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Anthraquinone profiles, antioxidant and antimicrobial properties of *Frangula rupestris* (Scop.) Schur and *Frangula alnus* Mill. bark

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ABSTRACT

Frangula rupestris and *Frangula alnus* are deciduous shrubs distributed in Balkan Peninsula. While the bark of *F. alnus* is medicinally widely used, little is known about chemical and biological properties of *F. rupestris*. In the present study, *F. rupestris* and *F. alnus* bark were evaluated for their reducing power, DPPH radical scavenging and chelating activity, as well as antioxidant activity in β-carotene-linoleic acid assay. In addition, phenolic content, anthraquinone profile and antimicrobial activity of *F. rupestris* and *F. alnus* bark were determined. The most represented anthraquinone derivatives in *F. rupestris* and *F. alnus* bark were physcion (0.11 mg/g) and emodin (2.03 mg/g), respectively. Both species demonstrated excellent antioxidant and antimicrobial activities with MIC values equal or lower than 2.5 mg/mL. The presented research indicates that both species may have health benefits as natural antioxidants and antimicrobial agents for use in functional foods or medicine.

1. Introduction

Reactive oxygen species (ROS) have many important functions in normal metabolism. Besides taking part in intracellular signalling and regulation, they are included in bacterial ingestion and killing by phagocytes. However, excess ROS can produce oxidative damage on cellular macromolecules, impair protein function, and trigger cell death. Consequently, ROS may contribute to pathogenesis and/or development of many diseases such as diabetes (Mokini, Marcovecchio, & Chiarelli, 2010), cancer (Valko, Rhodes, Moncola, Izakovic, & Mazura, 2006), and cardiovascular diseases (Singh & Jialal, 2006). Some of those ailments can be prevented or reduced by food rich in metabolites that can react with ROS and convert them into less reactive substances. For example, plant phenolics are multifunctional antioxidants which can act as reducing agents, free radical scavengers, metal chelators, and singlet oxygen quenchers thus inhibiting processes that can lead to membrane damage, ageing, heart disease, and cancer (Kaliora, Dedoussis, & Schmidt, 2006). It is well known that many medicinal and nutritional plants are rich sources of polyphenols which, through their antioxidant activity, exert positive effects on human health (Surveswaran, Cai, Corke, & Sun, 2007; Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2009). In order to evaluate the antioxidant capacity of plant materials several methods and assays have been conducted such as FRAP (ferric reducing/antioxidant power), DPPH (2,2-diphenyl-1-picrylhydrazyl), TRPA (total reducing power ability), ORAC (oxygen radical absorbance capacity), LDL (low density lipoprotein) oxidation and others. However different trends between the assays have been found (Oszmianski & Wojdylo, 2007; Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006). Hence it is pertinent to use several assays instead of a single one to evaluate and compare the antioxidant activity in food and plant extracts (Suárez-Jacobo et al., 2011).

Natural anthranoides are derivatives of 1,8-dihydroxyanthrone and thus also belong to the group of natural phenolic compounds. Anthraquinone derivatives and plants that contain them are widely used as mild laxatives (Hänsel & Sticher, 2007, chap. 26). Besides their purgative properties, anthraquinones possess antibacterial, antiviral (Andersen et al., 1991; Ifesan, Hamtasin, Mahabusarakam, & Voravuthikunchai, 2009), antifungal (Agarwal, Singh, Verma, & Kumar, 2000; Manojlović, Solujić, Sukdolak, & Milošev, 2005), antioxidant (Ammara et al., 2009) and anticancer (Fenig, Nordenberg, Beery, Sulkes, & Wasserman, 2004) properties. Some plants of *Frangula* Mill. genus are used in European and American traditional medicine as mild purgatives due to their anthraquinone content. *Frangula purshiana* (DC.) J.G. Cooper, is mostly used in American continent. Its bark contains a significant quantity of anthraquinone derivatives, especially monoglucosides and emodin (Hänsel & Sticher, 2007, chap. 26). On the other hand, barks and fruits of alder buckthorn (*Frangula alnus* Mill., *Rhamnaceae*; syn. *Rhamnus frangula* L.) have been used for centuries in folk and official European medicine as purgatives. Besides the mixture of emodine derivates,

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responsible for purgative activity, *F. alnus* bark contains tannins and peptide alkaloids (Hänsel & Sticher, 2007, chap. 26; Wichtl, 2004).

