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Artigo

A Simple and Efficient Protocol for the Knoevenagel Reaction of Benzylidenemalononitriles and the Evaluation of the Larvicidal Activity on *Aedes Aegypti*

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Um Protocolo Simples e Eficiente para a Reação de Knoevenagel de Benzilidenomalonitrilas e Avaliação da Atividade Larvicida em *Aedes Aegypti*

Resumo: Os mosquitos do gênero *Aedes* são responsáveis pela dengue, febre amarela, chikungunya e Zika. Embora importantes avanços surgiram no desenvolvimento de medidas alternativas para o controle de mosquitos, os inseticidas químicos continuam sendo uma parte vital dos programas de controle integrado. Nesse trabalho foram sintetizados uma série de benzilidenomalononitrilas em bons rendimentos (71-99%), usando apenas água e glicerol a temperatura ambiente. O estudo da atividade larvicida entre os derivados de benzilidenomalononitrilas mostrou que o composto **2e** (R = 4-Cl) apresentou melhor atividade larvicida (LC_{50} e LC_{90} de 9,42 e 15,02, respectivamente, em 24 h). Um estudo de *docking* molecular foi aplicado para identificar a interação do modo **2e** com os sítios de ligação na enzima acetilcolinesterase, o perfil de interação mostrou um valor de 48,9795, com cinco ligações em três diferentes aminoácidos.

Palavras-chave: *Aedes aegypti*; Química verde; Atividade larvicida; Docking molecular.

Abstract

Mosquitoes of the genus *Aedes* are responsible for dengue, yellow fever, chikungunya and Zika. Although important advances have emerged in the development of alternative methods for mosquito control, chemical insecticides remain a vital part of integrated control programs. In this paper were synthetized benzylidenemalononitrile derivatives in good yields (71-99%) using only water and glycerol at room temperature. A study of the larvicidal activity between benzylidenemalononitriles showed that the compound **2e** (R= 4-Cl) possesses excellent larvicidal activity (LC_{50} and LC_{90} of 9.42 and 15.02, respectively at 24 h). A study of molecular docking was applied to identify the type of interaction of compound **2e** with binding sites at the enzyme acetylcholinesterase. The profile of the interaction showed a score 48.9795 with five bonds at three different amino acids.

Keywords: *Aedes aegypti*; Green chemistry; Larvicidal activity; Molecular docking.

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A Simple and Efficient Protocol for the Knoevenagel Reaction of Benzylidenemalononitriles and the Evaluation of the Larvicidal Activity on *Aedes Aegypti*

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1. Introduction

Mosquitoes of the genus *Aedes* are responsible for dengue, yellow fever, chikungunya, and Zika.[1] Yellow fever, like dengue, is a viral hemorrhagic fever and can be lethal. Chikungunya virus is an alphavirus that belongs to the Semliki Forest Virus antigenic complex. More one million cases have been reported in the Americas since 2013. The first Brazilian case was confirmed in the Amapá federal state in 201.[2-4] Zika virus has generated a great deal of concern because of its associations with microcephaly and Guillain-Barré syndrom.[5,6] These pathogens collectively infect 100 million people every year, and over 2.5 billion people live in areas susceptible to these disease.[1,7,8]

Insecticides,[7,9] genetically modified mosquitoes,[10] and larvicides[11,12] can all control these vectors. However, controlling the mosquito vector at the larval stage is especially powerful because they can be easily targeted in breeding habitats, i.e., larvae are immobile.

Although important advances have emerged in the development of alternative measures for mosquito control, chemical insecticides remain a vital part of integrated control programs. The use the pyrethroid and organophosphate pesticides are common in the control of adult mosquitoes worldwid.[1,9] However, resistance to insecticides is a growing problem in vector control programs this resistance can be a consequence of various physiological variables.[13,14]

Concurrently, continuous monitoring of mosquito populations may play an important role in preventing or minimizing the development of resistance to effective insecticides. However, developing new products to combat insects is equally important. Therefore, the synthesis of new biologically active compounds for larvicidal control has attracted strong interest.

