# Amelioration of metabolic cardiovascular risk factors and ischemia/reperfusion injury: focus on natural substances

**PhD thesis**

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# List of publications

## 1. Publications directly related to the subject of the thesis

### Articles:

I. **Demján V.**, Kiss T., Siska A., Szabó M. R., Sárközy M., Földesi I., Csupor D., Csont T. Effect of *Stellaria media* tea on lipid profile in rats. *Evidence-Based Complementary and Alternative Medicine* 2020, 2020:5109328, doi: 10.1155/2020/5109328. [Q1, IF: 2.629]

II. **Demján V.**, Sója A., Kiss T., Fejes A., Gausz F. D., Szűcs G., Siska A., Földesi I., Tengölics R., Darula Zs., Csupor D., Pipicz M., Csont T. *Stellaria media* tea protects against diabetes-induced cardiac dysfunction in rats without affecting glucose tolerance. *Journal of Traditional and Complementary Medicine* 2021, doi: 10.1016/j.jtcme.2021.08.003 [D1]

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III. **Demján V.**, Gáspár R., Csont T. Effects of modulation of NMDA receptors in ischemia/reoxygenation injury of cardiac cells. *Cardiologica Hungarica*, 2020 Scientific Congress of the Hungarian Society of Cardiology, Experimental Cardiology II. section, Supplementum D, Vol 50., p. 163.

## 2. Other articles

I. Pipicz M., **Demján V.**, Sárközy M., Csont T. Effects of Cardiovascular Risk Factors on Cardiac STAT3. *International Journal of Molecular Sciences* 2018, 19(11):3572, doi: 10.3390/ijms19113572 [Q1, IF: 5.923 ]

# 1. Introduction

## 1.1. Cardiovascular diseases and ischemic heart disease

Cardiovascular diseases, particularly ischemic heart disease including acute myocardial infarction characterized by impaired blood supply are the leading cause of death worldwide. In the development of atherosclerosis and ischemic heart disease, non-modifiable and modifiable risk factors play a crucial role. The modifiable risk factors have greater clinical relevance and are key targets in cardiovascular prevention.

## 1.2. Hypercholesterolemia and diabetes: metabolic cardiovascular risk factors

Hypercholesterolemia and diabetes are particularly relevant because of their high prevalence, and because these metabolic diseases themselves can also worsen cardiac function independently of atherosclerosis.

Hypercholesterolemia is a metabolic disease characterized by elevated total cholesterol level in the blood. Cholesterol can accumulate in the walls of coronary blood vessels with plaque formation and as a consequence can lead to atherosclerosis worsening the blood supply of the heart causing oxygen and nutrient deficiency. In severe cases, sudden plaque rupture and subsequent thrombus formation can occur, leading to acute myocardial infarction.

Diabetes is a common metabolic disease characterized by elevated blood glucose level and impaired glucose tolerance. It has been reported that diabetes causes vascular functional and structural changes contributing to endothelial dysfunction and development of atherosclerosis. In the absence of proper therapy diabetes may lead to development of various macro- and microvascular complications, including diabetic cardiomyopathy.

## 1.3. Acute myocardial infarction and ischemia/reperfusion injury

Acute myocardial infarction is one of the major manifestations of ischemic heart disease. Occlusion of the coronary arteries is usually caused by thrombus formation due to ruptured atherosclerotic plaques, blocking the normal blood flow and therefore causing an ischemic injury. In clinical therapy, restoration of blood supply at an early stage with reperfusion is crucial to prevent further cell death and decrease infarct size. However, not only the ischemic period causes tissue damage, but reperfusion therapy itself can cause further cell death, so altogether an ischemia/reperfusion (I/R) injury develops.

Attenuation of the severity of I/R-induced cell damage with cytoprotective pharmacological agents is a promising therapeutical approach in the management of ischemic heart disease to improve clinical outcomes.

## 1.4. Natural substances as potential tools for prevention or treatment of ischemia/reperfusion injury

Medicinal herbs and endogenous molecules which can be synthetised in the body belong to the group of natural substances, which are promising agents in ameliorating risk factors of ischemic heart disease or inducing cytoprotection. Their advantages compared to synthetic drugs include better tolerability, less side effects and complex mechanisms of action.

