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Synthesis, molecular docking and insecticidal activity evaluation of chromones of date palm pits extract against *Culex pipiens* (Diptera: Culicidae)

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Abstract
Agricultural wastes may provide natural alternative to mosquitoes control because of their bioactive components. The current study involved evaluation of the toxicity of date palm pits extracted in methanol 70% against third larval instar of *Culex pipiens.* Methanolic extract of date palm pits *via* HPLC yielded two novel chromone flavonoid compounds; chromone 1 and chromone 2. These chromone derivatives were synthetically prepared under laboratory conditions. Results showed that chromone 1 and chromone 2 exhibited excellent insecticidal activity against third larval instar of *Cx. pipiens* after 24 hours of treatment at LC_{50} 32.359 and 38.717 ppm, respectively and great inhibition of AChE with value -61.48 and -60.45 %, respectively compared to control. Chromone 1 and chromone 2 were theoretically evaluated for interactions with acetylcholinesterase (AChE) using *in silico* molecular docking techniques. Both experimental and *in silico* results showed that chromone 1 exhibited higher activity than chromone 2. These results proved that, the chromone derivatives considered to be inhibitors for AChE enzyme in *Cx. pipiens*.

Keywords: Date palm pits, flavonoid, molecular docking, ache, *Culex pipiens*

Introduction
Mosquitoes mainly *Culex*, are the most important vectors because of their role in transmission a variety of human and animal diseases, causing millions of deaths every year [1, 2]. Among these diseases, malaria, yellow fever, dengue hemorrhagic fever, filariasis and Rift Valley fever which are endemic and epidemic in some areas of many countries [3]. The transmission of diseases can be interrupted by controlling the vectors [4-6]. The continuous use of traditional chemical insecticides leads to adverse effects such as developing of resistance, affecting the public health and reducing beneficial non-target biota. Retardation of these effects forced authors to search for new products that are ecofriendly and environmentally safe [7, 8].
The date palm is one of the major fruit trees in Egypt [9]. Date palm pits, considered as a source of antioxidants plants, produce different types of secondary metabolites (polyphenols, flavonoids and phytochemicals) that protect them from infections and harsh environments. These polyphenols often provide valuable bioactive properties to the plants and animals to maintain their functions and homeostasis as well as prevent diseases [10]. Moreover, it is clearly that crude or purified date palm pits extracts are efficacious for the control of insects [11-14].
The present study aimed to investigate the chemical composition and insecticidal effect of chromone 1 and chromone 2 extracted from date palm pits using 70% methanol and to understand the docking interactions of these chromone derivatives with the active site of acetylcholinesterase, AChE.

Materials and Methods
Plant material
The date palm pits were collected from the food factories, Qalyubia governorate, Egypt. The material was authenticated by Taxonomy Division of Botany department, Faculty of Science, Ain-shams University, Egypt.

Reagents and chemicals
HPLC grade solvents such as acetonitrile, acetic acid, methanol, hexane, ethyl acetate and chloroform were purchased from E. Merck (India) Limited. While, gallic acid, kaempferol, tannic acid, syringic acid, catechin, ferulic acid, and chromone were purchased from Sigma-Aldrich.

Extraction and fractionation
Samples of 500 gm. of date pits were dried under shade condition and then grinded in a food grinder. The dried samples were directly extracted by means of soxhlet apparatus using methanol 70% in ratio (1:3) as a solvent. The solvent extract of date palm pits were evaporated and dried under vacuum using a rotary evaporator at 60-70 ˚C. The residues were weighed and preserved at 4 ˚C until use [15].

Isolation of compounds
Date palm pits extract was subjected to column chromatography to isolate the active constituents as chromone derivatives 1 and 2 as reported before [16-19]. Other sub fractions passed through a preparative thin layer chromatography (TLC) and high performance liquid chromatography (HPLC)**.** Primary stock solutions of phenolic compounds (Gallic acid, Tannic acid and Ferulic acid, Saponin and Chromone) were prepared in methanol to obtain a concentration of 1 mg/ml. Isolated compounds of sub fractions were confirmed by comparing with standards through TLC, melting point (M.P) and HPLC data [20-22]**.** The chromone derivatives 1 and 2 were revealed with Fourier transform infrared spectrometer (FT-IR), proton nuclear magnetic resonance (^{1}HNMR) and electron impact mass spectroscopy (EI-MS).

Mass spectroscopy (EI-MS)
The MS was performed in positive electron ionization (EI) mode with ionization energy of 70 eV. The detector signal was recorded from 5 minutes onward after injection and ions were scanned across the range of 50–650 Amu.

Fourier transform infrared spectroscopy (FTIR)
Spectrophotometer (KBr discs) in the middle infrared region (400 cm^{-1} to 4000 cm^{-1}) was used to quantify the characteristic peaks of chromone derivatives isolated from date palm pits using Nicolet FT-IR (4100 Jasco-Japan).

Nuclear magnetic resonance spectroscopy (NMR)
^{1}H-NMR spectra recorded on a Bruker spectrophotometer (Germany) at 400 MHz using TMS as internal standard and with residual signals of the deuterated solvent δ = 7.26 ppm for $CDCl_3$ and δ = 2.51 ppm for DMSO-d6.

Synthetic route of chromone 1 and chromone 2 under laboratory conditions
Elucidation of chromone 1 and chromone 2 was done by green synthesis method via grinding of 2, 4 dihydroxyacetophenone, 4-hydroxy benzaldehyde and methyl (7E, 10E) - 7, 10- octadecadienoate as shown below in (Figure 1).

