Regulatory Toxicology and Pharmacology 73 (2015) 923–929

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Toxicity and antioxidant capacity of *Frangula alnus* Mill. bark and its active component emodin

Sandra Radić Brkanac [a], Marko Gerić [b], Goran Gajski [b], Valerija Vujčić [a], Vera Garaj-Vrhovac [b], Dario Kremer [c], Ana-Marija Domijan [c, *]

[a] Department of Biology, Faculty of Science, University of Zagreb, Rooseveltov Trg 6, 10000 Zagreb, Croatia
[b] Mutagenesis Unit, Institute for Medical Research and Occupational Health, Ksaverska c. 2, 10000 Zagreb, Croatia
[c] Department of Pharmaceutical Botany, Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, 10000 Zagreb, Croatia

ARTICLE INFO

Article history:
Received 30 July 2015
Received in revised form
16 September 2015
Accepted 18 September 2015
Available online 21 September 2015

Keywords:
Human peripheral blood lymphocytes
Cytotoxicity
Genotoxicity
Oxidative stress
Polyphenol content

ABSTRACT

In the present study toxicity of *Frangula alnus* Mill. bark, widely used as laxative, was investigated. Human peripheral blood lymphocytes (HPBLs) were treated with *F. alnus* bark extract or emodin (emodin is bark component with laxative property), and cytotoxicity, genotoxicity and parameters of oxidative stress were assessed. Also, polyphenol content of bark extract and antioxidant activity of the extract and emodin measured by DPPH, ABTS and FRAP methods were examined. The bark extract (500 μg/ml) produced cell death and DNA damage, while level of ROS changed at 250 μg/ml. Emodin induced cell death and DNA damage at 150 μg/ml and 200 μg/ml, respectively, and the increase of ROS was observed at 25 μg/ml. These results suggest that both, bark extract and emodin, are cyto/genotoxic to HPBLs and that oxidative stress is involved in the mechanism of their toxicity. The results on antioxidant activity showed that, unlike emodin, bark extract possess moderate antioxidant capacity (44.6%, 46.8% and 2.25 mmol Fe^{2+}/g measured by DPPH, ABTS and FRAP assay, respectively) that can be related to relatively high phenolic content (116.07 mg/g). However, due to toxicological properties use of *F. alnus* bark as well as emodin-containing preparations should be taken with caution.

1. Introduction

Bark of *Frangula alnus* Mill. (syn. *Rhamnus frangula* L.), also referred as *Frangulae cortex* (Council of Europe, 2008) is widely used as laxative and can be found as component of herbal laxative preparations. Laxative property of *F. alnus* bark has been attributed to the presence of anthraquinone glycoside derivatives, glucofrangulins and frangulins; glucofrangulins are anthraquinone diglycosides while frangulins are anthraquinone monoglycosides (EMEA, 2007; Maleš et al., 2010). The predominant anthraquinone of *F. alnus* bark is emodin (Kremer et al., 2012).

The group of anthraquinone-containing laxatives is known to cause melanosis coli and their possible role is also suggested in the development of colorectal adenomas and cancer (Siegers et al., 1993; Van Gorkom et al., 1999; Willems et al., 2003). However, studies on toxicity of bark of *F. alnus* are lacking. In WHO monograph it is stated that *F. alnus* bark and other laxatives containing anthraquinone glycosides should not be used continuously for longer than 1–2 weeks, because of the possible electrolyte imbalance (hypokalaemia) (WHO, 2004). More importantly, hypokalaemia could be aggravated by concomitant use of other medicinal products that induce the imbalance, such as diuretics (EMEA, 2007). Additional problem presents anthraquinone-containing laxative abuse (Van Gorkom et al., 1999).

Studies on cytotoxic and genotoxic potential of several anthraquinones such as emodin, aloe-emodin, and danthron, all of which are naturally occurring, pharmacologically active laxatives, suggest their possible genotoxic potential (Mueller et al., 1999; Mueller and Stopper, 1999; Nesslany et al., 2009). However, two-year feeding study on the toxic effects of emodin on experimental rodents showed that emodin is not carcinogenic (there was no evidence of carcinogenic activity in male rats and female mice, and equivocal evidence in female rats and male mice), although pathological changes in renal tubule were observed (NTP, 2001). Also, it is reported that metabolic activation is needed, and that metabolite 2-hydroxyemodin and not emodin acts as genotoxin (Masuda and Ueno, 1984; NTP, 2001).

* Corresponding author.
E-mail address: adomijan@pharma.hr (A.-M. Domijan).

http://dx.doi.org/10.1016/j.yrtph.2015.09.025
0273-2300/

Since toxicological data on *F. alnus* bark are lacking, the aim of this study was to explore toxicological profile of *F. alnus* bark collected in Croatia whose anthraquinone content is established (Kremer et al., 2012). In order to evaluate the impact of its active component emodin on toxicity of *F. alnus* bark, toxicological profile of emodin is also assessed. For toxicological studies human peripheral blood lymphocytes (HPBLs) were treated with *F. alnus* bark extract or with emodin and cytotoxicity, the induction of DNA strand-breaks and parameters of oxidative stress as possible mechanism of their toxicity were evaluated. Additionally, antioxidant capacities of *F. alnus* bark extract and emodin as well as polyphenol content of the bark extract were determined.

2. Materials and methods

2.1. Chemicals

Acridine-orange (AO), caffeic acid, catechin, dihydroethidium (DHE), dimethyl sulfoxide (DMSO), ethidium-bromide (EtBr), emodin, Folin–Ciocalteau reagent, gallic acid, histopaque, monochlorobiamine (MCB), low melting point (LMP) and normal melting point (NMP) agaroses, quercetin, RPMI 1640 medium, rat liver S9 mix, Triton X-100, vanillin, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution were purchased from Sigma (St Louis, MO, USA). All other chemicals used were laboratory-grade and were purchased from Kemika (Zagreb, Croatia).

