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In vitro and *in silico* inhibitory activity of angiotensin-converting enzyme 2 (ACE-2) and anti-inflammatory effects of natural componds from *Rheum officinale* roots

Pham Ngoc Khanh[1, 2, *], Tran Thu Huong[1], Nguyen Xuan Ha[1], Hoang Thi Ngoc Anh[1], Vu Thanh Loc[1], Do Huu Nghi[1], Do Thi Thao[3], Trinh Thi Thu Ha[1], Nguyen Phi Hung[1], Vu Thi Ha[1], Ludger A. Wessjohann[4], Nguyen Manh Cuong[1, 2, *]

[1]*Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Cau Giay, Ha Noi, Viet Nam*

[2]*Graduate University of Science and Technology, 18 Hoang Quoc Viet, Caugiay, Hanoi 122100, Viet Nam*

[3]*Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Ha Noi, Viet Nam*

[4]*Leibniz Institute of Plant Biochemistry, Weinberg 3, D-06120, Halle/Saale, Germany*

[*]Emails: *1.nmcuong_inpc@yahoo.com.vn, 2.khanhngoclila@gmail.com*

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Abstract. The chemical constituents of Rhubarb roots (*Rheum officinale*) were examined for their anti-ACE-2 and anti-inflammatory effects by preventing the production of NO and IL-6. Four natural compounds, including two stilbenes, rhaponticin (**1**) and rhapontigenin (**2**), and two anthraquinones, chrysophanol (**3**) and emodin (**4**), were isolated from the roots of Rhubarb. Rhaponticin (**1**) exhibited a potent inhibitory effect *in vitro* on ACE-2 with an IC_{50} value of 80.9 μM. In an *in silico* study, rhaponticin (**1**) had a binding energy value of −9.32 kcal/mol, lower than MLN-4760, on the ACE-2 enzyme. This compound generated hydrogen bonding and hydrophobic interactions with the key amino acid residues in the ACE-2 cavities. Emodin (**4**) exhibited inhibitory activity on NO production in primary cultures of macrophages RAW264.7, with an IC_{50} value of 20.9 μM. Emodin (**4**) also lowered the IL-6 pro-inflammatory cytokine level by 0.76-fold at a concentration of 100 μg/mL. Together with its NO inhibitory capacity, emodin (**4**) becomes the most potent anti-inflammatory candidate among the compounds tested.

Keywords: Rhubarb, *Rheum officinale*, rhaponticin, emodin, ACE-2.

Classification numbers: 1.1.1, 1.2.1, 1.1.6.

1. INTRODUCTION

The SARS-CoV-2 virus recently triggered a global pandemic, posing a significant threat due to the high number of infected individuals (over 775 million) and a high mortality rate (over 1 %, until April 24th, 2024) [1]. The SARS-CoV-2 virus enters the lung by binding its spike

protein to the angiotensin-converting enzyme 2 (ACE-2) protein receptor, which is expressed in a variety of human organs and tissues (lung, heart, kidney, liver, etc.). ACE-2 allows the virus to enter the cells, so it is associated with multi-organ injury in COVID-19, such as acute kidney, heart, liver, and lung injury, as well as pneumothorax [2].

There are a number of options in drug development that can be used to prevent and cure COVID-19 disorders. These include producing vaccines, blocking viral entrance through the ACE-2 receptors, inhibiting critical viral proteins (M^{pro}, RNA polymerase, S-protein, etc.), and suppressing the expression of pro-inflammatory factors (IL-1, IL-6, IL-10, IL-12, and TNF-α [1, 4 - 5].

Many herbal products and therapies containing medicinal plants have been utilized around the world for this purpose, and clinical studies have proven the effectiveness of some of these products in treating COVID-19 disorders. Some medicinal plant extracts, including *Lycoris radiata, Pyrrosia lingua, Artemisia annua,* and *Lindera aggregata,* exerted anti-SARS-CoV activity at 2.4 - 88.2 μg/mL [6]. The extracts of *Rheum officinale* Baill., *Polygonum multiflorum* Thunb. (root tubers), and *P. multiflorum* Thunb. (vines) with anthraquinone constituents inhibited the binding of S protein to ACE-2, with IC_{50} values ranging from 1 to 10 μg/mL [7].

In our primary work, several medicinal components of herbal products and isolated natural compounds were screened as ACE-2 inhibitors. Rhubarb (*Rheum officinale* Baill.), (Vietnamese name: Đại Hoàng) is one of these remarkable herbs that showed their potential activities in the prevention of the binding of virus protein via ACE-2 protein, thereby reducing the entry of the virus into the human body and controlling the inflammatory cascades of the body reaction that trigger the lung damage brought on by SARS-CoV-2.

Rheum belongs to the family Polygonaceae, which includes roughly 60 herbaceous perennials that grow in temperate and subtropical climates in Asia, Europe, and other parts of the world [8]. China is the distribution center of the genus, with 41 species and two variants accounting for three-quarters of the total *Rheum* species in the world [9]. In Viet Nam, *Rheum* plants are primarily found growing under the forest canopy in cool, high mountain areas (at least over 1000 meters above sea level), such as Sapa (Lao Cai) and Mau Son (Lang Son). In folk remedies, the shrub is used as a laxative for the treatment of constipation, in combination with licorice for the treatment of digestion disorders such as indigestion, flatulence, and abdominal distension. The first goal of our study was to determine the chemical compositions of this plant, and the second was to identify the potential of its pharmacological components with anti-COVID-19 properties exerted by reduced NO generation, IL-6 cytokine expression, and ACE-2 activity, as well as *in silico* activity against the ACE-2 enzyme.

