounds of essential oil acting as competitive inhibitors of *Cl*AChE1.
• Assays of acute toxicity and cytotoxicity showed that the essential oil is safe.

✉ Flávio Augusto Sanches Politi
flaviopoliti@hotmail.com

[1] Department of Organic Chemistry, Institute of Chemistry, University Estadual Paulista-UNESP, Rua Prof. Francisco Degni 55, Quitandinha, Araraquara, SP CEP 14800-060, Brazil

[2] Faculdade de Ciências Farmacêuticas, University Estadual Paulista-UNESP, Rod. Araraquara-Jaú Km 01, Araraquara, SP 14801-902, Brazil

[3] Centro de Pesquisa e Inovação em Biodiversidade e Fármacos, Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, SP 13563-120, Brazil

[4] Centro de Assistência Toxicológica (CEATOX), University Estadual Paulista-UNESP, Distrito de Rubião Júnior s/n, Botucatu, SP 18618-970, Brazil

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Introduction

Insects of the Cimicidae family (Heteroptera, Cimicomorpha) compose a small and distinct Hemiptera group (Criado et al 2011). Similar to insects of the Triatominae subfamily, cimicids arouse great interest in public health because of two

important evolutionary adaptations: hematophagy, common to all genera, and anthropophily, shared by only some of these species. The *Cimex* genus is the best known and studied; the species *Cimex lectularius* and *Cimex hemipterus* show a very close association with man, one of its primary hosts (Lai et al. 2016). The common bed bug *C. lectularius* L. presents a cosmopolitan standard that is distributed nearly worldwide excluding Antarctica (Rey 2002). During the 1990s, pest control professionals began reporting a global sudden increase in the number of infestations by bed bugs (Doggett et al. 2004a). In recent years, bed bug infestations have become increasingly recurrent, with exponential increases since the early 2000s (Boase 2001; Doggett 2005). The National Pest Management Association (NPMA) of the USA reported a 500 % increase in bed bug incidents (Doggett et al. 2004b). Approximately 67 % of US pest control companies reported increases in infestations (Gangloff-Kaufmann and Shultz 2003). Some areas of Europe showed a 10-fold increase in the incidence of infestation since 1999 (Doggett et al. 2004a). Infestations are reported in Spain (Ferrer and Sainz-Elipe 2005), Scandinavia (Kilpinen et al. 2008), Italy (Masetti and Bruschi 2007), Asia (Lee et al. 2008; Hirao 2010; Tawatsin et al. 2011), Africa (Omudu and Kuse 2010), Australia (Doggett et al. 2004a, 2011), and Brazil (Nascimento 2010).

The resurgence and spread of infestations may be due to an increase in population density at the periphery of metropolises, associated with poor quality of life in such areas (Neves 2005). Other factors supporting resurgence are their relatively fast biological cycles (Harlan 2006); suppression of natural predators such as spiders and ants (Ter Poorten and Prose 2005) and the incorrect use of insecticides such as carbamates (Zhu et al. 2010), pyrethroids (Romero et al. 2007; Lilly et al. 2009), and organophosphates (Tawatsin et al. 2011; Kilpinen et al. 2011) that inadvertently selects for highly resistant strains. Currently, the major importance in resistance research is being given to the molecular mechanisms of insecticidal resistance and correspondent resistance management, aiming to handle the dispersal of resistant plagues on a larger area (Naqqash et al 2016).

When Lilly et al. (2009) performed insecticide resistance tests comparing a modern strain with an ancient susceptible one, they found that the lethal concentration to eliminate 50 % of bed bugs (LC_{50}) was 1.4-million-fold higher with permethrin (third-generation pyrethroid) and approximately 430,000-fold higher with deltamethrin (fourth-generation pyrethroid) for the modern strain (Doggett and Russell 2009). Furthermore, they observed that when deltamethrin was applied directly to adults from the resistant strain of *C. lectularius*, only 60 % died after 10 days. When the same strain was maintained in contact with a surface treated with the pyrethroid, the mortality rate decreased to only 30 %.

In addition to the problems associated with blood loss and severe psychological damage (Doggett et al. 2012; Goddard and deShazo 2009), some authors have recently reopened discussions about the potential for bed bugs as vectors of human pathogens. Delaunay et al. (2011) described approximately 45 pathogens possibly transmitted by *C. lectularius*. Leulmi et al. (2015) demonstrated for the first time that bed bugs can acquire *Bartonella quintana*, maintain these microbes for more than 2 weeks and release them as viable organisms following a stercorarial shedding, thus transmitting the bacteria vertically to their progeny.

