Studies on bioactive 6-gingerol derivatives and thymoquinone-protoflavone hybrid molecules

Summary of Ph.D. Thesis

Sara Hassan Hassan Ahmed

Doctoral School of Pharmaceutical Sciences
Institute of Pharmacognosy
University of Szeged

Szeged, Hungary
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University of Szeged
Doctoral School of Pharmaceutical Sciences
Program of Pharmacognosy
Head: Prof. Dr. Judit Hohmann, DSc

Institute of Pharmacognosy
Supervisor: Prof. Dr. Attila Hunyadi, DSc

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INTRODUCTION

Cardiovascular diseases (CVDs) and cancer were classified as the first and second leading cause of death; respectively. Great efforts have been devoted toward discovering therapeutic agents against these pathologies and many drugs have reached the market, nevertheless, more efforts are still needed to reach the coveted goals.

Many therapeutic agents that reached the market are either natural or their development is inspired by naturally existing compounds. Scientists use the structure of natural agents reported to be potentially active against certain diseases to synthesize new derivatives. These derivatives are then subjected to bioactivity testing to compare them with the parent compounds and to characterize their pharmacophores. *In silico* studies for the compounds under development also greatly save time and cost invested in drug discovery research.

This thesis summarizes semi-synthetic work on three natural phenolic compounds: 6-Gingerol (6-G); from ginger, *Zingiber officinale* Rosc. (Zingiberaceae), protoapigenone (PA), from *Thelypteris torresiana* Gaud. (Thelypteridaceae), and thymoquinone (TQ) from *Nigella sativa* L. (Ranunculaceae). Compounds prepared from 6-G were tested for their CVD-related therapeutic potential. Further, the antitumor potential of TQ and PA inspired the preparation of new hybrid compounds containing these two natural products as key building blocks tested for their antiproliferative activity.

AIMS OF THE STUDY:

6-Gingerol derivatives:

In this part, we aimed to:

1. synthesize, purify, and characterize semi- or total synthetic derivatives of 6-G, and
2. in research collaboration, to study their AA-induced platelet aggregation inhibitory activity, COX-1 enzyme inhibitory activity, and antioxidant activity, and to assess their ADME behavior using experimental and *in silico* tools, and
3. to predict their interaction with the *h*-COX-1 enzyme utilizing *in silico* molecular docking.

Thymoquinone-protoflavone hybrids:

Here we decided to:

1. combine the TQ with different protoflavone derivatives into ester-linked hybrid structures,
2. in research collaboration, to assess the antiproliferative potentials of these compounds together with their parent fragments (alone and in combination) against HeLa, MCF-7, MDA-MB-231, and U-87 cell lines, and
3. to evaluate structure-activity relationships and interpret bioactivity data in view of the compounds' chemical stability.

METHODS

6-Gingerol derivatives; isolation, synthesis and testing

- **Isolation and synthesis**

Ginger extract was purchased from Xi'an Pincredit Bio-Tech Co., Ltd., Xi'an, China. 6-G (**1**) was purified from the crude extract using flash chromatography (Silica, gradient elution of 0–10% of acetone in *n*-hexane) and utilized to synthesize 6-shogaol (**2**), and subsequently 4,5-dihydro-6-shogaol (**7**); which used as starting material for synthesis of compound **64**. Compound (**1**) was also used for the synthesis of compounds **65** and **66**. Compound **47** was derived from vanillin (**67**) and 2,4-nonanedione (**70**), while compound **72** was from compound **47**. The same method was used for the synthesis of compound **71** only by replacing the vanillin with *p*-Hydroxybenzaldehyde; **68**. Compounds **47** or **71** were then utilized as starting materials for the synthesis of compounds **14-18**, per needed. Compound **81** was synthesized from reduced ferulic acid; compound **79**. Different synthetic methods, purification techniques, and structural elucidation methods are used to achieve the abovementioned compounds.

- **Biological Activity**

Antiplatelet Activity, COX-1 Inhibitory Activity, Physicochemical character, and blood–brain barrier specific permeability assays, antioxidant activity, and molecular docking were conducted according to the previous reports.

Thymoquinone-protoflavone hybrids; synthesis, stability testing and, bioactivity:

- **Synthesis**

Thymoquinone (**63**) was synthesized from thymol, which is then used as a starting material for the synthesis of compounds **83** and **84,** each was integrated into different ester hybrid derivatives (**89**–**96**) with the suitable protoflavone fragment (**62**, **86**–**88**) prepared as previously reported. The stability of the hybrid compounds was assessed through an enzymatic hydrolysis assay using a Porcine esterase enzyme.

- ***In vitro* antiproliferative activity**

A collection of gynecological cancer cell lines of human origin was used as *in vitro* models to study the antiproliferative effects of the evaluated compounds (the hybrids, their building fragments, and their experimental 1:1 combination). The colorimetric MTT assay was used to assess the ability of the prepared compounds to inhibit cellular proliferation. The data were calculated and evaluated using GraphPad Prism 9.5.1 (GraphPad Software, San Diego, CA, USA). The same procedure was performed using MRC-5 cells to determine the cancer selectivity of the most promising test compounds.

RESULTS AND DISCUSSION

6-Gingerol derivatives:

- **Chemistry:**

6-Gingerol was isolated from an ethanolic ginger extract via a feasible extraction process resulting in a pure product with up to 36% yield (**fig. 1**).

