



The antiproliferative potential of isolated emodin and aloe-emodin from *Rheum ribes* on different cancer cell lines

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Abstract

Herbal compounds are important resources that used in the treatment of many diseases, and are inspiring in drug design and development. *Rheum ribes*, a perennial native plant, has potentially diverse medicinal effects, including antioxidant, antibacterial and anti-tumor activity. The key molecules associated with anticancer activity of *R. ribes* in tumor formation still remains unclear. The present study was aimed to elucidate the antiproliferative effects of two purified compounds, emodin and aloe-emodin, from *R. ribes*. In our study, chromatographic separation was used to purification of compounds. Experimental 1D and 2D 1H and 13C-NMR spectra helped us to confirm the chemical structures of emodin and aloe-emodin. The viability of human glioblastoma cell line (U373), human breast carcinoma cells (MCF-7), and human colorectal cancer cell line (HT-29) were detected by WST-1 proliferation assay as dose- and time-dependent. The isolated emodin and aloe-emodin from *R. ribes*, reduced viability of human glioblastoma, breast and colorectal cancer cell lines in dose- and time-dependent manner significantly. These antiproliferative effects may contribute as a possible strategy to the development of new therapeutic approaches in the treatment of cancer.

Key words: *Rheum ribes*, emodin, aloe-emodin, cancer, glioblastoma

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Rheum ribes'ten izole edilen emodin ve aloe-emodinin farklı kanser hücre hatları üzerindeki antiproliferatif potansiyeli

Özet

Bitkisel bileşikler birçok hastalığın tedavisinde kullanılan ve ilaç tasarımı ve geliştirilmesinde ilham verici olan önemli kaynaklardır. Çok yıllık doğal bir bitki olan *Rheum ribes*, antioksidan, antibakteriyel ve anti-tümör aktivite dahil olmak üzere çeşitli potansiyel tıbbi etkilere sahiptir. *R. ribes*'in tümör oluşumundaki antikanser aktivitesi ile ilişkili anahtar moleküller hala belirsizliğini korumaktadır. Bu çalışmanın amacı, *R. ribes*'den saflaştırılan iki bileşik olan emodin ve aloe-emodinin antiproliferatif etkilerini aydınlatmaktır. Çalışmamızda bileşiklerin saflaştırılması için kromatografik ayırma kullanılmıştır. Deneysel 1D ve 2D 1H ve 13C-NMR spektrumları, emodin ve aloe-emodinin kimyasal yapılarını doğrulamamıza yardımcı oldu. İnsan glioblastom hücre hattının (U373), insan meme karsinom hücrelerinin (MCF-7) ve insan kolorektal kanser hücre hattının (HT-29) canlılığı, WST-1 proliferasyon analizi ile doza ve zamana bağlı olarak belirlendi. *R. ribes*'den izole edilmiş emodin ve aloe-emodin, insan glioblastomu, meme ve kolorektal kanser hücre hatlarının canlılığını doza ve zamana bağlı bir şekilde önemli ölçüde azaltmıştır. Bu antiproliferatif etkinlik, kanser tedavisinde yeni terapötik yaklaşımların geliştirilmesine olası bir strateji olarak katkıda bulunabilir.

Anahtar kelimeler: *Rheum ribes*, emodin, aloe-emodin, kanser, glioblastoma

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1. Introduction

Plants have been used for many centuries as nutrient, dietary supplementation, food additives, flavoring, and main source of drugs used in treatment of human sickness. In particular, the therapeutic properties of plants are currently being studied intensively for the development and discovery of newer therapeutics. Increasing incidence and high mortality rates have made highlighted the studies on the treatment of cancer in recent years. In cancer studies, phyto-compounds, which are compounds derived from plants, have an important position, because of their low or no cytotoxicity and clinical efficacy. Plant-derived compounds such as irinotecan, camptothecin, paclitaxel, vinblastine, combrestatin and etoposide, are used in clinical as chemotherapy drugs [1], and natural plant polyphenols, curcumin, quercetin, resveratrol, luteoline, gallic acid, hesperidine and genistein, etc., are also considered as promising adjuvants for cancer prevention and treatment [2].

