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# Prenylated flavanone derivatives isolated from *Erythrina addisoniae* are potent inducers of apoptotic cell death

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## ABSTRACT

Extracts of *Erythrina addisoniae* are frequently used in the traditional medicine of Western Africa, but only insufficient information about active compounds is available. From the stem bark of *Erythrina addisoniae*, three new (**1**, **2**, **4**) and three known (**3**, **5**, **6**) flavanones were isolated: addisoniaflavanones I and II, containing either a 2'',3''-epoxyprenyl moiety (**1**) or a 2'',3''-dihydroxyprenyl moiety (**2**) were shown to be highly toxic (MTT-assay: EC<sub>50</sub>-values of 5.25 +/- 13.5 and 8.5 +/- 15.2 μM, respectively) against H4IIE hepatoma cells. The cytotoxic potential of the other isolated flavanones was weaker (range of EC<sub>50</sub>-values between 15 and >100 μM). Toxic effects of addisoniaflavanone I and II were detectable after 3 h (MTT assay). Both compounds induced an apoptotic cell death (caspase 3/7 activation, nuclear fragmentation) in the hepatoma cells and, at high concentrations, also necrosis (membrane disruption: ethidium bromide staining). Formation of DNA strand breaks was not detectable after incubation with these compounds (comet assay). In conclusion, the new prenylated flavanones addisoniaflavanones I and II may be of interest for pharmacological purposes (e.g. potential use as cytostatic drugs) due to their high cytotoxic and pro-apoptotic potential.

## Key words:

Apoptosis, cytotoxicity, *Erythrina addisoniae*, addisoniaflavanone, prenylated flavonoids

**Abbreviations:** FBS, fetal bovine serum; HPLC, high performance liquid chromatography; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide; PBS, phosphate buffered saline; ROS, reactive oxygen species.

The genus *Erythrina* is known to contain prenylated flavonoid derivatives<sup>1</sup>, which were reported to possess interesting biological and pharmacological properties<sup>2</sup>. In traditional medicine, extracts prepared from *Erythrina* spp. are used against several important diseases in their respective area of distribution.<sup>3-5</sup> *Erythrina addisoniae* Hutch. & Dalziel, which occurs in Ghana and other West-African states, is used to treat dysentery, hepatitis, rheumatic disorders and pain, as well as swellings and cancer.<sup>6,7</sup> In a previous paper we reported the occurrence of bioactive pterocarpanes and flavanones from the stem bark of *E. addisoniae* showing cytotoxic activities in micromolar ranges.<sup>8,9</sup> Prenylated stilbenoids and isoflavanoids from the root bark of *Erythrina* species have also been reported to possess inhibitory activities against neuraminidases from influenza viruses and have shown activities against breast cancer lines and a commercially available protein tyrosine phosphatase 1B.<sup>10-12</sup> We now describe the isolation and structure elucidation of three new and three known prenylated flavanones and their cytotoxic activities against H4IIE hepatoma cells.

## RESULTS AND DISCUSSION

Purification of four major fractions of the dichloromethanic extract of the stem bark of *Erythrina addisoniae* Hutch. & Dalz. afforded six further compounds (**1-6**). The <sup>1</sup>H- and <sup>13</sup>C-NMR spectra of compound **1** showed resonances for 25 carbons and 28 protons. Nine carbon and seven proton signals were found at shift values characteristic for a 5,7-dihydroxy-2,3-dihydro-benzo[b]pyran-4-on moiety, representing the A- and C-ring moiety of flavanones.<sup>8,13</sup> Interpretation of the 2D-NMR data led to the assignment of six further carbon resonances to a 3,4,5-trisubstituted benzoyl moiety attached to C-2 of the pyranone ring, which represents the B-ring of a flavanone. The down shift of the resonance of C-4' ( $\delta$  157.0 ppm) indicated a 4'-OH group, accompanied by aliphatic substituents at positions C-3' and C-5'. Accordingly, five of the remaining ten carbon resonances could be identified as signals of a 3,3-dimethylallyl substituent (isoprenyl side chain) by means of the HMBC-, HMQC- and COSY-spectra. The five remaining <sup>13</sup>C resonances indicated a second isoprenyl derivative as substituent, its methylene and methine resonances significantly shifted upfield by  $\Delta$  53.9 and 54.6 ppm, respectively. Moreover the according proton resonances of the terminal methyl groups were shifted to a higher field. In comparison to the regular isoprenyl side chain the methylene protons were no longer represented by one signal ( $\delta$  3.04 and 2.75) indicating a stereochemical effect, which was reported to be characteristic for a 3,3-dimethylallylmethyl side chain.<sup>14-17</sup> Therefore, compound **1** could be identified as 3'-(2'',3''-epoxy-3''-methylbut-3''-enyl)licoflavanone by 1D and 2D NMR spectra. The structure was additionally confirmed by its mass spectra. This compound was found for the first time in nature and we suggest the name addisoniaflavanone I. The NMR spectra of compound **2** resembled those of **1**. Differences were found for the signals of the methylene and methine groups as well as of the terminal methyl groups of one side chain. The according proton signals of the methyl groups appeared in a higher field and the methylene and methine proton resonances were shifted upfield. These differences indicated a hydrolytic scission of the epoxide leading to a 2'',3''-dihydroxylated side chain, which could be confirmed by mass spectrometric data. Thus **2** was identified as the equally new natural product 3'-(2'',3''-dihydroxy-3''-methylbutyl)licoflavanone, for which the name addisoniaflavanone II may be suggested. In the case of compound **3** the NMR spectra showed analogy to the carbon and proton resonances of a 5,7-dihydroxy-flavanone with two isoprenyl substituents. One of the substituents could be identified as a 3,3-dimethylallyl side chain at C-5'. In comparison with **1** and **2** the <sup>13</sup>C NMR signals of C-3' and C-4' appeared at smaller shift values indicating a different isoprenyl substituent, a methylation of the C-4'-OH or a ring closure.