Benzylidenemalononitrile derivatives are versatile building blocks in the synthesis of biological and pharmacological molecules. For example, 1,3-diarylpyrazole derivatives have anti-inflammatory properties[15] and can be prepared via an intermediary in the synthesis of tyrphostins, which are active in cancer cell lines.[16] Similarly, aminopyridines are potent antibacterial agents[17] and the phenanthroline-*3*-carbonitrile precursor analogues have larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus*.[18]

The synthesis of benzylidenemalononitrile derivatives occurs via the Knoevenagel condensation. Synthesis in aqueous solvent has experienced remarkable growth, but this synthesis usually requires catalysts such as organic bases, Lewis acids,[19] ionic liquids,[20] organometallic catalysts,[21] and functionalized biopolymers under microwave irradiation.[22] In continuation of our work on new catalyst-free synthetic strategies,[23,24] we developed an efficient and easy methodology that use only water and glycerol at room temperature to prepare benzylidenemalononitrile derivatives with potential larvicial activity against *Aedes aegypti*.

2. Materials and methods

2.1. General methods

2.2. Gas Chromatography-Mass Spectrometry (GC-MS)

The reactions analyses were conducted using a gas chromatograph (GC2010 Ultra Shimadzu Corporation, Japan) equipped with an auto-sampler injection AOC-20i (Shimadzu). Electron capture detection used as detector (Shimadzu MS2010 Plus), electronic impact of 70 eV and fragments detected from 50-500 Da. Separations were performed on a fused silica capillary column (DB-5MS 5% 30 m × 0.25 mm internal diameter, 0.25 mm film thickness) in a stream of helium 1.0 mL min^{-1}. Injector temperature was 230 °C, ion source 200 °C, 270 °C of interface and split ratio 5. The oven temperature program started at 100 °C with

an increase of 7 °C min^{-1} to 200 °C and 20 °C min^{-1} to 300 °C lasting for 2 min.

2.3. Fourier Transform Infrared (FTIR)

FTIR spectra were recorded on a Bomen MB-100 spectrometer samples were prepared as thin films on KBr disks. The transmittance was expressed in cm^{-1} of band between 4000 to 400 cm^{-1}.

2.4. Nuclear Magnetic Resonance (NMR)

NMR spectra were recorded on an Agilent Technologies 500/54 Premium Shielded or Agilent Technologies 400/54 Premium Shielded spectrometer. The samples were solubilized in $CDCl_3$ (99.9%) or CD_3OD (99.9%) and chemical shifts expressed in ppm relative to internal standard TMS or deuterated solvents. The chemical shifts were given in ppm and coupling constants (*J*) in Hz. The description of signals includes: s = singlet, d = doublet, t = triplet and m = multiplet.

2.5. Chemical reagents

Benzaldehyde **1a** (99.5%), 4-methoxybenzaldehyde **1b** (98%), 3,4,5-trimethoxybenzaldehyde **1c** (99%), 4-hydroxy-3-methoxybenzaldehyde **1d** (98%), 4-chlorobenzaldehyde **1e** (97%), 4-fluorobenzaldehyde **1f** (98%), 3-nitrobenzaldehyde **1g** (98%) and malononitrile (99%) (Table 2) were purchased of Sigma-Aldrich and used without further purification. Glycerol (98%) and ethanol (99%) were purchased of Vetec. Deuterated solvents were purchased from Cambridge Isotope Laboratories.

