SUPPLEMENTARY MATERIAL

Tagetnoic acid, a new lipoxygenase inhibitor peroxy fatty acid from *Tagetes minuta* growing in Saudi Arabia

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Abstract

A new peroxy fatty acid, tagetnoic acid (**5**) [4-((3*S*,6*S*)-6-((3*E*,8*E*)-octadeca-3,8-dien-1-yl)-3,6-dihydro-1,2-dioxin-3-yl)butanoic acid] and four known metabolites: ecliptal (5-formyl-α-terthiophene) (**1**), 5-(4-hydroxybut-1-ynyl)-2,2`-bithiophene (**2**), 22,23-dihydrospinasterone (**3**), and stigmasterol (**4**) were separated from the *n*-hexane fraction of the aerial parts of *Tagetes minuta* L. (Asteraceae). Their chemical structures were verified using IR, UV, 2D and 1D NMR, and HRMS. Compounds **3**-**5** displayed potent lipoxygenase inhibitory potential with IC_{50}s 2.26, 1.83, and 1.17 μM, respectively compared to indomethacin (IC_{50} 0.89 μM). Moreover, molecular docking study revealed that the potent activity of **5** is due to H-bonding and hydrophobic interaction. The results of this study suggested that *Tagetes minuta* dietary consumption would be useful for the individuals at risk of acute and chronic inflammatory disorders.

Keywords: *Tagetes minuta*, Asteraceae, Tagetnoic acid, Peroxy fatty acid, Lipoxygenase inhibitor, Molecular docking

List of supplementary materials

List of abbreviations

1D, one dimensional nuclear magnetic resonance; 2D NMR, two dimensional nuclear magnetic resonance; BA, *n*-butyric acid moiety; COSY, correlation spectroscopy; GIT, gastrointestinal tract; DHD, 3,6-dihydro-1,2-dioxine; EIMS; electron impact mass spectroscopy; EO, essential oil; ESIMS, electron spray ionization mass spectroscopy; EtOAc, ethyl acetate; HMBC, heteronuclear multiple bond correlation; HRESIMS, high resolution electron spray ionization mass spectroscopy; HRMS, high resolution mass; HSQC, heteronuclear single quantum coherence; IR, infra-red; IC_{50}, inhibitory concentration 50; IL-1β, interleukin-1β; IL-6, interleukin-6; TLC, thin-layer chromatography; LOXs, lipoxygenases; NO, nitric oxide; ODD, octadeca-3,8-diene; NOESY, nuclear overhauser effect spectroscopy; PPARγ, activating peroxisome proliferator-activated receptor-γ; PUFAs, poly-unsaturated fatty acids; RP_{18}CC, reversed phase C18 column chromatography; SiO_2, silica gel; TNF-α, tumor necrosis factor-α; UV, ultraviolet.

General experimental procedures

Digital Melting Point 9100 Electrothermal instrument (Electrothermal Engineering Ltd, Essex, England) was utilized for measuring the melting points. A spectrophotometer Hitachi-300 (Kyoto, Japan) was utilized to get the UV spectra. Optical rotation was acquired using a PerkinElmer polarimeter 341 LC Model (PerkinElmer, Waltham, MA, USA). IR spectra were assessed on a 400-Shimadzu Infrared spectrophotometer (Kyoto, Japan). JEOL spectrometer (JMS-SX/SX 102A) was utilized to obtain the EIMS (Joel, Peabody, MA, USA). ESIMS spectra were estimated using a LCQ-DECA spectrometer (ThermoFinnigan, Bremen, Germany). HRESIMS was carried out by LTQ-Orbitrap spectrometer (ThermoFinnigan, Bremen, Germany). NMR spectra were recorded on Bruker Avance DRX 600 MHz spectrometer. RP_{18} (0.04-0.063 mm Merck), silica gel (SiO_2, 0.063-0.200 mm), and LiChrolut EN/RP_{18} extraction tube (6 mL, RP_{18}, 40-63 μm) (Merck, Darmstadt, Germany) were used for chromatographic separation. SiO_2 60 F_{254}

plates (0.2 mm, Merck, Darmstadt, Germany) were utilized for thin-layer chromatography (TLC). TLC analyses were performed using *n*-hexane/EtOAc (95:5, S_1) and *n*-hexane/EtOAc (90:10, S_2). 5-LOX kits, linoleic acid, and indomethacin were bought from Sigma-Aldrich (St. Louis, MO, USA).