### *1.4.1. Stellaria media*

Nowadays, apart from pharmacotherapy, medicinal plants are gaining popularity in prevention and treatment of various metabolic diseases including hypercholesterolemia and diabetes. In folk medicine, common chickweed (*Stellaria media*) tea is believed to improve general metabolism, normalize increased blood cholesterol level, lower blood glucose level, making it an adjuvant therapy for patients. Moreover, consumption of chickweed tea for lowering blood cholesterol and glucose level is recommended by some websites dealing with medicinal plants and health issues, too. The aboveground part of the herb has been applied in folk medicine as tea. According to the results of recent phytochemical and pharmacological studies, several *Stellaria* species have noteworthy pharmacological activities (e.g., antibacterial, anti-inflammatory, and antiallergic effects). Nevertheless, there is no firm experimental or clinical evidence supporting cholesterol-lowering and anti-diabetic effect of *Stellaria media* tea.

### *1.4.2. Kynurenic acid*

Endogenous molecules exerting cytoprotective properties have great potential in attenuating the developing I/R injury. A promising molecule with recently recognized cytoprotective effects is kynurenic acid, which is synthetised via the kinurenine pathway during degradation of the amino acid tryptophan. It has been reported to exert neuroprotective, anti-inflammatory and antioxidant effects and to attenuate infarct size in an *in vivo* mouse model of myocardial I/R injury. Yet, the underlying molecular mechanisms in the proposed cardioprotective effects remain unclear. One theoretical option is the receptor-mediated effect and modulation of downstream signaling pathways. Kynurenic acid acts as an antagonist on ionotropic glutamate receptors, such as N-methyl-D-aspartate (NMDA) receptor, which are abundant not only in the nervous system, but also in the heart. According to literature data, activation of the NMDA receptors worsened I/R injury in stroke and induced cardiomyocyte apoptosis. Based on these data, we hypothesised that NMDA receptor antagonism may be a possible mechanism explaining the cardioprotective effect of kynurenic acid.

# 2. Aims

The aim of the present thesis was to elucidate the effects of natural substances in prevention or treatment of ischemic heart disease using two different approaches: i) attenuating the severity of modifiable metabolic cardiovascular risk factors and their harmful cardiac consequences or ii) inducing cytoprotection in order to decrease I/R injury.

**1. Improving metabolic cardiovascular risk factors**

Management of modifiable risk factors plays a crucial role in prevention of ischemic heart disease including acute myocardial infarction. We focused on two major metabolic cardiovascular risk factors, hypercholesterolemia and diabetes. According to folk medicine, *Stellaria media* tea is thought to attenuate blood cholesterol and glucose levels; however, there is no firm scientific evidence to support these presumed effects. Our aim was to elucidate the potential therapeutic efficacy of *Stellaria media* tea in two different *in vivo* animal studies: i) in hypercholesterolemia model to describe the potential cholesterol-lowering effect of the tea, and ii) in experimental diabetes to elucidate its effects on glucose homeostasis. Apart from the potential efficacy on the severity of hypercholesterolemia and diabetes, we were also interested in the impacts of the herbal treatment on cardiac structure and function in these metabolic diseases.

**2. Inducing cardiocytoprotection in I/R**

Attenuation of I/R-induced cell death with cytoprotective agents is a promising therapeutic possibility to minimize cardiac injury. We focused on kynurenic acid, a natural, endogenous tryptophan metabolite that has been proposed to exert cardioprotection with unclear underlying molecular mechanisms. Therefore, our aim was to confirm the protective effect of kynurenic acid on cardiomyoblasts in an *in vitro* model of I/R and to test the potential involvement of NMDA receptor antagonism in the mechanism of action.

# 3. Materials and methods

## 3.1. Characterization of *Stellaria media* tea

### *3.1.1. Preparation of Stellaria media tea*

*Stellaria media* was dried, grounded and extracted with boiling water (1:10 w/v ratio) by ultrasonication. The highly dense extract was separated from solid particles by mechanical press, and the aqueous extract was lyophilized.