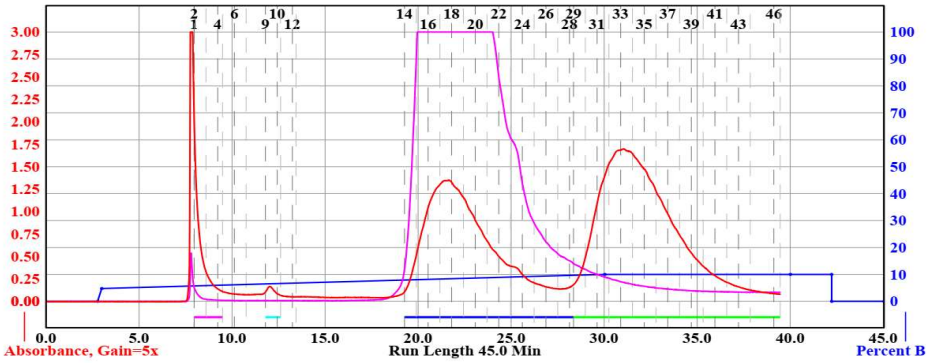
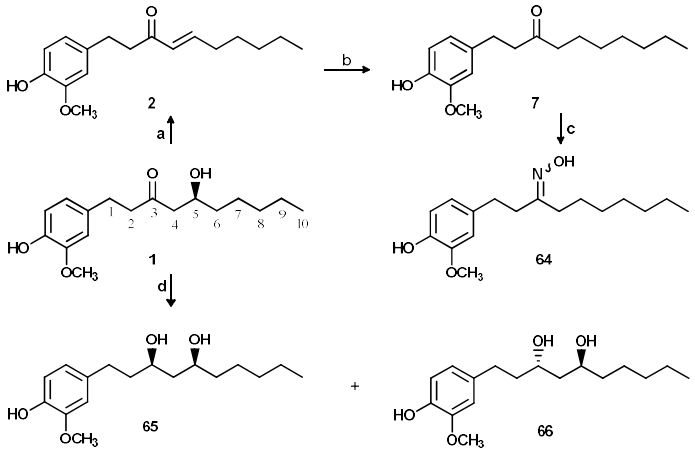


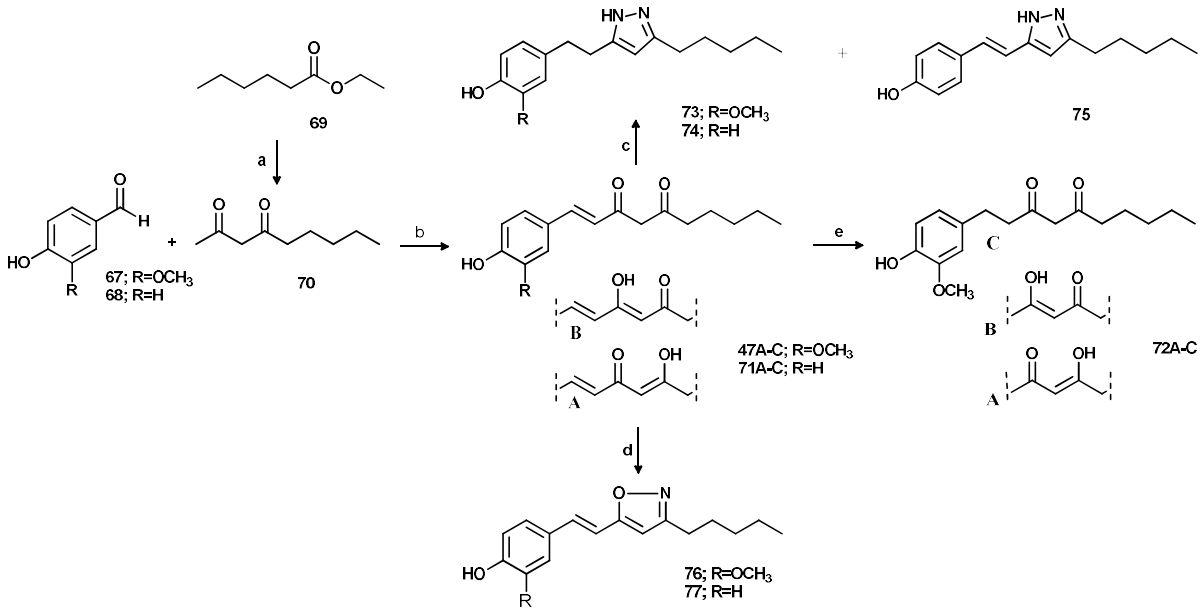
Figure 1: The process of 6-gingerol isolation from the extract via flash chromatography techni-que using Silica as a stationary phase and n-hexane: acetone as a mobile phase (λ_1=225, λ_2=366)

The purified 6-G was subsequently utilized as a starting material for the synthesis of five compounds (**2, 7,** and **64**–**66**). Except for compound **64**, all of them are naturally present in ginger root. Further derivatives were inspired by the structure of 6-G, and in total fourteen compounds were synthesized (**Schemes 1**, **2**, and **3**); eight of them are new (i.e., compounds **64**, **71**, **73**–**77**, and **81**) according to the SciFinder database.



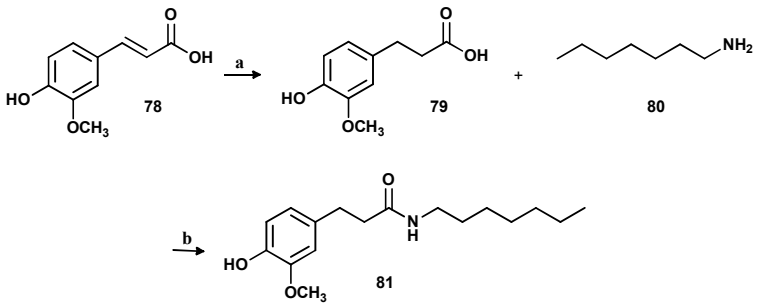
Scheme 1. Semi-synthesis of compounds **2**, **7**, and **64**–**66** from 6-gingerol (**1**). Reaction conditions: **a**. *p*TsOH/toluene/110 °C; **b**. H_2/Pd/C/EtOAc/r.t.; **c**. $NH_2OH.HCl$/EtOH/rt; **d**. $NaBH_4$/MeOH

Compound **2**, 6-shogaol was obtained through a dehydration process using P-TsOH, while 6-gingerdiol epimers, compounds **65** and **66** were gained in a 3:2 ratio mixture product upon 6-G reduction (**Scheme 1**). They were separated using the preparative HPLC technique (**Fig. 6**). Reductive amination of 6-G resulted in compound **64**, an oxime isostere of 6-paradol (compound **7**) and it was obtained as an isomeric mixture of both *E* and *Z* isomer. 6-Dehydrogingerdione (**47**) was utilized as a starting material for the synthesis of another three products (viz; **72**, **73**, and **76**) (**Scheme 2**). A similar set of compounds (**74**, **75,** and **77**) was synthesized from compound **71**.



