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

PhD thesis

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Szeged

2021

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]

Conference presentation:

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]

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1. List of Abbreviations

A	late diastolic transmitral flow velocity
Akt	protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ANOVA	analysis of variance
AUC	area under the curve
CK-MB	creatine kinase–myocardial band
DAPI	2-[4-(aminoiminomethyl)-phenyl]-1H-indole-6-carbo-ximidamide-hydrochloride
$dp/dt_{max, min}$	maximum and minimum of first derivatives of LVDP
DHE	dihydroethidium
D-PBS	Dulbecco's phosphate buffered saline
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
E	early diastolic transmitral flow velocity
E'	early diastolic mitral annular tissue velocity
ELISA	enzyme-linked immunosorbent assay
Erk	extracellular signal-regulated kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPR35	G-protein-coupled receptor 35
HbA1c	hemoglobin A1c
HChol	hypercholesterolemia
HDL	high-density lipoprotein
HPLC	high performance liquid chromatography
I/R	ischemia/reperfusion
ip.	intraperitoneal
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LDL	low-density lipoprotein
LVDP	left ventricular developed pressure
LVEDP	left ventricular end-diastolic pressure
NMDA	N-methyl-D-aspartate
OGTT	oral glucose tolerance test
ORAC	oxygen radical absorbance capacity
PBS	phosphate buffered saline
QE	quercetin equivalent
SEM	standard error of the mean
STAT3	signal transducer and activator of transcription 3
STZ	streptozotocin
TE	trolox equivalent
UPLC	ultra performance liquid chromatography

2. Summary

Background and aims

Cardiovascular diseases, particularly ischemic heart disease including acute myocardial infarction are among the leading causes of death worldwide; therefore, they are a relevant research area. Atherosclerosis-related impaired blood supply, i.e., ischemia causes nutrient and oxygen deficiency in the heart resulting in cell death, which is further enhanced by reperfusion itself, contributing to ischemia/reperfusion injury.

Management of modifiable risk factors is crucial in prevention of ischemic heart disease, where hypercholesterolemia and diabetes are particularly relevant metabolic cardiovascular risk factors. Nowadays, apart from pharmacotherapy, medicinal herbs are gaining popularity in prevention and treatment of these metabolic diseases. According to folk medicine, common chickweed (*Stellaria media*) tea is thought to attenuate blood cholesterol and glucose levels; however, there is no firm scientific evidence to support these presumed effects. Therefore our aim was to elucidate the potential therapeutic efficacy of *Stellaria media* tea in animal models of hypercholesterolemia and diabetes, and to describe the impacts of the herbal treatment on cardiac structure and function in these metabolic diseases.

Attenuation of ischemia/reperfusion-induced cell death with cytoprotective pharmacological 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. Our aim was to confirm the protective effect of kynurenic acid on cardiomyoblasts in an *in vitro* model of ischemia/reperfusion and to test the potential involvement of N-methyl-D-aspartate (NMDA) receptors in the mechanism of action, on which kynurenic acid exerts antagonist effects.

Materials and methods

For characterization of *Stellaria media* tea itself, flavonoid content, chemical composition and antioxidant activity were measured. We tested the effects of *Stellaria media* tea in two different *in vivo* rat models. Experimental hypercholesterolemia was induced by cholesterol-enriched diet and experimental diabetes was achieved by fructose-enriched diet supplemented with a single low-dose of streptozotocin. Parameters representing blood lipid profile and glucose homeostasis were measured, respectively. Cardiac structural or functional parameters were also assessed. In order to elucidate the underlying molecular mechanisms in cardiac alterations, common signaling pathways were examined.

In a separate set of experiments, we investigated the impacts of kynurenic acid in an *in vitro* model of ischemia/reperfusion on H9c2 cardiomyoblast cells. Then we tested the involvement of NMDA receptors in the effect. Cells were treated with an NMDA receptor agonist (NMDA), a synthetic antagonist (MK-801), or a combination of NMDA and the endogenous (kynurenic acid) or synthetic antagonist. Cell viability, oxidative stress and morphological alterations of cell nuclei were measured.

Key results

Stellaria media tea does not lower blood cholesterol level in hypercholesterolemia and does not influence cardiac function. In diabetes, *Stellaria media* treatment does not change elevated blood glucose level and impaired glucose tolerance; however, it improves cardiac output and cardiac work and prevents increased phosphorylation of cardiac signal transducer and activator of transcription 3 protein. The tea exerts a rather low direct antioxidant capacity; nevertheless, it contains various apigenin glycosides and presumably rutin, which may contribute to cardioprotection.

The cardiocytoprotective effect of kynurenic acid is confirmed on H9c2 cells. Moreover, NMDA treatment dose-dependently elevates cell death and ratio of cell nuclei with apoptotic morphological alterations which is diminished by co-treatment with antagonists. However, in comparison to kynurenic acid, MK-801 antagonist treatment alone does not influence cell viability.

Conclusions

In our studies, we tested two substances with natural origin in different aspects of managing ischemic heart disease. Our results did not confirm the blood cholesterol- and glucose-lowering effect of *Stellaria media* tea in the examined dose and animal models for two major modifiable metabolic risk factors. However, *Stellaria media* treatment can be promising in improving diabetic cardiac dysfunction. In the mechanism of cytoprotective effects of kynurenic acid in ischemia/reperfusion injury, NMDA receptor antagonism may not play a major role. Taken together, natural substances can be valuable candidates for future research for attenuating mortality and improving clinical outcomes of ischemic heart disease.

3. Introduction

3.1. Cardiovascular diseases and ischemic heart disease

Despite the promising tendency in improvements of outcomes with the help of new therapeutic strategies, cardiovascular diseases remain the leading cause of death worldwide[1]. Among cardiovascular diseases, particularly ischemic heart disease represents an epidemiological burden[2]. According to the Hungarian Central Statistical Office, almost a quarter of all deaths were caused by ischemic heart disease in 2019[3]. This disease group is usually characterized by atherosclerosis-related impaired blood supply, i.e., ischemia in the coronary arteries and therefore nutrient and oxygen deficiency, which leads to harmful structural and functional consequences in the heart.

In the development of ischemic heart disease, non-modifiable and modifiable cardiovascular risk factors play a crucial role (Figure 1). Male sex, elderly age, genetic factors, family history and ethnicity belong to the non-modifiable risk factors. The modifiable risk factors have greater clinical relevance and are key targets in prevention. They include e.g., hypercholesterolemia, diabetes, smoking, obesity, physical inactivity, fat and sugar rich diet, elevated blood pressure and psychosocial factors[4] (Figure 1).

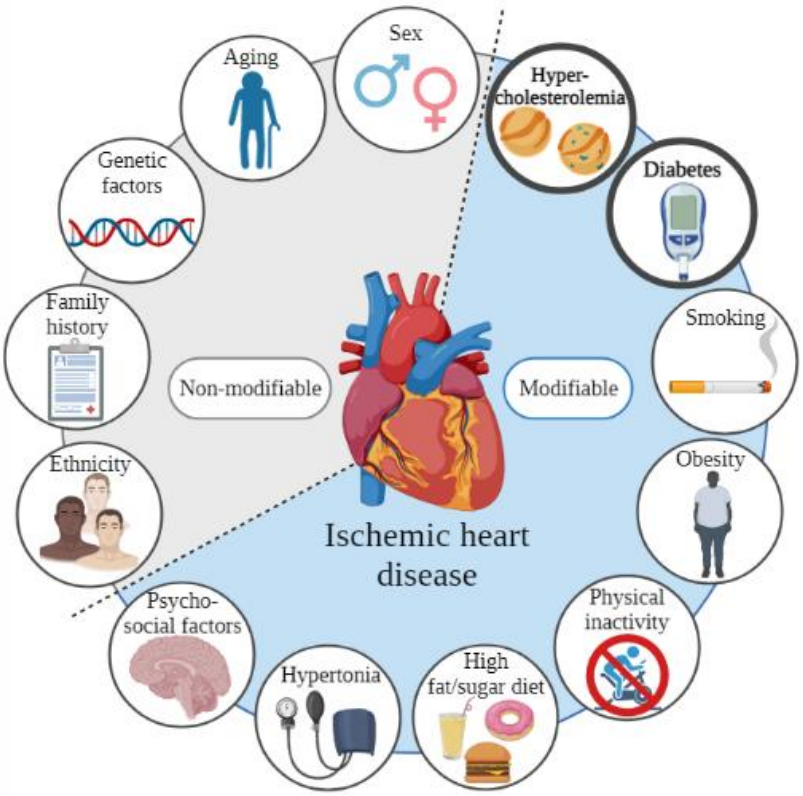

Figure 1: Non-modifiable and modifiable risk factors of ischemic heart disease

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3.2. Hypercholesterolemia and diabetes: metabolic cardiovascular risk factors

Attenuating the severity of modifiable risk factors is crucial in prevention of atherosclerosis and ischemic heart disease related negative cardiovascular outcomes.

Hypercholesterolemia and diabetes are particularly relevant modifiable metabolic cardiovascular risk factors because of their high prevalence, and because these metabolic diseases themselves can also worsen cardiac function independently of atherosclerosis. Due to the continuously increasing trend in hypercholesterolemia and diabetes morbidity, investigation of harmful consequences of the diseases and potential therapeutic interventions are relevant and current research areas[5]. Attenuation of these metabolic cardiovascular risk factors has been shown to decrease the incidence and severity of ischemic heart disease[6, 7].

