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## Reduction of anxiety-like and depression-like behaviors in rats after one month of drinking *Aronia melanocarpa* berry juice

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### ABSTRACT

The treatment of mood and anxiety disorders by nutraceuticals is gaining growing awareness. Berries of *Aronia melanocarpa* (Black chokeberry) and their extracts, exceptionally abundant in diverse phenolic compounds, have become famous for the highest *in vitro* antioxidant activity among fruits and notable health benefits (e.g. anti-diabetic, anti-inflammatory, cardioprotective). This study was designed to investigate the behavioral effects of month-long unlimited consumption of *Aronia* master juice (AJ) and/or juice reconstruct without polyphenols (RJ), in young male rats. AJ was initially evaluated for its content of phenolic compounds by spectrophotometric assays and HPLC-DAD. Rats that were supplied with three various water concentrations of AJ and RJ, respectively: 20%+0% (ARO group), 5%+15% (RAJ) and 0+20% (PLC), were compared with those which consumed only water (CTL). Daily drinking of AJ solutions was significantly elevated from the second or third week onward, which was most expressed in the ARO group. Only this group displayed behavioral variations, manifested by certain hyperactivity in open field tests and prominent reductions of anxiety-like behaviors in the elevated plus maze. The ARO rats also expressed an alleviation of depression-like behavior in forced swimming tests. These findings demonstrate the beneficial behavioral effects of the one-month-long free drinking of phenolic-rich AJ in rats (> 20 ml/kg b. mass daily) that may be recognized as stimulating, anxiolytic-like and antidepressant-like. The *in vitro* assays suggested that MAO-A/MAO-B inhibitions by the phenolic compounds of AJ might be the possible *in vivo* mechanisms for such behavioral actions.

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## 1. Introduction

Medical literature suggests that mental illnesses have become almost epidemic in the developed world.<sup>1</sup> Based on clinical documentation, it was estimated that nearly 20% of both the adult and youth population in the USA had some form of mental ailment in 2013.<sup>2</sup> Mood and anxiety disorders are two kinds of psychiatric diseases that attract most of the attention of the public and medical community, which is based on the evidence that more than half of mankind may be affected by either of these complaints during their life.<sup>1</sup> Besides psychotherapy, the first-line option in the management of these illnesses is pharmacotherapy, where a growing number of psychoactive drugs exerts a substantial degree of beneficial effects.<sup>3,4</sup> Unfortunately, most of these drugs are commonly associated with unwanted side-effects and many individuals exhibit refractory or intolerant responses.<sup>1,3,4</sup> Therefore, a certain part of research on psychiatric diseases has focused on the investigation of alternative therapies or adjunctive strategies, which could improve clinical outcomes.<sup>5</sup> The use of nutraceuticals is one of the main approaches to that effect.<sup>5-8</sup>

The *Aronia* shrub, a member of the *Rosaceae* family, has become a very popular cultivar in Eastern and Northern Europe, where it is grown as an ornamental plant and industrial source of natural coloring agents.<sup>9</sup> The berry of *Aronia melanocarpa* Elliott (Black chokeberry; the genus name *Aronia* has been replacing this common name) is rich source of polyphenols, especially anthocyanins, which are correlated with high antioxidant activity.<sup>9,10</sup> *Aronia* has a simple anthocyanin spectrum, consisting almost exclusively of cyanidin glycosides such as cyanidin-3-arabinoside and cyanidin-3-galactoside, but has a manifold higher anthocyanin content in comparison with other berry fruits.<sup>10,11</sup> Proanthocyanidins and hydroxycinnamic acids (chlorogenic and neochlorogenic acids) are also found in greater amounts while flavonols (quercetin glycosides) and flavan-3-ols (epicatechin) are usually present as minor components in *Aronia* berries.<sup>10,12</sup> A number of experimental and clinical studies revealed that anthocyanins themselves, such as anthocyanin-rich *Aronia* extracts, exhibited diverse potential health benefits, including cardioprotective, anti-diabetic and anti-inflammatory effects.<sup>9,13-15</sup> Some limited studies have also shown the effects of *Aronia* juices on the behavior and cognitive functions of experimental animals.<sup>16,17</sup> Our observation that certain other polyphenol-rich fruit extracts, although lower in phenolics when compared to *Aronia*, have provoked a number of animal and human studies that investigated their behavioral/psychological effects,<sup>18-21</sup> inspired us to explore those particular influences of the juice of *Aronia*.

This behavioral study was carried out on young male rats, which were provided for one month with unlimited consumption of water dilutions (20%, v/v) of one of the following: master *Aronia* juice (AJ) with a high concentration of phenolic compounds, juice reconstruct without phenolic compounds (RJ), or their combination (AJ:RJ=1:3), where the control group of animals was supplied with pure water. The influences of the drinking variants on the overall activity and locomotion, as well as on anxiety-like and depression-like behaviors, were evaluated using appropriate animal behavioral models and are discussed later. AJ was originally assayed for total phenolic constituents and proanthocyanidins, while the particular phenolic compounds were identified and quantified by the HPLC/DAD method. The capacity of AJ to inhibit *in vitro* monoamine-oxidase enzymes, MAO-A and MAO-B, was estimated and compared with analogous effects of the several phenolic compounds found in AJ. The relations between the potential *in vivo* MAO inhibition by the phenolic compounds of *Aronia* and the experimental behavioral parameters registered in this study are discussed.

## 2. Materials and methods

### 2.1 Materials

A commercial product ("Nutrika", Belgrade, Serbia), obtained by pressing of fresh *Aronia* berries and presented as a pure and concentrated *Aronia* master juice (AJ), was used to prepare drinking solutions in our lab. The manufacturer's analytical data for the AJ batch used in this study, regarding the nutritional values of the most abundant components of AJ, were used to prepare RJ. It contained the exact same amounts of particular sugars, organic acids, minerals and vitamins as AJ, but without the bioactive phenolics (in g/L): glucose 45.0, fructose 28.0, sorbitol 50.0, citric acid 1.21, K-citrate-monohydrate 6.0; in mg/L: vitamin C 29.2, vitamin B1 0.38, vitamin B2 0.71, vitamin B6 0.44, niacin 0.39, CaCl<sub>2</sub>·2H<sub>2</sub>O 559, MgCl<sub>2</sub> 550, NaCl 760). Both master beverages, AJ and RJ, were stored at 4°C during the study. All chemical reagents applied were of analytical grade and produced either by "Sigma" (USA) or "Serva" (Germany).

