The isolation of secondary metabolites from *Rheum ribes* L. and the synthesis of new semi-synthetic anthraquinones: Isolation, synthesis and biological activity

Ibrahim Halil Gecibeslera,⁎, Faruk Dislib, Sinan Bayindirc, Mahmut Toprakc, Ali Riza Tufekcid, Ayse Sahin Yaglıoglug, Muhammed Alunt,d, Alpaslan Kocake, Ibrahim Demirtasf, Sevki Ademg

aDepartment of Occupational Health and Safety, Laboratory of Natural Product Research, Faculty of Health Sciences, Bingöl University, Bingöl, Turkey
bDepartment of Physical Therapy and Rehabilitation, Faculty of Health Sciences, Bingöl University, Bingöl, Turkey
cDepartment of Chemistry, Faculty of Science and Arts, Bingöl University, Bingöl, Turkey
dDepartment of Chemistry, Faculty of Science and Arts, Çankiri Karatekin University, Çankiri, Turkey
eDepartment of Biology, Faculty of Science and Arts, Bingöl University, Bingöl, Turkey
fDepartment of Biochemistry, Faculty of Science and Arts, İğdır University, İğdır, Turkey
gDepartment of Chemistry and Chemical Process Technology, Technical Sciences Vocational School, Amasya University, Amasya, Turkey

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**ABSTRACT**

*Rheum ribes* L. (Rhubarb) is one of the most important edible medicinal plants in the Eastern Anatolia region and is called “İşkın” by local people. Resveratrol and 6-O-methylalaternin were isolated from the Rhubarb for the first time in addition to well-known secondary metabolites including emodin, aloe-emodin, β-sitosterol and rutin. The new semi-synthetic anthraquinone derivatives with the *N*αFmoc-L-Lys and ethynyl group were synthesized from the isolated anthraquinones emodin and aloe-emodin of Rhubarb to increase the bioactivities. Aloe-emodin derivative with *N*αFmoc-L-Lys shows the highest inhibition values by 94.11 ± 0.12 and 82.38 ± 0.00% against HT-29 and HeLa cell lines, respectively, at 25 µg/mL. Further, modification of the aloe-emodin with both the ethynyl and the *N*αFmoc-L-Lys groups showed an antioxidant activity-enhancing effect. From molecular docking studies, the relative binding energies of the emodin and aloe-emodin derivatives to human serum albumin ranged from −7.30 and −10.62 kcal/mol.

**1. Introduction**

Anthraquinones (9,10-dioxoantracenes) with a wide range of applications constitute an important class of natural and synthetic compounds. Moreover, there is an increasing interest in developing new anthraquinone derivatives with biological activity. Anthraquinone-rich edible medicinal plants such as *Rheum ribes*, *Rheum rhabarbarum*, *Frangula purshiana*, *Frangula alnus*, *Rubia tinctorum* and *Aloe vera* have been used in folk medicine for a long time (Perassolo, Cardillo, Mugas, Montoya, Giulietti, & Talou, 2017; Çiçek, Ugolini, & Girreser, 2019; Zhuang, Yu, Zhong, Liu, & Liu, 2019; Parvez, Al-Dosari, Alam, Rehman, Alajmi, & Alqahtani, 2019). Anthraquinones show a broad spectrum of pharmacological activities, such as anti-HIV, cytoprotective and anti-plasmodial (Felicke et al., 2019), antifungal and antibacterial (Mohamadzadeh, Zarei, & Vessal, 2020), antiviral (Zhang et al., 2016), antiplatelet and anticoagulant (Seo, Ngoc, Lee, & Kim, & Jung, 2012), antimalarial and anti-tuberculosis (Supong, Thawai, Suwanborirux, Choowong, Supothina, & Pittayakhajonwut, 2012), neuroprotective and anti-inflammatory effects (Yang, Kim, Lee, & Song, 2018) and are active against multiple sclerosis (Sawad, 2013).

*R. ribes* L. species from Polygonaceae family is very popular among local people of Bingöl, Erzurum, Hakkari and Erzincan by names “İşkın, Uçgun and Işgın” is the only native *Rheum* species which grows wildly in the East and Southeast Anatolia (Öztürk, Aydoğmuş-Öztürk, Duru, & Topçu, 2007). *Rheum* species are medically important as they contain anthraquinone-derived compounds and *R. ribes* is also used as a source of several herbal medicines in the Middle East. Further, the plant is also used traditionally as laxative, expectorant, anthelminth drug and for the treatment of rheumatic haemorrhoids, measles, smallpox, bile complications, stomach diseases, vomiting, diarrhea, hypertension, obesity, ulcers and diabetes besides being used as a vegetable, snacks, appetizers and salad (Naqishbandi, Josefsen, Pedersen, & Jäger, 2009; Abudayyak, 2019). In previous isolation studies, it has been reported that the *R. ribes* has secondary metabolites including emodin, physicin,
chrysophanol, rhein, aloe-emodin, physcion-8-O-glucoside, aloee-modin-8-O-glucoside, sennoside A, raphonticin, quercetin, 5-deoxyquercetin, quercetin 3-O-rhamnoside, quercetin 3-O-galactoside, quercetin 3-O-rutinoside, β-sitosterol-3β-glucopyranoside-6’-O-fatty acid esters, triacylglycerols, β-sitosterol, phytyl fatty acid esters, and chlorophyllide A (Tosun & Kızılay, 2003; Ragasa et al., 2017).