3.2.1. Hypercholesterolemia

Hypercholesterolemia is a metabolic disease characterized by elevated total cholesterol level in the blood. In Europe, on average 74.5% of men aged 40-59 years have higher than 5.2 mmol/L total blood cholesterol level[8]. In the blood, cholesterol can be transported with the help of lipoproteins. Low-density lipoprotein (LDL) transports cholesterol from the liver to the peripheral tissues and cells, while high-density lipoprotein (HDL) is responsible for the reverse cholesterol transport into the liver[9]. Higher level of LDL and lower level of HDL cholesterol represent a greater risk, increasing the probability of accumulation of cholesterol in the periphery[10].

Hypercholesterolemia has severe negative cardiovascular complications as cholesterol can accumulate in the walls of coronary blood vessels with plaque formation and it can lead to atherosclerosis. The plaques contain other lipids, cellular waste products, calcium deposits and fibrin as well[11]. As a consequence, the walls of coronary blood vessels thicken, while the diameter narrows worsening the blood supply of the heart causing oxygen and nutrient deficiency. In severe cases, sudden plaque rupture and subsequent thrombus formation can occur that blocks coronary arteries leading to acute myocardial infarction[12]. Moreover, hypercholesterolemia itself can lead to systolic and diastolic cardiac dysfunction without presence of endothelial dysfunction and atherosclerosis[13].

3.2.2. Diabetes

Diabetes is a common metabolic disease characterized by elevated blood glucose level and impaired glucose tolerance. The prevalence of diabetes for adults was approximately 171 million patients worldwide in 2000 and according to predictions, this number is expected to be doubled by 2030[5]. Approximately 90% of diabetic patients suffer from type 2 diabetes,

which is associated with obesity and is characterized by insulin resistance. In the absence of proper therapy diabetes may lead to development of various macro- and microvascular complications including diabetic cardiomyopathy, cataract, kidney failure, as well as neuronal damage[14]. Moreover, it has been reported that diabetes causes vascular functional and structural changes contributing to endothelial dysfunction and development of atherosclerosis[15].

Diabetic cardiomyopathy is characterized by diastolic and/or systolic dysfunction which can lead to heart failure without the presence of classic risk factors such as dyslipidaemia, hypertension, or coronary artery disease[16, 17]. Pathophysiological mechanisms involved in the development of diabetic cardiomyopathy include abnormal insulin metabolic signaling, hyperglycemia-induced oxidative stress, cardiac lipotoxicity, mitochondrial dysfunction, endoplasmic reticulum stress and cardiomyocyte death[16].

3.3. Acute myocardial infarction and ischemia/reperfusion injury

Acute myocardial infarction is one of the major manifestations of ischemic heart disease (Figure 2). Its diagnosis is established by elevated blood levels of biochemical necrosis markers (i.e., creatine kinase–myocardial band (CK-MB), cardiac troponin T), and electrocardiographical changes (i.e., ST elevation, pathologic q waves) or typical chest pain[18]. 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[19].

In clinical therapy, restoration of blood supply and abolishment of ischemia at an early stage with reperfusion therapy is crucial to prevent further cell death and decrease infarct size[20]. However, not only the ischemic period results in tissue damage, but reperfusion therapy itself can cause further cell death, so altogether we can talk about an ischemia/reperfusion (I/R) injury[21] (Figure 2).

Mechanisms of myocardial injury include diffuse cardiomyocyte loss (necrosis, apoptosis), oxidative stress, margination of nuclear chromatin and electrolyte alterations *etc*.[22]. 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.

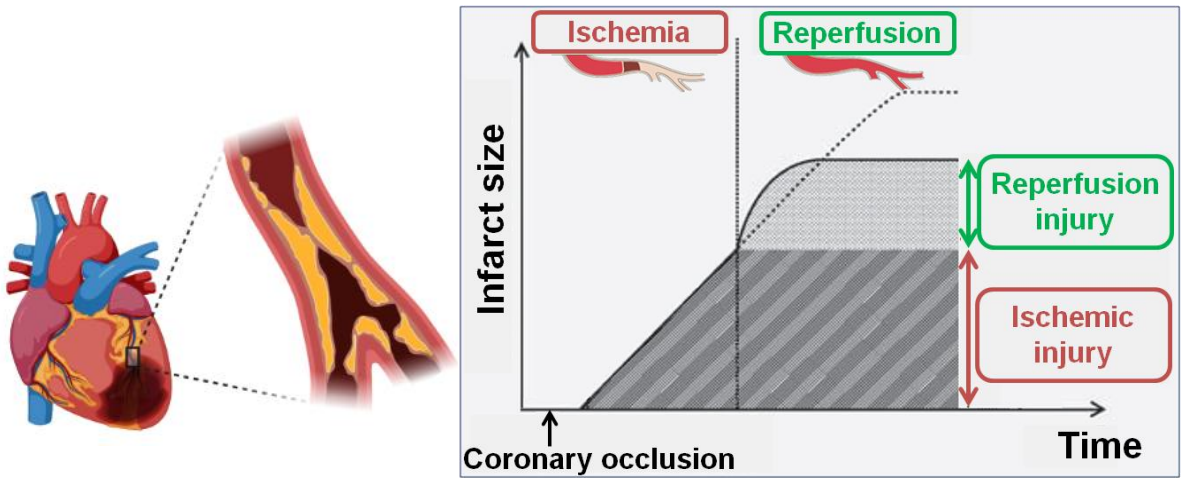

Figure 2: Acute myocardial infarction and ischemia/reperfusion injury[23]
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3.4. Natural substances as potential tools for prevention or treatment of ischemia/reperfusion injury

There are different definitions for natural substances in the literature. Natural substances include a wide variety of substances with natural origin, they can be products derived from plants via various extraction methods without chemical modifications or they can be broadly defined as chemicals produced by living organisms[24]. According to our term used in this thesis, medicinal herbs and endogenous molecules which can be synthetised in the body belong to the group of natural substances. Advantages of these natural substances compared to synthetic drugs include better tolerability, less side effects and complex mechanisms of action. They are promising agents in ameliorating risk factors of ischemic heart disease or inducing cytoprotection and have great relevance in this field. Moreover, they are often known for their antioxidant or anti-inflammatory effects and can serve as a starting step in drug development.

3.4.1. Herbal medicine

Nowadays, apart from pharmacotherapy, medicinal plants are gaining popularity in prevention and treatment of various metabolic diseases including hypercholesterolemia and diabetes. Consumption of these plants in form of tea, extract or dietary supplement is thought to be safer with less side effects than the synthetic medicaments due to their natural origin while exerting similar efficacy (Figure 3). There are some herbs with a well-described and scientifically proven cholesterol-lowering effects, such as ginger (*Zingiber officinale*)[25, 26] or artichoke leaf extract (*Cynara scolymus*)[27, 28]. Other herbs have been reported to exert anti-hyperglycemic properties, for instance aqueous leaf extract of stinging nettle (*Urtica*

dioica)[29, 30] or powdered fenugreek seeds (*Trigonella foenum graecum*)[31, 32]. However, the efficacy and safety of medicinal herbs are not always scientifically proven and their usage is often based only on tradition and presumed properties.

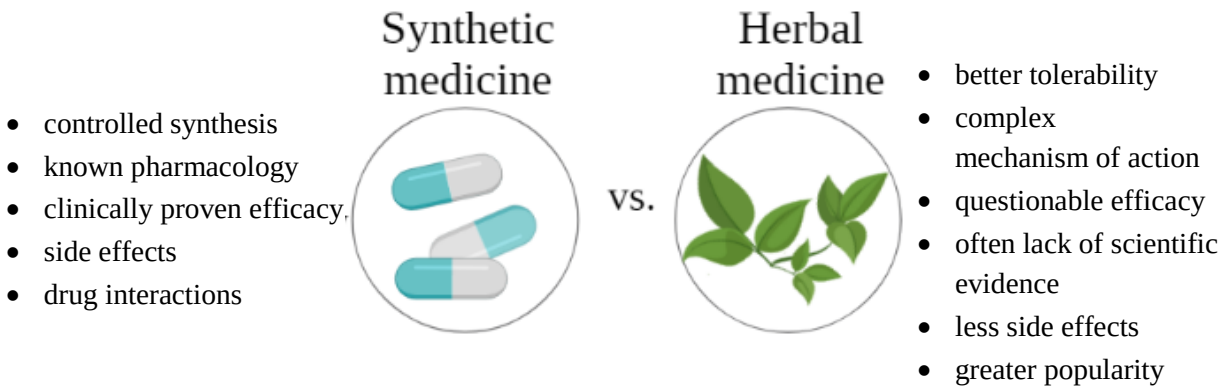

Figure 3: Standard pharmacotherapy and medicinal plants

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3.4.1.1. Stellaria media

Nowadays, in folk medicine common chickweed (*Stellaria media*; Figure 4) is becoming even more prominent in natural remedies. According to a popular Hungarian traditional healer, *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[33]. 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[34, 35].

The wide ecological tolerance and short-term vegetative reproduction period make chickweed a common and widespread species. In cool, moist, and moderately shaded environment, huge territories are covered by this plant; thus, its presence in cultivated fields is a serious agricultural problem.