According to the remaining proton resonances and the 2D NMR spectra it was found that the second prenyl moiety at C-3' formed a 2,2-dimethyl-3-hydroxy-tetrahydropyrane moiety. Compound **3** was identified as 2(*S*)-5,7-dihydroxy-5'-prenyl-[2'',2''-(3''-hydroxy)-dimethylpyrano]-(5'',6'':3',4')flavanone. This compound was already reported Cui et al. in the stem bark of *Erythrina abyssinica*<sup>18</sup>, all analytical data found by us are identical to those reported there. The NMR data of **4** also indicated a 5,7-dihydroxy-flavanone with a single 3,3-dimethylallyl side chain. In contrast to **1** and **2** the proton and carbon spectra of **4** showed two additional resonances typical for a methoxylation at C-4' and an aldehyde substituent. According to the 2D NMR spectra **4** could be identified as 3'-formyllicoflavone-4'-methylether which could be named addisoniaflavanone III. Compounds **5** and **6** were found to be Abyssinin II and Abyssinoflavanon V, previously isolated from *E. milbrandii*<sup>19</sup> and *E. abyssinica*.<sup>20</sup>

Each of the isolated substances shows substituents at C-3' and C-5'. In the case of **1**, **2**, **3** and **6** the molecules possess one 3,3-dimethylallyl side chain and a different substituent derived from a second 3,3-dimethylallyl moiety both ortho to the 4'-OH group. Biogenetic investigations have already been made for biotransformation of 3,3-dimethylallyl side chains in ortho-prenylated phenols.<sup>15</sup> The isoprenyl substituents seem to be part of a series of enzymatic reactions which start with a 3,3-dimethylallyl side chain and may lead via the 2,3-epoxy-3-methylbut-3-enyl derivative (**1**) to the hydroxylated side chain in compound **3** (see fig. 2). This possible biogenetic pathway also include 3'-(2-hydroxy-3-methyl-but-3-enyl)-licoflavanone-4'-methylether, 3'-(2-hydroxy-3-methyl-but-3-enyl)-abyssinone II and abyssinoflavanone VII, previously reported as constituents of *E. addisoniae*.<sup>8</sup> Side chains can also form 2,2-dimethylchromene derivatives by cyclization with phenolic hydroxyl groups as found in **3** and **6**. Eight of the 20 different partial structures formed by such prenyl side chains in natural products were found in *E. addisoniae* so far.<sup>7,8</sup> Their structures and substitution patterns could be unequivocally resolved through 2D-HMBC spectra.

Since *Erythrina* species are used to treat cancer in traditional medicine of western Africa, we analysed the cytotoxic potential of distinct compounds against H4IIE hepatoma cells (figure 2): Incubation of these cells with **1**, containing the epoxy-group, as well as **2** showed them to be highly cytotoxic. In the MTT assay their EC<sub>50</sub> values (24 h) were determined to be 5.25 +/- 13.5 µM and 8.5 +/- 15.2 µM, respectively. The toxicity of **3** was slightly lower, but in the same order of magnitude (EC<sub>50</sub> value (24 h): 14.7 +/- 7.2 µM). If the prenyl group in position 3' of the B-ring moiety is shortened, as found in **4**, the toxicity is significantly reduced (EC<sub>50</sub> value (24 h): 59 +/- 16.9 µM). If the prenyl group in position 3' of the B-ring moiety replaced by a methoxy group as found in **5**, the EC<sub>50</sub>-value is higher than 100 µM. If the prenyl group at C-5' is missing, in case of **6**, only slightly toxic effects were detectable (EC<sub>50</sub> of 63 +/- 18.1 µM). The compounds with the highest toxicity (**1** and **2**) were further investigated for the kinetic of cell death (figure 3): Compound **1** caused toxic effects after an incubation time of 3 h, showing significant reduction of cell viabilities at the lowest concentration analyzed (2.5 µM). A concentration of 10 µM caused an approximate 50% reduction of cell viability already after 3 h. A similar kinetic was found for **2**. Both compounds were further analysed for the mode of cell death: In case of apoptotic cell death, a shrinking of the cell, activation of specific enzymes, so-called caspases, results in the fragmentation of the nucleus, "blebbing" of the membrane and finally the fragmentation of the whole cell lead to apoptotic vesicles, which can be taken up by phagocytes. Analysing the mode of cell death caused by **1** and **2**, a significant activation of the caspase-3, a central apoptotic enzyme, was detectable (figure 4C). Incubation with 10 µM of **2** resulted in a 7.6-fold increase of the caspase-3-activity, in the case of **1** a similar increase (8.5-fold) already occurred at a concentration of 5 µM. The apoptotic mode of cell death was further confirmed using the life-dead assay (figure 4A): In case of **1**, the amount of apoptotic cells was determined to 20.8 +/- 4%, in case of **2**, the

amount of apoptotic cells was lower (5.1 +/- 1.8%). The life/death assay was further used to determine the amount of necrotic cells (cells with disrupted cell membranes): In case of **1**, 28.9 +/- 6.7% necrotic cells were determined, in case of **2**, 45.1 +/- 5.05% necrotic cells were found. Since induction of cell death (apoptotic or necrotic) might be caused by induction of DNA strand breaks, we determined the effect of the two compounds on the formation of DNA strand breaks using a single cell gel electrophoresis (comet assay). Despite significant toxicity after 3 h of incubation for the two compounds, no induction of DNA strand breaks was detectable up to concentrations of 10  $\mu$ M (figure 5). Our results clearly indicate that the addisoniaflavanones I and II (**1 and 2**) possess highly cytotoxic potential in hepatoma cells after relatively short incubation time (starting at 3 h) inducing predominantly an apoptotic cell death. These compounds may therefore be interesting for pharmacological purposes (e.g. potential use as cytostatic drugs).

