Dual activities of emodin – DNA protectivity *vs* mutagenicity

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ORIGINAL ARTICLE

Abstract

OBJECTIVES: Emodin is a bioactive anthraquinone that has diverse biological effects. It is also known as a biosynthetic precursor of hypericin. The purpose of this study was to assess mechanisms of potential genotoxic and antioxidant effects of emodin. We also investigated the potential genotoxic effect of photoactivated emodin.

METHODS: Potential genotoxicity was determined by the alkaline comet assay and the Ames test. The potential DNA protectivity of emodin was determined by the DNA-topology assay. On purpose to clarify molecular mechanism of its DNA protectivity against Fe^{2+}-induced DNA breaks, three different assays were used (Reducing power-, DPPH- and Fe^{2+}-chelating assay).

RESULTS: Using the alkaline comet assay and the Ames test we confirmed the genotoxic effect of both non-photoactivated and photoactivated emodin in a dose-dependent manner. Genotoxicity of photoactivated emodin did not differ from that obtained with non-photoactivated one. The DNA-topology assay revealed a DNA-protective activity of emodin. In the reducing power and DPPH assays emodin exhibited weak antioxidant activities. We did not observe any chelating activity of emodin in the Fe^{2+}-chelating assay.

CONCLUSIONS: We found out that emodin exhibited dual activities. On one side it was genotoxic inducing primary DNA lessions (determined by the comet assay) as well as gene mutations (determined by the Ames test). On the other side it exhibited DNA-protective activity (determined by the DNA-topology assay). Molecular mechanism underlying this DNA protective effect can be attributed to its free radicals scavenging and reducing activities.

INTRODUCTION

Plants have been used for centuries as a valuable source of natural products maintaining human health and their use can be of great significance in many aspects of human life (Miadokova *et al.* 2006;

Horvathova *et al.* 2008; Hudecova *et al.* 2010). However, different plant secondary metabolites could also be harmful when applied to humans. Therefore, it is necessary to carry out *in vitro* and *in vivo* tests in order to assess the potential risks of phytocompounds prior to their clinical appli-

cation. Anthraquinone emodin, identified in 17 plant families with many species (e.g. *Senna obtusifolia*, *Fallopia japonica*, *Rumex nepalensis*, *Rheum palmatum*) distributed worldwide, itself demonstrates numerous biological activities. It is also known as a biosynthetic precursor of hypericin – one of the bioactive constituents of *Hypericum perforatum* L. (Zobayed *et al.* 2006). Emodin possesses anti-inflammatory, antimicrobial, antiviral, antiangiogenic, antitumor, vasorelaxant, laxative, antidiabetic and antioxidant effects (Hsu & Chung 2012; Wei *et al.* 2013), but less attention has been paid to its genotoxic potential.

In this study, we investigated whether non-photo-activated or photoactivated emodin exhibits genotoxic effects using the comet assay and Ames test and potential DNA-protective activities employing the DNA-topology assay. In order to explain molecular mechanisms of emodin potential DNA-protective activities we used three tests suitable for an assessment of antioxidant activities. We evaluated its reducing power, DPPH radical scavenging activity, and Fe^{2+}-chelating ability.

MATERIAL AND METHODS

The study was performed with emodin (Figure 1) purchased from Sigma Aldrich (Bratislava, Slovakia), dissolved in dimethylsulfoxide (DMSO) or methanol to prepare 0.1 M stock solution.

The alkaline comet assay

The comet assay was performed according to Collins *et al.* (1996). Briefly: microscope slides were pre-coated with base layer of 100 µl of 1% NMP (normal melting point) agarose in water. Blood samples were collected by finger-prick into 1×PBS and left on ice for 30 min. Lymphocytes were isolated from the collected blood by standard centrifugation with Histopaque (Sigma-Aldrich) and the pellet of cells was re-suspended in 1% LMP (low melting point) agarose in PBS buffer. A volume of 80 µl of the cell suspension was layered on to a pre-coated slide. Cover slips were placed on the