Figure 4: Common chickweed (*Stellaria media*)

(source:[36])

Although chickweed has been consumed as salad and the aboveground part of the herb has been applied in folk medicine as tea[37, 38], its efficacy has not been investigated in clinical trials[39]. Moderate interest has been shown toward this plant from the middle of the last century. Because of the potential biological benefits, phytochemical and pharmacological studies have started to focus on species of the *Stellaria* genus. These studies are based mainly on *in vitro* or *in vivo* animal experiments. According to their results, several *Stellaria* species have noteworthy pharmacological activities (e.g., antibacterial, anti-inflammatory, and antiallergic effects)[40]. Nowadays, *Stellaria media*, mostly consumed as tea, is gaining popularity for its believed beneficial effects on blood lipid profile and glucose levels[41]. Nevertheless, there is no firm experimental or clinical evidence supporting its cholesterol-lowering and anti-diabetic effect.

3.4.2. Cytoprotective endogenous molecules

Endogenous molecules exerting cytoprotective properties have great potential in attenuating the developing I/R injury. They can be administered exogenously or their endogenous synthesis can be stimulated or their degradation can be inhibited to achieve the biological effects. In the literature, there are some natural substances with well-described cardiocytoprotective effects, such as the nucleoside adenosine[42] or the proteoglycan biglycan[43, 44]. A promising molecule with similar recently recognized cytoprotective effects is kynurenic acid, which is present in plants, mammals and humans as well[45, 46].

3.4.2.1. Kynurenic acid

Kynurenic acid is synthetised via the kinurenine pathway during degradation of the essential amino acid tryptophan (Figure 5). In the first step, tryptophan is converted to N-formyl-L-kynurenine that can be catalyzed by indoleamine 2,3-dioxygenase-1 and -2 enzymes in the liver or tryptophan-2,3-dioxygenase enzyme in the extrahepatic tissues[47]. Then N-formyl-L-kynurenine is converted by formamidase enzyme to L-kynurenine, which is the central intermediate molecule of the kinurenine pathway that can be further converted in three different directions. One of these is the formation of kynurenic acid by kynurenine aminotransferase enzymes[47] (Figure 5).

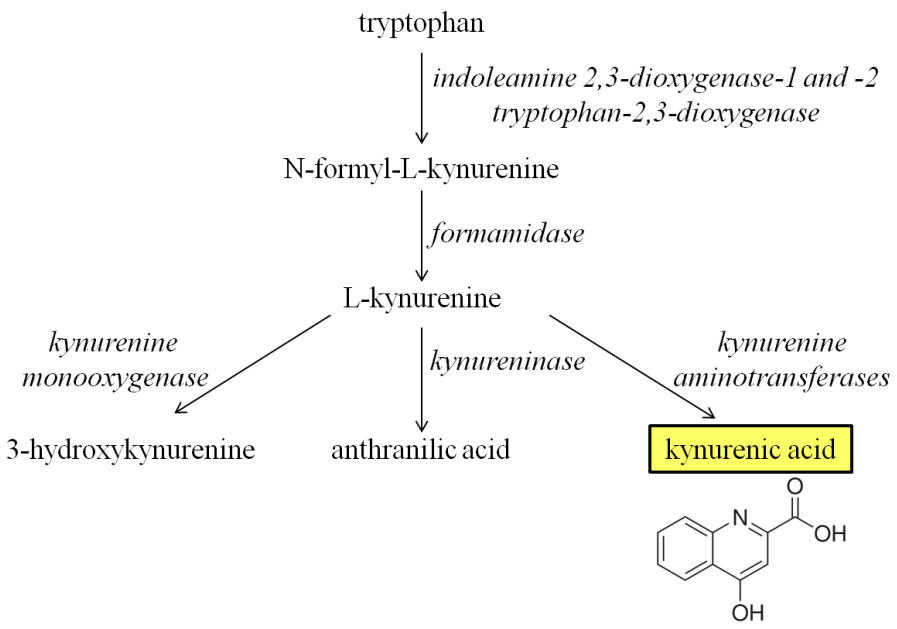

Figure 5: Simplified illustration of tryptophane degradation via the kynurenine pathway (prepared based on:[47])

In the literature, kynurenic acid has been reported to exert neuroprotective[48], anti-inflammatory[49] and antioxidant effects[50]. More importantly, it also has beneficial effects in the cardiovascular field. Olenchock *et al*. demonstrated that pretreatment with kynurenic acid attenuates infarct size in an *in vivo* mouse model of myocardial I/R injury[51]. Our research group got similar results in *in vitro* simulated I/R experiments on neonatal rat cardiomyocytes (data not shown). Yet, the underlying molecular mechanisms in the proposed cardioprotective effects remain unclear. One theoretical option is that kynurenic acid has direct anti-inflammatory and immunosuppressive properties which can be beneficial since in response to myocardial damage, complement system will be activated and cytokines will be released causing an inflammatory reaction accompanied by recruiting leukocytes to the heart[52]. Moreover, kynurenic acid exerts antioxidant effects attenuating oxidative stress which plays a role in the development of I/R injury[50, 53]. Another possibility 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), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and cainate receptors, as well as for nicotinic acetylcholine receptor, while it is an agonist for G-protein-coupled receptor 35 (GPR35) and aryl-hydrocarbon receptors[54]. As a first step, our research group focused on the potential involvement of NMDA receptors in the mechanism of action of kynurenic acid.

NMDA receptors are abundant not only in the nervous system, but also in peripheral organs, such as the heart, lung, kidney and pancreas[55]. The receptors belong to the ionotropic

glutamate receptors and have a heterotetramer structure containing two obligatory GluN1 subunits in combination with two GluN2 and/or GluN3 subunits[56]. Simultaneous binding of glycine and glutamate amino acids activates these ligand-gated ion-channel receptors. As the channel opens, Ca^{2+}, Na^{+} and K^{+} cations flow into the cells and this increased calcium influx influences various signaling pathways and the mitochondria[56].

NMDA receptor activity can be modulated both positively and negatively. Activator modulators increase the maximal receptor response or the affinity for the agonist, but have a different binding site from the agonist binding site[56]. Receptor antagonists may be sorted into three categories: competitive antagonists, which can bind to the same bindig site as the agonist, ion channel blockers or noncompetitive antagonists which have subunit-specific binding sites[56]. Kynurenic acid belongs to the noncompetitive group and is a glycine bindig site antagonist.

Overactivation of NMDA receptors results in excessive Ca^{2+} influx into the cells, activating various signaling pathways which may lead to excitotoxicity causing neuronal damage[57, 58]. This has been demonstrated to play a role in the pathomechanism of different neurodegenerative disorders, such as Alzheimer's disease[59]. According to literature data, activation of the NMDA receptors worsened I/R injury in stroke[60] and induced cardiomyocyte apoptosis[61]. Based on these data, we hypothesised that NMDA receptor antagonism may be a possible mechanism explaining the cardioprotective effect of kynurenic acid.

4. 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.

5. Materials and Methods

5.1. Characterization of *Stellaria media* tea

5.1.1. Preparation of Stellaria media tea

Stellaria media was harvested in Algyő (Hungary) by "Ezerjófű" Association in 2017. Voucher specimen (no: 882) was deposited in the herbarium of the University of Szeged, Faculty of Pharmacy, Department of Pharmacognosy. The drug was dried and stored at room temperature. The dried and grounded drug was extracted with boiling water (1:10 w/v ratio) for 15 minutes by ultrasonication. The highly dense extract was separated from solid particles by mechanical press, and the aqueous extract was lyophilized. Approximately 1.5 g lyophilizate was obtained from 10.0 g dried drug.

5.1.2. Total flavonoid content of Stellaria media tea and screening for flavonoids

Flavonoids are active compounds in several medicinal herbs and are related to anti-oxidative properties; therefore, the total flavonoid content of the lyophilized *Stellaria media* tea was determined in the Department of Pharmacognosy as quercetin equivalent (QE) using the aluminum chloride colorimetric method. The lyophilizate powder was dissolved in methanol to get a solution with a concentration of 1 mg/mL. For calibration curve 5, 10, 25, 50 μg/mL methanolic solutions of quercetin were prepared. As reagent, 2% aluminum chloride methanolic solution was used. Reaction mixtures were prepared by mixing 2 mL of solution and 2 mL of aluminum chloride, respectively. After 60 min of incubation at room temperature, the absorbance was measured against blank by applying UV-VIS spectrophotometer (Helios β ThermoSpectronic) at 420 nm. The total flavonoid concentration was calculated using a calibration plot ($R^2 = 0.9999$). The measurements were carried out in triplicate.

The flavonoid content of the lyophilized *Stellaria media* aqueous extract was screened by ultra performance liquid chromatography (UPLC). For this experiment 1 mg/mL methanolic solution was prepared from the lyophilizate. The presence of ubiquitous flavonoids in plants, namely apigenin, apigenin-7-glucoside, kaempferol, luteolin, quercetin, and rutin was screened by UPLC. Experiments were carried out on a Shimadzu Nexera X2 UHPLC liquid chromatography system equipped with a vacuum degasser, two binary pumps, mixer assembly, an auto sampler, a column temperature controller, and a diode array detector. Analysis was performed at 35 °C on a Kinetex XB-C18 column (100x3 mm, 2.6 μm, 100 Å, Phenomenex), with a mobile phase flow rate of 0.5 mL/min. The composition of mobile phase was water (A) and methanol (B), and gradient elution was applied (0 min: 20% B, 20 min:

40% B, 24 min: 55% B, 27 min: 70% B, 28 min: 70% B, 28.5 min: 20% B, 34 min: 20% B). The injection volume was set at 5 µL and the chromatographic profile was registered at 350 nm.

