Characterization of acetylcholinesterase from elm leaf beetle, *Xanthogaleruca luteola* and QSAR of temephos derivatives against its activity

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Insect acetylcholinesterase (AChE) is the principal target for organophosphate (OP) and carbamate (CB) insecticides. In this research, an AChE from third instar larvae of elm left beetle, *Xanthogaleruca luteola* was purified by affinity chromatography. The enzyme was purified 75.29-fold with a total yield of 8.51 %. As shown on denaturing SDS-PAGE, the molecular mass of purified AChE was 70 kDa. The enzyme demonstrated maximum activity at pH 7 and 35 °C. Furthermore, a series of temephos (Tem) derivatives with the general structure of P(O)–X–P(O) (1–44) were prepared, synthesized and characterized by ^{31}P , ^{13}C , ^1H NMR and FT–IR spectral techniques. The toxicity of 36 new Tem derivatives was screened on the third instar larvae and the compound compound 1,2 cyclohexane-N,N'-bis(N,N'-piperidine phosphoramidate) exhibited the highest insecticidal potential. The method of kinetic analysis is applied in order to obtain the maximum velocity (V_{max}), the Michaelis constant (K_{m}) and the parameters characterizing the inhibition type for inhibitors with >75% mortality in preliminary bioassay. The inhibition mechanism was mixed and inhibitory constant (K_i) was calculated as $4.70 \mu\text{M}^{-1}\text{min}^{-1}$ for this compound. Quantitative structure-activity relationship (QSAR) equations of these compounds indicated that the electron orbital energy has major effect on insecticidal properties.

Key words: QSAR, Biochemical properties, Acetylcholinesterase, Organophosphate

1. Introduction

AChE (EC 3.1.1.7) is a hydrolyzing enzyme that plays a vital role in cholinergic neurotransmission. This enzyme hydrolyses the neurotransmitter, acetylcholine (ACh) into choline and acetate [1]. AChE is the principal target of organophosphate (OP) and carbamate (CB) pesticides. These pesticides irreversibly bind to the serine amino acid in the active site of AChE. This inhibition results in sustained muscle impulses, insect convulsion, and even death [2]. Several biochemical and molecular studies have been focused to AChE properties in insect as regards the role of AChE in neurotransmission and its insensitivity to insecticides. So far, AChEs have been purified and characterized from some insect species including, *Diabrotica virgifera* [3], *Aphis gossypii* [4], *Spodoptera frugiperda* [5], *Bactrocera dorsalis* [6] and *Bombyx mandarina* [7]. Crude extracts from insect homogenization contain substantial amounts of contaminants and general esterases. These factors can affect the activity and biochemical properties of AChE in biochemical assay and one of the remedy for this problem is purification of AChE by chromatography techniques.

Due to the widespread and extensive use of pesticides in agriculture and health, its resistance has been significantly increased [2]. Therefore, by considering the resistance problem, numerous researches have focused on the need for safer, environmentally friendly pesticides and pesticides with novel mode of action. A rational method for achieving this objective is the application of QSAR (Quantitative Structure-Activity Relationship) models for the rapid prediction, saving costs and time for discovery of compound with new insecticidal activity. The QSAR is an appropriate tool in insecticide discovery to the extent that a mathematical equation was used to link the biological activity with a wide variety of physical or chemical parameters. So far, QSAR models have been successfully used for finding new compounds with pesticide activity [8; 9]

and some QSAR studies have been conducted for the screening of new organophosphates and carbamates [10; 11].

In this paper, an AChE from the elm leaf beetle was purified and its biochemical properties were characterized because of the importance of AChE inhibition by phosphoroamide (PHA) derivatives. . Furthermore, the median inhibitory concentration (I_{50}) and mechanism of inhibition of these compounds on AChE were determined after the screening of the PHA derivatives on third instar larvae. In addition, the relationship between their physico-chemical characteristics and insecticidal effect, as biological activity, were presented with equation.

2. Materials and Methods

2.1. Insect rearing

Eggs and larvae of *X. luteola* were collected from the elm tree in pesticide-free trees in the University of Guilan, Rasht, Iran. Insect colonies were reared in the laboratory conditions at 25 ± 2 °C; 16: 8 h (L: D); 75 ± 10 % RH in transparent plastic jars 10×20 cm [12].

2.2. Screening test

The synchronized third instars larvae were used in screening test. At the first, the concentrations of 5000 and 2500 mg/ml of PHA compounds, dissolved in DMSO: water (1:4), were prepared. Third instars larvae were immersed in solution made for 15 seconds. The treated insects were transferred on filter paper to drain. Control insects were dipped in DMSO: water (1:4). The treated insects were held in the laboratory conditions at 25 ± 2 °C; 16: 8 h (L: D); 75 ± 10 % RH in transparent plastic jars 10×20 cm with elm leaf as food. Mortality was counted after 24 h [13]. The compounds with mortalities higher than 75% were chosen and bioassayed again on third instar larvae.

2.3. Bioassays

The bioassay was conducted on the compounds with more than 75% mortality in the second concentration (i.e. 2500 mg/l) of screening test. The bioassay was carried out with concentrations of 310, 650, 1250 and 2500 mg/l and each third instar larvae were dipped in solution of different concentrations for 15 s. After that the insects drained on a filter paper and the treated insects were transferred to transparent plastic jars 10 × 20 cm containing fresh elm leaves. This experiment was repeated 5 times for each treatment and 10 larvae of the same age were used in each replicate. Mortality was recorded after 24 h and the LC₅₀s and their confidence limits were estimated using POLO-PC software [14].

2.4. Sample preparation and enzyme assays

The larvae alive after treatment with compounds 19, 30, 32 and 50 were homogenized in 200 µl phosphate buffer pH 7.0 containing triton X-100. The homogenates were centrifuged at 12000 ×g for 10 minutes at 4 °C. The supernatants were used as the enzyme source. AChE activity was determined according to Ellman *et al.* method [15] using acetylthiocholine iodide as substrate. Forty µl of supernatant was added to a tube containing 140 µl of phosphate buffer (20 mM; pH 7), 40 µl substrate (acetylthiocholine iodide), 20 µl DTNB. Increase in absorbance was recorded at 415 nm using a microplate reader (Awareness technology Stat fax 3200®). The enzyme velocity was converted to unit activity using the molar extinction coefficient of 13.6 mM cm⁻¹ for 5-thio-2-nitrobenzoate formed during the assay at 412 nm. AChE specific activity was expressed as units per mg of protein after measurement of protein by Bradford (1976) method [16], using bovine serum albumin (BSA) as standard. For esterase enzyme assay, 12.5 µl of supernatant was mixed with 50 µl of substrates (alpha-naphthyl acetate (α-NA) or beta-naphthyl acetate (β-NA)), 112.5 µl of the phosphate buffer and 50 µl of Fast Blue RR salt and the naphthol production was

monitored by measuring absorbance at 450 and 540 nm for α -NA and β -NA, respectively, using a microplate reader (Awareness Technology Inc, Stat Fax 3200®) as a kinetic mode [17].

2.5. Procedure for the synthesis of compounds

The synthesized method of compounds **12-24** and compounds **25-44** was presented to the references [18] and [19], respectively.

