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Aronia melanocarpa (Michaux) Elliot Fruit Juice Attenuates Acetaminophen-induced Hepatotoxicity on Larval Zebrafish Model

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ABSTRACT

Aronia melanocarpa (Michaux) Elliot (chokeberry) is a natural medicinal plant with a rich content of phenolic compounds such as procyanidins, anthocyanins, and phenolic acids. Chokeberry fruits are gaining worldwide popularity due to the strong bioactivities of their phenolic constituents, such as antioxidant, anti-inflammatory, anticancer, and liver-protective effects.

In the present study, total phenolic, flavonoid, and anthocyanin contents of chokeberry juice were determined via the Folin-Ciocalteu method, a spectrophotometric method based on $AlCl_3$ complexation, and pH differential method, respectively. Anthocyanin content was determined as 1.14% (equivalent to cyanidin-3-glucoside), while phenolic and flavonoid contents were measured as 5060.87 and 331.03 mg *per* 100 g of freeze-dried juice (equivalent to gallic acid and quercetin), respectively.

The hepatoprotective effects of chokeberry fruit juice were evaluated using a zebrafish *in vivo* model for acetaminophen (APAP)-induced liver injury. Zebrafish is an emerging *in vivo* liver injury model that enables hepatoprotective bioactivity screening of samples on live organisms.

The APAP-induced liver injury model was established by treating zebrafish larvae with 5 mM APAP from 2 days post fertilization (dpf) to 5 dpf. The hepatoprotective effect of chokeberry was evaluated via exposure to 1, 10, and 100 µg/mL of fruit juice. While chokeberry fruit juice did not cause any toxicity up to 100 µg/mL, it successfully reduced the injury induced by APAP when applied at 1 µg/mL concentration. To our knowledge, this is the first report evaluating the hepatoprotective effects of chokeberry using zebrafish *in vivo* liver injury model.

Keywords: Anthocyanin, Aronia, Chokeberry, Hepatoprotective, Zebrafish

1. Introduction

Aronia melanocarpa (Michx.) Elliot, commonly known as black chokeberry, is a deciduous shrub belonging to the Rosaceae family (Gurčík et al. 2023). The plant typically grows up to 1 - 2 meters tall and can spread 1 - 2 meters. The fruits of the plant are black, pea-sized berries that are edible but have a very astringent taste (Shahin et al. 2019; Ekiert et al. 2021).

Chokeberry is native to eastern North America, from Canada to the central United States. It grows in various habitats, including wetlands, swamps, and woodland edges (Moorhead & Rossell 2019). The plant has been cultivated as an ornamental plant and for its berries, which are used to make juice, wine, and other food products (Çelik et al. 2022).

In Turkiye, research on chokeberry cultivation started in 2012 at the Atatürk Horticultural Central Research Institute, and commercial production of chokeberry began in 2017 with the establishment of large orchards in the Marmara and Black Sea Regions (Akdemir 2022; Akdemir et al. 2023). Chokeberry fruits can be consumed fresh or processed, and their high market value has led to growing interest in chokeberry production in Turkiye. As production increases, the chokeberry industry is expected to expand further in the country.

Phytochemical studies showed that chokeberry fruits are rich in various nutrients, including vitamins C, E, and K, as well as bioactive compounds such as anthocyanins, flavonoids, and phenolic acids (Platonova et al. 2021). Anthocyanins are the water-soluble plant pigments that give the deep purple-black color of the fruits and are potent antioxidants (Khoo et al. 2017). The hepatoprotective bioactivity of anthocyanins has been reported by several research groups using *in vivo* rodent models (Wang et al. 2020). Nevertheless, the hepatoprotective potential of chokeberry has not been explored in the zebrafish liver injury model.

The purpose of the present study was two-fold; 1) to assess bioactivity potential of chokeberries harvested in Turkiye, 2) to test the hepatoprotective effects of chokeberry fruit juice, in the zebrafish hepatic injury model. To this end, total phenol, flavonoid and anthocyanin content of chokeberry juice were analysed. Next, the hepatoprotective bioactivity of fruit juice was evaluated using an *in vivo* zebrafish model featuring acetaminophen-induced liver injury.

To our knowledge, this study represents the first report demonstrating the liver-protective bioactivity of locally harvested chokeberry fruit juice using the zebrafish experimental model, shedding valuable insights for hepatoprotective drug discovery research.