5.1.3. Liquid chromatography-tandem mass spectrometry analysis of Stellaria media tea

In order to gain more information about the chemical composition, the aqueous extract was filtered through a 0.22 µm membrane filter and diluted tenfold with 0.1% formic acid and analyzed by data dependent liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis in the Biological Research Centre using a Waters MClass nanoUPLC system online coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer.

5 µl of the sample was injected onto a trapping column (Waters #186007496 m-Class Symmetry C18 column; ID: 0.180 mm, L: 20 mm, particle size: 5 µm, pore size: 100 µm) then separated on a reversed phase C18 column (Waters #186007484 BEH130 C18 column; ID: 0.075 mm, L: 250 mm, particle size: 1.7 µm, pore size: 130 Å, thermostated to 45 °C).

The effluent was analyzed by data dependent MS2 data acquisition. Both MS and MS2-HCD data were acquired with high mass accuracy and resolution using the Orbitrap analyzer. Following each MS spectrum (resolution: 120.000), HCD spectra were acquired applying stepped collision energy of NCE: 20, 40, and 60 (resolution: 15.000) on the five most intense precursors (mass range: m/z 240-1400, charge state: $z = 1–3$) in each cycle. A dynamic exclusion window of 5 s was applied. Abundant singly charged background ions (m/z 355.0699, 371.1012, 445.1200, 462.1466, 503.1075, 519.1388, 536.1654 and 610.1842) were excluded from MS2 data acquisition. External calibration to background ions m/z 371.1012 and 445.1200 afforded mass accuracy ~2ppm.

MS2 data were subjected to a spectral library search against the MzCloud database using the Compound Discovery software. The following criteria had to be fulfilled in order to consider an MS2 spectrum: minimum number of corresponding MS1 scans across the extracted ion chromatogram of the precursor: 12, maximum peak width: 1.5 min, minimum intensity: 100.000, minimum signal to noise ratio: 7. Search results with MzCloud best match score of at least 80 can be considered identical or structurally very similar (e.g., isomer) to the database entry. High „MzCloud best similarity match” scores indicate structural similarity to the database entry but different elemental composition (e.g., a flavonoid with identical aglycone but different glycan structure).

5.1.4. Measurement of in vitro antioxidant activity of Stellaria media tea

The *in vitro* antioxidant activity of the lyophilized *Stellaria media* aqueous extract was screened by two different assays in the Department of Pharmacognosy. The measurement of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging activity was carried out according to the method of Fukumoto *et al.* with some modifications[62]. The measurement was carried out on a 96-well microplate. Microdilution series of samples (1 mg/mL, dissolved in high performance liquid chromatography (HPLC) grade methanol) were made starting with 150 µL. To each well, 50 µl of DPPH reagent (100 µM, made with HPLC grade methanol) was added to gain 200 µL working volume. The microplate was stored at room temperature in dark conditions. The absorbance was measured after 30 minutes at 550 nm using a BMG Labtech FluoStar Optima plate reader. For the blank control HPLC grade methanol was used instead of the sample. As standard ascorbic acid (0.01 mg/mL, in HPLC grade methanol) was used. The evaluation of EC_{50} values were carried out with help of Graphpad Prism 6.05.

The oxygen radical absorbance capacity (ORAC) assay was carried out on a 96-well microplate according to the method of Mielnik *et al.*[63]. 20 µL of extracts (0.01 mg/mL) were mixed with 60 µl of 2,2'-azobis(2-methyl-propionamidine) dihydrichloride (12 mM final concentration, Sigma-Alrich, St. Louis, USA) and 120 µL of fluorescein solution (70 nM final concentration, Fluka Analytical, Japan), then the fluorescence was measured through 3 hours with 1.5 minute cycle intervals with BMG Labtech FluoStar Optima plate-reader. All the experiments were carried out in triplicate, as standard trolox ((±)-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid, Sigma-Aldrich, St. Louis, USA) was used. The antioxidant capacity was expressed as µmol Trolox Equivalent per g of dry extract (µmolTE/g), with help of GraphPad Prism 6.05.

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

5.2.1. Animals

The experiment conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (National Institutes of Health publication 85–23, revised 1996), 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 (purchased from Charles River Laboratories, Göttingen, Germany), weighing 270–324 g at the onset of the

experiments. Using only male rats in the studies was intentional as the hormonal changes during the menstrual cycle of females have been shown to influence serum lipids[64, 65] and it can influence cardiac performance as well. Rats were kept under standard climatic conditions (22 ± 2 °C room temperature, 12 h light/dark cycles), in pairs, in individually ventilated cages (Sealsafe IVC system) and had *ad libitum* access to tap water and laboratory rat chow.

5.2.2. Experimental design

After one week of acclimatization, rats were randomized into three groups: control (Control), hypercholesterolemia (HChol), and hypercholesterolemia+*Stellaria media* treatment (HChol+*Stellaria m.*). Rats in the control group (n = 8) received standard laboratory rat chow (Innovo Ltd., Isaszeg, Hungary). The other 16 rats were fed a special cholesterol-enriched diet, i.e., a standard laboratory rat chow supplemented with 2% (w/w) cholesterol (Hungaropharma, Budapest, Hungary), and 0.25% (w/w) sodium-cholate-hydrate (Sigma-Aldrich, St. Louis, USA) for 8 weeks to induce experimental hypercholesterolemia. We have chosen this cost-effective model of experimental hypercholesterolemia because our research group has previously accumulated extensive experience regarding the use of this model[66-68], and the lipoprotein profile of the cholesterol-fed rats (LDL/HDL ratio) is quite similar to that of humans. The diet of 8 animals receiving cholesterol-enriched chow was further supplemented with *Stellaria media* tea lyophilizate mixed into cookie balls (HChol+*Stellaria m.*) in order to examine the potential cholesterol-lowering effect of the plant. On the eighth week, rats were anesthetized with sodium pentobarbital (Euthasol, 50 mg/kg body weight, intraperitoneal (ip.), Produlab Pharma b.v., Raamsdonksveer, The Netherlands), the abdominal cavity was opened, and blood samples were taken from the abdominal aorta. Collected blood was allowed to clot and was centrifuged (2000×g, 20 min, 4 °C); then serum was separated for analysis of serum lipid profile to describe the efficacy of *Stellaria media* treatment. Before termination, transthoracic echocardiography was performed in order to evaluate the effects of experimental hypercholesterolemia and *Stellaria media* on cardiac morphology and function.

5.2.3. 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, calculated according to Nair and Jacob[69]. Rats in the HChol+*Stellaria m.* group received lyophilized *Stellaria media* tea mixed into cookie balls once a day. The recipe of cookie dough included 55% plain flour, 20% caster sugar and 25% water[70]. All animals received 2 g cookie dough/kg body weight per day. We have found in a pilot study that

administration of 2 g/kg body weight cookie dough for 7 days in control rats did not cause significant changes in levels of blood cholesterol, triacylglycerol, or glucose (data not shown). The dough was prepared once a week and kept at 4 °C until use. Individual portions of lyophilized *Stellaria media* tea were freshly mixed with the cookie balls right before administration. 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. We always made sure that the whole cookie ball was eaten, and the success rate of this technique was 100% during the experiment.

5.2.4. Measurements of serum lipid levels

To examine the effects of diet-induced hypercholesterolemia and *Stellaria media* treatment on the lipid profile, serum total cholesterol, HDL cholesterol and triacylglycerol levels were analyzed by using Roche Cobas 8000 analyzer system in the Department of Laboratory Medicine using enzymatic colorimetric assays from Roche (Mannheim, Germany). LDL cholesterol levels were measured using a kit from Diagnosticum, Budapest, Hungary, adapted to a plate reader (FLUOstar Optima, BMG), as described earlier[71].

5.2.5. Transthoracic echocardiography

Cardiac morphology and function were assessed by transthoracic echocardiography at week 8 as described previously[72-74]. Rats were anesthetized with sodium pentobarbital (Euthasol, 50 mg/kg body weight, ip.). Then, the chest was shaved and the rat was placed in a supine position onto a heating pad. Two-dimensional and M (motion) mode echocardiographic examinations were performed by the criteria of the American Society of Echocardiography with a Vivid IQ ultrasound system (General Electric Medical Systems, Boston, USA) using a phased array 5.0–11 MHz transducer (GE 12S-RS probe). Data of three consecutive heart cycles were analyzed (EchoPac Dimension software; General Electric Medical Systems, Boston, USA) by an experienced investigator in a blinded manner. The mean values of three measurements were calculated and used for statistical evaluation.

5.2.6. Statistical analysis

Statistical analysis was performed by using SigmaPlot 12.0 for Windows (Systat Software Inc). All values are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) or ANOVA on Ranks were used to determine the differences among the three experimental groups. $p<0.05$ was accepted as statistically significant difference, using Tukey *post hoc* test.

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

5.3.1. Animals

Altogether 30 adult (9-week old) male Wistar rats were used in this study (purchased from Charles River Laboratories, Göttingen, Germany), weighing 292–420 g at the onset of the experiments. Animal handling and ethical regulation were described in the 5.2.1 subsection of the thesis.