2.6. Synthesis of the benzylidenemalononitrile derivatives (2a-g)

The benzylidenemalononitrile products (**2a-g**) were prepared via Knoevenagel condensation (Table 2) from a mixture of appropriate aldehydes **1a-g** (3 mmol) and malononitrile (3.5 mmol) in 5 mL of water and glycerol (1:1) in a 25 mL round bottomed flask. The solution was maintained with magnetic stirring for 24 h at room temperature. The reaction was monitored by silica TLC plates, and bands were visualized under ultraviolet (UV) light, using hexane and acetyl acetate (7:3) as the eluent. At the end of the reaction, the precipitate was filtered and washed with ice water (50 mL). The product was recrystallized in ethanol overnight. All resulting compounds **1a-g** were obtained in good yields and characterized by melting point, ^{1}H NMR, FT-IR, and GC–MS analysis (Supplementary information).

2.7. Physical and spectroscopic data

2-benzylidenemalononitrile (**2a**): Molecular formula: $C_{10}H_6N_2$; MW: 154.05 g/mol; White solid; M.p. 85 °C; Yield 0.461 g (99%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.92 (m, 2H), 7.79 (s, 1H), 7.64 (m, 1H), 7.54 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 159.9, 134.6, 130.8, 129.6, 113.6, 112.5, 82.7; IR (KBr, cm^{-1}): 3032, 2224, 1683, 756, 677; MS (70 eV, %): *m/z* 154 (100), 127 (90), 103 (60).

2-(4-methoxybenzylidene)malononitrile (**2b**): $C_{11}H_8N_2O$; MW: 184.06 g/mol; Yellow solid; M.p. 122 °C; Yield 0.480 g (84%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.88 (d, *J*=8.0 Hz, 2H), 7.63 (s, 1H), 7.0 (d, *J*=8.0 Hz, 2H), 3.89 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): 164.72, 158.75, 133.14, 123.24, 114.80, 114.05, 112.84, 78.69, 55.69 ; IR (KBr, cm^{-1}): 3026, 2986, 2221, 1605; MS (70 eV, %): *m/z* 184 (100), 161 (13), 141 (25), 114 (42).

2-(3,4,5-trimethoxybenzylidene)malononitrile (**2c**): $C_{13}H_{12}N_2O_3$; MW: 244.08 g/mol; Yellow solid; M.p. 148 °C; Yield 0.724 g (99%); ^{1}H NMR (500 MHz, $CDCl_3$, ppm): 7.63 (t, *J*=0.5 Hz, 1H), 7.24 (s, 2H), 7.17 (d, *J*=0.5 Hz, 2H), 3.96 (s, 3H), 3.89 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$, ppm): 159.32, 153.35, 144.03, 125.89, 113.94, 113.15, 108.31, 80.59, 61.21, 56.34, 29.66.; IR (KBr, cm^{-1}): 3018, 2220, 1504, 1257, 1041. MS (70 eV, %): *m/z* 244 (100), 229 (74), 201 (37), 186 (21), 158 (32), 115 (32).

2-(4-hydroxy-3-methoxybenzylidene)malononitrile (**2d**): $C_{11}H_8N_2O_2$; MW: 200.02 g/mol; White solid; M.p. 136 °C; Yield 0.504 g (84%); ^{1}H NMR (500 MHz, CD_3OD, ppm): δ 7.94 (s, 1H), 7.57-7.56 (d, 1H, *J*=2.5 Hz), 7.43 (m, 1H), 7.07 (d, 1H, *J*= 8.5 Hz), 3.95 (s, 3H). ^{13}C NMR (126 MHz, CD_3OD, ppm): 159.76, 153.71, 146.92, 126.05, 124.67, 115.22, 114.45, 113.38, 111.19, 76.87, 55.05; IR (KBr, cm^{-1}): 3427, 3022, 2976, 2225, 226, 1026. MS (70 eV, %): *m/z* 200 (100), 157 (95), 129 (29), 102 (50).

2-(4-chlorobenzylidene)malononitrile (**2e**): $C_{10}H_5ClN_2$; MM: 188.01 g/mol; White solid; M.p. 176 °C; Yield 0.4455 g (79%); ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 7.88-7.85 (d, *J*= 8.5 Hz, 2H), 7.74 (s, 1H), 7.55-7.52 (d, *J*=8.5 Hz, 2H); ^{13}C NMR (126 MHz, $CDCl_3$, ppm): 158.26, 141.15, 131.83, 130.07, 129.27, 113.42, 112.32, 83.37; IR (KBr, cm^{-1}): 3095, 3032, 2225, 1683, 827. MS (70 eV, %): *m/z* 188 (71), 153 (100).