2. Material and Methods

2.1. Plant material

Chokeberries were collected during the fruiting season from Kamiloba region (Büyükçekmece/İstanbul) and deposited at Istanbul University-Cerrahpaşa Faculty of Pharmacy Herbarium (IUCPH; voucher no:220901). Fruits were crushed with a homogenizer (Stuart Scientific SHM2) and centrifuged at 3000 rpm for 3 min. The supernatant was freeze-dried and stored at -20 °C until the experiment.

2.2. Chemicals and reagents

APAP, Chem Cruz (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was commercially purchased. The main stock of APAP was prepared by solubilizing it in deionized water. All dilutions were carried out using E3 fish media. Low melting point agarose and tricaine methanosulfate (TMS) were purchased from Sigma (Sigma-Aldrich, DL, United States).

2.3. Total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu method, as described previously by Singleton and Rossi (Singleton & Rossi 1965), with slight modifications incorporated as per the recommendations of Dewanto et al. (2002). Through the calibration curve of gallic acid, the phenolics were determined and expressed as gallic acid equivalents (GAE, mg gallic acid/100g freeze-dried sample).

2.4. Total flavonoid content

Quantitative determination of total flavonoids was performed spectrophotometrically at 425 nm, following complexation with aluminum chloride. The total flavonoid content was determined and expressed as quercetin equivalents (QE, mg quercetin/100g sample) through the utilization of the calibration curve of quercetin.

2.5. Total anthocyanin content (TAC)

The determination of TAC was carried out using the pH differential method, as previously outlined by researchers (Giusti & Wrolstad 2001). Results were expressed as mg of cyanidin 3-glucoside per 100 mg sample.

2.6. Zebrafish maintenance and embryo collection:

A transgenic zebrafish line *lfabp:mCherry*, which was generated in Izmir Biomedicine and Genome Center (IBG) Cakan Lab, was used (Kwan et al. 2007). All adult zebrafish were maintained under a 14:10 light: dark cycle at 28.5 °C under standard feeding conditions at the IBG zebrafish facility.

Adult zebrafish were paired for mating in breeding tanks the day before collecting embryos, using dividers to separate males and females. The dividers were then removed, allowing the fish to spawn on the experimental day. Periodic checks were conducted, and embryos were collected at 5–10-minute intervals within an hour to minimize age variation resulting from multiple spawning events. Transgenic larvae were determined under a fluorescent microscope, embryos and larvae were maintained at 28.5±1 °C during experiments, and treatment was refreshed daily.

2.7. Exposure of larvae to apap and chokeberry fruit juice

Larvae were treated with APAP at a dose of 5 mM (755.8 mg/L) from 2 to 5 days post-fertilization (dpf) to generate a hepatic injury model. APAP also served as the positive control for this experiment. Exposure to chokeberry fruit juice (1; 10; 100 µg/mL in E3 media) was performed both as a single treatment (C) or in combination with 5 mM APAP (DT, dual treatment) for each concentration. Untreated larvae were only treated with fresh E3 media and used as the negative control. All treatments were refreshed daily and stopped at 5 dpf. Stereo-fluorescent images were taken, and the liver protectant effect of chokeberry fruit juice was determined via liver size measurements.

2.8. Statistical analysis

Comparisons of liver size changes between groups (n=10) were carried out using One-way analysis of variance (ANOVA), and p-values less than 0.05 were considered significant.

3. Results and Discussion

The phytochemical analysis revealed varying levels of anthocyanin, total phenolic, and flavonoid contents present in chokeberry juice (equivalent to cyanidin-3-glucoside, gallic acid, and quercetin, respectively), as shown in Table 1.

Table 1- Phytochemical content of chokeberry juice

Content	*Method*	*Reference compound*	*Determined**
Phenols	Folin-Ciocalteu	Gallic acid	5060.87
Anthocyanins	pH-differential method	Cyanidin-3-glucoside	1.14
Flavonoids	UV-spectroscopy	Quercetin	331.03

*, Determined concentrations are expressed as % (v/v) for anthocyanins and as mg/100g for phenolics and flavonoids

The anthocyanin content was determined as 1.14%, equivalent to cyanidin-3-glucoside, indicating a rich presence of these pigments responsible for the vibrant colors in fruits and also are known for their potential health benefits, including hepatoprotective bioactivities arising from the antioxidant and anti-inflammatory properties (Mohammed & Khan 2022). These findings align with prior research highlighting the potent antioxidant and anti-inflammatory properties of anthocyanins, suggesting a potential link to the observed hepatoprotective effects (Denev et al. 2018; Panjaitan et al. 2013).