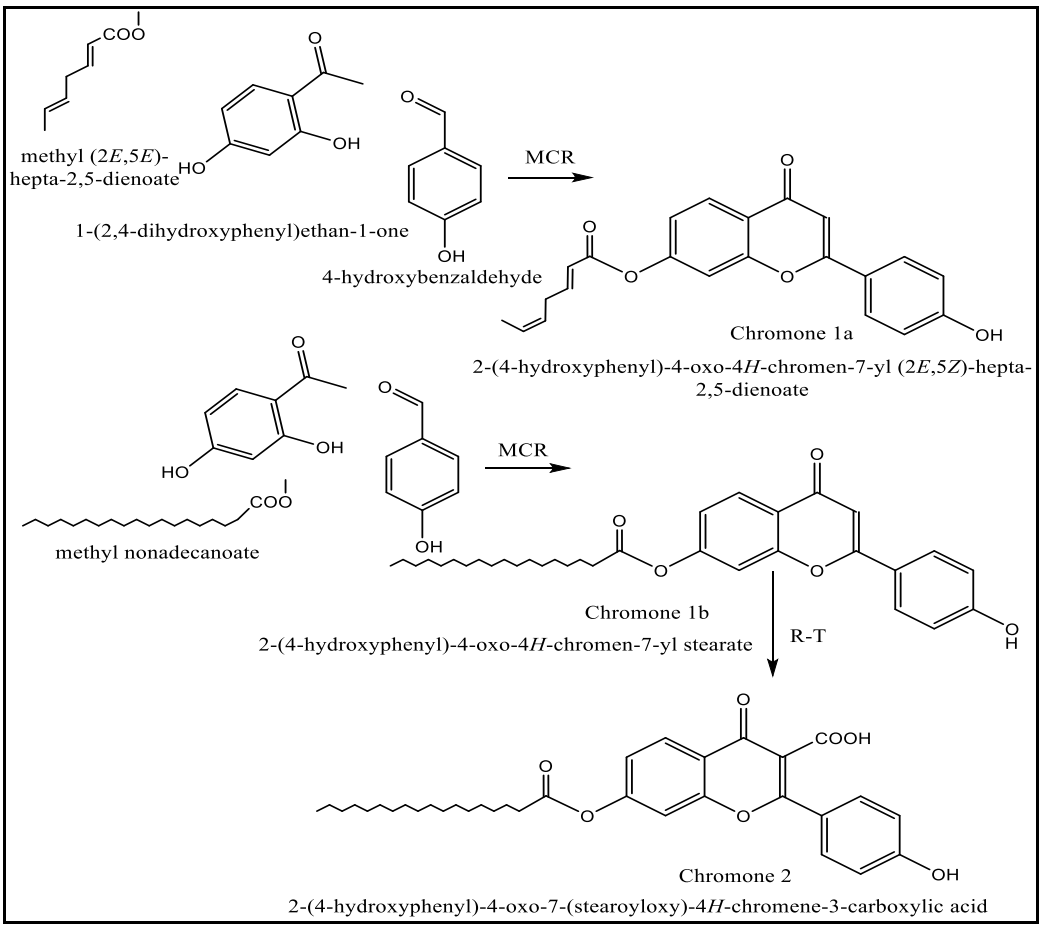


Fig 1: Outline the synthetic route of the chromone 1 and 2.

Insecticidal activity

Larvicidal activity of date palm pits methanolic extract and its chromone derivatives (chromone 1 and chromone 2) was evaluated against the third larval instar of *Cx. pipiens* under laboratory conditions (27±2 ˚C, RH 70±10% and 12-12 light-dark regime). Bioassay test was performed according to the standard World Health Organization larval bioassay test [23]. Mortality data were recorded after 48 hours of treatment and were used to estimate probit regression line and calculate LC_{50}, LC_{90}, slope function and X^2 using multiple linear regressions [24].

Estimation of acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was measured according to the method described by Simpson *et al* [25] using acetylcholine bromide (AChBr) as substrate.

Statistical analysis

Statistical analysis of the data was carried out according to the method of Lentner [26]. All the measurements were done in three replicates for each test and were expressed as mean ± SE. For statistical comparison among several means, one-way analysis of variance (ANOVA) was used at $P<0.05$.

In *silico* studies, homology modeling, active site identification and virtual screening studies

The comparative homology modeling studies are used to generate the three dimensional (3D) structure of the target enzyme in the absence of the reported structure of Acetyl cholinesterase (AChE) of *Culex pipiens* (House mosquito). The templates required for enzyme modeling are found by subjecting FASTA sequence to sequence comparison methods such as Basic Local Alignment Tool (BLASTp) [27] and Phyre2 [28] servers. The 3D models of the target enzyme AChE are built based on target-template alignment. The 3D structure of the target enzyme was energy minimized and refined using Swiss PDB-viewer (SPDBV) [29] and ModRefiner [30]. The stereochemical quality of the final homology model was assessed by standard protocols like Volume Area Dihedral Angle Reporter 1.8 (VADAR) [31], Protein structure analysis (ProSA), verify 3D and ERRAT server tools [32-34]. Binding site of AChE enzyme, which is responsible for the interactions of the target enzyme and ligand molecules, is identified theoretically using ProBiS [35] and MetaPocket 2.0 [36]. Molecular docking is a computational technique used to recognize the structures which bind well to the enzyme pocket [37]. Computer-aided virtual screening was carried out with two ligand compounds against the binding site of AChE. PyRx virtual screening tool [38] is used to screen the chromone derivatives against AChE enzyme through flexible docking option. To analyze the docking calculations, nine conformers are considered for each ligand-enzyme complex. The lowest binding energy score is chosen to identify the best binding mode of the docked compound to the target enzyme. The 2D and 3D molecular interaction models of the docked compounds-AChE complex involving H-bonds, Pi-Pi and Pi-sigma interactions are displayed using Accelrys Discovery Studio Visualizer software version 3.5 [39]. The absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of the docked molecules were identified using admetSAR program [40].

Results

Compounds Isolation

Date palm pits methanolic extract was chromatographed over silica gel column chromatography for the isolation of two pure compounds (Figure 2).