## EXPERIMENTAL SECTION

**General Experimental Procedures.** NMR spectra were measured in acetone- $d_6$  Uvasol (Merck) on a DRX 500 Bruker instrument operating at 500 MHz for  $^1\text{H}$ - and at 125 MHz for  $^{13}\text{C}$ -NMR. LC-MS were obtained using an Agilent LC HP 1100 combined with Thermoquest Finnigan LCQ Deca mass spectrometer, an APCI ion source and Thermoquest ESI. Knauer Eurospher 100 C-18 (5 $\mu\text{m}$ ; 227mm x 2 mm) column was eluted with HPLC-grade MeOH. For column chromatography silica gel 60 Merck was used. HPLC was performed on a Dionex P 580 equipped with an autosampler ASI-100 and STH 585, detection UVD 340 S. UV spectra were determined online via Dionex HPLC P 580. Optical rotations were recorded on a Perkin Elmer 241 MC polarimeter. TLC was performed on TLC plates precoated with silica gel 60 F<sub>254</sub> (Merck) and detected by anisaldehyde reagent. All tissue culture reagents were purchased from PAA Coelbe, cell culture dishes and multiwell plates were obtained from Falcon Heidelberg. All other chemicals were of analytical grade and were purchased from Sigma Deisenhofen or Merck Darmstadt.

**Plant Material.** The stem bark of *Erythrina addisoniae* Hutchinson & Dalziel (Leguminosae subfamily Papilionoideae) was collected in Ghana and identified by Augustina Addae at the Herbarium of the Forestry Research Institute of Ghana, Fumesua. There is a voucher specimen (4/27/02/02) on deposit.

**Extraction and Isolation.** The stem bark of *E. addisoniae* (500 g dried powder) was consecutively extracted with dichloromethane (DCM) and MeOH in a Soxhlet apparatus. After evaporation of the solvent a sticky DCM residue (75 g) was dissolved in MeOH, filtered and dried again. 9.0 g of the MeOH soluble part of the DCM extract were separated by column chromatography (CC) on Sephadex LH-20 with methanol as mobile phase. The collected eluates were combined to 4 fractions by monitoring with TLC (toluene/ EtOAc 6:4, detection anisaldehyde reagent). Fraction 2 was rich in flavonoids and then purified by CC on Sephadex LH-20 with methanol resulting in 12 subfractions. Sub fraction 2.7 was purified by CC on Si gel 60 using a n-hexane/ EtOAc gradient starting with 80 % n-hexane as eluent. Fraction 8, 11 and 13 of the resulting 15 sub fractions were purified by repeated CC on Si gel 60 and the compounds **4** (6.9 mg), **2** (0.9 mg) and **1** (1.1 mg) were obtained. Compounds **6** (2.3 mg) and **5** (2.3 mg) were elucidated by CC (Si gel) from sub fraction 2.15.3 and 2.15.11, respectively. After CC of fraction 2.16 on Si gel using toluene/ EtOAc 6:4 and repeated CC of 2.16.19 on Si gel compound **3** (2.7 mg) was obtained. All compounds were identified by their mass spectra and 1D- and 2D-NMR experiments.

**Cell culture and determination of cytotoxicity.** Metabolically active H4IIE rat hepatoma cells were grown in Dulbecco's modified Eagle's medium (4.5 g/L glucose, 2 mmol/L L-glutamine, 100 units/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin and 10% fetal bovine serum), in a humidified atmosphere (37°C, 5% CO<sub>2</sub>). The effect of isolated compounds on cell viability was determined using the MTT assay.<sup>21</sup> The cells were plated in 96-multiwell plates with 10,000 cells/well. The cells were allowed to attach for 24 h and then treated with different concentrations of the compounds for 24 h. After this treatment the medium was changed and the cells were incubated for 3 h under cell culture conditions with 0.7 mg/mL MTT. After this incubation the cells lysed with 50% ethanol / 49% water / 1% acetic acid. The concentration of reduced MTT as a marker for cell viability was measured at 560 nm.

### **Determination of apoptotic/necrotic cell death (life/dead-assay)**

Caspase-3/7-activity was measured using the Apo-ONE homogeneous caspase 3/7 assay (Promega) according to the manufacturer's protocol. Briefly, 50,000 cells/well were plated on 96-multiwell plates, allowed to attach for 24 h and treated with isolated compounds for 24 h. Then, 50  $\mu\text{l}$  of Apo-ONE Caspase-3/7-reagent was added and increase in fluorescence was measured at 37°C (excitation: 485 nm, emission: 535 nm).

The increase of fluorescence was analysed for 3 h. We further investigated nuclear fragmentation (Hoechst 33342 staining) as a further feature of apoptotic cell death, as well as ethidium bromide/acridine orange staining as feature of necrotic cell death according to Michels et al.<sup>22</sup>. The apoptotic/necrotic index (defined as percentage of cells with fragmented nuclei/ethidium bromide staining in a randomly selected visual field) was determined by analysing three cell culture dishes for each measurement (four visual fields counted per dish) in triplicate.

#### Determination of DNA strand breaks

For determination of DNA strand breaks the single cell gel electrophoresis ("comet") assay was performed according to Singh et al.<sup>23</sup> using alkaline conditions. H4IIE cells were seeded in a six-well ( $0.5 \times 10^6$ /well), incubated 24 h later with various concentrations of pterocarpan (3 h), then DNA single strand break formation was assessed (image length = head to tail distance).

#### Statistics

Data are given as mean  $\pm$  standard deviation (SD) of at least three independent experiments. The significance of changes in the test responses was assessed using an one-way ANOVA followed by LSD test (Analyse-it, Leeds, UK), differences were considered significant at  $p < 0.05$ .