Frangula rupestris (Scop.) Schur (syn. *Rhamnus rupestris* Scop.) is a species distributed in southeastern Alps, Transylvanian Alps and Carpathian Mountains. It is a deciduous shrub, growing to 0.5–1 m on slopes or fissures in limestone rocks (Šilić, 1990). While the chemical composition of *F. alnus* and *F. purshiana* are well documented, there is no published data about the chemical composition, application or therapeutic effects of *F. rupestris*. In addition, although *F. alnus* is one of the most common laxatives, its antioxidant activity was not researched in detail (Stef et al., 2009). Thus, the aim of this study was to research antioxidant and antimicrobial activity as well as the quantity of phenolic substances (anthraquinone profile, total phenols and flavonoids) in bark of *F. rupestris* and to compare it with the *F. alnus* bark.

2. Materials and methods

2.1. Chemicals and microorganisms

Emodin (>99%), rhein (>99%), chrysophanol (>99%), aloe-emodin (>99%) and physcion (>99%) were purchased from Extrasynthese (Genay, France) and their chemical structures are reported in Fig. 1. Butylated hydroxyanisol (BHA), DPPH, β-carotene, Folin–Ciocalteu reagent, linoleic acid, Tween-40 (polyoxyethylene sorbitan monopalmitate), ascorbic acid, quercetin and gallic acid were purchased from Sigma–Aldrich Chemical Co. (USA). Methanol (HPLC-grade) and formic acid (99%) were obtained from Carlo Erba Reagenti (Milan, Italy). Double-distilled water was obtained using a Millipore Milli-Q Plus water treatment system (Millipore Bedford Corp., Bedford, MA, USA). Other chemicals and solvents were of analytical grade.

Microbial species (*Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 10535, *Candida albicans* ATCC 10231, and *Microsporum gypseum* MFBF 3) from the stock cultures of microorganisms (Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb) were used. Sabouraud 2% (w/v)-glucose agar, Müller–Hinton agar and Müller–Hinton broth were supplied by Merck (Germany) and RPMI 1640 broth by the Sigma–Aldrich Chemical Co. (USA).

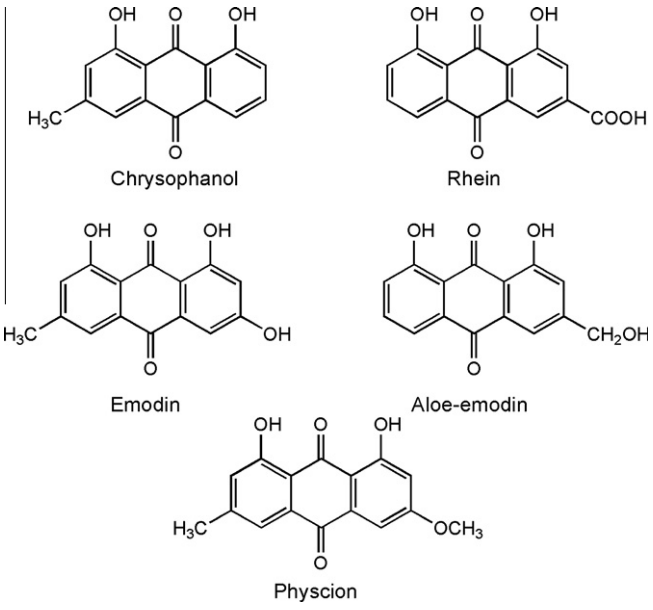

Fig. 1. Chemical structures of the five analytes assayed in *Frangula* barks.

2.2. Herbal material and extraction

Randomly selected samples of ten wild growing plants of *F. rupestris* (Scop.) Schur (syn. *R. rupestris* Scop.) and *F. alnus* Mill. (syn. *R. frangula* L.) were collected in September of 2009 in Croatia. Sample of *F. rupestris* was collected in locality Vaganac (44°19′N; 15°28′E; 700 m a.s.l.) while sample of *F. alnus* was collected in locality Hrastenica (46°38′N; 16°43′E; 164 m a.s.l.). About 200 g of bark was removed from the stem of each species, mixed to obtain randomly selected samples and dried for 2 weeks in a well-ventilated room, in one layer, protected from direct solar light. To limit oxidation and photo-oxidation, air-dried samples were placed in double paper bags, closed in a dark container and stored in a dry place at room temperature (22 °C) protected from light, and saved four months until the analysis (loss on drying 6.8% ± 0.1%, $n = 5$).