Extraction and isolation

The powdered aerial parts (1.2 kg) were macerated with MeOH (4 × 4 L). The total MeOH extract was concentrated to obtain 22.3 g. The later was suspended in distilled water (200 mL) and fractionated using *n*-hexane (5 × 500 mL) to yield *n*-hexane (2.7 g) and aqueous (19.6 g) fractions. The *n*-hexane fraction (2.7 g) was chromatographed on SiO_2 CC (130 g, 50 × 3 cm) using *n*-hexane/EtOAc gradient to obtain eight subfractions: TMH-1 to TMH-8. Subfraction TMH-2 (268 mg) was subjected to SiO_2 CC (20 g, 50 × 2 cm), eluting with *n*-hexane/EtOAc (99.1 to 93:7) to afford impure **1**. It was submitted to RP_{18} CC eluting with H_2O/MeOH gradient, then on LiChrolut EN/RP_{18} tube using H_2O/acetonitrile to yield **1** (11.9 mg). SiO_2 CC (15 g, 50 × 2 cm) of subfraction TMH-3 (311 mg) using *n*-hexane/EtOAc (99.2 to 90:10) gave impure **2**, which was purified on LiChrolut EN/RP_{18} tube using H_2O/MeOH gradient to get **2** (9.7 mg). Subfraction TMH-4 (274 mg) was treated as subfraction TMH-4 to give **3** (10.6 mg). SiO_2 CC of subfraction TMH-4 (590 mg) using *n*-hexane/EtOAc gradient to give impure **3** and **4**. Each one was purified on RP-18 CC (50 g × 50 × 2 cm, MeOH/H_2O gradient) to give **3** (9.2 mg) and **4** (14.5 mg). Subfraction TMH-6 (611 mg) was subjected to SiO_2 CC (20 g × 50 cm × 1 cm) using *n*-hexane/EtOAc gradient to give impure **5**. It was purified on RP-18 CC (50 g × 50 × 2 cm, MeOH/H_2O gradient) to give **5** (5.1 mg).

Spectral data of compounds 1-4

Ecliptal (5-Formyl-2,2`,5`,2``-terthiophene) **(1)**: Yellow amorphous powder; $^1$H NMR ($CDCl_3$, 600 MHz): $\delta_H$ 9.86 (H-6`), 7.67 (H-4), 7.27 (H-3``), 7.24 (H-4`), 7.23 (H-3,3`), 7.05 (H-4``,5``); ^{13}C NMR ($CDCl_3$, 150 MHz): δ_C 182.5 (C-6``), 146.6 (C-5), 141.6 (C-2``), 137.4 (C-2`), 136.4 (C-5`), 134.5 (C-2), 128.1 (C-3), 126.9 (C-4), 125.4 (C-5``),

124.7 (C-3``), 124.5 (C-4`), 124.0 (C-3`, 4``); ESIMS *m/z*: 277 $[M+H]^+$ (Ibrahim et al., 2016).

5-(4-Hydroxybut-1-ynyl)-2,2`-bithiophene **(2)**: Yellow amorphous powder; $^1H$ NMR ($CDCl_3$, 600 MHz): $\delta_H$ 7.22 (H-3`), 7.16 (H-5`), 7.05 (H-4), 7.01 (H-4`), 7.00 (H-3), 3.82 (H-4``), 2.73 (H-3``); ^{13}C NMR ($CDCl_3$, 150 MHz): δ_C 138.1 (C-2), 136.8 (C-2`), 132.4 (C-4), 127.9 (C-4`), 124.9 (C-3`), 124.1 (C-5`), 123.3 (C-3), 122.1 (C-5), 91.7 (C-2``), 75.5 (C-1``), 61.0 (C-4``), 24.2 (C-3``); ESIMS *m/z*: 235 $[M+H]^+$ (Ibrahim et al., 2016).