### 2.2 Analysis of phenolic compounds in AJ

The total phenolics in AJ were determined by a modified Folin-Ciocalteu method and presented in mg/L of gallic acid equivalents.<sup>22,23</sup> Identification and quantification of individual phenolic compounds were performed by chromatographic analysis (Agilent series 1100 HPLC instrument with a DAD detector) on a reverse phase Zorbax SB-C18 (Agilent) analytical column (250 mm x 4.6 mm i.d., 5 µm particle size).<sup>23,24</sup> Before its injection into the HPLC

system, AJ was filtered through a 0.45 µm membrane filter. The analysis of flavonoids and hydroxycinnamic acids was performed with a mobile phase that consisted of solvent A (1 % v/v solution of orthophosphoric acid in water) and solvent B (acetonitrile) using an elution gradient according to the previously published method: 90-80 % A 0-5min, 80 % A 5-20 min, 80-40 % A 20-30 min, 40- 0 % A 30-35 min.<sup>24,25</sup> The injection volume was 5 µl. Detection wavelengths were set at 260 and 350 nm, and the flow rate was 1ml/min. Quantification was performed using standardized calibration curves of flavonoids (rutin, hyperoside and isoquercetin) and hydroxycinnamic acids (chlorogenic and neochlorogenic acids). The anthocyanin analysis utilized a mobile phase that consisted of solvent A (10% of formic acid in water) and solvent B (acetonitrile) with an elution profile as follows: 99% A 0-0.5 min, 99-93% A 1-4 min, 93-90% A 4-7 min, 90-86% A 7-11 min, 86-75% A 11-15 min, 75-60% A 15-18 min, 60-25% A 18-22 min, 25% A 22-25 min. Detection wavelengths were set at 290, 350 and 520 nm, and the flow rate was 1 ml/min. The contents of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-arabinoside were determined using calibration curves, while cyanidin-3-*O*-galactoside and cyanidin-3-*O*-xyloside were quantified in equivalents of cyanidin-3-*O*-arabinoside. All experiments were repeated at least two times. The results are presented as mg/L of AJ.

Total proanthocyanidins were determined spectrophotometrically by a slightly modified vanillin assay.<sup>26</sup> Namely, 20 µl of the 2% AJ in methanol in duplicate, or a range of concentrations of (+)-catechin in methanol, were mixed with 100 µl of working vanillin reagent (0.5% vanillin and 4% HCl in methanol). After 20 min at 30°C, their absorbencies were measured at 500 nm (A500). The contents of proanthocyanidins in ARJ were expressed as (+)-catechin equivalents in mg/L.

### 2.3 Animals and treatments

Male adult Wistar rats, 4 months old, weighing 370–430 g, provided by the vivarium of the Institute for Biological Research, Belgrade, Serbia, were used in this study. The animals were kept in groups of 3-4 per cage (42 x 26 x 18cm), under controlled conditions (room temperature 23–25°C, 12 h light-dark cycle, food and drinking solutions *ad libitum*). All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes. They were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

Rats were randomly assigned to 3 experimental groups of 8 animals each, and one control group (N=12). The experimental groups were supplied with different drinking solutions, prepared daily as combinations of AJ and RJ in tap water (v/v): (1) ARO, contained 20% AJ; (2) RAJ, 5% AJ +15% RJ; (3) PLC, 20% RJ. The control group (CTL) consumed tap water. The rats were allowed to drink these

solutions or water for the next 34 days, without restrictions. The amounts of the consumed drinking solutions and food were estimated daily per cage and calculated weekly per experimental group. Individual body masses, which had been measured before (Day-1) and at the end of the study (Day35), were used to determine the average gain of body mass ( $\pm$ S.E.M.) for each experimental group. All animals were consuming commercial rat chow (Veterinarski zavod Subotica, Serbia; 20% of proteins). On the days: -1, 31, 32 and 34, appropriate behavioral tests were performed (09h-13h), when rat activities were registered in a quiet room with shaded illumination, by video recording. Most of the behavioral parameters were detected and analyzed by ANY-maze Video tracking system (4.13.; Stoelting, USA) while the remaining behavioral features were observed and recounted by a proficient examiner, who was unaware of group qualification of the rats. All parts of the apparatus that came in contact with the animals were cleaned with 10% alcohol at the end of each session.

## 2.4 Behavioral tests

**2.4.1 Open field test (OFT).** This test was used to estimate average ambulation of each rat on Day-1 and Day31 of the study, when they were allowed to move freely in a plastic open-box (44 x 44 x 35 cm). The movements of single rats in four adjacent boxes were simultaneously registered by a PC-connected camera positioned centrally above, for 20 min. The ANY-maze software evaluated animal activities according to two chosen parameters: (1) the measure of overall activity, presented by the percentage of total time when the animal was active, and (2) locomotion, by the distance traveled (in m).<sup>27</sup> The third analyzed parameter, (3) number of rearings (standing upright on the hind legs), which reflected vertical activity, was counted during observation of the video recordings.

**2.4.2 Elevated plus maze (EPM).** This is a typical test based on the innate fear of rodents of open and elevated spaces, which is applied to detect anxiety-like behaviors and to evaluate anxiolytic-like treatments in experimental animals.<sup>27</sup> The EPM apparatus consisted of a cross-shaped Plexiglas platform with two opposite open arms (OA, 50 x 10 cm) and two opposite closed arms (CA, 50 x 10 cm) with 40 cm walls, connected by a central platform (CP, 10 x 10 cm) and elevated 50 cm from the floor.<sup>27</sup> On the Day32, each rat was placed in a CP facing one of the CAs. Their movements were recorded for the next 5 min with a video camera mounted vertically above the apparatus. The level of rat anxiety was estimated by the periods spent in OA and number of entries into OA, which were quantified and presented in our test as % of OA time and % of OA entries.<sup>27,28</sup> Another two parameters, the number of entries in CA and the time of immobility, were also used, as they may reflect general activity and ambulation.<sup>28,29</sup> While these four parameters were estimated by Any-maze software, the other two ethological parameters were detected and counted by an observer: the number of

SAP (stretched attend posture; when a rat stretches its head and shoulders before returning to its initial position), which represented risk assessment, and the number of head dips (when a rat scans over the side of an OA towards the floor), as a reflection of exploratory behavior.<sup>28,30</sup> A random half of CTL animals (n=6), which were being supplied with pure drinking water, was considered as a primary control group with non-monitored behavior (CTL). They received saline (0.9% NaCl) 1ml/kg of b.mass (*ip*), 60 min before the test, and the same was applied to all rats in the experimental groups. The other 6 CTL rats were treated with 1 mg/kg of anxiolytic diazepam in saline, in the same manner as the other groups. They were considered as a reference treatment group with reduced anxiety-like behaviors (DZP).