2.5.1. General synthesis

Synthesis of bis phosphorus amido chalcogenides ligands with skeleton P (O) -NH-P (O):

The compound (C₆H₅O) 2P (O) NH (1 mmol) is added to a solution of 30 ml dry carbon tetrachloride with solvent (1 mmol) of phosphorous penta chloride gas. The reaction mixture is reflux for 18 h. After cooling the product reflux, (1 mmol) of formic acid is added to it. After 2 h of mixing of the solution to air at room temperature, the solvent was thrown and white precipitate of 30 ml carbon tetrachloride washing net (C₆H₅O) 2P (O) NHP (O) Cl was separated. In the next step, (1 mmol) of intermediate 2 acetonitrile or tetrahydrofuran amine (4 mmol) is added to 10 mL of solvent and was stirred for 8 hours at -5 to -8°C. Then mixing the solution to air deposition and thrown solvent and by washing with water, it was purified (Scheme 1)

(C₆H₅O)₂P(O)NHP(O)Cl₂ (1)

Powder sample; m.p. 163–165 °C. ¹H NMR (500.13 MHz, d₆-DMSO, 25°C, TMS); δ= 7.18 (t, 2 H, *para*-phenyl), 7.24 (d, 4 H, *ortho*-phenyl), 7.37 (t, 4 H, *meta*-phenyl), 8.33 (b, 1 H, NH) ppm. ¹³C NMR (125.77 MHz, d₆-DMSO, 25°C, TMS); δ= 120.41 (d, ³J_{PC} = 4.8 Hz, 4 C, *ortho*-phenyl), 124.72 (s, 2 C, *para*-phenyl), 129.45 (s, 4 C, *meta*-phenyl), 150.56 (d, ²J_{PC} = 6.7 Hz, 2 C, *ipso*-phenyl) ppm. ³¹P{¹H} and ³¹P NMR (202.45 MHz, d₆-DMSO, 25°C, H₃PO₄ external); δ= -3.87 (d, ²J_{PP} = 9.3 Hz), -7.10 (d, ²J_{PP} = 9.6 Hz) ppm. IR (KBr): $\tilde{\nu}$ = 2940.5 (N–H), 2720.1 (s), 1595.3 (s), 1491.7 (s), 1391.8 (s), 1256.7 (P=O), 1193.4 (P=O), 1008.5 (P–O), 762.8 (P–N), 497.3 (P–Cl).

(C₆H₅O)₂P(O)NHP(O)(NH₂)₂ (2)

powder sample; ¹H NMR (250.13 MHz, d₆-DMSO, 25°C, TMS); δ= 5.53 (m, 4 H, NH₂), 7.19 (t, 2 H, *para*-phenyl), 7.27 (d, 4 H, *ortho*-phenyl), 7.42 (t, 4 H, *meta*-phenyl), 8.33 (b, 1 H,

NH) ppm. ^{13}C NMR (62.89 MHz, d6-DMSO, 25°C, TMS); δ = 120.82 (d, $^3J_{\text{PC}}$ = 7.8 Hz, 4 C, *ortho*-phenyl), 125.13 (s, 2 C, *para*-phenyl), 129.79 (s, 4 C, *meta*-phenyl), 151.00 (d, 2 C, *ipso*-phenyl) ppm. $^{31}\text{P}\{^1\text{H}\}$ and ^{31}P NMR (101.25 MHz, d6-DMSO, 25°C, H_3PO_4 external); δ = -7.33 (d, $^2J_{\text{PP}}$ = 9.4 Hz), -9.35 (d, $^2J_{\text{PP}}$ = 9.8 Hz) ppm. IR (KBr): $\tilde{\nu}$ = 3483.7 (N-H), 3185.1 (N-H), 3063.4 (s), 1592.4 (s), 1488.6 (s), 1332.3 (s), 1219.6 (P=O), 1149.1 (P=O), 1027.0 (P-O), 932.1 (P-N).

(C₆H₅O)₂P(O)NHP(O)(NHC₂H₅)₂ (3)

powder sample; m.p. 143–145 °C. ^1H NMR (250.13 MHz, d6-DMSO, 25°C, TMS); δ = 1.21–1.23 (m, 6 H, NHC₂H₅), 2.73 (m, 4 H, NHC₂H₅), 4.99 (m, 4 H, NH), 7.15 (t, 2 H, *para*-phenyl), 7.29 (d, 4 H, *ortho*-phenyl), 7.32 (t, 4 H, *meta*-phenyl), 8.30 (b, 1 H, NH) ppm. ^{13}C NMR (62.89 MHz, d6-DMSO, 25°C, TMS); δ = 11.51 (s, 2 C, NHC₂H₅), 34.82 (s, 2 C, NHC₂H₅), 121.24 (d, $^3J_{\text{PC}}$ = 4.7 Hz, 4 C, *ortho*-phenyl), 124.47 (s, 2 C, *para*-phenyl), 129.75 (s, 4 C, *meta*-phenyl), 151.01 (d, 2 C, *ipso*-phenyl) ppm. $^{31}\text{P}\{^1\text{H}\}$ and ^{31}P NMR (101.25 MHz, d6-DMSO, 25°C, H_3PO_4 external); δ = -7.36 (d, $^2J_{\text{PP}}$ = 11.7 Hz), -8.55 (d, $^2J_{\text{PP}}$ = 11.9 Hz) ppm. IR (KBr): $\tilde{\nu}$ = 3153.1 (N-H), 2980.5 (s), 1593.3 (s), 1488.9 (s), 1359.9 (s), 1252.0 (P=O), 1202.4 (P=O), 1013.9 (P-O), 931.5 (P-N), 767.9 (P-N).

(C₆H₅O)₂P(O)NHP(O)(NHC₃H₇)₂ (4)

powder sample; m.p. 154–156 °C. ^1H NMR (250.13 MHz, d6-DMSO, 25°C, TMS); ^{13}C NMR (62.89 MHz, d6-DMSO, 25°C, TMS); δ = 11.50 (s, 2 C, NHC₃H₇), 21.19 (s, 2 C, NHC₃H₇), 39.00 (s, 2 C, NHC₃H₇), 121.21 (d, $^3J_{\text{PC}}$ = 4.7 Hz, 4 C, *ortho*-phenyl), 124.56 (s, 2 C, *para*-phenyl), 129.79 (s, 4 C, *meta*-phenyl), 151.81 (d, 2 C, $^2J_{\text{PC}}$ = 6.9 Hz, *ipso*-phenyl) ppm. $^{31}\text{P}\{^1\text{H}\}$ and ^{31}P NMR (101.25 MHz, d6-DMSO, 25°C, H_3PO_4 external); δ = -7.30 (m), -7.82 (m)

ppm. IR (KBr): $\tilde{\nu}$ = 3063.9 (N–H), 2968.5 (s), 1595.0 (s), 1490.8 (s), 1349.4 (s), 1215.6 (P=O), 1205.6 (P=O), 1010.4 (P–O), 937.6 (P–N), 763.1 (P–N).

(C₆H₅O)₂P(O)NHP(O)[N(CH₃)₂]₂ (5)

powder sample; m.p. 166–168 °C. ¹H NMR (250.13 MHz, d₆–DMSO, 25°C, TMS); δ = 2.40 (m, 12 H, N(CH₃)₂), 7.17 (t, 2 H, phenyl), 7.32 (t, 8 H, phenyl), 8.35 (b, 1 H, NH) ppm. ¹³C NMR (62.89 MHz, d₆–DMSO, 25°C, TMS); δ = 27.02 (s, 4 C, N(CH₃)₂), 121.12 (d, ³J_{PC} = 4.6 Hz, 4 C, *ortho*–phenyl), 124.67 (s, 2 C, *para*–phenyl), 129.36 (s, 4 C, *meta*–phenyl), 151.65 (d, 2 C, *ipso*–phenyl) ppm. ³¹P{¹H} and ³¹P NMR (101.25 MHz, d₆–DMSO, 25°C, H₃PO₄ external); δ = -7.23 (d, ²J_{PP} = 9.9 Hz), -8.14 (d, ²J_{PP} = 9.7 Hz) ppm. IR (KBr): $\tilde{\nu}$ = 3112.6 (N–H), 2757.1 (s), 1592.9 (s), 1491.0 (s), 1342.2 (s), 1244.6 (P=O), 1192.4 (P=O), 1052.3 (P–O), 932.6 (P–N), 771.3 (P–N).

