SUPPLEMENTARY MATERIAL

Phytochemical Constituents, *In-vitro* Anticancer Activity and Computational Studies of *Cymbopogon schoenanthus*

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Abstract

The cytotoxic effects of *Cymbopogon schoenanthus* L. aerial part ethanol extract were examined against some cancer cell lines, and HUVEC normal cell line using MTT assay. The ethanol extract was prepared by ultrasonic-assisted extraction and analyzed by GC-MS and HPLC. The extract was found to be rich in terpene compounds. The extract proved to be highly selective and effective against breast and prostate cancer cell lines (MDA-MB-435, MCF-7, and DU 145) with IC_{50} as low as 0.7913±0.14, 12.841±0.21, and 30.51±0.18µg/ml, respectively. *In silico* modeling was performed to investigate the binding orientation and affinity of the major identified compounds against Polo-like kinase (PLK1 protein) a cancer molecular target using molecular docking and molecular dynamic whereas eudesm-5-en-11-ol, piperitone, and 2,3-dihydrobenzofuran displayed better binding affinity and stability against PLK1 compared to the reference drug. These findings encourage further *in vivo* studies to assess the anti-cancer effects of *C. schoenanthus* extract and its components.

Keywords: *Cymbopogon schoenanthus*, terpenes, anticancer, molecular docking, molecular dynamics

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28 ## 1. Experimental

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30 ### *1.1 Plant Material Collection and Identification*

31 The fresh aerial parts of *C. schoenanthus* were picked up from a private garden in Gezira state,
32 Sudan. The voucher specimen of the plant sample was authenticated by Prof. Elhady Mohammed
33 Mohammed Ahmed, and was reserved under voucher number (1552020-Sc) in the herbarium of
34 Medicinal and Aromatic Plant Research Center, Faculty of Pharmacy, Gezira University, Sudan.

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36 ### *1.2 Chemicals and Reagents*

37 MTT assay reagents and chemicals included: MTT, DMEM, FBS, penicillin/streptomycin, and
38 trypsin-EDTA, were obtained from Gibco, USA. Phosphate buffered saline, DMSO, and authentic
39 reference materials for HPLC analysis including: gallic acid, catechin, chlorogenic acid, rutin, ellagic
40 acid, hesperidin, quercetin, kampeferol, and apigenin were procured from Sigma-Aldrich, Germany.
41 Other chemicals were of analytical grade.

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43 ### *1.3 Plant Extraction*

44 The ultrasonic-assisted extraction technique was used to extract the dry powdered plant material
45 (100 g) with 95% ethanol. The obtained extract was evaporated under reduced pressure by using a
46 rotary evaporator at a temperature not exceeding 60ºC to yield the residue of the extract.

47 ### *1.4 Derivatization of Plant Extract*

48 The extracted sample (up to 5 mg) was dissolved in hexene (1 mL) and 1% sulfuric acid in methanol
49 (2 mL) in a test tube with a condenser. The mixture was then refluxed for 24 hours at 50°C in a

50 stoppered tube. The esters were extracted with hexene (2 5 mL) and the layers were separated using
51 pasteur pipettes, water (5 mL) containing sodium chloride (5%) was then added. The hexene layer
52 was dried over anhydrous sodium sulfate after being rinsed with water (4 mL) that contained
53 potassium bicarbonate (2%) (Christie and Han 2012).

54 ### *1.5 Gas Chromatography- Mass Spectrometry (GC-MS) Condition*

55 GC-MS instrument (Model GC-MS-QP2010-Ultra, Shimadzu business, Japan) fitted with a
56 capillary column (Rtx-5ms-30.00 m × 0.25 mm × 0.25 µm) was utilized to determine the
57 phytochemical content of the ethanolic extract. The *C. schoenanthus* extract sample was injected
58 using the split mode, with the injection port temperature set at 300°C, helium flowed at 1.61 ml/min,
59 the oven temperature started at 50°C, then set to increase 10°C/min until reached 300°C, the ion
60 source temperature was 200°C and the interface temperature set at 250°C. Scan mode was used to
61 evaluate the samples, which had a mass range of 40–500 m/z. The mass spectrometer's ion source
62 temperature was 240ºC, and electron impact ionization was stabilized with a collision energy of 70
63 eV. The total run time was 32 minutes. The molecules were then defined using NIST`98 mass
64 spectral database.