5.3.2. Experimental design

After one week of acclimatization, rats were randomized into three groups: Control, Diabetes, Diabetes+*Stellaria m.* treatment. Rats in the control group (n = 10) received standard laboratory rat chow for 20 weeks. The other 20 rats were fed a special fructose-enriched diet, i.e., a standard laboratory rat chow supplemented with 60% (w/w) fructose (Floravita Ltd., Soltvadkert, Hungary) for 20 weeks to induce experimental prediabetes as described earlier[75] and were treated with a low-dose streptozotocin (STZ, Sigma-Aldrich, St. Louis, USA) injection (20 mg/kg body weight, ip.) on the 17th week to achieve experimental diabetes[76-78]. STZ is a pharmacological agent, which exerts toxic effects to the insulin-producing pancreas β-cells. In order to prevent STZ-induced hypoglycemia, drinking water containing 10% glucose (Molar Chemicals Ltd., Halásztelek, Hungary) was given to the animals after STZ injection. The diet of 10 animals receiving fructose-enriched chow was further supplemented with *Stellaria media* tea administered with oral gavage technique (Diabetes+*Stellaria m.*) in order to examine the potential effects of *Stellaria media* tea on the glucose homeostasis and cardiac function, while the other rats received equal amount of distilled water. One animal in the Control group and two animals in the Diabetes group were excluded due to technical problems occurring during the treatments. Fasting blood glucose level measurements were performed every 4 weeks, accompanied by oral glucose tolerance tests (OGTT) at weeks 12, 16 and 19. On the 20th week, rats were anesthetized with sodium pentobarbital (Euthasol, 50 mg/kg body weight, ip.), the abdominal cavity was opened, and blood samples were taken from the abdominal aorta. Then the rats were given 500 U/kg heparin (Heparibene injection, Ratiopharm, Germany) intravenously into the *vena cava inferior*, then pancreata and hearts were isolated. Isolated hearts were subjected to working perfusion according to Neely in order to evaluate cardiac function. Left ventricular heart tissue samples were snap frozen and stored at −80 °C until further biochemical analysis. Collected blood was allowed to clot and was centrifuged (2000×g, 20 min, 4 °C), then serum was separated for analysis of serum parameters.

5.3.3. Administration of Stellaria media tea

Stellaria media tea lyophilizate was prepared as described in the 5.1.1 subsection. The lyophilized *Stellaria media* powder was then dissolved in distilled water to achieve a final concentration of 100 mg/ml. The extract and the vehicle (distilled water) were stored at 4 °C and were brought to room temperature before administration. Rats in the Diabetes+*Stellaria media* group received *Stellaria media* tea (100 mg/kg body weight) with traditional oral gavage technique once a day since the onset of the experiment. The Control and Diabetes groups received equal amount of distilled water. The treatment was carried out at the same two-hour time range every day to minimize the possible influence of circadian rhythm.

5.3.4. Measurement of parameters reflecting endocrine and exocrine function of the pancreas

During the 20-week experiment, fasting blood glucose level and OGTT measurements were performed in blood samples from the *vena saphena* using blood glucose test stripes (Accu-Chek, Roche Hungary Ltd., Budapest, Hungary) after overnight fasting and the average of two measurements was calculated. By OGTT measurements, after the baseline blood sampling, 1.65 g/kg body weight glucose was administered to the rats by oral gavage technique and blood glucose levels were measured at 30, 60, 90 and 120 minutes after glucose administration in order to describe the glucose tolerance after a standard amount of glucose intake. Area under the curve (AUC) values were calculated. Normally the blood glucose concentration normalizes 120 minutes after glucose administration, but in case of diabetes, higher blood glucose and AUC values are expected.

At termination, fasting blood glucose levels were determined. Hemoglobin A1c (HbA1c) levels were analyzed by DCA Vantage Analyzer System (Siemens) provided by Diagnosticum Ltd. (Budapest, Hungary). HbA1c is the glycated form of hemoglobin which is synthetised by a non-enzymatic reaction in case of long-lasting high blood glucose concentrations. It is a great marker for describing the blood glucose state in the past 2-3 months, since it can only be broken down together with the erythrocytes. The blood glucose lowering hormone insulin was determined in the serum by enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (Mercodia, Ultrasensitive Rat Insulin ELISA). Insulin is synthesized by the pancreas β cells and will be secreted in response to higher blood glucose levels.

5.3.5. Ex vivo working heart perfusion

Cardiac performance and function was assessed in isolated working hearts, as described earlier[79-81]. Hearts were isolated and the aorta and left atrium were cannulated and initially perfused in Langendorff mode (at a constant pressure of 73 mmHg, 37 °C) with Krebs-Henseleit buffer containing 118 mM NaCl, 25 mM $NaHCO_3$, 4.3 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 11 mM glucose (all from Molar Chemicals Ltd., Halásztelek, Hungary), gassed with 95% O_2 and 5% CO_2[73]. Then, the perfusion system was switched to working mode according to Neely with recirculating buffer[82]. Hydrostatic preload and afterload were kept constant at 13 mmHg and 73 mmHg, respectively, throughout the experiments. Hearts were subjected to 10 minutes equilibration period before measurement (n = 8–10). Cardiac functional parameters including 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.

5.3.6. 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 technique as described earlier[83]. Briefly, powdered frozen left ventricular heart tissue samples were homogenized with radioimmunoprecipitation buffer supplemented with protease inhibitor cocktail. The homogenates were centrifuged (15000×g, 30 min, 4 °C) and supernatants were collected and stored at –80 °C until analysis. Protein concentration was determined using bicinchoninic acid assay. Proteins were separated by standard polyacrylamide gelelectrophoresis and then transferred to a nitrocellulose membrane. Membranes were blocked in 5% w/v bovine serum albumin (1 h, room temperature) and were incubated overnight at 4 °C with the following primary antibodies: phospho(Ser473)-Akt (1:500), total Akt (1:2000), phospho(Thr202/Tyr204)-Erk1/Erk2 (1:2000), total Erk1/Erk2 (1:1000), phospho(Tyr705)-STAT3 (1:1000), total STAT3 (1:2000), Bax (1:1000), Bcl-XL (1:1500). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10000) or tubulin (1:1000) were used as loading controls. The following day the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (40 min, room temperature) and chemiluminescent detection was used on X-ray film.

5.3.7. Statistical analysis

All values are presented as mean ± SEM. Repeated measures two-way ANOVA was applied for analysis of time-dependent 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.

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

5.4.1. H9c2 cell culture

H9c2 rat cardiomyoblasts were used in the *in vitro* experiments obtained from ATTC and cultured in Dulbecco's modified Eagle's medium (Lonza) supplemented with 10% v/v fetal bovine serum (EuroClone), 200 nM glutamine (Sigma-Aldrich, St. Louis, USA) and 1% v/v antibiotic/antimycotic solution (Sigma-Aldrich, St. Louis, USA). Cells were cultured in 25 cm^2 and 75 cm^2 tissue culture flasks and were subcultured each time they reached 70–80% of confluence. Cells from passages P18-20 were seeded at a density of 4×10^3 cells in 96-well plates for viability and oxidative stress measurements and at a density of 2×10^4 cells in 24-well plates with glass coverslips for cell nuclear morphology analysis and were used for the different experiments 2 days later.

5.4.2. Experimental design

H9c2 cells were exposed to simulated I/R protocol composed of 6 hours of simulated ischemia followed by 2 hours of simulated reperfusion. During the simulated I/R protocol, cells were treated with 64 μM kynurenic acid to confirm its cardioprotective effects. To examine the involvement of NMDA receptors in the cell death in I/R injury, cells were treated with an NMDA receptor agonist, N-methyl-D-aspartic acid (25–400 μM) or an NMDA receptor antagonist, (+)-MK-801 hydrogen maleate (0.47–120 μM) or a combination of NMDA (400 μM) and MK-801 (7.5 μM) or kynurenic acid (64 μM) during simulated I/R. Cell viability, oxidative stress and morphological alterations of cell nuclei were measured as end-points.

5.4.3. Simulated ischemia/reperfusion

Simulated I/R was used to mimic acute myocardial infarction *in vitro* and was carried out as described previously[44]. The protocol composed of 6 hours of simulated ischemia followed

by 2 hours of simulated reperfusion. During simulated ischemia, cells were covered with glucose-free hypoxic solution containing 119 mM NaCl, 5.4 mM KCl, 1.2 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 5 mM HEPES, 1.3 mM $MgSO_4$, 0.9 mM $CaCl_2$, 20 mM sodium-lactate and 0.1% bovine serum albumin, with pH adjusted to 6.4, and kept in a hypoxic incubator gassed with a mixture of 95% N_2 and 5% CO_2, where the oxygen tension was set to 0.4% to mimic both oxygen and nutrient deficiency. After 6 hours, cells were transferred back into the normoxic incubator and covered with differentiation medium to mimic reperfusion. To prove the effectiveness of simulated I/R as a stress factor, a control group of cells was covered with glucose-containing normoxic solution at physiological pH level[44].

5.4.4. Kynurenic acid, NMDA and (+)-MK-801 treatments

10 mM stock solution of kynurenic acid was prepared by dissolving kynurenic acid (Sigma-Aldrich, St. Louis, USA) in 0.1 M NaOH and setting its pH to 7.38–7.42. 64 μM concentration of kynurenic acid was applied, which was chosen based on previous results of our research group in neonatal rat cardiomyocyte experiments (data not shown). As vehicle, 0.1 M NaOH was used after pH adjustment to 7.38–7.42.