(C₆H₅O)₂P(O)NHP(O)[N(C₂H₅)₂]₂ (6)

powder sample; m.p. 164–167 °C. ¹H NMR (250.13 MHz, d₆–DMSO, 25°C, TMS); δ = 1.18 (m, 12 H, N(C₂H₅)₂), 2.80 (m, 8 H, N(C₂H₅)₂), 7.17 (t, 2 H, phenyl), 7.29–7.33 (t, 8 H, phenyl), 8.25 (b, 1 H, NH) ppm. ¹³C NMR (62.89 MHz, d₆–DMSO, 25°C, TMS); δ = 11.46 (s, 4 C, N(C₂H₅)₂), 41.62 (s, 4 C, N(C₂H₅)₂), 121.17 (d, 4 C, *ortho*–phenyl), 124.65 (s, 2 C, *para*–phenyl), 129.81 (s, 4 C, *meta*–phenyl), 150.65 (d, 2 C, *ipso*–phenyl) ppm. ³¹P{¹H} and ³¹P NMR (101.25 MHz, d₆–DMSO, 25°C, H₃PO₄ external); δ = -7.38 (d, ²J_{PP} = 8.9 Hz), -8.63 (d, ²J_{PP} = 8.2 Hz) ppm. IR (KBr): $\tilde{\nu}$ = 3066.1 (N–H), 2706.6 (s), 1595.5 (s), 1492.2 (s), 1356.9 (s), 1247.6 (P=O), 1200.9 (P=O), 1005.0 (P–O), 936.5 (P–N), 763.5 (P–N).

(C₆H₅O)₂P(O)NHP(O)[N(C₄H₉)₂]₂ (7)

oil sample; ^1H NMR (250.13 MHz, d6-DMSO, 25°C, TMS); δ = 0.85 (m, 12 H, N(C₄H₉)₂), 1.27 (m, 8 H, N(C₄H₉)₂), 1.57 (m, 8 H, N(C₄H₉)₂), 2.74 (m, 8 H, N(C₄H₉)₂), 7.17 (t, 2 H, phenyl), 7.31–7.33 (t, 8 H, phenyl), 8.29 (b, 1 H, NH) ppm. ^{13}C NMR (62.89 MHz, d6-DMSO, 25°C, TMS); δ = 14.07 (s, 4 C, N(C₄H₉)₂), 20.04 (s, 4 C, N(C₄H₉)₂), 28.64 (s, 4 C, N(C₄H₉)₂), 47.25 (s, 4 C, N(C₄H₉)₂), 121.17 (d, $^3J_{\text{PC}}$ = 4.7 Hz, 4 C, *ortho*-phenyl), 124.63 (s, 2 C, *para*-phenyl), 129.78 (s, 4 C, *meta*-phenyl), 151.65 (d, 2 C, *ipso*-phenyl) ppm. $^{31}\text{P}\{^1\text{H}\}$ and ^{31}P NMR (101.25 MHz, d6-DMSO, 25°C, H₃PO₄ external); δ = -7.34 (d, $^2J_{\text{PP}}$ = 9.1 Hz), -8.50 (d, $^2J_{\text{PP}}$ = 9.3 Hz) ppm. IR (KBr): $\tilde{\nu}$ = 3412.7 (N–H), 2961.8 (s), 2871.5 (s), 1594.8 (s), 1491.1 (s), 1386.8 (s), 1258.3 (P=O), 1198.7 (P=O), 1011.3 (P–O), 929.2 (P–N), 769.6 (P–N).

(C₆H₅O)₂P(O)NHP(O)(NC₄H₈O)₂ (8)

oil sample; ^1H NMR (500.13 MHz, d6-DMSO, 25°C, TMS); δ = 2.91 (m, 8 H, NC₄H₈O), 3.69 (m, 8 H, NC₄H₈O), 7.12 (t, 2 H, phenyl), 7.27 (d, 4 H, phenyl), 7.31 (t, 4 H, phenyl), 8.10 (b, 1 H, NH) ppm. ^{13}C NMR (125.75 MHz, d6-DMSO, 25°C, TMS); δ = 42.71 (s, 4 C, NC₄H₈O), 63.43 (s, 4 C, NC₄H₈O), 120.59 (d, $^3J_{\text{PC}}$ = 4.7 Hz, 4 C, *ortho*-phenyl), 124.14 (s, 2 C, *para*-phenyl), 129.30 (s, 4 C, *meta*-phenyl), 151.14 (d, $^2J_{\text{PC}}$ = 6.5 Hz, 2 C, *ipso*-phenyl) ppm. $^{31}\text{P}\{^1\text{H}\}$ and ^{31}P NMR (202.45 MHz, d6-DMSO, 25°C, H₃PO₄ external); δ = -4.81 (m), -5.43 (m) ppm. IR (KBr): $\tilde{\nu}$ = 3036.9 (N–H), 2724.3 (s), 1598.2 (s), 1486.8 (s), 1382.6 (s), 1250.3 (P=O), 1165.4 (P=O), 1074.2 (P–O), 919.1 (P–N), 774.0 (P–N).

(C₆H₅O)₂P(O)NHP(O)(NH–(NC₄H₈O)₂) (9)

powder sample; m.p. 183–186 °C. ^1H NMR (500.13 MHz, d6-DMSO, 25°C, TMS); δ = 2.44 (m, 8 H, NC₄H₈O), 3.34 (m, 8 H, NC₄H₈O), 5.46 (d, $^2J_{\text{PNH}}$ = 7.2 Hz, 2 H, NH), 7.08 (t, 2 H, phenyl), 7.21–7.31 (m, 4 H, phenyl), 7.36–7.39 (m, 4 H, phenyl), 8.47 (b, 1 H, NH) ppm. ^{13}C

NMR (125.76 MHz, d6-DMSO, 25°C, TMS); δ = 55.33 (s, 4 C, NC₄H₈O), 65.59 (s, 4 C, NC₄H₈O), 121.78 (d, 4 C, *ortho*-phenyl), 124.14 (s, 2 C, *para*-phenyl), 130.52 (s, 4 C, *meta*-phenyl), 151.48 (d, 2 C, *ipso*-phenyl) ppm. ³¹P{¹H} and ³¹P NMR (202.45 MHz, d6-DMSO, 25°C, H₃PO₄ external); δ = -5.23– -5.36 (m) ppm. IR (KBr): $\tilde{\nu}$ = 3112.6 (N–H), 2967.9 (s), 2861.9 (s), 1591.4 (s), 1492.3 (s), 1369.4 (s), 1241.2 (P=O), 1200.6 (P=O), 1001.0 (P–O), 936.2 (P–N), 758.4 (P–N).