65 ### *1.6 HPLC Analysis of Flavonoids and Phenolic acids*

66 The analysis of flavonoids and phenolic acids compounds was achieved on Waters 2690 HPLC
67 system equipped with a Waters 996 photodiode array detector and an analytical column C_{18}
68 (4.6x250mm, 5µm) at ambient column temperature. The separation was done using a mobile phase
69 consisting of 0.1 % phosphoric acid in water: acetonitrile following gradient elution at a flow rate
70 1 ml/min and wavelength adjusted at 280 nm for 80 minutes. The stock solution of 9 different
71 standards and extract were dissolved in methanol, filtered using 0.22µm syringe filter then 10 µl

72 of each sample was injected. Flavonoids and phenolic acids were identified by comparing their
73 retention times to that of a set of external standards that were analyzed under the same conditions.
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75 ### *1.7 Cell Lines and Cell Culture*

76 The cancer cells HCT-116, SKOV-3, DU 145, MCF-7, MDA-MB-435 as well as HUVEC normal
77 cells, were obtained from Nawah-Scientific, Cairo, Egypt and China Pharmaceutical University,
78 Nanjing, China, respectively. The cells were removed from a liquid nitrogen containers and quickly
79 were maintained in a DMEM medium supplemented with FBS (10%), penicillin (100 units/mL),
80 and streptomycin (100 mg/mL). All cells were placed in an incubator with controlled humidity and
81 temperature at 37 °C and maintained with carbon dioxide 5%.
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83 ### *1.8 Cell Viability Assay*
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85 A microculture of MTT assay was utilized to study the cell viability. By measuring the absorbance
86 of formazan crystals formed by metabolically viable cells, the MTT assay (5 mg/ml) affords a
87 quantitative estimation of the viable cells. DMSO was used to dissolve the herbal extract. For
88 interpolating the dosage inhibitory response curve, serial dilutions of stock solution were made for a
89 working concentration range of 10000 - 1 μg/ml. Each well of a 96-well plate was filled with viable
90 cells from each cell line in 100 μl of fresh culture media. Following the dosage regimen, 100 μl of
91 various concentrations of herbal extract per well were added after 24 hours of attachment. Incubation
92 was done at 37°C and 5% CO_2. A 96-well plate was cultivated for 72 hours before adding 20 μl of
93 MTT reagent to each well, placing it in a 37°C incubator for 4 hours, then removing the media and
94 adding 150 μl MTT reagent and agitating the cells for 15 minutes.

After discarding the media, the formazan crystals were measured in three independent experiments using a microplate spectrophotometer (BMGLABTECH®FLUOstar Omega, Germany) (Van de Loosdrecht et al. 1994).

1.9 Computational Studies

1.9.1 Ligand Preparation and Protein Preparation

The 3D structures of molecules that were identified by HPLC and GC-MS were acquired from the PubChem database. The molecules were then prepared by employing the (LigPrep, 2020) of Maestro and minimized using OPLS3e force field. The crystal structure of PLK 1 (PDB ID: 2RKU) was obtained from Protein Data Bank (PDB). The protein was prepared by using protein preparation wizard (PPW) of Maestro. PPW removed crystal water molecules and fixed missing atoms and side chains. The receptor grid generation module was utilized to determine the binding site of the protein around the pre-existing ligand.

The prepared compounds and protein were used for molecular docking, MM-GBSA binding free energy calculations, and molecular dynamics.

1.9.2 Molecular Docking and MM-GBSA Calculations

These studies were carried out for the compounds of plant extract to evaluate the strength of receptor-ligand interactions.

The grid for docking was generated using the coordinates of the co-crystalized ligand. Glide (Grid-based ligand docking with energetics) of Schrodinger was used for the docking study. XP (Extra Precision) docking mode of the glide which is the most accurate method was selected for the screening process.

119 MM-GBSA is a vital calculation method for combining free energy and quantifying docking
120 accuracy. The prime module of Schrodinger was used to estimate the free binding energies for
121 compounds using XP docking poses as inputs.

122 *1.9.3 Molecular Dynamic (MD)*

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124 Molecular dynamic (MD)studies were conducted to verify the stability of top-scoring compounds
125 bound to PLK 1 protein. Academic Desmond software by D. E. Shaw Research was used to
126 perform MD simulations. The system was solvated in TIP3P water molecules in an orthorhombic
127 box (10 Å x 10 Å x 10 Å). The simulation was conducted in 0.15M NaCl and minimized using
128 OPLS3e forcefield. All three systems were equilibrated at NVT and NPT ensembles at 300K to
129 ensure a fully converged system for a production run. The runs for simulation were conducted
130 using NPT ensemble at temperature and pressure values of 300 K and 1.01325 bar, respectively
131 for 50 ns. The RMSD and RMSF plots were used to analyze the whole period of simulations.