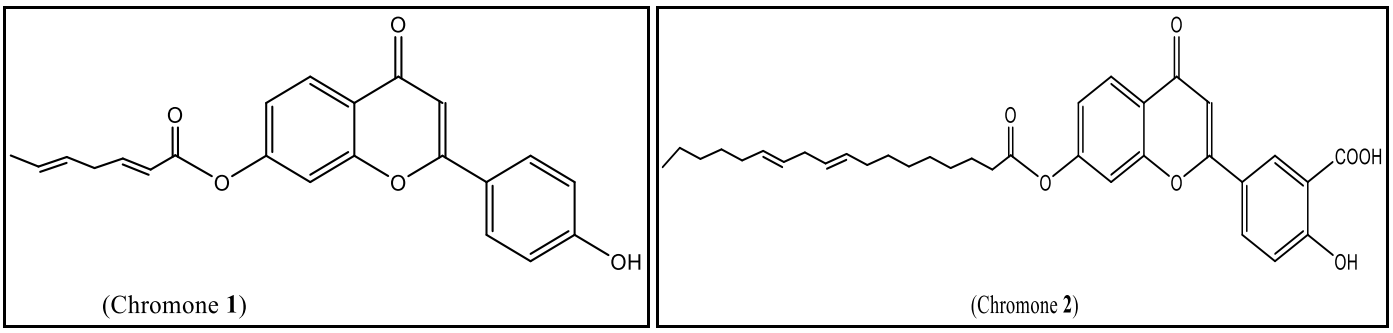


Fig 2: Outline the chemical flavonoid structures; chromone1 and chromone 2 isolated from date pits methanolic extract 70%.

Chromone 1: (2-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yl (9E, 12E)-octadeca-9, 12-dienoate) yellow crystalline powdered, m.p. 96-98ºC (Rf =0.31)Yield 70%. FT-IR (KBr) spectrum shows absorption bands at (in cm^{-1}): 1685 (CO), 3420, 3533 (OH) (Figure 5). The ^{1}H-NMR ($CDCl_3$) spectrums show signals in ppm at: 1.00-1.10 (m, 4H, $2CH_2$), 2.15 (s, 6H, $2CH_3$), 2.45-2.48 (m, 4H, $2CH_2$), 3.32 (t, 2H, CH_2), 4.03 (t, 2H, CH_2COO), multiplet at 7.44-7.73 (m, 2ArH, Pyrim), 10.2 (s, acidic OH proton which exchanged in D_2O). EIMS, m/z (relative intensity) 267 [M^+ 85.5%·], 260, 252, 208, 165, 146, 121. Elemental analysis; M.wt= 894 Calc. $C_{33}H_{40}O_5$, Calc. % C 76.71, H 7.83; found % C 76.47, H 7.54. (Figure 3 & 4).

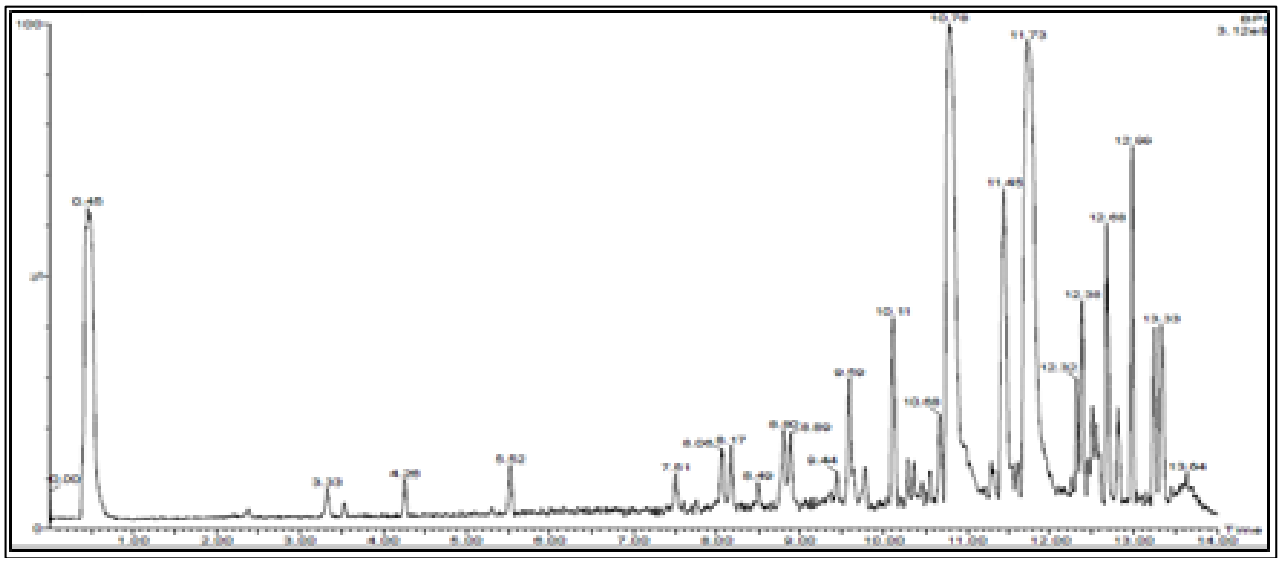


Fig 3: Outline HPLC and time retention of high % yield of chromone derivatives 1 and 2 as represented by LJ 10.78 and LJ 11.73 respectively.