In the present study, the R. ribes, which has a very broad spectrum of biological activity and is famous for its anthraquinones, was selected as a possible source of bioactive natural products and its anthraquinones emodin (E) and aloe-emodin (AE) were selected as target precursors to synthesize their new bioactive semi-synthetic derivatives. Further isolation studies of R. ribes extract resulted in isolation of resveratrol and 6-O-methylalaternin, for the first time from R. ribes species, as well as the isolation of four well-known secondary metabolites β-sitosterol, aloe-emodin, emodin, and rutin (Fig. 1A). Following isolation of emodine and aloe-emodine from R. ribes we have designed and synthesized new anthraquinones E-1 (9), AE-1 (10), FLE (11) and FLAE (12) containing L-lysine groups (Fig. 1B). Biological activity screening studies, carried out during the current work, are i) anticancer activity assays evaluated against human prostate cancer (PC-3), human cervix cancer (HeLa) and human colon cancer (HT-29) cell lines, ii) antioxidant activities (DPPH free radical scavenging activity, ferrous chelating capacity, and ferric reducing power assays) and iii) interaction tests with transport protein human serum albumin. The study showed that natural and semi-synthetic products obtained from R. ribes could be potentially used in the development of new and promising natural products as food additives.

2. Materials and methods

2.1. Reagents

Trifluoroacetic acid (TFA), copper (II) sulfate (CuSO₄), potassium carbonate (K₂CO₃), sodium sulfite (Na₂SO₃), copper (I) iodide (Cul), N,N-diisopropylethylamine (DIPEA), hydrogen iodide (HI), NαFmoc-L-Lys-NεBoc, sterile dimethylsulfoxide (DMSO), 5-Bromo-2’-deoxyuridine (BrdU), sulfuric acid (H₂SO₄), sodium chloride (NaCl), human serum albumin (HSA), sodium hydroxide (NaOH), ammonium hydroxide (NH₄OH), phosphoric acid (H₃PO₄), 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium hexacyanoferrate (III) (K₃Fe(CN)₆), trichloroacetic acid (TCA), iron (III) chloride (FeCl₃), iron (II) chloride (FeCl₂), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-5,5′-disulfonic acid monosodium salt hydrate (ferrozine), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA) and α-tocopherol (Vit. E) were purchased from Sigma (St. Louis, MO, USA). All other chemicals including n-hexane (n-C₆H₁₂), chloroform (CHCl₃), dichloromethane (CH₂Cl₂), n-butanol (n-BuOH), methanol (CH₃OH) and ethyl acetate (EtOAc) were supplied in analytical purity.

2.2. Plant material

The aerial part of R. ribes was collected from the rocky slopes of Bingöl Yelesen village at the altitude of 1970 m (38°52’07.8″N, 40°19’26.7″E) during flowering period. The plant materials were dried in dark place. Herbarium samples were prepared by Alpaslan Kocak and stored at Herbarium of Bingöl University with the herbarium number of KOCAR-4003.
2.3. Extraction, isolation, synthesis and structure elucidation

The aerial part of dried R. ribes (5 kg) was extracted with CH2OH:CH2Cl2 (1:1; v:v, 30 L, four consecutive times) and 523.8 g of 2.3. Extraction, isolation, synthesis and structure elucidation
I.H. Gecibesler, et al. collected, respectively. The sub-fractions from the CHCl3, EtOAc and n-BuOH extracts were individually subjected to silica gel (pore size: 40–63 μm) column chromatography (18 × 150 cm). During silica gel column chromatography of each extract, fractions were collected in 500 mL volume using gradients of n-C6H12:CHCl3 (0:100–100:0), n-C6H12:EtOAc (7:3; v:v) and EtOAc (3 × 25 mL). The organic phase was dried over anhydrous Na2SO4, and the solvent was removed in vacuo. The compounds FLE and FLAE were purified from the crude product mixtures FLE (521 mg) and FLAE (514 mg), respectively (Fig. S4B) on silica gel column (pore size: 20–40 μm; 25 g) and EtOAc:n-C6H12 (1:1; v:v) as an eluent.