Scheme 2. Preparation of gingerdione derivatives **47, 71,** and **72** and their heterocyclic analogues **73**–**77**. Reaction conditions: **a**. 1. NaH/Et_2O/Acetone/0 °C, 2. EtOH/HCl; **b**. B_2O_3/*i*$BuNH_2$/DMF/ 90 °C; **c**. H_6N_2O/HCl/EtOH/80 °C; **d**. NH_2OH.HCl/pyridine/ethanol/80 °C; **e**. H_2/Pd/C/EtOAc /rt. Major tautomers of compounds **47, 71,** and **72** are indicated with A, B, and C.

Compound **81** was synthesized as an amide derivative mimicking the structure of 6-G to test the effect of this functional group change in the activity (**Scheme 3**).



Scheme 3. Preparation of the amide analogue of 6-paradol (**81**). Reaction conditions: **a**. H_2/Pd /C/EtOAc/rt; **b**. DCC/DMAP/CH_2Cl_2/rt.

Structural elucidation for 6-G and its derivatives was performed through HRMS and NMR.

➢ Biological Activity

1. Antiplatelet activity

To assess the antiplatelet bioactivity of 6-G (**1**) and its derivatives, they were tested for their inhibitory effects on AA-induced platelet aggregation; a very important pathway in the platelet aggregation process. Except for compound **74**, all compounds showed better platelet-aggregation inhibitory activity compared to the positive control; aspirin (IC_{50} = 106 μM) (**Table 1**).

Table 1. Antiplatelet inhibition assay results. For the assay, washed human platelets were treated with 6-gingerol derivatives for 3 min and then stimulated with arachidonic acid (100 μM); data are presented as mean ± SEM, *n* = 3; aspirin was used as a positive control.

Compound	Antiplatelet IC_{50} (μM)	$LLE^{a}_{(Antiplatelet)}$
1	45.9 ± 5.1	1.46
2	2.8 ± 0.5	1.40
7	2.1 ± 1.0	1.56
64	5.2 ± 0.4	0.47
65	51.7 ± 2.7	1.26
66	45.1 ± 6.0	1.32
47(A) [b]	4.1 ± 1.0	1.89 [b]
71(A) [b]	71.7 ± 28.3	0.24 [b]
72(B) [b]	3.6 ± 0.9	1.94 [b]
73	4.1 ± 1.2	0.72
74	>100	-
75	3.5 ± 0.9	0.63
76	3.1 ± 0.9	1.08
77	32.0 ± 10.1	−0.27
81	35.9 ± 23.7	0.80
Aspirin	106.0 ± 20.2	2.58

[a] Ligand–lipophilic efficiency: LLE = pIC_{50}—log$P_{predicted}$. For LLE, green colouring indicates a satisfactory level ($LLE_{(Antiplatelet)} \geq 1.5$ and $IC_{50} \leq 10$ μM). [b] For the sake of strict characterization, the tautomer with the highest log*P* value (see **Table 3**) was included in the LLE calculation for compounds **47**, **71**, and **72**, i.e., the value for the worst possible case is shown in the table.

Regarding the structure-activity relationships, our results suggest an important role for the aromatic methoxy group, which is evident by comparing the IC_{50} values of **47** vs. **71**, **73** vs. **74**, and **76** vs. **77**. Intriguingly, compound **75** didn't follow this rule. This might highlight a possible role of the $\Delta^{1,2}$ olefin in some cases, e.g., when it is conjugated with a pyrazole ring. On the other hand, the 5-OH group substitution is greatly unfavourable; as suggested by the increased antiplatelet activity of compounds **2**, **7**, and **64**, as well as its oxidation (compound **72** vs. **1**) or replacement by a heterocycle (e.g., compound **73** vs. **1**).

Compound **81**, the amide derivative of compound **1** was only moderately active. Comparatively, replacing the 3-oxo group by an oxime group led to only a slight, ca. 2-fold decrease in the antiplatelet activity (IC_{50} = 5.2 μM).

Ligand-lipophilic efficiency (LLE) is an estimate of drug-likeness through linking potency and lipophilicity of a compound, defined as the pIC_{50} of interest minus the LogP of a compound. Notably, most of the compounds that showed better results in terms of drug-likeness (LLE) are naturally present in ginger root (**72**, **47**, **7**, **1,** and **2**).

2. COX-1 inhibitory activity:

As cyclooxygenase-1enzyme represents a key factor in the platelet aggregation process, we investigated the activity of our compounds against COX-1 using a fluorometric assay. Our results are -to a great extent-in harmony with the antiplatelet inhibition assay results (the IC_{50} datasets of **Table 2** give a linear correlation coefficient (R^2) value of 0.887, strongly suggesting the inhibition mechanism to be COX-1-mediated). Compounds **73, 7, 64, 76,** and **2** showed the best IC_{50s}, while compounds **77, 71,** and **74** were the least.

Table 2. Cyclooxygenase-1 (COX-1) inhibition assay results. Data are presented as mean ± SEM, n = 2.

Compound	COX-1 IC_{50} (μM)	$LLE^{a}_{(COX-1)}$
1	62.5 ± 23.8	1.30
2	9.8 ± 0.6	0.81
7	4.4 ± 0.2	1.26
64	5.2 ± 0.3	0.48
65	54.3 ± 6.5	1.27
66	76.2 ± 0.3	1.12
47(A) [b]	23.1 ± 9.3	1.14
71(A) [b]	>200	-
72(B) [b]	11.8 ± 5.4	1.53
73	3.6 ± 0.2	0.74
74	>200	-
75	17.5 ± 0.1	−0.04
76	5.85 ± 0.04	0.83
77	>200	-
81	>100	-

[a] Ligand–lipophilic efficiency: LLE = pIC_{50}—$\log P_{predicted}$. For LLE, green colouring indicates a satisfactory level ($LLE_{(COX\text{-}1)} \geq 1.0$ and $IC_{50} \leq 10$ μM). [b] For the sake of strict characterisation, the tautomer with the highest log*P* value (see **Table 3**) was included in the LLE calculation for compounds **47**, **71**, and **72**, i.e., the value for the worst possible case is shown in the table.

3. Physicochemical Character and Blood–brain Barrier Specific Permeability:

To assess the druggability of 6-G and its derivatives their ADME characteristics were investigated using *in silico* (lead optimization parameters and the Central Nervous System Multiparameter Optimisation (CNS MPO) compliance) and *in vitro* (determination of kinetic solubility and *in vitro* BBB permeability) approaches. A punch of descriptors was calculated (**Table 3**).

