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Protective effects of *Aronia melanocarpa* juices either alone or combined with extracts from *Rosa canina* or *Alchemilla vulgaris* in a rat model of indomethacin-induced gastric ulcers

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ABSTRACT

The aim of the study was to investigate the effects of four Aronia melanocarpa-based juices in a rat model of indomethacin-induced gastric ulceration.

The juices were: AM1 and AM2 (produced from aronia fruits at 20 °C and 60 °C, respectively), AMRC (a mixture of AM2 with Rosa canina extract) and AMAV (aronia juice with Alchemilla vulgaris). Male Wistar rats were used. Each of the juices (10 ml/kg) was administered for 10 days. Indomethacin (30 mg/kg) was injected subcutaneously and after 4 h, the effects were estimated.

Indomethacin caused heavy destructions of the gastric mucosa, increased the expression of Bax and decreased the expression of Bcl-2, induced a certain increase in lipid peroxidation and a slight decrease in gastric PGE2 content. The pretreatment with the juices reduced the severity of indomethacin-induced gastric lesions and antagonized the effects of indomethacin on apoptosis and lipid peroxidation. The highest was the protective effect of AMAV, the juice with the highest polyphenolic content.

The protective effect of Aronia melanocarpa-based juices against indomethacin-induced gastric lesions could be attributed to their polyphenolic contents. The mechanism involved to the highest extent in the protective effect of the juices was the inhibition of apoptosis.

1. Introduction

Gastric ulceration is a highly prevalent health problem affecting a large number of the population worldwide (Lanas et al., 2015). The imbalance between aggressive and protective factors is the major mechanism in the pathophysiology of peptic ulcer disease. The main aggressive factors are *Helicobacter pylori* and the nonsteroidal anti-inflammatory drugs (NSAIDs) (Zhang et al., 2014). With the increase in life expectancy, the incidence of musculoskeletal and cardiovascular diseases rises and, therefore, the use of NSAIDs increases (Kim, 2016). In NSAID users, the risk of complications of peptic ulcer is four times higher (Lanas et al., 2015). Indomethacin-induced ulcerogenesis in rats is a commonly used experimental model for investigation of the gastroprotective potential of different agents. Oxidative stress, inflammation and apoptosis are thought to be the most important mechanisms of gastropathy induced by NSAIDs (Pal et al., 2010; Sinha et al., 2015). Therefore, polyphenolic substances due to their antioxidant, anti-inflammatory and anti-apoptotic properties can be a valuable alternative to the conventional drugs in preventing and treating gastric ulcers (Bi et al., 2014). A large number of plants, rich in polyphenols, have been

Abbreviations: AM1, Aronia melanocarpa juice 1, obtained at 20 °C; AM2, Aronia melanocarpa juice 2, obtained at 60 °C; AMAV, Aronia melanocarpa juice combined with Alchemilla vulgaris extract; AMRC, Aronia melanocarpa juice combined with Rosa canina extract; COX, cyclooxygenase; HPLC, high performance liquid chromatography; Indo, indomethacin; NSAIDs, nonsteroidal anti-inflammatory drugs; ORAC, oxygen radical absorbance capacity; PG, prostaglandin; PP, percentage of protection; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; UI, ulcer index

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demonstrated to possess a pronounced gastroprotective effect (Awaad et al., 2013). A good example in this regard are the fruits of *Aronia melanocarpa* [Michx.] Elliot (black chokeberry). They are an extremely rich source of polyphenols with high antioxidant (Zheng and Wang, 2003; Bermudez-Soto and Tomas-Barberan, 2004; Wu et al., 2004; Oszmianski and Wojdylo, 2005; Jakobek et al., 2011; Denev et al., 2012; Valcheva-Kuzmanova et al., 2007, 2012a,b, 2014) and anti-inflammatory activities (Borissova et al., 1994; Zapolska-Downar et al., 2011). *Aronia melanocarpa* originates from North America and nowadays is also grown in Europe as an important industrial crop. It is a raw material for the production of a broad range of products, such as juices, nectars, syrups, teas, wines, spirits, food colorants, etc. (Strigl et al., 1995). In order to increase the polyphenol content of black chokeberry fruit products, we investigated the effect of different technological parameters, and demonstrated that the extraction temperature (20 °C, 40 °C, 60 °C or 80 °C) of juice pressing and nectar extraction has a profound effect on the polyphenol content and composition. This is very important, since variation of technological parameters during processing could result in functional foods with different chemical composition, rendering different biological activity (Denev et al., 2018). Certain medicinal plants are a rich source of antioxidant components and their addition to *Aronia melanocarpa* juice products could further increase their polyphenol contents and antioxidant activity. A good example in this respect is *Rosa canina* L. (rosehip) which has been known as a medicinal plant since ancient times. *Rosa canina* fruits are well-known for their high phenolic content, antioxidant and anti-inflammatory properties (Chrubasik et al., 2008; Kilicgun and Altiner, 2010; Denev et al., 2013). In a previous study, we revealed that the addition of a rosehip extract to chokeberry, blackberry, blackcurrants or elderflower extracts increased significantly their antioxidant activity measured by the oxygen radical absorbance capacity (ORAC) method (Kratchanova et al., 2014). Moreover, there was a synergistic increase in the antioxidant activity of the mixed extracts that reached up to 64% (Kratchanova et al., 2014). Therefore, it was interesting to investigate whether the observed synergistic effect in ORAC *in vitro* would be a prerequisite for an increased pharmacological activity *in vivo*. *Alchemilla vulgaris* L. (lady's mantle) is another medicinal plant particularly rich in phenolic compounds (Boroja et al., 2018). It is widely spread in Europe and Asia and had been used in the traditional medicine for treating ulcers, digestive problems, wounds and eczema, as well as gynecological disorders (Jarić et al., 2015). Our previous study revealed that *Alchemilla vulgaris* showed high antioxidant, antimicrobial and neutrophil-modulating activities (Denev et al., 2014). Thus, it is an appropriate source of polyphenols to further increase the polyphenol content of *Aronia melanocarpa* fruit juice if combined with it.

Aronia melanocarpa has been investigated for a wide range of pharmacological activities. Most recent investigations deal with the antioxidant and anti-inflammatory effects of aronia polyphenols and individual phenolic compounds (Denev et al., 2019), with the neuroprotective and memory improving effects of *Aronia melanocarpa* juice (Daskalova et al., 2019), as well as with the beneficial effects of aronia extracts in obesity (Kim et al., 2018; Lim et al., 2019). *Aronia melanocarpa* fruit juice has been shown to ameliorate the gastrointestinal damage in a rat model of indomethacin-induced gastric ulceration (Valcheva-Kuzmanova et al., 2005b) and in a rat model of colitis induced by 2,4,6-trinitrobenzenesulfonic acid (Valcheva-Kuzmanova et al., 2018). However, its gastroprotective effect in combination with other polyphenol-rich medicinal plants has not been addressed. Therefore, the aim of the current study was to investigate the protective effect of two *Aronia melanocarpa* fruit juices (juice AM1 and juice AM2) and two *Aronia melanocarpa*-based juices (juice AMRC and juice AMAV) in a rat model of indomethacin-induced gastric ulcers. Juices AM1 and AM2 were obtained from the same batch of fruit but at different extraction temperatures: juice AM1 – at 20 °C and juice AM2 – at 60 °C. These temperatures are often used industrially in black chokeberry fruits processing and give products that differ significantly in their polyphenol content and composition (Denev et al., 2018). Juice AMRC was *Aronia melanocarpa* fruit juice in combination with *Rosa canina* (rosehip) extract, and juice AMAV was produced from *Aronia melanocarpa* fruit juice to which *Alchemilla vulgaris* extract was added. Conceived this way, the study would help answering the following questions: 1) Is the technological processing resulting in *Aronia melanocarpa* fruit juices with different polyphenol content and composition a prerequisite for a difference in the gastroprotective effect? 2) Will the *in vitro* synergistic effect in the antioxidant activity of combined *Aronia melanocarpa* and *Rosa canina* extracts result in a better gastroprotective effect *in vivo*? 3) Will the enrichment of *Aronia melanocarpa* fruit juice with polyphenols from *Alchemilla vulgaris* lead to an improved gastroprotective effect?