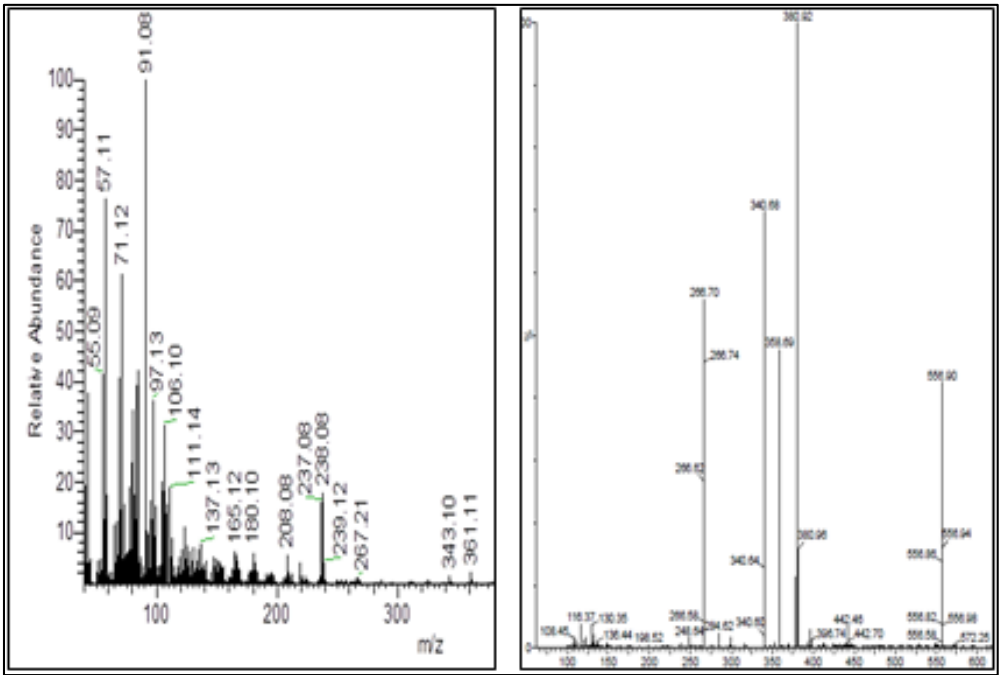


Fig 4: Outline mass spectra of the chromone 1 (left) and chromone 2 (right)

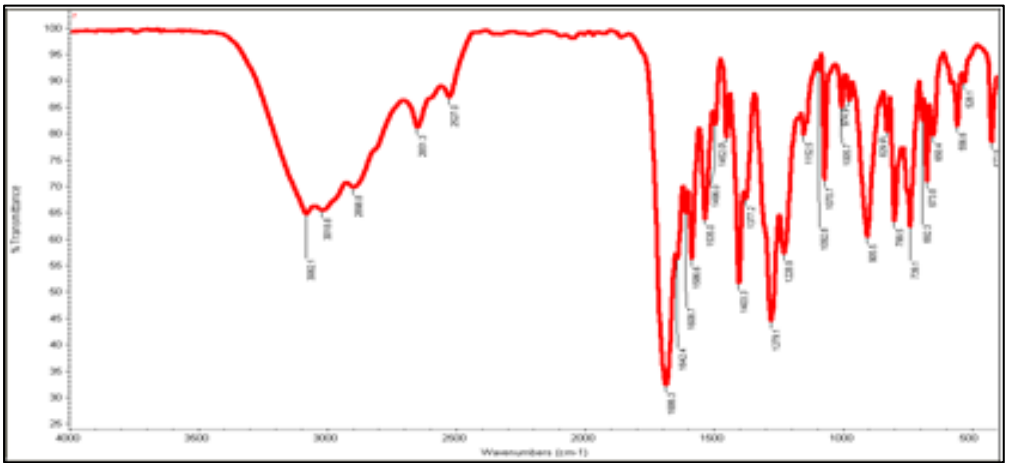


Fig 5: Outline IR spectrum of isolated chromone 1.

Chromone 2: (2-hydroxy-5-(7-((9E, 12E)-octadeca-9, 12-dienoyloxy)-4-oxo-4H-chromen-2-yl benzoic acid) yellow amorphous powdered, m.p. 110-112ºC (Rf = 0.23), yield 75%. FT-IR (KBr) spectrum shows absorption bands at (in cm^{-1}): 1700, 1685 (CO), 3453 (OH), 3220, 3133 (COOH) due to intramolecular hydrogen bond (Figure 6). The ^{1}H-NMR ($CDCl_3$) spectrums show signals in ppm at: 1.00-1.10 (m, 4H, $2CH_2$), 2.15 (s, 6H, $2CH_3$), 2.45-2.48 (m, 4H, $2CH_2$), 3.32 (t, 2H, CH_2), 4.03 (t, 2H, CH_2COO), multiplet at 7.44-7.73 (m, 2ArH, Pyrim), 10.2 (s, acidic OH proton which exchanged in D_2O). EIMS, 267[$M^{+\cdot}$], 260, 252, 208, 165, 146, 121. Elemental analysis; M.wt 894 Calc. $C_{34}H_{40}O_5$, Calc. % C 72.83, H 7.19; found % C 72.54, H 6.82. (Figure 3 & 4).