For determination of antioxidant activity extracts were prepared by ultrasonication of grounded herbal material (2.5 g) with methanol (7 mL) at 35 °C for 45 min. Upon filtration, herbal material was re-extracted twice. Combined filtrates were evaporated to dryness under reduced pressure. The extracts were stored at +4 °C in the dark until use. Extraction yield (amount of total extractable compounds), was calculated as weight of extract obtained from 100 g of sample. Bandelin SONOREX® Digital 10 P ultrasonic bath was used for ultrasonication and Perkin Elmer Lambda 25 spectrophotometer (Perkin Elmer, USA) for absorbance measurements.

2.3. Total phenol content

Total phenol content of extracts was determined by the Folin–Ciocalteau colorimetric method (Singleton, Orthofer, & Lamuela-Raventos, 1999). Briefly, 0.5 mL of the extract solution was mixed with the Folin–Ciocalteu reagent (0.5 mL) and 100 mg/mL Na_2CO_3 (0.5 mL). After 1 h of incubation at room temperature the absorbance was measured against water at 760 nm. The phenol content was calculated from calibration curve of gallic acid and expressed as gallic acid equivalents.

2.4. Total flavonoid content

Flavonoid content was determined by the method of Kumazawa, Hamasaka, and Nakayama (2004), with minor modifications. Briefly, to 0.5 mL of extract solution, 0.5 mL of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. The flavonoid content was expressed as quercetin equivalents (mg/g) from a calibration curve recorded for this standard. A solution of 0.5 mL of the extract diluted with 0.5 mL of ethanol served as the blank.

2.5. HPLC analysis

2.5.1. Sample extracts preparation

For determination of anthraquinone profile, the exhaustive ultrasonic extraction was performed on finely triturated bark samples (0.7120 g and 0.4019 g for *F. rupestris* and *F. alnus*, respectively) at room temperature in methanol (7.0 and 4.0 mL for *F. rupestris* and *F. alnus*, respectively). The extracts were treated with a solution of HCl 6 M (12 mL) to hydrolyse glycosides. The organic phases were quantitatively separated by liquid–liquid extraction (LLE) with ethyl acetate (20 mL), dried under vacuum, and stored at −20 °C until analysis.

2.5.2. HPLC conditions

HPLC analyses were performed according to previously described protocol (Genovese et al., 2010; Locatelli et al., 2009) on a Waters liquid chromatograph equipped with a model 600 solvent

pump and a 2996 photodiode array detector. Empower v.2 Software (Waters Spa, Milford, MA, USA) was used for acquisition of data. A C18 reversed-phase packing column (GraceSmart RP18, 4.6 × 150 mm, 5 μm; Grace, Deerfield, IL, USA) was used for the separation. The column was thermostated at 28 ± 1 °C using a Jetstream2 Plus column oven. The UV/Vis acquisition wavelength was set in the range of 200–500 nm. Analogue output channel A was set at wavelength 254 nm with a bandwidth of 9.6 nm. The qualitative analyses were achieved at wavelength of 435 nm. Flow-rate was 0.7 mL/min while the injection volume was 20 μL. The mobile phase was directly on-line degassed by using Degassex DG-4400 (Phenomenex, Torrance, CA, USA). Isocratic elution was performed using the mobile phase water–methanol (40:60, v/v, 1% formic acid). Formic acid was used to reduce peak tailing. All the prepared sample solutions were centrifuged and the supernatant was injected into HPLC. The quantification of anthraquinones was carried out using external calibration curves for each compound in the range of concentrations of 10–200 μM with seven concentration levels. Standards were prepared in HPLC mobile phase.

2.6. Antioxidant capacity

2.6.1. Free radical scavenging activity (RSA)

DPPH radical scavenging was monitored according to the method of Yen and Chen (1995) with minor modification. Briefly, 2.0 mL of extract solution was added to 2.0 mL of DPPH ethanolic solution (63 μg/mL). The mixtures were vortexed for 1 min and then left to stand at room temperature in the dark. After 30 min absorbance was read at 517 nm. RSA for the DPPH free radical was calculated using the following equation:

$$\mathrm{RSA} = \left(1 - \frac{A_{\mathrm{sample}}}{A_{\mathrm{control}}}\right) \times 100 \quad (1)$$

where A_{control} is the absorbance of the control (solution to which no antioxidant was added) and A_{sample} is the absorbance of the extract solution. Bleached DPPH solution, prepared by adding 2.0 mL of DPPH solution (63 μg/mL) to 2.0 mL BHA solution (1 μg/mL) was used as a blank. DPPH radical scavenging activity was calculated as EC_{50}, the concentration that scavenges 50% of DPPH free radical and thus has RSA = 50%. The antioxidant activity of BHA as a standard reference was also assayed.