2. Materials and methods

2.1. Chemicals

Indomethacin (Indo) was purchased from Acros Organics (Geel, Belgium). Before administration, it was prepared as a suspension in a vehicle (2 drops of Tween 80 per 5 mL distilled water). Folin-Ciocalteu's phenol reagent was obtained from Merck (Darmstadt, Germany). Cyanidin-3-O-galactoside chloride, cyanidin-3-O-arabinoside chloride and cyanidin-3-O-arabinoside chloride were purchased from Extrasynthese S.A. (Genay Cedex, France). Gallic acid, chlorogenic acid, 3,4-dihydroxy benzoic acid, p-coumaric acid, caffeic acid, ellagic acid, ferulic acid, catechin, rutin, naringin, naringenin, epicatechin, myrecetin, quercetin-3-glucoside, quercetin, kaempherol, glucose, fructose, sucrose, sorbitol, quinic acid, tartaric acid, malic acid, ascorbic acid, α-keto-glutaric acid, citric acid, shikimic acid, oxalic acid were purchased from Sigma Aldrich (Steinheim, Germany). All other solvents used were of analytical grade and purchased from local distributors.

2.2. Plant materials

Aronia melanocarpa berries were supplied by the licensed farmer Todor Petkov (Kazanlak, Stara Zagora district, Bulgaria) in the stage of full maturity in August 2017. Fresh fruits were packed in polyethylene bags, frozen immediately and stored at −18 °C until the time of juice production. For preparation of juices, 5 kg frozen fruits were defrosted at room temperature and homogenized with a laboratory blender.

Rosa canina (rosehip) fruits were harvested from the Rhodope mountains in the autumn of 2016, frozen at −18 °C and freeze dried for 96 h in Alpha 1–4 LDplus laboratory freeze drier. After that, dried rosehips were deseeded and stored in paper bags at room temperature until the time of production of juice AMRC.

Dried *Alchemilla vulgaris* herb, produced by SD “Sharkovi i sie” (Lot № 17, Feb 2017) was purchased from a local pharmacy in Plovdiv, Bulgaria.

2.3. Preparation of juices

2.3.1. Preparation of juice AM1 and juice AM2

For preparation of *Aronia melanocarpa* juices AM1 and AM2, 1 kg of fruit homogenate was transferred in a brown-glass bottle and incubated in a thermostatic shaker water bath (NUVE, Turkey) for 1 h, at either 20 °C or 60 °C. After that, mixtures were filtered through cheesecloth and the liquid phases were centrifuged (20 min, 6200 g) in a bench top centrifuge Megafuge 1.0R (Heraerus Instruments, Germany). The obtained supernatants were denoted as juice AM1 and juice AM2, for the juices obtained at 20 °C or 60 °C, respectively.

2.3.2. Preparation of juice AMRC

For the preparation of rosehip extract, 200 g rosehips husks (without seeds) were mixed with 800 mL of ultrapure water and homogenized in a laboratory blender. The homogenized sample was

transferred into a brown-glass bottle and subjected to an extraction in thermostatic shaker water bath (NUVE, Turkey) for 1 h at 60 °C. After that, the mixture was centrifuged (20 min, 6200 g) and the supernatant was denoted as rosehip extract. For preparation of juice AMRC, juice AM2 was mixed with rosehip extract in a ratio of 70:30 (v/v).

2.3.3. Preparation of juice AMAV

For preparation of uice AMAV, dry lady's mantle aerial parts were milled to fine powder in a laboratory mill. After that, 20 g of the milled herb were mixed with 1 kg *Aronia melanocarpa* fruit homogenate and extracted in a thermostatic shaker water bath (NUVE, Turkey) for 1 h at 60 °C. The mixture was centrifuged (20 min, 6200 g) and the supernatant was denoted as juice AMAV.

All extractions were performed once.

2.4. Phytochemical screening and measurement of antioxidant activity

2.4.1. High performance liquid chromatography (HPLC) analysis of sugars

HPLC determination of sugars was performed using an Agilent 1220 HPLC system (Agilent Technology, USA), with a binary pump and Refractive Index Detector (Agilent Technology, USA). The column was Zorbax Carbohydrate (5 μm, 4.6 × 150 mm, Agilent), connected to a guard column Zorbax Reliance Cartridge (Agilent), 80% (v/v) acetonitrile was used as an eluent, at a flow rate of 1.0 mL/min and temperature 25 °C. Results were calculated using standard curves (range 0.1 mg/mL – 10 mg/mL) built with the corresponding sugar standards and expressed as g/100 mL juice.

2.4.2. HPLC determination of organic acids

HPLC determination of organic acids was performed by an Agilent 1220 HPLC system (Agilent Technology, USA), with a binary pump and UV–Vis detector (Agilent Technology, USA). Organic acid separation was performed using an Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25 °C at 210 nm wavelength. The mobile phase was 25 mM phosphate (K_2HPO_4/H_3PO_4) buffer (pH 2.4), with a flow rate of 1.0 mL/min. Results were calculated using standard curves (range 0.001 mg/mL – 1 mg/mL) built with the corresponding organic acid standards and expressed as mg/100 mL juice.

2.4.3. HPLC analysis of phenolic compounds

HPLC analysis of phenolic components was performed as described by Denev et al. (2018), using an Agilent 1220 HPLC system (Agilent Technology, USA), with a binary pump and UV–Vis detector (Agilent Technology, USA). Separation was performed on an Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25 °C using a wavelength of 280 nm. The mobile phases used were 0.5% (v/v) acetic acid (A) and 100% acetonitrile (B), at a flow rate of 0.8 mL/min. The gradient elution started at 14% (v/v) B, linearly increased to 25% (v/v) B between the 6th and 30th min, and then to 50% (v/v) B at the 40th min. Results were calculated using standard curves (range 0.001 mg/mL – 0.1 mg/mL) built with the corresponding phenolic standards and expressed as mg/L juice.