2.2. Herbal material and extract preparation

Sample collection of *F. alnus* bark and analysis of its anthraquinone profile are described in Kremer et al. (2012). Bark samples were collected from ten wild growing plants randomly selected in locality Hrastelnica, Croatia (46 38′N; 16 43′E; 164 m a.s.l.) in September of 2009. About 200 g of bark was removed and mixed to obtain randomly selected sample. Bark sample was air-dried for two weeks in a well-ventilated room at 60% air humidity and room temperature (22 °C), single-layered and protected from direct sunlight. Its anthraquinone profile was: emodin (69.70%), physicon (25.90%), and chrysophanol (4.45%) (Kremer et al., 2012).

In this study to mimic possible human exposure aqueous solution of methanol extract of *F. alnus* bark was prepared. Briefly, the extract was made by ultrasonication of grounded herbal material (5 g) with methanol (14 ml) at 35 °C for 45 min. Homogenized and macerated plant material was first filtered through Munktel filter discs grade 388 and then through GHP Acrodisc with 0.45 μm membrane. Obtained extract was evaporated by rotary evaporator, dissolved in distilled water and stored at −18 °C in the dark until use. For ultrasonication Donau Lab Sonic DLS 660-T/H (Elma, Germany) ultrasonic bath was used.

2.3. Blood sampling and treatment

Whole blood samples were taken from a healthy male non-smoking donor (age 19). The donor had not been exposed to ionizing radiation for diagnostic or therapeutic purposes, or vaccinated and treated with drugs that might have interfered with the results of testing for a year before blood sampling. The subject gave informed consent to participate in this study. The study was approved by the Ethics Committee of Faculty of Pharmacy and Biochemistry, University of Zagreb, and observed the ethical principles of the Declaration of Helsinki. Whole venous blood was collected under sterile conditions in heparinised vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing lithium heparin as anticoagulant.

All experiments were conducted on the same blood sample in order to avoid inter-individual variations in results. The blood sample was treated either with *F. alnus* bark extract in the final concentrations of 10, 50, 100, 500, 750, 1000 and 1500 μg/ml prepared in redistilled water for 24 h or with emodin in the final concentrations of 5, 10, 25, 50, 100, 150 and 200 μg/ml (emodin was dissolved in DMSO; final DMSO concentration in blood samples was 1%) for 24 h. In each experiment a non-treated control was included. Testing was done with and without S9 metabolic activation (10%, v/v) which is routinely used in cytogenetic assays. Blood samples were incubated *in vitro* at 37 °C in a humified atmosphere with 5% CO_2 (Heraeus Heracell 240 incubator, Langenselbold, Germany).

2.4. Cell viability (cytotoxicity) assay

Indices of cell viability were established by differential staining of HPBLs with AO and EtBr (Duke and Cohen, 1992). Following treatment, HPBLs were isolated by Histopaque density centrifugation method (Singh, 2000). The slides were prepared using 200 μl of HPBLs and 2 μl of stain (AO and EtBr, in final concentration 100 μg/ml, 1:1, v/v, in phosphate-buffered saline, PBS). A total of 100 cells per replicate were examined with an epifluorescent microscope (Olympus BX-51, Tokyo, Japan). Cells were classified as follows: live cells with functional membrane, with uniform green staining of the nucleus, and necrotic cells with uniform orange (red) staining of the nucleus.

2.5. Alkaline comet (SCGE) assay

The alkaline comet assay was carried out as described by Singh et al. (1988) with minor modifications (Gajski et al., 2008). Briefly, after the exposure, 5 μl of whole blood was mixed with 100 μl of 0.5% LMP agarose and added to fully frosted slides pre-coated with 0.6% NMP agarose. Once the NMP agarose was solid, the slides were covered with 0.5% LMP agarose, and cells lysed (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl sulfoxide; pH 10) over night at 4 °C. After the lysis, the slides were placed in alkaline solution (300 mmol/l NaOH, 1 mmol/l Na_2EDTA; pH 13) for 20 min at 4 °C to allow DNA unwinding and subsequently electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized in 0.4 mol/l Tris buffer (pH 7.5) for 5 min 3 times, stained with EtBr (10 μg/ml) and analysed at 250 × magnification using an epifluorescence microscope (Zeiss, Göttingen, Germany) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, UK). The level of DNA damage was expressed as percentage of DNA in the tail, and a total of 100 randomly captured comets were examined from each slide.

2.6. Reactive oxygen species (ROS) and glutathione (GSH) assays

ROS and GSH level in HPBLs was determined using fluorescent probe DHE and MCB, respectively (Domijan and Abramov, 2011.). After the treatment, HPBLs were isolated as described above (Singh, 2000). Afterwards the HPBLs were incubated either with 10 μl of 10 μmol/l DHE for 25 min (to assess ROS) or with 10 μl of 10 μmol/l MCB (to assess GSH) at room temperature, protected from light. Dyes were prepared in PBS buffer; DHE is a membrane permeable compound that is oxidized to red fluorescent ethidium by the action of radicals mostly superoxide radicals ($O_2^{\cdot-}$), thus an increase of red fluorescence are suggestive of $O_2^{\cdot-}$ generation, while MCB forms a fluorescent adduct with GSH. The slides were examined under epifluorescent microscope Olympus BX-51

connected to the camera (Olympus DP70, Tokyo, Japan) at excitation 450–490 nm and emission 520 nm or more, and excitation at 380 nm and emission at >400 nm, for ROS and GSH assay, respectively. Images of the fluorescence were analysed using software (Lucida 6.0, Wirral, UK). A total number of at least 100 cells were counted in 10 fields of each slide. Identical conditions were kept in all experimental groups.