The phenolic content was measured as 5060.87 mg *per* 100 g of chokeberry juice and expressed as gallic acid equivalents. Phenolics are well-known for their ability to scavenge free radicals and protect against oxidative stress, contributing to potential liver-protectant effects (Tan et al. 2012; Zhang & Tsao 2016). Flavonoid content in the chokeberry juice was measured as 331.03 mg per 100 g juice, equivalent to quercetin. Flavonoids are a large group of bioactive compounds with antioxidant and anti-inflammatory activities, offering potential protection against various liver diseases (Akhlaghi 2016). Also, recent investigations have indicated that flavonoids, including quercetin, can induce hepatoprotective effects (Singh et al. 2014).

In the context of *in vivo* bioactivity testing, it was observed that APAP significantly impaired liver function and reduced liver size, thereby confirming the suitability of the APAP-induced hepatotoxicity larval model for our experiment. Notably, dual treatment (DT) with 1 µg/mL chokeberry fruit juice and 5 mM APAP for 96 h between 2-5 dpf has led to a significant increase in liver size in zebrafish larvae (Figure 1D and 2) compared to the group treated with sole 5 mM APAP (positive control). This result suggests that consuming chokeberries may provide partial protection against liver injury induced by APAP.

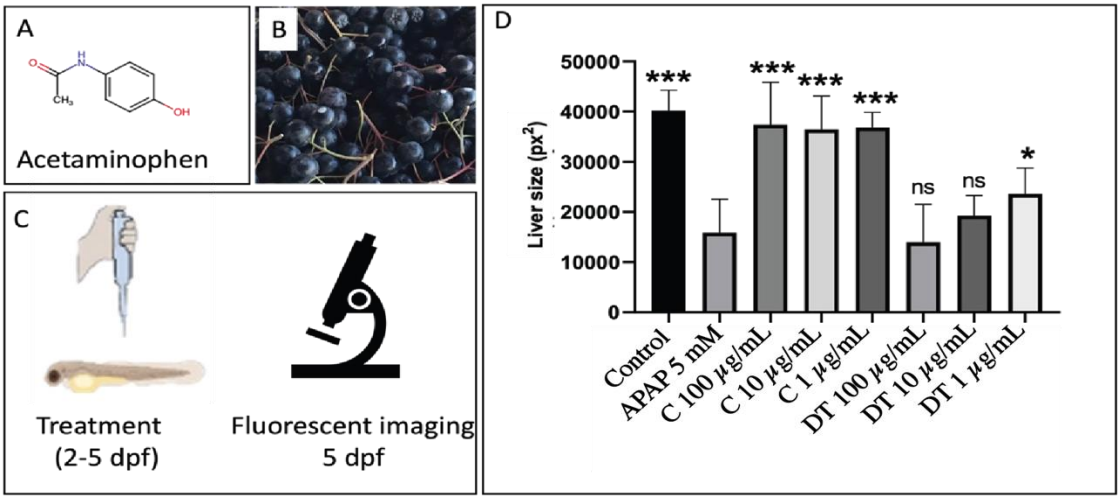

Figure 1- Treating zebrafish larvae between 2-5 days post-fertilization (dpf) with most-DILI agent, acetaminophen 5 mM, significantly decreased the liver size of which liver injury was alleviated with A) Chemical structure of APAP B) Chokeberry fruits C) Schematic illustration of experiment D) 2D measurement of liver size in different treatment groups. Control: untreated control group; APAP 5 mM: acetaminophen treated group; C: Chokeberries treated group; DT: Dual treatment with chokeberries in various concentrations and 5 mM APAP

The liver size of the untreated control was calculated as 40202.4 px^2 (square pixels), while the APAP 5 mM treated group had an average liver size of 15908.9 px^2, implying severe APAP-induced liver injury (Figure 1D). While all tested concentrations of chokeberry juice have shown more-less healed liver tissue, 1 µg/mL chokeberry juice demonstrated the best result with a statistically significant ($P<0.05$) increase in liver area (23633.7 px^2) compared to the APAP single-treated group. Single treatments of fruit juice seemed to be safe for liver health up to 100 µg/mL (Figure 1D and 2A).