Rheum species are known to have medical importance in the treatment of many diseases and disorders. *Rheum ribes* L., a member of the Polygonaceae family, is an important source in folk medicine. The medically important *Rheum* species are rich in anthracene-derived compounds [3]. *R. ribes* are used to treat laxative and joint disorders in Iran [4, 5]. The Polygonaceae family also includes eight genera and seventy species in the Turkey. *R. ribes* is the only species in the family represented by the Turkey [6]. This plant grows naturally Turkey, Lebanon and Iran and it famous with names “İşgın, Kurdish Banana and Uçgun”.

R. ribes' young twigs and stems consumed as the salad, fresh vegetable and appetizing by the local people especially in Turkey's eastern and south-eastern Anatolia region. Besides, roots, leaves, flowers and stems are also used by natives in this area to treat the bile ducts, stomach discomforts, diarrhea, hemorrhoids, measles and smallpox as well its use as an appetite enhancer [7, 8]. Further, ethnobotanists in Jordan reported that the root of *R. ribes* was used by the public in diabetes, hypertension, obesity, kidney stone and sand [9]. Water decoction from the *R. ribes* roots was shown to reduce blood sugar in mice when given to mice diabetic with alloxan, but not hypoglycemia when administered to healthy mice forming the control group [10]. It was shown that isolates of the roots, leaves and stems of *R. ribes* had anti-viral and anti-microbial effect activity [11-14].

Phytochemical studies performed by the researchers on *R. ribes* species were collected from different provinces, such as Hakkari and Erzincan in Turkey. It has revealed the existence of different types of secondary metabolites chrysofanol, rhein, emodin, aloe-emodin, aloe-emodin-8-O-glucoside, physcion, physcion-8-O-glucoside, sennoside A, rhaponticin quercetin, 5-desoxyquercetin, quercetin 3-O-rhamnoside, quercetin 3-O-galactoside and quercetin 3-O-rutinoside [15-17].

The objective of this study was to purify the bioactive compounds, emodin (E) and aloe-emodin (AE), from *R. ribes*, and to determine the anti-proliferative activities of these purified compounds first time. The anti-proliferative activities of E and AE was observed in the cancer cell models of human glioblastoma, breast and colorectal cancer, U373, MCF-7 and HT-29 cell lines, respectively. This study elucidates the anticancer potential of *R. ribes* E and AE phytochemicals as potential anti-tumor agents.

2. Materials and methods

2.1. Plant material

R. ribes species, which were grown at 2000 m and higher in the natural habitats of Yelesen village of Bingöl, Turkey, were collected from the field following the flowering period during April-May months. The collection of the species was devoted to collecting without destroying nature. The species identification of the plant material was made by Alpaslan Koçak and the herbarium specimen was stored with KOÇAK 4003 registration number. *R. ribes*, collected in a suitable form from the field, was spread on unprinted paper and dried in a damp and shaded environment. The dried material was milled in the laboratory type mill so as not to exceed 3 cm.

2.2. Chemicals and reagents

Dulbecco's modified eagle medium-high glucose (DMEM-HG), fetal bovine serum (FBS), penicillin/streptomycin and phosphate buffered saline (PBS) was obtained from Lonza (Walkersville, MD, USA). Tripan blue, tris HCl, trypsin-EDTA, dimethyl sulphoxide (DMSO), ethanol and methanol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide (H₂O₂), sodium hydroxide (NaOH) and glycine was purchased from Merck (Darmstadt, Germany). WST-1 [Water Soluble Tetrazolium-1; 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt] cell proliferation assay kit was obtained from Intron Biotechnology (Gyeonggi-do, Korea).

2.3. Extraction, fractionation and isolation process

Dried and ground plant material (5 kg) was taken in a glass flask and 10.0 L of methanol: chloroform (1:1; v/v ratio) was added and left to extraction for 2 days at the laboratory temperature. At the end of the second day, the extract

was filtered with the help of the Whatman no 4 filter paper and the remaining pulp was allowed to be extracted again with the same amount and ratio of solvent system. This procedure was repeated three more times. The extracts obtained by the extraction process were combined and the solvents they contained were removed under reduced pressure at a temperature of 35°C by means of the rotary evaporator. Removal of the solvent system from the extracts yielded 523.8 g of dry extract with a yield of 10.476%. The extraction yield was calculated by the following formula; % Extract yield = [Dry extract amount (g)/dry plant quantity (g)]x100. The dry extract was stored at +4°C by taking an amber flask for anticancer activity and isolation studies. For isolation, 50 g of methanol: chloroform dry extract was dissolved in 1L of distilled water. The aqueous extract was fractionated with chloroform (8x0.6 L). At the end of the fractionation, 15.63 g of dry chloroform extract was obtained as a result of the removal of chloroforms by rotary evaporator. Isolation process was continued with this part. For the flash chromatography system, the pharmaceutical glass column (5x60cm) containing sephadex LH-20 column filler. The methanol: chloroform (35:65; v: v) solvent system was used as mobile phase and operating conditions for elution collection were adjusted to 2 mL per minute. A total of 126 sub-fractions were eluted in each elution volume of 10 mL. As a result of the chromatographic separation, the emodin (68 mg) and aloe-emodin (56 mg) were obtained in the sub-fraction 37 and 75, respectively (Figure 1).