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133 ***1.10 Statistical Analysis***

134 The results are represented as an average plus or minus the standard error of the mean
135 (SEM). The statistical analysis was calculated using GraphPad Prism v5 order.

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169 *Table S1: Results of the GC-MS analysis of the Cymbopogon schoenanthus ethanol extract*

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Peak	Compound	Retention Time	Peak Percentage (%)
1	Ethyl orthoformate	3.409	3.71
2	2,3-Dihydrobenzofuran	9.391	11.84
3	Piperitone	10.010	3.55
4	Carvacrol	10.613	0.81
5	Pseudodiosphenol	10.785	0.34
6	2-Methoxy-4-vinylphenol	10.891	1.51
7	2-Cyclohexen-1-one, 4-hydroxy-3-methyl-6-(1-methylethyl)	12.595	0.61
8	Cyclohexanone, 2-isopropyl-2,5-dimethylexanone	12.905	0.66
9	Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)	14.771	42.53
10	Eudesm-4(14)-en-11-ol	15.422	1.25
11	γ-Eudesmol	15.906	6.13
12	3-Eudesmen-11-ol	16.268	14.61
13	Eudesm-5-en-11-ol	18.181	7.81
14	Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-yl ester	19.372	0.21
15	Palmitic acid	19.822	1.03
16	Oleic Acid	21.727	0.53
17	Stearic acid	21.968	0.14
18	Formic acid, 3,7,11-trimethyl-1,6,10-dodecatrien-3-yl ester	22.084	0.22
19	12-Isopropyl-1,5,9-trimethyl-4,8,12-cyclotetradecatriene-1,2-diol	22.348	0.10
20	γ-Sitosterol	23.921	1.24
21	Octadecanophenone	24.099	0.26
22	Diisooctyl phthalate	25.447	0.58
23	α-Tocopherol	26.161	0.33

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179 *Table S2: GC-MS of major identified compounds of Cymbopogon schoenanthus derivatized*
180 *extract*

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Peak	Compound	Retention Time	Peak Percentage (%)
1	Caprylic acid methyl ester	10.178	0.22
2	Methyl 6-methyloctanoate	11.717	0.28
3	Thymol methyl ether	12.650	0.39
4	2-Cyclohexen-1-one, 3-methyl-6-(1-methylet	13.140	1.21
5	Hexadecane	13.520	0.12
6	Carvacrol	13.973	0.79
7	Decanoic acid, methyl ester	14.470	0.13
8	Cyclohexane, 2,4-diisopropenyl-1-methyl-1-vinyl	15.937	1.33
9	gamma.-Elemene	16.736	1.20
10	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a	17.269	0.12
11	Eudesma-4(14),11-diene	17.814	0.50
12	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)	18.023	0.31
13	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)-	18.302	0.58
14	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	18.448	1.44
15	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	18.630	0.15
16	Tridecanoic acid methyl ester	19.035	10.91
17	Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)	19.058	5.60
18	.gama.-eudesmol	20.550	11.68
19	Cubenol	20.646	1.37
20	4.beta.H,5.alpha.-Eremophil-1(10)-ene, 11-(t	20.805	2.92
21	alpha.-Eudesmol	20.938	11.62
22	9,19-Cyclolanostan-24-one, 3-acetoxy-25-methoxy	21.101	3.22
23	Tridecanoic acid, 12-methyl-, methyl ester	21.751	0.26
24	Eudesm-4(14)-en-11-ol	21.852	0.50
25	Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy	22.626	0.42
26	Phthalic acid, butyl undecyl ester	24.111	3.52
27	Palmitic acid, methyl ester	24.905	5.80
28	Cyclopentanetridecanoic acid, methyl ester	26.304	0.14
29	Linoleic acid, methyl ester	27.334	5.04
30	9-Octadecenoic acid, methyl ester	27.430	7.53
31	Methyl stearate	27.713	1.44
32	Eicosanoic acid, methyl ester	30.304	0.60
33	Pentacosanoic acid, methyl ester	32.716	0.81
34	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl)methyl]cyclopropyl]methyl]cyclopropyl]methyl]-, methyl ester	37.029	1.15