2.6.2. The reducing power of the extracts

The reducing power of extracts was determined according to the method of Yen and Chen (1995). Briefly, extracts (0.2–1.0 mg) were dissolved in 1.0 mL of distilled water and mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (10 g/L). The mixtures were incubated at 50 °C for 20 min. After incubation, 2.5 mL of trichloroacetic acid (100 g/L) was added to the mixtures. Following that, samples were centrifuged for 10 min (360 × *g*). Aliquots of 2.5 mL of the upper layer were combined with 2.5 mL of water and 0.5 mL of ferric chloride solution (1 g/L). Absorbance of the reaction mixture was read spectrophotometrically at 700 nm.

2.6.3. β-Carotene-linoleic acid assay

The antioxidant activity of extracts was evaluated in a β-carotene-linoleic acid system, according to the previously described procedure (Zovko Končić, Kremer, Karlović, & Kosalec, 2010). Briefly, into a round-bottom flask containing 20 mg of linoleic acid and 200 mg of Tween 40, 1 mL of β-carotene solution in chloroform (0.2 mg/mL) was added. After removing chloroform in a rotary evaporator, 50 mL of aerated distilled water was added to the oily residue with vigorous stirring. Aliquots (5 mL) of obtained emulsions were transferred to a series of tubes containing 2 mg of extract or 0.5 mg of BHA (positive control) dissolved in 1 mL of 0.4% (w/v) Tween 40 solution. A tube with 1 mL of 0.4% Tween 40 without the extract, served as the negative control. Solution of 2 mg of the extract in 6 mL of Tween 40 solution served as the blank for the corresponding extract. After addition of the emulsion to the tubes, they were placed in a water bath at 50 °C for 2 h. During that period, the absorbance of each sample was measured at 470 nm at 15 min intervals, starting immediately after sample preparation ($t = 0$ min) until the end of the experiment ($t = 120$ min). The rate of β-carotene degradation (R) was calculated according to the first order kinetics. The percent of antioxidant activity (ANT) was calculated using the equation:

$$\mathrm{ANT} = \frac{R_{\mathrm{control}} - R_{\mathrm{sample}}}{R_{\mathrm{control}}} \times 100 \quad (2)$$

where R_{control} and R_{sample} are bleaching rates of the negative control and the antioxidant (plant extract or BHA), respectively.

2.6.4. Chelating activity (ChA)

The chelation of iron(II) ions was studied as described by Decker and Welch (1990). An aliquot of the extract in methanol (1.1 mL) was added to 100 μL of 0.70 mM $FeCl_2$. After 5 min, the reaction was initiated by adding 400 μL of 1.40 mM ferrozine. Following 10 min incubation at room temperature, the absorbance at 562 nm was recorded. The control contained all the reaction reagents except the extract or quercetin (chelating standard). The Fe^{2+}-chelating activity (ChA) was calculated using the equation below:

$$\mathrm{ChA} = \left(1 - \frac{A_{\mathrm{sample}}}{A_{\mathrm{control}}}\right) \times 100 \quad (3)$$

where A_{control} is the absorbance of the negative control (solution to which no extract was added) and A_{sample} is the absorbance of the extract solution. Chelating activity was expressed as $ChEC_{50}$, the concentration that chelates 50% of Fe^{2+} ions and thus has ChA = 50%.

2.7. Antimicrobial susceptibility assay

Before analysis, extracts were diluted with 96% (v/v) ethanol to final concentration 20 mg/mL. The same concentration was used in diffusion assay. Serial of twofold diluted extracts from 10 mg/mL to 4.89 μg/mL was used for micro-dilution broth assay. Inoculums were prepared with fresh cultures of microbial strains, cultured on tryptic-soy agar for 18 h (48 h for fungi) at 37 °C. Yeast and dermatophyte species were cultivated on Sabouraud 2%-dextrose agar with the addition of chloramphenicol (50 mg/L) for 48 h at 37 °C. Density of inoculum was set to 0.5 McFarland units using a nephelometer (ATB 1550, bioMérieux, France). The final concentrations of bacteria were 1.5×10^8 CFU (colony forming units)/mL, and fungi 5×10^6 CFU/mL.