**2.4.3 Forced swimming test (FST).** This test is based on the observations that rats forced to swim in an inescapable space express characteristic behavior of immobility, when they are floating in water and making only those movements that are necessary to keep their heads afloat. Such immobility reflects a state of despair or a depression-like form of behavior that can be reduced by human antidepressant drugs.<sup>27</sup> Increases in active responses, such as climbing or swimming, and reduction in immobility, are considered as behavioral profiles consistent with an antidepressant-like action<sup>31</sup> In this adaptation of the original FST,<sup>27,32</sup> each rat was trained on Day33 for 12 min, by being forced to swim in an open glass cylinder (R=20cm, L=48 cm), containing 25 cm of water maintained at 23-25°C. On Day34, rats were individually placed into the water tank for 6 min and their activities were registered from a vertical position by video camera. The active periods of swimming (defined as movement throughout the swim chamber) and climbing (upward-directed movements of the forepaws along the side of the swim chamber), together with the immobile-periods, were later observed, registered and summarized by a trained observer, using ANY-maze software. One hour before their individual test, a random 6 CTL rats and each rat of the 3 experimental groups were injected with saline (*ip*; 1 ml/kg b. mass), while another 6 CTL rats were injected with 15 mg/kg of antidepressant imipramine in the same manner. The first group represented the basic control group (CTL) and the second group was considered as the reference treatment group with reduced depression-like behaviors (IMI).

## 2.5 MAO assays

The effects of *Aronia* juice and phenolic compounds on *in vitro* MAO activity were evaluated using MAO-Glo Assay V1401 (Promega; USA) as described elsewhere.<sup>33</sup> This two-step bioluminescent assay was performed in 96-well flat-bottom assay plates where the purified rat liver mitochondrial fraction was used as a source of MAO enzymes.<sup>34</sup> In the first step, the mitochondrial microsomes were incubated with a substrate (concentrations were set at their Km values of 40µM and 4µM for MAO-A

and MAO-B, respectively) and various concentrations of the phenolic compounds (0.1 $\mu$ M-100  $\mu$ M/1mM) or AJ, for 1 h at room temperature. The dilutions of AJ in the assay were adjusted to the concentration of total phenolic compounds determined for AJ (6.484 g/L), so that their ranges of concentrations in the assay were 0.01 - 100  $\mu$ g/mL. The reaction buffer for the MAO assay contained 100 mM Hepes (pH 7.5) and 5% glycerol, and for MAO-B, the buffer additionally contained 10% dimethyl sulfoxide (DMSO), which was used to inhibit MAO-A and stimulate MAO-B activity. In the second step, after the addition of reconstituted luciferin detection reagent used to generate and stabilize the luminescence, the mixtures were incubated at room temperature for 20 minutes. Pargyline was used as a reference compound for both assays. The luminescent signal was measured using a Chameleon chemiluminescence reader (Hidex, Turku, Finland). The results were presented as IC<sub>50</sub> values in  $\mu$ g/mL of AJ phenolics, or in  $\mu$ M for the particular phenolic compounds.

### 2.6 Data presentation and statistical analysis

Experimental data are presented as mean  $\pm$  S.E.M., in relation to the number of animals. Statistical analyses of all experimental data, as well as the determination of IC<sub>50</sub> values for MAO inhibition, were done by the Prism software (5.0.; GraphPad software, USA). Intergroup differences were evaluated by either 2-way (OFT) or 1-way ANOVA, followed by Newman-Keul's post-hoc test for multiple comparisons (if  $p < 0.05$ ). Two-way Student's t-test was used to compare homologous Day31 and Day-1 values (OFT).

## 3. Results

### 3.1. Characterization and quantification of phenolic constituents in AJ

The qualitative and quantitative analyses of AJ are presented in Table 1. Only the identified phenolic compounds with concentrations  $> 10$  mg/L are provided.

### 3.2. Quantitative parameters of eating, drinking and body mass gain

The average daily consumptions of drinking solutions in the experimental groups during this 5-week study are represented in Table 2. They are shown in mL per animal, instead of ml per body mass, considering the variable gains in body mass during the study. Analysis by 2-way-ANOVA revealed a significant influence of the week of the study ( $F(4,120)=28.03$ ,  $p < 0.0001$ ) and of the contents of drinking solutions ( $F(3,120)=41.31$ ,  $p < 0.0001$ ), but also of their interaction ( $F(12,120)=8.78$ ,  $p < 0.0001$ ). These effects are reflections of the greatly enhanced drinking of solutions with *Aronia* phenolics, where only two groups supplemented with AJ showed significant elevations of daily

drinking from the 2<sup>nd</sup> (RAJ) or 3<sup>rd</sup> week (ARO) onward. The consumed amount of the drinking solution in the ARO group was 50-70% higher vs. CTL, and about 40-60% vs. PLC, throughout the 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> week, while this increase in the RAJ group went up to 30%.

Table 3 shows the overall consumptions of the liquids and food in the experimental groups during the five weeks of the study. Also, this table provides the consumed quantities of the total phenolic compounds, proanthocyanidins and anthocyanins in g per kg of b. mass in each experimental group. These calculations are based on the analytical data presented in Table 1 and the volumes of the solutions that were drunk weekly (Table 2). The volume-mass transformations are based on the mass densities of 1.10, 1.09 and 1.02 g/mL, estimated for AJ, RJ and all drinking mixtures, respectively. Standard deviations of these values were not obtained since it was not possible to track individual consumptions within the group. The variations of the average rat chow consumptions among the groups were within 5%, which may indicate that they are not significant. Otherwise, the amounts of the drinking solutions consumed in the groups RAJ and ARO were between 13% and 30% higher than the amounts in groups CTL and PLC, which could be referred to as significant elevations. The calculated quantities of the phenolics that were consumed in the ARO group were about 4.5 times larger than in the RAJ group. Supplementary calculations for the period of the first 4 weeks of the study pointed out that the average daily phenolic consumptions per kg of rat body mass were: 30.8 and 133.0 mg/kg/day in the RAJ and ARO groups, respectively. During the 5<sup>th</sup> week of the study, when all behavioral tests were performed, these amounts were 33.0 (RAJ) and 168.0 (ARO) in mg/kg/day. The variations of the body mass gains among the groups were significant [F(3,32)=5.535,  $p < 0.0035$ , 1-way-ANOVA], with conspicuous 60-70% enlargements in the ARO group when matched with the CTL and RAJ groups, but not in comparison with the PLC group.