(C₆H₅O)₂P(O)NHP(O)(NH–(NC₅H₁₀)₂) (10)

powder sample; m.p. 170–172 °C. ¹H NMR (500.13 MHz, d6-DMSO, 25°C, TMS); δ = 1.34 (m, 4 H, NC₅H₁₀), 1.57 (m, 8 H, NC₅H₁₀), 2.86 (m, 8 H, NC₅H₁₀), 7.11 (t, 2 H, phenyl), 7.26 (d, 4 H, phenyl), 7.31 (t, 4 H, phenyl), 8.00 (b, 1 H, NH) ppm. ¹³C NMR (125.75 MHz, d6-DMSO, 25°C, TMS); δ = 23.86 (s, 2 C, NC₅H₁₀), 55.38 (s, 4 C, NC₅H₁₀), 57.61 (s, 4 C, NC₅H₁₀), 121.76 (d, 4 C, *ortho*-phenyl), 124.02 (s, 2 C, *para*-phenyl), 130.46 (s, 4 C, *meta*-phenyl), 151.58 (d, 2 C, *ipso*-phenyl) ppm. ³¹P{¹H} and ³¹P NMR (202.45 MHz, d6-DMSO, 25°C, H₃PO₄ external); δ = -5.21 (m) ppm. IR (KBr): $\tilde{\nu}$ = 3371.6 (N–H), 3207.3 (s), 2865.5 (s), 1595.6 (s), 1490.3 (s), 1315.6 (s), 1244.1 (P=O), 1198.3 (P=O), 1064.3 (P–O), 932.3 (P–N), 764.8 (P–N).

(C₆H₅O)₂P(O)NHP(O)(NH(C₆H₅)₂) (11)

powder sample; m.p. 135–137 °C. ¹H NMR (250.13 MHz, d6-DMSO, 25°C, TMS); δ = 6.54–6.65 (m, 2 H, N–H), 7.06 (t, 2 H, phenyl), 7.22 (t, 2 H, phenyl), 7.27 (m, 8 H, phenyl), 8.30 (m, 4 H, phenyl), 8.41 (m, 4 H, phenyl), 8.40 (b, 1 H, NH) ppm. ¹³C NMR (62.89 MHz, d6-DMSO, 25°C, TMS); 114.05 (m, 4 C, *ortho*-HNC₆H₅), 120.37 (d, ³J_{PC} = 4.8 Hz, 4 C, *ortho*-OC₆H₅), 124.67 (m, 2 C, *para*-OC₆H₅), 128.71 (m, 2 C, *para*-HNC₆H₅), 129.50 (m, 4 C, *meta*-OC₆H₅), 130.05 (m, 4 C, *meta*-HNC₆H₅), 136.48 (s, 2 C, *ipso*-HNC₆H₅), 150.48 (d, ²J_{PC} = 6.6 Hz, 2 C,

ipso-OC₆H₅) ppm. ³¹P{¹H} and ³¹P NMR (101.25 MHz, d₆-DMSO, 25 °C, H₃PO₄ external); δ = -6.44 (d, ²J_{PP} = 9.9 Hz), -9.53 (d, ²J_{PP} = 9.9 Hz) ppm. IR (KBr): $\tilde{\nu}$ = 3020.5 (N-H), 2926.4 (s), 1592.1 (s), 1491.8 (s), 1360.9 (s), 1250.8 (P=O), 1202.0 (P=O), 1047.6 (P-O), 947.5 (P-N), 760.2 (P-N).

2.6. AChE purification

Synchronized third instar larvae were homogenized in phosphate buffer (20 mM; pH 7.0) containing 0.1% triton X-100 using a hand-held glass homogenizer. The homogenate was centrifuged at 14,000 ×g for 30 min at 4 °C twice. Supernatant was used for AChE purification.

The procainamide-based AChE affinity column was used for purification of AChE. The matrix was prepared based on the method of Pasteur et al. (1997) [20] using ECH Sepharose 4B. Procainamide (1.36 g procainamide in 10 ml water) were mixed with EEDQ solutions (0.124 g EEDQ in 10 ml ethanol) and then 20 ml ECH Sepharose 4B were added. The coupling reaction was done by gently mixing the solution at 4 °C for 12 h. The procainamide-coupled Sepharose 4B was packed to a 1×8.5 cm glass column and washed successively with 50% ethanol, 20 mM Tris-HCl buffer (pH 7.0), and 1 M NaCl solution. The column then was equilibrated with 0.05 M phosphate buffer (pH 7.0) containing 0.1% (v/v) triton X-100 and 0.05 M NaCl (PTS buffer) [21]. The supernatant loaded on a procainamide-based AChE affinity column and enzyme eluted with a flow rate of 0.5 ml/min. AChE activities in fractions were measured based on the Ellman *et al.* method [15] as mentioned above.

2.6.1. Determination of pH optimum and effect of temperature on AChE activity

The pH profiles of the AChE were characterized in a universal buffer comprising phosphate, borate and acetate (40 mM of each) adjusted to various pHs (pH 3 to 12) by adding HCl or NaOH for acidic and basic pH values, respectively. Before determining AChE activity, the en-

zyme and buffer mixtures were incubated at different pHs for 4 min. Then the enzyme activity was measured as kinetic mode based on the Ellman *et al.* method [15]. Also, the activity of AChE was measured by incubating the reaction mixtures in optimal pH value (20 mM phosphate buffer) at different temperatures ranging from 15 to 65 °C with 10 °C intervals.

2.6.2. Inhibition assay of insect AChE

Inhibition assays were conducted by the pre-incubating the purified AChE with different concentrations of PHA derivatives for 10 minutes. Afterward, the substrate was added to the reaction mixture containing phosphate buffer, pH 7.0 and DTNB. Residual activity was measured kinetically at 412 nm as mentioned above. Median inhibition concentration (IC_{50}) and IC_{25} were calculated by probit analysis using the POLOPC software [14].

2.6.3. Mechanism of enzyme inhibition by compounds

To investigate the mechanism of inhibition, the effect of IC_{50} and IC_{25} concentrations of each inhibitor were investigated on AChE activity in presence of different concentrations of the substrate (10, 5, 2.5, 1.25 and 0.625 mM). The K_m and V_{max} values in presence and absence of inhibitors were estimated from Lineweaver–Burk plots. Also, the inhibitory constant K_i is calculated [22].

2.7. Statistical analysis

The LC_{50} values and 95% confidence intervals were calculated from probit regressions using the POLO-PC computer program [14]. One-way analysis of variance (ANOVA) was employed for data analysis [23]. Differences between treatments were evaluated using Tukey's test and were considered significant when the probability level $p \leq 0.05$.

QSAR studies were conducted for identifying the effect of physicochemical parameters on the AChE inhibition activity [24]. The stepwise multiple linear regression procedure is a popular me-

thod in QSAR studies for selection descriptors. The MLR method was performed by the software package SPSS 16.0 for selection of the descriptors [25]. The electronic and structural descriptors are obtained by either the quantum chemical calculations, theoretical and experimental studies. The electronic descriptors comprised the energy of frontier orbital (E_{HOMO} and E_{LUMO}), electrophilicity (ω), polarizability (PL), the charge difference between the atoms in functional groups and the net atomic charges (Q). Similarly, the structural descriptors are hydrophobic coefficient ($\log P$), dipole moment (μ) and molecular volume (Mv). The toxicities of phosphorhydrazide analogues are expressed in terms of $\log(1/I_{50})$ as an anti-cholinesterase activity. The descriptor values were related with toxicity using MLR analysis. MLR of descriptors selected for biological activity, leads to the problem of multicollinearity. This problem can be solved by using the principal component analysis (PCA). These linear combinations form a new set of variables, called principal components (PCs), which are mutually orthogonal. The first PC contains the largest variance and the second new variable contains the second largest variance, and so on. The variable selection in this PCA study was done by using the Fisher's weights. The descriptors with higher correlation coefficient and lower correlation ($|r| < 0.5$) to $\log(1/I_{50})$ were selected to carry out stepwise MLR analysis and to optimize the QSAR equation [26]. All quantum chemical calculations were carried out by using the Gaussian 03 program package [27].