22,23-Dihydrospinasterone **(3)**: White needles; m.p. 159-161 $^{\circ}C$; ^1H NMR ($CDCl_3$, 600 MHz): δ_H 5.21 (H-7), 1.01 (H-19), 0.93 (H-21), 0.87 (H-29), 0.85 (H-26), 0.82 (H-27), 0.57 (H-18); ^{13}C NMR ($CDCl_3$, 150 MHz): δ_C 212.2 (C-3), 139.7 (C-8), 117.1 (C-7), 56.1 (C-17), 54.9 (C-5, 14), 48.7 (C-9), 45.7 (C-24), 44.4 (C-4), 43.4 (C-13), 42.9 (C-10), 39.5 (C-1), 38.9 (C-2), 38.2 (C-12), 36.7 (C-20), 34.5 (C-22), 30.2 (C-6), 29.3 (C-16), 29.2 (C-25), 26.3 (C-23), 24.8 (C-28), 23.2 (C-15), 22.8 (C-11), 21.6 (C-21), 19.8 (C-26), 19.2 (C-27), 12.4 (C-19), 12.1 (C-29), 11.8 (C-18); EIMS *m/z*: 412 $[M]^+$ (Ibrahim, 2014).

Stigmasterol **(4)**: White needles; m.p. 176-177 $^{\circ}C$; IR (KBr) ν_{max} 3494, 2938 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz): δ_H 5.34 (H-6), 5.17 (H-22), 5.01 (H-23), 3.53 (H-3), 1.01 (H-19), 0.93 (H-21), 0.85 (H-29), 0.81 (H-26), 0.79 (H-27), 0.68 (H-18); ^{13}C NMR ($CDCl_3$, 150 MHz): δ_C 140.6 (C-5), 138.2 (C-22), 129.4 (C-23), 121.8 (C-6), 71.9 (C-3), 56.7 (C-14), 56.0 (C-17), 50.1 (C-9), 45.8 (C-24), 42.5 (C-13), 42.1 (C-4), 40.7 (C-20), 39.8 (C-12), 37.3 (C-1), 36.6 (C-10), 33.9 (C-7), 31.9 (C-2), 31.7 (C-8), 29.1 (C-25), 28.3 (C-16), 26.1 (C-28), 24.3 (C-15), 23.1 (C-21), 21.1 (C-11), 19.8 (C-26), 19.4 (C-27), 18.8 (C-19), 12.1 (C-29), 11.9 (C-18); EIMS *m/z*: 412 $[M]^+$ (Mohamed and Ibrahim, 2007).

Molecular modeling

The modeling experiments were performed using SYBYL_X software (Tripos, St.Louis) versions 2.0 or 2.0._64. The 5-LOX crystal structure was downloaded from the Brookhaven website (www.rcsb.org) (PDB: 3V99). The Biopolymer Preparation tool was used for the protein preparation under the following parameters order: H-addition, H-

bonding; Termini treatment, Protonation type of histidines, according to H-bonding donor or acceptor; Side chain Bumps, Fix by Lovell method. At the end of the preparation, brief staged energy minimization was carried out to the amino acid residues only under the following parameters: Iterations, 100; Initial minimization, None; Force Field, MMFF94s; Charges, MMFF94; Dielectric constant, Constant; Non-Bonding Cutoff, 8.0 Å. The 2D chemical structures were illustrated by ChemDraw Professional 15.0 and saved as SDF file. Then these structures were prepared for docking by converting them to the 3D structures using the Concord protocol of SYBYL_X and saved as SLN files. Surflex Docker was utilized for docking under the Dock Ligands protocol of SYBYL_X. The co-crystallized ligand extracted and the protomol initiated. The binding modes of the docked ligands were then evaluated and compared to the co-crystallized ligand to identify the possible differences and similarities. Maestro academic software was used to generate 3D and 2D figures.