### 3.3. Open field test

Two of the parameters evaluated by OFT before the start of the study, the distance traveled (Fig. 1A) and the active time (Fig. 1B), did not show significant variations among the experimental groups [F(3,32)=0.901,  $P=0.451$  and F(3,32)=1.309,  $P=0.288$ , respectively], while the influences of the one-month treatments on these parameters were evident on Day31 [F(3,32)=3.917,  $P=0.017$  and F(3,32)=2.924,  $P=0.0488$ , respectively]. The post-hoc test revealed significant differences between the groups: ARO and CTL (+35%,  $p < 0.05$ ), as well as for ARO vs. RAJ (+46%,  $p < 0.05$ ), regarding the distance traveled. Also, active time was enhanced in the ARO group in comparison with both the CTL (+33%,  $p < 0.05$ ) and RAJ (+32%,  $p < 0.05$ ) groups. The fluctuation of these parameters in the same experimental group between Day31 and Day-1 were verified by the Student's t-test, which revealed significant increases of both distances traveled (+22%,  $p=0.040$ ; Fig. 1A) and active times (+20%,  $p=0.032$ ; Fig. 1B) only in the ARO group. The rearings recorded in OFT (Fig. 1C) were not significantly

altered on both Day-1 and Day31 [ $F(3,32)=1.231$ ,  $P=0.315$  and  $F(3,32)=1.139$ ,  $P=0.348$ , respectively], but there were almost significant elevations of this parameter in the RAJ (+56%,  $p=0.053$ ) and ARO (+40%,  $p=0.059$ ) groups, between Day31 and Day-1.

### 3.4. Elevated plus maze

EPM was used on Day32 of the experiment to determine the effects of treatments on the anxiety-like forms of rat behavior (Fig. 2). Two parameters of EPM that reflect the opposite to such behavior were significantly altered: % of time spent in OA ( $F(4,31)=5.874$ ,  $p=0.0012$ ; Fig. 2A), with a significant increase in ARO vs. PLC (+215%;  $p<0.05$ ) and vs. RAJ (+150%;  $p<0.05$ ), but not vs. CTL (+77%;  $p>0.05$ ), and % of OA entries ( $F(4,31)=10.4$ ,  $p<0.0001$ ; Fig. 2B), which was more strongly expressed in the ARO group (+176% vs. CTL, and +132 vs. PLC;  $p<0.001$ ) than in the DZP group (+105% vs. CTL, and +72 vs. PLC;  $p<0.05$ ). Two parameters of EPM that may reflect general activity and ambulation, the number of entries in CA (Fig. 3C) and the time of immobility (Fig. 3D), were not substantially affected by the treatments ( $F(4,31)=0.321$ ,  $p=0.861$  and  $F(4,31)=2.307$ ,  $p=0.080$ , respectively). It was the same with the number of SAP (Fig. 3E), which represented risk assessment ( $F(4,31)=0.321$ ,  $p=0.861$ ). On the contrary, the number of head dips (Fig. 3F), as a reflection of exploratory behavior, was clearly influenced by the treatments ( $F(4,31)=4.584$ ,  $p=0.005$ ), with significant elevations in the ARO group vs. the CTL (+156%;  $p<0.05$ ), PLC (+105%;  $p<0.05$ ) and RAJ (+125%;  $p<0.05$ ) groups.

### 3.5. Forced swimming test

This test was used as a regular model for screening the antidepressant-like action of the treatments. The percentages of time when animals were immobile during 6 min in the cylindrical water container, which represented a depression-like form of behavior on Day34, are shown in Fig. 3A. There was a very significant effect of the treatments on the immobile periods in the FST ( $F(4,31)=11.57$ ,  $p<0.0001$ ). The post-hoc test revealed that this parameter was significantly diminished in both the ARO (-28% vs. CTL, and -23% vs. PLC;  $p<0.001$ ) and IMI (-21% vs. CTL, and -16% vs. PLC;  $p<0.01$ ) group. The influence of the treatments on the periods of active swimming, which may be interpreted as the modulation of serotonin (5-HT) neurotransmission,<sup>32</sup> also appeared to be very significant ( $F(4,31)=21.15$ ,  $p<0.0001$ ; Fig.3B). This was an outcome of exceptionally intensified swimming in the ARO group (+133% vs. CTL, and +93% vs. PLC;  $p<0.001$ ), and of a lesser swimming increase in the IMI group (+61% vs. CTL, and +33% vs. PLC;  $p<0.05$ ). There was a modest effect of the treatments on climbing behavior ( $F(4,31)=3.90$ ,  $p<0.011$ ; Fig 3C), which indicated the levels of noradrenaline (NE) neurotransmission,<sup>32</sup> whereas only the rats that received imipramine exhibited an increase in this parameter ( $p<0.05$ , IMI vs. CTL or PLC).

### 3.6. MAO assays

The abilities of AJ to inhibit MAO-A or MAO-B enzymes were evaluated by the MAO-Glo Assays (Table 4). The  $IC_{50}$  values (50% inhibitory concentration of the phenolic constituents in  $\mu\text{g/L}$ ) were calculated in relation to the determined AJ concentration of the phenolics (0.65%). These data were compared with the estimated  $IC_{50}$  values (in  $\mu\text{M}$ ) for some of the phenolic compounds that had been detected in AJ, and pargyline, as a referent MAO inhibitor. The MAO inhibiting potency of AJ was more prominent for MAO-A than for MAO-B enzymes, while quercetin was the only one among the tested compounds that showed substantial MAO-A inhibition (Table 4).