2.4.4. HPLC determination of anthocyanins

Anthocyanins were determined by an Agilent 1220 HPLC system (Agilent Technology, Palo Alto, Ca), with a binary pump and UV–Vis detector (Agilent Technology, USA). Wavelength of 520 nm was used. Anthocyanins were separated using an Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25 °C. The mobile phases used were 5% (v/v) formic acid (A) and 100% methanol (B) at a flow rate of 1.0 mL/min. The gradient elution started at 15% (v/v) B and linearly increased to 30% (v/v) B at the 20th min. Results were calculated using standard curves (range 0.001 mg/mL – 1 mg/mL) built with the corresponding anthocyanin standards and expressed as mg/L juice.

2.4.5. Total polyphenols analysis

Total polyphenols were determined by the method of Singleton and Rossi (1965), using the Folin-Ciocalteu's reagent. Gallic acid was used for the calibration curve and the results were expressed as gallic acid equivalents (GAE) per liter juice.

2.4.6. Oxygen radical absorbance capacity (ORAC) assay

ORAC was measured according to the method of Ou et al. (2001). ORAC analyses were carried out on FLUOstar OPTIMA plate reader (BMG Labtech, Germany) with an excitation wavelength of 485 nm and emission wavelength of 520 nm. The results were expressed as μmol of Trolox equivalents per liter (μmol TE/L).

2.5. Animals

The experiment was carried out on 72 male Wistar rats (weight 200–250 g) housed in plastic cages in a well ventilated room maintained at 22 ± 1 °C and on a 12/12 light/dark cycle. The rats were fasted 24 h before the ulcer induction but had free access to water. Throughout the rest of the time, the animals had free access to food (normal pelleted diet) and drinking water.

All experimental procedures were conducted according to the national laws and policies, in conformity with the international guidelines (EU Directive, 2010/63/EU for animal experiments).

The experimental protocol was approved by the Bulgarian Food Safety Agency (Document № 204/07.07.2017).

2.6. Experimental procedure

The experimental animals were divided into 6 groups, each of 12 rats: control, Indo, AM1 + Indo, AM2 + Indo, AMRC + Indo and AMAV + Indo. In the course of 10 days, the control and Indo groups were pretreated orally with distilled water (10 mL/kg) while groups AM1 + Indo, AM2 + Indo, AMRC + Indo and AMAV + Indo were pretreated with the respective juice at a dose of 10 mL/kg. On the 10th day, 1 h after the pretreatment, control rats were injected subcutaneously with the vehicle for indomethacin (2 mL/kg) and the rest of the groups were injected with indomethacin (30 mg/kg as a 2 mL/kg suspension). After 4 h, gastric ulcer formation was estimated.

2.7. Tissue preparation and macroscopic assessment of gastric lesions

On the 10th experimental day, 4 h after ulcer induction, the animals were anesthetized with diethyl ether. For biochemical investigations, blood from the sublingual veins was collected and serum was prepared by centrifugation of blood at 2000 rpm for 10 min. The serum was stored at −20 °C until analysis.

After the decapitation of the animals, laparotomy was performed. The stomach was removed, opened along the great curvature and washed in saline. After spreading of the stomach over the pad, macroscopic observation of the mucosal lesions was performed. Ulcer number was counted. The length and width of each lesion was measured for calculation of ulcer area. Five petechiae were considered as a 1-mm^2 lesion.

Stomach samples were taken and fixed in 10% neutral-buffered formaldehyde solution for histopathological examination. Samples of stomach tissue were frozen. Later, homogenates were prepared from them for biochemical investigations.

2.8. Calculation of ulcer index and percentage of protection

The severity of lesions was scored (Dekanski et al., 1975) as follows: 0 = no damage; 1 = blood at the lumen; 2 = pinpoint erosions; 3 = one to five small erosions < 2 mm; 4 = more than five small erosions < 2 mm; 5 = one or three large erosions > 2 mm; 6 = more than three large erosions > 2 mm.

Table 1
Chemical composition and antioxidant activity of *Aronia melanocarpa* fruit juices (juice AM1 and juice AM2) and *Aronia melanocarpa*-based juices containing *Rosa canina* extract (juice AMRC) or *Alchemilla vulgaris* extract (juice AMAV).

Ingredient	Juice AM1	Juice AM2	Juice AMRC	Juice AMAV
Dry solids (%)	21.7	22.0	17.1	26.0
Organic acids (mg/100 mL)				
Quinic acid	323.1 ± 26.1	325.2 ± 27.5	282.6 ± 19.1	507.2 ± 31.2
Malic acid	240.0 ± 22.6	239.1 ± 25.1	193.3 ± 12.1	323.8 ± 16.9
Ascorbic acid	78.8 ± 6.4	78.0 ± 5.9	66.9 ± 4.1	107.3 ± 6.5
Citric acid	33.8 ± 3.7	37.2 ± 3.9	117.2 ± 4.9	68.6 ± 3.5
Oxalic acid	1.9 ± 0.2	1.9 ± 0.2	2.3 ± 0.2	7.3 ± 0.2
Tartaric acid	3.1 ± 0.3	2.4 ± 0.2	2.4 ± 0.2	7.1 ± 0.3
Succinic acid	–	–	0.61 ± 0.1	10.47 ± 0.8
Sugars (g/100 mL)				
Fructose	3.45 ± 0.21	3.58 ± 0.28	3.01 ± 0.16	4.53 ± 0.29
Glucose	2.88 ± 0.2	2.80 ± 0.27	2.33 ± 0.15	3.74 ± 0.31
Sorbitol	10.33 ± 0.91	10.58 ± 0.70	9.09 ± 0.54	10.84 ± 0.68
Sucrose	0.12 ± 0.02	0.10 ± 0.01	0.12 ± 0.01	0.13 ± 0.01
Phenolic compounds (mg/L)				
Total polyphenol content	7772.7 ± 321.1	11237.4 ± 456.2	10802.1 ± 218.3	15929.1 ± 356.7
Flavonoids				
Quercetin	90.4 ± 8.7	49.6 ± 3.2	42.2 ± 2.5	76.7 ± 5.2
Quercetin-3-β-glucoside	103.6 ± 6.4	228.8 ± 11.0	192.5 ± 10.5	363.2 ± 19.4
Rutin	593.1 ± 21.3	446.5 ± 12.5	382.2 ± 15.6	2614.0 ± 189.5
Catechin	–	–	1230.4 ± 78.9	1731.5 ± 95.2
Epicatechin	251.4 ± 14.2	408.2 ± 25.6	383.3 ± 29.1	858.6 ± 50.2
Anthocyanins total	863.8	2125.0	1359.1	2148.4
Cyanidin-3-galactoside	638.5 ± 21.0	1498.4 ± 102.3	991.5 ± 45.6	1507.0 ± 58.2
Cyanidin-3-glucoside	44.4 ± 4.1	120.1 ± 8.7	78.9 ± 3.6	133.5 ± 9.8
Cyanidin-3-arabinoside	177.5 ± 13.2	501.9 ± 31.8	285.4 ± 14.5	502.3 ± 45.6
Cyanidin-3-xyloside	2.73 ± 0.2	4.59 ± 0.2	3.25 ± 0.3	5.51 ± 0.5
Phenolic acids				
Chlorogenic acid	1142.9 ± 81.2	1375.6 ± 80.3	1262.9 ± 56.2	1809.7 ± 103.8
Neochlorogenic acid	1305.2 ± 102.8	1543.1 ± 111.2	1499.2 ± 96.5	2027.0 ± 131.8
Caffeic acid	–	–	49.8 ± 4.1	184.0 ± 9.8
3,4-Dihydroxy-benzoic acid	–	–	181.1 ± 9.0	321.8 ± 16.5
ORAC, μmol TE/L	81256 ± 6545	122545 ± 9849	138569 ± 10253	168456 ± 10458

Results are presented as means from at least three measurements ± standard deviations (SD).