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184 *Table S3: IC_{50} values and selectivity indexes (SI) of antiproliferative activity of Cymbopogon*
185 *schoenanthus aerial part ethanol extract*

HUVEC	MDA-MB-435		MCF-7		DU 145		HCT-116		SKOV-3	
IC_{50}	**IC_{50}**	**SI**	**IC_{50}**	**SI**	**IC_{50}**	**SI**	**IC_{50}**	**SI**	**IC_{50}**	**SI**
223.4	0.7913±0.14	282.32	12.841±0.21	17.40	30.51±0.18	7.32	206.32±0.13	1.08	502.629±0.12	0.44

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188 *Table S4: XP Docking scores and MM-GBSA free binding energies of Cymbopogon*
189 *schoenanthus compounds against 2RKU protein target*

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NO	Compound	2RKU (kcal/mol)	
		docking score	MMGBSA dG Bind
1	Eudesm-5-en-11-ol	-7.12	-49.03
2	Piperitone	-7.058	-47.21
3	2,3-Dihydrobenzofuran	-6.512	-29
4	Eudesm-4(14)-en-11-ol	-6.335	-51.15
5	dl-.alpha.-Tocopherol	-6.105	-59.79
6	Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)	-5.796	-40.20
7	Onvansertib	-6.194	-45.80

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192 Figure S1: GC-MS chromatogram of the *Cymbopogon schoenanthus* ethanol extract.

193 Figure S2: GC-MS chromatogram of the *Cymbopogon schoenanthus* derivatized extract.

194 Figure S3: HPLC chromatogram of the *Cymbopogon schoenanthus* ethanol extract.

195 Figure S4: The 2D and 3D interactions with 2RKU protein:

196 A. 2-Eudesm-5-en-11-ol B. Piperitone C. 2,3-Dihydrobenzofuran

197 Figure S5: Protein ligand RMSD:

198 A. 2-Eudesm-5-en-11-ol B. Piperitone C. 2,3-Dihydrobenzofuran
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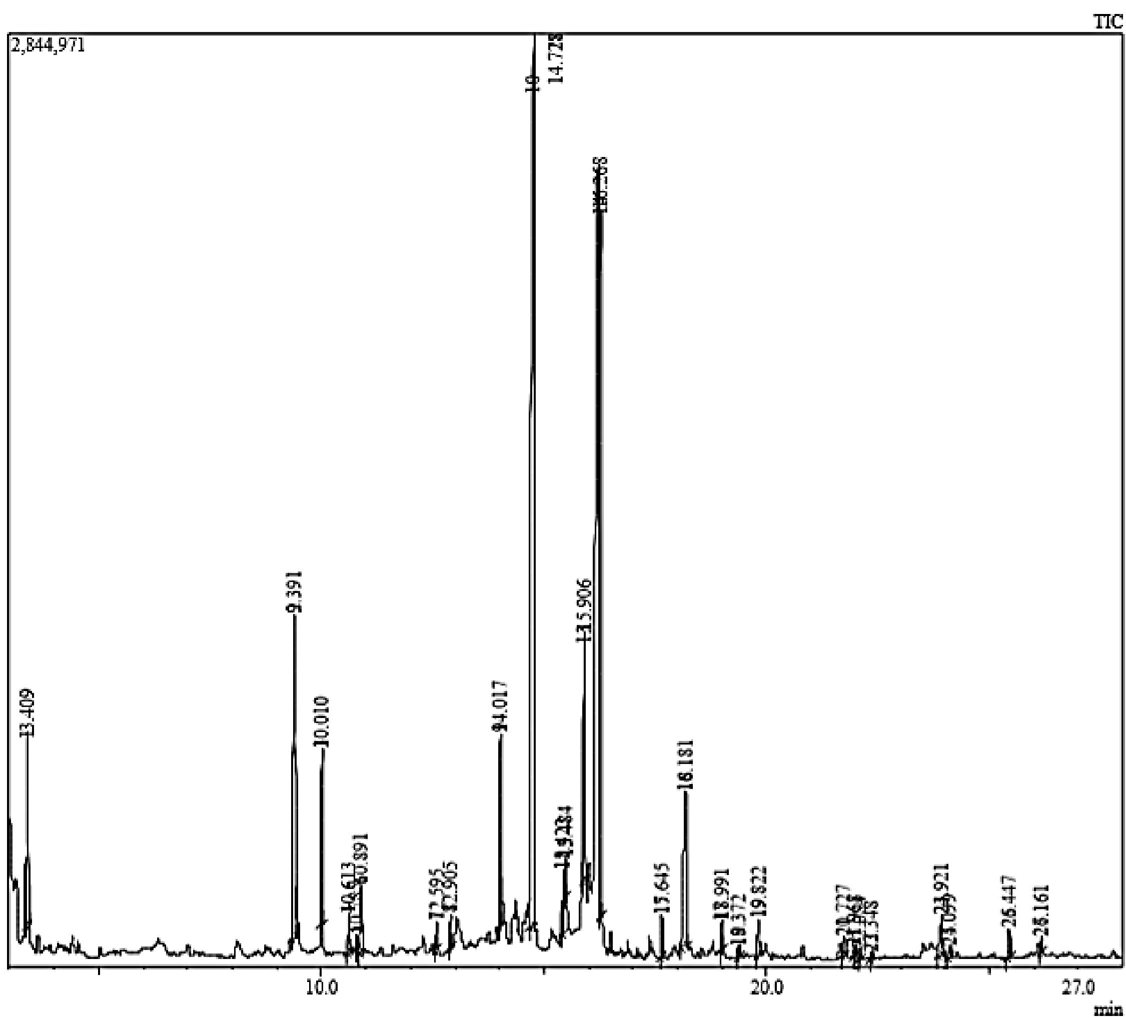