## 4. Discussion

The results of this study demonstrate that month-long consumption of diluted AJ with a high level of phenolic compounds may induce significant behavioral variations in male rats, which were expressed as somewhat increased locomotion and reduced anxiety-like and depression-like forms of behavior. These behavioral modifications are related to unlimited drinking of 20% AJ water dilution during 30 days (Table 2). The concept of this study was quite different from the typical nutritional-behavioral experimental studies, where specific diets, rich in active ingredients, or purified natural compounds, are provided to animals by monitored oral administrations. The approach applied here allowed the animals to control their drinking habits in line with their general behavior, which was analogous to human living patterns. Accordingly, it was found that the adjunction of AJ in drinking water induced very significant elevations of the daily drinking amounts, from the 2<sup>nd</sup>/3<sup>rd</sup> week onward, which was more pronounced in the ARO than in the RAJ group (Table 2). Only the average group consumption of either mixture was being tracked here, since it was not possible to control and determine individual drinking amounts. The progressive consumptions of both drinking mixtures with AJ over time revealed that the rats developed increased affinity for this juice starting from the 2<sup>nd</sup>/3<sup>rd</sup> week of the experiment. A possible explanation for this may be increased thirst, which could be induced by the bitter-astringent taste of AJ.<sup>35</sup> However, the main source of bitterness in AJ should be amygdalin,<sup>9</sup> which has been reported to have thirst-quenching capabilities.<sup>36</sup> Moreover, in our pilot test, which was performed on 5 rats that were allowed to drink 20% mixture of AJ in water for 20 days, the average 30%-increment of the solution volume that was drunk on Day20 (vs. Day-1), was completely reduced on Day21 (+2% vs. Day-1), when the rats were supplied with water only (data unpublished). This information should not exclude a temporary induction of thirst by sustained AJ consumption, whereby the increased affinity for the particular component(s) of *Aronia* juice may be another option.

The dietary effects of intensified drinking in this study seem to promote body mass gain only for the ARO rats, when compared to the CTL rats. It may be supposed that the highest amount of the drinking solution that was drunk by the ARO rats (+11% vs. RAJ and +25% vs. PLC) was the reason for their body mass gain, since the consumption of rat chow was not enhanced in this group (Table 3). On the other hand, the PLC group showed certain elevations in both the rat chow eating and body mass gain (Table 3). A similar dietary study, where *Aronia* extract did not alter food intake and water consumption in rats that were fed a fructose-rich diet during 6 weeks while their body mass gain and epididymal adipose weight were reduced, suggests that *Aronia* might be beneficial in preventing or decreasing obesity and the metabolic syndrome.<sup>37</sup> Another nutritional experiment showed a slight decrease in diet utilization and growth for the rats on *Aronia* polyphenolic diets relative to the control group.<sup>38</sup> In an analogous human study, when 100 ml of polyphenol-rich *Aronia* juice was consumed by healthy women, they showed no changes in body weight or in habitual dietary intake after 3 months on this regimen.<sup>39</sup> The discrepancies in the registered dietary effects of *Aronia* between these and our study are coherent, having in mind the different concepts related to unlimited vs. restricted consumptions of *Aronia* products. Taking all this into account, it seems that the registered increase in affinity for the diluted AJ and its extensive consumption solely contributed to the enhanced body mass gains of the ARO rats, since this research did not show that the free drinking of *Aronia* juice could stimulate appetite and food intake.

The behavioral tests in this study were performed after the one-month drinking regimen, during 4 consecutive days (31-34). ARO was the only experimental group with significant behavioral variations in this period. These effects should be the result of drinking large amounts of phenolic-rich AJ, where the calculated average amount consumed daily in the first 4 weeks was about 22 g/kg b. mass (133 mg/kg/daily of phenolic constituents). During the 5<sup>th</sup> week, when the behavioral tests were performed, the ARO rats took over 30 g/kg of AJ daily (168 mg/kg/daily of phenolics), which was about 5 times the AJ amount consumed in the RAJ group (6 g/kg). The behavioral consequences, registered by OFT in the ARO group on Day31, were increased field activity and locomotion in relation to both the Day-1 and CTL group (Fig. 1A & B), while the multiplication of rearings was almost significant (+40% vs. Day-1,  $p=0.059$ ; Fig. 1C). On the other hand, these parameters of activity were not significantly higher in the ARO vs. PLC group (Fig. 1), which suggested that the phenolic compounds were not solely responsible for that effect. A comparative analysis of the results generated by OFT and EPM indicates that the supposed hyperactivity of the ARO group may be alternatively explained as an expression of the exploratory behaviors, which could be reinforced by the reduction of

innate anxiety. This assumption has its roots in the facts that no hyperactivity of the ARO group was found in EPM (evaluated by the number of CA entries and the percentage of immobility time; Fig. 2 C&D), while an elevation of the parameters that are opposite to the anxiety-like behaviors (the percentage of time spent in OA, the percentage of OA entries; Fig. 2 A&B) come together with an increased number of head dips in the ARO group (Fig. 2F), which reflected exploratory behaviors.<sup>28</sup> The enhancements of these three EPM parameters in the ARO group were exhibited toward three other experimental groups (CTL, PLC and RAJ), whilst they were similar to the acute effects of the anxiolytic diazepam (DZP; 1mg/kg). This evidence implies that only the intensive everyday consumption of AJ may induce anxiolytic-like effects in rats, which are detectable by EPM. The results of FST, presenting the reduction of immobile periods in ARO in comparison to the other three groups (Fig. 3 A), which was more convincing than in the group acutely treated with the antidepressant imipramine (IMI; 15 mg/kg), suggest that everyday consumption of AJ may also elicit antidepressant-like effects in rats. Since it has been shown that the antidepressant-like actions in FST, which were mediated by the stimulation of 5-HT neurotransmission, induced the increase of active swimming period, whereas the augmentation of NE neurotransmission was related to the enhanced climbing activity,<sup>32</sup> the here registered activation of active swimming, but not of climbing in the ARO group (Fig 3 B&C), suggests that this antidepressant-like effect in rats may proceed from the stimulation of the neurotransmission mediated by 5-HT and not by NE. The absence of both the increased climbing in FST and rat hyperactivity in EPM may testify against a possibility that the OFT-referred ambulatory stimulation in ARO could simulate antidepressant like effect in FST. Nevertheless, to clearly disprove (or not) this plausibility, more sensitive and distinctive behavioral evaluation of the antidepressant effect must be performed in the further research on *Aronia* extracts. In any case, such reductions of the anxiety-like and depression-like behaviors, and a possible psycho-stimulation in animals, which followed a sustained AJ consumption, may be interpreted as beneficial from the human viewpoints.<sup>40,41</sup>