2.7. Polyphenolics assays

The polyphenolic compounds were assessed in *F. alnus* bark extract prepared in final concentration of 1000 μg/ml. Standard phenolic compounds were prepared in the same concentration.

Total soluble phenols (TP) of *F. alnus* bark extract were determined with Folin–Ciocalteau reagent according to Zhishen et al. (1999) using gallic acid as a standard phenolic compound. 0.02 ml of extract solution was diluted with 0.1580 ml of distilled water and then 0.1 ml of Folin–Ciocalteau reagent was added. Afterwards, 0.3 ml Na_2CO_3 (1.88 mol) was added, and the mixture was incubated for 30 min at 45 °C. The absorbance of the mixture was measured at 765 nm. The TP content was calculated from the calibration curve and expressed as gallic acid equivalents.

The content of total flavonoids (TF) of *F. alnus* bark extract was determined with $AlCl_3$ according to the method described by Zhishen et al. (1999) using quercetin as a standard. To 0.2 ml of the diluted extract solution 0.06 ml $NaNO_2$ (5%) was added. After 5 min incubation, 0.06 ml $AlCl_3$ (10%) was added and mixture was incubated at room temperature for additional 6 min. Afterwards, 0.4 ml NaOH (1 mol) and distilled water were added to the mixture. The absorbance of reaction mixture was read at 415 nm. The TF content was calculated from the calibration curve and expressed as quercetin equivalents.

Total hydroxycinnamic acids (THA) and total flavonols (TFL) of *F. alnus* bark extracts were measured according to the method of Howard et al. (2003) using caffeic acid and quercetin as standards. 0.25 ml of the extract was mixed with 0.25 ml HCl (1 g/l; prepared in 950 g/l ethanol) and 4.55 ml HCl (2 g/l). The absorbance of the solution was read at 320 and 360 nm, respectively. THA and TFL contents were calculated from the corresponding calibration curves and expressed as caffeic acid and quercetin equivalents, respectively.

Proanthocyanidins (TPA) of *F. alnus* bark extracts were measured according to the vanillin-HCl assay described by Sun et al. (1998) using catechin as a standard. Vanillin solution (0–300 μg/ml in methanol) was mixed with 0.25 ml of the extract and 0.75 ml of HCl (conc.). Following 15 min incubation, the absorbance of reaction mixture was read at 500 nm. TPA content was calculated from the calibration curve and expressed as catechin equivalents.

All absorbance measurements were performed on spectrophotometer (Analytik Jena Specord 40, Analytik Jena, USA).

2.8. Antioxidant activity assays

The antioxidant activity was assessed in *F. alnus* bark extract or emodin (emodin was dissolved in DMSO) both in final concentration of 1000 μg/ml. Standard compounds were prepared in the same concentration.

Total antioxidant capacity (free radical scavenging activity) was measured using a stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) following the method of Germano et al. (2002) with slight modification. Briefly, 0.05 ml of tested compound (the bark extract or emodin) was added to 0.95 ml of freshly prepared 0.1 mmol ethanolic DPPH solution and incubated in the dark for 30 min at 25 °C. The decrease in absorbance was measured at 517 nm. The radical scavenging activity was calculated from the equation:

$$\%\ inhibition = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 was the absorbance of the control (blank, without tested compound) and A_t was the absorbance in the presence of the tested compound.

The ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) assay was carried out according to Re et al. (1999). *F. alnus* bark extract or emodin (0.02 ml) was added to ABTS solution (2.0 ml), incubated for 6 min at 25 °C and absorbance was read at 734 nm. The radical scavenging capacity was calculated as percent of DPPH inhibition, according to equation described above.

The FRAP (Ferric Reducing Antioxidant Potential) assay was used to estimate the antioxidant activity of *F. alnus* bark extract or emodin, according to the original method of Benzie and Strain (1999). The bark extract or emodin (0.05 ml) was mixed with the freshly prepared FRAP reagent (0.95 ml) and absorbance was read at 593 nm after 4 min reaction time. A calibration curve was constructed for ferrous sulphate $FeSO_4 \times 7H_2O$ and the results were expressed as mmol Fe^{2+}.

The absorbance measurements of the antioxidant activity assays were performed on spectrophotometer (Analytik Jena Specord 40, Analytik Jena, USA).

2.9. Statistical analysis

Results of the comet assay, ROS and GSH levels are presented as means ± standard error, while results for cell viability, polyphenolic compounds content and antioxidant activity as means ± standard deviation. All results were evaluated using Statistica 12.0 program package (StatSoft, Tulsa, OK). In order to normalize the distribution and equalize the variances of the comet assay data, a logarithmic transformation was applied. Multiple comparisons between groups were done by means of ANOVA on log-transformed data. Post-hoc analyses of differences were done by the Newman–Keuls test. As for the cell viability and parameters of oxidative stress (ROS and GSH), statistical significance was analysed using the Student's t-test. Results of polyphenolic compounds content and antioxidant activity were subjected to one-way analysis of variance (ANOVA) for comparison of means, and significant differences were calculated according to Duncan's multiple range test. Data were considered statistically significant at $P < 0.05$.

3. Results

3.1. Cytotoxicity and genotoxicity of F. alnus bark extract and emodin

The whole blood was treated with *F. alnus* bark extract (concentration range from 0 to 1500 μg/ml; for 24 h) and cell viability and DNA damage were determined. The concentration of 500 μg/ml of the bark extract significantly reduced cell viability (Fig. 1A; $P < 0.05$) and the same concentration (500 μg/ml) significantly increased DNA damage in HPBLs assessed with the alkaline comet assay (Fig. 1C; $P < 0.05$).