N-methyl-D-aspartic acid (Sigma-Aldrich, St. Louis, USA) was dissolved in phosphate buffered saline (PBS, VWR International Ltd., Debrecen, Hungary) to achieve a 4 mM concentration in the stock solution. A concentration range of 25–400 μM was used based on literature data in the field of neurology. PBS alone served as vehicle control.

The NMDA receptor antagonist (+)-MK-801 hydrogen maleate (Sigma-Aldrich, St. Louis, USA) was dissolved in PBS and stock solution with 1.2 mM concentration was prepared. MK-801 treatment was applied in a concentration range of 0.47–120 μM. Vehicle group received PBS only.

All the treatments were maintained during the entire simulated I/R protocol.

5.4.5. Viability assay

At the end of the simulated I/R protocol, calcein assay was used to measure cell viability[44, 84]. Calcein AM (Promokine) dye is converted to green-fluorescent calcein following hydrolysis by intracellular non-specific esterases in living cells. Cells were washed with warm Dulbecco's phosphate buffered saline (D-PBS, Sigma-Aldrich, St. Louis, USA), and then incubated for 30 min with 1 μM calcein AM dissolved in dimethyl sulfoxide and diluted in D-PBS. After washing with D-PBS, fluorescence intensity was measured at excitation and emission wavelengths of 490/520 nm using a plate reader (Fluostar Optima, BMG). Viability measurements were repeated minimum 3 times with at least 7-14 biological

replicates. 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.

5.4.6. Oxidative stress measurement

To investigate simulated I/R-induced cellular superoxide production, dihydroethidium (DHE, Sigma-Aldrich, St. Louis, USA) staining was performed as described previously[44]. When oxidized, DHE can intercalate within the DNA and emits red fluorescence. During the 2-hour simulated reperfusion, cells were covered with medium supplemented with 10 μM DHE while maintaining the NMDA receptor modulator treatments. At the end of the protocol, after washing, fluorescence intensity was measured at excitation and emission wavelengths of 530/620 nm using a plate reader (Fluostar Optima, BMG) followed by detection of cell viability with calcein assay. The fluorescence intensity of DHE was corrected to cell viability.

5.4.7. Analysis of cell nuclear morphology

For the assessment of simulated I/R-induced apoptotic morphological changes of cell nuclei, they were visualized by 2-[4-(aminoiminomethyl)phenyl]-1H-indole-6-carboximidamide hydrochloride (DAPI) staining. At the end of the simulated I/R protocol, cells on coverslips were fixed in 4% paraformaldehyde (20 min, room temperature) and then were permeabilized with 0.3% Triton X-100-containing PBS (20 min, room temperature). Coverslips were washed with PBS three times and blocked in PBS-Tween-20 containing 5% bovine serum albumin (30 min, room temperature). Cell nuclei were stained with DAPI solution (1:10000, 10 min, room temperature). Coverslips were covered with Mounting Medium and cells were visualized with Nikon Eclipse Ti-E microscope (Nikon Instruments Inc., USA) using the same exposition time for all samples. Pictures were analysed by Image J software. The number of cell nuclei with apoptotic morphological alterations, such as bigger, disintegrated nuclei, apoptotic bodies, cellular fragments or smaller condensed nuclei were counted and normalized to the total number of cell nuclei expressed in % of the vehicle group.

5.4.8. 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.

6. Results

6.1. Characterization of *Stellaria media* tea

6.1.1. Total flavonoid content and flavonoid screening

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

6.1.2. Liquid chromatography-tandem mass spectrometry analysis

In order to gain more information about the chemical composition of the tea, an LC-MS/MS analysis was performed. In the analysis, several components have been detected. These data were subjected to a spectral library search against the MzCloud database which revealed altogether five possible components out of the ten most intense sample components, which appeared to be various glycosylated apigenin-derivatives. MS2 spectrum of m/z 595.1660 displayed reasonable resemblance (MzCloud best match: 62.9) to MzCloud spectral library entries to two isomeric compounds, 6-arabinosyl-8-galactosylapigenin (corymboside) and 6-β-d-glucopyranosyl-8-β-D-ribopyranosylapigenin (schaftoside), while MS2 spectrum of m/z 595.1660 showed good agreement (MzCloud best match: 82.8) with apigenin-6,8-di-C-glucoside (vicenin). Two further components, m/z 757.2195 and 933.2657 showed similarity to MS2 data acquired on 2″-O-α-l-rhamnopyranosyl-isovitexin, a compound with the same apigenin base structure. The molecular mass of these two components were 178.1 and 354.1 Da higher compared to the database entry. Six further components (m/z 274.2737, 535.1448, 679.2974, 677.2817, 611.1608 and 381.0793) did not show any resemblance to MzCloud entries indicating that these components were not included with an MS2 spectrum in the spectral library.

6.1.3. In vitro antioxidant activity

The lyophilized powder was evaluated for antioxidant activity using DPPH and ORAC assays. The lyophilized aqueous extract of *Stellaria media* exerted 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 μmolTE/g in the ORAC study.

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

6.2.1. Serum lipid parameters and body weight

Lipid levels were measured from serum in order to validate the development of diet-induced hypercholesterolemia by the end of an 8-week feeding protocol. Total cholesterol concentration was significantly elevated in the HChol and the HChol+*Stellaria media* groups compared to the control group; however, there was no significant difference between HChol and HChol+*Stellaria media* values (Figure 6a). Triacylglycerol levels showed no significant difference due to cholesterol-enriched diet or *Stellaria media* treatment (Figure 6b). Similarly to total cholesterol, serum LDL cholesterol concentration was significantly higher in the HChol group, which was not affected by *Stellaria media* tea lyophilizate (Figure 6c). Serum HDL cholesterol level was significantly higher in the HChol group compared to control values; however, *Stellaria media* treatment did not affect significantly HDL cholesterol level (Figure 6d). These results suggest that *Stellaria media* tea lyophilizate does not have cholesterol-lowering effect.

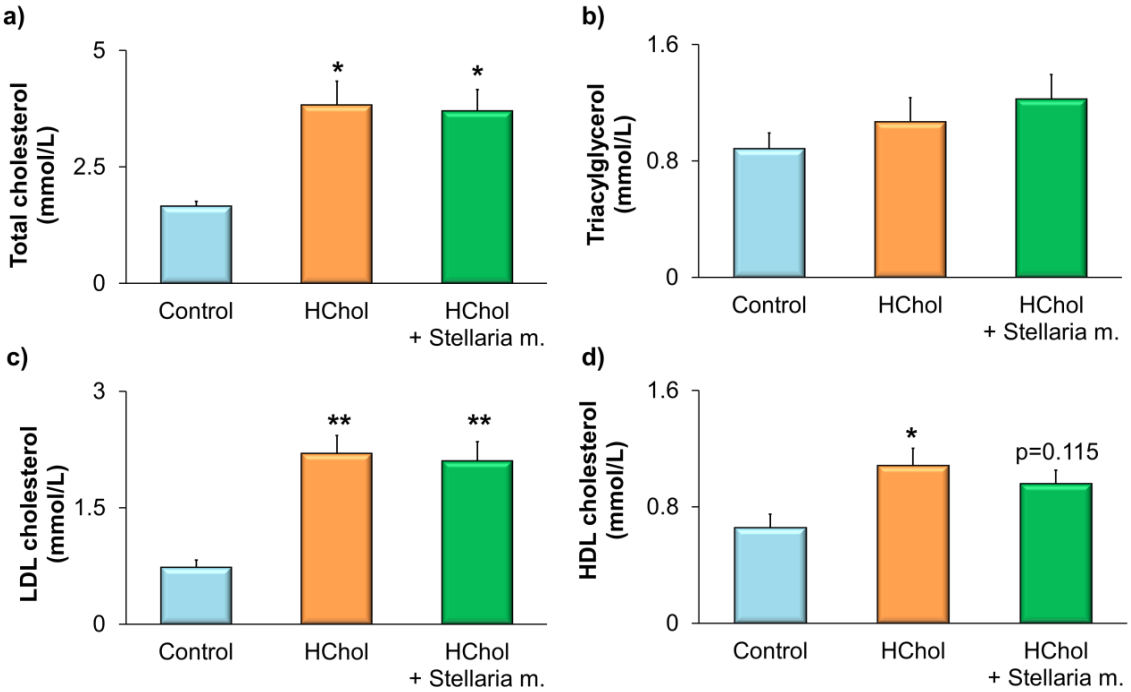

Figure 6: (a) Serum total cholesterol, (b) serum triacylglycerol, (c) LDL cholesterol, and (d) HDL cholesterol levels in rats fed either cholesterol-enriched (HChol), cholesterol-enriched + *Stellaria media* (HChol+*Stellaria m.*), or normal diet (Control). Results are means ± SEM (n = 8 in each group), analyzed by one-way ANOVA with Tukey *post hoc* test. ∗p<0.05 vs. Control, ∗∗p<0.01 vs. Control.

Body weight showed a continuous increase from 305±4 g at the onset of the experiment to 505±13 g at week 8 in the control group fed a normal diet (Figure 7a). Neither cholesterol-enriched diet nor *Stellaria media* treatment affected body weight significantly at any time points (Figure 7a). Weight gain during the 8-week feeding protocol was also not affected significantly by any of the treatments (Figure 7b).