The evolution of strains highly resistant to most chemicals has accelerated the search for alternative methods to control bed bugs, e.g., the use of heat in the form of steam for controlling localized infestations (Puckett et al. 2013) or the use of essential oil-based pesticides (Singh et al. 2014; Wang et al. 2014). The use of natural products as a source of lead compounds for drug design is a scientifically exciting topic of great interest to insecticide manufacturers. The advantages of botanical insecticides, especially essential oils, include the synergistic effects of its components, low environmental impact, and lower production costs (Yunes et al. 2001). Successful examples of formulations can be found in the work of Solomon et al. (2012) who used a microencapsulated essential oil of citronella as mosquito repellent, and Oyedele et al. (2002) who suggested the incorporation of an essential oil extracted from lemongrass into ointments and/or cream formulations with various bases or liquid paraffin solutions to decrease rapid volatilization.

The insecticide and repellent properties of essential oils from different species of the *Tagetes* genus have been described (Vasudevan et al. 1997; Politi et al. 2013). In this work, we investigated the biological activity and safety profile of a *Tagetes patula* L. (Asteraceae) essential oil (EO) as a promising insecticide against common bed bugs. To better understand the mechanism of action of this complex matrix in the insect, we also conducted molecular modeling for three major components identified in the EO docked in the catalytic site of *C. lectularius* acetylcholinesterase 1 (*Cl*AChE1).

Materials and methods

C. lectularius

Adult common bed bugs of both genders were obtained from colony CTA 001 (origin: Nova Paulicéia, Gavião Peixoto (SP), 20 adults and 6 nymphs, 27 June 2006), maintained in the insectary of the Laboratory of Parasitology, School of Pharmaceutical Sciences, UNESP, Araraquara (SP), Brazil. The insects are fed twice a week in Wistar rats (*Rattus norvegicus*) and are kept in plastic rearing containers with

cardboard harborages in an environmental chamber (27–28 °C, 70 % humidity, 12:12 h light and dark photoperiod).

Plant material

Aerial parts of *T. patula* (stems, leaves, and flowers) were obtained from the Collection of Medicinal and Aromatic Plants (CPMA) of the Multidisciplinary Center for Chemical, Biological and Agricultural Research (CPQBA), State University of Campinas (UNICAMP). The propagation was performed using seeds from the Top Seed Garden line (Agristar®). A voucher specimen was deposited in the CPQBA Herbarium with the process number 1421.

Cell line

The naturally immortalized human keratinocyte cell line (HaCaT) was obtained from the Rio de Janeiro Cell Bank (BCRJ) and cultured in the Laboratory of Cytology and Cell Biology at the School of Pharmaceutical Sciences, UNESP, Araraquara (SP), Brazil.

Essential oil extraction

The EO extraction was performed by hydrodistillation using Clevenger (Unividros®) equipment coupled to a heating mantle. After straining to remove organic matter, approximately 200 g of plant material was weighed and transferred to a 6-L flask that was then filled to half-volume with distilled water. Extraction occurred over 3 h. The oil was collected in a beaker, and trace water was removed by adding anhydrous sodium sulfate (Na_2SO_4, Synth®). The procedure was repeated to obtain approximately 3 mL of pure essential oil.

GC-MS analysis

Chemical analysis of the plants was performed by gas chromatography coupled to mass spectrometry (GC-MS) with a Shimadzu® QP-2010 equipped with an AOC5000 autosampler and an EN-5MS capillary column (5 % diphenyl, 95 % dimethylsilicone, 30 m × 0.25 mm; film thickness 0.25 μm, SGE Analytical Science). Helium was used as the carrier gas (1.3 mL min^{-1}). A total volume of 1.0 μL of a 10 % solution of the oil was injected in hexane onto an injector heated to 240 °C operating in split mode (split ratio 1:60). The oven temperature ramped from 60 to 240 °C at a heating rate of 3 °C min^{-1}. The mass detector was operated in electronic ionization mode (70 eV), the ionization source was 250 °C, the transfer line was 260 °C, and acquisition achieved by scanning from m/z 40 to 400. The results were expressed as relative area (percent area) and linear retention indices were calculated by injection of a series of *n*-alkanes (C8–C40, Sygma®). For the proposed identification, the retention rate of each constituent was calculated using the Van Den Dool and Kratz Eq. 1:

$$RI = \left(\frac{T_x - T_n}{T_{n+1} - T_n} + n\right) \times 100 \quad (1)$$

Where RI = the temperature-programmed retention index of the interesting compound; T_n and T_{n+1} = the retention times (min) of the two standard *n*-alkanes containing n and $n+1$ carbons; T_x = retention time of the compound of interest; and n = number of carbons in the first bracketing *n*-alkane. The identification was confirmed by comparison of the calculated retention index (using homologous hydrocarbon series) with literature retention indices (NIST atomic spectroscopy database).