Having the score for each animal, the mean score for each group was calculated.

For each group, the following equation was used to calculate the ulcer index (UI) (Adinortey et al., 2013):

UI = total ulcer score/number of animals.

The percentage of protection (PP) for each pretreated group was calculated as follows (Adinortey et al., 2013):

PP = (UI of Indo group – UI of the pretreated group) x 100/UI of Indo group.

2.9. Histopathological investigation

Fixed specimens of the stomachs were embedded in paraffin, cut into sections and placed on microscope slides. Staining with hematoxylin and eosin (H & E) was used for the histopathological examination which was performed by light microscopy.

2.10. Immunohistochemical assays

The deparaffinized and dehydrated 4 μm sections were treated for 5 min with 3% hydrogen peroxide solution for peroxidase activity inhibition. For antigen retrieval, they were incubated for 20 min at 97 °C in the preheated EnVision FLEX Target Retrieval Solution. After cooling, the slides were placed in diluted FLEX Wash Buffer at room temperature for 1–5 min. Sections were stained using FLEX protocol in Dako Autostiner. The following antibodies were used: Bcl-2 (N-19) and Bax (C-20) (Santa Cruz, CA, USA) at a dilution of 1:50. Finally, peroxidase activity was estimated by the diaminobenzidine-tetrachloride H_2O_2-method. Counterstaining was performed using Mayer's hematoxylin. Negative controls were incubated with non-immune sera instead of primary antibody.

A morphometric method was used to assess semi-quantitatively the tissue contents of Bax and Bcl-2. On the basis of the occurrence of immune deposits, the content was scored as follows: 3 – strong, 2 – moderate, 1 – weak, 0 – lacking. From each sample, 150 cells were scored for Bax and Bcl-2. The average score of Bax and Bcl-2 expression was calculated for each sample.

2.11. Biochemical assays

2.11.1. Thiobarbituric acid reactive substances (TBARS)

Membrane lipid peroxidation was monitored my measurement of thiobarbituric acid reactive substances (TBARS) in rat gastric mucosal homogenates after the method of Ohkawa et al. (1979). Aurius 2021 UV-VIS spectrophotometer, Cecil Instruments Ltd, UK, was used. The color produced by the reaction of thiobarbituric acid with lipid peroxides was measured at 532 nm. Gastric tissue was homogenized using ice cold Tris/HCl, 50 mM, pH 7.4 (1:10). The homogenates were centrifuged (2000 rpm, 10 min, 4°C) and the supernatants were used for the biochemical assays. The TBARS levels were expressed as nmol/g tissue. The standard was malondialdehyde, the major product obtained from membrane fatty acids during their peroxidation.

2.11.2. Prostaglandin E_2 (PGE_2)

Minced gastric tissue was weighed, and homogenized with ice-cold phosphate-buffered saline (0.01M, pH = 7.4) at a ratio of 1:9. The homogenates were centrifuged for 5 min at 5000 × *g* and the supernatant was used for determination of PGE_2 activity using a commercial ELISA kit (Fine Test, Wuhan Fine Biotech Co., LTD, China). The optical density was measured at 450 nm using a microplate reader (800 TS, Bio-Tek Instruments,USA) and the results were presented as pg/g tissue.

2.12. Statistical analysis

Results are expressed as mean ± S.E.M. Using GraphPad Prism statistical software, data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison post hoc test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Phytochemical screening and ORAC assay

The results from the phytochemical screening and the ORAC activities of the investigated juices are presented in Table 1. The four juices had very high contents of polyphenolic compounds presented by flavonoids and phenolic acids. From the flavonoids, the highest was the content of anthocyanins (cyanidin glycosides). Phenolic acids were presented by neochlorogenic acid and chlorogenic acid in the four juices while caffeic acid and 3,4-dihydroxy-benzoic acid were present only in juices AMRC and AMAV. The total polyphenols and the individual polyphenolic ingredients were the highest in juice AMAV, followed by juice AM2, juice AMRC and juice AM1. The polyphenolic profile of juice AMAV is characterized by a very high content of rutin, epicatechin and catechin. From the results, it is evident that the difference in the extraction temperatures for juice AM1 and juice AM2 did not have a significant effect on the content of sugars and organic acids. However, it had a profound effect on the content of secondary metabolites – polyphenols and anthocyanins. In juice AM2, extracted at 60 °C, the contents of total polyphenols and total anthocyanins were respectively 45% and 146% higher in comparison with juice AM1. This is very important, since variation of technological parameters during processing could result in functional foods with different chemical composition, rendering different biological activity. The addition of the rosehip extract to juice AM2 decreased the contents of dry solids and sugars in the resulting juice AMRC. Besides, *Rosa canina* fruit extract decreased slightly the total polyphenol content but increased the ORAC of AMRC juice. Lady's mantle extract enriched aronia juice in sugars and organic acids, and increased its dry solids content to 26%. The effect on the phenolic content was even more significant, resulting in almost 16 g/L total polyphenols in juice AMAV. This was the product with the highest ORAC value, as well. The polyphenolic profile of juice AMAV is characterized by a very high content of rutin, epicatechin and catechin. Additionaly, it is richer in phenolic acids in comparison with other investigated products.

3.2. Macroscopic evaluation of gastric lesions

No gastric mucosal lesions were detected in the control rats. In Indo group, multiple mucosal lesions were observed in the glandular part of the stomach. They were widespread, linear and varied most often from 1 to 4 mm in length, bleeding at the moment of observation. The mean ulcer number in the Indo group was 12.53 ± 2.5 (Fig. 1A), and the mean ulcer area was 24.26 ± 6.5 mm^2 (Fig. 1B). Pretreatment with juice AM1, juice AM2, juice AMRC and juice AMAV reduced the ulcer number by 38.8%, 78.7%, 51.4% and 84.8%, respectively, and the ulcer area by 47.6%, 66.0%, 46.8% and 94.6%. The results for ulcer number were significantly different from Indo group for groups AM2 + Indo ($p < 0.01$), AMRC + Indo ($p < 0.05$) and AMAV + Indo ($p < 0.001$) (Fig. 1A). The reduction of ulcer area was significant in AMAV + Indo group ($p < 0.01$) in comparison with Indo group (Fig. 1B) (see Fig. 2).

3.3. Ulcer index and percentage of protection

Using scoring criteria for assessment of the severity of mucosal lesions (Dekanski et al., 1975), the mean ulcer score, the ulcer index (UI) and percentage of protection (PP) were calculated. The results are presented in Table 2.