2. MATERIALS AND METHODS

2.1. Materials

The roots of Dai Hoang (*Rheum officinale* Baill.) were collected at Sapa district, Lao Cai province, in December 2020. The samples were identified by the botanist Prof. Dr. Tran The Bach (Institute of Ecology and Biological Resources (IEBR), VAST). A voucher specimen (C-664) is deposited in the herbarium of the Department of Bioactive Compounds and Chemical Analysis, Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology, Ha Noi, Viet Nam.

2.2. General experiment

^{1}H-NMR (500 MHz or 600 MHz) and ^{13}C NMR (125 MHz or 150 MHz) spectra were measured on a Bruker AVANCE 500 and 600 spectrometer. Column chromatography (CC) was carried out on silica gel (Si 60 F_{254}, 230–400 mesh, Merck). All solvents were distilled before use. Precoated plates of silica gel 60 F_{254} were used for analytical purposes. Compounds were visualized under UV radiation (254–365 nm) and by spraying plates with 10% H_2SO_4, followed by heating with a heat gun. All other chemicals were commercial products of reagent grade.

The enzyme inhibition of the test substances was measured under the manufacturer's instructions for the ACE-2 inhibitor screening kit (MAK378, Merck KGaA, Darmstadt, Germany), which utilizes the ability of an active ACE-2 to cleave a synthetic 4-methoxycoumarin-7-acetic acid (MCA)-based peptide substrate to release a free fluorophore. Dulbecco's modified eagle medium and its supplements, including fetal bovine serum (FBS), were purchased from GIBCO, Invitrogen, USA. The production of nitric oxide (NO) was measured using the Griess Reagent System kit (Promega Cooperation, WI, USA). MLN-4760, an ACE-related carboxypeptidase inhibitor, was used as a positive control. The mouse IL-6 ELISA kit was purchased from R&D Systems (St. Louis, MO, USA). LPS (lipopolysaccharide), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], and dimethylsufoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The RAW264.7 murine macrophage cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA).

2.3. Isolation of natural compounds from Rhubarb roots

The dried powdered roots of Rhubarb were extracted with methanol (at room temperature, 3 times x 2 L, for 2 days each time) and concentrated under decreased pressure to yield a crude MeOH extract. This extract was then suspended in water and successively partitioned with organic solvents of increasing polarity. The resulting fractions were concentrated to give the corresponding fractions, including *n*-hexane (4.6 g), dichloromethane (DCM fraction, 6.9 g), ethyl acetate (EtOAc fraction, 50 g), and water (70 g).

The DCM fraction (6.9 g) was subjected to column chromatography (CC) through silica gel (40–63 μm) with gradient solvents of *n*-hexane: EtOAc (6:1, 4:1, 2:1, 1:1) to afford 3 fractions (frs.) C1, C2, and C3. The sub-fraction C1 was further separated on a silica gel column, eluting with a gradient of *n*-hexane: EtOAc (50:1, 40:1, 30:1, 20:1, 10:1, 1:1) to yield compound **3** (chrysophanol, 10 mg). The sub-fraction C2 was further separated on a silica gel column, eluting with a gradient of *n*-hexane: EtOAC (8:1, 6:1, 4:1, 3:1, 2:1, 1:1) to obtain compound **4** (emodin, 15 mg).

The EtOAc fraction (20.0 g) was subjected to CC with silica gel (40–63 µm) and eluting with the solvent system *n*-hexane: EtOAc (3:1, 2:1, 1:1) to afford 5 fractions E1–E5. The sub-fraction E3 was further separated on a silica gel column, eluting with a gradient of *n*-hexane: EtOAc (3:1, 2:1, 1:1) to obtain two fractions E3.1 and E3.2. Compound **1** (rhaponticin, 12 mg) was yielded by recrystallization from the E3.2 sub-fraction. The sub-fraction E4 was subjected to CC through silica gel with gradient solvents of diclomethane and methanol (20:1, 10:1, 1:1) to obtain compound **2** (rhapontigenin, 119.8 mg).

Spectroscopic data of isolated compounds

1 (Rhaponticin): Colorless crystall, soluble in organic solvents, $C_{21}H_{24}O_9$ (M = 420.14); ^{1}H-NMR (600 MHz, MeOD , δ ppm): 9.28 (1H, s, OH), 8.77 (1H, s, OH), 7.04 (1H, brs, H-2′), 6.99 (1H, d, J = 16.8 Hz, H-7), 6.86 (1H, d, *J* = 16.2 Hz, H-8), 6.91 (1H, d, *J* = 8.4 Hz, H-5′),

6.96 (1H, brs , H-6′), 6.81 (1H, brs, H-6), 6.64 (1H, brs, H-2), 6.48 (1H, brs, H-4), 4.92 (1H, d, *J* = 7.2 Hz, H-1′′), 3.88 (3H, s, 4′-OCH_3). ^{13}C-NMR (150 MHz, MeOD, δ ppm): 160.47 (s, C-5), 159.59 (s, C-3), 149.08 (s, C-4′), 147.71 (s, C-3′), 141.24 (s, C-1), 132.12 (s, C-1′), 129.95 (d, C-7), 127.52 (d, C-8), 120.17 (d, C-6′), 113.67 (d, C-2′), 112.71 (d, C-5′), 108.43 (d, C-4), 107.14 (s, C-2) , 104.26 (s, C-4), 102.41 (d, C-1′′), 78.23 (s, C-3′′), 78.05 (s, C-5′′), 74.95 (s, C-2′′), 71.48 (s, C-4′′), 62.59 (d,C-6′′), 56.42 (q,4′-OCH_3).