slides to ensure even spread. Slides were kept at 4°C to solidify the gel. After removing the cover slips, the cells were treated with different concentrations of emodin (0.25–2 µg/mL) for 1 h at 37°C. 100 µM hydrogen peroxide (H_2O_2) was used as a positive control. For a negative control, the cells were left untreated, in fresh 1×PBS buffer solution for 1 h at 37°C. In case of photoactivated samples, following the incubation with emodin, the cells were washed with fresh PBS and placed on top of a specially modified lamp composed of eleven L18W/30 fluorescent tubes (Osram, Berlin, Germany; with maximum emission range 530–620 nm) for activation (25 min, which equals to a dose of 3.65 J/cm²). Cells were lysed by immersing slides into the lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, pH 10 and 1% Triton X-100) at 4°C for 1 h. After lysis, the slides were transferred into an electrophoretic tank containing fresh and chilled alkaline electrophoretic solution (300 mM NaOH, 1 mM Na_2EDTA, pH>13), and left for 20 min at 4°C to allow the DNA to unwind. The electrophoresis was conducted for 30 min at 4°C at 25 V and 260–320 mA. The slides were neutralized by washing one time in 1×PBS (5 min) and one time in dH_2O (5 min), and stained with ethidium bromide (20 µg/mL). Using fluorescence microscope (OLYMPUS BX 51), 100 random nucleoids were scored in each sample at magnification 400×. Depending on the relative intensity of DNA fluorescence in the comet tail, each comet was scored from 0 to 4 (0 = undamaged, 4 = >80% DNA in the tail). The final percentage of DNA damage was calculated from the total score for each sample.

Ames (Salmonella typhimurium) assay

This assay was performed according to the revised method by Maron and Ames (1983). Two experimental protocols of the pre-incubation Ames assay were used. *Salmonella typhimurium* tester strains TA97, TA98, and TA100 were obtained from the Czech Collection of Microorganisms (Brno, Czech Republic). As positive controls, 9-aminoacridine (5 µg/plate; Serva) was used for TA97; 4-nitroquinoline 1-oxide (0.5 µg/plate; Serva) was used for TA98; and sodium azide (2 µg/plate; Serva) was used for TA100. The assay was conducted in test tubes containing 0.1 mL of overnight bacterial culture (approximately 1×10^8 cells/mL, cultivated in 50 mL of LB medium), top agar and the tested emodin (0.025; 0.1; 0.5 µg/plate) or the positive mutagens. The plates for the negative control contained 100 µL of DMSO. Test tubes containing emodin were incubated for 1 h with bacteria. After the 1 h of incubation, mutagens and top agar were added, and the contents were plated on minimal bottom agar plates. *his⁺* revertants were counted after 72 h of incubation at 37°C on a Biotran III Colony Counter (New Brunswick Scientific Co.). For each bacterial strain there were three plates for the negative control, three plates for the appropriate positive control and three plates for emodin applied alone and together
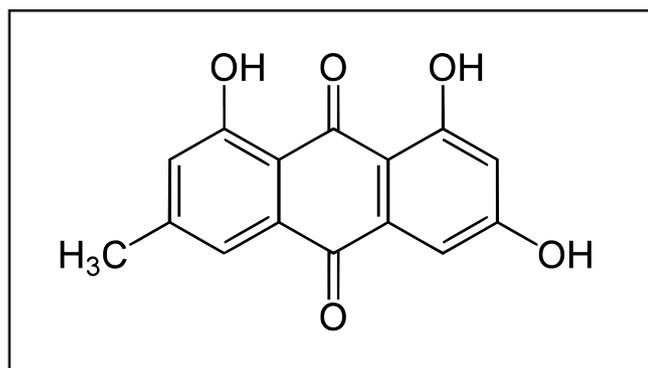
Fig. 1. Structural formula of emodin (6-methyl-1,3,8-trihydroxyanthraquinone).

with mutagens. Each value is the mean ± SD of three separate experiments, nine plates together.

DNA-topology assay

The method of electrophoretically monitored DNA-damaging activity and DNA-protective activity was described in detail previously (Cipak *et al.* 2006). The reaction mixture (final volume of 10 μL) contained 200 ng of plasmid DNA (pBR322) and either Fe^{2+} (10 μM) alone, or emodin alone, or combinations of emodin with Fe^{2+}. Specific details of agent concentrations are given in the legend to Figure 5. DNA breaks were assayed by measuring the conversion of supercoiled plasmid DNA (form I) to relaxed circular (form II). Topological changes of DNA molecules correspond with the electrophoretical mobility of DNA topoisomers. Analysis of DNA modifications was made by the agarose gel electrophoresis (1.5% agarose, 1 h/60 V). The DNA was visualized by staining with EtBr (1 mg/mL) and UV illumination (UV Transilluminator Mini-BISPro, DNR Bio Imaging Systems Ltd.).