In general, all compounds complied with the components of Lipinski's rule of 5 (Ro5). To investigate the distribution of the A-C tautomeric states of derivatives **47**, **71**, and **72**, Chemaxon Ltd.'s freely available Tautomer Generator plugin was used. The results demonstrated that the enol form is the predominant form (**A**:**B**, ~30:60%). The p*Ka,acid* parameters for these compounds refer to the proton-dissociation behaviour of the enol and dione C-H acid functions in comparison to the aromatic OH function of the other gingerols (**1**, **2**, **7**, and **64**–**66, 73-81**).

In terms of lipophilicity, though all compounds have logP <5, nevertheless, based on the two-level risk classification created by the lead likeness and CNS MPO criteria ($\log D_{pH7.4}$), **2**, **7**, and **64** and **73**–**77** exceed (magenta: high violation), and **81** approaches (yellow: moderate violation) the $\log D_{pH7.4}$ violation limit. The enol forms **A**/**B (47A**, **71A**, **72B)** are more lipophilic than the dione forms (**C**), which was also reflected in their corresponding CNS MPO values. Based on CNS MPO values; compounds **1**, **2**, **7**, **65**–**66**, **47**, **71**, and **72** (the **C** tautomer form), **76** and **81** (green–yellow) can be classified as suitable candidates for further CNS-targeted preclinical studies.

Experimental kinetic solubility (PBS, pH 7.4) and *in vitro* BBB-specific permeability (PAMPA-BBB) of the compounds were then measured. Using a three-level categorization for the kinetic solubility values, compounds **1**, **2**, **65**, **66**, **72**–**74**, and **81** were in the acceptable range (greater than 100 μM). PAMPA-BBB

study was only applied to these gingerols due to the limitation of poor solubility of other gingerols. Compounds **1**, **2**, **65**, **66**, and **81**; marked in green ($P_{e,BBB} \geq 25 \cdot 10\text{-}7$ cm/s) showed increased BBB permeability. On the contrary, the increased hydrophilic character of compound **72** may compromise its BBB permeability.

Table 3. *In silico* and experimental data for physicochemical and BBB permeability characterization. Results are given as mean ± standard error of the mean (SEM); $n = 3$.

		Predicted Values [a]					Experimental Data	
Cmpds ID	Tautomer Distribution (%) [b]	p$K_{a,base}$/ p$K_{a,acid}$ [c]	log$P$/log$D$ $_{pH7.4}$	TPSA	HBD/ HBA	CNS MPO [d] [161]	Kinetic Solubility [e] (µM)	PAMPA-BBB [e'] P_e ($\cdot 10^{-7}$ cm/s)/MR (%)
1		-/10.0	2.9/2.9	66.8	2/4	5.06	>500	35.2 ± 2.4/23.8 ± 1.5
2		-/10.0	4.2/4.2	46.5	1/3	4.18	110.2 ± 3.8	31.7 ± 3.6/20.9 ± 3.9
7		-/10.0	4.1/4.1	46.5	1/3	4.19	45.2 ± 2.7	-/11.0 ± 0.7
64		-/10.1	4.8/4.8	62.1	2/4	3.60	75.1 ± 7.3	-/4.0 ± 11.1
65		-/10.1	3.0/3.0	69.9	¾	4.72	>500	33.9 ± 2,5/9.1 ± 7.6
66		-/10.1	3.0/3.0	69.9	¾	4.72	460.7 ± 10.3	27.4 ± 1.6/18.5 ± 2.7
47A	31	-/8.3	**3.5**/3.5	66.8	2/4	4.81		
47B	60	-/8.3	3.2/3.2	66.8	2/4	4.53	54.0 ± 1.3	-/-
47C	9	-/8.7	3.3/3.3	63.6	¼	5.01		
71A	38	-/8.3	**3.9**/3.8	57.5	2/3	4.62		
71B	54	-/8.4	3.4/3.4	57.5	2/3	4.15	15.6 ± 0.1	-/-
71C	8	-/8.9	3.5/3.5	54.4	1/3	4.75		
72A	31	-/8.7	3.4/3.4	66.8	2/4	4.58	314.6 ± 23.7	-/-
72B	60	-/8.7	**3.5**/3.4	66.8	2/4	4.56		
72C	9	-/9.4	3.3/3.3	63.6	¼	4.98		
73		3.8/10.1	4.7/4.7	58.1	2/4	3.67	276.7 ± 5.5	15.8 ± 0.8/33.6 ± 4.6
74		3.9/10.1	4.9/4.9	48.9	2/3	3.57	202.9 ± 5.5	20.7 ± 4.3/3.1 ± 5.7
75		3.1/10.0	4.8/4.8	48.9	2/3	3.59	89.4 ± 3.1	-/-
76		-/9.9	4.4/4.4	55.5	¼	4.04	10.9 ± 2.0	-/-
77		-/9.8	4.8/4.8	46.3	1/3	3.87	<LOD	-/-
81		-/10.1	3.6/3.6	58.6	2/4	4.36	482.6 ± 18.7	32.2 ± 3.4/12.1 ± 5.2
Aspirin		-/3,5	1.4/-1.7	63.6	¼	5.75	-	-

[a] Predicted values calculated by ACD/Labs Percepta software, [b] Generated by Chemaxon Tautomer Generator, [c] strongest acidic pK_a, [d] CNS MPO were determined using predicted p$K_{a,basic}$, log$P$/log$D_{pH7.4}$ (classic and consensus settings, respectively), TPSA and HBD values,[e/e'] after 2 h/4 h, at 37 °C in PBS, pH 7.4. *Colours of classification systems*: log$D_{pH7.4}$: moderate violation (yellow) ≥ 3.5, high violation (magenta) ≥ 4.0; CNS MPO: low (magenta) ≤ 4.0, moderate (yellow) ≤ 4.7, good (green) > 4.7; Kin.Sol.: low (magenta) ≤ 100, moderate (yellow) ≤ 300, good (green) > 300; Increased BBB-permeability (green) > 25.