Impregnated paper disk test

For the preparation of test solutions, we accounted for the density of the essential oil ($d_{EO} = 0.73$ g mL^{-1}). Solutions were prepared to 100, 50, 25, 12.5, and 6.25 mg mL^{-1} using 2 % Tween 80 (*v*/*v*). Cypermethrin (Tagros®, 93 % (*v*/*v*)) was used as the positive control. The concentration was converted into parts per million (1.0 ppm = 1.0 mg L^{-1}), and the purity content was corrected to obtain test solutions of 100, 200, 400, 800, and 1600 ppm using acetone as the solvent (Kweka et al. 2009). For the negative control, two groups were prepared: one treated with acetone and another treated with 2 % Tween 80 solution. Exactly 1.0 mL each of these solutions was applied to cellulose filter paper (9 cm diameter), which remained at room temperature for approximately 30 min to dry. The filter papers were placed in Petri dishes, to which 20 adult common bed bugs per group were subsequently introduced. The bugs were fed 24 h before the assay. Each treatment was replicated two times. The plates were maintained in a biochemical oxygen demand (BOD) environmental chamber (25 °C, RH 80 %, 12 h/12 h photoperiod), and the results were visualized at 24, 48, and 72 h, counting as dead any insect unable to move after stimulation. In the case of more than 5 % mortality in the control group (Tawatsin et al. 2011), final mortality was corrected using Abbott (1925).

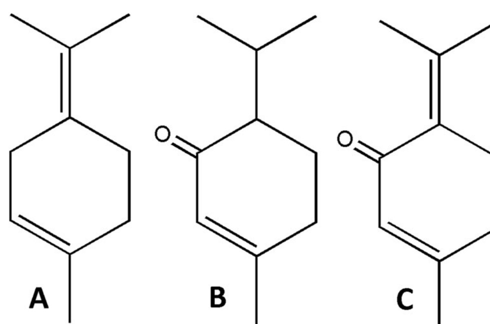

Fig. 1 Molecular structures of major compounds of the essential oil of *T. patula*. **a** α-terpinolene. **b** Piperitone. **c** Piperitenone

Table 1 Chemical composition of *T. patula* L. essential oil obtained by GC-MS

RI	Component	Percent
933	α-pinene	0.46
948	Camphene	0.04
971	Sabinen	0.78
977	β-pinene	0.05
987	β-myrcene	0.17
1006	α-phellandrene	0.23
1016	α-terpinene	0.04
1022	*o*-cymene	0.36
1028	Limonene	14.48
1033	*cis*-β-ocimene	4.51
1043	*trans*-β-ocimene	0.38
1046	Dihydrotagetone	0.96
1056	γ-terpinene	0.15
1086	α-terpinolene	15.48
1088	*p*-cymenene	0.81
1098	β-linalool	0.56
1133	Cosmene	0.20
1135	Epoxyocimene	0.24
1147	*trans*-tagetone	2.41
1176	*p*-1,8-menthadien-4-ol	1.54
1178	Terpinen-4-ol	0.55
1183	*p*-cymen-8-ol	3.60
1192	α-terpineol	0.19
1239	Carvone	0.07
1249	Piperitone	11.52
1281	2-camphanol acetate	0.18
1294	Thymol	0.08
1308	2-methoxy-4-vinylphenol	0.46
1334	Piperitenone	12.74
1350	Eugenol	0.13
1356	Piperitenone oxide	0.84
1375	Geranyl acetate	0.49
1376	α-copaene	0.16
1386	6,8-nonadien-2-one, 6-methyl-5-(1-methylethyldiene)	0.19
1419	Caryophyllene	3.78
1452	*cis*-β-farnesene	0.30
1454	Humulene	0.54
1480	Germacrene D	0.52
1519	Zingiberene	0.15
1495	Bicyclogermacrene	0.18
1519	δ-cadinene	0.16
1559	Nerolidol	0.20
1576	Spathulenol	2.76
1580	Caryophyllene oxide	6.10
1634	Isospathulenol	0.12
1654	α-cadinol	0.07
1837	Neophytadiene	1.66
–	NI	8.6

RI retention index relative to *n*-alkanes on the EN-5 MS capillary column, *NI* not identified

Oral and dermal acute toxicity

The acute oral toxicity test was performed according to protocol 420, adopted by the Organization for Economic Cooperation and Development (Organization for Economic Co-operation and Development (OECD) 2001), to obtain LC_{50} using male Wistar rats weighing approximately 200 g. Groups of five animals were submitted to a single oral administration at fixed doses of 2000 and 4000 mg kg^{-1} of the essential oil. Propylene glycol 30 % (*v*/*v*) was used as a control. The solutions were orally administered with the aid of syringe and gavage. The animals were individually placed in polyethylene cages and observed every 30 min for the first 24 h and then daily, verifying the incidence and severity of any abnormalities, including mortality and clinical alterations (change in behavior, gross lesions, body weight changes, diarrhea, lethargy, sleep, pain, tremors, convulsions, or any other clear sign of toxicity). After 14 days, all surviving animals were killed with overdose of anesthetic (xylazine/ketamine) intraperitoneally, proceeding to necropsy to identify visible or microscopic pathologic modifications.