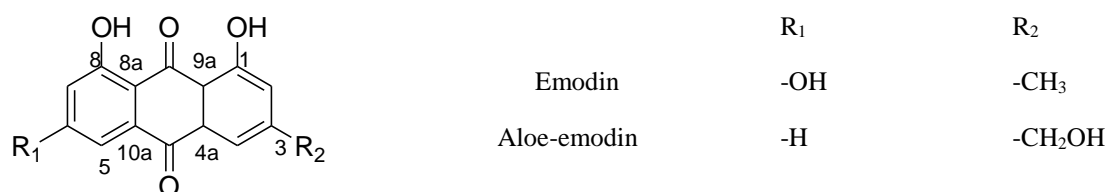


Figure 1. Chemical structures of emodin and aloe-emodin from *R.ribes*

2.4. Cell lines and cell culture conditions

Human glioblastoma cells (U373), breast cancer cell line (MCF-7) and colorectal adenocarcinoma cells (HT-29), were obtained from the Central Laboratory Research and Application Center of Bingol University, Turkey. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C [18].

2.5. Cell viability

The dose- and time-dependent antiproliferative effects of E and AE from *R. ribes*, was measured using the WST-1 cell proliferation assay kit according to manufacturer's protocol (Intron Biotechnology, Gyeonggi-do, Korea), *in vitro*. Cells (5 × 10³ cells/well) were seeded into 96-well culture plates and incubated at 37°C and 5% CO₂ overnight. The plates were treated with various concentrations (1, 10, 25, 50, and 100 µg/mL) of E and AE for 24 and 48 hours. The control groups were exposed to 0.05% DMSO containing media. Culture medium was replaced by fresh medium and 10 µl of WST-1 reagent were pipetted into wells. After 3 h incubation period, the amount of soluble formazan dye produced by metabolically active cells was measured with an ELISA microplate reader (SpectraMax 384 Plus, Molecular Devices, USA) at 450 nm as absorbance (optical density (OD)). The mixture of WST-1 reagent and culture medium without cells was evaluated as a background control. To determine percentage of cell survival that formula was used: % cell survival = OD_{test}/OD_{control} × 100%. The half maximal inhibitory concentration (IC₅₀) value for each test group was calculated according to the findings.

2.6. Statistical analysis

Each assay was performed in three experiments and mean ± standard deviation (SD) values were calculated. Statistical analysis of the findings were evaluated by t-test and one-way ANOVA test using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). *P* values <0.05 was assumed to be statistically significant.

3. Results

3.1. Chemical characterization

The ¹H and ¹³C NMR spectra of E and AE compounds were analyzed using 600 MHz NMR spectrometer (Bruker AVANCE). 7 mg of each compound was taken to NMR tube and dissolved in 450 µL of DMSO-*d*₆. The chemical shifts of compounds that consistent with the literature was documented in the table 1 [19, 20]. Also chemical structures of emodin and aloe-emodin confirmed by experimental 1D and 2D ¹H and ¹³C-NMR spectra.

Table 1. The ^{13}C -NMR chemical shifts (δ ; ppm) of the emodin, aloe-emodin and they comparison with literature data (150 MHz, DMSO- d_6)

	Emodin (Experimental)	Emodin [19]	Aloe-emodin (Experimental)	Aloe-emodin [20]
C1	166.03	166.44	161.76	161.8
C2	108.39	108.17	117.51	117.7
C3	161.87	162.14	154.14	154.5
C4	109.43	109.36	124.78	125.1
C5	124.59	124.51	121.06	121.4
C6	148.71	148.76	137.72	138.0
C7	120.93	121.23	119.74	120.0
C8	164.90	166.17	162.06	162.1
C9	190.19	190.43	191.97	191.9
C10	181.85	181.34	181.70	182.1
C4a	135.58	135.47	133.64	134.7
C8a	113.85	113.90	114.73	114.9
C9a	109.22	109.36	116.18	116.4
C10a	133.29	133.10	133.41	133.5
-OCH ₃	21.96	21.50		63.3
-CH ₃			62.50	