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202 Figure S1: GC-MS chromatogram of the *Cymbopogon schoenanthus* ethanol extract.

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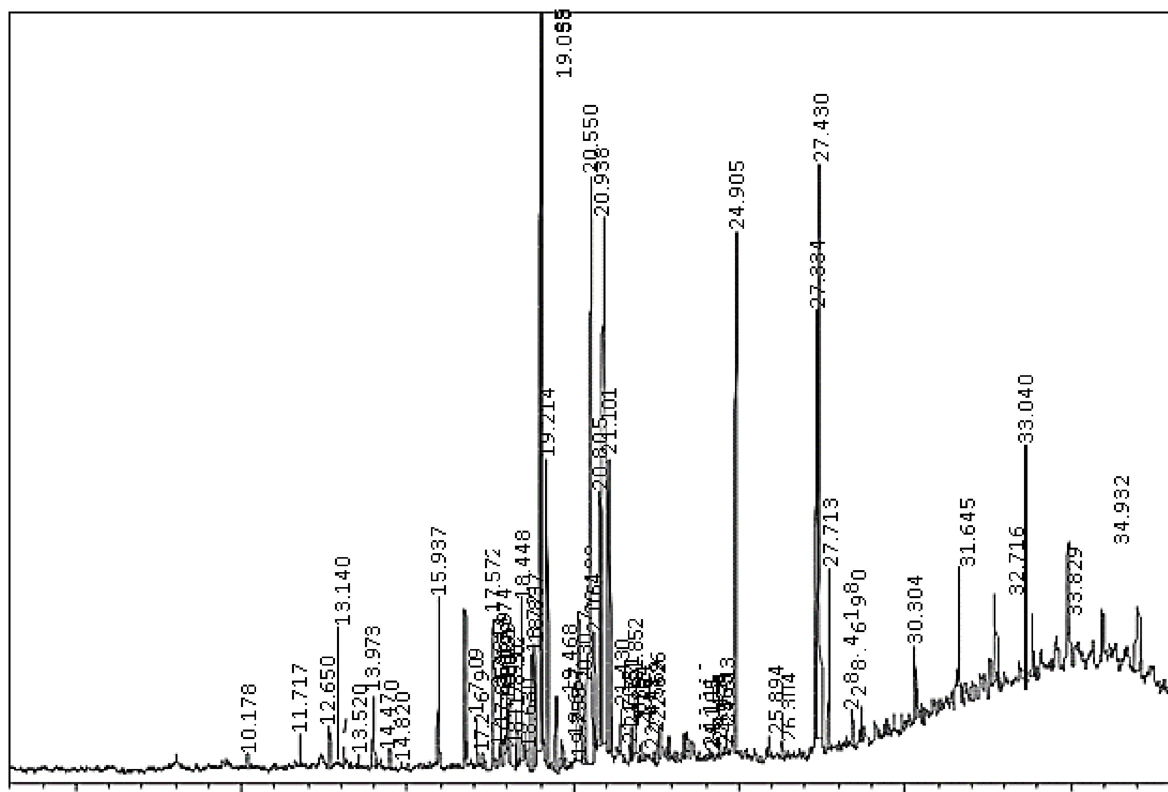

Figure S2: GC-MS chromatogram of the *Cymbopogon schoenanthus* derivatized extract.