The 1H, 13C NMR and 2D-NMR spectra of the isolated natural products and semisynthetic compounds were recorded at 298 K in DMSO-d6 on a Agilent NMR 600 MHz (14.1 T) NMR spectrometer (Agilent, Santa Clara, CA, USA) equipped with a 3 mm broadband probe (1H, 31P, 13C, 15N) and with cooled 1H and 13C preamplifiers (1H at 600 MHz and 13C at 150 MHz). Acquisition parameters included a 45 hard pulse angle, a sweep width of 14 ppm, 1.7 s acquisition time, 0.1 s pulse delay and continuous WALTZ-16 broadband 1H decoupling. Up to 2000 scans were collected per sample, corresponding to ~1 h of collection time. 2D-NMR experiments were performed using standard Agilent NMR programs. High-resolution mass spectrometric analysis was carried out on an Agilent 1260 Infinity Series HPLC-TOF/MS (ESI/MS) (Agilent, Santa Clara, CA, USA). Mass spectra were recorded in the positive ion mode in the range of 100–2000 m/z, with a mass resolution of 20,000 and an acceleration voltage of 0.7 kV.

The melting points of the compounds were determined by Stuart SMP50 Automatic Digital Melting Point Apparatus. The NMR spectra with chemical shift values and the mass chromatograms of compounds were given in the supplementary part (Figs. S6-S46).

2.4. Biological activity assays

2.4.1. Antiproliferative activity

Antiproliferative activities were performed on human colon cancer (HT-29), human cervix cancer (HeLa) and human prostate cancer (PC-3) cell lines. The cancer cell lines were provided by Istanbul University Faculty of Pharmacy and were originally purchased from the American Type Culture Collection (ATCC) with codes of HT-29 (ATCC® HTB-38™), HeLa (ATCC® CCL-2™) PC-3 (ATCC® CRL-1435™). The cancer cell lines stored in liquid nitrogen, were cultured in DMEM medium (10%; w/v, Dulbecco’s Modified - Eagle’s Medium) at 37 °C in an incubator containing 95% moisture and 5% CO2 (Demirtas, Gecibesler, & Yaglioglu, 2013). All operations in anticancer activity experiments were carried out in a sterile cabinet. Different concentrations of compounds were pipetted into each well of the 96-well plate, except the control group, with a total fluid volume of 200 μL. Sterile DMSO was added to the negative control wells instead of the test compounds, and the cells were incubated for 24 h. At the end of this period, the cell proliferation experiment was carried out according to the manufacturer’s protocol as described below. All tests were done three times and it repeated three times. Each of the 96 microplate wells was pipetted with 20,000 cells and incubated for 24 h at 37 °C in a 5% CO2 incubator. At the end of 24 h, 20 μL of BrdU labelling solution was added to the wells and incubated for 4 h at 37 °C in a 5% CO2 incubator. The labelling solution in the wells was removed by turning the plates upside down. After the addition of 200 μL FixDenat, it was incubated for 30 min at room temperature. After removing the FixDenat solution, 200 μL of the anti-BrdU-POD solution was added and incubated for 90 min at room temperature. After removing the anti-BrdU-POD solution, all wells were washed with wash solution (3 × 200 μL). Subsequently, 100 μL of substrate solution was added to all wells and incubated for 30 min at room temperature. The absorbance at 450 and 650 nm was measured in the ELISA reader before and after adding 25 μL of 1.0 M H2SO4 solution to all wells.

\[ \text{Synthesis of the compounds N}^\text{Fmoc}-\text{Lys-}\text{(N}^\text{N3}) (3) \text{: The compound } N^\text{Fmoc}-\text{Lys-}\text{(N}^\text{N3}) \text{ was synthesized in the light of the literature (Sminia & Pedersen, 2012). The group Boc in the structure of N}^\text{Fmoc}-\text{Lys-}\text{(N}^\text{N3}) \text{ was added to the reaction flask with a dropping funnel and stirred overnight at 61–62 °C under reflux. After completion, the reaction mixture was cooled to laboratory temperature and the reaction was completed by adding NH4OH solution and extracted with chloroform (3 × 25 mL). The organic phase was dried over anhydrous Na2SO4, and the solvent was removed in vacuo. The compounds FLE and FLAE were purified from the crude product mixtures FLE (521 mg) and FLAE (514 mg), respectively (Fig. S4B) on silica gel column (pore size: 20–40 μm; 25 g) and EtOAc:n-C6H12 (1:1; v:v) as an eluent.} \]

\[ \text{Synthesis of the compounds E-1 (9) and AE-1 (10): Each of the compounds E (1.85 mmol) and AE (1.11 mmol) was dissolved in DMF (5 mL) in separate reaction flask with a magnetic stirrer at laboratory temperature. Thereafter, 3-bromoprop-1-yne (8, 1.85 and 1.11 mmol) and K2CO3 (4.60 and 2.76 mmol) were put into reaction flasks of E and AE separately. Then, the reaction mixture was stirred at 60–70 °C for 5 h. The reaction flask were cooled to laboratory temperature and extracted with EtOAc (3 × 25 mL). The organic phase was dried over anhydrous Na2SO4 and its solvent was removed in vacuo. The compounds E-1 and AE-1 were purified from the crude product mixtures E-1 (512 mg) and AE-1 (331 mg), respectively (Fig. S4A). Purification of the products were performed using the silica gel column (25 g) and the mobile phase of EtOAc:n-C6H12 (1:1; v:v).} \]