Emodin (in concentration range from 0 to 200 μg/ml; for 24 h) significantly decrease cell viability at 150 μg/ml (Fig. 1B; $P < 0.05$), and 200 μg/ml of emodin induced significant increase of DNA damage in HPBLs (Fig. 1D; $P < 0.05$).

In experiments in which S9 fraction was used together with *F. alnus* bark extract or emodin, no statistically significant increase

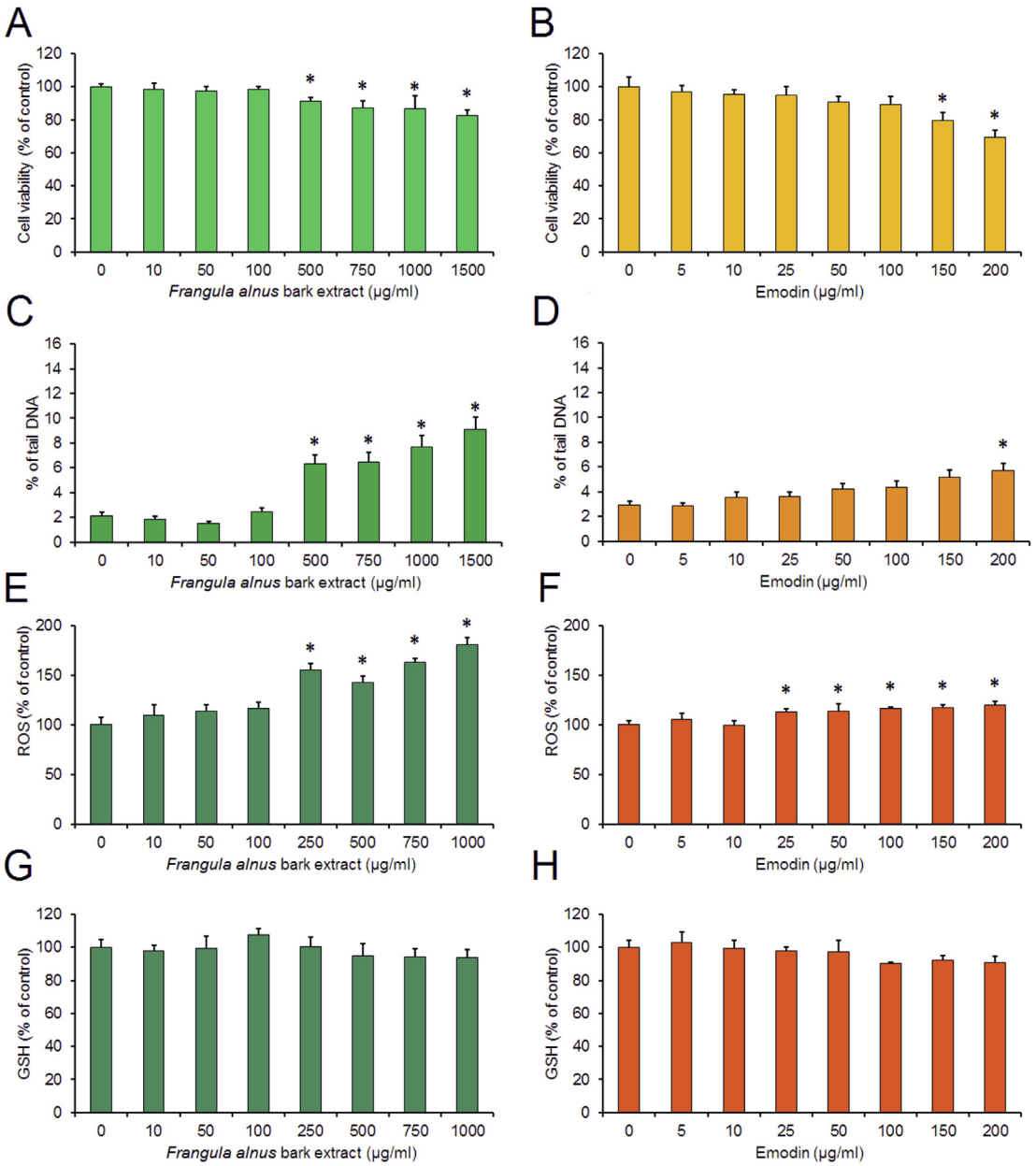

Fig. 1. Effects of *Frangula alnus* Mill. bark extract on: (A) cell viability, (C) DNA damage (E) reactive oxygen species (ROS) and (G) glutathione (GSH) level; effect of emodin on: (B) cell viability, (D) DNA damage (F) ROS and (H) GSH level in human peripheral blood lymphocytes (HPBLs). Cells were exposed to different concentrations of *F. alnus* bark extract or emodin for 24 h, and cell viability, DNA damage, and ROS and GSH levels were assessed. DNA damage in the cells was assessed with the alkaline comet assay and is expressed as % of DNA tail, while ROS and GSH levels were assessed with the fluorescent probes. Cell viability, ROS and GSH levels were expressed as % of control (control set at 100%). *$P < 0.05$.

in cell death or DNA damage compared to corresponding control or compared to treatment without S9 fraction was observed (data not shown).

3.2. Oxidative stress induced by F. alnus bark extract and emodin

As biomarkers of oxidative stress ROS and GSH levels were followed. The level of ROS in HPBLs treated with *F. alnus* bark extract (0–1000 μg/ml; 24 h) was assessed by use of fluorescent probe DHE (Fig. 2A–C). In these experiments lower concentrations of tested substance were used since oxidative stress was expected at concentrations that did not have effect on cytotoxicity or genotoxicity (since oxidative stress as mechanism of toxicity should precede these events). The concentration of 250 μg/ml of the bark extract induced an increase of ROS level (Fig. 1E; $P < 0.05$). The bark extract had no significant effect on GSH level in HPBLs assessed with fluorescent probe MCB (Fig. 2D–F), but slight decrease of GSH level could be observed at concentrations that induced significant increase of ROS level (Fig. 1G).