Cylinder-plate diffusion method was used for zones of inhibition determination. Briefly, 1 mL of inoculum was mixed with approximately 22 mL of Müller–Hinton agar for bacterial strains, and Sabouraud 2%-glucose agar for the yeast. After cooling the inoculated agars at room temperature for maximum 25 min, holes ($d = 6$ mm) were made with stainless steel cylinders. Fifty microlitres of extracts were dropped into holes. In order to accelerate the diffusion of extracts into agar, plates were incubated at +4 °C for 1 h and then at +37 °C for 18 h under aerobic conditions. The diameter of the inhibition zone around each hole was measured and recorded.

Minimal inhibitory concentration (MIC) were determined by the twofold micro-dilution method in Müller–Hinton broth for bacterial strains and RPMI 1640 pH 7.0 for yeast according to the *Clinical and Laboratory Standards Institute* (formerly NCCLS)

M7-A6 and M-27A recommendations (National Committee for Laboratory Standards, 1997a, 1997b), respectively. MIC was defined as the lowest concentration of extract that allows no more than 20% growth of the microbes which is seen as a decreased number of colonies after removing the loop with 10 μL of each dilution on tryptic-soy agar or Sabouraud 2%-glucose agar and incubation at 37 °C for 18 h. All measurements were performed in duplicate, and expressed as mean.

2.8. Statistical and mathematical analyses

The experiments for determination of antioxidant capacity were performed in triplicate and the results were expressed as mean ± SD. Statistical comparisons were made using a Student's *t*-test. Statistical analyses were performed using the JMP V6 from SAS software (SAS Institute, Cary, NC, USA). A value of $p < 0.05$ was considered to indicate statistical significance.

3. Results and discussion

3.1. Extraction yield, total phenol and total flavonol contents

Polyphenols are the large group of phytochemicals that are gaining acceptance as being responsible for the health benefits associated with plants. The contents of total phenolics and flavonoids as well as the amount of dry matter obtained from the herbal material (extraction yield) in the methanolic extracts of two *Frangula* species barks are summarised in Table 1. *F. alnus* contained approximately 2.5 times more extractable substances than *F. rupestris*. The amount of total phenolics in extract of *F. alnus* was more than twice higher than the amount of phenols in *F. rupestris* extract. Similarly, the amount of total flavonoids in *F. alnus* is almost twofold higher than the amount of those substances in *F. rupestris*. The data in Table 1 also illustrates that more than half of the extracted phenolic substances in both extracts were flavonoids.

3.2. Chromatographic analyses of anthraquinone

Fig. 2a shows the separation of mixture containing five anthraquinone standards for the quantitative analysis at 254 nm and concentration of 200 μM. A robust baseline separation was achieved in 80 min. Under these conditions the retention time of analytes were 11.66 (±0.54), 18.92 (±0.86), 36.80 (±1.54), 42.03 (±2.09) and 76.66 (±1.87) min for aloe-emodin, rhein, emodin, chrysophanol, and physcion, respectively. Fig. 2b and c shows chromatographic profiles of extracts of *F. rupestris* and *F. alnus*, respectively. Table 2 reports the calibration parameters obtained in this study.

Anthraquinone profile of *F. rupestris* and *F. alnus* barks consisted of emodin, chrysophanol and physcion while aloe-emodin and rhein were below limit of quantification (10 μM) (Table 3). Even though *F. alnus* and *F. rupestris* belong to the same genus, the qualitative and quantitative composition of the anthraquinones in both barks was significantly different. In accordance with previous data (Wichtl, 2004) the most represented anthraquinone aglycone in *F. alnus* was emodin (69.7%). However, the main anthraquinone in *F. rupestris* was the methyl derivative of emodin, physcion (60.7%). Total amount of anthraquinones was significantly greater in *F. alnus* (2.895 mg/g) than in *F. rupestris* (0.181 mg/g).