2 (**Rhapontigenin**): Ivory-white crystalls, then turning to brown, soluble in organic solvents, $C_{15}H_{14}O_4$ (M = 258.27); ^{1}H-NMR (500 MHz, MeOD , δ ppm): 7.02 (1H, d, *J* = 2.0 Hz, H-2′), 6.94 (3H, m, H-8, H-5′ & H-6′), 6.81 (1H, d, *J* = 16.5 Hz, H-7), 6.47 (2H, d, *J* = 2.0 Hz, H-2 & H-6), 6.19 (1H, t, *J* = 2.5 & 4.5 Hz, H-4), 3.87 (3H, s, 4′-OCH_3). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 159.66 (s, C-5 & C-3), 148.99 (s, C-4′), 147.72 (s, C-3′), 141.12 (s, C-1), 132.25 (s, C-1′), 129.37 (d, C-7), 127.88 (s, C-8), 120.02 (d, C-2′), 113.62 (d, C-6′), 112.78 (d, C-5′), 105.88 (d, C-2 & C-6), 102.83 (d, C-4), 61.54 (q, 4′-OCH_3).

3 (**Chrysophanol**): Bright yellow solid, soluble in organic solvents (MeOH, CH_2Cl_2); $C_{15}H_{10}O_4$ (M = 254.24); ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 12.10 (1H, s, 1-OH), 11.99 (1H, s, 8-OH), 7.81 (1H, dd, *J* = 1.0 & 8.5 Hz, H-5), 7.64 -7.66 (2H, m, H-6 & H-4), 7.28 (1H, dd, *J* = 1.0 & 8.5 Hz, H-7), 7.09 (1H, d, *J* = 1.0 Hz, H-2), 2.33 (3H, s, 3-CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 192.58 (s, C-9), 182.01 (s, C-10), 162.76 (s, C-1), 162.46 (s, C-8), 149.36 (s, C-3), 136.96 (d, C-6), 133.33 (s, C-4a), 124.57 (d, C-7), 124.38 (d, C-2), 121.37 (d, C-4), 115.91 (s, C-8a), 121.37 (d, C-4), 133.96 (s, C-10a), 113.78 (s, C-9a), 22.27 (q, 3-CH_3).

4 (**Emodin**): Orange solid, soluble in organic solvents (MeOH, CH_2Cl_2); $C_{15}H_{10}O_5$ (M = 254.24); ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 12.04 (1H, s, 1-OH), 11.96 (1H, s, 8-OH), 7.43 (t, 1H, *J* = 0.5 & 1.5 Hz, H-5), 7.11 (dd, *J* = 1 & 1.5 Hz, 1H, H-7), 7.07 (d, 1H, *J* = 2 Hz, H-4), 6.55 (d, *J* = 2 Hz, H-2), 2.47 (s, 3H, 6-CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$, δ ppm): 189.56 (s, C-9), 181.8 (s, C-10), 165.55 (s, C-3), 164.38 (s, C-8), 161.34 (s, C-1), 148.12 (s, C-6), 134.98 (s, C-10a), 132.7 (s, C-4a), 124.0 (d, C-2), 120.35 (d, C-4), 113.25 (s, C-9a), 108.82 (s, C-8a), 107.83 (d, C-7), 21.42 (q, 6-CH_3).

2.4. *In vitro* ACE-2 inhibitory effects

The ACE-2 inhibitory effects of isolated compounds, MeOH extract, and the EtOAc fraction (at 100 → 6.25 μg/mL) were evaluated *in vitro* as described previously [1].

2.5. In vitro pro-inflammatory inhibitory assays

To test the anti-inflammatory effects of the studied compounds, RAW 264.7 cells induced by *E. coli* lipopolysaccharide were utilized as an experimental model.

2.5.1. Inhibitory effect on NO production in LPS-stimulated RAW 264.7 cells

Murine RAW264.7 macrophages were cultured in DMEM containing 2 mM HEPES, 1.0 mM sodium pyruvate, 10% heat-inactivated FBS, streptomycin (100 μg/mL), and penicillin (100 IU/mL) at 37 °C with 5% carbon dioxide in fully humidified air. Cells were frequently subcultured after 3-5 days at a ratio of 1:3. The experiment was proceeded as described previously [4].

2.5.2. In vitro inhibitory effects of the tested samples on production of the cytokine IL-6

RAW 264.7 cells were put into the experimental wells of 96-well plates with the right amount of cells (5×10^4 cells in 190 μL of medium) and incubated at 37 °C with 5 % CO_2 overnight. The sample in 10 % DMSO (10 μL) was introduced into the wells in different concentrations. Several wells without reagent but with cells (190 μL) and 10 μL of 10 % DMSO were used as negative controls. The culture medium (FBS-free) was used as a blank sample. After 2 h, LPS was added at a concentration of 1 μg/mL to all experimental wells. After 24 h, the cell culture supernatants were collected, and the presence of IL-6 in the culture medium was determined by strictly following the manufacturer's instructions for the mouse IL-6 ELISA kit (R&D Systems , St. Louis, MO, USA). The assay detects and measures endogenous mouse IL-6 protein levels. The pro-inflammatory cytokine levels (in pg/mL) were calculated using the OD values and the standard reference curve. The following formula was used to determine the fold change in cytokine expression, and the findings are shown as the mean SD from three experiments [10].

$$\text{Fold change } = \frac{OD_{Sample} - OD_{Blank}}{OD_{DMSO} - OD_{Blank}} = \frac{cytokine\ level\ in\ sample\ treated\ cells}{cytokine\ level\ in\ untreated\ cells} \quad (1)$$

2.6. Cell viability assay

An 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay (Celltiter 96-Aqueous One Solution Assay, Promega, Madison, WI, USA) was used to analyze the effect of compounds on cell viability [4].

2.7. Statistical analysis

All the experiments were repeated three times, and all results were expressed as the mean ± SD (n = 3 - 5). The analysis of the data was accomplished by ANOVA followed by Tukey's test using GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA) and the SDSS program. A *p*-value between < 0.05 and 0.01 was considered significant.