The level of ROS in HPBLs after the treatment with emodin (0–200 μg/ml; 24 h) was significantly increased already at 25 μg/ml (Fig. 1F; $P < 0.05$). Emodin had no significant effect on GSH level in HPBLs, however, a decrease in GSH level could be observed at concentration that induced an increase of ROS level indicating that oxidative stress is involved in the mechanism of emodin toxicity (Fig. 1H).

Since cytotoxicity and genotoxicity experiments with S9 fraction indicated that no toxic metabolites of *F. alnus* bark extract or of emodin are produced, in these experiments S9 fraction was not used.

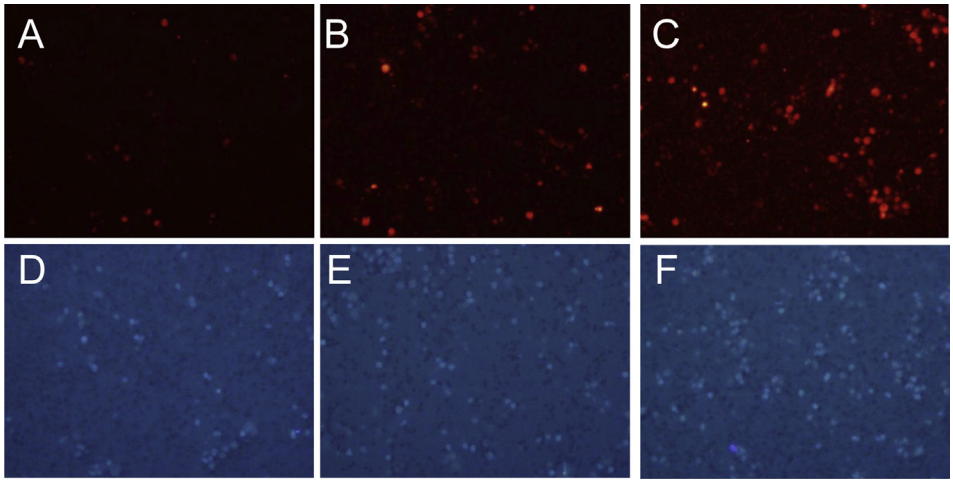

Fig. 2. The level of reactive oxygen species (ROS; A–C) and glutathione (GSH; D–F) in human peripheral blood lymphocytes (HPBLs) treated with *Frangula alnus* Mill. bark extract (0–1000 μg/ml; 24 h). Level of ROS was assessed using fluorescent probe dihydroethidium (DHE; red fluorescence) and GSH level using monochlorobiamine (MCB; blue fluorescence). A, D – control; B, E – 500 μg/ml; C, F – 1000 μg/ml. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Total polyphenol content of *F. alnus* bark extract

The contents of TP, TF, THA, TFL and TPA of the *F. alnus* bark extract are presented in Table 1. These data illustrates that more than half of the extracted phenolic substances in the bark extract were flavonoids. Additionally, TPAs were detected in *F. alnus* bark extract. The production of proanthocyanidins (condensed tannins) is rarely studied in plants though this group of flavonoids are considered to be the most powerful antioxidants responsible for the antioxidant activity of wine, teas and fruits (Matkowski, 2008).

3.4. Antioxidant activity of *F. alnus* bark extract and emodin

Results of antioxidant activity of *F. alnus* bark extract and emodin measured with DPPH, ABTS and FRAP assays are presented in Table 1. Antioxidant capacity of *F. alnus* bark (1000 μg/ml) assessed with DPPH and ABTS assays indicate that *F. alnus* bark has moderate antioxidant activity. The moderate antioxidative activity of bark extract is confirmed with FRAP assay. On the other hand, emodin (1000 μg/ml) showed low or no antioxidant capacity measured with DPPH and ABTS assays, as well as with FRAP assay.

4. Discussion

Nowadays interest for herbal/natural products is increasing, however in most cases clinical data and toxicological studies of such preparations are lacking (Silva et al., 2008). In EU several drugs based on *F. alnus* bark are available on the market (Van Gorkom et al., 1999; Vitalone et al., 2012). Since laxative effect of preparations based on *F. alnus* bark (as well as other anthraquinone-containing laxatives) is exerted by damaging epithelial cells that can lead to development of colorectal carcinoma, only short-term use of such preparations in case of occasional constipation is recommended (Van Gorkom et al., 1999; WHO, 2004; EMEA, 2007). Although Nusko et al. (2000) in a prospective case control study on patients on colonoscopy found no connection in anthraquinone-containing laxative use and colorectal carcinoma, more recent Italian surveillance on reports of suspected adverse reactions associated with herbal laxatives (anthraquinone-containing laxatives) showed that such preparations should be used only for limited period of time (Vitalone et al., 2012). In present study we systematically approach to the problem of possible toxic effect of *F. alnus* bark on human health from the aspect of cyto/genotoxicity and separately investigated toxicity of *F. alnus* bark and its active component emodin. The toxicological study (cytotoxic and genotoxic potential of tested substances) was performed on primary human cells, HPBLs, using widely accepted biomarkers for the evaluation of genome damage after exposure to different physical and/or chemical agents as well as to wide range of natural products (Collins et al., 2008; Dusinska and Collins, 2008; Garaj-Vrhovac and Gajski, 2009). To check possible metabolic activation of *F. alnus* bark or emodin, in toxicological studies S9 fraction was included.