4. Antioxidant activity:

The antioxidant activity of ginger root extract and its constituents was well reported. We investigated the antioxidant effects of 6-G derivatives in this study using different models. Compound **65** showed the most promising antioxidant activity. Besides its DPPH-scavenging properties, its ORAC value was more than twice as much as that of Trolox. Notably, compound **66**, the 3-epimer of **65**, has only about half of the activity of compound **65** in the DPPH assay and was also weaker in terms of ORAC.

Table 4. The antioxidant activity results of 6-gingerol and its derivatives. Values are given as mean ± standard error of the mean (SEM). ORAC assay results are given in Trolox equivalents (TE), $ONOO^-$ scavenging, and xanthine oxidase (XO) inhibition assay results are given in % inhibition at concentrations of 500 and 100 µM, respectively; $n = 2$ for DPPH; $n = 3$ for ORAC, $ONOO^-$ and XO.

Compounds	DDPH		ORAC TE	$ONOO^-$ Scavenging (%)	XO Inhibition (%)
	IC_{50} (μM)	LLE [a]			
1	8.92 ± 0.46	2.15	1.30 ± 0.04	<5.0	<5.0
2	11.41 ± 0.49	0.75	1.10 ± 0.03	<5.0	<5.0
7	9.43 ± 0.16	0.93	1.36 ± 0.02	<5.0	<5.0
64	8.56 ± 0.07	0.27	0.47 ± 0.09	<5.0	10.56 ± 2.30
65	6.51 ± 0.28	2.19	2.30 ± 0.05	<5.0	<5.0
66	13.82 ± 0.03	1.86	1.12 ± 0.03	<5.0	<5.0
47	9.04 ± 0.19	1.54	1.98 ± 0.12	<5.0	<5.0
71	>200	-	2.89 ± 0.49	<5.0	<5.0
72	10.86 ± 0.69	1.46	2.60 ± 0.06	<5.0	<5.0
73	16.16 ± 0.46	0.09	0.44 ± 0.07	<5.0	10.13 ± 1.65
74	>200	-	1.00 ± 0.02	<5.0	12.30 ± 0.90
75	18.98 ± 1.63	−0.08	2.88 ± 0.16	<5.0	16.01 ± 4.05
76	8.13 ± 0.21	0.69	0.77 ± 0.20	38.40 ± 3.05	<5.0
77	>100	-	1.76 ± 0.06	5.64 ± 1.31	<5.0
81	14.07 ± 0.21	1.25	1.62 ± 0.004	<5.0	<5.0
allopurinol	-	-	-	-	98.80 ± 0.11

[a] Ligand–lipophilic efficiency: LLE = pIC_{50}—$\log P_{predicted}$. For LLE, green colouring indicates a satisfactory level (LLE ≥ 1.5 and IC_{50} ≤ 10 μM).

5. Molecular docking:

To predict the interaction of these compounds with the COX-1 enzyme, a molecular docking approach was implemented using AutoDock4. The *h*-COX-1 enzyme crystal structure was retrieved from the Protein Data Bank (PDB) database (ID: 6Y3C). The binding pocket was identified according to L. Tóth et al. the new isoxazole-derivative; compound **76** achieved the best binding affinity (-9.5 Kcal/mol) (**fig. 2**).

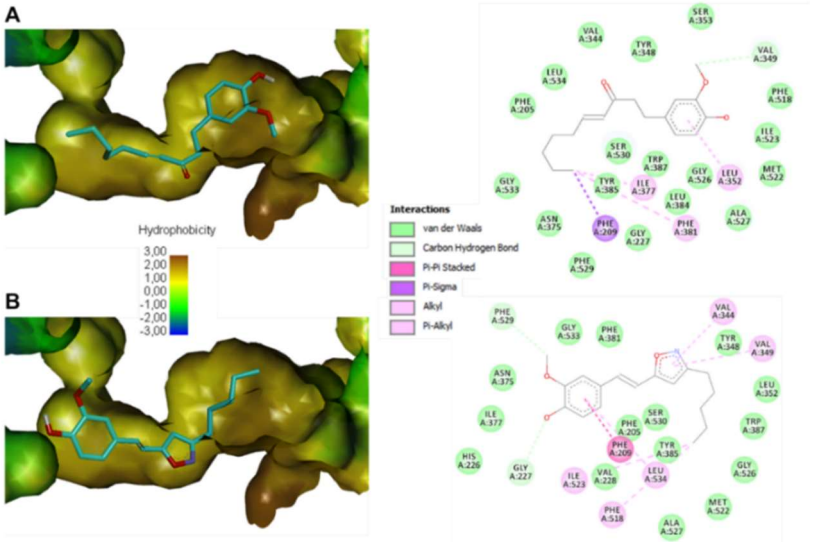
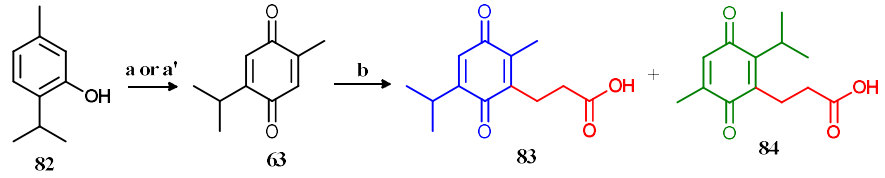


Figure 2. The binding mode of compounds **2** (**A**) and **76** (**B**) on *h*-COX-1 enzyme (PDB ID: 6Y3C) visualized with Discovery studio visualizer (21.1.0.20298); 3D orientation with the enzyme's hydrophobicity surface to the left and 2D representation of the observed interactions to the right. Colour codes for **A** and **B** are identical.

Protoflavone-thymoquinone hybrids:

- **Chemistry:**

TQ (**63**) was synthesized from thymol (**82**) using two methods (**Scheme 4**): the first using mCPBA, while the second utilizing PIFA as an oxidizing agent. The second method using PIFA method resulted in a higher yield from the first purification step, compared to two or more needed in the case of the first (using mCPBA), As far as we have searched, this is the first report for the synthesis of TQ from thymol using PIFA as an oxidizing agent.