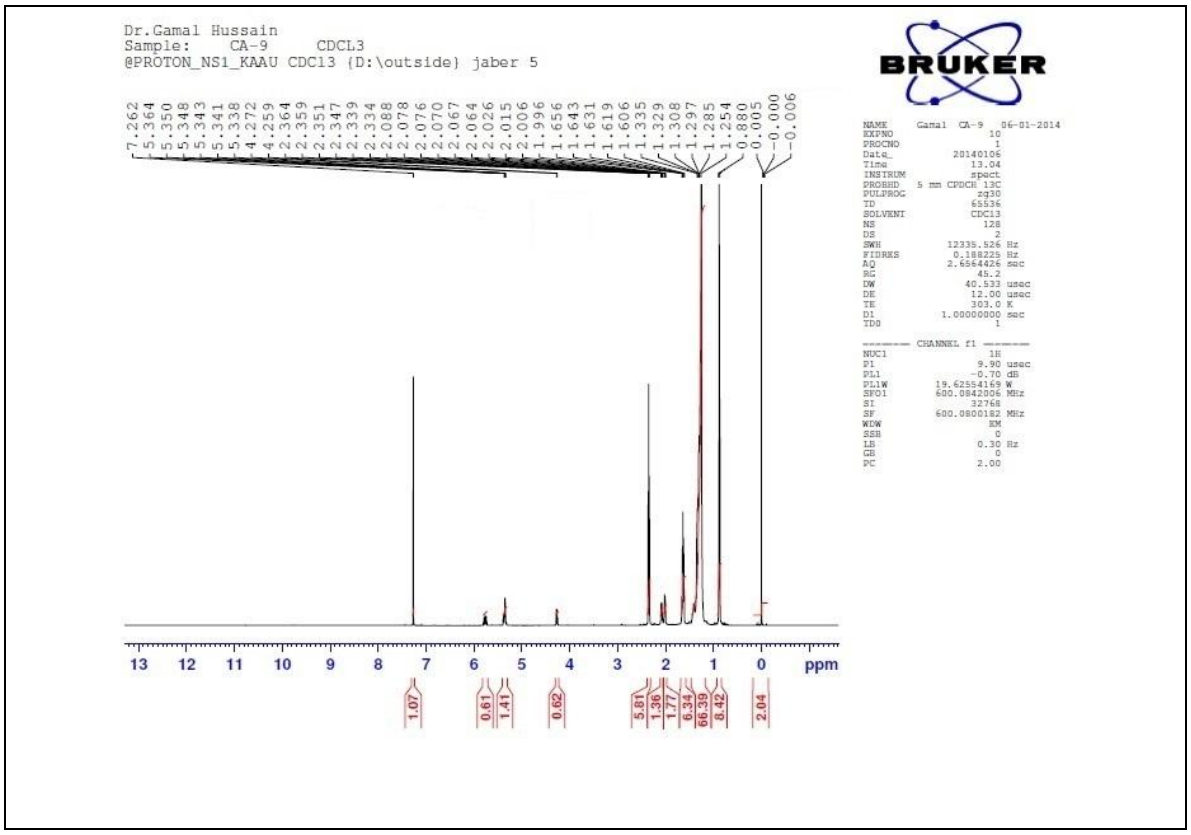

Figure S1. ^{1}H NMR spectrum of compound **5** (600 MHz, $CDCl_3$).