2-(4-fluorobenzylidene)malononitrile (**2f**): $C_{10}H_5FN_2$; MW: 172.04 g/mol; White solid; M.p. 135°C; Yield 0.4128 g (80%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.98-7.96 (m, 2H), 7.75 (s, H), 7.27-7.23 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): 166.11 (d, $^1J_{C-F}$ =207 Hz), 157.98, 133.37, 127.18, 117.18, 113.40, 112.22, 82.05; IR (KBr, cm^{-1}): 3074, 3041, 2229, 1508. MS (70 eV, %): *m/z* 172 (100), 145 (96), 121 (54).

2-(4-nitrobenzylidene)malononitrile (**2g**): $C_{10}H_5N_3O_2$; MW: 199.04 g/mol; White solid; M.p. 106 °C; Yield 0.423 g (71 %); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 8.38-8.36 (d, *J*= 8.8 Hz, 2H), 8.07-8.04 (d, *J*= 8.8 Hz, 2H), 7.87 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): 56.82, 150.33, 135.76, 131.27, 124.60, 112.58, 111.55, 87.52; IR (KBr, cm^{-1}): 3045, 2225, 1527, 1356. MS (70 eV, %): *m/z* 199 (75), 153 (100), 141 (33), 123 (86).

2.8. Larvicidal activity

2.8.1. Collection and maintenance of target vector

The larvicidal experiments used third instar larvae of *Aedes aegypti* Rockefeller strain from the Arthropoda Laboratory of the Federal University of Amapá. The assay was conducted under controlled conditions with a temperature between 25 ± 2 °C, relative humidity of 75 ± 5%, and photoperiod of 12 hours.

2.8.2. Larvicidal activity in vivo

Compounds **2a-g** were dissolved in dimethylsulphoxide (DMSO) at different concentrations (15.0, 12.5, 10.0, 5.00, and 2.50 ppm) from a stock solution of 1.500 ppm. Negative controls were performed using distilled water containing the same amount of DMSO (1%). All experiments were performed in triplicate with 10 larvae in each replicate. The mortality rate of the larvae was determined at 24 and 48 h of exposure. Larvae were considered dead when they did not respond to stimuli or did not rise to the solution surface relative to the control. The bioassay experiments were conducted according to World Health Organization (WHO) standards with modifications.[25]

2.9. Statistical analysis

The lethal concentration LC_{50} and LC_{90} (determined in 24 and 48 h incubation) for compound **2e** were calculated using Probit analysis with Software StatGraphic Centurium XV version 15.2.11. When the control mortality of the treated groups was between 5-20%, the analysis was corrected according to WHO[25] formula: mortality (%) = X − Y/X × 100, where X = percent survival in the untreated control and Y = percent survival in the treated sample.

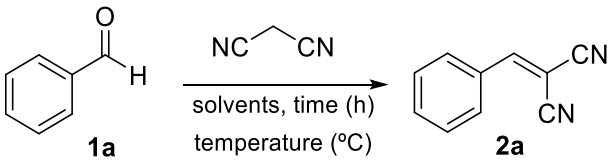
2.10. Molecular docking between the compound 2e and the acetylcholinesterase

The crystallographic structure of acetylcholinesterase from *Drosophila melanogaster* deposited in complex form under code 1DX4 with a resolution of 3.64 Å was selected from the Protein Data Bank (PDB). To validate the docking, the Mean Square Deviation (RMSD) was calculated using the Discovery Studio (DS) Visualizer for the crystallographic structure, and the docking simulation was performed using GOLD 4.1. The RMSD was considered relevant for results below 2 Å; the RMSD for 1XD4 complex ligand was 0.7732 Å.[26] The amino acids of the active site were selected as described in the literature: TYR71; TRP83; GLY149; PHE330; Y370; TRP472; and HIS480.[27]