3.4. Histopathological investigation

The microscopic appearance of the stomach wall of the control rats was quite normal (Fig. 3A). The lesions evoked by indomethacin were manifested by erosions comprising about 2/3 of the mucosal layer, filled with blood and haematin materials (Fig. 4).

In the rats pretreated with the four juices, the gastric erosions were more superficial and in some cases only bleeding and focal desquamation of the superficial epithelium or zonal destructions of gastric glands were found (Fig. 3C, D, 3E and 3F).

3.5. Immunohistochemical assays

The Bax and Bcl-2 expression scores, measured semi-quantitatively, are presented in Fig. 4.

The results from the immunohistochemical assay showed that in the control group, Bax was expressed in the cytoplasm of all epithelial cells with the highest expression in the lower one-third of the mucosa (Fig. 5A). In Indo group, Bax expression score was significantly increased ($p < 0.001$ vs. control) (Fig. 4A) and the increase was most prominent in the lower two-thirds of the mucosa (Fig. 5B). In the groups AM1 + Indo, AM2 + Indo and AMRC + Indo, the Bax expression was high (Fig. 5C, D and 5E). The semi-quantative measurement showed that the Bax expression scores for these groups were significantly lower in comparison with Indo group ($p < 0.001$ vs. Indo) (Fig. 4A) but remained significantly higher than that of the control group ($p < 0.001$ vs. control) (Fig. 4A). The lowest was the Bax expression in AMAV + Indo group, similar to that of the control group (Fig. 5F), and the Bax expression score was not significantly different from the control one (Fig. 4A).

Bcl-2 was expressed in the cytoplasm of all epithelial cells in the control group, the highest being the expression in the lower two-thirds of the mucosa (Fig. 6A). The Bcl-2 expression in Indo group was significantly lower ($p < 0.001$) than that of the control group (Fig. 4B), especially in the lower two-thirds of the mucosa (Fig. 6B). In all pretreated groups (AM1 + Indo, AM2 + Indo, AMRC + Indo and AMAV + Indo), the expression of Bcl-2 was increased (Fig. 6C, D, 6E and 6F). The Bcl-2 expression scores of AM1 + Indo and AM2 + Indo groups were significantly higher ($p < 0.01$ and $p < 0.001$, respectively) than that of Indo group and did not differ significantly from that of the Control group (Fig. 4B) while the Bcl-2 expression scores for AMRC + Indo and AMAV + Indo groups were even significantly higher ($p < 0.001$) than that of the control group (Fig. 4B).

3.6. Thiobarbituric acid reactive substances (TBARS)

In Indo group, gastric TBARS concentration increased by 30.8% in comparison with that of the control group but the result was not significantly different (Fig. 7). In comparison with Indo group, the reduction of TBARS in gastric tissue was respectively by 40.3% in group AM1 + Indo, 25.6% in group AM2 + Indo, 42.3% in group AMRC + Indo and 32.3% in group AMAV + Indo. Thus, the decrease was statistically significant for AMRC + Indo group ($p < 0.05$ vs. Indo group) (Fig. 7).

3.7. Prostaglandin E_2 (PGE_2)

PGE_2 concentration was reduced by 7% in Indo group in comparison with that of the Control group. The pretreatment of rats with the juices had the opposite effect but all these changes in PGE_2 concentration were not statistically significant (Fig. 8).

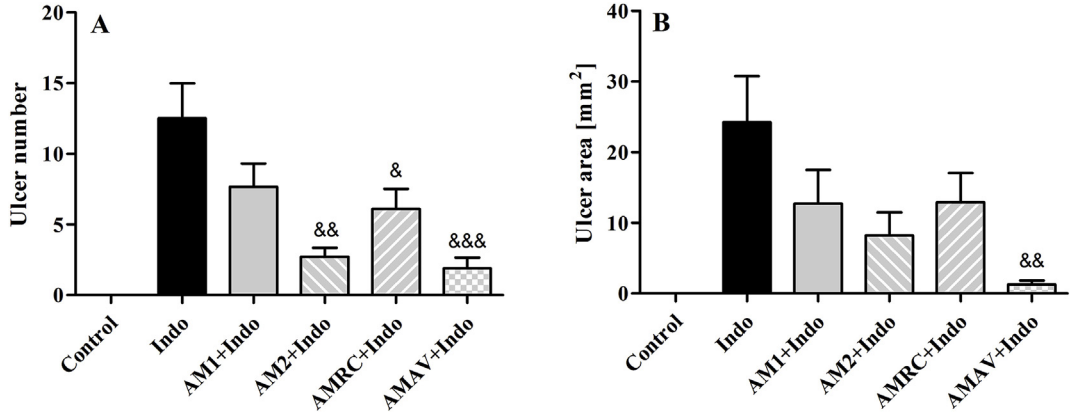

Fig. 1. Effects of *Aronia melanocarpa* juices either alone (juices AM1 and AM2) or combined with extracts from *Rosa canina* or *Alchemilla vulgaris* (juices AMRC and AMAV) on the number (panel A) and area (panel B) of gastric mucosal lesions in a rat model of indomethacin-induced ulceration; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. Indo group.

4. Discussion

This study investigated the effects *Aronia melanocarpa* juices either alone or combined with extracts from *Rosa canina* or *Alchemilla vulgaris* on the severity of indomethacin-induced gastric ulcers in rats. This model was chosen because of the high prevalence of NSAIDs-induced gastric damage (Huang et al., 2002). In the current experiment, indomethacin caused a severe gastric ulceration proven by a number of macroscopic indices, histopathological and immunohistochemical investigations. Nonsteroidal anti-inflammatory drugs have been reported to disrupt the hydrophobic barrier properties of the gastric mucus layer as well as to disrupt membrane phospholipids and induce pore formation (Darling et al., 2004; Lichtenberger et al., 2006). All these changes allow acid diffusion into cells leading to apoptosis, necrosis and ulcer formation. The results from the immunohistochemical investigation proved the induction of apoptosis as an important mechanism underlying the ulcerogenic effect of indomethacin. In this experiment, indomethacin had prominent effects on expression of proteins engaged in regulation of apoptosis. It increased the expression of Bax (Figs. 4 and 5) and decreased the expression of Bcl-2 (Figs. 4 and 6). An overexpression of Bax promotes cell death while Bcl-2 overexpression suppresses apoptosis and enhances cell survival (Qiao et al., 2011). Bcl-2 protein forms heterodimers with Bax, thus antagonizing its function (Xia and Talley, 2001).

A very important mechanism underlying the ulcerogenic effect of

Table 2
Ulcer score, ulcer index (UI) and percentage of protection (PP) in a rat model of indomethacin-induced ulceration and pretreatment with juice AM1, juice AM2, juice AMRC and juice AMAV; $p < 0.01$ vs. Indo group.