Reducing power assay

The reducing capacities of emodin were determined according to Horvathova *et al.* (2012). 1 mL of different concentrations of emodin in methanol were mixed with 2.5 mL of a 0.2 M phosphate buffer (pH 6.6) and 2.5 mL 1% (w/v) solution of potassium ferricyanide $[K_3Fe(CN)_6]$. The mixtures were incubated at 50 °C for 20 min. Afterwards; 2.5 mL of a 10% (w/v) trichloracetic acid solution was added to each mixture and centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of the upper layer of each supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of a 0.1% (w/v) $FeCl_3$ solution, and the absorbance was recorded at 700 nm spectrophotometrically (GENESYS 10 Bio, Spectronic). Gallic acid was used as a positive control. The higher absorbance of the reaction mixture indicates the higher reducing power.

DPPH radicals scavenging activity

The scavenging activity of emodin against DPPH (1,1-diphenyl-2-picrylhydrazyl) free radicals were measured using a modified DPPH assay (Locatelli *et al.* 2009) as follows: 1 mL methanolic solution of DPPH• in the concentration of 0.05 mg/mL was added to 50 μL of various concentrations of emodin or gallic acid used as a positive control. After 25 min of incubation at room temperature, the absorbance at 517 nm was measured spectrophotometrically (GENESYS 10 Bio, Spectronic) and the scavenging of the DPPH• by emodin was calculated according the formula:

$$\text{Scavenging of DPPH radicals (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the control reaction, containing all reagents except the tested compounds,

and A_{sample} is the absorbance of tested compounds. Methanol was used as a blank.

Fe^{2+}-chelating activity

The chelating activity (ChA) of emodin toward ferrous ions was conducted as described previously (Rajić *et al.* 2010). Briefly, 50 μL of 0.25 mM $FeCl_2$ solution was added to 150 μL of emodin solution. The reaction was initiated by adding 100 μL of 1 mM ferrozine solution. After 10 min of incubation at room temperature the absorbance at 545 nm was recorded using spectrophotometer (GENESYS 10 Bio, Spectronic). A reaction mixture containing 150 μL of methanol instead of emodin solution served as a negative control. Na_2EDTA was used as the positive chelating standard. ChA was calculated according to the formula:

$$\text{Ferrous ion chelating ability (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ (absorbance of the negative control, e.g. without test compound) and A_{sample} (absorbance of the sample solution). Chelating activity was expressed as $ChEC_{50}$, the concentration that chelates 50% of Fe^{2+} ion.

Statistical analysis

The results represent the mean of three experiments ± standard deviation. The significance of differences between means was evaluated by the Student's t-test: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

RESULTS AND DISCUSSION

The comet assay was used for the evaluation of primary DNA damages induced by emodin. The alkaline comet assay on lymphocytes revealed that both non-photoactivated and photoactivated emodin exerted genotoxic activity in a dose-response manner (Figures 2–3). Similar results were obtained by Go *et al.* (2007) on mouse L5178Y cells and Li *et al.* (2010) on TK6 cells. On the other hand, Wamer *et al.* (2003) showed that activation of emodin with UV led to higher DNA damage.

The Ames assay was employed for gene mutations assessment. We used three *S. typhimurium* strains (TA97, TA98 and TA100). As follows from Figure 4, emodin exerted mutagenic activities in a dose-response manner. These results correspond with that obtained with other bacterial strains (Wehner *at al.* 1979; Go *et al.* 2007).