2.8. Molecular docking

Molecular docking studies were performed with active ingredients isolated from Rhubarb. The study was carried out with AutoDock Vina software, as described previously [1, 4].

2.9. Pharmacological properties and ADME prediction

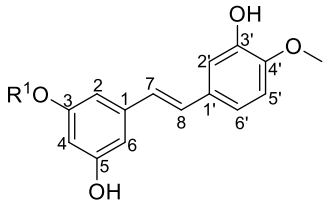
The molecules violating pharmaceutical-like properties, ADME (Absorption, Distribution, Metabolism, and Excretion), and Lipinski's rule of five (LoF) [11] were also determined. ADME tools are useful for predicting the physicochemical properties, pharmacokinetics, and drug-likeness of potential drug candidates, particularly during the preclinical phase. This study utilized the open-access SwissADME and pkCSM-pharmacokinetics web servers to investigate ADME profiles of phytochemicals from *Rheum officinale*.

3. RESULTS AND DISCUSSION

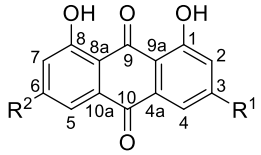
3.1. Isolation of natural compounds

Four natural compounds were isolated from the roots of *Rheum officinale*, including two stilbene compounds, rhaponticin (**1**) and rhapontigenin (**2**), and two anthraquinones,

chrysophanol (**3**) and emodin (**4**). Their structures were identified by NMR and MS spectral analysis and comparison with the literature (Figure 1) [12-15].



Rhaponticin (**1**) R^1=β-D-glc
Rhapontigenin (**2**) R^1=H



Chrysophanol (**3**) R^1=CH_3, R^2=H
Emodin (**4**) R^1=OH, R^2=CH_3

Figure 1. Four compounds isolated from the roots of *Rheum officinale.*

3.2. *In vitro* anti-inflammatory effects based on the inhibition of NO and IL-6 production

Table 1. The results of *in vitro* ACE-2, IL-6, and NO inhibitory effects of extracts and compounds from Rhubarb roots.

		ACE-2 inhibition			NO inhibition		IL-6 fold change	% Survival rate	
No.	Samples	(%)*	IC_{50} (μg/mL)	IC_{50} (μM)	(%)#	IC_{50} (μM)	fold change*	(%)#	IC_{50} (μM)
1	MeOH extract	17.4 ± 0.33	> 100		ND	ND	ND	ND	ND
2	EtOAc fraction	20.95 ± 0.24	> 100		ND	ND	0.52 ± 0.01	83.61	57.50[a]
3	Rhaponticin (**1**)	74.67 ± 0.35		80.9 ± 0.09	ND	ND	0.53 ± 0.01	ND	ND
4	Rhapontigenin (**2**)	ND	ND	ND	34.75	> 100	ND	88.15	
5	Chrysophanol (**3**)	22.23 ± 0.94		>100	30.34 ± 1.51	> 100	2.31 ± 0.2	86.95	>100
6	Emodin (**4**)	18.17 ± 0.22		>100	87.22 ± 3.24	20.9 ± 1.6	0.76 ± 0.06	22.04	>100
7	Enzyme control (-)	0		0					
8	Inhibitor control (+)	99.73 ± 0.22	0.033						
9	L-NMMA				90.22 ± 1.13	31.37±0.33			

* At sample concentration of 100 μg/mL; # At sample concentration of 20 μg/mL; ND – means not determined; [a]IC_{50} at μg/mL

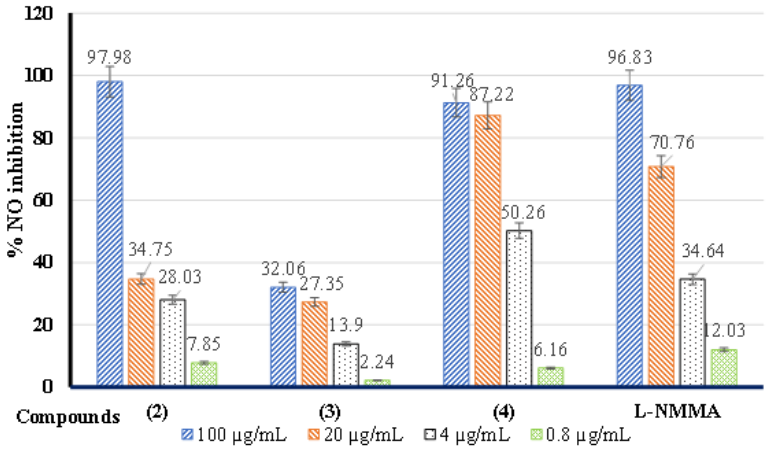


Figure 2. Inhibition of NO production by rhapontigenin (**2**), chrysophanol (**3**), and emodin (**4**), isolated from Rhubarb roots.

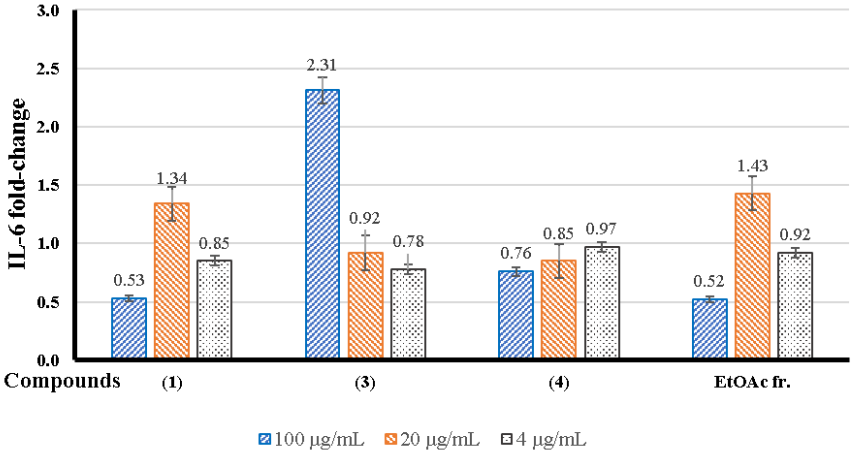


Figure 3. Fold change in IL-6 production of natural compounds isolated from Rhubarb roots.