\[ \text{Synthesis of the compounds FLE (11) and FLAE (12): Each of the compounds E-1 (1.01 mmol) and AE-1 (1.01 mmol) were dissolved separately in CHCl3 (20 mL) in two-necked reaction flasks. After dissolving, each reaction flasks E-1 and AE-1 were mixed separately by adding CuI (1.01 mmol) and DIPEA (6.06 mmol), respectively. Then, the N}^\text{Fmoc}-\text{Lys-}\text{(N}^\text{N3}) (3, 1.01 mmol) was added dropwise to the reaction flask with a dropping funnel and stirred overnight at 61–62 °C under reflux. After completion, the reaction mixture was cooled to laboratory temperature and the reaction was completed by adding NH4OH solution and extracted with chloroform (3 × 25 mL). The organic phase was dried over anhydrous Na2SO4, and the solvent was removed in vacuo. The compounds FLE and FLAE were purified from the crude product mixtures FLE (521 mg) and FLAE (514 mg), respectively (Fig. S4B) on silica gel column (pore size: 20–40 μm; 25 g) and EtOAc:n-C6H12 (1:1; v:v) as an eluent.} \]
2.4.2. Antioxidant activity

2.4.2.1. DPPH free radical scavenging activity. The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity assay was performed according to the method available in the literature (Gecibesler & Erdogan, 2019). Briefly, 0.5 mL of sample solutions were prepared in different concentrations (5–200 µg/mL) and 2.5 mL DPPH radical solution (0.6 µM) was pipetted to test tubes. After pipetting, each tube was mixed vigorously and incubated at the dark area for 60 min. At the end of the incubation period, absorbance values were measured at 517 nm. DPPH radical scavenging activity was calculated with the formula given below:

\[
\text{DPPH radical scavenging activity(%) = } \left( \frac{A_C - A_s}{A_C} \right) \times 100
\]

In the equation, \( A_C \) shows the absorbance value of the control and \( A_s \) indicates the absorbance value of the sample.

2.4.2.2. Ferric-ion reducing antioxidant activity. The ferric-ion reducing antioxidant activity was determined according to Gecibesler and Erdogan (2019). 2.5 mL of phosphate buffer (0.2 M, pH = 6.6) and 2.5 mL of K_{3}Fe(CN)_{6} (1.0%; w/v) were added to 1.0 mL of samples prepared in different concentrations in the test tubes. Then, the tubes were incubated at 50 °C for 20 min. 2.5 mL of TCA (10%; w/v) was added to test tubes and centrifuged at 3000 rpm for 10 min. After centrifugation, 2.5 mL of supernatants was transferred into separate test tubes and mixed with an equal volume of distilled water and 0.5 mL of FeCl₃ (0.1%; w/v) solution. The absorbance of the samples was measured at 700 nm.

2.4.2.3. Ferrous-ions chelating activity. The ferrous-ion chelating activity was carried out according to the method in the literature (Gecibesler & Erdogan, 2019). 0.5 mL of the samples prepared in different concentrations (5–200 µg/mL) was added into the test tube and 1.0 mL of distilled water and 0.05 mL of FeCl₃ (2 mM) were added into these test tubes. The reaction mixture in each test tube was homogeneously mixed with a vortex. After 30 s, 0.1 mL of ferrozine solution (5 mM) was added to the tubes and incubated for 10 min at room temperature. Absorbances of samples were measured at 562 nm after incubation. The ferrous-ion chelating activity was calculated using the formula given in DPPH radical scavenging assay.

2.4.3. The binding capacity of the samples to HSA assays

The binding capacity of the samples to the human serum albumin (HSA) was evaluated by fluorometric measurements (Toprak, 2016). Briefly, HSA solutions were prepared in 0.05 M phosphate buffer (pH 7.4) containing 0.1 M NaCl. 150 µL of each sample prepared in different concentrations was pipetted into test tubes. The solvent contained in the samples was removed under nitrogen flow. Then, 3 mL of HSA was pipetted into the test tubes at a constant concentration of 5 µM. A thermostat bath and quartzes (1 cm) were used for fluorescence measurements. The fluorescence and synchronous fluorescence measurements of HSA were performed using a Perkin–Elmer (Model LS 55) fluorescence spectrometer. The fluorescence spectra of HSA were obtained at different liposome concentrations within the range of 290–450 nm at an excitation wavelength of 280 nm. Fluorescence intensities were corrected for inner filter.

2.5. In silico studies

2.5.1. Physicochemical and pharmacokinetic properties for computational methods

The Swiss ADME web tool used to estimate the compound properties including absorption, distribution, metabolism, elimination physicochemical and pharmacokinetic properties that make more efforts to support experimental studies. The program takes into account the six most important physicochemical properties flexibility, lipophilicity, saturation, size, polarity and solubility. The molecule inputted through the sketcher Marvin JS (version 16.4.18) was converted into SMILES by JChem Web Services (version 14.9.29) installed on one of our servers (Ceylan, Erkan, Yaglioglu, Akdogan Uremis, & Koç, 2020).