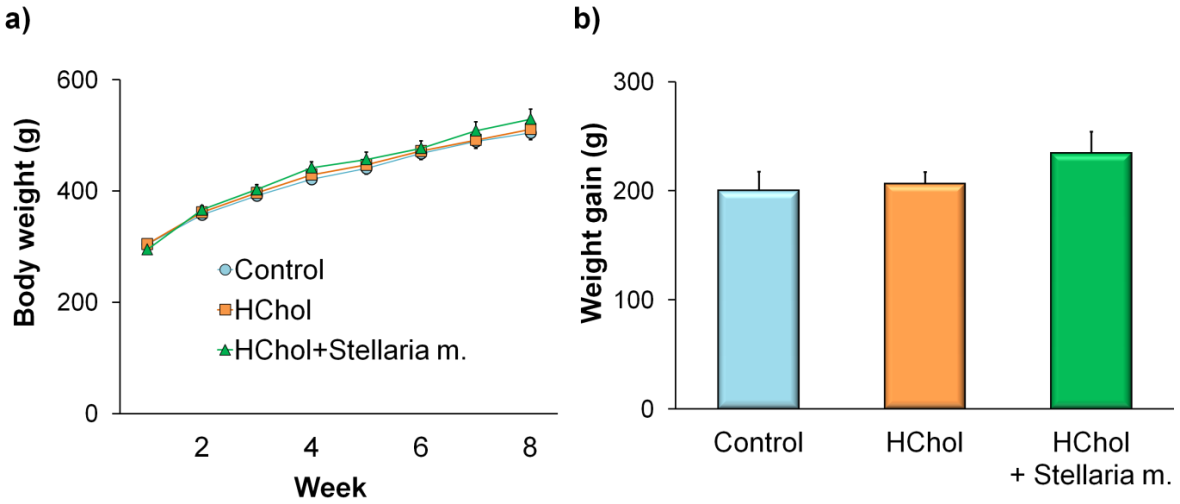

Figure 7: (a) Body weight and (b) weight gain during 8 weeks in the control group (blue spheres) and rats fed with cholesterol-enriched diet (HChol) (orange squares) or cholesterol-enriched diet with *Stellaria media* extract (HChol+*Stellaria m.*) (green triangles). Results are means ± SEM (n = 8 in each group), analyzed by one-way ANOVA with Tukey *post hoc* test.

6.2.2. Heart function assessed by transthoracic echocardiography

Transthoracic echocardiographic measurements performed at the end of the feeding protocol showed that diet-induced hypercholesterolemia did not alter cardiac morphology or function compared to controls (Table 1). In hypercholesterolemic rats *Stellaria media* treatment did not affect cardiac morphology as there were no differences in systolic and diastolic wall thickness parameters (Table 1). Parameters related to systolic and diastolic cardiac function including left ventricular end-diastolic and end-systolic volume, stroke volume, ejection fraction, heart rate, early (E) and late (A) diastolic transmitral flow velocity, early diastolic mitral annular tissue velocity (E'), E/A and E/E' ratios were also not influenced significantly by diet-induced hypercholesterolemia and *Stellaria media* treatment (Table 1).

Parameter (unit)	View/ Mode	Control	HChol	HChol+ *Stellaria m.*	Significance
Anterior wall thickness-systolic (mm)	short axis/ MM	3.57 ± 0.11	3.41 ± 0.17	3.10 ± 0.12	ns
Anterior wall thickness-diastolic (mm)		2.20 ± 0.11	2.22 ± 0.17	2.16 ± 0.11	ns
Inferior wall thickness-systolic (mm)		3.85 ± 0.11	3.46 ± 0.15	3.69 ± 0.09	ns
Inferior wall thickness-diastolic (mm)		2.14 ± 0.09	2.13 ± 0.14	2.19 ± 0.13	ns
Posterior wall thickness-systolic (mm)	long axis/ MM	3.82 ± 0.08	3.68 ± 0.12	3.62 ± 0.09	ns
Posterior wall thickness-diastolic (mm)		2.19 ± 0.03	2.39 ± 0.13	2.52 ± 0.19	ns
Septal wall thickness-systolic (mm)		3.79 ± 0.06	3.62 ± 0.12	3.54 ± 0.17	ns
Septal wall thickness-diastolic (mm)		2.50 ± 0.11	2.24 ± 0.10	2.35 ± 0.11	ns
Left ventricular end-diastolic volume (µl)	4CH/ 2D	127 ± 15	129 ± 13	105 ± 13	ns
Left ventricular end-systolic volume (µl)		49 ± 8	53 ± 5	46 ± 6	ns
Stroke volume (µl)		79 ± 8	76 ± 8	59 ± 8	ns
Ejection fraction (%)		63 ± 2	59 ± 1	56 ± 3	ns
Heart rate (1/min)		343 ± 14	367 ± 8	370 ± 11	ns
MV E velocity (m/s)		0.80 ± 0.06	0.87 ± 0.03	0.79 ± 0.03	ns
MV A velocity (m/s)[a]		0.42 ± 0.03	0.74 ± 0.13	0.52 ± 0.09	ns
E/A[a]		1.75 ± 0.06	1.24 ± 0.24	1.65 ± 0.30	ns
MV E' (m/s)		0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	ns
E/E'		16.90 ± 2.19	14.25 ± 2.34	15.98 ± 3.06	ns

Table 1: Effect of *Stellaria media* on left ventricular morphological and functional parameters at the end of 8-week diet. Transthoracic echocardiographic measurement values in rats fed either cholesterol-enriched (HChol), cholesterol-enriched+*Stellaria media* (HChol+*Stellaria m.*), or normal diet (Control). Results are means ± SEM (n = 8 in each group; [a] n = 3–3–4 in corresponding groups), analyzed by one-way ANOVA or ANOVA on Ranks. 2D: two dimensional; 4CH: four chambers view; MM: M (motion) Mode; MV: mitral valve; E and A: early and late diastolic transmitral flow velocity; E': early diastolic mitral annular tissue velocity; ns: not significant.

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

6.3.1. Fasting blood glucose, glucose tolerance and body weight

Fasting blood glucose levels were measured every 4 weeks since the onset of the experiment, accompanied by OGTT measurements at weeks 12, 16 and 19. In fasting blood glucose levels and glucose tolerance, there was no significant difference among the three groups until week 16 (Figure 8a). On the 17th week, the rats in the Diabetes and Diabetes+*Stellaria media* groups were injected with a low dose of STZ (20 mg/kg body weight, ip.), while the Control group was treated with equal amount of vehicle (citrate buffer). Following the STZ injection a significant elevation in fasting blood glucose levels could be observed in the Diabetes group compared to the Control group, achieving an experimental diabetes state (Figure 8a). Although on week 18 the blood glucose elevation in the

Diabetes+*Stellaria media* group was significantly lower compared to the Diabetes group, this difference was faded away by week 19 (Figure 8a). According to our OGTT results, there was a significant elevation in the AUC levels in the Diabetes group compared to the Control group on week 19 (Figure 8b) representing an impairment of glucose tolerance. *Stellaria media* treatment did not affect glucose intolerance in diabetes (Figure 8b).

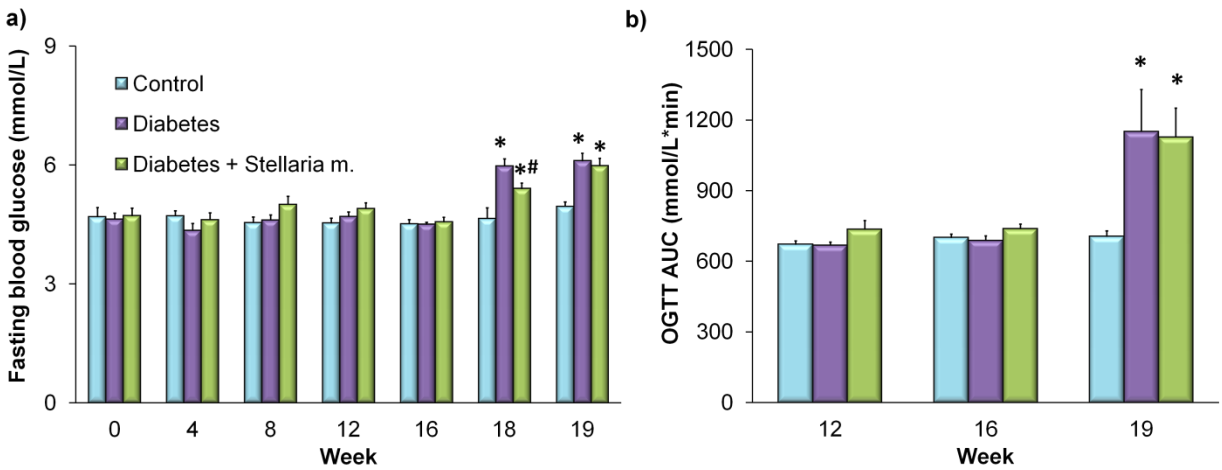

Figure 8: (a) Fasting blood glucose levels and (b) area under the curve (AUC) values of oral glucose tolerance test (OGTT) measurements. Results are means ± SEM (n = 8–10), analyzed by one-way ANOVA followed by Holm-Sidak *post hoc* test,*p<0.05 vs. Control, #p<0.05 vs. Diabetes.