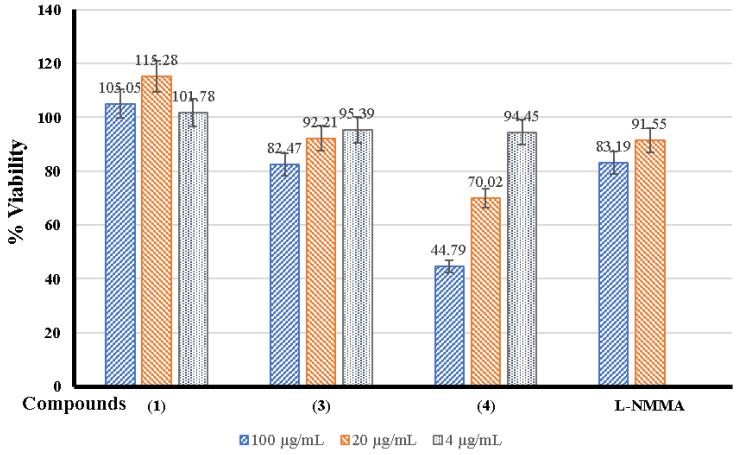


Figure 4. The survival rate of the Rhubarb natural compounds on RAW 264.7 cells using MTT method. Data represent mean values of triple determinations ± SEM.

The natural compounds isolated from Rhubarb roots were evaluated for their inhibitory effects on ACE-2, IL-6, and NO overproduction *in vitro* and *in silico* to serve as a scientific basis for their application as a multifunctional agent in the treatment of SARS-CoV-2 and diseases caused by oxidative stress and inflammation.

The results in Figure 2 show that at a concentration of 4 μg/mL, the compounds (**2-4**) could inhibit NO production with values of 28.0, 13.9, and 50.3%, respectively. At a concentration of 20 μg/mL, compounds (**2**) and (**4**) had a good ability to block NO production by 34.7 and 87.2%, respectively (Figure 2). Chrysophanol (**3**), at 20 μg/mL, only partially inhibited the generation of NO by 27.3%. Emodin (**4**) has the ability to inhibit NO at a value of 87.2, 50.2, and 6.1% at concentrations of 20, 4, and 0.8 μg/mL, respectively. With an IC_{50} value of 20.9±1.6 μM, emodin (**4**) was found to be the best at blocking NO, much better than the positive control compound L-NMMA (31.3 μM). However, the inhibitory NO production capacity of emodin might be affected by its low cell viability percentage (Table 1 and Figure 2).

Therefore, in order to prevent the cytotoxic effects of all tested samples at the highest concentration (100 μg/mL), the seeding cell concentration was raised to 5 x 10^4 cells/well for the IL-6 inhibitory experiment. The results displayed in Figure 3 showed that the levels of pro-inflammatory cytokines changed in the presence of the test samples. Both the EtOAc fraction and rhaponticin (**1**) at 20 μg/mL had significant effects on the IL-6 level, changing it by 1.43 and 1.34-fold, respectively. They reduced their IL-6 levels to 0.92- and 0.85-fold, respectively, at concentrations of 4 μg/mL. Especially, chrysophanol (**3**) could induce higher levels of pro-inflammatory cytokine IL-6 to 2.31-fold at 100 μg/mL and also suppress that to 0.78-fold at 4 μg/mL. Emodin (**4**), interestingly, changed the IL-6 levels by 0.76-, 0.85-, and 0.97-fold at three tested concentrations. Among the isolated compounds, emodin (**4**) appears to be the most powerful IL-6 inhibitor due to its evident inhibitory effect on IL-6 production at all experimental concentrations. In addition, chrysophanol (**3**) was also able to suppress IL-6 production by 0.78-fold at the lowest concentration (4 μg/mL). All the isolated compounds from the Rhubarb roots also showed no cytotoxicity vs. RAW264.7 cells at the 4–20 μg/mL concentration range with more than 70% cell survival (data not shown) (Figure 4).

3.3. *In vitro* ACE-2 inhibitory effects

The results of the *in vitro* ACE-2 inhibitory effects of extracts and compounds from Rhubarb roots are presented in Table 1. The results show that the MeOH extract and EtOAc fraction inhibited the ACE-2 activity at concentrations of 100 μg/mL by 17.4 ± 0.33 % and 20.9 ± 0.24 %, respectively. Rhaponticin (**1**) exhibited an inhibitory effect on the ACE-2 enzyme with an IC_{50} value of 80.9 ± 0.09 μM. Other compounds showed no-to-low inhibitory effects on the ACE-2 enzyme (Table 1).

3.4. Molecular docking simulation

Molecular docking is a common method for predicting the direction, affinity, and interaction of ligands within a protein binding pocket. In this study, we first evaluated the docking parameters by running a re-docking simulation for the co-crystallized MLN-4760 with the ACE-2 protein, followed by a real molecular docking simulation for the compounds under study. Before the validation phase, the correctness of the docking methodology was verified using the root mean square deviation (RMSD) value. Figure 5 shows the significant overlap between the redocked ligand and the co-crystallized ligand, MLN-4760. The redocking result

demonstrated the high reliability of the docking technique, with an RMSD of 1.01 (< 2 Å) between the coordinates of the MLN-4760 redock and the original co-crystallized one (Fig. 5).