A brief glance at reviews in biomedical literature reveals a substantial number of studies presenting the effects of natural phenolic-rich sources, mainly of the flavonoid group, on the behavior of experimental animals and humans.<sup>42,43</sup> Hence, there are only several studies where *Aronia* juices/extracts were investigated in such a manner. The group of Valcheva-Kuzmanova found by passive avoidance tests that one-month consumption of *Aronia* juice (up to 10 ml/kg b. mass daily) could improve memory in rats, while the social interaction test suggested some anxiolytic-like effects.<sup>16,17</sup> However, they did not find any effects on locomotion in OFT.<sup>16</sup> This may be in line with our evidence, where the RAJ group, with an approximate daily consumption of AJ in the range 5-6

ml/kg, was not overactive. The ARO group, which showed increased locomotion in our study, received more than 20 ml/kg of AJ daily. Experiments with other polyphenol-rich fruit extracts exhibit particular behavioral and cognitive effects. It has been reported that low-molecular proanthocyanidins from grapes, applied to mice for 7 days in doses of up to 50 mg/kg, exerted antidepressant-like effects in behavioral despair tests but did not affect locomotor activity.<sup>19</sup> The proposed antidepressant mechanism was dose-dependent inhibition of MAO-A, and, to a lesser extent, of MAO-B, which induced marked increases of serotonin, as well as of dopamine and noradrenaline, in the frontal cortex, hippocampus and hypothalamus.<sup>19</sup> Another study using *in vitro* MAO assays did not find significant effects of proanthocyanidins B1 and B2, but revealed that anthocyanidins and anthocyanidin-3-glycosides inhibited the activities of both MAO-A and MAO-B in the low micromolar range.<sup>20</sup> The well-known medical applications of MAO-A inhibitors as antidepressants and, in drug combinations, as anti-anxiety drugs,<sup>44</sup> combined with growing evidence that flavonoids may inhibit MAO enzymes,<sup>45</sup> has motivated us to evaluate AJ and some of its phenolic constituents by *in vitro* MAO assays (Table 4). These assays revealed reliable MAO-A inhibition by AJ ( $IC_{50}=2.2 \mu\text{g}$  of *Aronia* phenolics per L), which was expressed in relation to the determined concentration of AJ phenolic compounds (6.48 g/L). The reconstituted phenolic-free *Aronia* juice, RJ, did not affect MAO enzymes in the assay (data not included in Table 4). The levels of MAO inhibition, generated by the specific phenolic compounds in these assays, mostly corresponded to the literature data<sup>20,45,46</sup> where quercetin appeared to be the most potent MAO-A inhibitor ( $IC_{50}=0.72 \mu\text{M}$ ). However, this flavonol was found as aglycon only in traces in AJ, while the most abundant phenolics in AJ showed poor or insignificant interactions with MAO enzymes (Table 4). Obviously, it is challenging to estimate the participation of individual phenolic compounds in the resulting MAO inhibiting potency of AJ, especially with a reasonable presumption that there was a synergistic action of the various phenolics. Also, there is the question of the exact AJ phenolic composition that was being consumed throughout the study, knowing that the qualitative/quantitative analyses had been performed on fresh juice, before the start of the experiment. Considering both, the evidence that anthocyanin stability in commercial *Aronia* juices was satisfactory ( $t_{1/2}=6-7$  weeks at 4°C, 30-50 weeks at 25°C),<sup>47</sup> while all other phenolic compounds in *Aronia* juices were found to be much more stable than anthocyanins,<sup>48</sup> together with the duration of our experiment and the handling of AJ, the stable concentrations of AJ phenolics is prospective throughout this study.

The greatest challenge is to extrapolate these *in vitro* data to the complexity of a living organism. Dietary polyphenols are highly sensitive to the mild alkaline conditions in the small intestine and a good proportion of these compounds can be transformed into other unknown and/or undetected structural forms with different chemical properties and, consequently, different bioaccessibility, bioavailability and biological activity.<sup>49</sup> Anthocyanins are poorly absorbed in the small intestine, so

they are recovered in blood and found in urine in nanomolar concentrations, with the concern that conjugation (glucuronidation and methylation) affects the biological activity of anthocyanins *in vivo*.<sup>10,50</sup> Proanthocyanidin particles that may be absorbed through the gut barrier can be up to trimer size and they constitute less than 5% of their total amount.<sup>10</sup> So, these two groups of polyphenols which are present in moderate amounts in AJ are the least well absorbed. Chlorogenic and neochlorogenic acids are found in significant concentrations in *Aronia*, but esterification decreases their intestinal absorption.<sup>10</sup> Monomeric flavanols and flavan-3-ols, like those usually detected in *Aronia* ((-)-epicatechin and quercetin) are more efficiently absorbed than anthocyanins, but their amounts in AJ are very low and it is not very likely that they have a significant influence on plasma polyphenol concentrations.<sup>10,49</sup> On the other hand, quercetin glucosides, such as isoquercetin, have been shown to be much more quickly absorbed and more bioavailable than the smaller quercetin aglycone, or the larger quercetin glycosides such as rutin.<sup>51,52</sup> The findings that glucoside chains are submitted to highly effective hydrolysis in the small intestine endothelium indicate that most of the ingested flavanols may enter circulation as quercetin aglycones.<sup>52</sup> So the therapeutic profiles of all flavanols found in *Aronia* may correspond to that of quercetin. Another and still open query is related to the brain bioavailability of polyphenols, dependent on their passing through the blood-brain barrier,<sup>53</sup> which is, however, beyond the scope of this study. At all events, it is plausible that the combinations of the original and transformed phenolic compounds of AJ may attain rat circulation and pass the blood-brain barrier in such concentrations that they could produce certain neurochemical effects by synergism. A part of these effects would be achieved by the central MAO inhibition (especially of MAO-A). It may result preferentially in the up-regulation of central serotonin and not of noradrenaline levels. Furthermore, the previously registered direct influences of particular bioflavonoids on the neurotransmitter reuptake processes,<sup>18,21</sup> GABA receptors,<sup>45</sup> even on acetyl-cholinesterase,<sup>54</sup> suggest that these additional interactions may contribute to the behavioral alterations observed in ARO rats in this study. Only further investigations may answer some of the questions regarding the exact central influence of the phenolic constituents of *Aronia*.

## 5. Conclusions

The present findings demonstrate significant behavioral variations in male rats after one month of unlimited drinking of diluted AJ (20%), when the average daily consumption of *Aronia* phenolic compounds exceeded 130 mg/kg b. mass. These changes were registered in animal models of behavior and recognized as somewhat increased locomotion and the reduction of anxiety-like and depression-like forms of behavior. Although this study suggests that the central MAO-A inhibition by the *Aronia* phenolic compounds may be important for such behavioral effects, further research would shed more light on these interactions.