The dermal acute toxicity test was performed according to protocol 402 (Organization for Economic Co-operation and Development (OECD) 2004). The fur of the rats was shaved on the dorsal region behind the head, and after 24 h, the solutions were applied with the aid of a syringe. In the first 4 h of exposure, the animals were placed in acrylic boxes (20 × 7 × 8 cm), to avoid product ingestion. The animals were transferred to polyethylene cages and observed every 30 min for the first 24 h and then daily. The protocols for both trials were submitted to the Ethics Committee on Animal Use (CEUA)-Instituto de Biociências (UNESP), Botucatu (SP), and accepted under protocol number 2013/606. The work was carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

Cytotoxicity assay

The cytotoxicity of the EO of *T. patula* was tested using the sulforhodamine B (SRB) assay (Skehan et al. 1990). Briefly, 96-well plates were pre-incubated with culture medium

Table 2 Mortality rate of mixed-gender adult common bed bugs (*Cimex lectularius*) exposed to cypermethrin at different concentrations

Cypermethrin (mg mL^{-1})	24 h		48 h		72 h		Mortality[a] ± SD
	Live	Dead	Live	Dead	Live	Dead	
1.6	05	15	01	19	01	19	94.4 (±1.169)a
0.8	09	11	0	20	0	20	100 (±2.345)a
0.4	13	07	10	10	06	14	66.7 (±1.602)a,b
0.2	11	09	08	12	08	12	38.9 (±1.329)b,c
0.1	13	07	13	07	13	07	27.7 (±0.548)c,d
Acetone	18	02	18	02	18	02	10 (±1.095)d
Total	20		20		20		

[a] The percentage of mortality was corrected by Abbott's formula (1925), and the values represent the mean of two assays performed at different times. Values followed by the same lowercase letters (a–d) do not exhibit significant differences by the Student-Newman-Keuls test ($p \leq 0.05$)

(Dulbecco's modified minimum essential medium (DMEM) supplemented with bovine fetal serum 10 % (*v*/*v*), 100 μL $well^{-1}$) for 24 h (37 °C, 5 % CO_2 atmosphere). After 24 h, the adhesion and growth of cells were observed. The medium was discarded and the test samples (EO at 200, 100, 50, 25, 12.5, and 6.25 μg mL^{-1}) and controls (doxorubicin 20 μg mL^{-1} as positive control and vehicle control) were added to each well at a cell count of 1.4×10^4 cell mL^{-1}. After 24 h of incubation, the cells were fixed with trichloroacetic acid (TCA; 100 μL $well^{-1}$) and refrigerated for 1 h at 4 °C. The TCA was removed, and 0.4 % SRB dye (*v*/*v*) was added (50 μL $well^{-1}$). After 20 min, the plates were washed with 1 % acetic acid solution (*v*/*v*) and dried at 25 °C. The unbound dye was removed after washing, dyestuff bound to the protein was solubilized in basic media (Tris base, 10 mM, pH 10.5), and optical density at 570 nm was determined using a microplate absorbance reader (iMark™, BioRad). The percentage of living cells was calculated according to Eq. 2:

$$\text{Survival} = \left(\frac{M_{\text{AbsT}} - M_{\text{AbsNC}}}{M_{\text{AbsVC}} - M_{\text{AbsNC}}} \right) \quad (2)$$

where M_{AbsT} = mean absorbance value of the tested sample; M_{AbsNC} = mean absorbance value of the negative control; and M_{AbsVC} = mean of absorbance value of the vehicle control.

Homology modeling of *Cl*AChE1

The structural model of *Cl*AChE1 was built by homology modeling on the basis of the closest homologous protein with a crystal structure available in the Protein Data Bank (PDB). The *Cl*AChE1 amino acid sequence was obtained from the GenBank database (accession AEN69455.1). The template structure was the crystallographic human butyrylacetylcholinesterase (*Hs*BuChE) at 2.7 Å resolution (PDB ID 4TPK), which showed 48 % sequence identity to the target protein and 93 % coverage. The pairwise sequence alignment was performed using ClustalW2, and 48 models were constructed using MODELLER (version 9.10) default parameters. The best model was selected based on the lowest DOPE energy and the validation step included Ramachandran-plot analysis using PROCHECK. The RMSD value between the main-chain atoms of the model and template was calculated by structural alignment of the template and the predicted structure in Pymol 1.7.