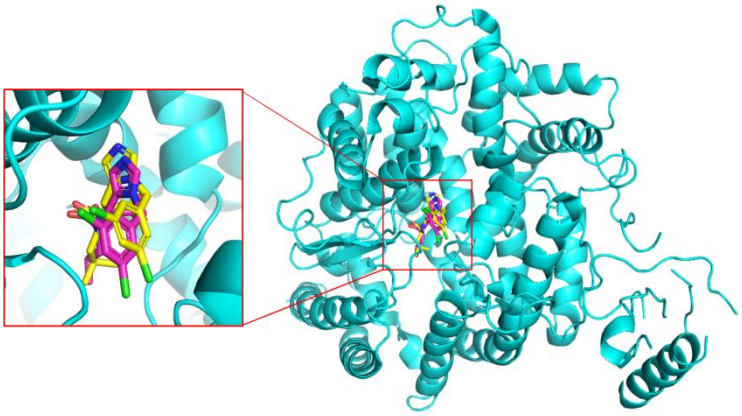


Figure 5. The redocked pose of the co-crystallized ligand MLN-4760 in ACE-2. The cyan color represents the pose of MLN-4760, while the magenta was the best re-docking pose obtained by AutoDock4 (RMSD = 1.01 Å).

Next, the bioactive stilbene rhaponticin was docked into the active-site pocket of the ACE-2 protein to further understand its mechanism of action. Rhaponticin had a negative binding energy of −9.342 kcal/mol, indicating significant interactions with the ACE-2 protein binding site, and had a lower one than the ACE-2 inhibitor MLN-4760 (−9.2 kcal/mol). The interaction properties of rhaponticin and MLN-4760 were analyzed and are represented using the Discovery Studio Visualizer software, as shown in Figure 6 and Table 2.

3.5. Predicting the drug potential of the research compounds

The drug-likeness parameters according to Lipinski's criteria are presented in Table 3. The results show that the four compounds all meet the Lipinski criteria for oral bioavailability.

3.6. Discussion

Research on ACE-2 enzyme inhibition for the prevention and treatment of inflammatory diseases in general and COVID-19 viral pneumonia in particular is very important nowadays [1-2]. ACE-2 plays a catalytic role in converting Ang-I and Ang-II to angiotensin, which binds to the G-protein-coupled receptor MAS to exert a range of functions in multiple organs and systems (vasodilation, vascular protection, anti-fibrosis, anti-proliferation, and anti-inflammation) [16]. When the SARS-CoV-2 virus binds to ACE-2, it prevents ACE-2 from performing its normal function to regulate angiotensin II (ANG II) signaling. As a result, the function of ACE-2 is "inhibited," leading to the overproduction of ANG II, which is the most representative bioactive peptide in the renin-angiotensin system (RAS) and widely participates in the progression of cardiovascular diseases (hypertension, myocardial infarction, heart failure, etc.) and lung injury in COVID-19 patients [17].

The catalytic domain has one active site: the zinc metallopeptidase domain, which shows ~ 42 % sequence identity with the amino domain of ACE. The spike (S) protein of SARS-CoV-2 mediates entry by binding to the ACE-2 receptor present on the cell surface, followed by fusion of the viral envelope with the host cell membrane. The impact of the epidemic caused by the

new strain of coronavirus (SARS-CoV-2) is very heavy, seriously affecting life and socio-economic conditions both in our country and around the world. Therefore, the study of antiviral activity related to the anti-inflammatory evaluation of active medicinal plants is of great importance.

For example, *Isatis indigotica* root extract and its phenolic derivatives hesperetin and sinigrin had the potential to inhibit coronavirus 3CL with IC_{50}s of 8.3 μM and 217 μM, respectively [18]. An aqueous extract of the anthocyanin-rich fraction of *Hibiscus sabdariffa* species inhibited ACE activity with an IC_{50} of 91.2 μg/mL [19]. The coumarin osthole compound extracted from many medicinal plants and herbs, such as *C. monnieri, Angelica pubescens*, and some plants in the subclass Leguminnosae and Compositae, has the ability to prevent LPS-induced acute lung injury and improve survival rate in mice by affecting ACE expression [20]. Luo *et al.* reported that the MeOH extract and EtOAc fraction of *Rheum palmatum* L. roots had high protease activity against SARS-CoV 3CL with IC_{50} values of 17.4 ± 0.33 and 20.1 ± 0.24 % g/mL, respectively [21].

Rhaponticin (**1**) exhibited a remarkable inhibitory effect on the ACE-2 enzyme with an IC_{50} value of 132 μM, while other tested samples showed no-to-low inhibitory effects on the ACE-2 enzyme (Table 1). A literature review, on the other hand, found that emodin, an anthraquinone most popular in *Rheum* and *Polygonum* plants, significantly blocks the interaction between S protein and ACE-2 (with an IC_{50} value of 200 μM) and also the infectivity of a S protein-pseudotyped retrovirus to Vero E6 cells [6]. Other anthraquinones, like gluco-aurantioobtusin from the MeOH extracts of *Cassia tora*, exhibited significant inhibitory properties against ACE activity with an IC_{50} value of 30.24 μM [22]. TS-1276, anthraquinone, and its derivative emodin were also found to exhibit an inhibiting effect on the binding of the S protein to ACE-2 [23]. Chen *et al*. also reported that rhein and emodin 8-*O*-β-D-glucoside showed good ACE-2 inhibitory effects with IC_{50} values of 18.3 and 22.5 μM, respectively [24].

Similarly to these results, our work shows that emodin is not cytotoxic at normal test concentrations [25]. Emodin showed no or a low inhibitory effect on the ACE-2 enzyme, but had the highest NO inhibition at 4 μg/mL. Emodin (**4**) suppressed NO production with an IC_{50} value of 20.9 μM. Chrysophanol (**3**), at 100 μg/mL, inhibited the generation of NO only partially (32%). This is like what chrysophanol did to inducible nitric oxide synthase (iNOS)-mediated NO production in macrophages treated with LPS [25], where it didn't have any major effects.