### *3.1.2. Total flavonoid content of Stellaria media tea and screening for flavonoids*

The total flavonoid content of *Stellaria media* tea was determined as quercetin equivalent (QE) using the aluminum chloride colorimetric method. The presence of ubiquitous flavonoids, namely apigenin, apigenin-7-glucoside, kaempferol, luteolin, quercetin, and rutin in the aqueous extract was screened by ultra performance liquid chromatography (UPLC).

### *3.1.3. Liquid chromatography-tandem mass spectrometry analysis of Stellaria media tea*

The tea was analyzed by data dependent liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a nanoUPLC system online coupled to a mass spectrometer. The effluent was analyzed by MS2 data acquisition. MS2 data were subjected to a spectral library search against the MzCloud database using the Compound Discovery software.

### *3.1.4. Measurement of the antioxidant activity of Stellaria media tea*

DPPH assay was used for free radical scavenging activity measurement. Oxygen radical absorbance capacity (ORAC) assay was applied, then the fluorescence was measured. Trolox was used as standard. The result was expressed as μmol Trolox Equivalent/g of dry extract.

## 3.2. Investigating the effects of *Stellaria media* tea on hypercholesterolemia and cardiac function

### *3.2.1. Animals*

The experiment conforms to the Guide for the Care and Use of Laboratory Animals, and the regulations of the Hungarian Act No. XXVIII of the year 1998 on protection and care of animals were strictly followed. The study was approved by the local animal ethics committee of the University of Szeged (XV.1181/2013). 24 adult (8-week old) male Wistar rats were used in the experiments, weighing 270–324 g at the onset of the experiments. Rats were kept under standard climatic conditions ($22 \pm 2$ °C room temperature, 12 h light/dark cycles), in pairs, in individually ventilated cages and had *ad libitum* access to tap water and rat chow.

### *3.2.2. Experimental design*

Rats were randomized into control, hypercholesterolemia (HChol), and hypercholesterolemia+*Stellaria media* treatment (HChol+*Stellaria media*) groups. Rats in the control group ($n = 8$) received standard laboratory rat chow. The other 16 rats were fed a

special cholesterol-enriched diet, supplemented with 2% cholesterol, and 0.25% sodium-cholate-hydrate for 8 weeks to induce experimental hypercholesterolemia. The diet of 8 animals was further supplemented with *Stellaria media* tea lyophilizate mixed into cookie balls (HChol+*Stellaria media*). On the eighth week, rats were anesthetized with sodium pentobarbital (Euthasol, 50 mg/kg body weight, ip.), and blood samples were taken from the abdominal aorta for analysis of serum lipid profile. Before termination, echocardiography was performed in order to evaluate the effects of experimental hypercholesterolemia and *Stellaria media* on cardiac morphology and function.

#### *3.2.3. Administration of Stellaria media tea*

The dose of *Stellaria media* tea was 100 mg/kg body weight as it is considered as equal to human daily dose. Once a day, rats in the HChol+*Stellaria media* group received lyophilized *Stellaria media* tea mixed into cookie balls. The recipe of cookie dough (2 g/kg body weight/day) included 55% plain flour, 20% caster sugar and 25% water. During the one-week long acclimatization period, the rats were habituated to the cookie balls in order to prevent neophobia and were trained to accept the cookie balls voluntarily in their home cages.

#### *3.2.4. Measurements of serum lipid levels*

Serum total cholesterol, HDL cholesterol, and triacylglycerol levels were analyzed by enzymatic colorimetric assays from Roche. LDL cholesterol levels were measured using a kit adapted to a plate reader.

#### *3.2.5. Transthoracic echocardiography*

Cardiac morphology and function were assessed by transthoracic echocardiography. Two-dimensional and M mode examinations were performed with a Vivid IQ ultrasound system using a phased array 5.0–11 MHz transducer. Data of three consecutive heart cycles were analyzed then the mean values were calculated and used for statistical evaluation.

#### *3.2.6. Statistical Analysis*

All values are presented as mean ± SEM. One-way ANOVA was used to determine the differences among the three experimental groups. $p<0.05$ was accepted as statistically significant difference, using Tukey *post hoc* test.

### 3.3. Testing the effects of *Stellaria media* tea on diabetes and heart function

#### *3.3.1. Animals*

Altogether 30 adult (9-week old) male Wistar rats were used in this study, weighing 292–420 g at the onset of the experiments. Animal handling and ethical regulation were described in the 3.2.1 subsection of the thesis.