Molecular docking

Molecular docking and scoring protocols implemented in Surflex (Jain 2003) were used to investigate the binding mode

Table 3 Mortality rate of mixed-gender adult common bed bugs (*Cimex lectularius*) exposed to the essential oil of *Tagetes patula* at different concentrations

Essential oil (mg mL^{-1})	24 h		48 h		72 h		Mortality ± SD
	Live	Dead	Live	Dead	Live	Dead	
100	08	12	02	18	0	20	100 (±2.041)a
50	05	15	03	17	03	17	85 (±1.722)a
25	11	09	08	12	08	12	60 (±1.975)a,b
12.5	16	04	12	08	10	10	50 (±1.633)a,b
6.25	19	01	18	02	17	03	15 (±0.894)b,c
2 % Tween 80	20	0	20	0	20	0	0 (±0.000)c
Total	20		20		20		

Values followed by the same lowercase letters (a–d) do not exhibit significant differences by the Student-Newman-Keuls test ($p \leq 0.05$). The data correspond to the mean of two assays performed at different times.

Table 4 LC_{50} and LC_{90} values of cypermethrin and essential oil of *Tagetes patula* on common bed bug *Cimex lectularius*

Sample	LC_{50} mg mL^{-1} (LCL–UCL)[a]	LC_{90} mg mL^{-1} (LCL–UCL)[a]	Standard error	Chi-square	*p* value	R[b]
Cp	0.17 (0.13–0.21)	0.65 (0.49–1.02)	0.02	1.276	0.734	0.734
EO	15.85 (12.61–19.44)	55.44 (41.89–84.21)	1.75	1.448	0.694	0.820

Cp cypermethrin, *EO* essential oil of *T. patula*

[a] 95 % confidence limits (*LCL* lower control limit; *UCL* upper control limit)

[b] *R* correlation coefficient (deaths × dose)

of the major EO components (Fig. 1) in the *Cl*AChE1 catalytic site. Hydrogen atoms were added in standard geometry using the biopolymer module implemented in SYBYL X. Histidine, glutamine, and asparagine residues within the catalytic site were verified for possible flipped orientation, protonation, and tautomeric states. The binding site was defined by the ProtoMol protocol (threshold = 0.5 and bloat = 3) and included residues 38–44, 102–103, 162–163, 253–259, 264–265, 268, 302–308, 312, and 330. The docking procedures were repeated 20 times for each putative ligand. Visual inspection was employed to select the most likely representative conformation of the EO major compounds.

Statistical analysis

The results of insecticidal tests were submitted to analysis of variance (ANOVA), and the means were compared using the Student-Newman-Keuls (SNK) test ($p \leq 0.05$), using ASSISTAT 7.7™ software. To calculate the lethal concentration (LC_{50} and LC_{90}), probit analysis was performed using Statplus® software (AnalystSoft, 2009). The degree of adjustment of the data was evaluated using Pearson's chi-square test.

For the cytotoxic assay, the results are expressed as the mean ± standard error (SE) of three separate experiments. Statistical analyses were performed using Graphpad Prism™ (version 5.01). The data were subjected to one-way ANOVA, followed by Tuckey's HSD test.

Results

Steam distillation of the dried plant material yielded 2.8 μL/g of a yellowish oil. GC-MS analysis of the EO of *T. patula* revealed 47 compounds representing 91.4 % of the total (Table 1). The main compounds were α-terpinolene (15.48 %), limonene (14.48 %), piperitenone (12.74 %), and piperitone (11.52 %). Some typical compounds previously detected in the genus *Tagetes* also appear in good amount, such as spathulenol (2.76 %) (Cruz et al. 2014) and *trans*-tagetone (2.41 %) (De Feo et al. 2004). In addition, other insecticide compounds were identified in minor proportion, such as β-linalool (0.56 %) (Chang et al. 2009), α-pinene (0.46 %) (Wang et al. 2009), and carvone (0.07 %) (Tripathi et al. 2003).

Tables 2 and 3 present the results of the biological test in detail. The essential oil of *T. patula* proved to be as effective as the synthetic insecticide. At its highest concentration, the EO showed 100 % mortality. The acetone used to prepare the pyrethroid dilutions showed 10 % mortality, while the 2 % Tween 80 (*v*/*v*) used to prepare the dilutions of EO, did not cause any death. Thus, the mortality in the group treated with cypermethrin was corrected using Abbott's formula. The LC_{50} and LC_{90} were obtained from the probit analysis (Finney method) and are shown in the Table 4. Next, to verify whether the inhibitory activity was due to a cytotoxic effect, we evaluated the cytotoxicity of the EO to the HaCat cell line. The evaluated LC_{50} was 37.06 μg mL^{-1} (Fig. 2). At 50 μg mL^{-1}, the EO was as cytotoxic as the positive control ($p < 0.001$), with cell viability of 28 %. Finally, to assess the EO safety profile, in vivo acute toxicity was evaluated. The collected data indicated that there was no death at any of the used concentrations for either oral or dermal exposure. The assessed LC_{50} values for oral and dermal exposure were >4000 mg kg^{-1}. Regarding side effects, the only observation was that the animals were slightly lethargic within the first 24 h of administration of the tested agents. However, as the controls were also lethargic, it is likely that this effect was caused by the vehicle. After necropsy on the fifteenth day, no major pathology was found.