Interleukin-6 (IL-6) can activate intercellular signal transmission and exchange by acting on cell receptors via autocrine or paracrine pathways, completing the necessary biological tasks of cells [26]. The pathology of COVID-19 is closely linked to the high expression of IL-6, leading to life-threatening multiple organ dysfunction [27]. Therefore, inhibiting IL-6 production could potentially block the cytokine-release syndrome and organ damage, making it a potentially effective strategy for COVID-19 treatment [28]. Interestingly, emodin (**4**) may be the chemical with the highest potential for IL-6 inhibition among the compounds studied. Even at the lowest concentration (4 μg/mL), it showed a slight effect (0.97-fold). Similarly to previous publications, Fan et al. (2016) also found that emodin possessed IL-6 inhibitory activity with an IC_{50} value of 5.97 μM [29].

In our experiment, chrysophanol (**3**) was the most remarkable of the isolated *Rheum* constituents, as it can inhibit IL-6 to the lowest level (0.78-fold). Su-Jin et al. found that administration of chrysophanol reduced the levels of IL-6 in the colon tissues of mice induced colitis by dextran sulfate sodium (DSS). Chrysophanol inhibited IL-6 production by 32 % (ca. 0.3-fold) [30].

Table 2. Calculated binding affinities of the studied compounds in the active site of ACE-2 protein.

#	**Compound**	**Binding energy ΔG (kcal/mol)**	**Hydrogen bonds to**	**Hydrophobic interactions with**
1	Rhaponticin	−9.342	AsnA149, AspA269, GluA406	ProA346, PheA274, HisA345, ThrA445
2	Rhapontigenin	−7.552	ThrA445, AspA367, ProA346, HisA345	PheA274, HisA374, ArgA273
3	Chrysophanol	−8.379	GluA406, Arg518	ArgA273, PheA274, HisA345, HisA374, ProA346
4	Emodin	−8.149	AspA367	PheA274
5	MLN-4760	−9.2	ProA346, ThrA371, GluA375, HisA505, TyrA515	MetA360, LysA363, ArgA273, TyrA510, ArgA514, ArgA518

The ACE-2 binding energy values of the four compounds that were isolated from the roots of Rhubarb ranged from − 9.342 to − 8.149 kcal/mol, showing substantial interactions with the protein binding site. The ACE-2 reference inhibitor MLN-4760 was found to have a binding energy value of − 9.20 kcal/mol, whereas the stilbene rhaponticin (**1**) had a lower value of − 9.32 kcal/mol (Table 2).

*Table 3***.** Predicting the drug-like potential according to Lipinski criteria (oral bioavailability) and the toxicity of research compounds - calculated values. Level 1 = highly toxic, level 6 = non-toxic.

Compound Name	**MW**	**H- bond donors**	**H-bond acceptors**	**LogP**	**Molar Refractivity**	**TPSA (Å^2)**	**LD_{50} (mg/kg)**	**Toxicity Prediction**
Rhaponticin (**1**)	420.41	6	9	0.63	106.50	149.7	1380	4
Rhapontigenin (**2**)	258.27	3	4	2.98	74.37	69.92	2400	5
Chysophanol (**3**)	254.00	2	4	2.18	67.82	74.60	5000	5
Emodin (**4**)	270.00	3	5	1.89	69.48	94.83	5000	5
Rolipram	275.00	1	4	2.62	76.25	47.56	600	4
Celecoxib	381.00	2	4	4.79	90.32	86.36	1400	4

As shown in Table 2 and Figure 6, the positive control substance MLN-4760 interacts with several major amino acids in the active-site region of ACE-2, including hydrophobic interactions with MetA360, LysA363, ArgA273, TyrA510, ArgA514, and ArgA518 as well as hydrogen interactions with ProA346, ThrA371, GluA375, HisA505, and TyrA515 [31].

Just like the standard inhibitor MLN-4760, two compounds (**2** and **3**) had hydrophobic interactions with the acid amine residue ArgA273. Compounds **1** and **4** did not have these interactions. Notably, all four compounds had π-π T-shaped hydrophobic interactions with the acid amine residue PheA274 of the S1′-subsite of chain A [31]. Furthermore, distinct from the other three compounds and MLN-4760, rhaponticin (**1**) exhibited hydrogen interactions with AsnA149, AspA269, and GluA406. It is noted that this compound is glued to the active-site pocket of ACE-2 through hydrogen bonding and hydrophobic interactions. Thus, the calculations support that rhubarb is a potential ACE-2-directed and anti-inflammatory plant.