Our results indicate that *F. alnus* bark extract is cyto- and genotoxic to HPBLs (in concentration above 500 μg/ml), and that by addition of S9 fraction no toxic metabolites of *F. alnus* bark are produced in the course of our study. On the other hand, bark extract at concentration of 250 μg/ml did not induce cytotoxicity and genotoxicity, but caused significant increase of ROS production indicating that the mechanism of toxicity and genotoxicity of *F. alnus* bark is at least partially through oxidative stress.

Similarly as *F. alnus* bark, emodin was cytotoxic (150 μg/ml) and genotoxic (200 μg/ml) to HPBLs, and its toxicity is observed at lower concentrations than that of *F. alnus* bark. Our previous study on anthraquinone content of *F. alnus* bark revealed that emodin is the

Table 1
Level of total phenols (TP), total flavonoids (TF), total hydroxycinnamic acids (THA), total flavonols (TFL) and total proanthocyanidins (TPA) of *Frangula alnus* Mill. bark extract, and DPPH radicals (%), ABTS radicals (%) and ferric reducing capacity (mmol Fe^{2+}/g) of the bark extract and emodin.

Extract/compound	TP (mg/g)	TF (mg/g)	THA (mg/g)	TFL (mg/g)	TPA (mg/g)	DPPH (%)	ABTS (%)	FRAP (mmol Fe^{2+}/g)
F. alnus bark[a]	116.1 ± 3.3	75.2 ± 1.5	61.8 ± 0.3	58.3 ± 0.5	56.7 ± 0.3	44.6 ± 0.8	46.8 ± 1.2	2.25 ± 0.07
Emodin[a]	–	–	–	–	–	2.4 ± 0.1	0.3 ± 0.0	0.49 ± 0.09
Standard[a]	–	–	–	–	–	94.9 ± 0.0	98.0 ± 0.1	72.5 ± 1.00

[a] 1000 μg/ml.

predominant anthraquinone (around 70%) in the bark extract and that it is present in concentration of 2.03 mg/g (Kremer et al., 2012). This indicates that emodin, although present in low amount in the bark of *F. alnus*, greatly contributes to its toxicity. This observation is in line with study of Helmholz et al. described in EMEA (2007) in which dose-dependent increase in the mutation rate for emodin and for *F. alnus* bark extract was found, and similarly in that study emodin had higher mutagenic potency than the extract itself. Emodin, as well as other herbal originating anthraquinones, was positive in the Ames test (Westendorf et al., 1990; NTP, 2001), and toxic, genotoxic and mutagenic in eukaryotic cells (Mueller et al., 1999; Mueller and Stopper, 1999). Emodin induced micronuclei in Syrian hamster embryo cells at concentrations from 13.75 to 25 μg/ml (Gibson et al., 1997), dose-dependent increase of micronuclei (significant increase at 74 μmol/l; corresponding to 20 μg/ml) in mouse lymphoma L5178Y cells (Mueller et al., 1996) and in the same cell line (L5178Y cells) emodin induced moderate increase in relative mutation frequency at concentration of 30 μmol/l (that correspond to 8.1 μg/ml) (Mueller et al., 1999). On the other hand, on L5178Y cells concentration of 56 μmol/l of emodin (equal to 15.1 μg/ml) did not induce an increase in comet assay tail intensity (Mueller et al., 1999), but that concentration is 10 times lower than the one that significantly increased tail intensity in our study. In a recent study *in vitro* and *in vivo* experiments showed that emodin is embryotoxic (Chang et al., 2012). It is implied that emodin toxicity/genotoxicity is exhibited by its biotransformation to more toxic metabolite (Masuda and Ueno, 1984; Masuda et al., 1985). However, study of Mueller et al. (1999) showing that emodin induces gene mutation and micronucleus formation in mouse lymphoma L5178Y cells in the absence of S9 fraction indicate that for toxic effect of emodin no metabolic activation is required. Results of our study confirmed that observation, since in our experiments with S9 fraction level of cell death or DNA damage was comparable to emodin treatment alone. In our study emodin at concentration of 25 μg/ml increased production of ROS in HPBLs but did not showed cytotoxic and genotoxic effects, confirming its pro-oxidant properties already tested for increasing sensitivity of tumour cells to anticancer drug (Yang et al., 2004). Our study confirmed cytotoxic and genotoxic potential of emodin and more importantly we showed that *F. alnus* bark extract and emodin are cytotoxic and genotoxic to primary human cells.

In several studies beneficial properties of *F. alnus* bark have been reported such as antifungal (Manojlovic et al., 2005), antiviral (Sydiskis et al., 1991), antibacterial (Kremer et al., 2012) and antioxidant (Kabić et al., 2008; Kremer et al., 2012). Therefore, our next step was to check antioxidant capacity of the same *F. alnus* bark extract that was used for toxicological studies. In order to compare antioxidant property of *F. alnus* bark extract and emodin, antioxidant capacity of emodin was determined. Additionally, polyphenols content of *F. alnus* bark extract was assessed.

The *F. alnus* bark extract showed relatively good antioxidant capacity assessed by DPPH, ABTS and FRAP assays. These results are comparable with only few studies testing antioxidant property of *F. alnus* bark (Kabić et al., 2008; Kremer et al., 2012). In both mentioned studies antioxidant properties of the bark extract were attributed to its high phenolic and flavonoids content. This is probably the case in our study as well, since in tested *F. alnus* bark extract we found high level of phenolic compounds. On the other hand, our results showed low antioxidant activity of emodin, which implies that emodin is not contributing to *F. alnus* bark antioxidant capacity. Several studies suggest that emodin is good scavenger of free-radical species (Yen et al., 2000; Vargas et al., 2004). Although, Tian and Hua (2005) found that aloe-emodin (another anthraquinone) has low antioxidant activity. Such discrepancy in results can be explained by the difference in model tests, methods used and/or concentration of substance.