*3.3.2. Experimental design*

Rats in the control group (n=10) received standard laboratory rat chow. The other 20 rats were fed a special fructose-enriched diet, supplemented with 60% fructose for 20 weeks and were treated with a low-dose streptozotocin (STZ) injection (20 mg/kg body weight, ip.) on the 17$^{th}$ week to achieve experimental diabetes. The diet of 10 animals was further supplemented with *Stellaria media* tea administered with oral gavage technique (Diabetes+*Stellaria media*). Fasting blood glucose levels were measured every 4 weeks, accompanied by oral glucose tolerance tests (OGTT) at weeks 12, 16 and 19. On the 20$^{th}$ week, rats were anesthetized and blood samples were taken from the abdominal aorta for analysis of serum parameters, then pancreata and hearts were isolated. Isolated hearts were subjected to Neely working perfusion. Left ventricular heart tissue samples were snap frozen and stored at −80 °C until further biochemical analysis.

*3.3.3. Administration of Stellaria media tea*

*Stellaria media* tea lyophilizate was prepared as described in the 3.1.1 subsection. The lyophilized powder was then dissolved in distilled water (100 mg/ml). Rats received *Stellaria media* tea (100 mg/kg body weight) with oral gavage technique once a day since the onset of the experiment. The Control and Diabetes groups received equal amount of distilled water.

*3.3.4. Measurement of serum parameters reflecting endocrine function of the pancreas*

Fasting blood glucose level and OGTT measurements were performed in blood samples from the *vena saphena* using test stripes after overnight fasting. By OGTT measurements, 1.65 g/kg body weight glucose was administered by oral gavage and blood glucose levels were measured at 30, 60, 90 and 120 minutes after and area under the curve (AUC) values were calculated. Haemoglobin A1c (HbA1c) levels were analyzed by DCA Vantage Analyzer System. Serum insulin was determined by enzyme-linked immunosorbent assay technique.

*3.3.5. Ex vivo working heart perfusion*

Hearts were isolated, the aorta and left atrium were cannulated and initially perfused in Langendorff mode with Krebs-Henseleit buffer. Then, the perfusion system was switched to working mode according to Neely with recirculating buffer. Heart rate, coronary flow, aortic flow, cardiac output, left ventricular developed pressure (LVDP) and its first derivatives ($dp/dt_{max}$ and $dp/dt_{min}$), and left ventricular end-diastolic pressure (LVEDP) were measured.

*3.3.6. Western blot analysis*

Phosphorylation of signal transducer and activator of transcription 3 (STAT3), protein kinase B (Akt) and extracellular signal-regulated kinase (Erk) proteins, and activation of Bax and Bcl-XL proteins were detected by Western blot.

*3.3.7. Statistical analysis*

All values are presented as mean ± SEM. Repeated measures two-way ANOVA was applied for analysis of body weight change. One-way ANOVA was used to determine the differences among the three experimental groups and $p<0.05$ was accepted as statistically significant difference, using Holm-Sidak *post hoc* test. In the analysis of the working heart perfusion and Western blot results, those data which were out of mean ± 2SD range were excluded in order to minimize the effect of extremities.

## 3.4. Investigating the impact of kynurenic acid and NMDA-receptor modulation in simulated ischemia/reperfusion

*3.4.1. Experimental design*

H9c2 cardiomyoblasts cells were exposed to simulated I/R protocol, which mimics acute myocardial infarction *in vitro*. Cells were treated with 64 μM kynurenic acid to confirm its cardioprotective effects. To examine the involvement of NMDA receptors, cells were treated with an NMDA receptor agonist, NMDA (25–400 μM), an NMDA receptor antagonist, MK-801 (0.47–120 μM) or a combination of NMDA+MK-801 or NMDA+kynurenic acid.

*3.4.2. Simulated ischemia/reperfusion*

During the 6 hours of simulated ischemia, cells were covered with glucose-free hypoxic solution with pH adjusted to 6.4, and kept in a hypoxic incubator. Then the cells were transferred back into the normoxic incubator for 2 hours and covered with differentiation medium. A control group of cells was covered with glucose-containing normoxic solution at physiological pH level.