3.2. Antiproliferative effects of emodin and aloe-emodin from *R. ribes*

The dose- and time-dependent antiproliferative activities of E and AE were assessed in U373, MCF-7 and HT-29 cells. The increasing concentrations of E and AE were treated cell lines. After 24 and 48 h treatment, the viability of the cells were observed with WST-1 cell proliferation assay. The GraphPad Prism 8 software was used to estimate the half-maximal inhibitory concentrations (IC₅₀).

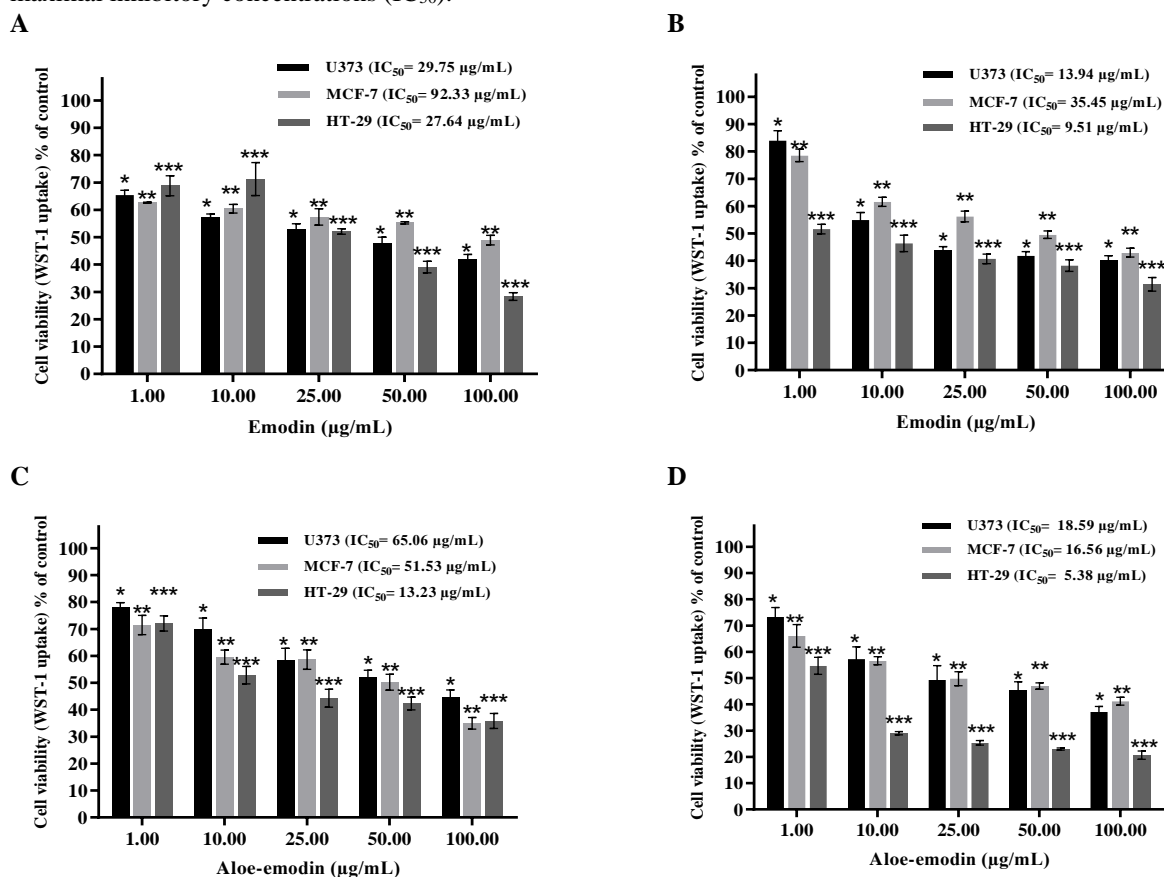


Figure 2. Emodin and aloe-emodin, from *R. ribes*, decreases the viability of cancer cells *in vitro*. U373, MCF-7 and HT-29 cells, grown in conditions which as described in materials and methods. (A) The cancer cells were treated for 24 h with different concentrations of E. (B) U373, MCF-7 and HT-29 cells were treated for 48 h with various concentrations of E. (C) The cells were treated with different AE concentrations for 24 h. (D) U373, MCF-7 and HT-29 cells were treated with various concentrations of AE for 48 h. The GraphPad Prism 8 programme was used to calculate the IC₅₀ values for E and AE. Asterisks indicate statistical significance by comparison of treatment groups with control cells (100%, cells treated with 0.05% DMSO containing media). The results are mean \pm SD of three experiments (U373 * p <0.001; MCF-7 ** p <0.001 and HT-29 *** p <0.001, when compared to control).