The ability of emodin to induce or prevent DNA breaks was evaluated using the DNA-topology assay (Figure 5). The assay responds sensitively to a metal occurrence and a free-radical generation in reaction medium (Cipak *et al.* 2001). Fe^{2+} ions induce DNA damage (DNA breaks) via Fenton-like reaction that results in a free radicals formation. Such DNA breaks subsequently change the plasmid topology. Thus, the assay is based on the electrophoretic detection
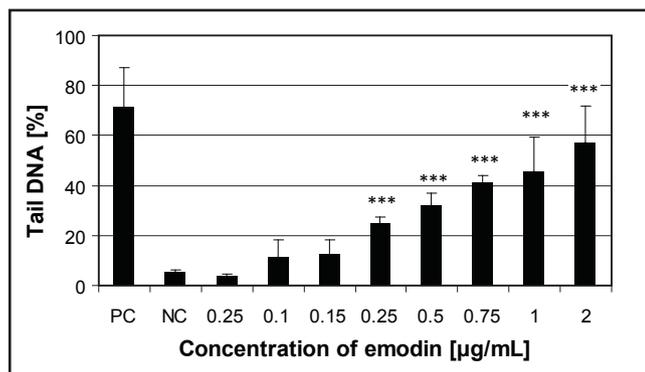
Fig. 2. Potential genotoxic activities of different concentrations of non-photoactivated emodin tested on human lymphocytes using the comet assay. Legend: NC – PBS used as a negative control; PC – positive control (100 µM H_2O_2). ***$p \leq 0.001$.
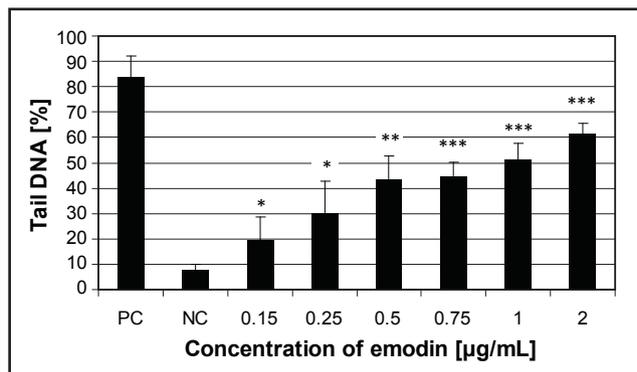
Fig. 3. Potential genotoxic activities of different concentrations of photoactivated emodin tested on human lymphocytes using the comet assay. Legend: NC – PBS used as a negative control; PC – positive control (100 µM H_2O_2). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

of changes induced in the plasmid DNA topology by Fe^{2+}. Emodin itself did not change the mobility of the supercoiled plasmid DNA topoisomers (form I), similarly as the supercoiled plasmid DNA used as a control. Moreover, we observed that emodin was not able to fully protect plasmid DNA against Fe^{2+}-induced DNA breaks especially with concentrations 0.25–5 µM as in these samples we could still detect band corresponding to a relaxed circular form (Form II). However, the ability of emodin to protect plasmid DNA increased in a concentration-dependent manner (Figure 5).
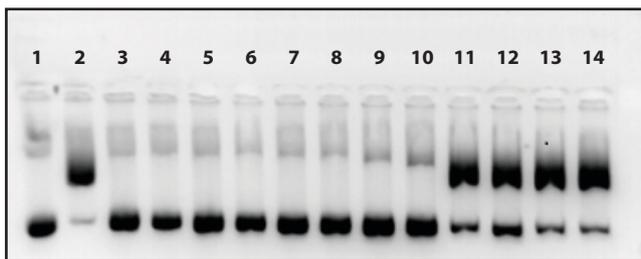
As we proved DNA-protective activity of emodin using the DNA-topology assay, we decided to assess the molecular mechanisms underlying this protective activity. The potential antioxidant effect of emodin was investigated using the Reducing power assay, the DPPH• scavenging assays, and the Fe^{2+}-chelating assay.

The measurement of the electron-donating activity of tested compounds in the Reducing power assay was used as an indicator of emodin`s potential antioxidant properties. However, emodin showed a low reducing power capacity as compared to gallic acid, which is known to be a very strong reducing agent, having a strong electron donating capacity (Figure 6).