To shed light on the possible mechanism of action of the EO, molecular modeling studies were conducted. First, a homology model of the putative molecular target *Cl*AChE1 was built. A

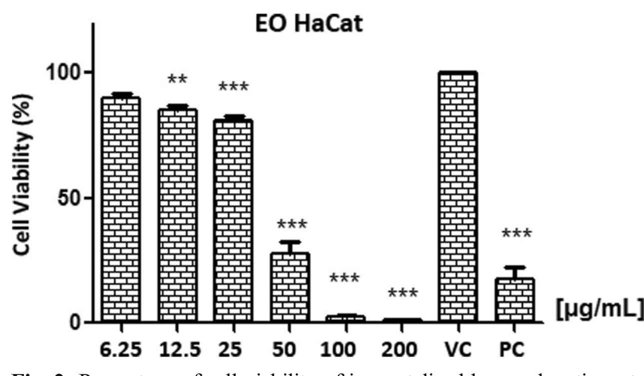

Fig. 2 Percentage of cell viability of immortalized human keratinocyte (*HaCaT*) with the essential oil of *Tagetes patula* using the sulforhodamine B assay. The results are expressed as the mean ± standard error (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. vehicle-treated control)

reliable 3D model of the target protein was constructed on the basis of its amino acid sequence and structural information from the *Hs*BuChE protein structure (PDB ID 4TPK) (Fig. 3a). The best model comprised a monomeric structure of 596 amino acid residues, with a root-mean-square deviation (RMSD) of 0.25 Å over Cα for 602 equivalent residues of *Hs*BuChE (Fig. 3b). The structure corresponds to an alpha and beta protein (a/b), with mainly parallel beta sheets (beta-alpha-beta units) in a three-layer fold of a/b/a. *Cl*AChE1 has four substituted residues that are related to ligand binding: the Phe307 and Tyr347 residues (Val288 and Ala328 in *Hs*BuChE, respectively) are located in the cavity interior, and Trp299 and Tyr352 (Ala277 and Gly333 in *Hs*BuChE, respectively) lie in the cavity entrance (Fig. 3c, d).

To verify whether the major EO compounds (Fig. 1) would fit into the *Cl*AChE1 binding site, molecular docking studies were employed. The modeled binding modes indicate favorable interactions between the major EO compounds and *Cl*AChE1 catalytic site residues (Fig. 4). Specifically, the natural products bind near the key residues constituting the catalytic triad site (Ser200, Glu307, and His440), choline-binding site (Trp84), and oxyanion hole (Gly118, Gly119, and Ala201), establishing favorable hydrophobic interactions with