E and AE exhibited dose- and time-dependent effects on viability of all cell lines. As shown in Fig 2, the decreases in percentage of viability were statistically significant in all treatment groups compared to control (U373 * $p < 0.001$; MCF-7 ** $p < 0.001$; and HT-29 *** $p < 0.001$). Treatment with E at 1 $\mu\text{g/mL}$ significantly reduced viability in all three cell lines in 24 h treatments, however, the decrease in viability of U373 and MCF-7 cells was milder at 48 hours treatment of the same concentration. The IC_{50} values of E, at 24 h treatment, were 29.75, 92.33 and 27.64 $\mu\text{g/mL}$ for U373, MCF-7 and HT-29 cell lines, respectively (Fig 2A). The required concentrations of E to inhibit the growth of 50% of cells (IC_{50}) for U373, MCF-7 and HT-29 were 13.94, 35.45 and 9.51 $\mu\text{g/mL}$ at 48 hours (Fig 2B). These results indicate that, 48 h treatment with E reduces IC_{50} values by more than half, compared to 24 h treatment. On the other hand, the viability of the cells decreased significantly in treated with AE at 1 $\mu\text{g/mL}$, which was the lowest concentration, but at the highest concentration, 100 $\mu\text{g/mL}$, the viability of all cells was the lowest at 48 hours of treatment. AE treatment at 100 $\mu\text{g/mL}$ in 48 h, reduced the viability of U373 human glioblastoma cells, MCF-7 breast cancer cell line and HT-29 colorectal adenocarcinoma cells to 37.1, 41.2 and 20.7%, respectively (Fig 2D). The IC_{50} values of AE were 65.06, 51.53, 13.23 $\mu\text{g/mL}$ and 18.59, 16.56, 5.38 $\mu\text{g/mL}$ in U373, MCF-7 and HT-29 cell lines, at 24 and 48 h treatment, respectively (Fig 2C, Fig 2D). IC_{50} values in 48 h AE treatment groups, also decreased approximately three-fold compared to 24 h treatments.

4. Conclusions and discussion

Cancer causes huge losses as a major health problem all over the world. Many new therapeutics and clinical treatments have been proposed to combat this disease. In this context, herbal-based treatment approaches stand out in the fight against cancer with their low toxic effects and efficacy. The therapeutic potential of medicinal plants has recently aroused a great interest in the treatment and prevention of many diseases, such as cancer [21]. The anticarcinogenic effect of *Rheum* genus have been shown in several studies. It was determined that *R. officinale* water extract was significantly growth inhibitory on A549 and MCF-7 cell lines and IC_{50} values were 620 and 515 $\mu\text{g/mL}$ [22]. *R. emodi* water and methanol extracts have been reported to show anticancer activity against two human carcinomas (MDA-MB-435S breast cancer and Hep3B liver carcinoma cell lines) [23]. *R. ribes* ethanol, water and methanol extracts significantly reduce the viability of HCT-116, A2780, MCF-7 and PC-3 cells, especially at concentrations of 25, 50 and 100 $\mu\text{g/mL}$, and in the same cell lines the IC_{50} values were determined between 14.2-42.2 $\mu\text{g/mL}$ [24]. In another study, ethanolic extract of *R. ribes* was showed significantly cytotoxic activity against SW742 and MCF-7 cells, and this extract inhibited the half of the cells at concentrations of 46.4 and 51.3 $\mu\text{g/mL}$, respectively [25]. *R. ribes* butanol fraction exhibited antiproliferative activity on MCF-7 human breast cancer cells, and IC_{50} value was determined as 36 $\mu\text{g/mL}$ [26]. *R. ribes* is particularly rich in anthraquinone compounds, and emodin and aloe-emodin are among the main components [15-17]. In this study, the anti-proliferative effects of two anthraquinone derivatives, emodin (1,3,8 trihydroxy-6-methylanthraquinone) and aloe-emodin (1,8-dihydroxy-3-hydroxymethyl-anthraquinone), which isolated from *R. ribes*, were investigated on different cancer cell lines.