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**Table 1** Quantities of phenolic compounds in *Aronia melanocarpa* fruit juice (AJ). Data expressed as mean values (n = 2 or 3), standard error <8% of mean values

Phenolic compounds	Amount (mg/L)
Total phenols <sup>a</sup>	6484
Total proanthocyanidins <sup>b</sup>	240
Phenolic acids <sup>c</sup>	
Chlorogenic acid	1389
Neochlorogenic acid	1057
Anthocyanins <sup>c</sup>	
Cyanidin-3-O-glucoside	21
Cyanidin-3-O-galactoside	301
Cyanidin-3-O-arabinoside	101
Cyanidin-3-O-xyloside	13
Flavonols <sup>c</sup>	
Quercetin-3-O-glucoside (isoquercetin)	53
Quercetin-3-O-galactoside (hyperoside)	97
Quercetin-3-O-rutinoside (rutin)	194

<sup>a</sup> Folin-Ciocalteu method, concentration based upon gallic acid as standard.

<sup>b</sup> determined spectrophotometrically by the vanillin assay, concentration based upon (+)-catechin as standard.

<sup>c</sup> single compounds determined by HPLC/DAD. The flavonoids (rutin, hyperoside and isoquercetin), cyanidin-3-O-galactoside and hydroxycinnamic acids (chlorogenic and neochlorogenic acids) were used as standards.

**Table 2** Daily consumptions of drinking solutions in 5 weeks of the experiment

Experimental group: Week	CTL	PLC	RAJ	ARO
1 <sup>ST</sup>	30.33 ± 1.73 <sup>a</sup>	30.00 ± 1.47 <sup>a</sup>	33.25 ± 1.11 <sup>a</sup>	30.24 ± 2.35 <sup>a</sup>
2 <sup>ND</sup>	39.50 ± 3.28 <sup>a*</sup>	35.70 ± 5.06 <sup>a</sup>	39.28 ± 1.94 <sup>a*</sup>	35.60 ± 0.40 <sup>a</sup>
3 <sup>RD</sup>	32.88 ± 1.25 <sup>a</sup>	39.10 ± 1.64 <sup>b*</sup>	42.40 ± 1.14 <sup>b**</sup>	57.42 ± 0.66 <sup>c***#</sup>
4 <sup>TH</sup>	35.70 ± 1.65 <sup>a</sup>	35.88 ± 1.80 <sup>a</sup>	46.37 ± 2.19 <sup>b***</sup>	55.17 ± 3.05 <sup>c***#</sup>
5 <sup>TH</sup>	34.10 ± 2.10 <sup>a</sup>	36.78 ± 1.09 <sup>a</sup>	43.73 ± 0.24 <sup>b**</sup>	58.50 ± 0.76 <sup>c***#</sup>

Experimental groups have been limitlessly supplied with tap water (CTL), and water dilutions of: 20% RJ (PLC), 5% AJ+15% RJ (RAJ), and 20% AJ (ARO). Values of the average daily consumptions of drinking solutions (in ml *per* animal) for each of 5 weeks of the experiment are presented as mean ± S.E.M., which reflected daily variations within a week (N=7 or 6). Values with different letters in the same row are significantly different from each other (P < 0.05), while those that share the same letters are not (P > 0.05). The column statistics is presented with symbols: \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001 vs. 1st week. #, p<0.001 vs. 2nd week. Newman-Keuls multiple comparisons test was applied after 2-way-ANOVA.

**Table 3** Cumulative consumptions of drinking solutions, AJ, total phenolics, proanthocyanidins, anthocyanins and rat chow, and average rat body mass gains in the experimental groups

Experimental group:	CTL	PLC	RAJ	ARO
Consumption (g/kg b.mass) of:				
Drinking solution	2813	2906	3273	3645
AJ	0	0	176	786
Phenolics	0	0	1.037	4.636
Proanthocyanidins	0	0	0.039	0.172
Anthocyanins	0	0	0.071	0.315
Rat chow	1775	1817	1718	1731
Body mass gain (% of b. mass on Day-1)	4.8 ± 0.5	6.9 ± 0.6*	5.1 ± 0.7	8.1 ± 0.9* #

Overall consumptions throughout 34 days of the experiment are presented for the rat chow and for each of the drinking solutions, where the consumed amounts of these solutions, AJ, total phenolics, proanthocyanidins and anthocyanins of AJ, are shown in grams per kg of average body mass [(Day-1 + Day35) / 2]. Mass densities of AJ and RJ were 1.10 and 1.09 g/mL, respectively, while they were 1.02 g/mL for all drinking solutions except tap water. Average body mass gains in each experimental group was estimated on Day35 and presented as percentage of body mass on Day-1 ± S.E.M. (N=8, or N=12 for CTL) \*, p<0.05 vs. CTL; #, p<0.05 vs. RAJ by post hoc Newman-Keuls multiple comparisons test.

**Table 4** *In vitro* MAO-A and MAO-B inhibition by AJ and the specific phenolic compounds

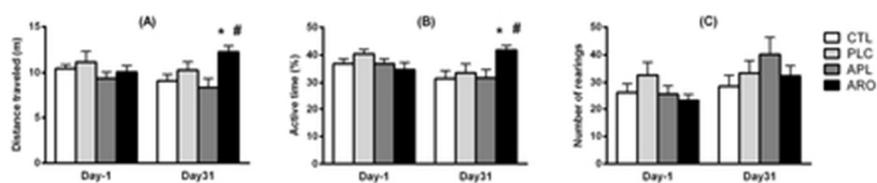
COMPOUNDS	IC <sub>50</sub> MAO A <sup>a</sup>	IC <sub>50</sub> MAO B <sup>a</sup>
AJ (µg/L) <sup>b</sup>	2.2	58.4
Chlorogenic acid (µM)	ND <sup>c</sup>	ND <sup>c</sup>
Cyanidin-3-O-glucoside (µM)	96.3	209
Cyanidin-3-O-arabinoside (µM)	80.8	477
Quercetin (µM)	0.72	170
Rutin (µM)	260	105
(+)-Catechin (µM)	38.5	22.0
Pargyline (µM) <sup>d</sup>	6.7	0.22

<sup>a</sup> Each inhibitory activity is expressed as the mean of 50% inhibitory concentration (IC<sub>50</sub>) of two experiments, which was obtained by an interpolation of the concentration-inhibition curves

<sup>b</sup> Adjusted to the concentration of phenolics in AJ, determined by Folin-Ciocalteu method

