The influence of fruit components of *Viburnum opulus* on lipid metabolism – *in vitro* studies

Nina Pietrzyk, MSc

Supervisor: Anna Podsędek, PhD, DSc

Auxiliary supervisor: Małgorzata Zakłos-Szyda, PhD

Abstract

The constantly growing incidence of metabolic diseases, including non-alcoholic liver disease, obesity and type 2 diabetes, prompts scientists to search for new therapeutics with activity to improve lipid metabolism. However, pharmacological treatment of lipid-related metabolic disorders is difficult and carries a large number of side effects. Therefore, in recent years, there has been an intensification of research on natural phytochemicals and their use in the prevention of metabolic diseases. Among the bioactive components of plants, phenolic compounds deserve special attention. They can influence lipid homeostasis by inhibition of the activity of lipolytic enzymes, reduction of lipid accumulation and free fatty acid uptake, influence of mitochondrial potential and ATP production due to the antioxidant activity. The ability of phenolic compounds to reduce the lipogenesis and gluconeogenesis processes, which are significantly disturbed in the state of fatty liver and adipose tissue, is particularly important. High concentrations of free fatty acids may be lipotoxic to cells of peripheral tissues such as skeletal muscles.

Guelder rose (*Viburnum opulus* L.) is one of the plants rich in bioactive compounds. In Eastern Europe, *V. opulus* fruit are used to make jams, preserves and tinctures, while in Turkey they are used to produce a fermented non-alcoholic drink with health-promoting properties. The biological activity of *V. opulus* fruits results from their rich composition, which includes phenolic compounds, such as flavonoids, anthocyanins and proanthocyanidins, as well as vitamin C, carotenoids and iridoids. So far, only a few *in vitro* studies confirmed the anti-inflammatory, anti-obesity and antidiabetic properties of the guelder rose fruit.

In this study, the cytoprotective properties of preparations obtained from the fruits of *V. opulus* against human hepatocytes (HepG2 cell line) and rat myoblast (L6 cell line) were determined. The evaluation of biological activity was carried out on four starting preparations of guelder rose fruit, namely juice obtained from fruits, phenolic compounds isolated from the juice as a result of solid phase extraction and two preparations obtained from fruit pomace after methanol-acetone or acetone extraction. The tested preparations were characterized by a high content of phenolic compounds, and the analysis of the phenolic profile using the UPLC-PDA-Q/TOF-MS method confirmed the presence of flavanols, flavonols, anthocyanins and hydroxycinnamic acids, as well as chlorogenic acid as the dominant phenolic compound. *V. opulus* fruit preparations were characterized with high antioxidant activity in the studied *in vitro* models.

The effect of *V. opulus* fruit preparations on lipid metabolism and their cytoprotective effect was determined for cells grown under standard conditions and for induced steatosis with usage of palmitic and oleic acids, and their mixtures. In the case of cells with steatosis, the preparations reduced oxidative stress, reduced lipid accumulation, and increased the cellular level of glucose uptake.

In order to elucidate the molecular mechanism of action of phenolic compounds of *V. opulus* fruit in HepG2 cells, the levels of proteins significantly involved in lipid metabolism were assessed using the Western blott in terms of the lipogenesis process - the process of lipid synthesis playing a major role in the development of non-alcoholic fatty liver disease, obesity and insulin resistance. The level analyzis of protein kinase AMPK, as well as SREBP-1c, ACC and FAS involved in lipogenesis was performed. The conducted research confirmed the ability of *V. opulus* fruit preparations to increase the level of AMPK phosphorylation (increased level of pAMPKα Thr172) and to reduce the level of SREBP-1c, ACC and FAS also during the co-incubation with fatty acids, which caused a drastic increase in the level of these lipogenic proteins. Simultaneously, the co-incubation of HepG2 cells with preparations and fatty acids increased the level of pSREBP-1c and pACC. Additionally, a high Pearson

correlation coefficients confirmed that phosphorylation of SREBP-1c and ACC resulted from the increased AMPK activity. The obtained results confirmed that activation of AMPK was performed with involvement of LKB-1 kinase, which phosphorylated Thr172 in the catalytic subunit of AMPK. Importantly, a decrease in the ATP level after cells incubation with extracts was observed, which may suggest allosteric activation of AMPK by AMP as a result of an increased intracellular AMP/ATP ratio. It can be suspected that reduction of lipid accumulation in hepatocyte cells incubated with *V. opulus* fruit preparations under induced steatosis conditions was regulated by AMPK.