**Addisoniaflavanone I (1)** was obtained as yellowish oily residue,  $[\alpha]_D^{20}$  -16.1 (*c*, 1.98 MeOH); UV  $\lambda_{\max}$  230, 286 nm (MeOH); HPLC  $R_t$  29.90 min (linear gradient from 5 – 100% MeOH against nanopure H<sub>2</sub>O with 0.1% phosphoric acid at 35 min); <sup>1</sup>H NMR (500 MHz in Me<sub>2</sub>OD-*d*<sub>6</sub>, *J* in Hz)  $\delta$  12.17 (1H, s, 5-OH), 7.12 (1H, d, *J* = 2.5 Hz, H-6'), 7.07 (1H, d, *J* = 2.5 Hz, H-2'), 5.93 (2H, s, H-6 and 8), 5.37 (1H, dd, *J* = 12.7, 2.8 Hz, H-2), 5.27 (1H, d, *J* = 7.4 Hz, H-2'''), 3.80 (1H, dd, *J* = 7.9, 5.7 Hz, H-2''), 3.26 (2H, m, H-1'''), 3.04 (1H, dd, *J* = 6.0, 9.8 Hz, H-1''), 3.03 (1H, dd, *J* = 12.9, 16.7 Hz, H-3<sub>ax</sub>), 2.75 (1H, dd, *J* = 3.2, 7.9 Hz, H-1'), 2.66 (1H, dd, *J* = 13.6, 3.2 Hz, H-3<sub>eq</sub>), 1.71 (3H, s, H-4'''), 1.68 (3H, s, H-5'''), 1.37 (3H, s, H-4''), 1.30 (3H, s, H-5''); <sup>13</sup>C NMR (125 MHz in Me<sub>2</sub>OD-*d*<sub>6</sub>)  $\delta$  197.3 (C-4), 167.4 (C-7), 165.3 (C-5), 164.4 (C-9), 157.0 (C-4'), 132.7 (C-3'''), 130.8 (C-1'), 130.3 (C-5'), 126.9 (C-2'), 126.7 (C-6'), 123.7 (C-2'''), 122.0 (C-3'), 103.2 (C-10), 96.8 (C-6), 95.8 (C-8), 80.2 (C-2), 78.1 (C-3''), 69.8 (C-2''), 43.4 (C-3), 32.3 (C-1''), 29.8 (C-1'''), 26.2 (C-5''), 25.9 (C-5'''), 20.7 (C-4''), 17.9 (C-4'''); MS: 425 [M + H]<sup>+</sup>.

**Addisoniaflavanone II (2)** was obtained as yellowish oily residue,  $[\alpha]_D^{20}$  -14.1 (*c*, 0.45 MeOH); UV  $\lambda_{\max}$  238, 288 nm; HPLC  $R_t$  28.37 min (linear gradient from 5 – 100% MeOH against nanopure H<sub>2</sub>O with 0.1% phosphoric acid at 35 min); <sup>1</sup>H NMR (500 MHz in Me<sub>2</sub>OD-*d*<sub>6</sub>, *J* in Hz)  $\delta$  12.18 (1H, s, 5-OH), 7.19 (1H, s, H-6'), 7.08 (1H, s, H-2'), 5.93 (2H, s, H-6 and 8), 5.39 (1H, dd, *J* = 12.8, 2.8 Hz, H-2), 5.31 (1H, d, *J* = 7.6 Hz, H-2'''), 4.65 (1H, t, *J* = 7.9 Hz, H-2''), 3.27 (2H, d, *J* = 7.6 Hz, H-1'''), 3.23 (2H, m, H-1''), 3.17 (1H, dd, *J* = 12.9, 17.0 Hz, H-3<sub>ax</sub>), 2.66 (1H, dd, *J* = 17.2, 2.8 Hz, H-3<sub>eq</sub>), 1.71 (3H, s, H-4'''), 1.69 (3H, s, H-5'''), 1.24 (3H, s, H-4''), 1.22 (3H, s, H-5''); <sup>13</sup>C NMR (125 MHz in Me<sub>2</sub>OD-*d*<sub>6</sub>)  $\delta$  197.3 (C-4), 167.3 (C-7), 165.3 (C-5), 164.4 (C-9), 159.3 (C-4'), 132.7 (C-3'''), 130.2 (C-1'), 128.3 (C-3'), 127.4 (C-2'), 123.0 (C-2'''), 123.3 (C-5'), 121.8 (C-6'), 103.2 (C-10), 96.8 (C-6), 95.8 (C-8), 90.1 (C-2''), 80.4 (C-2), 71.6 (C-3''), 43.6 (C-3), 31.2 (C-1''), 28.9 (C-1'''), 26.1 (C-5''), 25.9 (C-5'''), 25.2 (C-4''), 17.9 (C-4'''); MS: 443 [M + H]<sup>+</sup>.

**Addisoniaflavanone III (4)** was obtained as yellowish oily residue,  $[\alpha]_D^{20}$  -302 (*c*, 0.095 MeOH); UV  $\lambda_{\max}$  240, 260, 290 nm; HPLC  $R_t$  28.68 min (linear gradient from 5 – 100% MeOH against nanopure H<sub>2</sub>O with 0.1% phosphoric acid at 35 min); <sup>1</sup>H NMR (500 MHz in Me<sub>2</sub>OD-*d*<sub>6</sub>, *J* in Hz)  $\delta$  12.14 (1H, s, 5-OH), 10.36 (1H, s, CHO), 9.71 (1H, s, 7-OH), 7.82 (1H, d, *J* = 2.2 Hz, H-2'), 7.73 (1H, d, *J* = 2.2 Hz, H-6'), 5.99 (1H, d, *J* = 2.2 Hz, H-6),