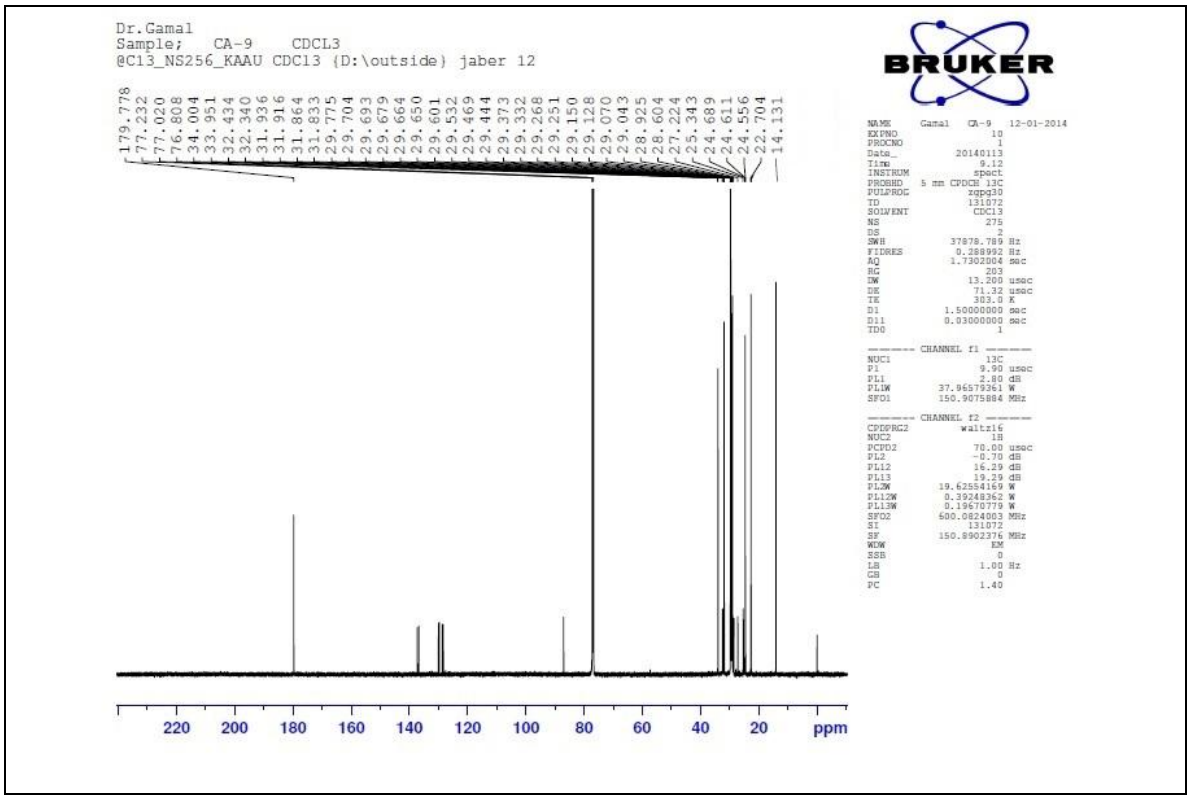

Figure S2. ^{13}C NMR spectrum of compound **5** (150 MHz, $CDCl_3$).

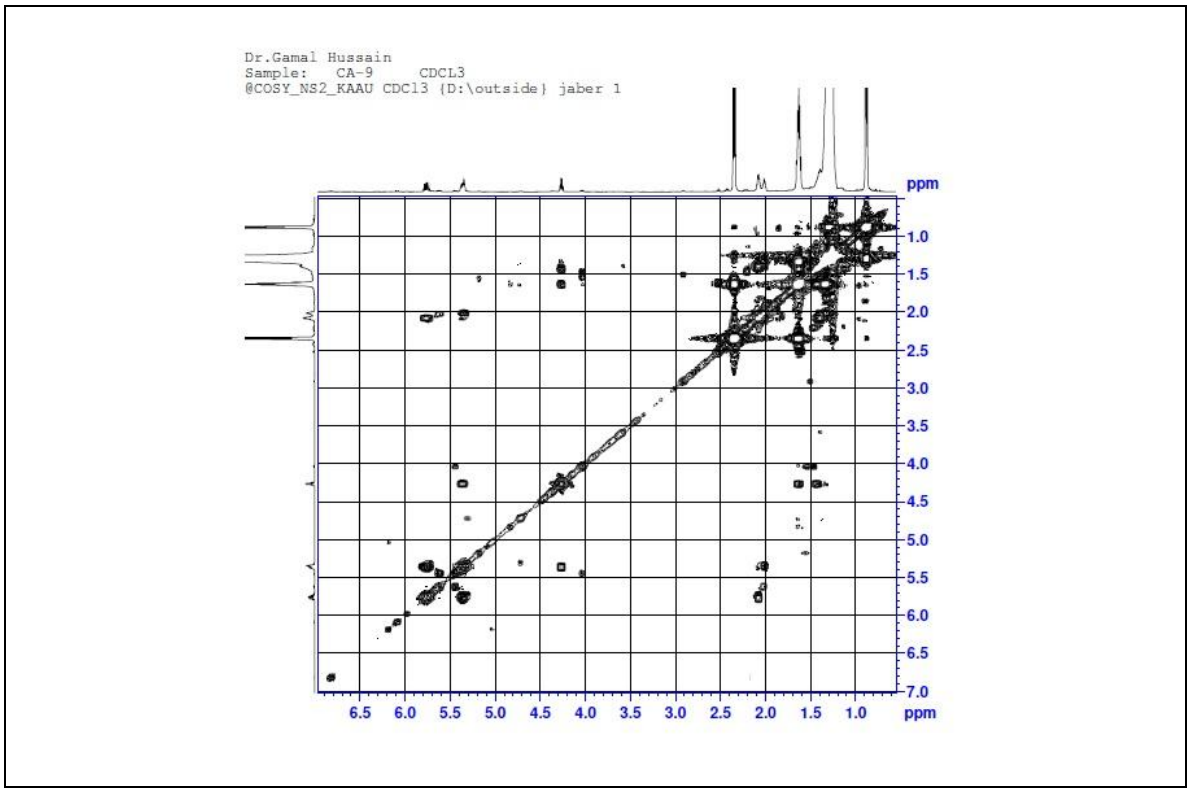

Figure S3. ^{1}H-^{1}H-COSY spectrum of compound **5** ($CDCl_3$).