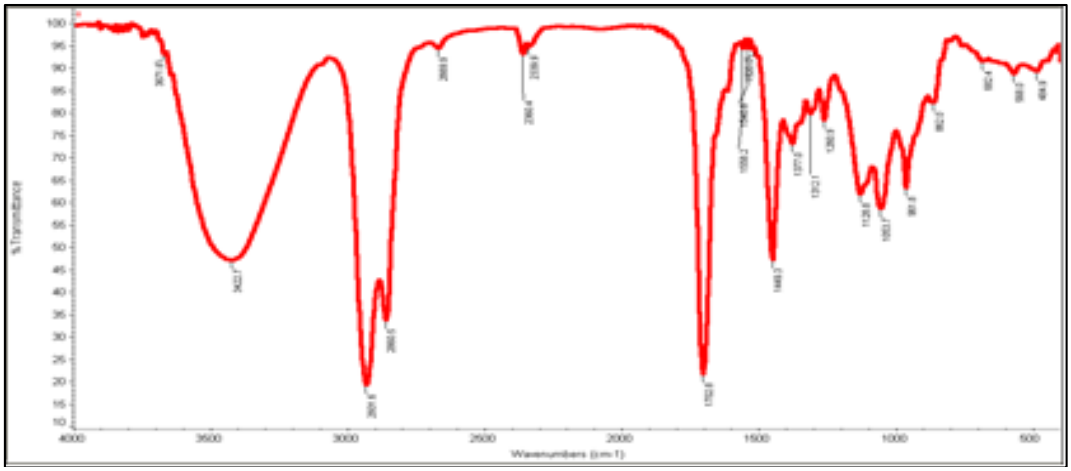


Fig 6: Outline IR spectrum of isolated chromone 2

Bioassay on the third larval instar

The data in (Table 2) show a high percentage mortality of chromone 1 than chromone 2 compared with control. The LC_{50} and LC_{90} of chromone 1 were (32.359 and 167.040 ppm) and chromone 2 with LC_{50} and LC_{90} (38.717 and 224.209 ppm), respectively.

Table 1: Assignment of the most characteristic Infrared bands for chromone 1& chromone 2

Peak position (cm^{-1}) (stretching frequency)	Assignment
1740-1750	Ester group (COO)
1705-1690	Acid (C=O)
1670-1660	CO- stretching frequency amide
2918-2884	C-H aliphatic symmetrical and asymmetrical
3100-3050	C-H aromatic or olefinic bond
3500-3100	O-H (broad due to hydrogen bond)

Table (2): Larvicidal activity of date palm pits methanolic extract, chromone 1 and chromone 2 against *Cx. pipiens* larvae

Tested compounds	LC_{50} (co. limits)	LC_{90} (co. limits)	Slope function ± SE	X^2
Chromone 1	32.359 (27.699-37.639)	167.040 (122.973-261.934)	1.79 ± 0.19	1.79
Chromone 2	38.5175 (33.003-45.8861)	224.209 (155.343-392.42)	1.68±0.19	2.80
Date palm pits methanolic extract	120.028	777.76	2.41± 0.27	26.42

Effect of Chromone derivatives on acetyl cholinesterase (AChE) activity

Results in (**Table 3)** showed a highly significant inhibition in AChE activity in larvae treated with Chromone 1 and Chromone 2 when compared with control. The highest reduction of AChE activity was observed after treatment with Chromone 1 (536 µg AChBr/ml/minute), followed by Chromone 2 (550.66 µg AchBr/ml/minute) as compared to control (1392.33 µg AchBr/ml/minute). The activity of acetylcholinesterase was significantly decreased by -61.47 % and -60.45%, after treatment with chromone 1 and 2, respectively.

Table (3): Acetylcholinesterase activity in *Cx. pipiens* larvae treated with date palm pits methanolic extract, chromone 1 and chromone 2

Tested compounds	Acetylcholinesterase µg AChBr released/ minute/g.b.wt± SE	Activity ratio
Chromone 1	536 ±27.22 [b]	-61.48
Chromone 2	550.66 ±27.96 [b]	-60.45
Control	1392.33 ±66.75 [a]	

Within a column, different letters mean significant differences at $p<0.05$ level of probability

***In silico* studies**

The three dimensional structure of the enzyme was generated using Modeller 9.11 [41]. It contains 24 α-helices and 16 β-strands as shown in (Figure 7). The validation of 3D structure was carried out using different tools. The stereochemical quality of the target enzyme AChE was predicted using Ramachandran plot from VADAR tool. The plot statistics (Figure 8) showed 91.3% of the total residues in the most favored region, 4.5% in additionally allowed regions, and 4.2% in generously allowed region, which implies a good 3D model quality. The ProSA server results a Z-score value of -10.12 for the refined structure (Figure 9 & 10), predicting the 3D model of AChE to be a good quality. Furthermore, The ERRAT score could give an overall quality factor for non-bonded atomic interactions, and a score of greater than 50 is acceptable [42]**.** Figure 11 represents 89.625 % as the results of ERRAT, which is considered to be good to use this model. In addition, (Figure 12) shows the results of Verify 3D program, which describes the compatibility of 3D atomic model of AChE with its own amino acid sequence (1D) based on the residue environment [43]. The result gives a value of 77.36%, from which it may be inferred that this model is of good quality. Binding site of the target enzyme is determined using computational prediction tools like ProBiS (Enzyme Binding Site) and Meta Pocket tools. The results show that the amino acid residues Asp200, TRP212, TYR249, SER250, Tyr258 and Tyr456 are the active site residues which are responsible for binding to the ligand molecules. Our study involves docking of Chromone derivatives with AChE using PyRx virtual screening tool. Nine conformers were considered for each ligand-enzyme complex, and the lowest docking energy was chosen to identify the preferable binding mode [44] of the docked compound in AChE as in (Figure 13). The molecular docking interaction models of the two ligand molecules and the target enzyme were illustrated in (Table 4 and Figure 14). The molecular interaction results like H-bonds, π-π and Pi-sigma are represented in (Figure 14). The admet SAR prediction tool was used to calculate the pharmacokinetic parameters of the docked molecules as illustrated in (Table 5). ADMET properties reveal that the chromone derivatives have better Human Intestinal Absorption (HIA) score, good Blood-Brain Barrier (BBB) values. All chromone derivatives displayed negative AMES toxicity test and negative carcinogenicity test.