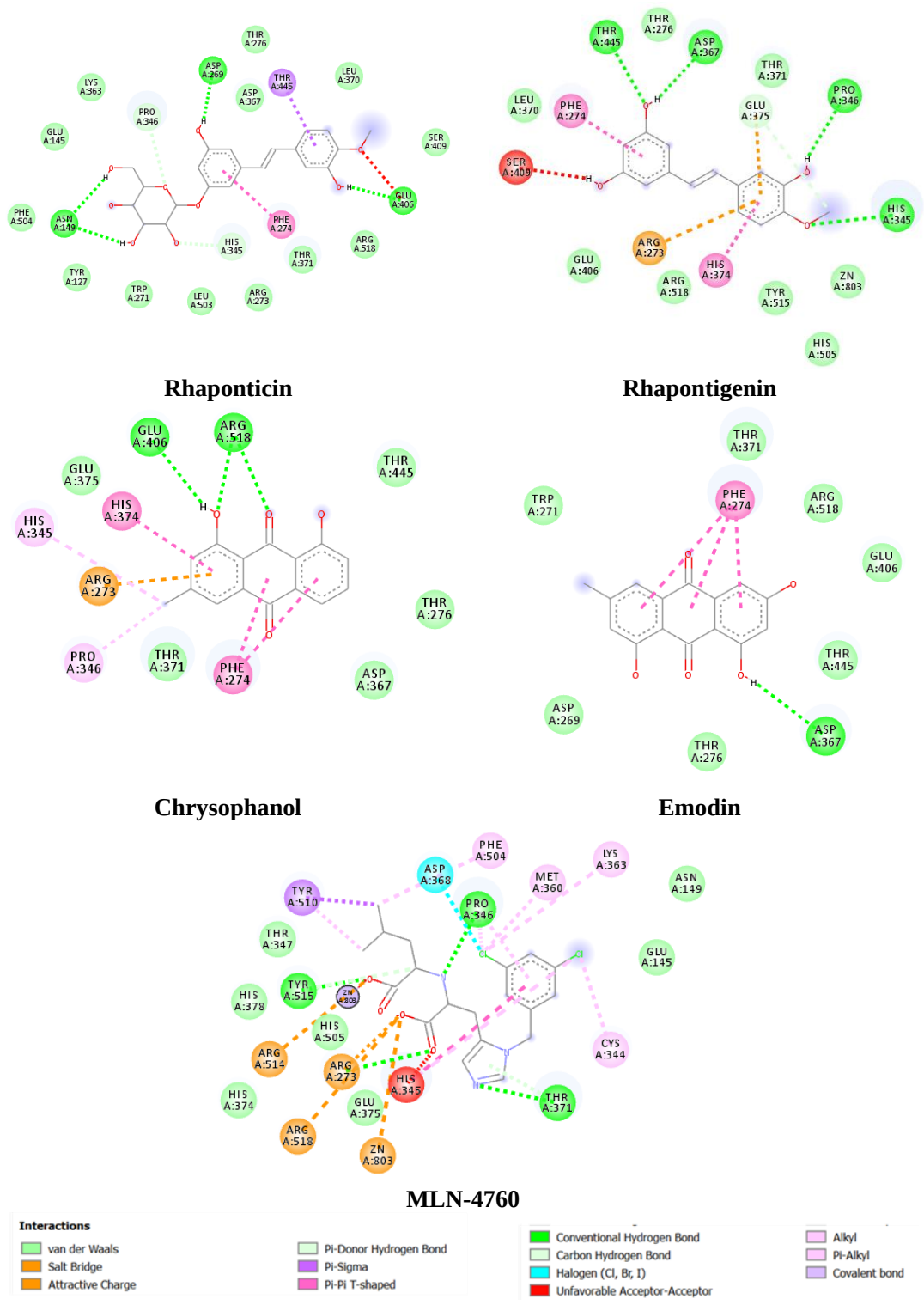


Figure 6. Molecular interaction analysis of natural compounds within the binding cavity of ACE-2 protein.

Table 3. Predicting the drug-like potential according to Lipinski criteria (oral bioavailability) and the toxicity of research compounds - calculated values. Level 1 = highly toxic, level 6 = non-toxic.

Compound Name	**MW**	**H- bond donors**	**H-bond acceptors**	**LogP**	**Molar Refractivity**	**TPSA ($Å^2$)**	**LD_{50} (mg/kg)**	**Toxicity Prediction**
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Rolipram	275.00	1	4	2.62	76.25	47.56	600	4
Celecoxib	381.00	2	4	4.79	90.32	86.36	1400	4

In the field of new drug research and development, Lipinski's Rule of Five is applied to evaluate the oral bioavailability of a compound. This simplified rule, formulated by Christopher A. Lipinski in 1997, includes five criteria: Molecular mass less than 500 Daltons; high lipophilicity (indicated by a LogP index less than 5); less than 5 donor hydrogen bonds; less than 10 (2x5) acceptor hydrogen bonds; and a molecular refractivity in the range of 40 – 130 [32]. This rule describes the molecular properties relevant for oral bioavailability to some extent and also to the pharmacokinetics of drugs in the human body, including absorption (A), distribution (D), metabolism (M), and excretion (E) (ADME). However, this rule does not predict whether a compound is pharmacologically active or toxic. This rule plays an important role and is used throughout drug development when a pharmacologically active compound is optimized step by step with the goal of increasing bioavailability, but it is still necessary to ensure that the physicochemical, stability, and toxicological properties of the compound match the criteria for becoming a drug. Compounds that fit Lipinski's rule tend to require fewer clinical trials and thus have a higher chance of becoming commercial drugs. Applying the above Lipinski's rule and conducting screening for the studied substances, we have the results presented in Table 3, showing that all four compounds from Rhubarb meet Lipinski criteria.

4. CONCLUSIONS

From the roots of Dai Hoang (*Rheum officinale* Baill.), four natural compounds, including two stilbenoids, rhaponticin (**1**) and rhapontigenin (**2**), together with two anthraquinones, chysophanol (**3**) and emodin (**4**), were isolated and structurally elucidated. These compounds have been studied for their anti-inflammatory effects on the basis of inhibiting ACE-2 enzyme activity and inhibiting NO and IL-6 production. The results show that compound rhaponticin (**1**) exhibited an inhibitory effect on the ACE-2 enzyme with an IC_{50} value of 80.9 μM. In a molcular docking study, rhaponticin also showed higher binding affinity than the reference compound MLN-4760. Combining *in silico* analysis and *in vitro* experiments shows that rhaponticin has potential anti-ACE-2 activity. Emodin (**4**) expressed anti-inflammatory activity by its inhibitory capacity on NO and IL-6 production. All four compounds from rhubarb meet the Lipinski criteria for excellent bioavailability. This is the first study of the anti-ACE-2 activity of the Rhubarb root extract and its constituents.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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