Group	Ulcer score (mean ± S.E.M)	UI	PP (%)
Control	0	0	–
Indo	4.41 ± 0.45	4.41	–
AM1 + Indo	3.17 ± 0.61	3.17	28.12
AM2 + Indo	3.00 ± 0.60	3.00	31.97
AMRC + Indo	3.75 ± 0.59	3.75	14.97
AMAV + Indo	1.40 ± 0.54$^{\&\&}$	1.40	68.25

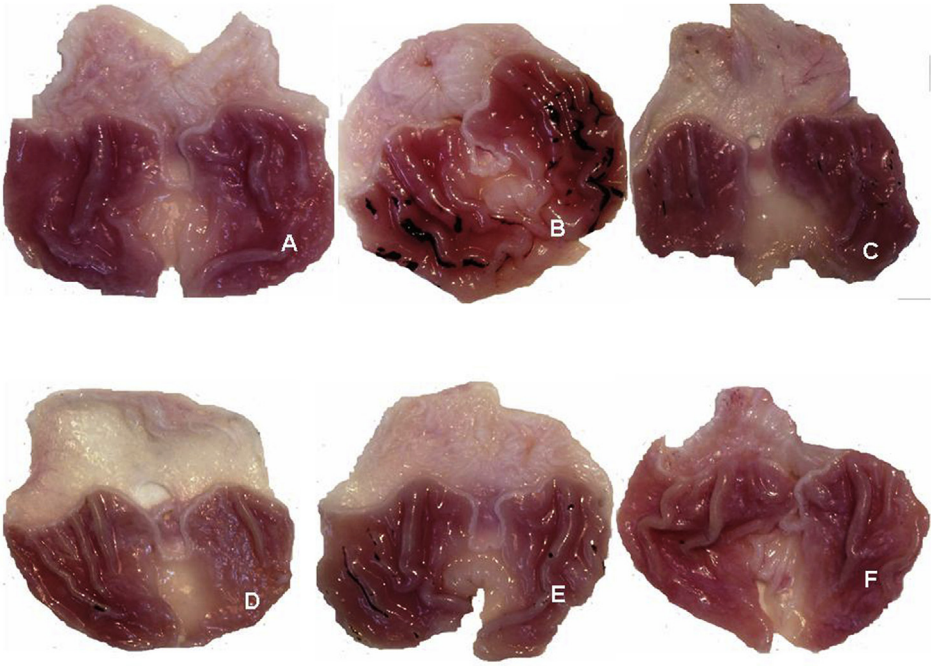

Fig. 2. Macroscopic appearance of stomachs in a rat model of indomethacin-induced ulceration: A. Control – Normal gastric mucosa; B. Indo – Hemorrhagic ulcerations; C. AM1 + Indo; D. AM2 + Indo; E. AMRC + Indo; F. AMAV + Indo. Reduction of hemorrhagic lesions in all pretreated groups (C, D, E, F).

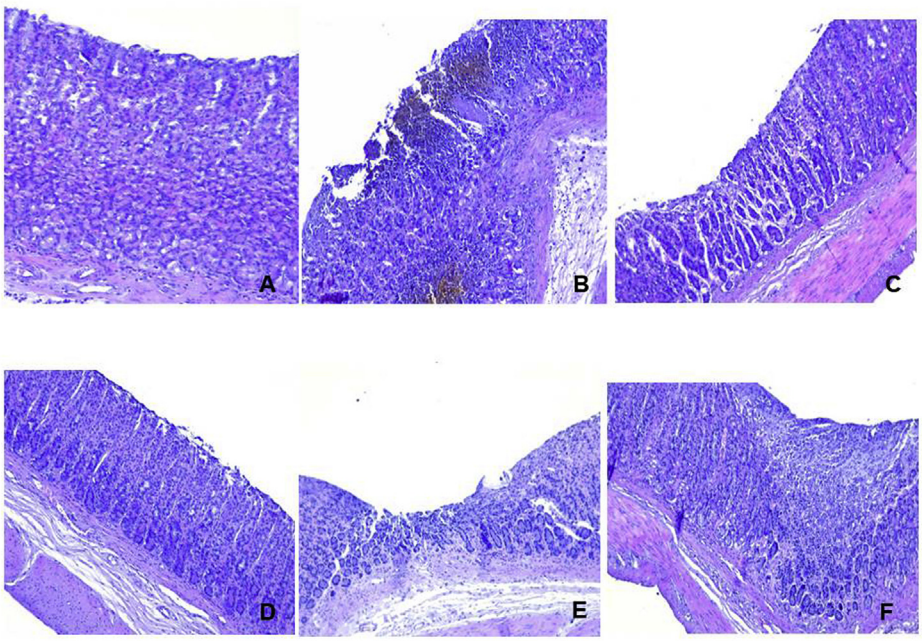

Fig. 3. Microscopic appearance of stomach wall in a rat model of indomethacin-induced ulceration: A. Control – Normal gastric mucosa; B. Indo – Hemorrhages and deep erosions; C. AM1 + Indo – Zonal destructions of gastric glands; D. AM2 + Indo – Desquamation of the superficial epithelium; E. AMRC + Indo – Superficial mucosal erosion; F. AMAV + Indo – Zonal destructions of gastric glands. H & E staining; magnification ×200.

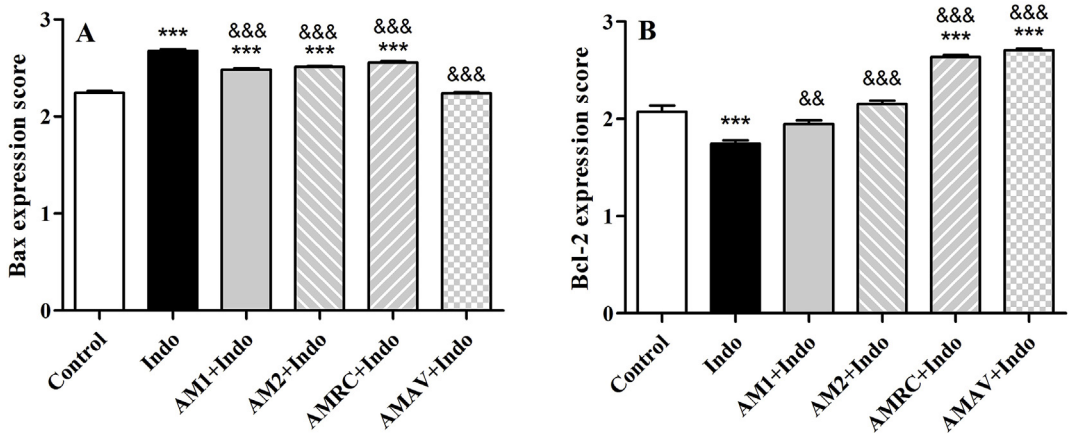

Fig. 4. Effects of *Aronia melanocarpa* juices either alone (juices AM1 and AM2) or combined with extracts from *Rosa canina* or *Alchemilla vulgaris* (juices AMRC and AMAV) on the expression score of Bax (panel A) and Bcl-2 (panel B) in gastric mucosa in a rat model of indomethacin-induced ulceration; ***$p < 0.001$ vs. Control group; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. Indo group.

NSAIDs is their ability to inhibit the production of gastroprotective prostaglandins (PGs) due to the inhibition of cyclooxygenase (COX). Studies have demonstrated that indomethacin suppresses the gastric PG synthesis (Shorrock and Rees, 1992). In the present experiment, PGE_2 concentration was non-significantly reduced by indomethacin (Fig. 8). This is in accordance with the results of other authors (Saad et al., 2002) who have concluded that the high enough inhibition of COX by indomethacin needs a much longer time and does not occur 6 h after a single dose.