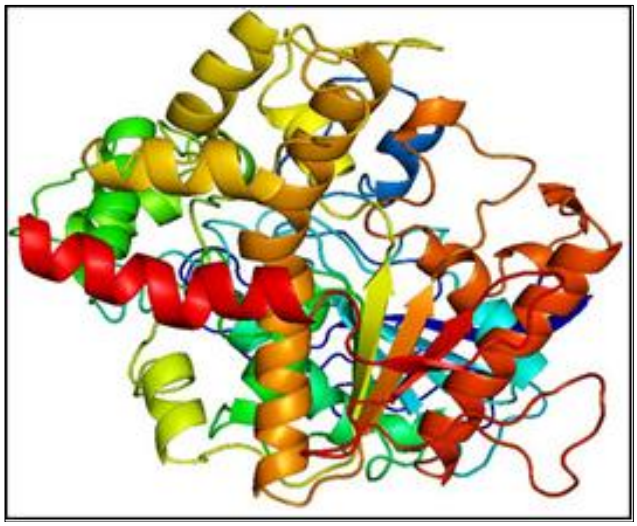


Fig 7: Three dimensional (3D) model of AChE, containing 24 α-helices and 16 β-strands.

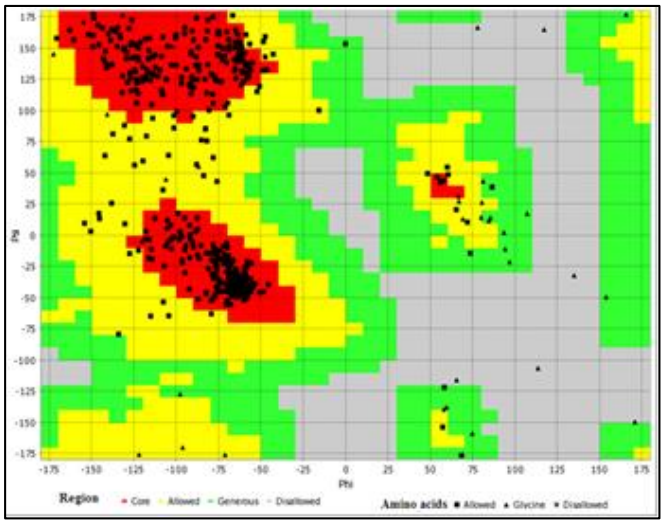


Fig 8: Ramachandran plot analysis of AChE. The red area represents the most favourable region of amino acid residues; the yellow region is additionally allowed and the generously allowed residues are in the green region.

	No. of residues	**%**
Residues in most favored regions [A, B, L]	610	91.3
Residues in additional allowed regions [a, b, l, p]	30	4.5
Residues in generously allowed regions [~a, ~b, ~l, ~p]	28	4.2
Residues in disallowed regions	0	0.0
Total number of non-glycine and non-proline residues	668	100
Number of end-residues (excl. Gly and Pro)	8	
Number of glycine residues (shown as triangle)	19	
Number of Proline residues	7	
Total number of all residues	702	

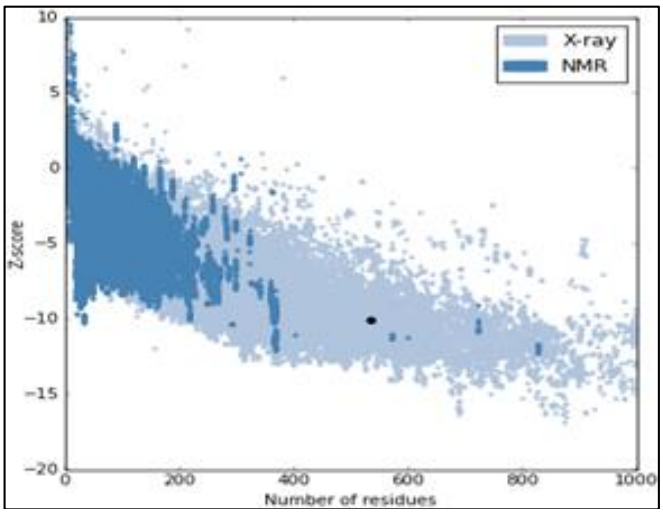


Fig 9: The local model quality of the AChE. The enzyme Z-score (-10.12) falls in the range of the Z-score for PDB enzymes whose structures are determined by NMR (dark blue region) and X-ray crystallography (light blue region), which indicates a good quality model.

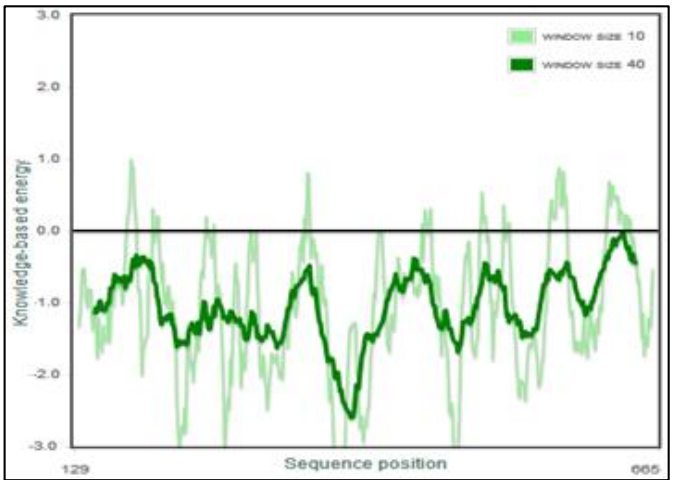


Fig 10: The Pro SA of the 3D model of AChE enzyme. The energy of maximum amino acid residues is within the negative region, indicating a good quality model

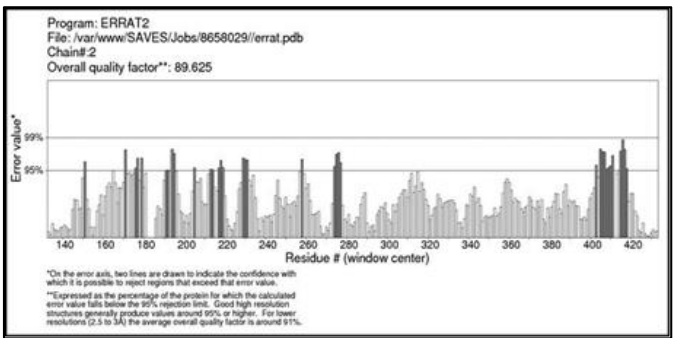


Fig 11: ERRAT plot of AChE enzyme showing 89.625% overall quality factor.