The DPPH assay is a test commonly used to examine the antioxidant activity of some compounds and the tendency of isolated pure compounds to act as hydrogen atom donors. We found out that emodin was able only slightly to scavenge DPPH radicals in comparison with gallic acid (Figure 7). Similar results were obtained
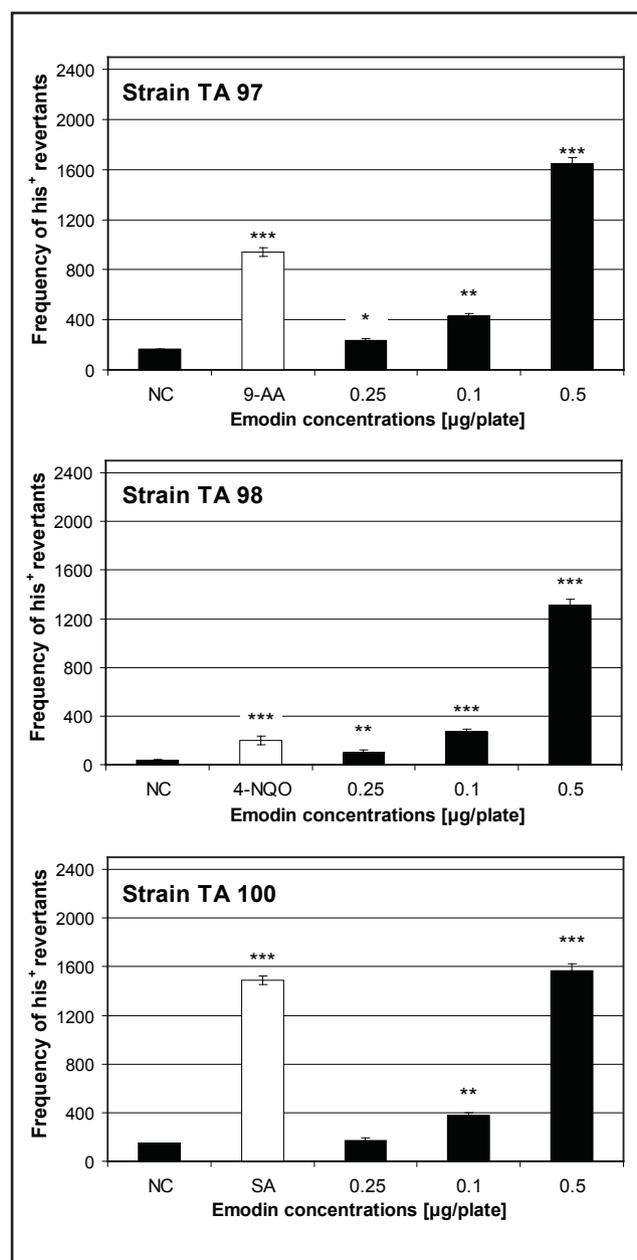