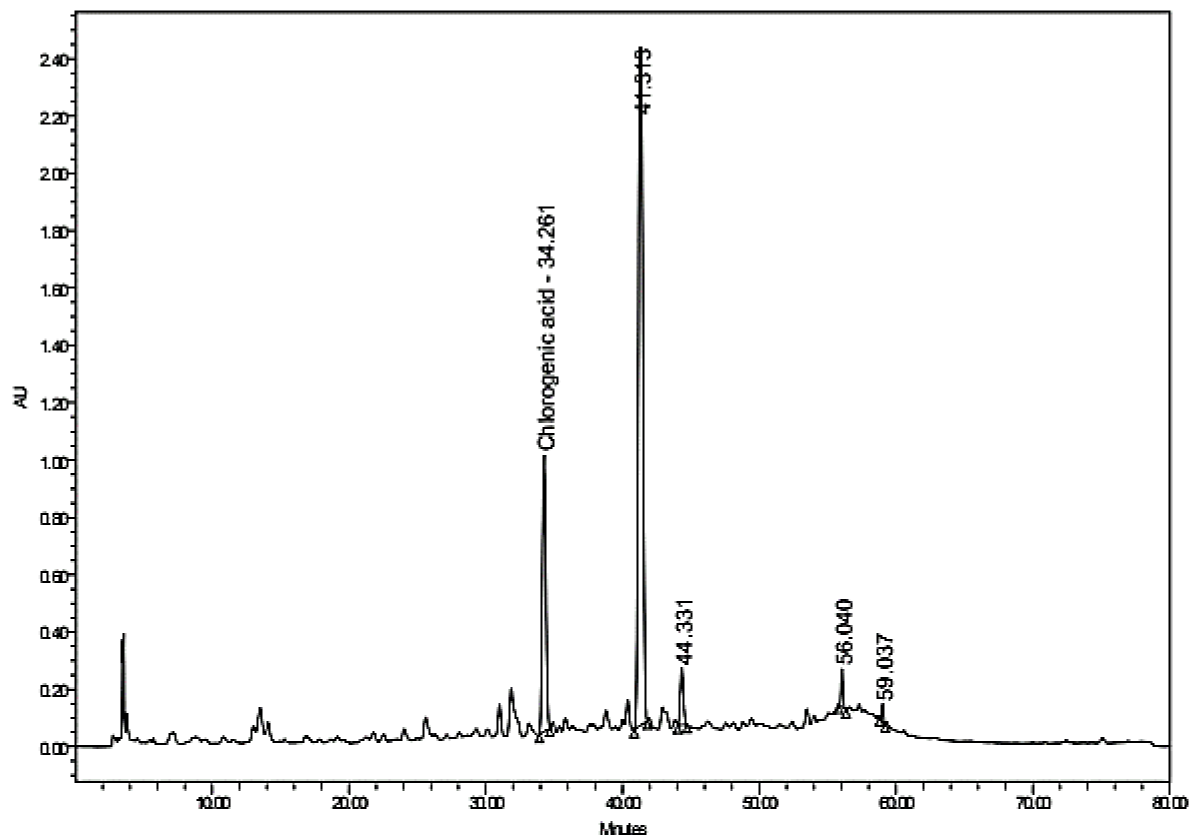

Figure S3: HPLC chromatogram of the *Cymbopogon schoenanthus* ethanol extract.

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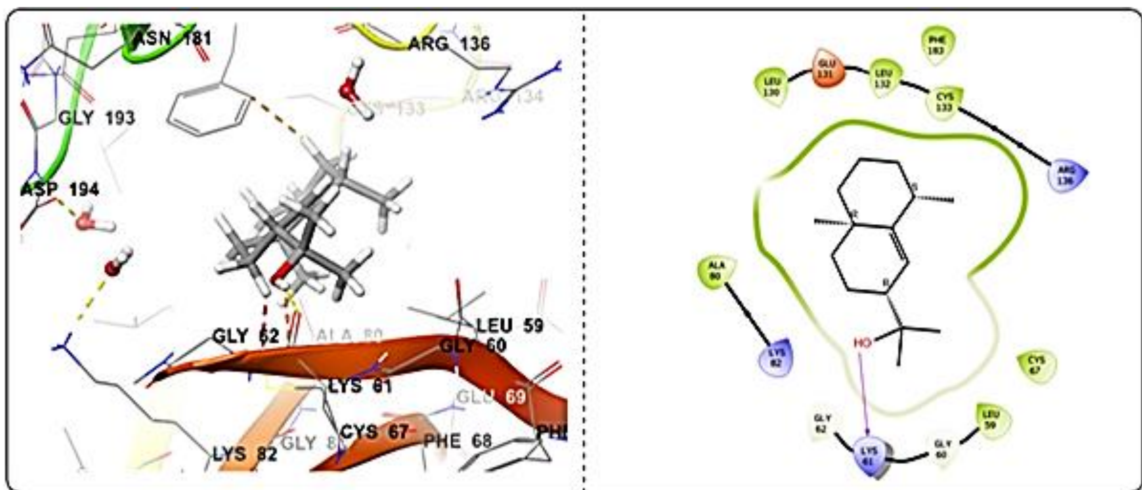