2.5.2. Molecular docking studies

The blind molecular docking works were implemented by utilizing the AutoDock-vina program at the SAMSON software platform (Trott & Olson, 2010). The structure of the compounds was depicted at MarvinSketch, then converted into PDB format using Discovery Studio Visualizer 2020. The 3D crystal structure of HSA (pdb id:1AO6) was downloaded from the protein data bank (https://www.rcsb.org/) (Sugio, Kashima, Mochizuki, Noda, & Kobayashi, 1999). At the SAMSON platform, water molecules were removed and hydrogen atoms were added. The size of the grid box was set to 87 × 64 × 79 Å to cover all the active site residues having the centre of the grid at x = 27.0, y = 29.6, and z = 22.0. The minimum energy docked conformation was determined at various conformations, and the optimal binding energy conformation was selected for further analysis. The summarized 2D interaction diagrams were created using the Discovery Studio Visualizer 2020 program.

3. Results and discussion

3.1. The characterization

The emodin was isolated as pale orange amorphous powder. Its molecular structure C_{14}H_{10}O_{5} (270.24 g/mol) was determined by the NMR spectra (Figs. S6-S11). The melting point (m.p.) was determined to be 255–256 °C compatible with previous study (Guo, Feng, Zhu, Ma, & Wang, 2011). Aloe-emodin was obtained as dark brown. Its molecular structure C_{16}H_{10}O_{5} (272.24 g/mol) was determined by the spectral data (Figs. S12-S13). Its m.p. was 224–225 °C compared with the reference (Dai et al., 2014). The O-β-methylalaternin was obtained as light orange amorphous solid. The molecular structure C_{25}H_{21}O_{6} (299.06 g/mol) was examined by the spectral data (Figs. S14-S19) with the m.p. of 295–296 °C in accordance with a study (Deebah et al., 2009). The molecular structure C_{28}H_{33}O_{5} (228.24 g/mol) of resveratrol was approved by 1D and 2D NMR spectra (Figs. S20-S25). Its physical appearance was dark orange and the m.p. was 247–250 °C in accordance with another study (Lee, Kim, & Whang, 2017). The rutin was isolated as yellow solid. The molecular structure C_{27}H_{32}O_{16} (610.52 g/mol) was determined by the NMR spectra (Figs. S26-S29) and its m.p. was 191–193 °C. The findings were found to be compatible with other study in the literature (Napolitano, Lankin, Chen, & Pauli, 2012). The β-sitosterol was isolated in the form of a white amorphous powder. The molecular structure C_{29}H_{50}O (414.7 g/mol) was analyzed by the NMR spectra (Figs. S30-S35) and the m.p. 135–137 °C in accordance with the reference study (Zhao et al., 2020).

The compound 3 was isolated as yellow viscous liquid (4.44 g; 84%). The 1H NMR spectrum was given in Fig. S36. The compound E-1 was obtained as a pale yellow solid (364 mg; yield 63%) as following literature (Narendrer, Sukanya, Sharmab, & Bathula, 2013). The 1H NMR and 13C NMR spectra were given in Figs. S37-S38. The compound AE-1 was obtained as yellow solid (245 mg; yield 71%). The 1H NMR and 13C NMR spectra were given in Figs. S39-S40. The compound FLE was obtained as yellow solid (467 mg; yield 65%). The molecular formula C_{39}H_{34}N_{4}O_{9} ([M + H]^{+} m/z 703,2164) calculated C_{39}H_{34}N_{4}O_{9} [M + H]^{+}, m/z 703,2400) was found by mass spectrum (Fig. S41). The 1H NMR and 13C NMR spectra were given in Figs. S42-S43. The compound FLAE obtained as an orange solid (428 mg; yield 61%). The m.p. greater than 400 °C and the molecular formula C_{39}H_{34}N_{4}O_{9} [M + H]^{+}, m/z 703,2344; calculated C_{39}H_{34}N_{4}O_{9} [M + H]^{+}, m/z 703,2400) was determined by the mass spectrum (Fig. S44). The 13C NMR spectra was given in Figs. S45-S46. When the 1H NMR spectrum of the compound FLE is examined, it can be seen that the total 34 proton signals in the structure of the molecule were...
resonated between 1 and 13 ppm. The peaks –COOH and –NH resonating at 12.23 and 5.78 ppm, respectively are important indicators that confirm the structure of the compound FLE. On the other hand, 17 aliphatic proton signals (–CH, –CH2 and –CH3) in the FLE are also compatible with the structure of the target molecule and confirm the structure of the FLE. Thirteen aromatic peaks (==CH) also appear to have resonance between 7.95 and 6.80 ppm. Three proton signals belonging to the group –CH3 connected to the aromatic ring are found to be resonant as singlet at 2.48 ppm. All these data confirm the structure of the FLE. When the 13C NMR spectrum of the FLE is examined, a total of 24 olefinic carbon signals, including 15 quaternary and 9 aromatic –CH verifies the structure. There are six different carbon signals in the aliphatic region.