5.96 (1H, d,  $J = 1.9$  Hz, H-8), 5.62 (1H, dd,  $J = 12.6, 3.2$  Hz, H-2), 5.32 (1H, t,  $J = 7.3$  Hz, H-2"), 3.96 (1H, s, OMe), 3.47 (1H, d,  $J = 7.3$  Hz, H-1"), 3.18 (1H, dd,  $J = 17.0, 12.6$  Hz, H-3<sub>ax</sub>), 2.84 (1H, dd,  $J = 17.3, 3.2$  Hz, H-3<sub>eq</sub>), 1.75 (3H, s, H-4"), 1.71 (3H, s, H-5"); <sup>13</sup>C NMR (125 MHz in Me<sub>2</sub>OD-*d*<sub>6</sub>)  $\delta$  197.0 (C-4), 190.4 (CHO), 167.8 (C-7), 165.7 (C-5), 164.4 (C-9), 162.7 (C-4'), 138.1 (C-5'), 136.7 (C-1'), 135.6 (C-6'), 134.4 (C-3"), 130.8 (C-3'), 125.6 (C-2'), 123.3 (C-2"), 103.7 (C-10), 97.5 (C-8), 96.4 (C-6), 79.6 (C-2), 65.0 (OMe), 43.8 (C-3), 28.9 (C-1"), 26.2 (C-5"), 18.4 (C-4"); MS: 383 [M + H]<sup>+</sup>.

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## List of Figure Legends

### Figure 1. Structures of isolated compounds

**1**: addisoniaflavanone I, **2**: addisoniaflavanone II, **3**: 2(*S*)-5,7-dihydroxy-5'-prenyl-[2'',2''-(3''-hydroxy)-dimethylpyrano]-(5'',6'':3',4')flavanone, **4**: addisoniaflavanone III, **5**: abyssinin II, **6**: abyssinoflavanon V

### Figure 2: Cytotoxicity of the isolated compounds in H4IIE cells

H4IIE cells were incubated with prenylflavanones for 24 h, then MTT reduction as a marker of cell viability was measured (absorbance at 560 nm). Results are expressed as viable cells in percent of control. Data are means  $\pm$  SD (n=3), \*:p<0.05 vs. corresponding control (DMSO).

### Figure 3. Toxic effects of addisoniaflavanone I and addisoniaflavanone II.

Time course of toxicity: H4IIE cells were incubated with addisoniaflavanone I (A) and addisoniaflavanone II (B) for different time points, then MTT reduction as a marker of cell viability was measured (absorbance at 560 nm). Results are expressed as viable cells in percent of control. Data are means  $\pm$  SD (n=3), \*:p<0.05 vs. corresponding control (DMSO).

### Figure 4: Induction of apoptosis/necrosis in H4IIE cells.

(A) H4IIE cells were incubated with addisoniaflavanone I and addisoniaflavanone II for 24 h, then the apoptotic index (amount of cells showing apoptosis-specific nuclear fragmentation) was detected (left graph) as well as the amount of necrotic cells (cells showing nuclear staining with ethidium bromide as marker of membrane disruption) (right graph). Data are means  $\pm$  SD (n=3), \*:p < 0.05 vs. corresponding control (DMSO). (B) representative pictures for nuclear fragmentation are shown: left picture: DMSO-treated cells, right picture: cells treated with 5  $\mu$ M addisoniaflavanone I for 24 h. (C) Apoptosis was further confirmed by detection of caspase 3/7 activity which was measured using homogeneous Apo-ONE assay (Promega). Results are expressed as increase in relative fluorescence units (rfu) for 3 h  $\pm$  SD (n=3), \*:p<0.05 vs. control (DMSO).

### Figure 5: Induction of DNA strand breaks in H4IIE cells

H4IIE cells were incubated with addisoniaflavanone I and addisoniaflavanone II for 2 h, then the formation of DNA strand breaks was detected using the comet assay. Results are expressed as average image length ( $\mu$ m). Data are means  $\pm$  SD (n=3), \*:p<0.05 vs. corresponding control (DMSO). Representative pictures are of the comet assay: (a) control, (b) positive control: 500  $\mu$ M H<sub>2</sub>O<sub>2</sub>.