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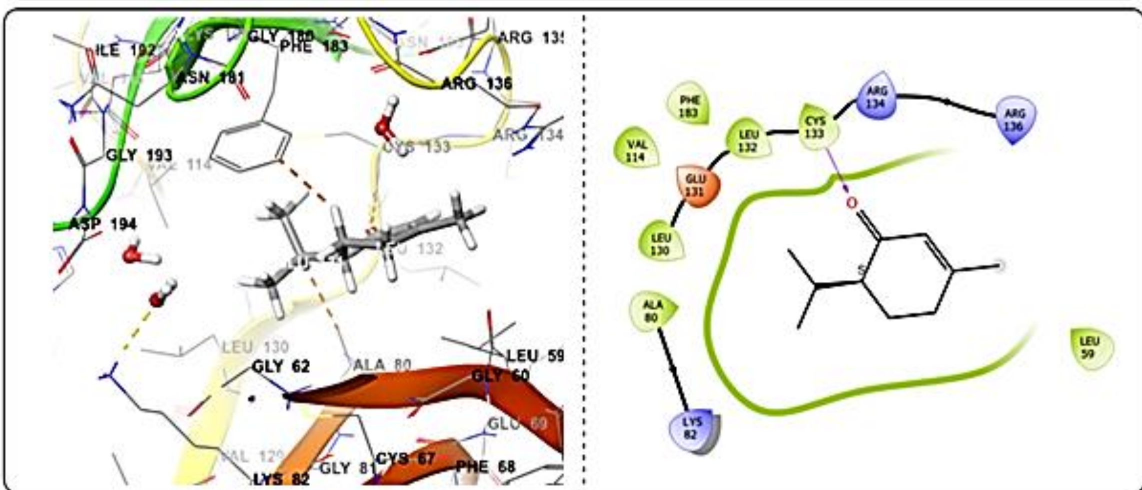

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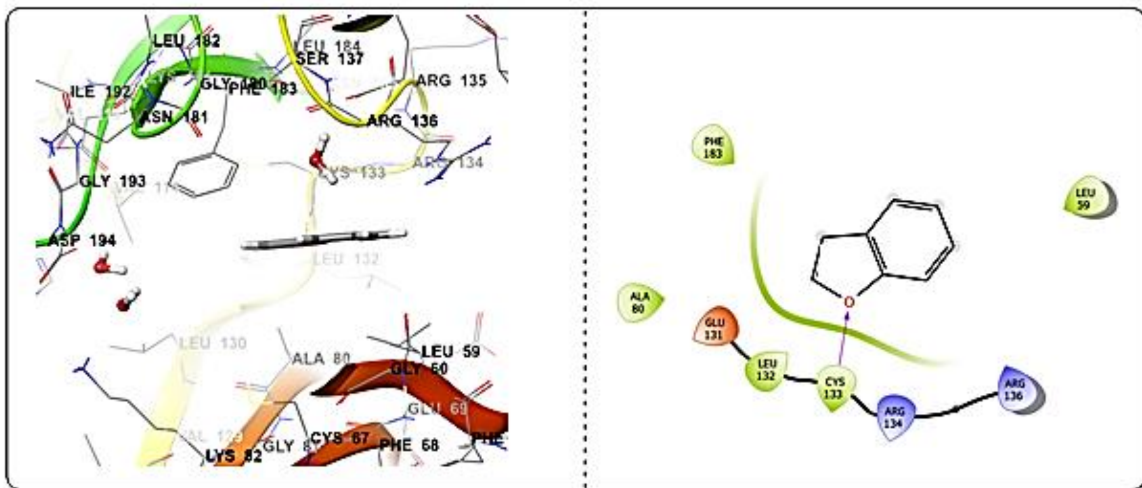