3.3. Antioxidant activity

Antioxidant activities of the extracts varied depending on the applied test. DPPH assay is simple, quick and commonly used to assess the antioxidant activity of plants and natural compounds, which act as free radical-scavengers or hydrogen donors *in vitro* (Chen et al. 2011). The scavenging effects of *F. alnus* and *F. rupestris* on DPPH free radicals are shown in Table 1. The extracts demonstrated appreciable scavenging properties against DPPH radicals. In the present study, *F. alnus* exhibited greater efficiency and scavenged 50% of DPPH free radicals at a concentration of 21.7 μg/mL while the EC_{50} of *F. rupestris* extract was approximately 50% higher, indicating lower activity. In β-carotene and linoleic acid assay, heat induces formation of a free radical from linoleic acid (Frankel, 1998). The radical then reacts with β-carotene, which undergoes rapid discoloration. Presence of an antioxidant can reduce the extent of β-carotene destruction by reacting with the linoleate free radical or any other free radical formed within the system. Both extracts demonstrated excellent antioxidant properties in this assay and they significantly reduced the degradation of β-carotene (Fig. 3). The activity of *F. alnus* extract was significantly greater than the activity of *F. rupestris* (Table 1).

Literature reports suggest that the antioxidant activity of plant herbs is associated with their reducing power, which terminates free radical chain reactions (Singh & Rajini, 2004). In the present study, reducing power of the *Frangula* spp. barks was evaluated measuring the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form. The reducing power of the investigated extracts was linearly increasing with the concentration (Fig. 4). Slopes of the trend lines (SRP) were calculated (Table 1). Coefficients of determination (r^2) for *F. rupestris* and *F. alnus* extracts were 0.9998 and 0.9996, respectively. *F. alnus* demonstrated significantly better reducing power than *F. rupestris*. In the Fenton reaction, hydroxyl radical production is directly related to the concentration iron or other transition ions (Galey, 1997). Since hydroxyl radicals are among the most harmful reactive oxygen species in biological systems, chelating activity of a compound contributes to its antioxidant properties. Even though the extracts were not as strong Fe^{2+} chelators as quercetin, both investigated extracts demonstrated notable chelating properties. However, the activity of *F. alnus* was significantly more pronounced than the activity of *F. rupestris*.

It seems that, among plants of closely related genera *Rhamnus*, flavonoids (Ammara et al., 2009) and other phenolic compounds

Table 1
Extraction yield (EY), total phenols (TP), total flavonoids (TF), radical scavenging activity (EC_{50}), antioxidant activity in β-carotene-linoleate test (ANT), slope of trendline in reducing power assay (SRP) and metal chelating activity ($ChEC_{50}$) of *Frangula rupestris* and *F. alnus* extract.

Extracts/species	EY[a] (%)	TP[b] (mg/g)	TF[b] (mg/g)	EC_{50}[b] (μg/mL)	ANT[b] (%)	SRP[b] (mg^{-1})	$ChEC_{50}$[b] (μg/mL)
F. rupestris	6.2	30.8 ± 1.4[A]	17.4 ± 0.7[A]	33.0 ± 0.2[A]	61.2 ± 5.5[A]	1.48 ± 0.05[A]	1313.5 ± 145.7[A]
F. alnus	15.6	62.3 ± 1.0[B]	30.7 ± 0.8[B]	21.7 ± 0.2[B]	75.3 ± 1.5[B]	2.85 ± 0.03[B]	2318.2 ± 164.3[B]
Standard	–	–	–	[c]2.6 ± 0.0[C]	[c]86.2 ± 2.7[C]	[d]8.80 ± 0.41[C]	[e]219.2 ± 4.7[C]

[A–C]Differences within column (samples connected by the same capital letter are statistically different at $p < 0.05$).
[a] Experiment was performed once.
[b] Values are means ± SD ($n = 3$).
[c] Standard: BHA.
[d] Standard: ascorbic acid.
[e] Standard: quercetin.