3. Results

3.1. Spectroscopic study

The ^{31}P NMR chemical shift at room temperature in DMSO appears as two separated peaks. For example, $\delta^{31}\text{P}$ and $^2J_{\text{PP}}$ values of compounds $(\text{C}_6\text{H}_5\text{O})_2\text{P}(\text{O})\text{--NH--P}(\text{O})(\text{NHC}_2\text{H}_5)_2$ (**3**) and $(\text{C}_6\text{H}_5\text{O})_2\text{P}(\text{O})\text{--NH--P}(\text{O})(\text{NHC}_6\text{H}_5)_2$ (**11**) reveals in the rang -7.36 ppm and $^2J_{\text{PP}} = 11.7$ Hz on

other -8.55 ppm and $^2J_{PP} = 11.9$ Hz for **3** as well as in the rang -6.44 ppm and $^2J_{PP} = 9.9$ Hz on other -9.53 ppm and $^2J_{PP} = 9.9$ Hz for **11** (Fig. 1).

Fig. 1. The ^{31}P NMR chemical shift at room temperature in DMSO

Acidic proton of N-H group of P(O)–NH–P(O) moiety in ^1H NMR spectra of compounds **9** and **10** exhibited as a broad peak due to resonance between two phosphoryl groups in the range 8.00 ppm to 8.47 ppm, respectively. The analysis of the IR spectra indicated that the fundamental $\nu(\text{P}=\text{O})$ stretching modes for compounds **1–11** appeared at the range 1202–1250 cm^{-1} . Moreover, the N–H stretching frequencies for all compounds were observed at the range of 2940–3483 cm^{-1} .

3.2. Screening compounds on third instar larvae of *X. luteola*

In this research, the toxicities of 36 temephos derivative (Scheme 2) were bioassayed on *X. luteola* larvae in order to find novel insecticide. Screening test with concentration of 5000 mg/l indicated that twelve compounds exhibited mortality more than fifty percent. The result of the second screening test revealed that when the concentration of 2500 mg/l was used, four compounds had more than 75% mortality (Table 1).

24	(CH ₃ CH ₂ O) ₂ P(O)N(C ₆ H ₁₀)NP(O)(OCH ₂ CH ₃) ₂	65	30	25.17	[18]
28	(C ₆ H ₅ O) ₂ P(O)NH-(CH ₂) ₂ -N[P(O)(OC ₆ H ₅) ₂]N-(CH ₂) ₂ -HNP(O)(OC ₆ H ₅) ₂	50	10	95.11	[19]
28	(C ₆ H ₅ O) ₂ P(O)NH-(CH ₂) ₂ -OP(O)(OC ₆ H ₅) ₂	40	10	104.70	[19]
30	(C ₆ H ₅ O) ₂ P(O)NH-CH(CH ₃)-CH ₂ -HNP(O)(OC ₆ H ₅) ₂	85	85	18.88	[19]
32	(C ₆ H ₅ O) ₂ P(O)N(CH ₃)-(CH ₂) ₂ -(CH ₃)NP(O)(OC ₆ H ₅) ₂	95	90	8.03	[19]
33	(C ₆ H ₅ O) ₂ P(O)NH-(CH ₂) ₃ -HNP(O)(OC ₆ H ₅) ₂	40	10	99.46	[19]
34	(C ₆ H ₅ O) ₂ P(O)NH-CH ₂ -C(CH ₃) ₂ -CH ₂ -HNP(O)(OC ₆ H ₅) ₂	30	-	205.50	[19]
35	(C ₆ H ₅ O) ₂ P(O)NH-(CH ₂) ₄ -HNP(O)(OC ₆ H ₅) ₂	10	-	163.99	[19]
37	(C ₆ H ₅) ₂ P(O)NH-HNP(O)(C ₆ H ₅) ₂	50	10	86.46	[19]
38	(C ₆ H ₅) ₂ P(O)NH-(CH ₂) ₂ -HNP(O)(C ₆ H ₅) ₂	60	15	252.62	[19]
39	(C ₆ H ₅) ₂ P(O)NH-CH(CH ₃)-CH ₂ -HNP(O)(C ₆ H ₅) ₂	20	-	99.46	[19]
40	(C ₆ H ₅) ₂ P(O)N(CH ₃)-(CH ₂) ₂ -(CH ₃)NP(O)(C ₆ H ₅) ₂	30	-	208.24	[19]
41	(C ₆ H ₅) ₂ P(O)NH-(CH ₂) ₃ -HNP(O)(C ₆ H ₅) ₂	10	-	150.24	[19]
42	(C ₆ H ₅) ₂ P(O)NH-CH ₂ -C(CH ₃) ₂ -CH ₂ -HNP(O)(C ₆ H ₅) ₂	10	-	152.17	[19]
43	(C ₆ H ₅) ₂ P(O)NH-(CH ₂) ₄ -HNP(O)(C ₆ H ₅) ₂	5	-	114.63	[19]
44	(C ₆ H ₅) ₂ P(O)N(C ₄ H ₈)NP(O)(C ₆ H ₅) ₂	10	-	151.23	[19]
45	(C ₆ H ₅) ₂ P(O)NH(C ₆ H ₁₀)HNP(O)(C ₆ H ₅) ₂	60	20	20.18	[19]
47	(C ₆ H ₅) ₂ P(O)O-(CH ₂) ₂ -OP(O)(C ₆ H ₅) ₂	5	-	184.11	[19]
49	(C ₄ H ₈ N) ₂ P(O)NH(C ₆ H ₆)NHP(O)(NC ₄ H ₈)	80	30	18.53	[19]
50	(C ₅ H ₁₀ N) ₂ P(O)NH(C ₆ H ₆)NHP(O)(NC ₅ H ₁₀)	100	95	6.15	[19]
51	(C ₆ H ₁₂ N) ₂ P(O)NH(C ₆ H ₆)NHP(O)(NC ₆ H ₁₂)	85	40	27.22	[19]

^a This work

In the second bioassay, only the toxicities of four compounds were evaluated on last instar larvae (Table 2). Among these compounds, compound 50 with the lowest I₅₀ has revealed the best insecticidal properties as the LC₅₀ of it was lesser.

Table 2. Susceptibility of last larval instar of *X. luteola* to temephos derivative compounds after 24 h.

Compounds	Number of treated larvae	LC ₅₀ (mg/l) (95% confidence limit)	Slope± SE	X ² (df)
19	250	1423.05 (998.06-1858.05)	1.19±0.31	1.87 (2)
30	250	1230.40 (931.16-1663.17)	1.84±0.3	1 (2)
32	250	952.61 (621.13-1015.44)	1.78 ±0.29	0.07 (2)
50	250	511.47 (341.26-674.06)	1.9 ± 0.3	1.5 (2)

As these compounds affected AChE and general esterases, so the activities of these enzymes were determined in the larvae treated with different concentrations (Fig. 2).

Fig. 2. Inhibition (%) of AChE (A), α - esterase (B) and β - esterase (C) form third instar larvae treated with different concentrations in vivo conditions. The inhibition percentage was calculated as follow: [(activity in control – activity in treated insect)/activity in control] *100.

* Means followed by different letters within columns indicate significant differences between concentrations, Tukey's test ($p < 0.05$).

3.3. AChE purification

Some non target serine esterases, the carboxylesterases, confer protection against toxicants. Sometimes, these protective esterases are sensitive to inhibition by organophosphates, and at some cases, even more sensitive than the AChE at target site. These enzymes demonstration limited kinetic efficiency and serves as pesticide sequestration. With the use of chromatography, the impurities in homogenate crude were easily removed from the extraction sample. Due to inhibition of general esterases by temephos derivative compounds, the AChE was purified using affinity chromatography. Therefore, in gathering of information regarding the effect of temephos derivative compounds on the AChE, it has been purified by affinity chromatography and the results obtained were summarized in Table 3 and Fig. 3. As shown in table 3, AChE has been purified up to 75.29 times.