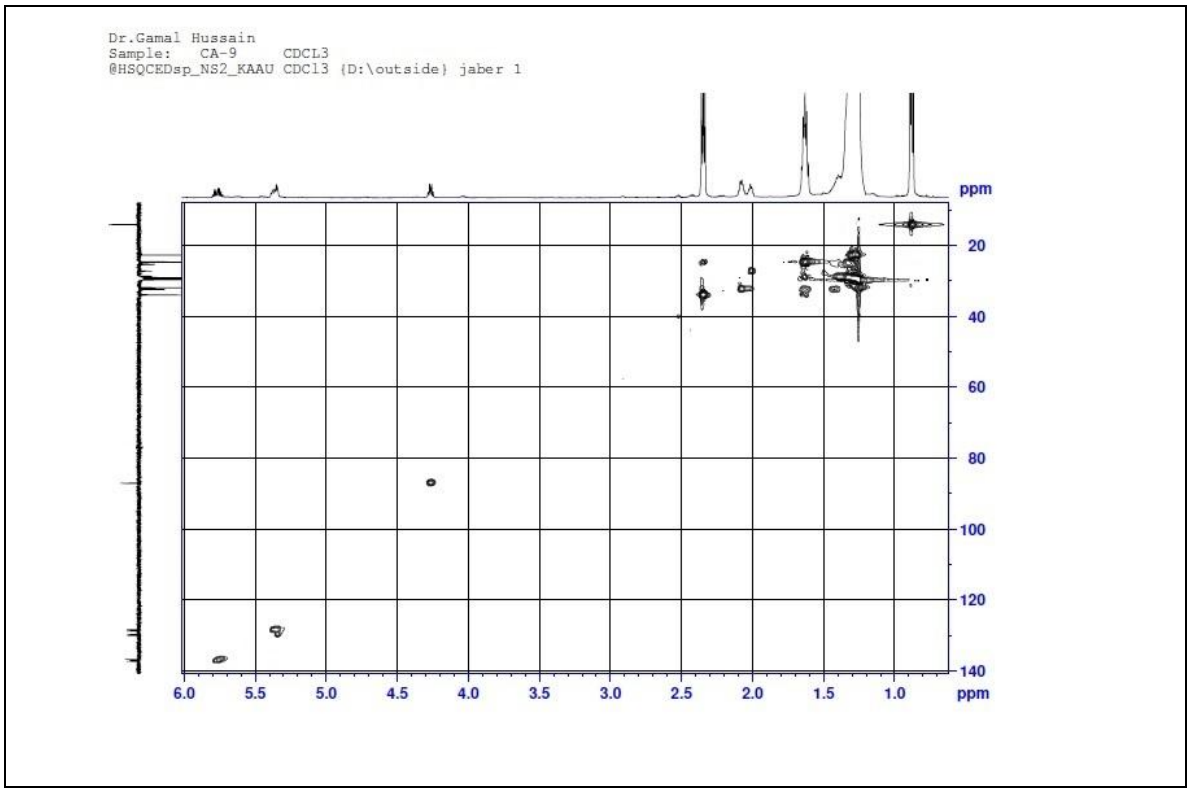

Figure S4. HSQC spectrum of compound **5** ($CDCl_3$).