*3.4.3. Viability assay and oxidative stress measurement*

Calcein assay was used to measure cell viability. Cell death was determined as percentage of simulated I/R-induced cell death, calculated as the difference between viability values of normoxic and vehicle treated hypoxic groups. To investigate simulated I/R-induced cellular superoxide production, dihydroethidium staining was performed.

*3.4.4. Analysis of cell nuclear morphology*

Apoptotic morphological changes of cell nuclei (bigger, disintegrated nuclei, apoptotic bodies, cellular fragments or smaller condensed nuclei) were visualized by DAPI staining.

*3.4.5. Statistical analysis*

All values are presented as mean ± SEM. One-way ANOVA was used to determine the differences among the experimental groups and $p<0.05$ was accepted as statistically significant difference, using Fisher LSD *post hoc* test. In the analysis, those data which were out of mean ± 2SD range were excluded in order to minimize the effect of extremities.

# 4. Results

## 4.1. Characterization of *Stellaria media* tea

### *4.1.1. Total flavonoid content and flavonoid screening*

The total flavonoid content determined by means of UV-VIS absorbance was 9.88±0.10 mg QE/gram. The flavonoid screening by means of UPLC afforded identification of rutin, based on comparison of its retention time and UV spectrum with a reference standard.

### *4.1.2. Liquid chromatography-tandem mass spectrometry analysis*

Several components have been detected. Five out of the ten most intense sample components appeared to be various glycosylated apigenin-derivatives with a spectral library search against the MzCloud database.

### *4.1.3. In vitro antioxidant activity*

*Stellaria media* tea exerted a rather low direct antioxidant capacity in both assays: $EC_{50}$ 168.30 ± 11.06 μg/L in the DPPH assay and 0.97 ± 0.16 μmol TE/g in the ORAC study.

## 4.2. *Stellaria media* tea does not lower blood cholesterol and has no effect on heart function in hypercholesterolemia

### *4.2.1. Serum lipid parameters and body weight*

Total and LDL cholesterol concentration was significantly elevated in the HChol and the HChol+*Stellaria media* groups; however, there was no significant difference between HChol and HChol+*Stellaria media* values. Serum HDL cholesterol level was significantly higher in the HChol group compared to control values; however, *Stellaria media* treatment did not affect it significantly. Triacylglycerol levels showed no significant differences. Neither cholesterol-enriched diet nor *Stellaria media* treatment affected weight gain and body weight significantly.

### *4.2.2. Heart function assessed by transthoracic echocardiography*

Diet-induced hypercholesterolemia and *Stellaria media* treatment did not alter cardiac morphology or function compared to controls in any of the measured parameters.

## 4.3. *Stellaria media* tea does not influence glucose homeostasis in diabetes; however, it improves diabetes-induced cardiac dysfunction

### *4.3.1. Fasting blood glucose, glucose tolerance and body weight*

A significant elevation in fasting blood glucose and AUC levels was observed in the Diabetes group, achieving an experimental diabetes state. *Stellaria media* treatment did not affect fasting hyperglycemia and glucose intolerance. Body weight increase was reduced in the last 6 weeks in the Diabetes group; this effect was not significant with *Stellaria media*.

*4.3.2. Parameters reflecting endocrine function of the pancreas*

At the end of the 20-week experiment, elevated fasting blood glucose, non-significantly increased HbA1c and serum insulin levels decreased by approximately 20% indicated impaired endocrine pancreatic function in the Diabetes group. *Stellaria media* tea failed to improve these parameters.

*4.3.3. Working heart perfusion*

Aortic flow, cardiac output and cardiac work were significantly impaired in the Diabetes group, indicating diabetic adverse effects on the heart. *Stellaria media* treatment significantly improved cardiac output and cardiac work. LVEDP showed a tendency of elevation in the Diabetes group; however, *Stellaria media* had no prominent effect on this parameter.

*4.3.4. Cardiac signaling pathways*

The phosphorylation of STAT3 was significantly elevated in the Diabetes group which was attenuated by *Stellaria media* treatment. There were no significant differences in Bax and Bcl-XL proteins and in the phosphorylation of Akt and Erk proteins.