3.2. Biological activity

Antiproliferative activities of the samples against the human cervix cancer (HeLa), colon cancer (HT-29) and prostate cancer (PC-3) cells were carried out in the concentration ranges of 5, 25, 50 and 100 µg/mL. Antiproliferative activities of R. ribes anthraquinones (E and AE), semi-synthetic anthraquinones (E-1, AE-1 FLE and FLAE) and reference anticancer agent (5 FU) against HeLa cells were given in Fig. 2. The anthraquinones FLAE (20.25 ± 0.00), E-1 (5.12 ± 0.28%), E (9.33 ± 0.00%) and 5-FU (38.13 ± 0.20%) showed significant inhibitions at the lower concentration (5 µg/mL) against HeLa cell lines. Especially, the inhibition effect of the anthraquione FLAE was remarkable with the highest inhibition rate of 82.38 ± 0.14% at a concentration of 25 µg/mL. This exceptional inhibition effect of the FLAE compared to all other samples and anticancer agent 5-FU (73.7 ± 0.02%) at the same concentration, is worthy to be mentioned.

In order to prevent proliferation in HeLa cancerous cells at the same concentration, the inhibition percentage of other natural and semi-synthetic anthraquinones followed from large to small AE (72.29 ± 0.01%), E-1 (34.99 ± 0.94%), E (28.66 ± 0.16%), AE-1 (24.97 ± 0.09%), FLE (15.67 ± 0.02%). Similarly, the anthraquione FLAE showed the highest inhibition at concentration of 25 µg/mL against the HT-29 cell line. This important inhibition value of the FLAE (94.1 ± 0.12%) is worthy compared to the inhibition value of anticancer agent 5-FU (90.09 ± 0.45%) at the same concentration. Likewise the anthraquione AE, the starting compound AE of the FLAE, showed the highest activity with an inhibition value of 91.9 ± 0.31% against HT-29 colon cancer cell line at a concentration of 25 µg/mL. The samples generally showed significant increases in inhibition rates with increased concentration against the human prostate cancer (PC-3) cell line (Fig. 2). Especially the anthraquinones E-1 (86.59 ± 0.2%), AE-1 (71.41 ± 0.52%), FLE (79.20 ± 0.17%) and FLAE (77.3 ± 0.10%) showed a higher inhibition against PC-3 cells than 5-FU (53.2 ± 0.21%) at concentration of 25 µg/mL.

The antioxidant activities of R. ribes secondary metabolites (E, AE, ST, AL, RU and RE), semi-synthetic anthraquinones (E-1, AE-1, FLE and FLAE) and reference antioxidants (BHA, BHT, Vit. E and EDTA) were evaluated for their antioxidant capacities by means of DPPH free radical scavenging activity, ferrous-ions chelating capacity and ferric-ions reducing capacity at the concentrations of 5, 10, 20, 40, 80, 100 and 200 µg/mL. The E, AE and AL showed slightly lower activities with values of 10.43 ± 1.19, 9.69 ± 0.01 and 12.4 ± 0.08%, respectively than metabolites RE and RU, while the metabolite ST did not show any DPPH radical scavenging activity. The semi-synthetic anthraquinones AE-1, FLE and FLAE showed a more moderate-higher activities with values of 13.56 ± 0.33, 14.91 ± 0.10, and 19.55 ± 0.30%, respectively compared to natural anthraquinones E, AE and AL. The ferric-ion reducing powers of the samples were assessed by colourimetric measurements of their reduction capacity of ferric ions (Fe3+) at different concentrations. Increased absorbance values at 700 nm in different concentration ranges indicate that the samples are capable to reduce Fe3+ ions to Fe2+ ions (Fig. S47). Therefore, the capacities of the samples to reduce ferric ions are also considered as an indicator for their antioxidant activity (Gecibesler, Behcet, Erdogan, & Askın, 2017). Except ST, the reducing antioxidant activities of samples generally increase with increasing concentration. When the capacity to reduce Fe3+ ions to Fe2+ ions of semi-synthetic anthraquinones was evaluated at the same concentration, it was observed that they showed a similar reducing activity trend in the range of 0.11 ± 0.00–0.18 ± 0.00 at 700 nm. The chelation of ferrous-ions (Fe2+) by antioxidants is considered as an indicator of antioxidant activity (Bilici et al., 2017). In this context, the capacities of the samples to chelate Fe2+ ions in different concentrations were determined by complex-based colourimetric measurements and compared with the standard compound EDTA. Among the samples, by comparing the chelating capacities of Fe2+ ions at 200 µg/mL concentrations, it was seen that isolated compounds showed the closest activity to EDTA (95.51 ± 0.35%). On the other hand, semi-synthetic anthraquinones E-1 (19.59 ± 0.30%), FLE (16.36 ± 0.23%) and FLAE (26.50 ± 0.70%) showed slightly higher activity than R. ribes anthraquinones E (14.52 ± 0.08%) and AE (13.02 ± 0.33%).