Table 3. Purification of AChE from *X. luteola* by procainamide- based affinity chromatography

Purification steps	Total Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	Total activity ($\mu\text{mol}/\text{min}$)	(%) Product	Purification factor
Crude	5	5.30	26.5	0.204	5.413	100	1
Affinity chromatography	2	0.015	0.03	15.36	0.461	8.51	75.29

Fig. 3. Distribution pattern of protein and AChE activity over the fractions obtained following the affinity chromatography of crude enzyme. Fraction no. 6.0 contained the highest enzymatic activity using ATChI as the substrate.

To determine the molecular weight of purified enzyme, sample was charged in SDS-electrophoresis gel accompanied by protein marker. The single band was detected in the gel and the molecular mass of the purified AChE was calculated 70 kD (Fig.4).

Fig.4. The SDS-PAGE of AChE purification from *X. luteola*.
Marker; crude extract; affinity chromatography, respectively.

3.3.1. PH and temperature effects on AChE activity

The effects of pH on AChE activity purified from *X. luteola* showed high peak at pH 7.0 (Fig. 5). Significant difference in the AChE activity was found between the pHs 6.0, 7.0 and 8.0. In

higher or lower than optimal pH, enzyme activity decreased significantly and at pH 10 was decreased to less than 30%. AChE exhibited the most activity at 35 °C and approximately 70% of its activity was maintained at 45 °C. In other temperatures examined in this research, enzyme activity was reduced to less than 20%.

Fig. 5. The effect of pHs (A) and temperatures (B) on the mean relative activities of AChE purified from *X. luteola*. Different letters indicate that the activity of enzyme in different pH are significantly different from each other by Tukey's test ($p < 0.05$).

3.3.2. Determination of I_{50} , kinetic parameters and mechanism of inhibition

Inhibitory effects of temephos derivatives on purified enzyme were examined and median inhibition concentrations were expressed in Table 4 using the crude extract and purified AChE as enzyme source.

Table 4. Inhibitory effect (IC_{50} values) of different inhibitors on crude extract and purified AChE from *X. luteola*

Compound	I_{50} of crude (95% confidence limit) mg/l	I_{50} of purified enzyme (95% confidence limit) mg/l
19	20.17 (16.10-25.36)	9.19 (8.01-12.31)
30	18.88 (16.43-20.54)	6.24 (3.50-6.94)
32	8.03 (6.99-9.66)	2.96 (1.08-3.46)
50	6.15 (5.63-8.22)	2.02 (1.55-3.78)

The ratio of I_{50} of crude extracts to I_{50} of purified enzyme was 2.19, 2.9, 2.17 and 3.40 for the compound No. **19**, **30**, **32** and **50**, respectively. The insect general esterases are sensitive to inhi-

bition by organophosphates and also, these enzymes hydrolyze the acetylthiocholine iodide, AChE substrate. These reasons can explain the differences in I_{50} s obtained by crude extract and purified AchE. The effect of two concentrations of compounds, i.e. I_{25} and I_{50} , are examined on AChE activity in order to determine the type of inhibitory mechanisms and kinetic parameters are determined for each of them by Hyper software. With regard to these parameters, inhibition type and K_i of inhibitors were calculated and the results were presented in Table 5.

Table 5. Kinetic parameters and inhibitory effect of IC₂₅ and IC₅₀ of temephos derivatives on the AChE purified from *X. luteola*

Compounds		K_m^{app} μM	V_{max}^{app} $\mu m/min/mg$ protein	K_i $\mu M^{-1} min^{-1}$
	control	60.19±1.32	52.40±1.18	-
19	IC ₂₅	135.12±2.21	39.87±1.54	0.625
	IC ₅₀	94.74±1.38	20.09±2.22	
30	IC ₂₅	95.62±2.4	30.37±1.67	1.04
	IC ₅₀	72.04±0.73	16.15±1.05	
32	IC ₂₅	99.05±1.58	25.21±2.34	3.69
	IC ₅₀	65.76±1.70	12.29±0.74	
50	IC ₂₅	89.83±2.52	19.92±1.12	4.70
	IC ₅₀	64.81±1.03	10.79±0.85	

3.4. QSAR analysis of Temephos derivatives

Different quantum chemical properties as biological and affiliates descriptor were calculated using Gaussian software and Log (1/LC₅₀) parameter, are presented in Table 6.

Table 6. Quantum-chemical and theoretical descriptors for compounds computed by Gaussian 03 program.

No.	Electronic descriptors									Structural descriptors		log(1/I ₅₀)
	Charge				Polarizability		Frontier molecular orbital			Lipoph.	Steric	
	$Q_{P(1)}$ (a.u)	$Q_{P(2)}$ (a.u)	Q_X (a.u)	Q_N (a.u)	$PL_{P=X}$	PL_{N-H}	E_{HOMO}	E_{LUMO}	ω	μ (Debye)	Mv	
9	2.341	2.482	-1.112	-1.343	3.453	1.819	-0.252	-0.031	0.090	6.153	443.414	-1.95
6	2.399	2.481	-1.120	-1.346	3.519	1.795	-0.242	-0.030	0.087	8.545	300.932	-2.06
10	2.342	2.482	-1.117	-1.343	3.459	1.817	-0.247	-0.031	0.089	7.874	385.289	-1.88
12	1.995	1.993	-0.669	-1.018	2.664	1.448	-0.249	-0.011	0.142	2.640	257.626	-2.27
13	1.995	1.997	-0.673	-1.016	2.670	1.446	-0.248	-0.011	0.141	3.687	246.621	-2.27
14	2.015	2.016	-0.657	-0.893	2.673	----	-0.243	-0.005	0.128	3.927	277.857	-1.85
15	2.002	2.002	-0.670	-1.015	2.672	1.438	-0.249	-0.006	0.133	6.528	234.969	-1.99