3. Results and discussion

The reaction of benzaldehyde **1a** with 1 equiv. of malononitrile at room temperature in water for 24 h resulted in trace yield of Knoevenagel adduct **2a** (Table 1, entry 1). When the reaction was performed using a mixture of water and glycerol (1:1) as solvents and at room temperature the product **2a** was formed with 99% yield (Table 1, entry 2). Glycerol is a green co-solvent in several reactions in organic synthesis. Beyond to increase the solubility of organic reagents, facilitates the separation of the reaction product. The yield using glycerol as solvent at 24 h was of only 54% (Table 1, entry 3) due to glycerol's high viscosity. However, when the reaction was performed in a mixture of water and glycerol at 50 ºC for 12 h (Table 1, entry 3), the product **2a** was formed with 75% yield. These experiments showed that the success of the reaction was dependent on the solvent effects.

Table 1. Optimization of synthesis of benzylidenemalononitrile **2a** via the Knoevenagel condensation using water and glycerol as solvents



1a **2a**

Entry	Time (h)	Solvents	Temperature (ºC)	Yield (%) **2a**
1	24	Water	r.t.	Trace
2	24	Water:Glycerol	r.t.	99
3	24	Glycerol	r.t.	54
4	12	Water:Glycerol	50	75
5	12	Water:Glycerol	r.t.	90

r.t. = room temperature (30 ºC ± 2 ºC)

The water and glycerol mixture at room temperature outperforms conventional organic solvents like toluene, DMF, and ethanol. The condition reaction (Table 1, entry 2) encouraged us to apply this method for the synthesis of others benzylidenemalononitrile compounds (Table 2). Aldehydes **1a-g** with different functional groups were used to evaluate the influence of the substituent groups. In all cases, the formation of benzylidenemalononitrile compounds **2a-g** occurred via the Knoevenagel reaction.

All Knoevenagel adducts **2a-g** were obtained in good yields (71-99%). In general, the aromatic aldehydes readily condensed with malononitrile. Xu and co-authors reported the formation of these compounds in excellent yields of 99%; however, this reaction occurred in the presence of 10% polystyrene-supported DABCO as the catalyst in methanol at room temperature for 60 min .[28] In another study, Sonawane and co-authors showed the formation benzylidenemalononitrile compounds in 98% yield using 5 mol% $Ni(NO_3)_2.6H_2O$ at room temperature in water for 20 min.[29] Compounds **2a-g** were characterized by FTIR, GC-MS, and NMR and compared to literature data (Supplementary Information).

The benzylidenemalononitrile compounds **2a-g** were tested against *Aedes aegypti* larvae at different concentrations (15, 12.5, 10, 5, and 2.5 ppm). Of these, the compound **2e** had the most promising larvicidal properties (Figure 1).

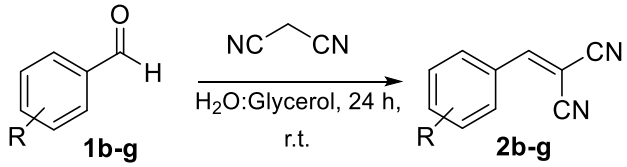
Electron-rich compound such as 2-(4-hydroxy-3-methoxybenzylidene)malononitrile **2c** exhibited low larvicidal activity profile. Similar data was observed for 2-(3,4,5-trimethoxybenzylidene)malononitrile **2d**. The compound **2b** showed better larvicidal mortality in comparison to their analogues approximately 30% at 12 ppm with 48 h of incubation.