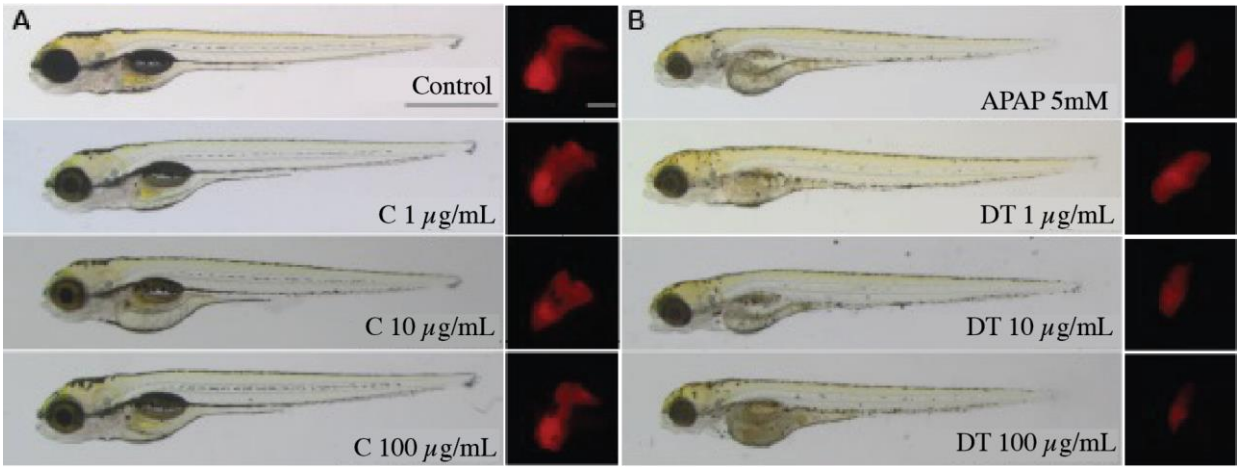

Figure 2- Chokeberry fruits displayed hepatoprotective bioactivity against APAP-induced liver injury on 5 dpf zebrafish larvae. The single treatment of APAP resulted in severe hepatic damage, while chokeberry dual treatment with APAP alleviated APAP-induced liver injury." A) The larvae treated with chokeberry fruit juice showed no discernible visual differences compared to the untreated control group. B) APAP (5mM=755.8 µg/mL) exposure severely reduced liver size and caused liver injury, while the combination of various concentrations of chokeberry fruit juice with the same dose of APAP partially reversed this injury. Control: untreated control group; APAP 5 mM: acetaminophen treated group; C: Chokeberries treated group; DT: Dual treatment with chokeberries and 5 mM APAP. Scale bars for whole larva and liver images: 1 mm and 200 µm, respectively

This study represents the first report to explore the hepatoprotective potential of chokeberry extract in a zebrafish model, despite previous evidence of its hepatoprotective bioactivity in rodents. Furthermore, our results present the potential utility of chokeberries grown in Turkiye due to their bioactivity.

Previously, the hepatoprotective effects of chokeberry extract against APAP-induced liver injury were established in a rat model, where daily oral administration of APAP at doses ranging from 2.5 to 10 mL/kg demonstrated notable mitigation of elevated liver serum biomarkers ALT and AST induced by APAP treatment (Valcheva-Kuzmanova et al. 2014). According to another research of same group, carbon tetrachloride (CCl_4)-induced elevation of AST and ALT activities in rats was reversed with anthocyanin administration (Valcheva-Kuzmanova et al. 2004). The co-administration of anthocyanins extracted from chokeberry was shown to decrease aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities, bilirubin levels, and urea in the blood serum in rats with cadmium chloride-induced liver injury (Kowalczyk et al. 2003). Similarly, a study using a mouse model of chronic alcoholic liver injury, showed that chokeberries have hepatoprotective effects by restoring the AST/ALT ratio and reducing pathological damage to the liver and other organs (Wang et al. 2020).

Although the liver-protective activity of chokeberry has been demonstrated in other *in vivo* models, the use of the zebrafish model is reported for the first time. The zebrafish model aligns with the principles of the '3R' framework, which emphasizes the reduction, refinement, and replacement of animal experiments (Canedo et al. 2022). Zebrafish's genetic similarity to humans and its transparent embryos provide a useful screening platform for real-time visualization of liver function and response to treatments (Goessling & Sadler 2015). Moreover, zebrafish serve as a valuable model for studying liver diseases due to conserved tissue functions, shared liver cell types, and regulatory networks (Cakan-Akdogan et al. 2023; Vliegenthart et al. 2014).