16	2.007	2.009	-0.682	-1.021	2.691	1.445	-0.248	-0.011	0.141	1.981	302.083	-1.53
17	2.005	2.008	-0.681	-1.016	2.689	1.443	-0.246	-0.010	0.138	6.738	250.477	-2.22
18	2.026	2.028	-0.665	-0.895	2.693	----	-0.241	-0.006	0.065	1.585	345.518	-1.72
19	2.002	2.020	-0.679	1.032	2.879	1.453	-0.249	-0.005	0.132	8.640	334.661	-0.91
20	2.000	2.021	-0.663	-1.054	2.684	1.494	-0.247	-0.010	0.069	8.427	301.243	-1.50
21	2.470	2.472	-1.120	-1.031	3.592	1.453	-0.269	-0.008	0.147	7.228	284.028	-2.30
22	2.473	2.491	-1.128	-1.050	3.619	1.502	-0.259	-0.007	0.070	13.056	262.414	-2.40
23	2.481	2.481	-1.074	-0.892	3.555	----	-0.248	-0.006	0.066	0.055	260.397	-2.10
24	2.476	2.476	-1.121	-1.012	3.597	1.428	-0.266	-0.004	0.139	5.611	256.666	-1.41
28	2.457	2.465	-1.093	-1.021	3.560	1.453	-0.257	-0.031	0.091	6.428	322.689	-1.97
28	2.462	2.545	-1.090	-1.007	3.635	1.433	-0.260	-0.031	0.092	7.434	305.300	-2.01
30	2.465	2.466	-1.098	-1.026	3.559	1.451	-0.256	-0.031	0.091	6.492	319.661	-1.03
32	2.472	2.485	-1.095	-0.878	3.580	----	-0.213	-0.057	0.116	6.097	406.507	-0.78
33	2.467	2.477	-1.090	-1.010	3.567	1.439	-0.256	-0.030	0.090	8.430	379.314	-1.99
34	2.467	2.477	-1.109	-1.050	3.586	1.502	-0.254	-0.031	0.091	7.761	341.167	-2.13
35	2.458	2.467	-1.094	-1.015	3.561	1.435	-0.255	-0.030	0.091	4.123	380.173	-2.21
37	2.032	2.032	-1.119	-0.800	3.151	1.196	-0.265	-0.058	0.126	6.179	275.395	-1.93
38	2.030	2.049	-1.082	-1.012	3.112	1.417	-0.257	-0.055	0.120	8.542	327.663	-2.40
39	2.120	2.133	-1.084	----	3.193	----	-0.266	-0.058	0.126	5.386	369.664	-2.26
40	2.037	2.049	-1.085	-1.010	3.122	1.424	-0.256	-0.054	0.120	7.277	340.937	-1.99
41	2.053	2.067	-1.086	-0.868	3.139	----	-0.246	-0.055	0.118	4.265	370.064	-2.31
42	2.054	2.056	-1.120	-1.017	3.139	1.479	-0.257	-0.056	0.122	11.497	367.251	-2.17
43	2.056	2.061	-1.121	-0.016	3.136	1.474	-0.255	-0.056	0.121	10.918	428.576	-2.18
44	2.051	2.056	-1.065	-1.009	3.112	1.409	-0.256	-0.047	0.110	2.040	406.652	-1.82
45	2.071	2.071	-1.068	-0.874	3.139	----	-0.244	-0.049	0.110	0.268	382.940	-1.18
47	2.047	2.051	-1.088	-1.056	3.135	1.412	-0.251	-0.055	0.119	13.328	359.233	-1.30
49	2.371	2.375	-1.078	-0.967	3.455	0.973	-0.196	-0.018	0.064	7.202	398.202	-1.26
50	2.365	2.369	-1.084	-0.970	3.453	1.385	-0.198	-0.019	0.065	7.139	351.385	-1.01
51	2.363	2.371	-1.092	-0.989	3.463	1.388	-0.195	-0.017	0.065	6.585	491.352	-1.43

The next step is that the equation was calculated based on the relationship between structure-activity of temephos derivatives and inhibition of AChE from elm leaf beetle.

$$p(IC_{50}) = -2.436Q_{P(1)} - 2.191Q_{P(2)} + 3.754Q_X + 0.071Q_N + 4.333PL_{P=X} + 0.138PL_{N-H} + 9.879E_{HOMO} + 2.087E_{LUMO} - 1.047\omega - 0.014\mu + 0.001Mv - 0.748$$

$$n = 36; R^2 = 0.419; R_{Adj}^2 = 0.430; S_{reg} = 0.429; r = 0.637, F_{statistic} = 1.112 \quad (1)$$

In this equation the R^2 was high and regression error, r and the VIF were low, which signifies that the alignment error had been between independent descriptors. That is why PCA was used to eliminate this error.

$$PC1 = 0.442Q_{P(1)} + 0.447Q_{P(2)} - 0.409Q_X - 0.166Q_N + 0.472PL_{P=X} + 0.100E_{HOMO} - 0.091E_{LUMO} - 0.304\omega + 0.24848\mu + 0.314Mv \quad (2a)$$

$$PC2 = -0.230Q_{P(1)} - 0.217Q_{P(2)} - 0.324Q_X + 0.273Q_N + 0.02PL_{P=X} - 0.559E_{HOMO} - 0.656E_{LUMO} + 0.211\omega + 0.164\mu + 0.185Mv \quad (2b)$$

These two components have 43.58, and 25.08% of the total variance accounted for 100% of the variance. Therefore, the result of the above equation is that electron variables will have

greater effect than structural variables on the dependent descriptor. Based on the above described equation, the PL_{N-H} of modifiers will be eliminated, because the ratio is lower than 2.0. As such, the equation between the structures and biological activity will be achieved.

$$p(IC_{50}) = -1.870Q_{P(1)} - 2.071Q_{P(2)} + 4.997Q_X + 0.029Q_N + 4.502PL_{P=X} + 12.402E_{HOMO} - 5.767E_{LUMO} + 1.337\omega - 0.008\mu + 0.001Mv - 0.069$$

$$n = 36; R^2 = 0.615; S_{reg} = 0.407; r = 0.525, F_{statistic} = 2.011 \quad (3)$$

The equation also has high linearity error tolerance in order to eliminate this error. Descriptors $PL_{P=X}$ and $Q_{P(1)}$ which has a high VIF is also removed and 8, 24 and 34 of the compounds were deleted and the following equation was obtained.

$$p(IC_{50}) = 0.830Q_{P(2)} + 1.392Q_X + 0.398Q_N + 14.961E_{HOMO} - 11.798E_{LUMO} - 1.927\omega - 0.004\mu + 0.001Mv + 1.471$$

$$n = 33; R^2 = 0.892; S_{reg} = 0.315; r = 0.225, F_{statistic} = 3.121 \quad (4)$$

Table 7. VIF^a values of experimental and theoretical QSAR equations

Independent variables	Eq. 1	Eq. 3	Eq. 4
$Q_{P(1)}$	112.856	103.660	-
$Q_{P(2)}$	124.675	69.200	4.589
Q_X	81.905	76.640	7.784
Q_N	4.525	3.592	1.320
$PL_{P=X}$	308.119	294.236	-
PL_{N-H}	3.385	-	-
E_{HOMO}	3.333	1.888	1.340
E_{LUMO}	7.537	4.589	5.989
ω	3.921	1.947	2.003
μ	1.853	1.242	1.197
Mv	3.444	2.990	2.332

a $VIF = 1/(1 - R_i^2)$; where, R_i is the multiple correlation coefficient of the i th independent variable on all of the other independent variables.

One of the best statistical methods to communicate internally between independent variables and AChE inhibited compounds is correlation matrix that is presented in the table 8. Some of the regression coefficients were close to 0.70, showing that they were closely correlated. The evaluation of the correlation coefficient of E_{HOMO} versus μ ($r = -0.61$) shows that E_{HOMO} has the higher contribution to the dipole moment descriptor; this implies that the frontier molecular orbital controlled process plays a very important function in the interaction of the compounds with insect AChE. Thus, decreasing the energy of the HOMO can greatly increase the μ and subsequently the value of $P(\text{IC}_{50})$.