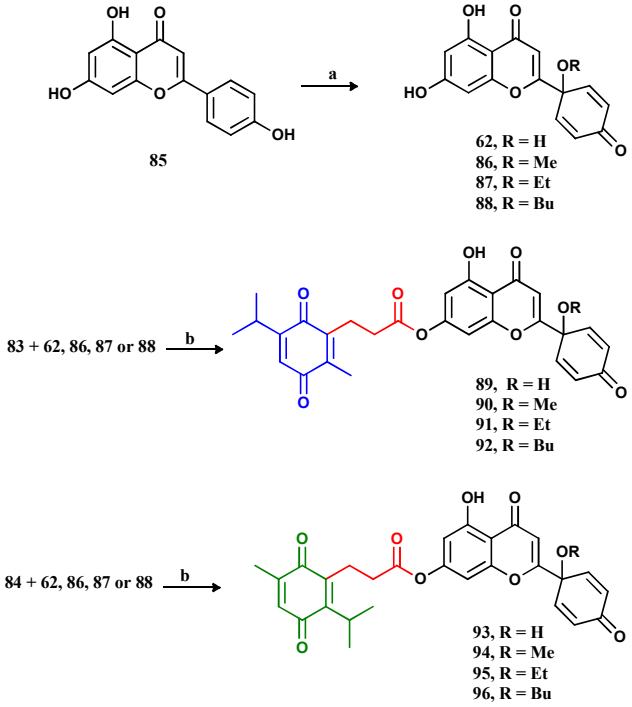


Scheme 4. Preparation of TQ (**63**) and its derivatives; compounds **83** and **84**. Reaction conditions: **a.** mCPBA/$CHCl_3$/rt **a`.** PIFA/AcN: H_2O/rt/1hr **b.**$C_4H_6O_4$/ $AgNO_3$/$(NH_4)_2S_2O_8$/AcN/H_2O/ 100°C

TQ adducts; compounds **83** and **84** were then synthesized using succinic acid (**Scheme 4**) and separated via preparative HPLC technique Though this reaction has been reported before, to our knowledge, this is the first time to report that it results in an isomeric mixture and to successfully isolate the isomers.

The protoflavones; **62**, and **86-88** were synthesized according to the method reported before by Hunyadi et al. Eight hybrid compounds were then synthesized containing different 1'-R groups in the protoflavone B-ring.

HRMS and 1D and 2D NMR techniques were used for the structural elucidation of all compounds. All the protoflavones and the hybrids possess the aromatic region peaks characteristic of protoflavone B-ring hydrogens with a ^1H-^1H coupling constant of around 10.



Scheme 5. Preparation of the protoflavone part (**62** and **86–88**) and the corresponding hybrids (**89–96**). Reaction conditions: **a.** PIFA/AcN: ROH/70 °C/1hr **b.** DDC/DMAP/dry DCM/rt

The hybrids: compounds **90** and **94** were tested for their stability. Kinetic studies demonstrated a tendency to hydrolysis by many solvents, especially methanol. The stability for these compounds was also tested in PBS, porcine esterase enzyme, MEM media over time compared to a blank, and total hydrolysis was noted after 1, 3 and, 24 hrs.

- **Antiproliferative assay:**

The activity of the hybrid molecules and their building blocks was assessed against HeLa, MCF-7, MDA-MB-123, and U-87 cell lines. In general, the protoflavone part is much more active than the TQ part with PA (**62)** demonstrating the most promising results among all compounds against MCF-7 cell line (IC_{50} = 0.66 μM), while its corresponding hybrid compound **93** superseded all against the rest of the cell lines. Though most of the activity of this compound could be attributed to the protoflavones fragment; compound **62** (**fig. 7**), nevertheless, this compound was more selective toward MDA-MB-231 compared to MCF-7 cell line with ca. double activity in the former. Interestingly, this selectivity was more evident in terms of TQ with ca. triple activity in the former, which might suggest that this character of compound **93** was inherited from the TQ fragment. All hybrids and protoflavones demonstrated comparable or stronger activity than the positive controls; cisplatin and temozolomide (**table 5**).

Table 5. Antiproliferative effects of thymoquinone-protoflavone compounds (hybrids [**90-96**] and their building blocks [**63, 83,** and **84** and **86-89**]) on tested cancer cell lines.

Compounds	Calculated IC_{50} ± SEM; [μM][a]			
	MDA-MB-231	MCF-7	HeLa	U-87
63	7.02 ± 0.17	23.97 ± 1.37	> 100	39.07 ± 3.53
83	38.17 ± 2.81	> 100	> 100	77.72 ± 1.61
84	12.44 ± 0.37	> 100	> 100	88.73 ± 5.17
62	0.57 ± 0.07	0.66 ± 0.06	1.80 ± 0.09	1.73 ± 0.11
86	2.23 ± 0.13	3.95 ± 0.30	5.51 ± 0.21	7.03 ± 0.11
87	1.22 ± 0.03	2.50 ± 0.11	2.83 ± 0.12	1.73 ± 0.06
88	0.82 ± 0.05	2.01 ± 0.07	1.88 ± 0.10	1.50 ± 0.12
89	1.27 ± 0.04	1.65 ± 0.07	2.00 ± 0.26	6.16 ± 0.49
90	2.25 ± 0.13	2.66 ± 0.11	3.51 ± 0.17	7.63 ± 0.46
91	2.15 ± 0.08	3.21 ± 0.07	2.35 ± 0.15	8.22 ± 0.73
92	0.99 ± 0.05	1.68 ± 0.16	1.40 ± 0.25	3.43 ± 0.25
93	0.52 ± 0.02	1.20 ± 0.03	1.06 ± 0.08	1.16 ± 0.20
94	3.53 ± 0.17	5.44 ± 1.32	6.78 ± 0.28	18.89 ± 3.42
95	1.98 ± 0.06	4.11 ± 0.34	1.71 ± 0.16	6.06 ± 0.40
96	1.075 ± 0.08	2.70 ± 0.09	3.08 ± 0.46	8.65 ± 1.15
Cis[b]	9.71 ± 0.51	6.55 ± 0.77	16.01 ± 2.00	9.13 ± 1.79
TMZ[c]	—	—	—	388.2 ± 43.0