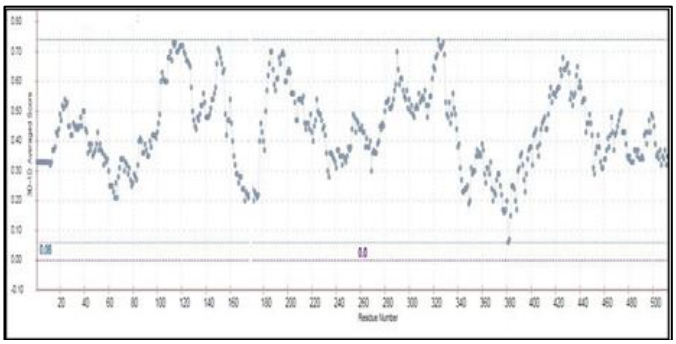


Fig 12: The Verify_3D value of AChE

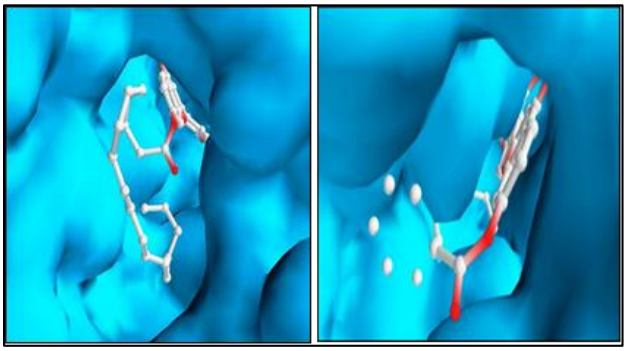


Fig 13: Schematic representation of the best docked poses of chromone derivatives (1 and 2) with AChE.

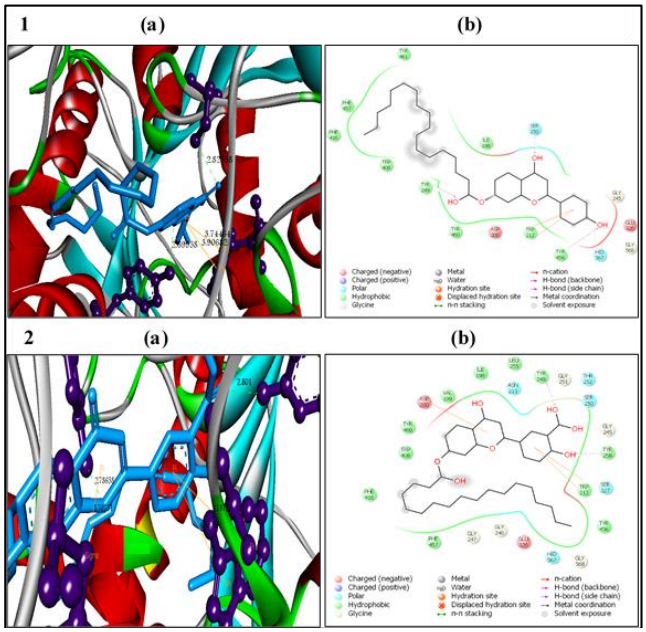


Fig 14: *In silico* molecular docking of the chromone derivatives 1 & 2 with the target enzyme3 Dimensional representation of the AChE-ligand complex. The binding residues are shown in violet ball and stick and the hydrogen bonds are represented by green dotted lines. The ligand molecules are shown in cyan stick model.2 Dimensional representation, the amino acids are shown in 3 letter code, and H-bonds in pink dotted lines, Pi-Pi and Pi-sigma interactions are shown in orange line.

Table 4: Molecular interactions and interacting residues of the AChE with Chromone derivatives. The chromone derivatives with the best binding energy are represented with docking interactions in the table showing H-bonding, Pi-Pi, and Pi–sigma interactions. Phenolic moiety is represented in red square while the pyran moiety is in green cycle.

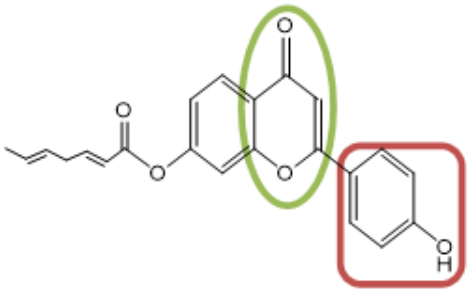
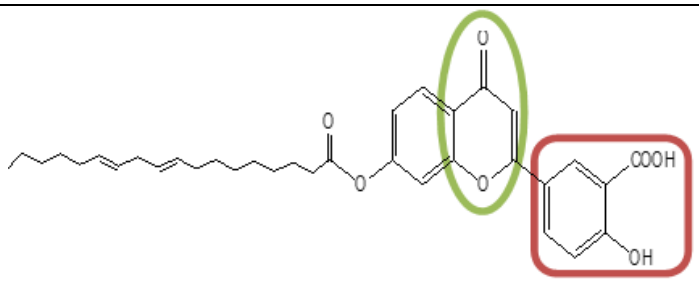
S. No.	Compound structure	Binding energy k.cal/mol	Docked complex (amino acid –ligand) interactions	Bond Distance (Å)
1	 Chromone 1	-7.0	**Hydrogen bonds** TYR456:OH---ligand 1 TYR249:OH---ligand 1 SER250:OH---ligand 1 **Pi–Pi interactions** TRP212---ligand 1 TRP212---ligand 1	 2.811 2.699 2.825 3.906 3.744
2	 Chromone 2	-8.2	**Hydrogen bonds** TYR249:OH ---ligand 2 TYR258:OH---ligand 2 **Pi–Pi interactions** TRP212---ligand 2 TRP212---ligand 2 **Pi-sigma interactions** ASP200---ligand 2	2.786 2.801 4.550 5.137 3.707

Table 5: ADMET proprieties of the chromone derivatives.