**4.4. Kynurenic acid exerts cytoprotective effect on cardiac cells in simulated ischemia/reperfusion independently of NMDA receptor modulation**

*4.4.1. Effects of kynurenic acid on cell viability and oxidative stress in simulated I/R*

Simulated I/R protocol induced a significant increase in cell death and oxidative stress compared to the normoxic control group. 64 μM kynurenic acid improved these alterations significantly.

*4.4.2. Effects of NMDA treatment and its combination with antagonists on cell viability and oxidative stress in simulated I/R*

100, 200 and 400 μM NMDA treatment during simulated I/R significantly enhanced cell death. In accordance with cell death, compared to the vehicle group, NMDA treatment increased the ratio of cell nuclei showing apoptotic morphological alterations. These changes were diminished with the combined treatment with antagonists. In settings of oxidative stress measurement, NMDA treatment and combination of NMDA and the two antagonists caused a similar trend as in case of cell death, however, these alterations did not reach the level of statistical significance.

*4.4.3. Effect of the NMDA receptor antagonist, MK-801 on cell viability in simulated I/R*

In comparison to kynurenic acid, the inhibition of NMDA receptors by MK-801 antagonist did not attenuate simulated I/R-induced cell death significantly in any of the applied concentrations, suggesting that the cytoprotection elicited by kynurenic acid may be NMDA receptor independent.

# 5. Discussion and conclusion

In this thesis, we investigated the role of natural substances (a medicinal herb and an endogenous molecule) in prevention or treatment of ischemic heart disease using two different approaches: by influencing metabolic cardiovascular risk factors and their cardiac consequences, or by inducing cytoprotection in order to decrease the I/R-induced cell death.

## 5.1. New findings

The novel findings of the present thesis can be summarised as follows:

- *Stellaria media* tea does not lower blood cholesterol level and does not influence cardiac function in hypercholesterolemia;
- *Stellaria media* tea has beneficial effects on diabetes-induced cardiac dysfunction: it improves impaired cardiac output and cardiac work; moreover, it prevents the diabetes-induced increase in cardiac STAT3 phosphorylation;
- The beneficial cardiac effect of *Stellaria media* tea is independent of the modulation of diabetes severity as it does not influence fasting hyperglycemia or glucose intolerance in diabetes;
- NMDA receptor antagonism is not the main mechanism in the cardiocytoprotective effect of kynurenic acid in simulated ischemia/reperfusion.

*Stellaria media* is a nowadays popular medicinal herb for lowering blood cholesterol and glucose levels. However, our findings do not support the rationale for using chickweed tea in these indications as the tea does not alter blood lipid profile in experimental hypercholesterolemia and does not improve glucose homeostasis in diabetes. In the literature, the antiobesity effect of ethanolic and methanolic extracts of *Stellaria media* was examined and administration of 400 mg/kg body weight methanolic extract was found to decrease triacylglycerol levels in different obesity models, but it did not affect total cholesterol levels. The effect of *Stellaria media* on the severity of diabetes was examined specifically by only one study in the literature. Ethanolic leaf extract of *Stellaria media* in a dose of 100–400 mg/kg/day administered by intraperitoneal injection has been shown to attenuate hyperglycemia in a 21-day alloxan-induced diabetic rat model. In contrast, Chidrawar *et al.* found that ethanolic extract of *Stellaria media* was ineffective to decrease hyperglycemia in both cafeteria-diet- and progesterone-induced obesity models. However, they also found that 200 and 400 mg/kg methanolic extract significantly attenuated serum glucose levels in these models. It should be noted that intraperitoneal application of ethanolic and methanolic

extracts of *Stellaria media* has limited translational value in the view of the human consumption of this medicinal plant.

Diabetic cardiomyopathy is one of the major consequences of diabetes. In our study, aortic flow, cardiac output and cardiac work were significantly decreased in the Diabetes group in comparison to the Control group. *Stellaria media* tea treatment significantly improved cardiac output and cardiac work, suggesting that *Stellaria media* tea may have beneficial effects on the heart in a diabetic state.