FIGURE 1

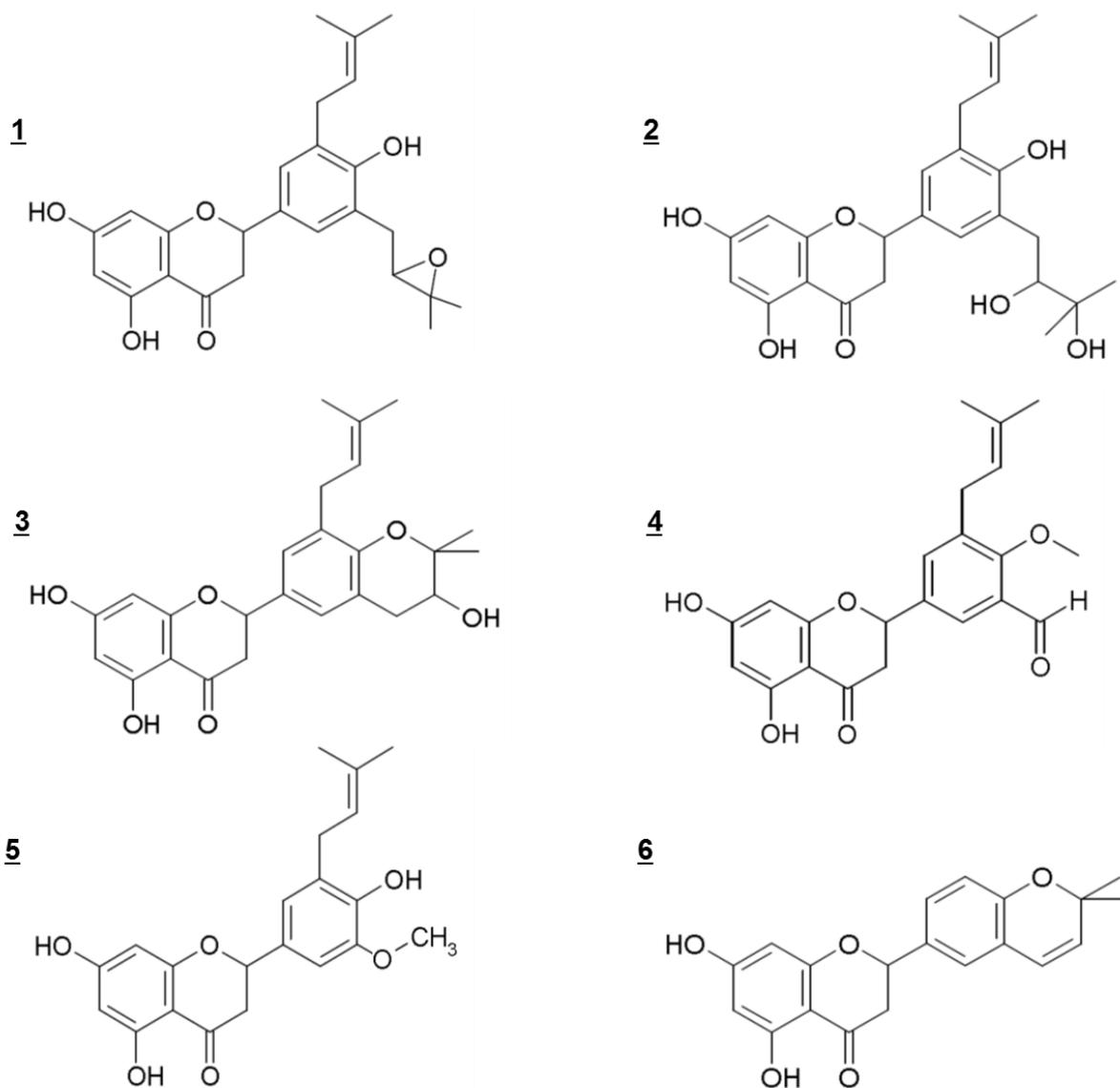


FIGURE 2

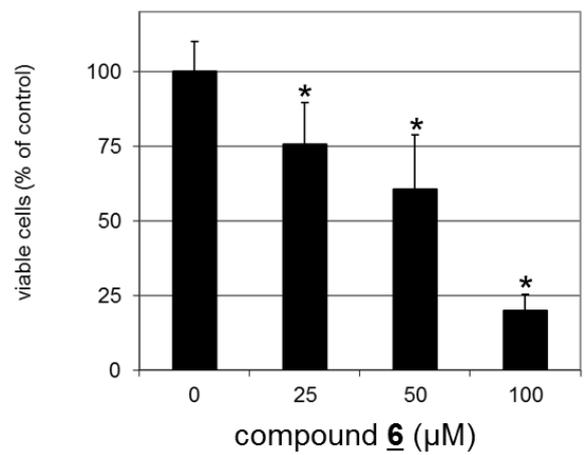
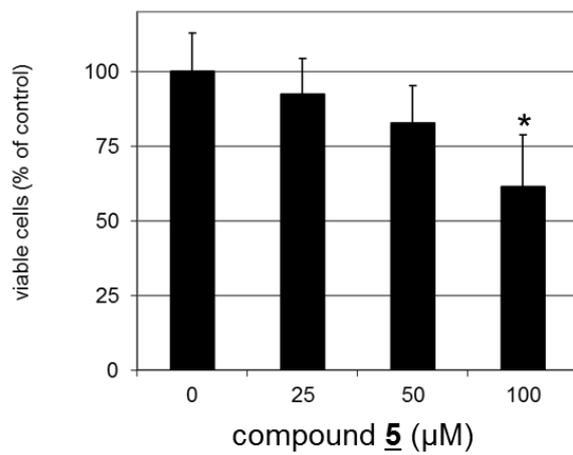
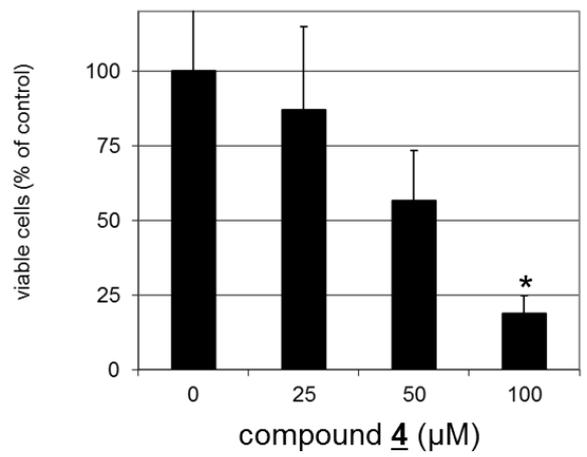
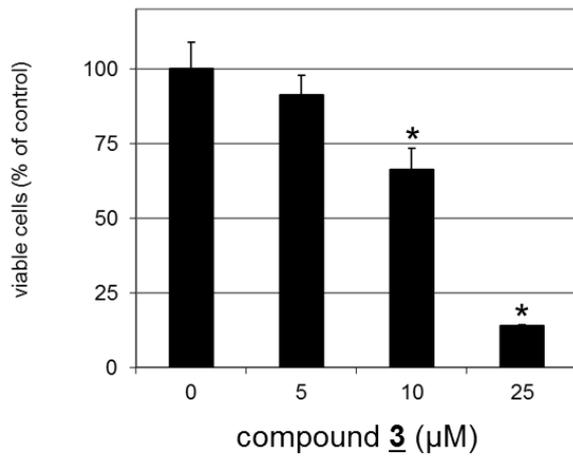
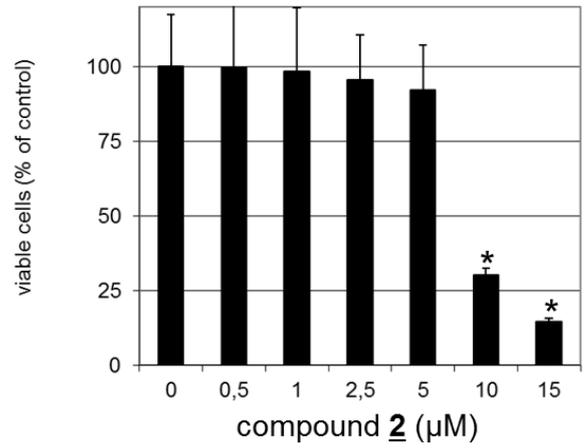
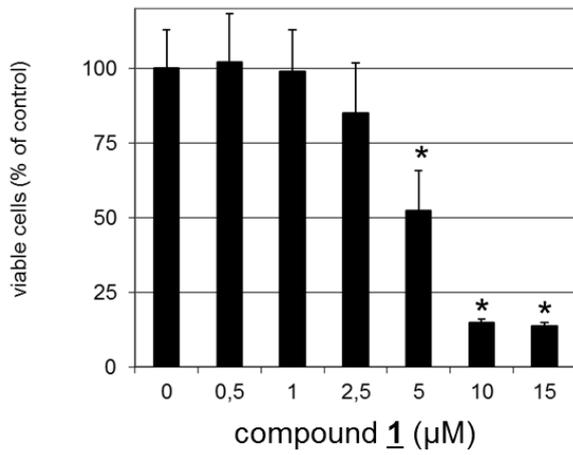