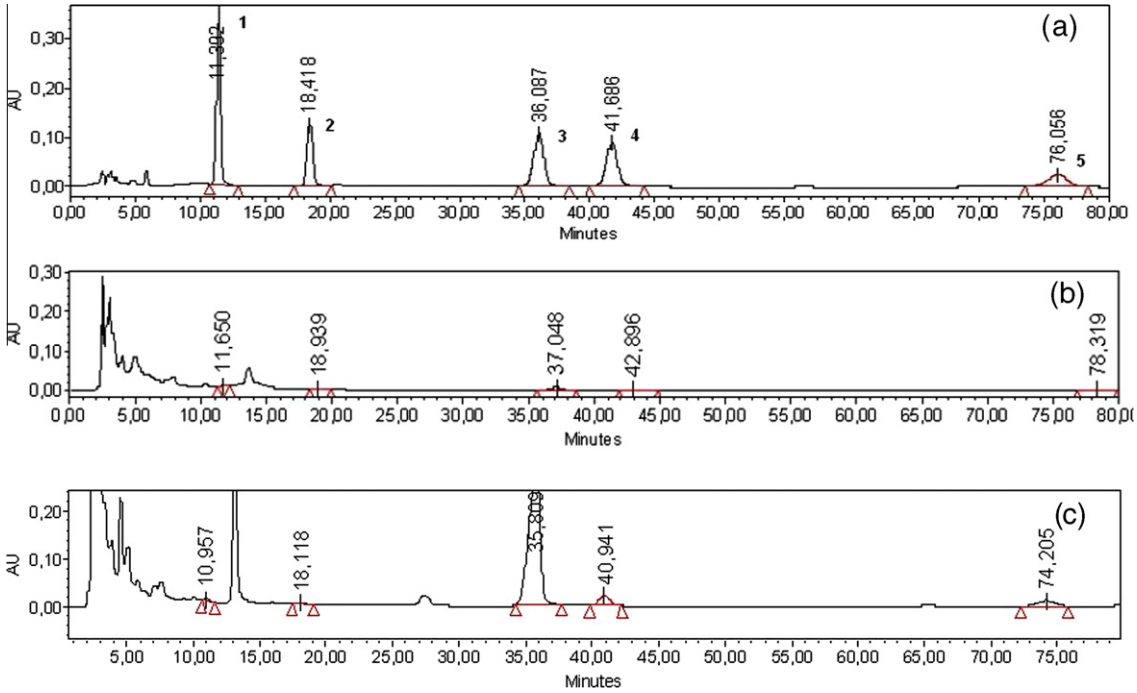

Fig. 2. HPLC–UV/Vis chromatograms of (a) a mixture solution of anthraquinone standards: (1) aloe-emodin; (2) rhein; (3) emodin; (4) chrysophanol; (5) physcion; concentrations: 200 μM. (b) Bark extracts of *Frangula rupestris* and (c) *F. alnus*; concentrations: 35 mg bark/mL solvent.

Table 2
Calibration parameters.

	Aloe-emodin	Rhein	Emodin	Chrysophanol	Physcion
Slope	38,720	20,040	29,920	26,990	6541
Intercept	−59,000	−68,860	−14,5000	−185,700	−66,400
r^2	0.9980	0.9958	0.9959	0.9899	0.9955
r	0.9990	0.9979	0.9979	0.9949	0.9977

r^2 = determination coefficient; r = correlation coefficient.

Table 3
Anthraquinones profiles of *Frangula rupestris* and *F. alnus* bark extracts.

Species	Aloe-emodin	Rhein	Emodin	Chrysophanol	Physcion	Total
Anthraquinones concentration (μM)						
F. rupestris	<LOQ	<LOQ	17.4	10.0	39.4	66.8
F. alnus	<LOQ	<LOQ	750	50.9	265	1066
Relative abundances of anthraquinones (%)						
F. rupestris	<LOQ	<LOQ	25.5	13.8	60.7	100
F. alnus	<LOQ	<LOQ	69.7	4.45	25.9	100

LOQ = Limit of quantification (10 μM).

(Ng, Lin, & Lu, 2007) are at least partly responsible for antioxidant activity of plant material. Additionally, anthraquinones were found to be strong natural antioxidants (Zhang et al., 2005). In this study, not only *F. alnus* extract was superior antioxidant than the extract of *F. rupestris*, but it was also richer in all the investigated types of phenolic compounds. Therefore, it is reasonable to assume that those compounds play an important role in antioxidant activity of studied *Frangula* species.