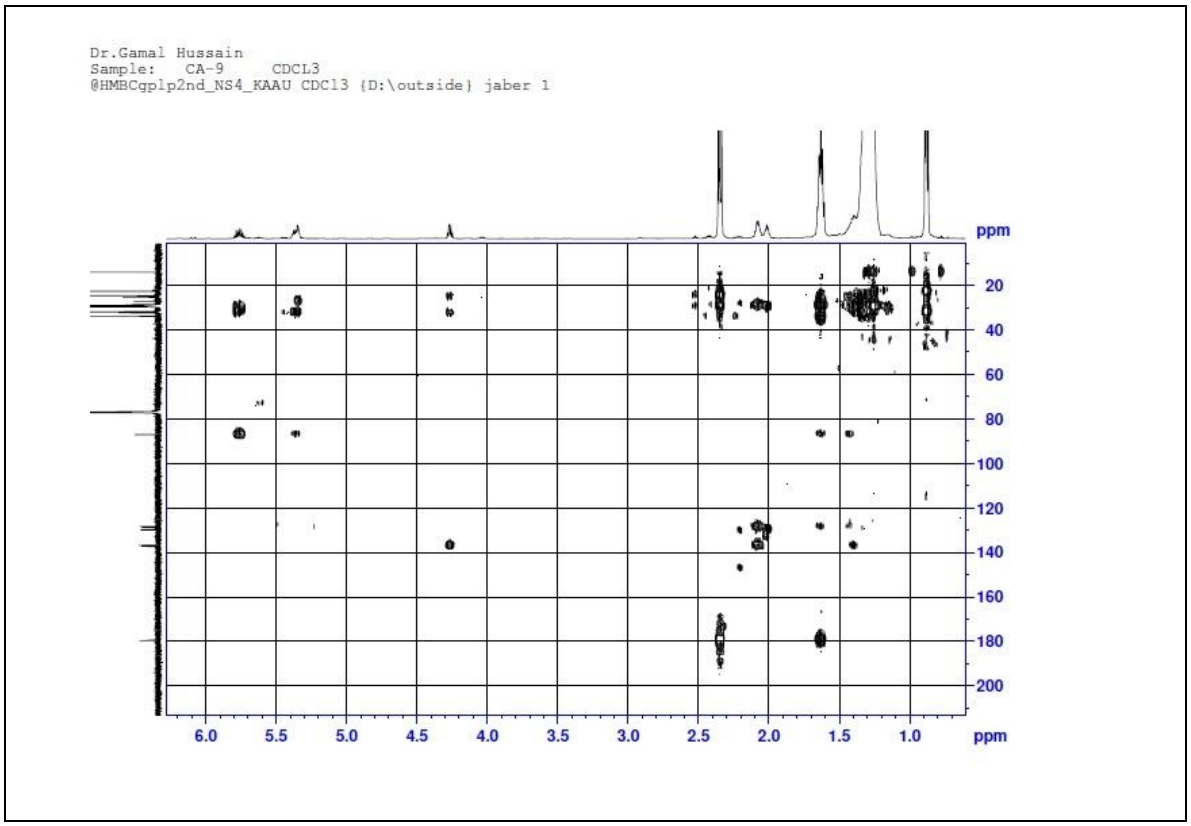

Figure S5. HMBC spectrum of compound **5** ($CDCl_3$).

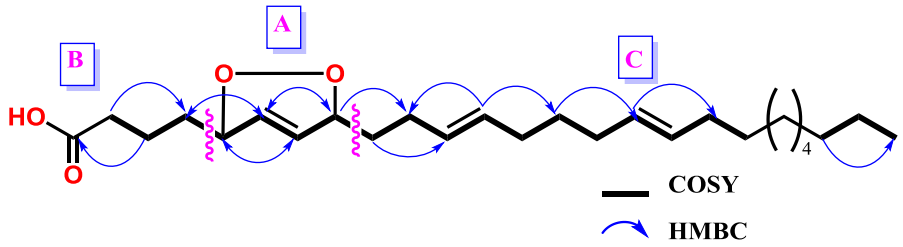

Figure S6. Substructures A-C and some key ^{1}H-^{1}H COSY and HMBC correlations of **5**.

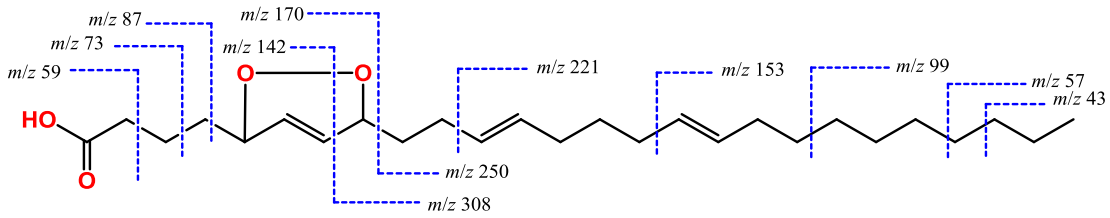

Figure S7. Possible fragmentation pattern of **5**.