Glioblastoma is a malignant brain tumor that affects stem/progenitor cells in the brain and it has the rapidly spread potential in adults [27]. Ismail *et al.* demonstrated that aloe-emodin inhibits proliferation of U87 malignant glioma cells in a dose and time dependent manner and has an IC_{50} value of 58.6 $\mu\text{g/mL}$ in 24 h treatment and 25.0 $\mu\text{g/mL}$ in 48 h treatment [28]. In accordance with this study, we found that aloe-emodin, that isolated from *R. ribes*, was decreased the viability of U373 glioblastoma cells in a dose and time dependent manner, and the IC_{50} value was 65.1 $\mu\text{g/mL}$ and 18.6 $\mu\text{g/mL}$ at 24 and 48 h treatment, respectively. We have also demonstrated that isolated emodine significantly inhibits the viability of U373 glioblastoma cells depending on increasing time and concentration.

Breast cancer is the most common cancer among women and accounts for 9.6% of cancer-related deaths worldwide [29]. Kang *et al.* determined the IC_{50} value of the emodin and aloe-emodin isolated from *R. palmatum* for 48 h in MCF-7 breast cancer cells as 16.4 and 12.6 $\mu\text{g/mL}$, respectively [30]. Similarly, we found the IC_{50} value as 16.6 $\mu\text{g/mL}$ for *R. ribes'* aloe-emodin in MCF-7 cells at 48 h treatment. However, isolated emodin from *R. ribes* was had a milder antiproliferative effect than aloe-emodin. It has been reported that, emodin and aloe-emodin significantly inhibited the proliferation of MCF-7 cells in a dose and time dependent manner and show anticancer activity [31]. We can also say that, emodin and aloe-emodin show anticancer activity depending on dose and concentration, and these two compounds make important contributions to *R. ribes* anticancer potential.

Colorectal cancer is the second cause of cancer-related deaths in both men and women [32]. Suboj *et al.* treated WiDr, RKO, SW 480 and SW 620 colon adenocarcinoma cells for 48 h with commercially AE, and showed that AE has a dose-dependent antiproliferative effect and it was arrested the cell cycle in the G2/M phase [33]. Similarly, in our study, AE exhibited dose-dependent inhibition in the proliferation of HT-29 colorectal cancer cells. In addition, both emodin and aloe-emodin, that isolated from *R. ribes*, significantly inhibited the proliferation of HT-29 colorectal adenocarcinoma cells, even at very low concentrations.

Overall, these results indicate that, isolated emodin and aloe-emodin from *R. ribes* have a significant anti-proliferative effect in human breast, colon and glioma cancer cell lines, and therewithal suggesting that these isolated compounds from *R. ribes* have potential therapeutic efficacy in the prevention and treatment of cancer. However, further investigations are needed to clarify the molecular mechanisms of these isolated compounds.