Another well enough accepted mechanism of NSAIDs-induced ulcerogenesis is the oxidative stress (Matsui et al., 2011; Kwiecień et al., 2015; Bjarnason et al., 2018). Reactive oxygen species (ROS) cause a destruction of cellular membranes by inducing lipid peroxidation. Ion trapping causes accumulation of NSAIDs in gastric epithelial cells and a subsequent uncoupling of mitochondrial oxidative phosphorylation takes place. This results in depletion of intracellular adenosine triphosphate and generation of ROS (Orrenius, 2007). Polymorphonuclear cells infiltrating the gastric mucosa also produce ROS (Grisham and Granger, 1988). In this study, there was a slight but not significant increase of TBARS in indomethacin-treated rats (Fig. 7). Thus, oxidative stress might contribute but is not a leading mechanism in the current indomethacin-induced ulceration. This finding was in accordance with the histopathological results which did not demonstrate a high infiltration of gastric mucosa with polymorphonuclear cells but the prevailing changes were the destructive and necrotic processes.

The pretreatment of rats with the four juices ameliorated the severity of the ulcer model which was demonstrated by macroscopic indices as well as by histopathological and immunohistochemical investigations. Pretreatment of rats reduced the ulcer number and ulcer area, as well as the ulcer score and ulcer index (Fig. 1, Table 2). According to the ulcer number, the protection was in the following order: AMAV > AM2 > AMRC > AM1. These results correlate very well with the polyphenol content of the tested products. Juice AMAV, characterized by the highest total polyphenol content, showed the best gastroprotective effect. Regarding the ulcer number, the effect of juice AM1 was not significant but the histopathological investigation proved a protective effect as the lesions in AM1 + Indo group were more superficial in comparison with those in the Indo group (Fig. 3). However, the complex morphometric and histopathological evaluation showed that the least was the protection by juices AMRC and AM1, and these

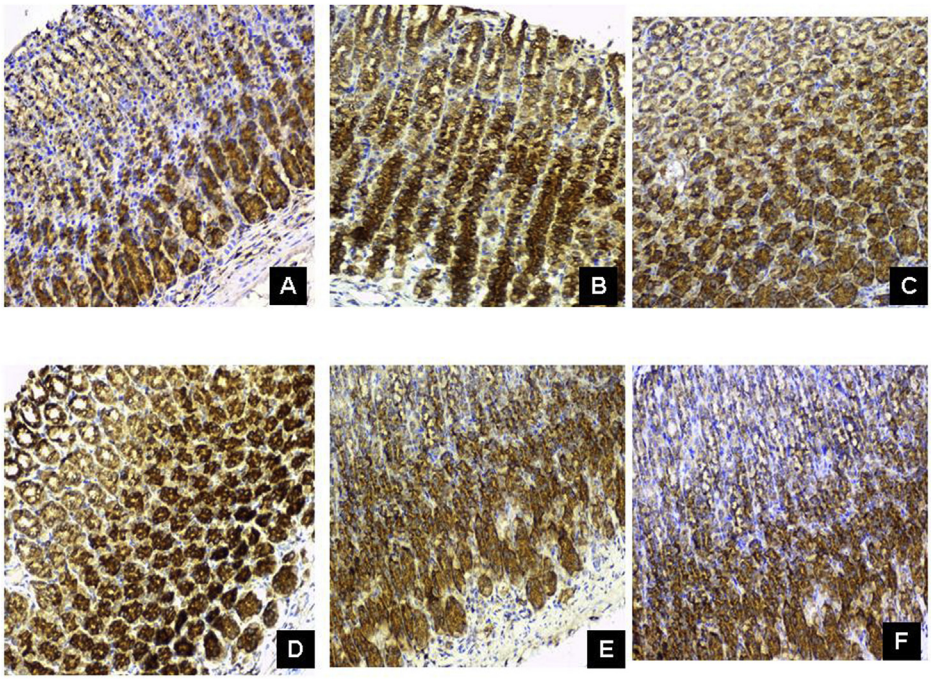

Fig. 5. Bax expression in gastric mucosa in a rat model of indomethacin-induced ulceration; A. Control – Bax positive cells predominantly in the basal part of the glands; B. Indo – Diffuse and intensive Bax expression throughout the glands; C. AM1 + Indo, D. AM2 + Indo, E. AMRC + Indo – High level of Bax expression (C, D, E); F. AMAV + Indo – Bax expression predominantly in the basal part of the glands; Imminohistochemistry; magnification × 200.

were the juices with the lowest content of polyphenolic substances. The differences in the phytochemical composition of juice AM1 and juice AM2, which were produced from the same aronia fruits, but at different temperatures, led to a difference in the observed gastroprotective effects. This demonstrates that the parameters of the technological processing are very important, not only for the chemical composition of the functional beverages but for their biological activities as well.

In this experiment, probably the most important mechanism underlying the gastroprotective effect of the juices was the inhibition of apoptosis. The four investigated juices antagonized the effects of indomethacin on apoptosis markers. They caused a highly significant decrease of Bax and increase of Bcl-2 expression in gastric tissue (Figs. 4–6). In this respect the highest was the activity of juice AMAV.

The antioxidant effects of the juices might also account for the beneficial effects of the treatments. *Aronia melanocarpa* friut juice has been demonstrated to possess a powerful antioxidant activity (Valcheva-Kuzmanova et al., 2007). *Aronia melanocarpa* polyphenols act as radical scavengers (Zheng and Wang, 2003; Jakobek et al., 2011; Valcheva-Kuzmanova et al., 2012a, 2014) and increase the endogenous antioxidant defenses in experimental models of organ damage and toxicity (Valcheva-Kuzmanova et al., 2004, 2012b). In the present experiment, indomethacin did not cause a high increase of lipid peroxidation and correspondingly, the effect of the pretreatment with the juices was not very pronounced (Fig. 7). However, there was a statistically significant decrease in TBARS in gastric tissue in AMRC + Indo group in comparison with Indo group. This result is very interesting, since it indicates that the observed effect on TBARS is not due only to the total polyphenol content of the investigated samples. More probably