3.4. Antimicrobial activity

The results of antimicrobial activity of methanol extracts of bark of *F. rupestris* and *F. alnus* are shown in Table 4. In diffusion assay, only *S. aureus* was found to be susceptible to both of extracts with equal zones of inhibition (12 mm). The noticeable difference in antimicrobial activity was detected against *P. aeruginosa*, where only *F. alnus* extract showed zone of inhibition of 14 mm. Because of some disadvantages of diffusion assay, such as poor diffusion of bio-active compounds through agar, broth micro-dilution assay

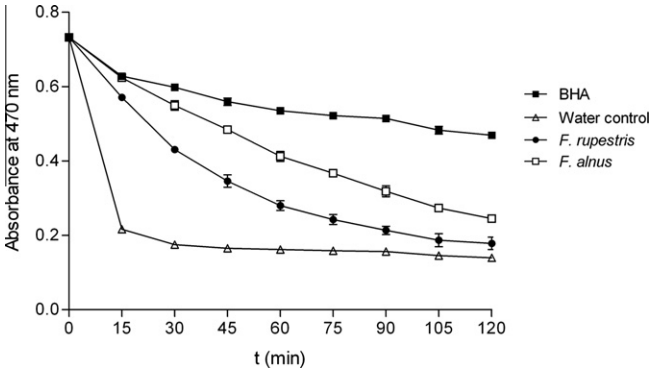

Fig. 3. Inhibition of β-carotene-linoleic acid emulsion bleaching by the extracts and BHA. Values are means ± SD (n = 3).

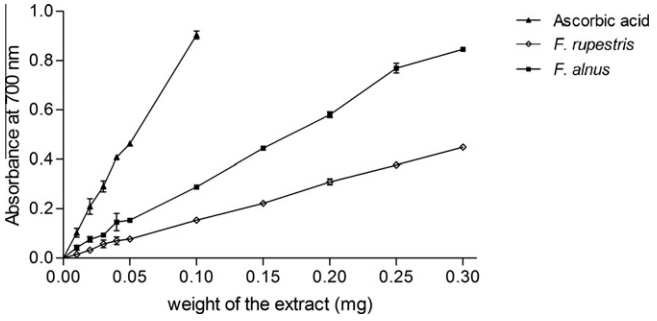

Fig. 4. Reducing powers of the extracts and ascorbic acid. Values are means ± SD (n = 3).

Table 4
Antimicrobial activity of *Frangula rupestris* and *F. alnus* extracts.

Extract	*Staphylococcus aureus* ATCC 6538		*Pseudomonas aeruginosa* ATCC 27853		*Escherichia coli* ATCC 10535		*Candida albicans* ATCC 10231		*Microsporum gypseum* MFBF 3	
	ZI[a]	MIC[b]	ZI	MIC	ZI	MIC	ZI	MIC	ZI	MIC
F. rupestris	12	0.625	ND	1.250	ND	2.500	ND	0.625	ND	0.625
F. alnus	12	0.625	14	1.250	ND	2.500	ND	0.625	ND	0.625
Control	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ATCC = American type culture collection; MFBF = Collection of Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb; ZI = zone of inhibition; MIC = zone of inhibition; ND = not detected; control = solvent of 96% (v/v) ethanol.
[a] Expressed in mm as mean of two independent measurements.
[b] Expressed in mg/mL as mean of two independent measurements.

was performed. Both methanol extracts showed antimicrobial activity against all microbial species tested with MIC values equal or lower than 2.5 mg/mL. There were no differences between antimicrobial activity of *F. rupestris* and *F. alnus*. The most sensitive microbes were gram-positive bacteria (*S. aureus*), yeast (*C. albicans*) and dermatophyte species (*M. gypseum*) with MIC values of 0.625 mg/mL.

To the best of our knowledge, this is a first report of *in vitro* antimicrobial activity of methanol extracts of *F. rupestris* and *F. alnus*. Due to the presence of anthraquinones in methanol extracts of bark of both investigated species, the observed antimicrobial activity can be directly connected with their contents.

4. Conclusions

In this study anthraquinone profiles of methanol extracts of *F. rupestris* and *F. alnus* bark were analysed using HPLC analyses. Anthraquinone profile of both species consisted of emodin, chrysophanol and physcion. Several *in vitro* assays were applied to evaluate the antioxidant potential of methanol extracts of bark from *F. rupestris* and *F. alnus*. In all the applied assays both extracts, and especially *F. alnus*, showed excellent antioxidant properties. In addition, *F. alnus* was richer in total phenols, flavonoids and anthraquinones. The extracts of both species showed antimicrobial activity against tested microbes in diffusion and dilution assays. There were no differences between antimicrobial activity of *F. rupestris* and *F. alnus*. The presented research indicates that moderate use of *F. alnus* and *F. rupestris* may be useful in disorders related to oxidative stress and microbial infections. In addition, it seems that the health benefits of *F. alnus* may extend beyond well-established use as purgative.

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