S. No.	Compound	Molecular Weight (g/mol)	Blood-Brain Barrier (BBB+)	Human Intestinal Absorption (HIA+)	Caco-2 Permeability (Caco2+)	AMES toxicity	Carcinogenicity
1	Chromone 1	516.68	0.847	0.994	0.613	Nontoxic	Non carcinogenic
2	Chromone 2	560.69	0.509	0.977	0.569	Nontoxic	Non carcinogenic

Discussion

Food waste is one of the great paradoxes of our life, and it is wasting resources to produce food [45]. Some research trends are to explore the use of waste from food industry and the waste utilization could provide economic gain to the farmers, industry, food security, environmental safety, and sustainability [37, 38]. Therefore, their utilization is highly desired by the date processing industries in developing their value added products [40, 41]. Date pits have the potential to be used as a supplement for antioxidants in nutraceutical, pharmaceutical, and medicinal products [42, 43]. The present study reported that date palm pits methanolic extract had insecticidal activity due to the presence of tannins, flavonoids, saponins, sterols and phenols [46]. Fraction of chromone derivatives were analyzed for their active mosquitocidal constituents by some known phenolic and flavonoid compounds by HPLC and GC-MS. The HPLC and GC-MS both chromatographic analyses showed that fraction contained chromones in the higher amount. Chromone derivatives are a well-known allelochemical and exhibited good insecticidal activity [47]. Moreover, in the present study, chromone 1 and 2 were found to have significant larvicidal activity at LC_{50}= 32.36 and 38.52 ppm, respectively. On the other hand, the synthetic pathway adopted to obtain the chromone 1 and chromone 2 was established based on their elemental analyses and spectral data. Multicomponent reaction (MCR) condensation of aromatic aldehyde e.g. 4-hydroxy benzaldehyde with 2.4-dihydroxyacetophenone and fatty ester e.g. methyl (2E, 5E)-hepta-2, 5-dienoate and Methyl (7E, 10E)-7, 10-octadecadienoate afforded the chromone derivatives 1 and 2, respectively in good yield according to the reported procedure [48]. The IR spectra (cm^{-1}) of compound 1 was characterized by the presence of OH stretching bands at 3422, CH (aliphatic) stretching bands at 2931-2860 and along with C=O band at 1702 and 1692 corresponding to carbonyl of ester and chromone respectively. Reaction of chromone 1 with chloroform in the presence of sodium hydroxide under Reimer–Tiemann reaction condition afforded the chromone 2. The structure of compound 2 was confirmed by the analytical and spectral data. The IR spectra (cm^{-1}) of chromone 2 showed the broad IR bands at 3341 cm^{-1} may be attributed to the ν (O–H) mode of surface moisture on the KBr disc or O-H hydrogen bonds, CH (aliphatic) stretching bands at 2925 and along with C=O band at 1746 and 1705-1650 cm^{-1} corresponding to carbonyl of ester and carbonyl of acid-chromone respectively. This broad band (1705-1650 cm^{-1}) pointed to the intramolecular hydrogen bond of the carboxylic and hydroxyl groups in the salicylic moiety. ^{1}H-NMR spectrum of chromone 1 for this group exhibited multiplet bands corresponding to the twenty-five aliphatic protons at

the range 0.85-1.8 ppm, singlet band corresponding to the four ethylenic protons at the range 5.35-5.51 ppm, multiplet of seven aromatic protons at the range 6.86-7.21ppm and exchangeable acidic protons at 9.42 ppm. Further evidence was gained from their mass spectra that showed the correct molecular ion peaks beside some of abundant peaks. Also, the synthetic pathway for the preparation of chromone 1 and chromone 2 was proved [49, 50].

The obtained results of AChE activity showed significantly inhibition after treatment with chromone 1 and 2. Similarly, several natural products have been shown to be inhibitors of AChE [51]. Also by using AChE extracted from *Rhizopertha dominica*, the inhibition of AChE activity was obtained by 10^{-3} M levels of the essential oils from the plants belonging to the labiatae family [52].

AChE is of interest because it is the target site for organophosphorous and carbamate insecticides in the central nervous system, and its role in cholinergic synapses is essential for insects and higher animals [53]. The AChE inhibition in larvae treated with both chromone 1 and 2 suggests that these chromones may prevent any message to be sent to the receptor and thus the insect becomes without neural orientation. In addition, inhibition of AChE causes accumulation of Ach at the synapses, so that the post synaptic membrane is in a state of permanent stimulation, which results in paralysis, ataxia, general lack of co-ordination in the neuromuscular system and eventual death [54, 55]. The molecular docking analysis suggests that the chromone derivatives can be used as potent inhibitors for AChE enzyme. From the aforementioned results, ADMET properties reveal that the chromone derivatives have better Human Intestinal Absorption (HIA) score [56], which means that the compounds could be better absorbed. The compounds have good Blood-Brain Barrier (BBB) values [57]. All chromone derivatives displayed negative AMES toxicity test which means that the ligand molecules are non-mutagenic [58]. Also, they displayed negative carcinogenicity test. These ligand molecules can be considered as potent antagonists against AChE.

Conclusion

In conclusion, there is an urgent need of herbal insecticide for economically important crops in developing countries. Date pit is a waste material for a new source of chromone derivatives that has significant insecticidal activity for mosquito control. The present study clearly demonstrated that the *in silico* molecular docking interactions of the chromone derivatives and AChE showed good binding energy scores. There is strong correlation between the results of molecular modeling and the biological effects which suppose that the tested compounds with phenolic and pyran moieties may be useful for designing new and safe inhibitors against AChE enzyme. These finding may suggest that chromone 1 and chromone 2 are neuro toxic compounds toward *Cx. pipiens*.

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