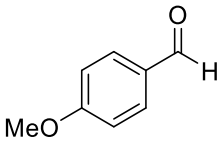
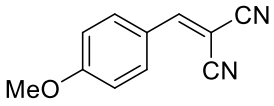
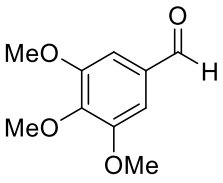
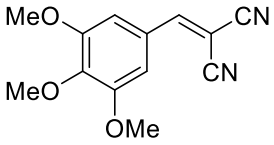
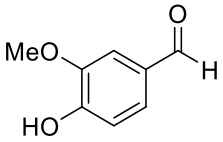
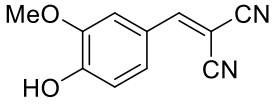
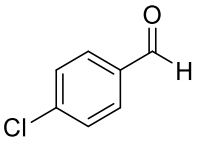
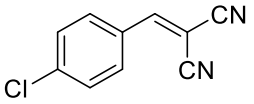
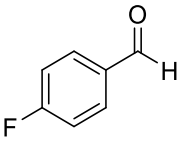
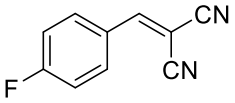
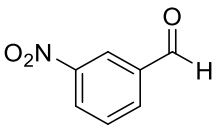
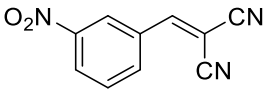
Among the halogenated compounds, the 2-(4-chlorobenzylidene)malononitrile **2e** had the best larvicidal mortality with LC_{50} values of 9.42 and 9.44 at 24 h and 48 h, respectively. The LC_{90} were 15.02 and 15.05 at 24 h and 48 h, respectively for the adduct **2e** (Table 3). This result confirmed the strong influence of the electronic and steric effects on the substituents on the benzylidenemalononitrile derivatives in the larvicidal activity of *Aedes aegypti*. Similarly, Da Silva and coworkers studied the larvicidal activity in a biurets series. They noted that activity for the fluorine *para*-substituent was lower than the chlorine.[30]

The 2-(4-fluorobenzylidene)malononitrile **2f** showed a mortality rate lower than 5%. The 2-(3-nitrobenzylidene)malononitrile **2g** has a strongly electron withdrawing substituent attached to the aromatic ring, and it did not exhibit mortality against *Aedes aegypti* larvae at 12 ppm.

Acetylcholinesterase (AChE) plays an important role in transmitting the signal/message to central nervous system, and it a critical mosquito enzyme, and AChE is the molecular target for many insecticides including organophosphate and carbamate compounds.[34] Thus, molecular docking was used to identify the mode of interaction between linkers at the enzyme or receptor/binding site via specific key interactions. This can predict the binding affinity between the protein-linker complexes.[35]

Table 1. Synthesis of benzylidenemalononitrile derivatives **2b-g** via Knoevenagel reaction in water and glycerol at room temperature for 24 h.



Entry	Aldehyde (**1b-h**)	Product (**2b-g**)	Yield (%)	M.p. [M.p. of literature]
1	 **1b**	 **2b**	87	122 ºC [114 - 116ºC] [32]
2	 **1c**	 **2c**	84	136 ºC [134-136 ºC] [32]
3	 **1d**	 **2d**	99	148 ºC [149-150 ºC] [33]
4	 **1e**	 **2e**	79	170 ºC [166-167 ºC] [33]
5	 **1f**	 **2f**	80	130ºC [125 - 126 ºC] [20]
6	 **1g**	 **2g**	71	113 ºC [107-109 ºC] [33]