Due to the fact that disturbed glucose metabolism contributes to the deepening of the process of cells steatosis and their insulin resistance, the levels of proteins involved in the isnulin receptor signaling pathway were analyzed – its substrate IRS-1 and the phosphorylated form (pIRS-1), glucose transporter GLUT4 and PTP-1B – the negative regulator of the pIRS-1 which dephosphorylates the insulin receptor. Incubation of HepG2 cells with palmitic acid resulted in an increased level of PTP-1B and decreased level of pIRS-1. Co-incubation of cells with tested preparations resulted in increased IRS-1 phosphorylation (higher pIRS-1 level) and decreased PTP-1B level. No change in GLUT4 level was observed, but the improvement of glucose uptake, an increase in glycogen levels and a decrease in the level of glucose released into the cell medium were detected. The results suggest that in addition to the positive effect on lipid metabolism, incubation of HepG2 cells with *V. opulus* fruit preparations increased the sensitivity of cells to insulin under the steatosis conditions.

Due to the fact that chlorogenic acid accounted for nearly 70% of the pool of all identified phenolic compounds in the fruit of guelder rose, it was decided to assess whether the activity of the tested preparations was the result of the activity of chlorogenic acid alone. Incubation of HepG2 and L6 cells with concentrations of chlorogenic acid corresponding to the concentration in the used non-cytotoxic concentrations of preparations showed its much lower activity compared to the original preparations (or lack of the activity). This suggests a potential synergism of *V. opulus* phenolic compounds. This was confirmed by further studies on the fractions of the most active preparation (purified juice) obtained after its separation on the Sephadex® LH-20. None of the 5 obtained fractions (containing different groups of phenolic compounds) showed a similar or higher activity towards HepG2 and L6 cells in comparison to the purified juice.

The key aspect of this doctoral dissertation was to determine the activity of fresh juice (as a preparation with potential application in the food industry) and purified juice (as a bioactive component of functional food or dietary supplements) after the process of simulated *in vitro* digestion. As a result of the purification of the obtained digestive mixtures by solid phase extraction, phenolic fractions of the digested preparations were obtained, for which the bioactivity was determined and compared to the crude preparations of *V. opulus* juice. The fresh juice preparation losted its cytoprotective activity, while the purified juice preparation reduced lipid accumulation and free fatty acid uptake, decreased reactive oxygen species and increased glucose analog uptake under "normal" conditions and with FFA-induced steatosis. However, the *in vitro* digestion process of the purified juice reduced its activity by nearly 50%. These studies allowed to confirm the preservation of the cytoprotective activity of *V. opulus* phenolic compounds under the conditions of FFA-induced steatosis after digestion process, and at the same time may suggest their functionality. The active fraction of phenolic compounds in the purified *V. opulus* juice were mostly derivatives of hydroxycinnamic acids, including chlorogenic acid and its isomers - neochlorogenic acid and cryptochlorogenic acid. The remaining phenolic compounds (flavanols, flavonols and anthocyanins) identified in the digested preparation were completely or partially degraded during the *in vitro* digestion process.

The results presented in this dissertation confirm the propriety of using preparations made from the *V. opulus* fruit (mainly purified juice, rich in phenolic compounds) in the prevention of metabolic diseases related to disturbed lipid metabolism. *V. opulus* fruit can be an excellent raw material for enriching functional foods or pharmaceutical preparations in the form of dietary supplements.