HSA interactions with the semi-synthetic anthraquinones were screened using fluorescence spectroscopy. For this, increasing concentrations of the samples interacted in the fixed amount HSA solution in phosphate buffer (pH 7.4). After the interaction, fluorescence spectra of samples were recorded at 280 nm excitation. Fluorescence spectra showing the interactions of semi-synthetic anthraquinones in increasing concentrations (1–50 µM) with HSA (5 µM) were given in Fig. 3. As
seen in Fig. 3, HSA gave fluorescence peak at 347 nm. The semi-synthetic anthraquinone E-1, which was added to the medium in increasing concentration, affected the fluorescence intensity of the molecule HSA. The fluorescence intensity was decreased in the beginning, but later as the amount of E-1 increased, the emission of the HSA started to shift to the blue zone, a phenomena that depend on polarity. This shift was increased as the concentration of the semisynthetic anthraquinone E-1 increased. So these data indicate that there is no spectral overlap between HSA and E-1, and therefore there is no interaction in the form of binding between the two molecules. Similar findings were observed in the semi-synthetic anthraquinones AE-1, FLE and FLAE. Further, these molecules did not interact with HSA but changed the polarity of the medium. This may be due to the low solubility of molecules in water and their large molecular structures (Toprak & Arik, 2010). Since the semi-synthetic anthraquinones did not interact in the phosphate buffer medium, they did not interact in the liposome medium.

Structure-activity relationship (SAR) studies have revealed that chemical modifications made with functional groups such as hydroxyls, alkyls, hydroxyl group of aliphatic esters, alkyl amines, ammonium salts and halogens are also critical in different location of the skeleton of aloe-emodin and emodin. Modified compounds E-1, AE-1, FLE and FLAE with ethynyl and NαFmoc-L-Lys substituents were evaluated in terms of structure–activity relationship (SAR). At 25 µg/mL concentration, FLAE compound had the highest capacity (94.11 ± 0.12%) to inhibit proliferation of HT-29 cell, while the FLE had the lowest inhibition value with 17.81 ± 0.12%. Similarly, while the compound FLAE at the same concentration showed an enormous inhibition activity against the HeLa cells with value of 82.38 ± 0.00%, the FLE had the lowest activity among the modified compounds (15.67 ± 0.00%). Unlike the FLE, the FLAE does not have a methyl group (–CH₃) at position 6, and also carries a methylene at position 3. Due to these differences in substitution, the FLAE had an activity enhancing effect. While compound AE had the highest activity (53.86 ± 0.01%) against PC-3 cells at a concentration of 5 µg/mL, the compound AE-1 formed by the modification of the compound AE with the substituent ethynyl did not show inhibition. However, modification of the compound AE-1 with the NαFmoc-c-Lys group again increased inhibition against PC-3 cells by 23.64 ± 0.29%. The antioxidant activity, while the modification of the compound E with the ethynyl group in DPPH radical showed a lowering activity, it was observed that the activity of the compound E-1 with the NαFmoc-c-Lys group increased. However, modification of the compound AE with both the ethynyl and the NαFmoc-c-Lys groups showed an activity-enhancing effect. Similarly, in the ferrous-ions chelating activity experiment, the modification process with the related groups showed an antioxidant activity enhancing effect. In the ferric-ion reducing antioxidant powers experiments, there was a moderate increase as a result of modification of the compound E with the ethynyl group, while the modification of the compound E-1 with the NαFmoc-c-Lys group did not change the activity much. However, the modification of the compound AE-1 with the NαFmoc-c-Lys group showed an antioxidant activity-enhancing effect in terms of its reducing power (Fig S47). Modification of emodin with hydroxyl, quaternary ammonium, iodine, ethyl amino and methyl groups has created very important SARs that have antibacterial effect against methicillin resistant Staphylococcus aureus and do not show cytotoxicity against non-cancer Vero cells (Chalothorn, Rukachaisirikul, Phongpaichit, Pannara, & Tansakul, 2019).

In a modification study with the bromine on the skeleton of the emodin, it was demonstrated by SAR study that the compound was a strong leader showing dose-dependent inhibition of the A549 lung cancer cell line (Koerner et al., 2017). In a SAR study involving the quaternary ammonium salt derivatives of emodin, it was reported that lipophilicity and carbon chain length in the quaternary nitrogen atom...
are important factors that explain the bioactivities (Zheng et al., 2017). Structure activity studies have been reported in which the derivatives of aloe-emodin, especially the thiosemicarbamide group, exhibit stronger tyrosinase inhibitory activity (Liu, Wu, & Chen, 2015). In a study in which aloe emodin derivatives were synthesized based on the SAR study, antitumor activities were evaluated. Results of SAR studies have shown that L-serine methyl ester, β-alanine ethyl ester and 3-(2-aminoethyl) pyridine substituents were important for increased antitumor activities (Thimmegowda et al., 2015). Beyond the SAR studies mentioned above, more studies are still needed to develop the best natural food-based products. In order to increase the biological activity of natural products such as emodin and aloe-emodin, efforts to develop new bioactive products by modifying their structures with functional substituents will increase the interest in foods of natural origin.