This model is characterized by its rapid reproduction and cost-effectiveness, enabling us to conduct experiments on a large scale without incurring excessive costs. This scalability is particularly advantageous in hepatoprotection research, where the efficient screening of numerous plant extracts is paramount.

Our study addressed the issue of non-dose-dependent bioactivity of chokeberry within the tested concentrations (Figure 1D). The increasing amount of chokeberry juice with 5 mM APAP displayed gradually decreased hepatoprotection, implying a reverse-dose response relationship. Higher concentrations, 200, 400, and 800 µg/mL (data not shown) of chokeberry juice were lethal for larvae when applied as dual treatment with 5 mM APAP, while, interestingly, single treatments of the same concentrations did not cause any toxicity nor lethality. The safety of single treatments and toxicity in higher doses in dual treatment both imply the possibility of interactions between chokeberry and APAP (Yang et al. 1992). It can be speculated that the mechanism of this drug-nutrient interaction may arise from the competition of APAP and the phytochemical content of

chokeberry juice for the same metabolic pathway. APAP is primarily metabolized in the liver through two pathways, glucuronidation, and sulfation, which also have roles in metabolizing various components in chokeberry juice (Płatosz et al. 2021; van Rongen et al. 2016). It is known that sulfotransferases and glucuronidation enzymes catalyze the metabolic reactions of chokeberry fruits and APAP. However, when the dose of APAP exceeds the capacity of these pathways, a small amount of the drug is metabolized by the cytochrome P450 enzyme system, specifically CYP2E1, into a toxic metabolite called NAPQI (van Rongen et al. 2016). If glutathione levels are depleted, or NAPQI production is excessive, the toxic metabolite can bind to liver proteins and cause cell damage, leading to liver injury. The depletion of glutathione might have taken place in this study due to the high concentrations of chokeberry consumption. It is possible that chokeberry occupied the glutathione metabolic pathway, leading to increased competition for the same metabolic enzymes between APAP and chokeberry. As a result, APAP was forced to take the alternative metabolic pathway known as the CYP system, which results in toxic metabolites. Further investigation is needed to clarify the underlying mechanism of chokeberry fruit metabolism.

The chokeberry used in the present study was locally obtained from Büyükçekmece/İstanbul, a field located close to the city center, which provides advantages of transportation to national and international markets. The phenolic, flavonoid, and anthocyanin content of the chokeberry juice can be considered as medium-high level when compared to different juice and dry fruit samples in the literature and market (Tolić et al. 2015). According to our findings the hepatoprotective activity is strikingly high when the strong injury capacity of APAP in the liver is considered. It is plausible to think that optimization of harvest, juice extraction and bottling processes can increase the bioactivity potential of these locally sourced chokeberries and provide an advantage to local farmers. The zebrafish hepatic injury model used here can be used to assess the *in vivo* activity of end products to reach the ideal conditions.

4. Conclusions

We investigated the potential liver protective and healing effects of chokeberry juice using an *in vivo* zebrafish APAP-induced liver injury model (2-5 dpf) and observed promising outcomes, particularly the hepatoprotective effect of chokeberry juice with the concentration of 1 µg/mL. The total phenolic, anthocyanin, and flavonoid content of chokeberry tested here might play a crucial role in protecting liver cells from oxidative damage, thus contributing to the observed positive effects on liver health. These groups of compounds are known to modulate various cellular processes, reduce inflammation, and enhance antioxidant defense mechanisms, which could be beneficial in mitigating liver injury induced by APAP. Thus, the phytochemical findings and demonstration of chokeberries' hepatoprotective effects on zebrafish provide valuable insights into the potential mechanisms underlying their hepatoprotective properties, for which further investigations are needed. By harnessing the advantages of the zebrafish model, we not only expand the scope of our research on the hepatoprotective effects of chokeberry in relation to its phenolic content but also accelerate the screening of pure compounds and plant extracts for their bioactivity potential using liver-specific transgenic zebrafish models.

The chokeberries collected from Büyükçekmece/İstanbul showed promising potential for hepatoprotective activity due to their medium-high levels of phenolic, flavonoid, and anthocyanin content. Optimization of the harvest, juice extraction, and bottling processes has the potential to further increase the bioactivity of these chokeberries, providing a significant advantage to local farmers. Zebrafish is a useful model for screening the hepatoprotective activities of plant extracts and natural products.

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