Acknowledgements

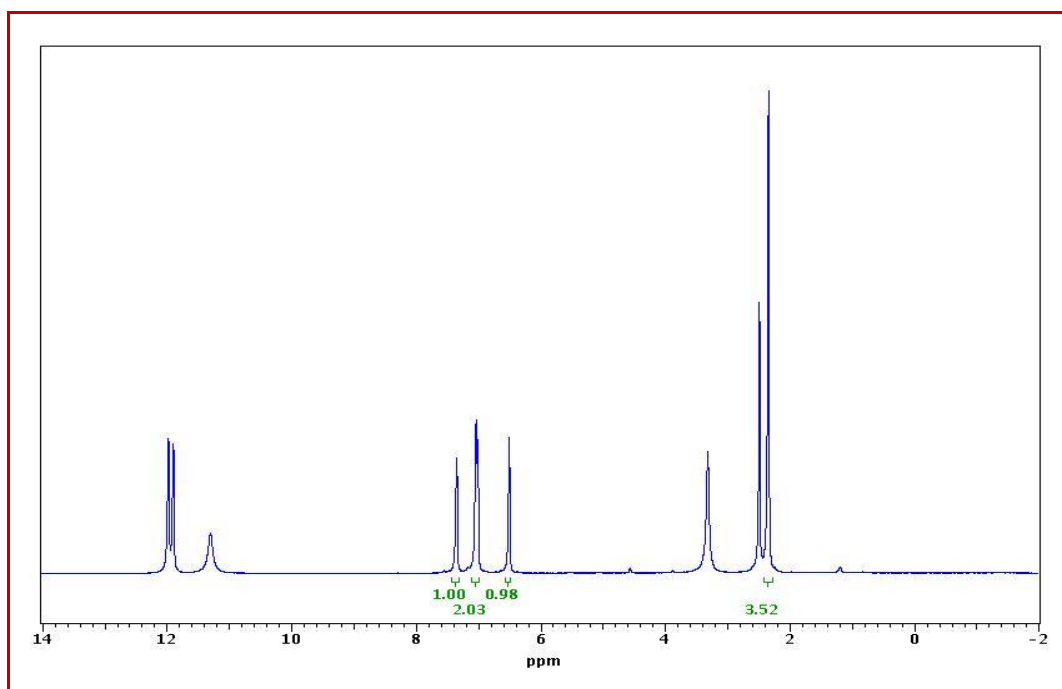
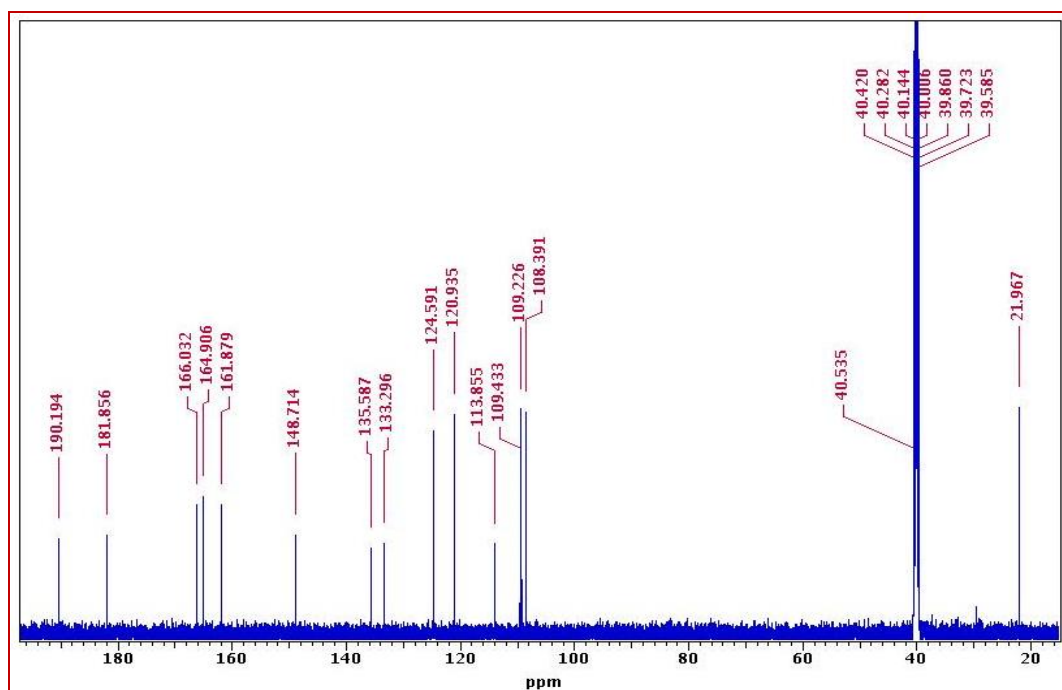
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Supplementary data

Figure S1. ¹H-NMR spectrum of the emodin compound (¹H-NMR: 600 MHz; DMSO-d₆)Figure S2. ¹³C-NMR spectrum of the emodin compound (¹³C-NMR: 150 MHz; DMSO-d₆)