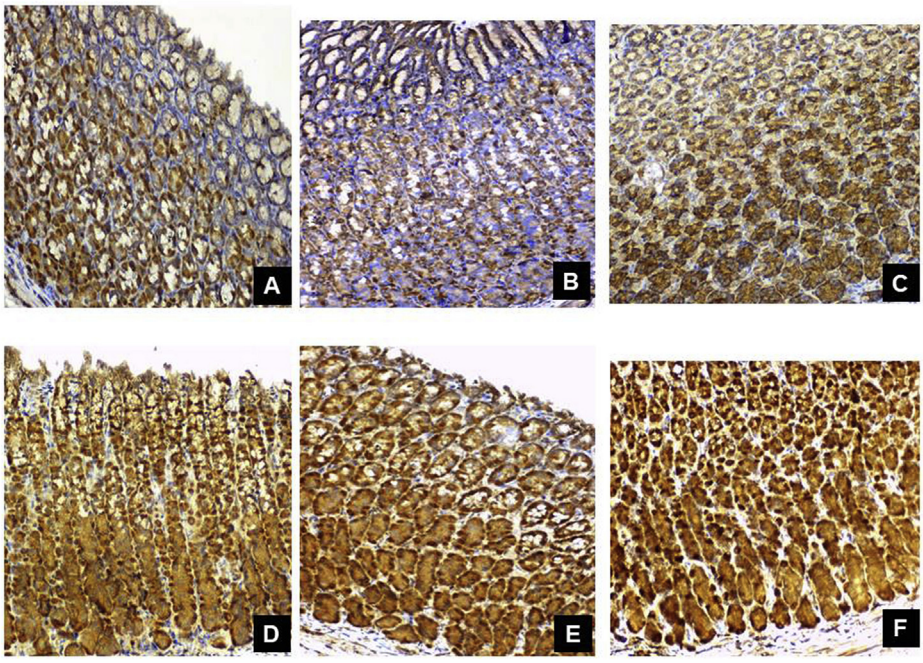

Fig. 6. Bcl-2 expression in gastric mucosa in a rat model of indomethacin-induced ulceration; A. Control – Bcl-2 expression mainly in the lower two-thirds of the gastric mucosa; B. Indo – Decreased Bcl-2 expression; C. AM1 + Indo, D. AM2 + Indo, E. AMRC + Indo, F. AMAV + Indo – Diffuse and intensive Bcl-2 expression throughout the glands in all pretreated groups (C, D, E, F); Imminohistochemistry; magnification × 200.

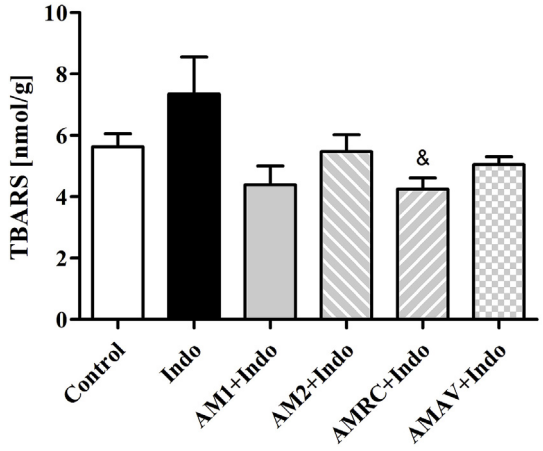

Fig. 7. Effects of *Aronia melanocarpa* juices either alone (juices AM1 and AM2) or combined with extracts from *Rosa canina* or *Alchemilla vulgaris* (juices AMRC and AMAV) on the level of thiobarbituric acid reactive substances (TBARS) in gastric mucosa in a rat model of indomethacin-induced ulceration; $^{\&}p < 0.05$ vs. Indo group.

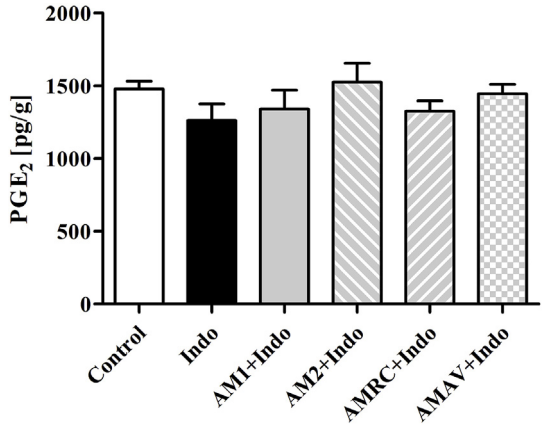

Fig. 8. Effects of *Aronia melanocarpa* juices either alone (juices AM1 and AM2) or combined with extracts from *Rosa canina* or *Alchemilla vulgaris* (juices AMRC and AMAV) on PGE_2 level in gastric tissue in a rat model of indomethacin-induced ulceration.

it is due to some other components present in rosehips. This juice as a combination of chokeberry fruit juice and rosehip extract revealed a synergism in the *in vitro* antioxidant activity.

Another mechanism that might contribute to the gastroprotective effect of the juices might be an effect on mucus production and properties. A previous experiment with *Aronia melanocarpa* fruit juice in a model of indomethacin-induced gastric ulcers has demonstrated an increase of the mucus production by the juice (Valcheva-Kuzmanova et al., 2005b). There are other investigations showing that polyphenolic substances increase mucus production that is not accompanied by an increase in PGE_2 level (Galati et al., 2003; Laloo et al., 2014). Apart from this, cross-linking of mucins by polyphenols is expected to improve the barrier properties of the gastric mucus (Georgiades et al., 2014). In this experiment, PGE_2 did not participate in the gastroprotection. The juices did not cause significant changes in PGE_2 concentration in gastric tissue (Fig. 8). Even a decrease in PGE_2 concentration could be expected on the basis of literature data of inhibition of COX enzymes (COX-1 and COX-2) by *R. canina* hip powder extracts (Jäger et al., 2007), *Alchemilla vulgaris* extract (Boroja et al., 2018) and aronia berry extracts (Ohgami et al., 2005; Jeong, 2008; Kang et al., 2017).

The effect of the juices on gastric secretion was not investigated in this study. However, an anti-secretory effect might be another possible mechanism staying behind the ulceroprotection by the juices. *Aronia melanocarpa* fruit juice has been shown to elevate the gastric pH and to reduce the volume of gastric content and the total acid output in an experimental model of ulcerogenesis induced by immobilization stress and pylorus ligation (Valcheva-Kuzmanova et al., 2005a). Polyphenolic ingredients of plants such as quercetin and anthocyanins, have been reported to reduce gastric histamine content by inhibition of the enzyme histidine decarboxylase and thus, to reduce gastric acid secretion (Alarcón de la Lastra et al., 1994; Nitta et al., 2017; Uno et al., 2017). Quercetin and anthocyanins were important ingredients of the four investigated juices (Table 1). Especially high was the total anthocyanin content of juice AM2 and juice AMAV, the juices with the most pronounced effects in this study. Phenolic acids such as caffeic, ferulic, gallic, cinnamic and coumaric, have been demonstrated to inhibit H^+/K^+-ATPase, the membrane protein responsible for gastric acid secretion (Siddaraju and Dharmesh, 2007a, 2007b). Caffeic acid was found in juice AMRC and even at a higher concentration in juice AMAV (Table 1). All these individual polyphenolic ingredients (quercetin, anthocyanins and caffeic acid) might contribute to the gastroprotective effects of the juices through a possible anti-secretory mechanism.

In conclusion, in this study the two *Aronia melanocarpa* fruit juices (juice AM1 and juice AM2) and the two *Aronia melanocarpa* juices combined with *Rosa canina* or *Alchemilla vulgaris* extracts (juice AMRC and juice AMAV) reduced the severity of indomethacin-induced gastric damage. The effects of the juices were proven by macroscopic and microscopic indices, as well as by immunohistochemical investigations. The highest was the protection from the juice AMAV which was with the highest content of phenolic substances. The mechanism involved to the highest extent in the gastroprotective effect of the juices was the inhibition of apoptosis.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.110739

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