*Stellaria media* has been reported to contain active metabolites e.g., phenolic compounds, flavonoids or steroid saponins that may play a role in pharmacological activities. Analysis of flavonoid screening indicated rutin being a possible component in the *Stellaria media* tea in our present study. The beneficial cardiovascular effect of rutin in diabetes has been already proposed. Some studies demonstrated that rutin alleviates diabetic cardiomyopathy and improves left ventricular dysfunction in STZ-induced diabetes and in high-carbohydrate, high-fat diet models. LC-MS/MS analysis of the extract afforded identification of various glycosylated apigenin-derivatives, which finding is in accordance with literature data. The glycosylated flavonoid derivatives can be poorly absorbed in the intestines directly; therefore, during digestion there is a deglycosylation step, and after that only the aglycone part of the molecule will be absorbed therefore being responsible for the biological effects. The aglycone part of these components is apigenin, which has already been reported to alleviate STZ-induced diabetic cardiomyopathy and to exert protective effects against cardiac dysfunction in myocardial infarction in diabetic rats, suggesting that apigenin-derivatives may contribute to the beneficial cardiac effects of *Stellaria media* tea.

We revealed that *Stellaria media* prevents diabetes-induced STAT3 phosphorylation in the heart. In the literature, STAT3 is proposed as a key mediator of diabetes-induced cardiac dysfunction and diabetes can increase its phosphorylation. However, it was reported that attenuation of the enhanced cardiac STAT3 activation may improve diabetes-induced cardiac dysfunction, therefore it may play a role in the beneficial cardiac effect of *Stellaria* media tea. Interestingly, the possible presence of rutin in *Stellaria media* tea may be a feasible explanation for the observed effects in our study, as in the literature rutin was suggested to exert cytoprotection by inhibiting STAT3 phosphorylation.

As a future perspective, it would be worthwhile testing the effects of *Stellaria media* tea in a developed hypercholesterolemic or diabetic state, which may have greater clinical relevance. Moreover, the lack of a group receiving only herbal treatment without cholesterol- or fructose-enriched diet can be considered as a possible limitation of our studies. Nevertheless, further investigation of STAT3 signaling pathway could identify downstream

targets or direct cause-effect mechanisms. Applying other doses of the tea, using a different extraction method or combination with standard therapies may also contribute to a deeper knowledge. It would be worth studying the effects of *Stellaria media* tea not only in a rat model, which might differ from the human metabolism, but in humans as well to show we can observe functional cardiac improvement in diabetic patients who drink chickweed tea regularly.

Apart from attenuating the severity of modifiable risk factors, another therapeutical approach is to induce cytoprotection with pharmacological agents to ameliorate cell death in I/R injury. We examined the involvement of NMDA receptors in the proposed cardioprotective effect of kynurenic acid, an endogenous metabolite. According to our results, NMDA receptor activation worsens ischemia/reperfusion injury. This finding is in accordance with literature data, as in the myocardium, NMDA receptor activation has been reported to induce mitochondrial dysfunction, oxidative stress and apoptosis in neonatal rat cardiomyocytes and activation of the receptors facilitated atrial fibrillation in rats. Moreover, according to literature data, NMDA receptor antagonism has been shown to have protective effects in the heart. However, in our studies, we did not confirm the beneficial effect of NMDA receptor antagonism in attenuating simulated I/R-induced cell damage. In contrast to kynurenic acid, MK-801 treatment alone did not attenuate cell death significantly, suggesting that the NMDA receptor antagonism might not be the main mechanism in the protective effect of kynurenic acid.

As the cardioprotective effect of kynurenic acid seems to be independent from NMDA receptor antagonism, one may speculate about the other possible receptors and modulation of downstream signaling pathways in the mechanism. Apart from NMDA receptor antagonism, kynurenic acid acts as an agonist on the G-protein-coupled receptor 35 (GPR35) and aryl-hydrocarbon receptor. Activation of GPR35 has been implicated to be involved in cardiovascular diseases and to inhibit pro-inflammatory signals. In response to hypoxia, mRNA and protein levels of GPR35 increased in neonatal mouse cardiomyocytes and myocardial GPR35 expression was induced due to acute myocardial infarction in *in vivo* mouse models. Aryl-hydrocarbon receptor pathway has been shown to play an important role in the development and function of the cardiovascular system. Activation of aryl-hydrocarbon receptors have been reported to mediate cardioprotective effects against doxorubicin-induced cardiotoxicity. Moreover, it was shown that the expression of these receptors increased after myocardial I/R injury in cardiomyocytes, suggesting that these receptors might play a role in myocardial I/R injury. One may further speculate about the other receptor-independent possibilities in the protective effect of kynurenic acid. Antioxidant property seems to be a