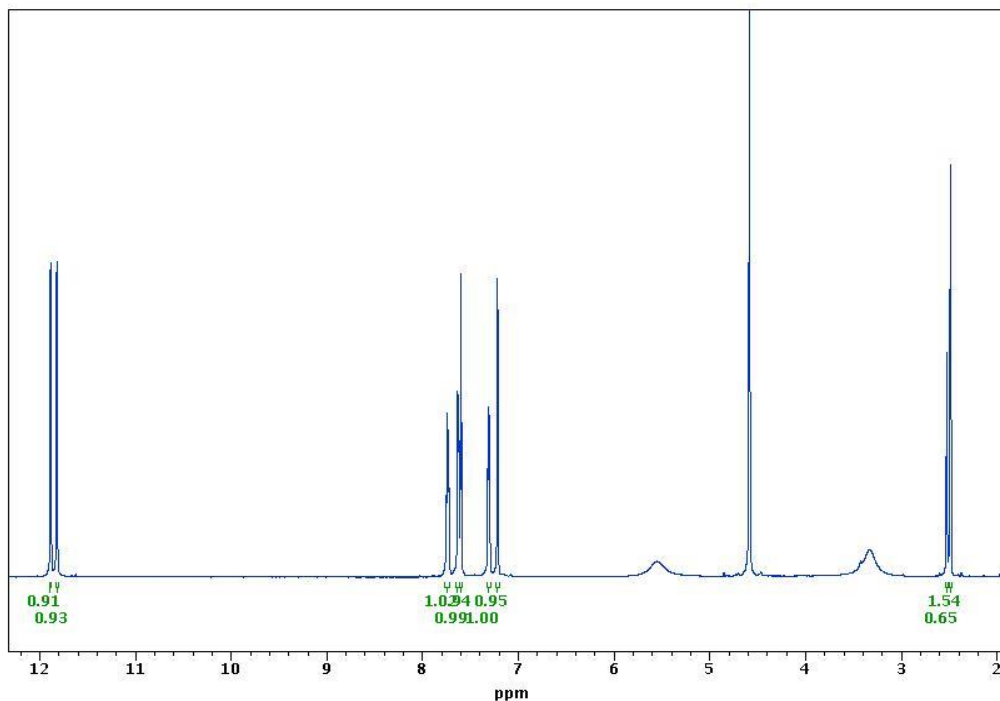


Figure S3. ¹H-NMR spectrum of the aloe-emodin compound (¹H-NMR: 600 MHz; DMSO-d₆).

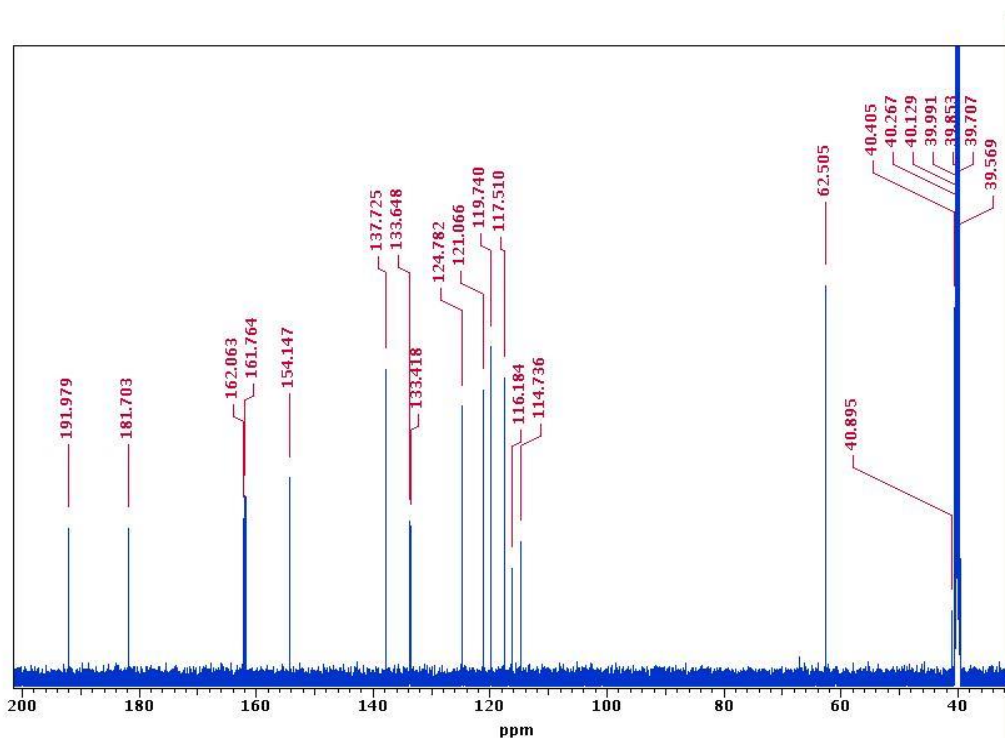


Figure S4. ¹³C-NMR spectrum of aloe-emodin compound (¹³C-NMR: 150 MHz; DMSO-d₆).