[a] Mean value from two independent measurements with three parallel wells

[b] cisplatin

[c] temozolomide

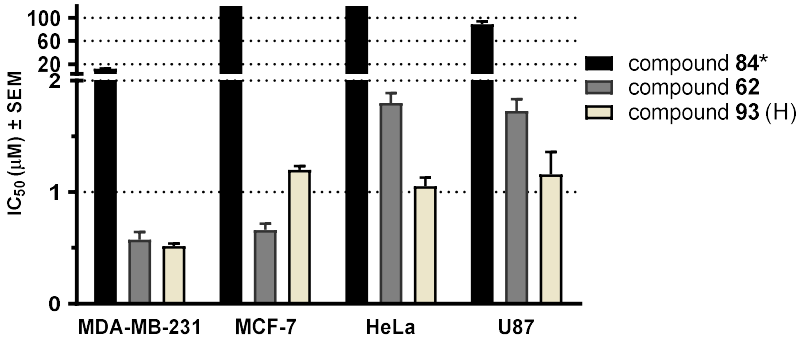


Figure 3. Calculated IC_{50} values of the hybrid (H) compound **93** and its building blocks (compounds **84** and **62**) on the tested cancer cell lines. *Calculated IC_{50} values of compound **84** on MCF-7 and HeLa cells were more than 100 μM.

In addition, the experimental combinations (1:1 mixture of building blocks) were tested on the U-87 cells for their antiproliferative activity for comparison with the corresponding hybrid compounds (**Table 6**).

Table 6. Calculated IC_{50} values of the structural combination (hybrid compounds) and experimental combination of the corresponding thymoquinone and protoflavone building blocks on the U-87 cells. Statistical analysis was performed by using unpaired t-test, * $p<0.05$; *** $p<0.001$ as compared to the corresponding 1:1 fragment mixture.

Compounds		Calculated IC_{50} ± SEM; [μM][a]	
Hybrid	1:1 mixture[b]	Hybrid	1:1 mixture
89	**83 + 62**	6.16 ± 0.49***	2.22 ± 0.43
90	**83 + 86**	7.63 ± 0.46*	10.03 ± 0.87
91	**83 + 87**	8.22 ± 0.73***	3.97 ± 0.23
92	**83 + 88**	3.43 ± 0.25***	1.73 ± 0.08
93	**84 + 62**	1.16 ± 0.20***	3.68 ± 0.35
94	**84 + 86**	18.89 ± 3.42	11.14 ± 1.02
95	**84 + 87**	6.06 ± 0.40	5.82 ± 0.61
96	**84 + 88**	8.65 ± 1.15***	2.90 ± 0.18
-	**63 + 62**	-	3.32 ± 0.54
-	**63 + 86**	-	11.04 ± 0.94
-	**63 + 87**	-	5.89 ± 0.28
	63 + 88	-	0.97 ± 0.42

[a] Mean value from two independent measurements with three parallel wells

[b] Each fragment was administered at the given concentration

The IC_{50} values showed at least a two-fold difference when compared to that of the hybrid compounds. Intriguingly, the hybrids; compounds **93** and **90** exhibited significantly better results compared to their corresponding experimental combinations with IC_{50} values of 1.16 and 7.63 μM, respectively, compared to 3.68 and 10.03 μM for the experimental combinations. These results might highlight a potential synergistic effect of their building blocks (**fig. 4**), which in turn supports the importance of the synthesis of hybrid compounds as a useful strategy to discover new compounds with superior antiproliferative activity compared to the experimental combinations on U-87 cells, considering possible solutions for the stability issues mentioned before.

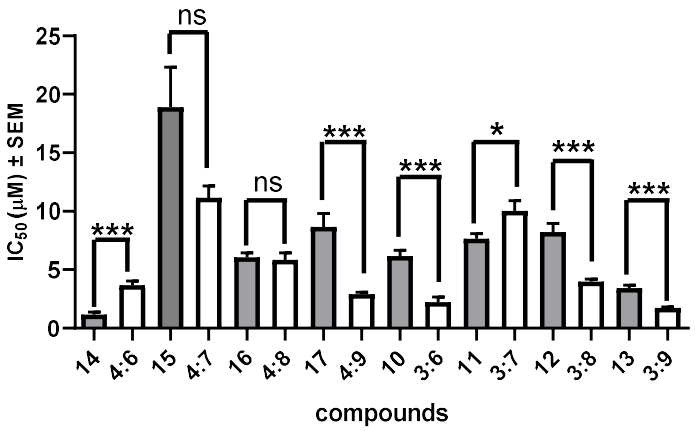


Figure 4. Calculated IC_{50} values of hybrid compounds (■) compared to that of their corresponding experimental combinations (□) determined on U-87 cell line. Statistical analysis was performed by using unpaired t-test.

The antiproliferative assay was also conducted on non-cancerous human lung fibroblast cells (MRC-5) to determine the cancer selectivity of compound **93**, its building blocks (**84** and **62**), their experimental mixture (**84** + **62** in 1:1 ratio), and two additional protoflavone derivatives (**87** and **88**); results shown in table below.

Table 7. Antiproliferative effects of selected compounds on MRC-5 non-cancerous human lung fibroblast cell line and the calculated selectivity indices (SI) of the tested compounds against U-87 glioblastoma cell lines; SI = IC_{50} (MRC-5)/IC_{50} (U-87).

Compound	Calculated IC50 ± SEM; [μM][a]		SI
	MRC-5	U-87	
84	40.25 ± 1.04	88.73 ± 5.17	0.45
62	2.62 ± 0.20	1.73 ± 0.11	1.51
87	3.79 ± 0.25	1.73 ± 0.06	2.19
88	3.56 ± 0.12	1.50 ± 0.12	2.37
93	5.10 ± 0.21	1.16 ± 0.20	4.40
84 + 62 (1:1 mixture)	4.72 ± 0.10	3.68 ± 0.35	1.28
TMZ[b]	1094 ± 45.1	388.2 ± 43.0	2.82

[a] Mean value from two independent measurements with three technical replicates each.

[b] Positive control; TMZ: temozolomide

The selectivity index of compound **84** is lower than 1, which means that it exhibited a stronger antiproliferative effect on MRC-5 cells than on U-87 cells. Cancer selectivity of the remaining test compounds has been proven to be higher than 1, i.e., they were more potent against U-87 cell lines than MRC-5 cells. Encouragingly, the hybrid **93** demonstrated the best cancer selectivity (SI=4.40) among the tested compounds, far exceeding that of the experimental mixture of its building blocks (SI=1.28) and also exceeding that of temozolomide (SI=2.82).