Table 8. Correlation matrix for $p(\text{IC}_{50})$ and selected parameters in eqn (4)

	$Q_{\text{P}(2)}$	Q_{X}	Q_{N}	E_{HOMO}	E_{LUMO}	ω	μ	M_v
$Q_{\text{P}(2)}$	1.000							
Q_{X}	-0.62	1.000						
Q_{N}	-0.31	0.23	1.000					
E_{HOMO}	0.19	0.16	-0.01	1.000				
E_{LUMO}	0.05	0.41	0.15	0.12	1.000			
ω	-0.57	0.34	0.24	0.17	-0.12	1.000		
μ	0.15	-0.33	0.51	-0.61	-0.23	-0.05	1.000	
M_v	0.2	-0.51	0.08	0.25	-0.58	-0.26	0.11	1.000

4. Discussion

4.1. The effect of compound on AChE and detoxification enzyme

Temephos is one of the renowned insecticides belonging to bis-phosphate family (Fig. 6). It is a non-systematic insecticide recommended for the control of a wide range of pests [28]

Fig. 6. Chemical structure of temephos

Due to the side effects, costly and time-consuming for the synthesis of new insecticide, it is necessary to find a suitable alternative compound for their replacement [29]. We recommend the designing and synthesis of new derivatives of the phosphate, in hopes of finding a compound with the lowest side effects and best insecticidal properties. The result indicated that all compounds could inhibit AChE activity more than the other enzymes, since the AChE is the key target for these compounds. Measuring enzyme's activities in treated larvae show that compound **50** exhibited higher inhibitory effect than other compounds. The most potent inhibitor to the least was **50>32>30>19**, as indicated by the relative enzyme activity values (Figs. 2), which show that AChE and general esterases are inhibited by these inhibitors. The *in vivo* inhibition results were dose dependent. This implies that increasing the concentration of inhibitors will decrease the enzymes activities. Esterases are the detoxification enzymes in insect which hydrolysis the external and internal compounds by adding a water molecule. These enzymes sequester or degrade insecticide esters before they get to their target sites on the nervous system [30].

4.2. Biochemistry of purified AChE

Some attempts have been made to purify or characterize the Insect's AChEs, and consequently, various information regarding their biochemical properties are currently available [5; 6; 7; 21; 31; 32; 33; 34]. In most cases, the affinity chromatography was employed for purifying this enzyme in some insects and the various isoforms with different molecular weights were detected by reducing and non-reducing gel electrophoresis. These insects typically have 2 or 3 isoforms in non-reduction gel with molecular weight between 130-140 KDa and one or more subunit in reduction gel with molecular weight between 60-94 KDa. In majority of these species, purification percentage during purification activity has been calculated above 200%. Thus, the SDS-PAGE analysis showed a single 70 kDa band for *X. luteola* as has been reported in the AChEs of other

insects. Therefore, the molecular weight of the AChE was nearly the same as those purified from *Drosophila melanogaster* (56 kDa) [35], *Haematobia irritans* (54 kDa) [36], *Leptinotarsa decemlineata* (65 kDa) [32; 37], *Rhyzopertha dominica* (56 kDa) [38; 39], *Galleria melonella* (60 kDa) [33], *Schizaphis graminum* (72 kDa) [21] and *Aphis gossypii* (63.5 kDa) [4].

Optimal pH for insect AChE activities was reported as 6 to 9.0. Activity of AChE purified from houseflies revealed high activities at pHs 7-9 [40], while the optimum pH for AChE purified from cotton aphid (*A. gossypii*) was 7.2 [4]. Activity of AChE in housefly increases with increase in temperature from 20 to 45 °C, but with much increase in temperature, it decreases greatly [40]. In the case of *A. gossypii*, it is similar to housefly and shows the highest activity at 45 °C. Temperature required for AChE maximum activity purified from *B.dorsalis* was estimated at 35 °C. Of course, there are significant differences between 35 and 45 °C, but with temperature increase up to 65 °C, enzyme activity decreases greatly [6].

V_{max}^{app} Values of compounds have decreased compared with the control situation and also, their K_m^{app} values have increased compared with control. These results indicate that inhibitory mechanisms of AChE by temephos compounds were mixed type. Among these compounds, compound 50 had the highest insecticidal effect and highest inhibitory potency on AChE, i.e high K_i . Thus, among this group of compounds, compound number 50 is a suitable model for designing of new compounds with insecticidal properties.

4.3. QSAR analysis

In equation 4, the inhibitory potential has a great influence on electronic descriptors like E_{HOMO} and E_{LUMO} molecular orbital parameters. E_{HOMO} and E_{LUMO} with a value of + 14.961 and - 11.798 are the most affected by inhibition parameter. Positive and negative sign shows that the two variables respectively increase and decrease the amount of E_{HOMO} and E_{LUMO} parameter

combinations, and also increased the inhibition of compound. The last equation reveals that descriptor ω , leaves the atom net charge and the net charge of phosphorus atom in order of importance with the inhibitory potential.

In table 8, the regression coefficients are greater than 0.70 which is a sign of close relationship between the variables [19]. The result of correlation matrix of relationships showed that E_{HOMO} have the greatest influence regression coefficient with inhibitory potential compared to other descriptors and the equation also expresses the highest interrelationships between this parameter and ω with $r = -0.61$ and the least interrelationships between this parameter and Q_N with $r = -0.01$.

4.4. Comparison of QSAR model of human with QSAR model of *X. luteola* on temephos derivatives

Inhibitory potential of AChE of human on derivatives were studied. Result revealed that side aromatic substitutions links between P-N, P-O and P-C, phenyl rings increases the combined inhibition of human acetylcholinesterase. The QSAR model of AChE of human denoted that independent variables $Q_{(N1)} > \log P > E_{\text{LUMO}} > Q_{(P1)}$ respectively have greatest inhibitory effect on the control of the human acetyl cholinesterase. Matrix relationship between $\log P$ and $Q_{(N1)}$ with $r = -0.650$ shows that hydrophobicity properties of the molecules had great effect on the electronic properties; therefore they could have effect on inhibition of human AChE [19].

$$\log(1/IC_{50}) = +0.175\mu + 0.845Q_P + 29.699Q_N - 12.903E_{\text{LUMO}} - 0.019M_v + 27.921\omega - 0.124$$

$$n = 10; R^2 = 0.922; S_{\text{reg}} = 0.438; F_{\text{statistic}} = 9.473$$

But as cited in equations of pest QSAR, parameter of $E_{\text{HOMO}} > E_{\text{LOMO}} > \omega > Q_X > Q_{(p2)}$ respectively have the greatest effect on inhibition of AChE from elm leaf beetle and the highest interrelationships was between E_{HOMO} and ω . By comparing the two equations it could be concluded that a

different variable of this class of phosphorus compounds has inhibition impact on AChE from human or insect. Primary efficacy variable of both equations is an electron property, and its impact on the human equation is dependent on structural features, but it is affected by other electronic features in the second equation. In another study involving the use of QSAR models on organ phosphorus, pesticides could be of help in synthesizing derivatives of this compound that have demonstrated better insecticidal properties. The equations of QSAR presented that electron descriptors have played a vital role on insecticidal effect. Compositions by this model was designed and tested in silicon. The results revealed that the insecticidal activity of newly synthesized compounds is greater than the existing insecticides [41]. Insecticidal effect of another class of derivatives was investigated on the AChE of housefly. This study was conducted on resistant strains of *Musca domestica*. The equation of the 36 combinations of PHA derivatives showed that the hydrophobic and electron orbitals properties are able to play a crucial role in the application of insecticidal activity on resistant strains [42]. These results established that the electronic features have more important insecticidal effect than any other descriptors in PHA derivatives compounds. It is therefore expected that with the change in the electron described, it could be design and the synthesis of PHA derivatives compounds are more favorable than the existent compound.

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Compound	19	30	32	50
LC ₅₀	1423.047	1230.40	952.61	511.47
(mg/l) (95%confidence limit)	(998.06-1858.05)	(931.1-1663.2)	(621.1-1015.4)	(341.2-674.0)
Slope ± Standard error	1.24±0.41	1.03±0.95	1.55±0.25	1.21±0.55

In vivo

In vitro

$$\log(1/IC_{50}) = +0.175\mu + 0.845Q_P + 29.699Q_N - 12.903E_{LUMO} - 0.019Mv + 27.921\omega - 0.124$$

$$n = 10; R^2 = 0.922; S_{reg} = 0.438; F_{statistic} = 9.473$$