Use of anthraquinone-containing laxatives is approved by health agencies, including WHO, however only short-term use of such preparations is advised (WHO, 2004; EMEA, 2007). Results of our study indicate that use of laxatives based on *F. alnus* bark or emodin is not harmless and might pose a threat to human health. This is even more important since exact composition and the amount of active component in such preparations is rarely known. Herbal composition depends on harvest season, plant origin and drying processes; some constituents during drying (oxidation) could transform to more toxic compounds (Silva et al., 2008). Additionally, plants belonging to same genus can have significantly different composition of anthraquinones. For example, main anthraquinone in *Frangula rupestris* (Scop.) is physicon (Kremer et al., 2012). Therefore, taking results of our study into account, higher level of emodin in plant material used for such preparations can lead to undesired effects. This is especially important in cases of self-medication or in abuse of anthraquinone-containing laxatives, such as use for weight loss. In many cases weight-reducing products contain anthraquinones, and users are unaware of their presence (Van Gorkom et al., 1999). In recent years use of herbal/natural products is increasing due to belief that they are much safer than synthetic drugs (Silva et al., 2008). Therefore, it is very important that composition of preparations based on *F. alnus* bark is determined, labelled correctly, and that their short-term use is highlighted.

To the best of our knowledge, this is the first report showing that *F. alnus* bark extract, although having moderate antioxidant properties, is cytotoxic and genotoxic to human cells and that its mechanism of toxicity is probably through oxidative stress. The biologically active component of *F. alnus* bark, emodin, was found to greatly contribute to its toxicity while simultaneously that anthraquinone exhibited low antioxidant activity. Taken together, these results indicate that use of *F. alnus* bark and herbal preparation based on *F. alnus* bark or emodin in treatment of constipation should be taken with caution.

Conflicts of interest

Authors have declared no conflicts of interest.

Acknowledgements

We wish to thank the Department of Plant Physiology at the Zagreb University Faculty of Science and the Institute for Medical Research and Occupational Health, Zagreb for financial support for the experimental part of the study.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2015.09.025.

References

Benzie, I.F.F., Strain, J.J., 1999. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 299, 15–27.

Chang, M.-H., Huang, F.-J., Chan, W.-H., 2012. Emodin induces embryonic toxicity in mouse blastocysts through apoptosis. Toxicology 299, 25–32.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Štětina, R., 2008. The comet assay: topical issues. Mutagenesis 23, 143–151.

Council of Europe, 2008. Frangula Bark. European Pharmacopoeia, sixth ed., pp. 1949–1950 Strasbourg, France.

Domijan, A.-M., Abramov, A.Y., 2011. Fumonisin B_1 inhibits mitochondrial respiration and deregulates calcium homeostasis – implication to mechanism of cell