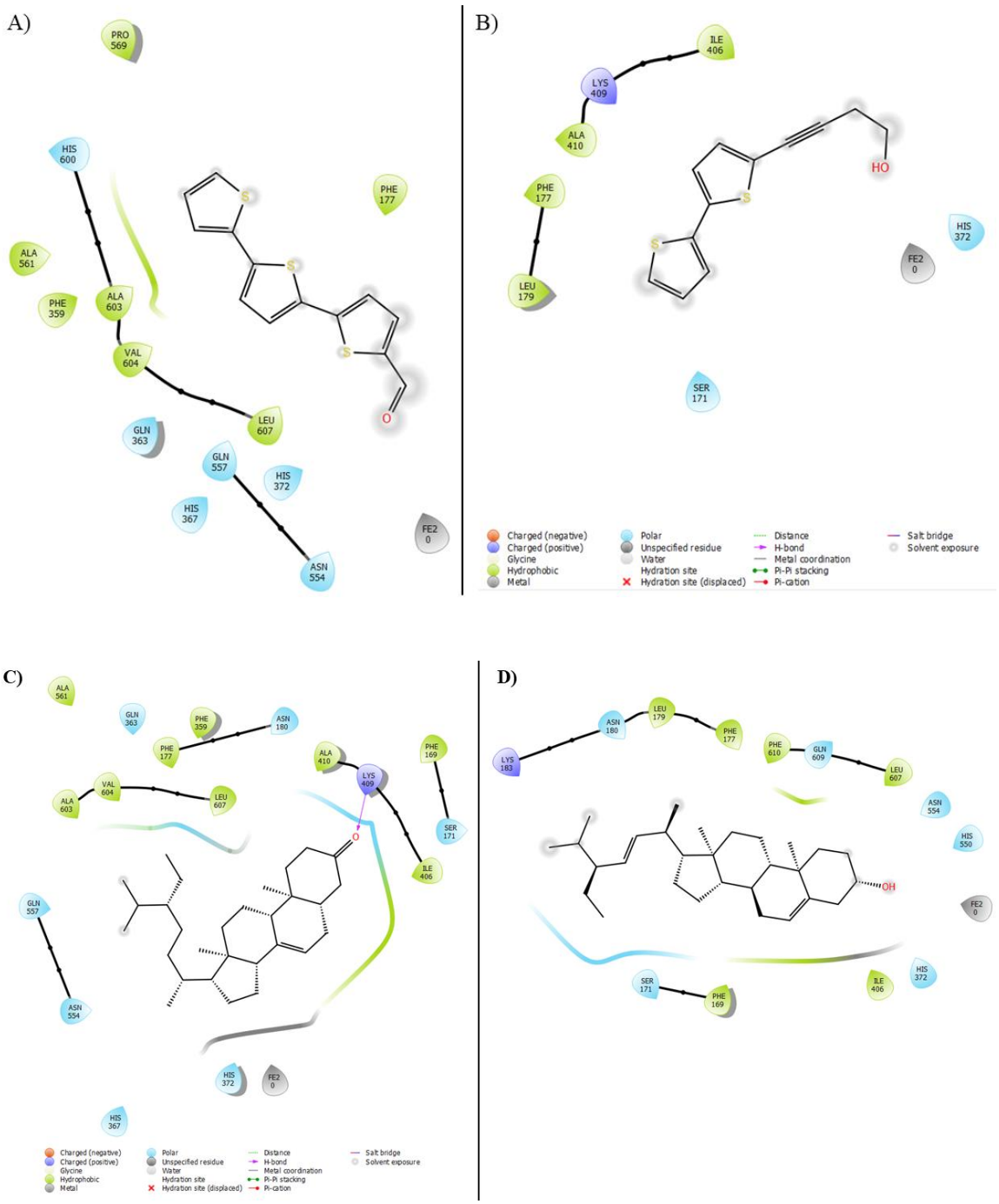

Figure S8. 2D diagram of 5-LOX inhibitors binding mode and residues involved in binding. A) **1**, B) **2**, C) **3**, D) **4**.

Table S1. The Pharmacokinetic parameters of the tested 5-LOX inhibitors.

Inhibitor No.	Mwt	#Rotatable bonds	#H-bond acceptors	#H-bond donors	MR	TPSA	Log P	Log S	Lip. v
1	276.4	3	1	0	76.33	101.79	4.35	-4.62	0
2	234.34	2	1	1	66.34	76.71	3.58	-4.14	0
3	412.69	6	1	0	132.27	17.07	7.21	-7.46	1
4	412.69	5	1	1	132.75	20.23	6.96	-7.46	1
5	420.63	19	4	1	127.5	55.76	6.76	-6.44	1

Mwt: Molecular weight; **TPSA**: Topological polar surface area; Log P: Calculated lipophilicity; Lip.V: Number of violations of Lipinski rule.