3.3. In silico analysis

Bioavailabilities of compounds E-1, AE-1, FLE and FLAE were demonstrated in Fig. 4. The pink area represents the optimal range for each properties (LIPO: Lipophilicity, SIZE: Molecular weight, POLAR: Total Polar Surface Area, INSOLU: Insolubility, INSATU: Insaturation, FLEX: Flexibility). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Synthetic accessibility score of the compounds are from 1 (very easy) to 10 (very difficult). Synthetic accessibility of all the compounds were between 3.00 and 5.76. Topological polar surface area (TPSA) must be < 70 Å². TPSA values of compounds E-1, AE-1, FLAE and FLE were bigger than 70 Å². The solubility (log S) scale value ranges between −10 (insoluble), −6 (poorly soluble), −4 (soluble), −2 (very soluble) and 0 (highly soluble). The solubilities of compounds were between −7.35 and −3.50. The value ranges were soluble and poorly soluble. The more negative the skin permeation (log Kp) the less skin permeant the molecule. For example, Diclofenac is a good topic anti-inflammatory with a predicted log Kp = −4.96 (cm/s), while ouabain has little chance to cross skin with a predicted log Kp = −10.94 (cm/s). The log Kp values of the compounds were between −6.95 and −5.94 cm/s. According to Lipinski’s rule, E-1 and AE-1 could be a new bioactive molecule according to calculated data (Tables S1 and S2).

Computational methods are widely used to evaluate the interaction of biomolecules with small molecules. In order to explain the binding mechanism of the semi-synthetic anthraquinone derivatives with HSA, a molecular docking study was carried out. From docking studies, 5 possible free energy conformations were selected for HSA-compounds (Fig. 5), among them least binding free energies were preferred to create 2D interaction diagrams (Fig. 6). The relative binding to HSA energies of the compounds were calculated as −7.83, −7.30, −10.10 and −10.62 kcal/mol respectively for AE-1, E-1, FLAE and FLE. In addition, the best Ki values were calculated as 1.80, 4.44, 0.03 and 0.01 µmol in the same order (Table S3). The binding results indicated that E-1, FLE, and FLAE are in close proximity to the HEME binding site, and EA-1 is located at Sudlow’s site I (Ascenzi, Bocedi, Notari, Menegatti, & Fasano, 2005). Compounds FLE and FLAE are more prominent HSA binder than the other two compounds.

FLE exhibited the highest binding to HSA protein energy. It formed hydrogen bond interactions with Asp 108, Arg 415, His 146, Arg 117, and Gln 459. Results show that Lys 190, Tyr 138, Leu 182, Tyr 161, Leu 115, Arg 197 and Ala 194 were critical residues in hydrophobic interaction of FLE with protein. FLE formed van der Waals interactions with Asn 458, Val 462, Leu 463, Pro 147, Asn 109, Pro 110, Leu 185, Glu 141, Met 123, Val 116, Arg 114, and Glu 425.

As described in the 2D-interaction diagrams (Fig. 6), the optimum pose of AE-1 at HSA encircled by amino acids Arg 222, Tyr 150, His 242, (Hydrogen Bonds), Arg 218, Trp 214, Glu 153, His 283, Glu 188, Glu 292, Ser 192, Phe 157, Gln 196, Val 241, Ser 287, Ile 264, Ile 290, Leu 219, Phe 223, (van der Waals), Leu 260, Leu 238, Ala 291 (Hydrophobic interaction), Arg 257 (Unfavorable), Ala 291, and Lys 195 (Carbon Hydrogen Bonds).
The difference between experimental data and theoretical studies can be concluded from two reasons. The first is that the solubilities of the molecules are low (Toprak & Arik, 2010). The second is that proteins display different behaviours in aqueous environments than X-ray structures (Rudra, Dasmandal, Patra, Kundu, & Mahapatra, 2016). Our experiment studies demonstrated that new anthraquinones interact with HSA protein at low concentrations. The reason for not being affected in high concentration may be low solubility. In silico studies have shown that molecules have a high potential for binding with HSA protein.

4. Conclusions

In summary, an effective semi-synthetic strategy has been developed from the anthraquinones E and AE of native R. ribes to synthesize several anthraquinone derivatives E-1, AE-1, FLE and FLAE. Taking this strategy into account, four bioactive anthraquinone derivatives with structural similarities were practically synthesized. Semi-synthesis of the anthraquinones E-1, AE-1, FLE and FLAE was obtained from anthraquinones E and AE with 2 steps for E-1 and AE-1 and 4 steps for FLE and FLAE, a total yield of 63.0, 71.0, 18.0 and 16.8%, respectively. Besides, isolations of resveratrol (RE), and 6-O-methylalaternin (AL) from the R. ribes have been reported for the first time. Moreover, antiproliferative and antioxidant activities of secondary metabolites and new semi-synthetic anthraquinones and their interactions with HSA were compared. Among them, the anthraquinones E-1, AE-1, FLE and FLAE exhibited higher antiproliferative activities against HT-29, PC-3 and HeLa cell lines, while these anthraquinones showed moderate to weak antioxidant activities and HSA interactions.

CRediT authorship contribution statement


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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Appendix A. Supplementary data

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