toxicity. Int. J. Biochem. Cell Biol. 43, 897–904.
Duke, R.C., Cohen, J.J., 1992. Morphological and biochemical assays of apoptosis. In: Coligan, J.E., Kruisbeal, A.M. (Eds.), Current Protocols in Immunology. John Willey & Sons, New York, NY, pp. 1–3.
Dusinska, M., Collins, A.R., 2008. The comet assay in human biomonitoring: gene-environment interactions. Mutagenesis 23, 191–205.
European Medicines Agency (EMEA)/Committee on Herbal Medicinal Products (HMPC), 2007. Evaluation of Medicines for Human Use. Assessment Report on *Rhamnus frangula* L. cortex. Doc. Ref.: EMEA/HMPC/76306/2006. London, UK.
Gajski, G., Garaj-Vrhovac, V., Oreščanin, V., 2008. Cytogenetic status and oxidative DNA-damage induced by atorvastatin in human peripheral blood lymphocytes: standard and Fpg-modified comet assay. Toxicol. Appl. Pharmacol. 231, 85–93.
Garaj-Vrhovac, V., Gajski, G., 2009. Evaluation of the cytogenetic status of human lymphocytes after exposure to a high concentration of bee venom in vitro. Arch. Ind. Hyg. Toxicol. 60, 27–34.
Germano, M.P., Pasquale, R.D., Valeria, D.A., Catania, S., Silvari, V., Costa, C., 2002. Evaluation of extracts and isolated fraction from Capparis spinosa L. buds as an antioxidant source. J. Agric. Food Chem. 50, 1168–1171.
Gibson, D.P., Brauninger, R., Shaffi, H.S., Kerckeart, G.A., LeBoeuf, R.A., Isfort, R.J., Aardema, M.J., 1997. Induction of micronucleus in Syrian hamster embryo cells: comparison to results in SHE cell transformation assay for National Toxicology Program test chemicals. Mutat. Res. 392, 61–70.
Howard, L.R., Clark, J.R., Brownmiller, C., 2003. Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. J. Sci. Food Agric. 83, 1238–1247.
Kabić, D., Mišan, A., Sakač, M., Psodorov, Đ., Milovanović, I., 2008. Medical plant mixtures as additives in functional bakery food production. Food Process. Qual. Saf. 35, 119–123.
Kremer, D., Kosalec, I., Locatelli, M., Epifano, F., Genovese, S., Carlucci, G., Zovko Končić, M., 2012. Anthraquinone profiles, antioxidant and antimicrobial properties of *Frangula rupestra* (Scop.) Schur and *Frangula alnus* Mill. bark. Food Chem. 131, 1174–1180.
Maleš, Ž., Kremer, D., Gašpar Randić, Z., Randić, M., Hazler Pilepić, K., Bojić, M., 2010. Quantitative analysis of glucofrangulins and phenolic compounds in Croatian Rhamnus and Frangulin species. Acta Biol. Cracovien. Ser. Bot. 52, 108–113.
Manojlovic, N.T., Solujic, S., Sukdolak, S., Milosev, M., 2005. Antifungal activity of Rubia tinctorum, Rhamnus frangula and Caloplaca cerina. Fitoterapia 76, 244–246.
Masuda, T., Ueno, Y., 1984. Microsomal transformation of emodin into a direct mutagen. Mutat. Res. 125, 135–144.
Masuda, T., Haraikawa, K., Morooka, N., Nakano, S., Ueno, Y., 1985. 2-Hydroxyemodin, an active metabolite of emodin in the hepatic microsomes of rats. Mutat. Res. 149, 327–332.
Matkowski, A., 2008. Plant in vitro culture for the production of antioxidants – a review. Biotechnol. Adv. 26, 548–560.
Mueller, S.O., Eckert, I., Lutz, W.K., Stopper, H., 1996. Genotoxicity of the laxative drug components emodin, aloe-emodin and danthron in mammalian cells: toposisomerase II mediated? Mutat. Res. 371, 165–173.
Mueller, S.O., Schmitt, M., Dekant, W., Stopper, H., Schlatter, J., Schreier, P., Lutz, W.K., 1999. Occurrence of emodin, chrysophanol and physicon in vegetables, herbs and liquors. Genotoxicity and anti-genotoxicity of anthraquinones and of whole plants. Food Chem. Toxicol. 37, 481–491.
Mueller, S.O., Stopper, H., 1999. Characterisation of the genotoxicity of anthraquinones in mammalian cells. Biochem. Biophy. Acta 1428, 406–414.
Nesslany, F., Simar-Meintieres, S., Ficheux, H., Marzin, D., 2009. Aloe-emodin-induced DNA fragmentation in the mouse in vivo comet assay. Mutat. Res. 678, 13–19.
NTP Technical Report, 2001. Toxicology and Carcinogenesis Studies of Emodin in F344/N Rats and B6C3F1 Mice. NTP TR 493, NIH Publication No. 01-3952. National Toxicology Program, Research Triangle Park, NC.
Nusko, G., Schneider, B., Schneider, I., Wittekind, C., Hahn, E.G., 2000. Anthranoid laxative use is not a risk factor for colorectal neoplasia: results of a prospective case control study. Gut 46, 651–655.
Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26, 1231–1237.
Siegers, C.-P., von Hertzberg-Lottin, E., Otte, M., Schneider, B., 1993. Anthranoid laxative abuse – a risk for colorectal cancer? Gut 34, 1099–1101.
Silva, C.R., Monteiro, M.R., Rocha, H.M., Ribeiro, A.F., Caldeira de Araujo, A., Leitao, A.C., Bezerra, R.J.A.C., Padula, M., 2008. Assessment of antimutagenic and genotoxic potential of senna (*Cassia angustifolia* Vahl.) aqueous extract using *in vitro* assays. Toxicol. In Vitro 22, 212–218.
Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, L.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.
Singh, N.P., 2000. Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. Mutat. Res. 455, 111–127.
Sun, B., Ricardo da Silva, J.M., Spranger, I., 1998. Critical factors of vanillin assay for catechins and proanthocyanidins. J. Agric. Food Chem. 46, 4267–4274.
Sydiskis, R.J., Owen, D.G., Lohr, J.L., Rosler, K.H.A., Blomster, R.N., 1991. Inactivation of enveloped viruses by anthraquinones extracted from plants. Antimicrob. Agents Chemother. 35, 2463–2466.
Tian, B., Hua, Y., 2005. Concentration-dependence of prooxidant and antioxidant effects of aloin and aloe-emodin on DNA. Food Chem. 91, 413–418.
Van Gorkom, B.A.P., de Vries, E.G.E., Karrenbeld, A., Kleibeuker, J.H., 1999. Review article: anthranoid laxatives and their potential carcinogenic effects. Alimen. Pharmacol. Ther. 13, 443–452.
Vargas, F., Díaz, Y., Carbonell, K., 2004. Antioxidant and scavenging activity of emodin, aloe-emodin, and rhein on free-radical and reactive oxygen species. Pharm. Biol. 42, 342–348.
Vitalone, A., Menniti-Ippolito, F., Raschetti, R., Renda, F., Tartaglia, L., Mazzanti, G., 2012. Surveillance of suspected adverse reactions to herbal products used as laxatives. Eur. J. Clin. Pharmacol. 68, 231–238.
Westendorf, J., Marquardt, H., Poginsky, B., Dominiak, M., Schmidt, J., Marquardt, H., 1990. Genotoxicity of naturally occurring hydroxyanthraquinones. Mutat. Res. 240, 1–12.
WHO, 2004. Cortex Frangulae. In: WHO Monographs on Selected Medicinal Plants, vol. 2. WHO, Geneva.
Willems, M., van Buuren, H.R., de Krijger, R., 2003. Anthranoid self-medication causing rapid development of melanosis coli. Neth. J. Med. 61, 22–24.
Yang, J., Li, H., Chen, Y.-Y., Wang, X.-J., Shi, G.-Y., Hu, Q.-S., Kang, X.-L., Lu, Y., Tang, X.-M., Guo, Q.-S., Yi, J., 2004. Anthraquinones sensitize tumor cells to arsenic cytotoxicity in vitro and in vivo via reactive oxygen species-mediated dual regulation of apoptosis. Free Radic. Biol. Med. 37, 2027–2041.
Yen, G.-C., Duh, P.-D., Chuang, D.-Y., 2000. Antioxidant activity of anthraquinones and anthrone. Food Chem. 70, 437–441.
Zhishen, J., Mengcheng, T., Jianming, W., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64, 555–559.