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234 Figure S4: The 2D and 3D interactions with 2RKU protein:

235 **A**. 2-Eudesm-5-en-11-ol **B**. Piperitone **C**. 2,3-Dihydrobenzofuran

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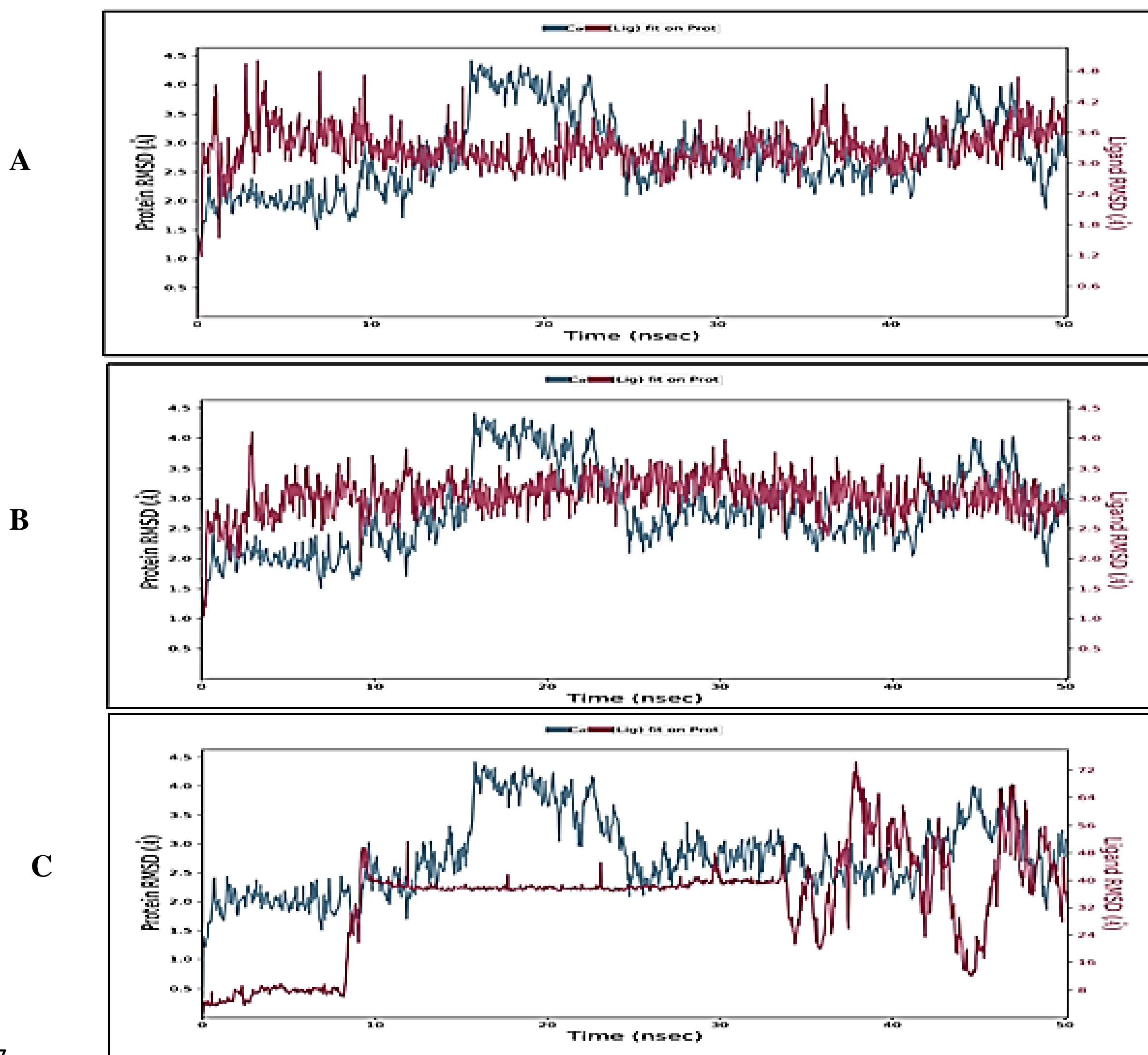

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239 Figure S5: Protein ligand RMSD:

240 **A**. 2-Eudesm-5-en-11-ol **B**. Piperitone **C**. 2,3-Dihydrobenzofuran

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