feasible approach, since kynurenic acid treatment attenuated the oxidative stress marker dihydroethidium fluorescence intensity in our study. This is in accordance with literature data, where kynurenic acid has been reported to exert antioxidant properties. Based on these literature data, involvement of GPR35 or aryl-hydrocarbon receptors or antioxidant property seems to be a feasible approach in the underlying mechanism behind the proposed cardioprotective effect of kynurenic acid; however, further studies are needed to address these issues.

We tested the effects of kynurenic acid and NMDA receptor modulation in *in vitro* experiments. In the future, it would be worth examining and hopefully demonstrating the proposed cardioprotective effect in *ex vivo* isolated hearts or in *in vivo* models of acute myocardial infarction. Moreover, additional studies are needed to further explore the underlying molecular mechanisms, the involvement of other receptors and signaling pathways associated with the cardioprotective effects of kynurenic acid.

Taken together, natural substances may have beneficial effects and relevance in prevention or treatment of ischemic heart disease and therefore they can be valuable candidates for future research for attenuating mortality and improving clinical outcomes of ischemic heart disease.

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# Társszerzői lemondó nyilatkozat

Alulírott **Dr. Kiss Tivadar**, mint megosztott első szerző nyilatkozom arról, hogy **Dr. Demján Virág** doktorjelölt szerepe az alábbiakban megjelölt közös publikációnk eredményeinek elérésében meghatározó volt. Kijelentem, hogy a publikációt a jelölt teljes mértékben felhasználhatja PhD értekezéséhez. Tudomásom szerint ezen eredményeinket más még nem használta fel tudományos fokozat megszerzéséhez, illetve ezt a jövőben sem teszi.

Az értekezésben felhasználásra került közös publikáció:

Demján V., Kiss T., Siska A., Szabó M. R., Sárközy M., Földesi I., Csupor D., Csont T. Effect of *Stellaria media* tea on lipid profile in rats. *Evidence-Based Complementary and Alternative Medicine* 2020, doi:10.1155/2020/5109328

.....

Dr. Kiss Tivadar

Szeged, 2021.10.05.

# Társszerzői lemondó nyilatkozat

Alulírott **Dr. Gáspár Renáta** és **Dr. Halmi Dóra**, mint megosztott első szerzők nyilatkozunk arról, hogy **Dr. Demján Virág** doktorjelölt szerepe a beadás előtt álló közös publikációnk alábbiakban megjelölt eredményeinek elérésében meghatározó volt és kijelentjük, hogy ezen eredményeket a jelölt felhasználhatja PhD értekezéséhez. Tudomásunk szerint ezen eredményeinket más még nem használta fel tudományos fokozat megszerzéséhez, illetve ezt a jövőben sem teszi.

Az értekezésben felhasználásra került eredmények:

- 64 μM kinurénsav kezelés hatása H9c2 sejtek calcein festéssel vizsgált életképességére és dihidroetídium festéssel vizsgált oxidatív stressz mértékére szimulált iszkémia/reperfúziós modellben
- NMDA kezelés, valamint NMDA+kinurénsav és NMDA+MK-801 kombinált kezelések hatása H9c2 sejtek calcein festéssel vizsgált életképességére, dihidroetídium festéssel vizsgált oxidatív stressz mértékére, valamint DAPI festéssel vizsgált sejtmagmorfológiai eltérésekre szimulált iszkémia/reperfúziós modellben
- MK-801 kezelés hatása H9c2 sejtek calcein festéssel vizsgált életképességére szimulált iszkémia/reperfúziós modellben

A beadás előtt álló közös publikációnk adatai:

Gáspár R., Halmi D., Demján V., Diószegi P., Igaz N., Juhász L., Z. Poles M., Patai R., Polgár T.F., Vincze A., Pipicz M., Kiricsi M., Csont T. The effect of kynurenic acid on ischemia/reperfusion injury, apoptosis and mitochondrial function in cardiomyocytes

Dr. Gáspár Renáta ..... ..... Dr. Halmi Dóra

Szeged, 2021.10.05.