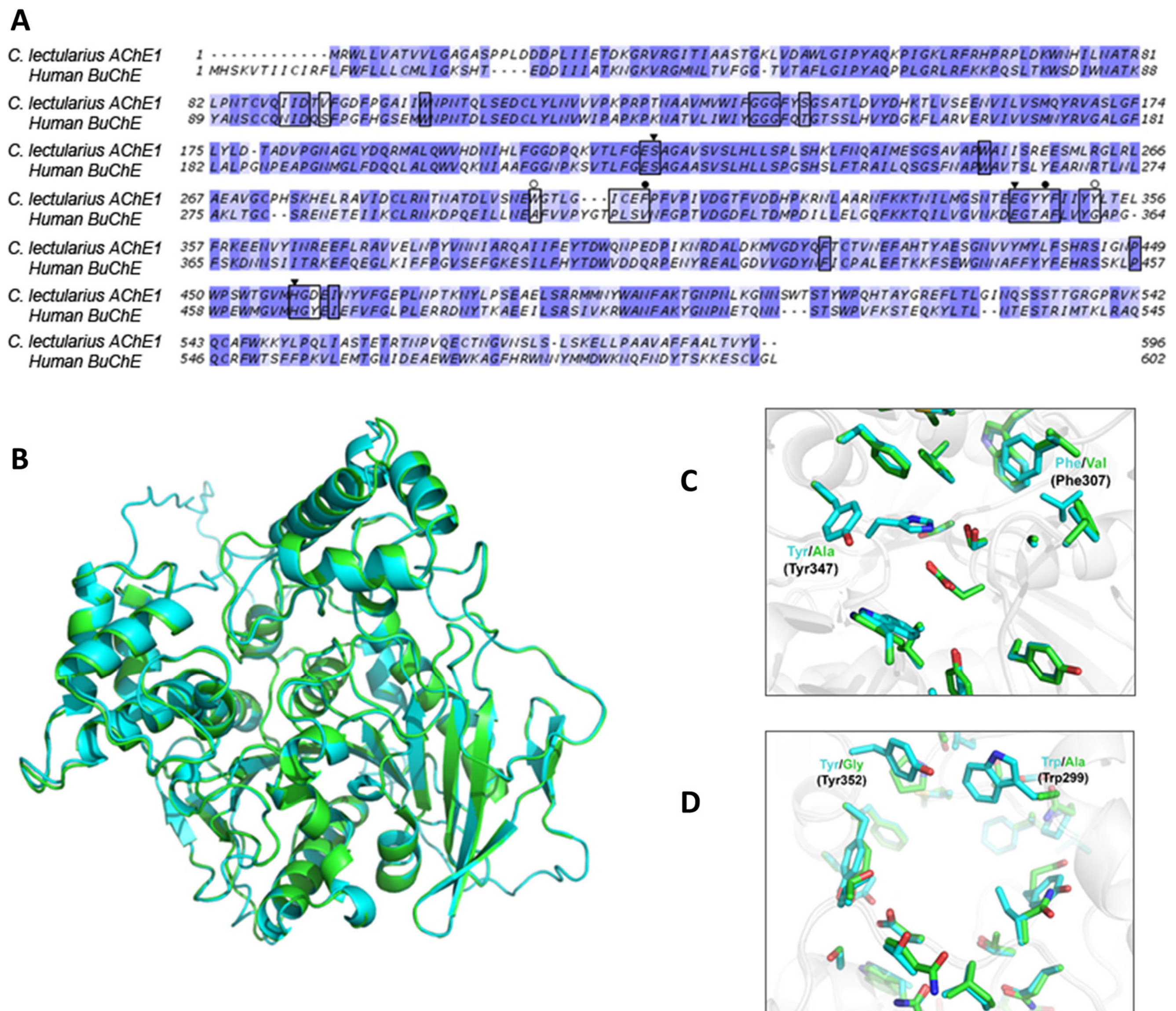

Fig. 3 Homology modeling of *Cimex lectularius* acetylcholinesterase 1. **a** Amino acid alignment of *H. sapiens* butyrylcholinesterase (template sequence) and *C. lectularius* acetylcholinesterase 1 (target sequence). Residues are highlighted in purple according to the substitution matrix BLOSUM62 by Jalview (1). Binding site residues are indicated by *boxes*, catalytic triad residues are indicated by *filled triangles*, and replaced residues are indicated by *filled circles* (cavity interior) and *open circles* (cavity entrance). **b** Structural alignment of template *Hs*BuChE (*green*) and *Cl*AChE1 model (*cyan*) (RMSD = 0.25 Å). **c** Catalytic site comparison indicating the significant residue replacements Phe307 and Tyr347 in *Cl*AChE1. **d** Binding site entrance comparison highlighting the significant residue replacements of Trp299 and Tyr352 in *Cl*AChE1

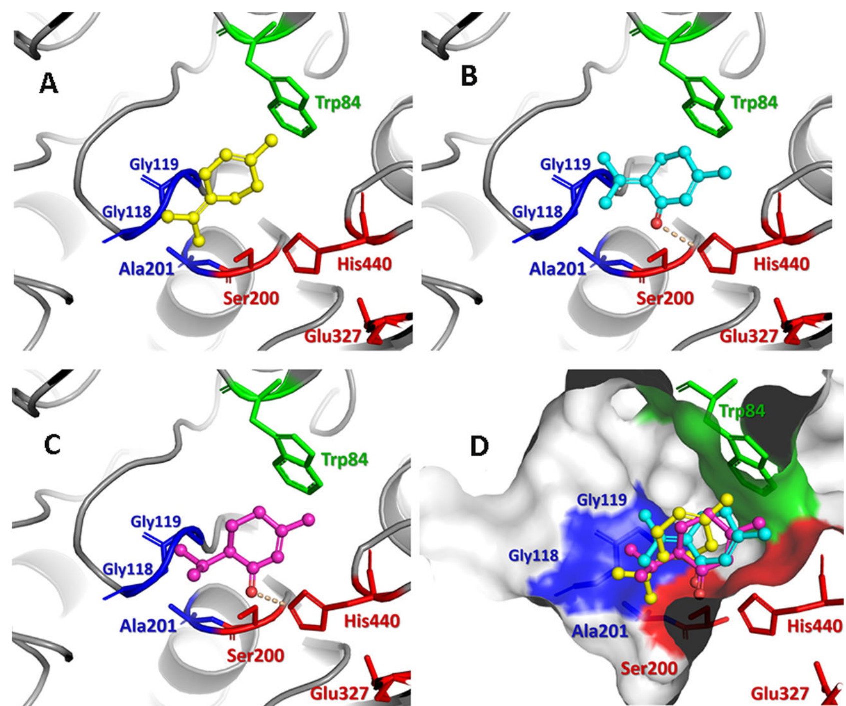

Fig. 4 Modeled binding mode of the major compounds of *Tagetes patula* essential oil (*EO*) into the *Cl*AChE1 catalytic site. **a** α-terpinolene. **b** Piperitenone. **c** Piperitone. **d** Clipped view of the *Cl*AChE1 catalytic site and structural superposition of the predicted binding mode of the major EO compounds. Residues constituting the catalytic triad site (Ser200, Glu307, and His440), choline-binding site (Trp84), and oxyanion hole (Gly118, Gly119, and Ala201) are indicated as *red*, *green*, and *blue sticks*, respectively

the main chain of Gly118, Gly119, and the side chain of Trp84. In the case of piperitenone and piperitone (Fig. 4b, c), a hydrogen bond is predicted between the carbonyl substituent and the side chain of His440.