SUMMARY

The work presented in this thesis can be briefly summarized as follows.

6-Gingerol derivatives: Fifteen compounds have been synthesized, eight of them are new (**64**, **71**, **73**—**77**, **81**), while the rest are naturally present in ginger root (**1**, **2**, **7**, **47**, **65, 66**, and **72**). Chemical changes were introduced into the structure of 6-gingerol (the functional groups of the aliphatic side chain and/or the aromatic methoxy group) through different synthetic strategies, and various chromatographic techniques were used to purify the products. The potential cardiovascular protective and antioxidant effects of the compounds were tested by *in vitro* bioassays combined with *in silico* and *in vitro* pharmacokinetic analysis and *in silico* molecular docking study. Compounds **7**, **2**, **76**, **75**, and **72** showed the most promising antiplatelet activity among all. A linear correlation coefficient (R^2) value of 0.887 for the COX-1 inhibitory activity results suggested this pathway to be responsible for the antiplatelet action. Considering the results of the pharmacokinetic analysis and molecular docking of those compounds, compound **2** may be pointed out as the most promising lead worthy for further preclinical studies, while compounds **7**, **47** and the new semisynthetic compound **76** are hits that require further structural optimization to improve their aqueous solubility. Concerning the antioxidant activity, Compounds **65**, **76**, **64**, **1**, and **47** were the most active in the diphenyl-2-picrylhydrazyl (DPPH) scavenging capacity assay, while compounds **71** and **75** showed the best activity in the ORAC TE assay.

Thymoquinone-protoflavone hybrids: Eight new ester-linked hybrid compounds (**89**—**96**) have been synthesized and tested for their antiproliferative activity, along with their corresponding fragments **25**, **62**, **83**, **84**, and **86**—**88** (alone and in combination) against four human cancer cell lines viz. HeLa, MCF-7, MDA-MB-231 and U-87. TQ and the protoflavones were semi-synthesized from their naturally occurring thymol and apigenin, respectively. After introducing a linker moiety into TQ, the adducts were coupled to different protoflavones through DCC-catalyzed esterification. The chemical stability of the hybrids was unfortunately rather poor in the cell culture medium, showing complete hydrolysis in MEM after one hour upon incubation at 37°C. Despite this character, some encouraging antitumor properties were revealed. The bioactivity of the hybrid molecules was mostly attributed to the protoflavones part (**62**, **86**—**88**), as TQ and it is adducts showed much weaker activity. Still, the hybrid compound **93** showed the best activity (ranging from 0.51—1.2 μM) among all hybrids and protoflavones against all cell lines except for MCF-7. Compound **93** also showed the highest selectivity against the TNBC cell line MDA-MB-231 compared to MCF-7, and the highest tumor selectivity against U-87 glioblastoma vs. MRC-5 fibroblasts. Despite their relatively low chemical stability, the hybrid compounds **90** and **93** acted significantly stronger against U-87 cells than the 1:1 combination of their corresponding fragments. This encourages further studies into such hybrid molecules, which, after a necessary improvement of their chemical stability, may provide valuable new antitumor agents.

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LIST OF PUBLICATIONS RELATED TO THE THESIS:

This thesis is based on the following publications:

I. **Ahmed, S. H. H.**, Gonda, T., Agbadua, O. G., Girst, G., Berkecz, R., Kúsz, N., Tsai, M-C., Wu, C.-C., Balogh, G.T., Hunyadi, A. (2023). Preparation and Evaluation of 6-Gingerol Derivatives as Novel Antioxidants and Antiplatelet Agents

Antioxidants, 12, 744 IF 7.7 (Q1/D1)

II. **Ahmed, S. H. H.**, Bizhar, A. T., Gonda, T., Girst, G., Szőri, K., Berkecz, R., Zupkó, I.**,** Minorics, R., Hunyadi, A. (2024). Preparation of Thymoquinone-Protoflavone Hybrid Molecules as Potential Antitumor Agents.

PLoSONE, doi: 10.1371/journal.pone.0291567 IF 3.8 (Q1)

Another related publication:

Ahmed, S.H.H., Gonda, T., Hunyadi, A. (2021). Medicinal chemistry inspired by ginger: exploring the chemical space around 6-gingerol.

RSC Advances, 11(43): p. 26687-26699. IF 4.0 (Q1)

PRESENTATIONS AND POSTERS RELATED TO THE THESIS:

I. **Ahmed, S. H. H.**, Gonda, T., Berkecz, R., Kúsz, N., Hunyadi, A. Isolation and synthesis of 6-gingerol and 6-gingerdione derivatives. 2nd Symposium of Young Researches on Pharmacognosy (**2021**). doi: 10.14232/ syrpharmacognosy.2021.a8.

Szeged, Hungary.

II. **Ahmed, S. H. H.**, Gonda, T., Agbadua, O. G., Girst, G., Berkecz, R., Kúsz, N., Tsai, M-C., Wu, C.-C., Balogh, G.T., Hunyadi, A. 6-gingerol derivatives and thymoquinone-protoflavones hybrids: natural antioxidants as building blocks of new bioactive compounds. 3rd Symposium of Young Researches on Pharmacognosy (**2022**). doi: 10.14232/syrpharmacognosy.2022.a7.

Szeged, Hungary.

III. **Ahmed. S. H. H.**, Bizhar A. Tayeb, Tímea Gonda, Gábor Girst, Kornél Szőri1, Róbert Berkecz, István Zupkó, Renáta Minorics, Attila Hunyadi. Thymoquinone-protoflavone hybrids: Studies into anticancer potentials. 4th Symposium of Young Researches on Pharmacognosy (**2023**). doi: 10.14232/syrmpnpr.2023.7.

Szeged, Hungary.

IV. Poster; **Ahmed, S. H. H.**, Gonda, T., Agbadua, O. G., Girst, G., Berkecz, R., Kúsz, N., Tsai, M-C., Wu, C.-C., Balogh, G.T., Hunyadi, A. 6-Gingerol Derivatives as Promising Antiplatelet Leads. 70th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA) (**2022**). Planta Med. 88(15): p. P-218.

Thessaloniki, Greece.