FIGURE 3

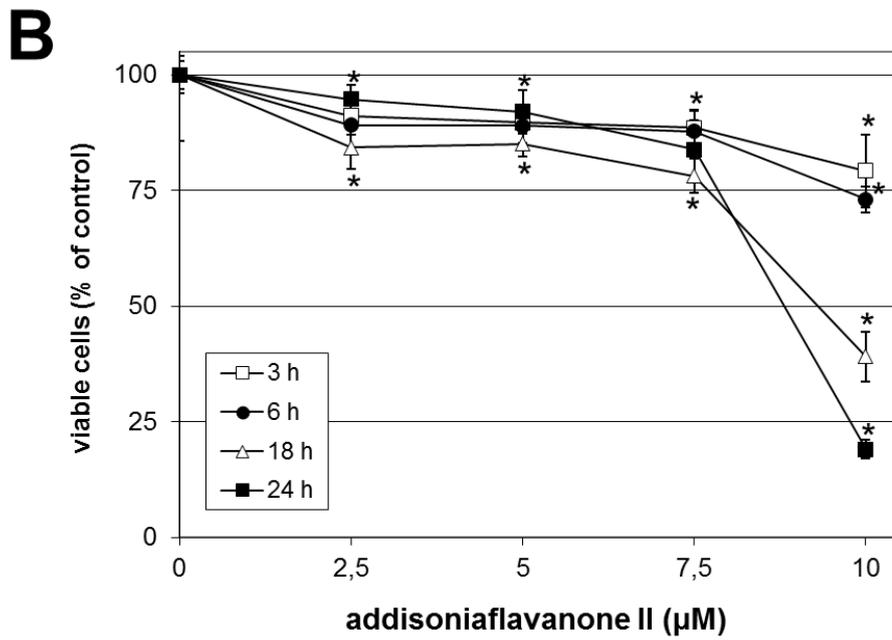
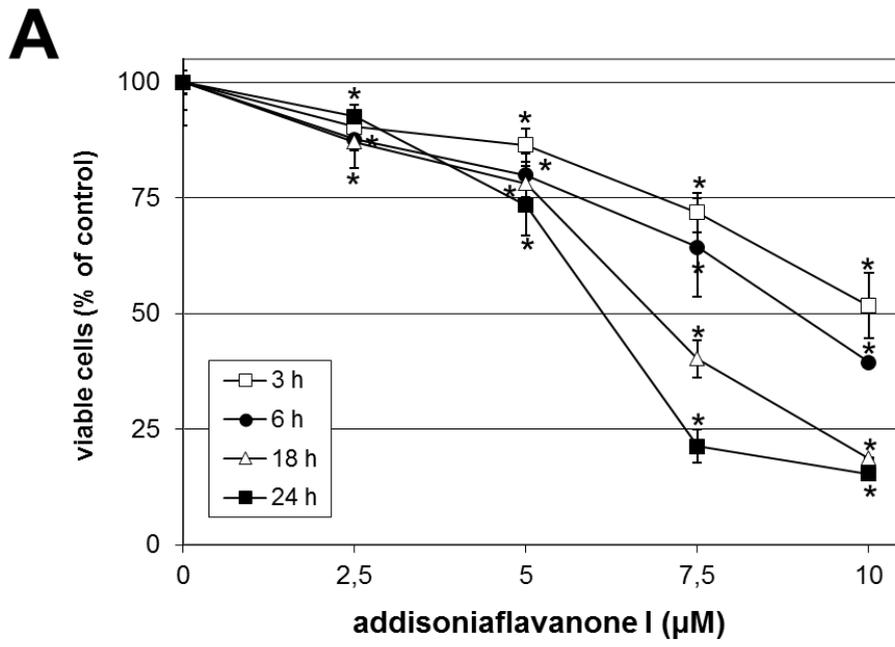