Discussion

Resistance developed by bed bugs to synthetic insecticides, as reported by Romero et al. (2007), and few references regarding susceptibility tests using crude plant extracts, makes the results described in this work quite significant. It is possible that the essential oil of *T. patula* provides an environmentally attractive alternative for the control of populations of *C. lectularius*, showing similar effects to cypermethrin in doses approximately 100-fold greater, which is significant if we consider that it is an unpurified complex matrix. We expect that major compounds extracted from essential oil may be even more effective.

The phytochemical constitution of the essential oil (Table 1) attests to a rich composition of monoterpenes, sesquiterpenes, and secondary metabolites closely related to the insecticide potential shown here (Viegas-Júnior 2003). Most reports indicate that these compounds show inhibitory and growth retardant effects, maturation damage, reduction of reproductive capacity, appetite suppression, or direct toxicity (Viegas-Júnior 2003). Oxygenated terpenoids are reported to have higher activity than those not oxygenated; among oxygenated terpenoids, the biological activity varies greatly by chemical groups and/or unsaturation (Pavela 2008). The EO of *T. patula* contains geranyl acetate, a monoterpenoid that is the acetate ester derivative of geraniol, which is found in the formulation of several essential oil-based pesticides such as Bed Bug Fix (NuSafe Floor Solutions Inc.), EcoRaider (Reneotech Inc.), Essentria (Envincio LLC), Rest Assured (ES & P Global LLC), and Stop Bugging Me (Rocasuba Inc.).

The mechanism of action of essential oils in insects remains unclear, but some reports have shown that some compounds present in such matrices can be neurotoxic (Coats et al. 1991; Kostyukovsky et al. 2002; López and Pascual-Villalobos 2010). Re et al. (2000) reported that linalool, a monoterpenoid present in the essential oil of *T. patula*, exhibits activity on central nervous system similar to synthetic organophosphate and carbamate insecticides by interfering with the release of acetylcholinesterase. Acetylcholinesterase (AChE) is a serine hydrolase vital for regulating the neurotransmitter acetylcholine (ACh) in both vertebrates and invertebrates (Toutant 1989). Due to the emergence of insecticide resistance in insects, AChE has been intensively investigated. Hence, with the goal of investigating the molecular basis for the evaluated biological activity of the EO against *C. lectularius*, we selected this enzyme as a target for modeling studies. In bed bugs, three acetylcholinesterase genes are well described (i.e., *clace1*, *clace2*, and *clsce*), with conserved motifs including a catalytic triad, a choline-binding site and an acyl pocket. The transcription levels of *Clace1* in the central nervous system are higher than the other two genes (Seong et al. 2012). Hwang et al. (2014) reported that the relatively higher correlation between the in vitro *Cl*AChE1 inhibition and the in vivo toxicity of organophosphates and carbamates suggests this enzyme as the most relevant toxicological target. We built a homology model of *Cl*AChE1 using

*Hs*BuChE as a template (Fig. 3). The *Cl*AChE1 model has a sequence identity of 60 % in binding site residues compared with the human homologue (Fig. 3a). As a consequence of these replacements, the *Cl*AChE1 binding site is smaller and presents a narrower entrance, indicating significant structural differences that might be explored for selective ligand design.

According to the modeled binding mode, the EO major compounds α-terpinolene, piperitenone, and piperitone have favorable polar and hydrophobic interactions with key residues in the catalytic site of *Cl*AChE1. In this conformation, the natural compounds block the choline substrate from binding, acting as competitive inhibitors of *Cl*AChE1, and ultimately impairing the important role of acetylcholinesterase in regulating neurotransmission in bed bugs. The accumulation of acetylcholine in synapses causes nervous hyperactivity and consequent collapse of the CNS, justifying the high mortality observed in the in vitro assay.

In conclusion, the essential oil of *T. patula* proved to be a safe and effective alternative against adult forms of the bed bug *C. lectularius* and can be used in consortium with currently available chemicals. Molecular modeling studies suggested that essential oil major compounds competitively inhibit the acetylcholinesterase of *C. lectularius*. Due to the emergence of resistance to chemical pesticides and related environmental problems, the results reported here indicate a promising novel approach to the development of an ecologically attractive product to control *C. lectularius*.

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