Fig. 4. Potential mutagenic activities of emodin tested on *S. typhimurium* using the Ames test. Legend: NC – negative control; 9-AA – 9-aminoacridine (5 µg/plate) used as a positive control for the strain TA97; 4-NQO – 4-nitroquinoline 1-oxide (0.5 µg/plate) used as a positive control for the strain TA98; SA – sodium azide (2 µg/plate) used as a positive control for the strain TA100. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Fig. 5. DNA protective activity of emodin studied in the DNA-topology assay – electrophoretic monitoring of changes induced in the structure of plasmid DNA by Fe^{2+} and changes in DNA topology after the emodin application. Lower band represents supercoiled plasmid DNA (form I), upper band relaxed circular DNA (form II). Lanes 1-14; 1: pBR322; 2: pBR322 + Fe^{2+}; 3: pBR322 + 50 µM emodin (E); 4: pBR322 + 25 µM E; 5: pBR322 + 5 µM E; 6: pBR322 + 2.5 µM E; 7: pBR322 + 0.5 µM E; 8: pBR322 + 0.25 µM E; 9: pBR322 + Fe^{2+} + 50 µM E; 10: pBR322 + Fe^{2+} + 25 µM E; 11: pBR322 + Fe^{2+} + 5 µM E; **12**: pBR322 + Fe^{2+} + 2.5 µM E; **13**: pBR322 + Fe^{2+} + 0.5 µM E; 14: pBR322 + Fe^{2+} + 0.25 µM E.
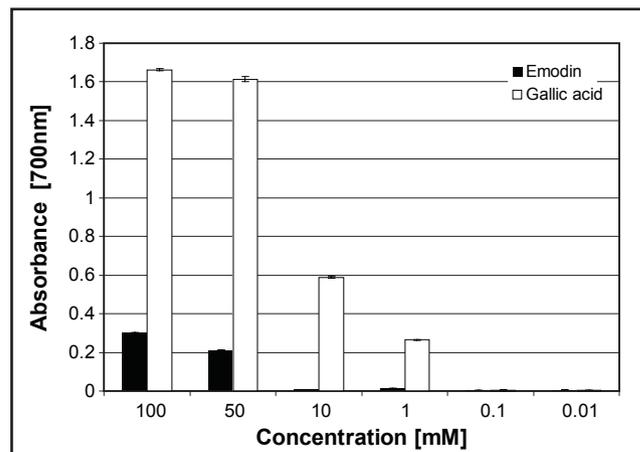
Fig. 6. Reducing power activity of emodin (black columns) in comparison with gallic acid used as a positive control (white columns). Each value is expressed as mean ± standard deviation (n=3).
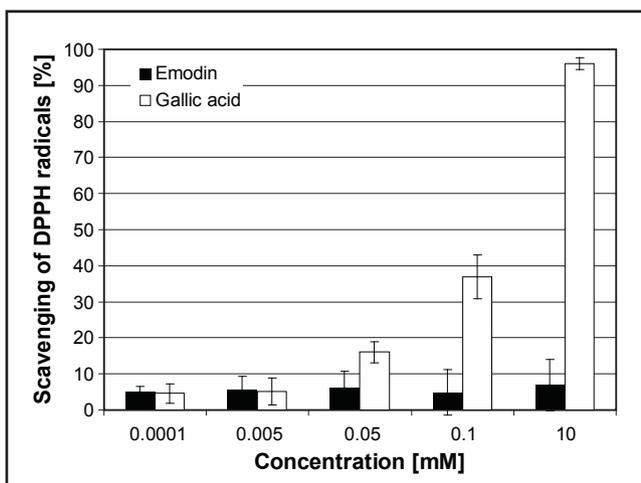
Fig. 7. DPPH radical scavenging activity of emodin (black columns) in comparison with gallic acid used as a positive control (white columns). Each value is expressed as mean ± standard deviation (n=3).
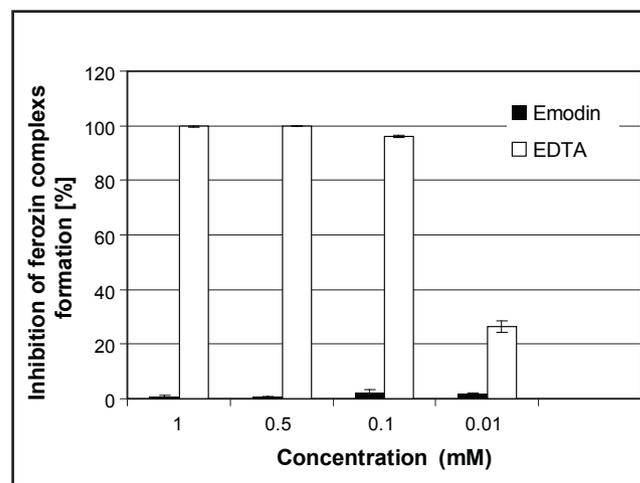
Fig. 8. Chelating activity of emodin (black columns) in comparison with ETDA used as a positive control (white columns). Each value is expressed as mean ± standard deviation (n=3).

by Mellado *et al.* (2013) and Wei *et al.* (2013), but Waly *et al.*(2013) showed strong DPPH• scavenging activity of emodin. We suggest that this activity of emodin can be influenced by more factors, e.g. natural or synthetic origin of emodin, plant species from which emodin was isolated, concentrations used etc.

One of the mechanisms of antioxidative action is a chelation of transition metals, thus preventing catalysis of hydroperoxide decomposition and Fentotype reactions (Gordon, 1990). The metal ion chelating capacity is significant since it reduces the concentration of transient metals that catalyze oxidative changes in DNA, lipids, proteins and other cellular components. We revealed that emodin did not chelate Fe^{2+} ions (Figure 8) suggesting that the mechanism of plasmid DNA protection is based on a mechanism independent of their Fe^{2+}-chelating properties.

We showed that emodin protected plasmid DNA against Fe^{2+}-induced DNA breaks in a dose-response manner. We propose that the protective effect of emodin is based on its hydroxyl radical scavenging activity that was already proved by Yen *et al.* (2000). Moreover we proved that its DNA-protective activities were not mediated by the chelation of Fe^{2+} ions.

Altogether, despite emodin possesses many positive properties, several limitations exist in its use, e.g. emodin causes skin, eye and respiratory irritations. Moreover, its proved genotoxic activity should be taken into account in future therapeutic applications.

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