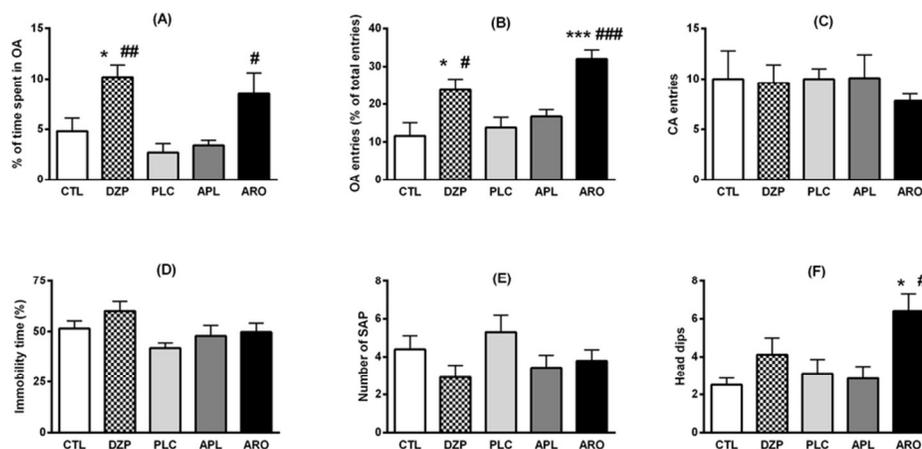
<sup>c</sup> Not determined as the significant inhibition (> 50 %) was not detected for the concentrations of up to 1mM

<sup>d</sup> Used as a comparative MAO inhibitor

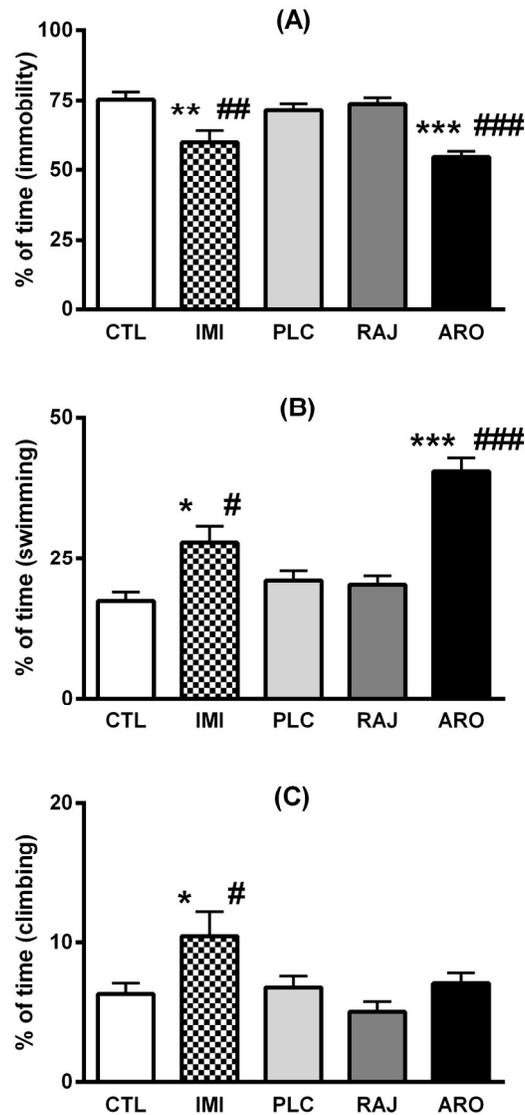


**Fig. 1** Effects of month-long unlimited drinking of either tap water (CTL), 20% RJ (PLC), 5% AJ+15% RJ (RAJ), or 20% AJ (ARO), on rat behavior in OFT. The overall rat activities were registered during 20 min, before the start of the experiment (Day-1) and on Day31 of the study. Three OF parameters, evaluated by ANY-maze software, are presented: (A) distance traveled, (B) percentage of active time, and (C) number of rearings, which are expressed as group mean  $\pm$  S.E.M. values (N=12 or 8). \*  $p < 0.05$ , vs. CTL, by One-way ANOVA post hoc Newman-Keuls multiple comparison test. #  $p < 0.05$ , by two-way Student's t-test, Day31 vs. Day-1.

38x8mm (300 x 300 DPI)

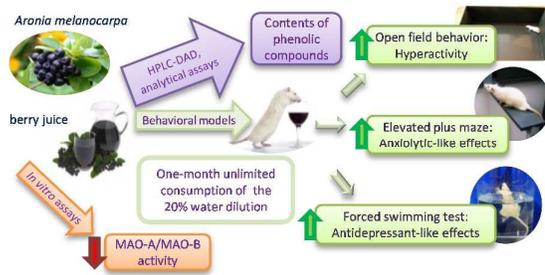


**Fig. 2** Effects of month-long unlimited drinking of the experimental mixtures (details about the experimental groups are in the caption of Fig 1.) on rat behavior in EPM. Each rat had been injected (*ip*; 1 ml/kg b.mass), one hour before the test was performed on Day32, with either 1 mg/kg diazepam (DZP; random 6 of 12 rats scheduled to drink only tap water) or saline (all other rats in the experimental groups and CTL). The rat behavioral parameters in EPM (as mean  $\pm$  S.E.M. N=6 or 8) were recorded for 5 min and evaluated with ANY-maze software or by observation, (A) percentage of time spent in OA, (B) percentage of OA entries, (C) CA entries, (D) percentage of immobility time, (E) number of stretched attend postures (SAP) and (F) number of head dips. \*  $p < 0.05$ , \*\*\*  $p < 0.001$  vs. CTL; #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$  vs. PLC, using 1- way ANOVA followed by Newman-Keuls post-hoc tests. 82x40mm (300 x 300 DPI)



**Fig. 3** The influence of one month of free drinking of the experimental mixtures on the proportion of the periods of (A) immobility, (B) active swimming, and (C) climbing, during 6 min in FST. (The same experimental groups are presented as in Figs. 1 and 2). One hour before the test was performed on Day34, rats had been injected (*ip*; 1 ml/kg b.mass) with either 15 mg/kg imipramine (IMI; random 6 of 12 rats on water consumption) or saline (all other rats of CTL and the experimental groups). Results are expressed as mean  $\pm$ S.E.M. (N=6 or 8). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. CTL; #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$  vs. PLC using 1- way ANOVA followed by Newman-Keuls post-hoc tests.

152x290mm (300 x 300 DPI)



The application of three animal models of behavior in young male rats reveals that the month-long drinking regimen of diluted berry juice of *Aronia melanocarpa* may have certain stimulating, anxiolytic-like and antidepressant-like effects, which could be related to the central MAO inhibition by *Aronia* phenolic compounds.