FIGURE 4

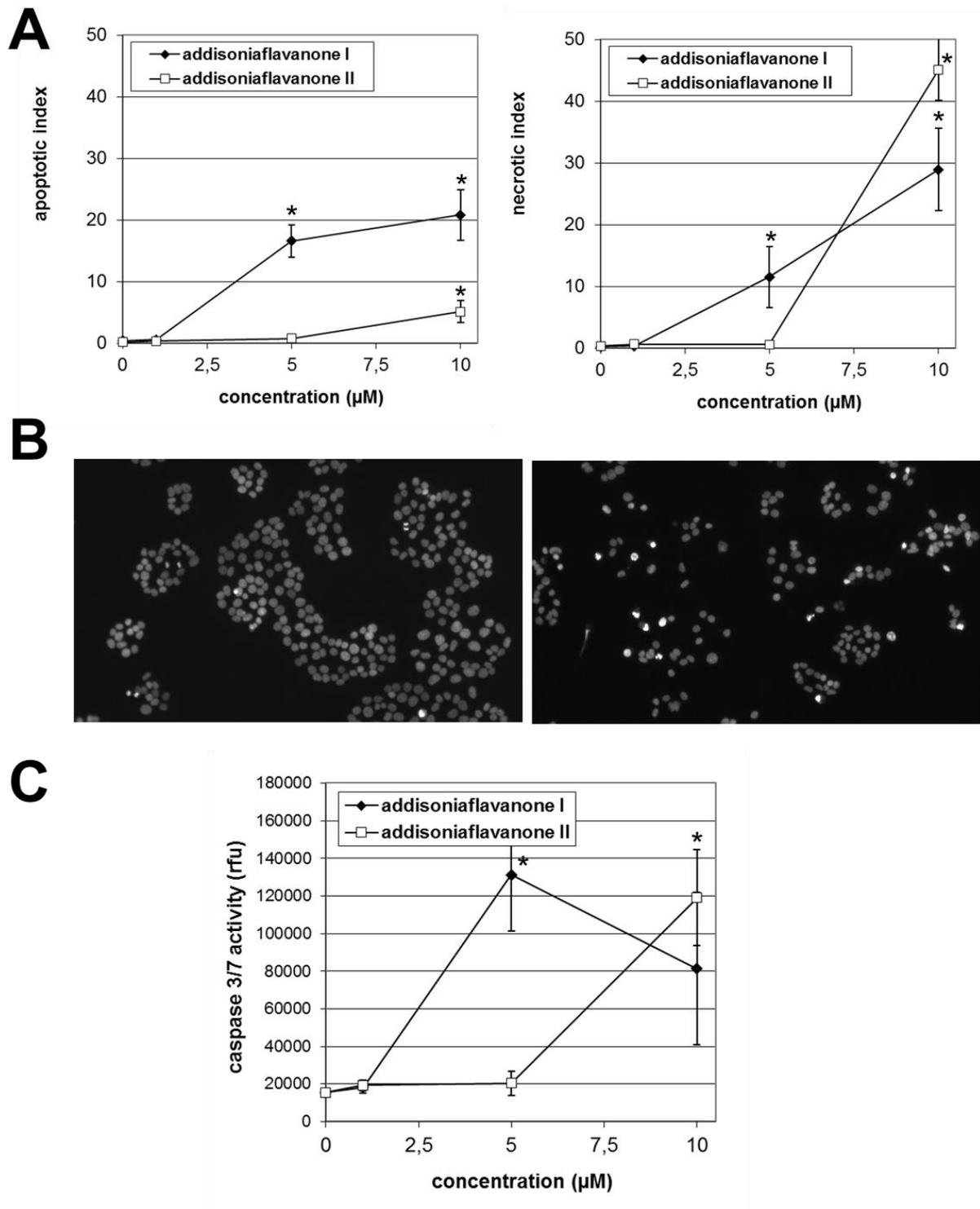
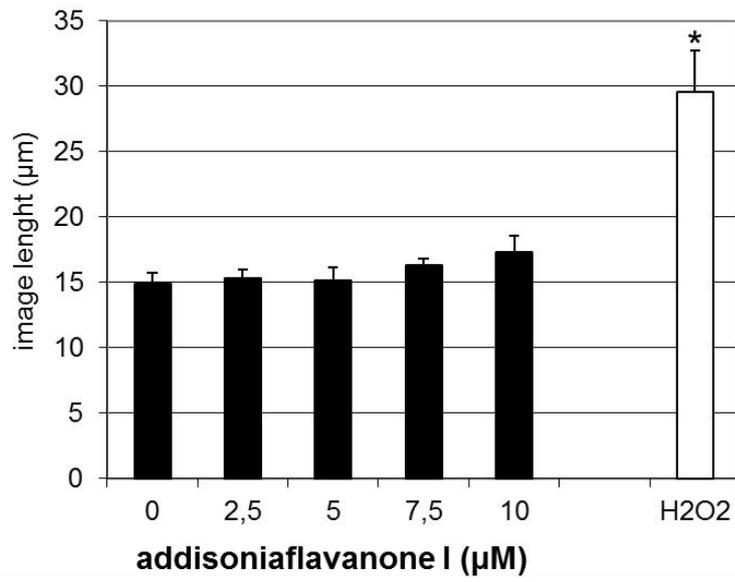
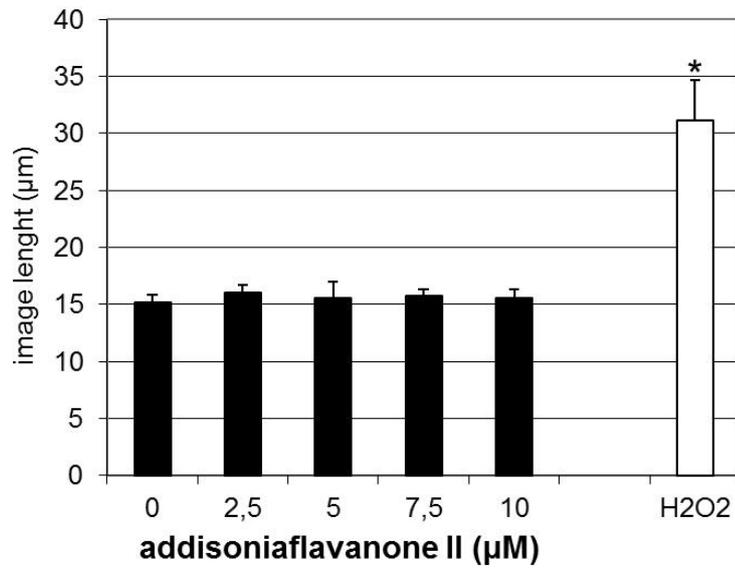


FIGURE 5

**A**



**B**



**C**