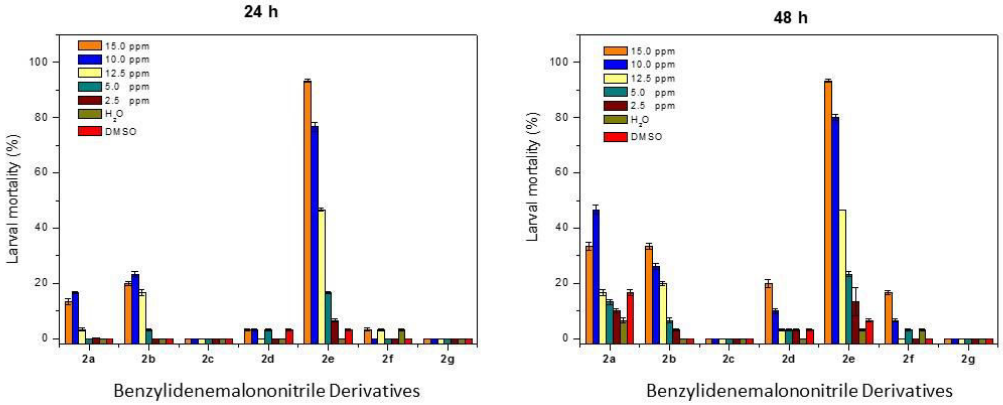


Figure 1. Larvicidal activity of benzylidenemalononitrile derivatives on *Aedes aegypti* larvae at 24 h and 48 h

Table 3. Larvicidal activity (LC_{50} and LC_{90}) of 2-(4-chlorobenzylidene)malononitrile **2e** by *Aedes aegypti*

Adduct **2e**	*Aedes aegypti*	
	24 h	48 h
LC_{50}*	9.42	9.44
C.L.**	7.32 – 11.44	7.34 – 11.47
LC_{90}*	15.02	15.05
C.L.**	12.70 - 20.14	12.72 - 20.19

*LC_{50} and LC_{90} in ppm. **C.L. = Confidence limit.

Figure 2 shows the profile of interaction between the compound **2e** and the amino acid residues of an AChE. The score was 48.9795 with five bonds at three different amino acids. These included HIS480, which is a hydrogen bond between the H16 atom of the adduct **2e** and O3458 of the HIS480 amino acid with a distance of 2.51 Å. Hydrophobic interactions were also observed between the TYR370 amino acid with the aromatic ring and chlorine atom Cl11 with a distance of 4.06 Å this also interacted with aromatic ring (4.38 Å) of the ligand **2e**. There were two hydrophobic interactions between TRP83 and the aromatic ring of compound **2e** and with the pyrrolidine and the aromatic rings, and the distances were 3.75 Å and 4.65 Å, respectively.

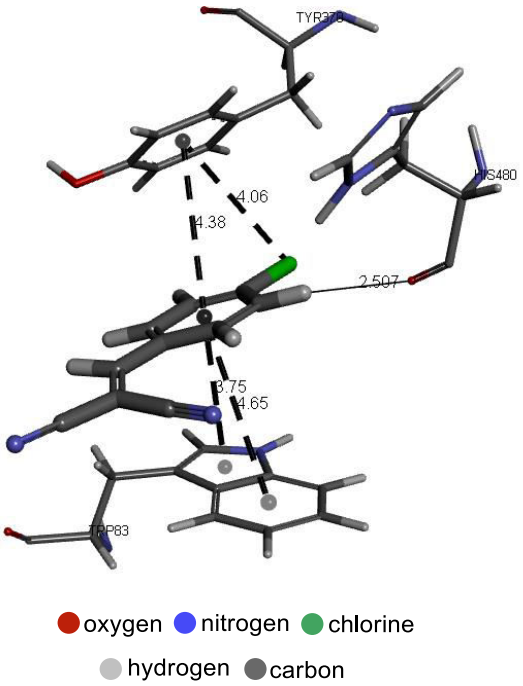


Figure 2. Simulation of molecular docking between the enzyme AChE (represented by the amino acids of the active site) and compound **2e**

4. Conclusion

In this study, new synthetic strategies using water and glycerol as the solvents for the preparation of benzylidenemalononitrile derivatives offered good yields in the room temperature. The larvicidal activity of these compounds were evaluated on *Aedes aegypti* larvae. The LC_{50} value was 9.42 at 24 h for adduct **2e**. These results suggested that compound **2e** can be obtained from an eco-friendly reaction in good yields and is a potential larvicidal molecule.

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