Body weight was measured every 3 days in order to calculate *Stellaria media* tea dosage and to monitor weight gain. It showed a continuous increase from 356±7 g at the onset of the experiment to 482±16 g at week 20 in the Control group fed with standard laboratory rat chow (Figure 9a). Compared to the controls, fructose-enriched diet plus STZ injection reduced body weight increase in the last 6 weeks in the Diabetes group; however, this effect was not significant in the Diabetes+*Stellaria media* group (Figure 9a). Weight gain during the feeding protocol was significantly lower in the Diabetes group compared to the Control group (Figure 9b). Similarly, there was a tendency of decrease in the Diabetes+*Stellaria media* group compared to the controls; however, it did not reach the level of statistical significance (Figure 9b).

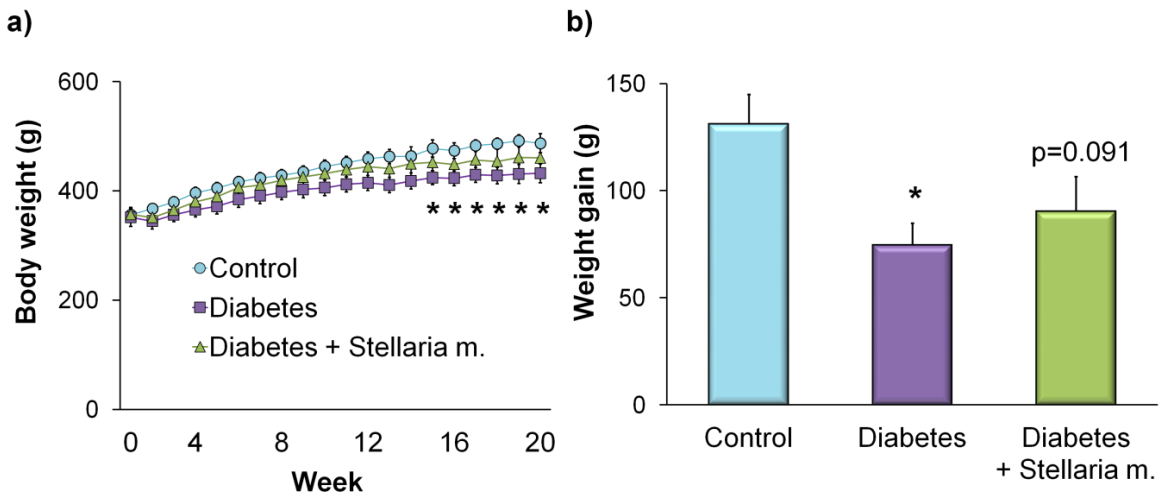

Figure 9: (a) Body weight and (b) weight gain during 20 weeks in the Control group (blue spheres), Diabetes group (purple squares) and Diabetes+*Stellaria media* group (green triangles). Results are means ± SEM (n = 8–10), analyzed by repeated measures two-way ANOVA or one-way ANOVA with Holm-Sidak *post hoc* test,*p<0.05 Diabetes vs. Control.

6.3.2. Parameters reflecting endocrine and exocrine function of the pancreas

At the end of the 20-week experiment, elevated fasting blood glucose, non-significantly increased HbA1c (3.6±0.1% in Control and 4.3±0.5% in Diabetes, respectively) and serum insulin levels decreased by approximately 20% indicated impaired endocrine pancreatic function (Figure 10a,b,c) in the Diabetes group. *Stellaria media* tea failed to improve these parameters (Figure 10a,b,c), and HbA1c was 4.6±0.4% in this group.

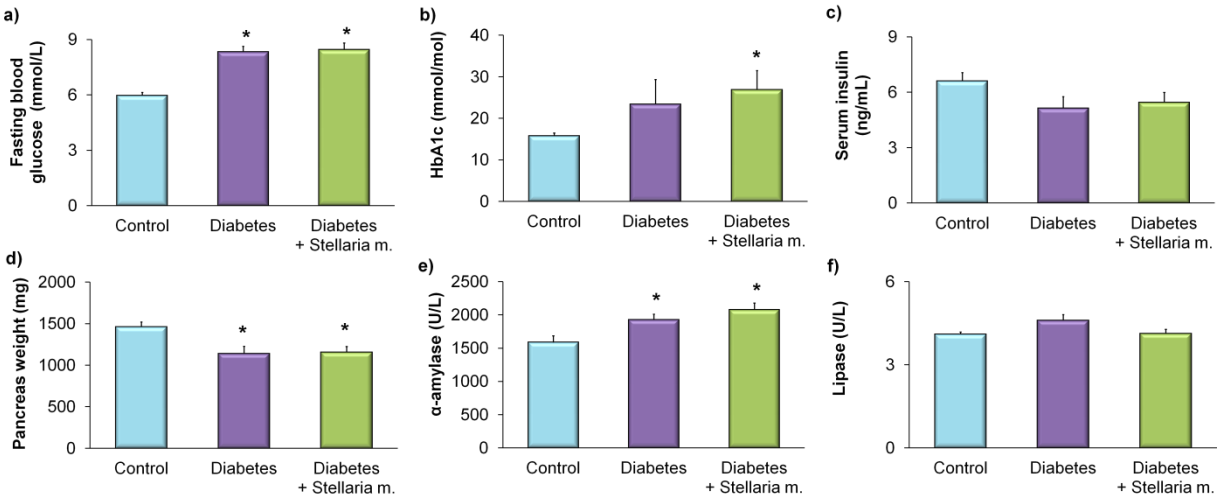

Figure 10: Parameters representing pancreatic function at week 20: (a) fasting blood glucose at termination, (b) HbA1c levels, (c) serum insulin levels, (d) pancreas weight, (e) enzyme activities of α-amylase and (f) lipase. Results are means ± SEM (n = 8–10 except for serum insulin measurement where n = 6–8), analyzed by one-way ANOVA followed by Holm-Sidak *post hoc* test,*p<0.05 vs. Control.

Pancreas weight was significantly lower in the Diabetes group compared to the Control group (Figure 10d), showing that there might have been pancreatic damage due to the fructose-enriched diet plus STZ injection. *Stellaria media* did not affect this alteration (Figure 10d). The serum activity of α-amylase was significantly elevated both in the Diabetes and Diabetes+*Stellaria media* groups (Figure 10e), while there was no difference among the three groups in the activity of lipase enzyme (Figure 10f).

6.3.3. Working heart perfusion

Cardiac dysfunction is a frequent consequence of diabetes; therefore, we assessed cardiac performance in isolated hearts subjected to working perfusion. Aortic flow, cardiac output and cardiac work, reflecting systolic heart function, were significantly impaired in the Diabetes group compared to the Control group (Figure 11a,b, Table 2), indicating diabetic adverse effects on the heart.

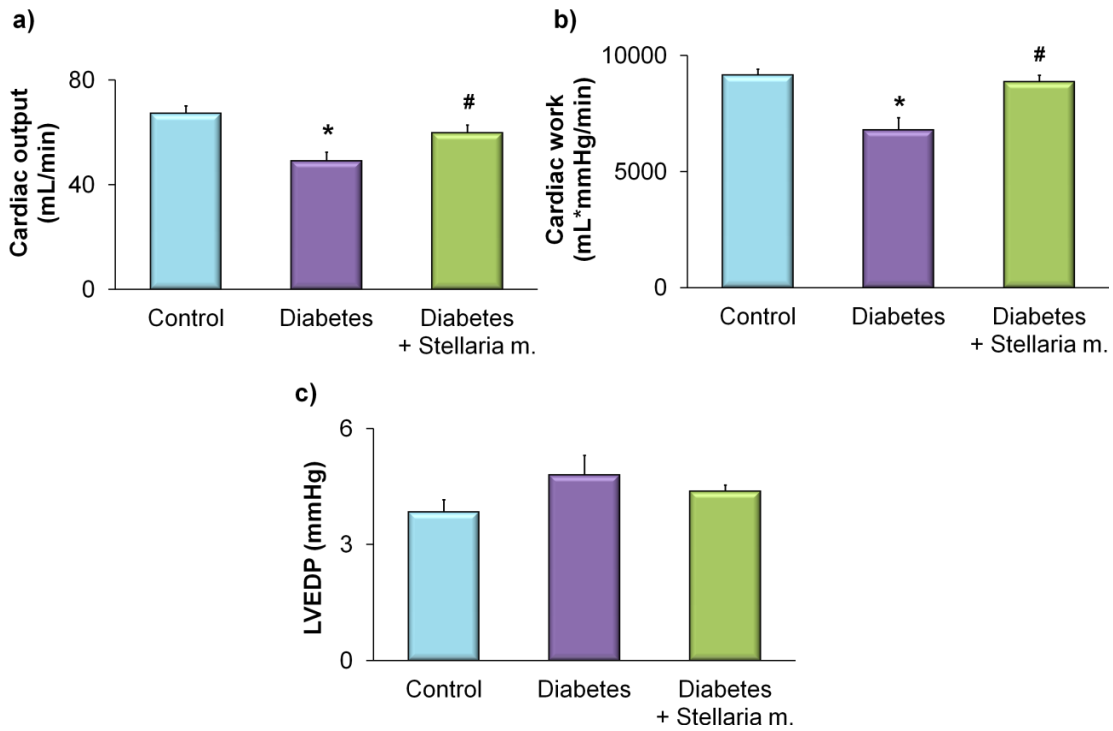

Figure 11: Cardiac function in isolated hearts subjected to working perfusion according to Neely: (a) cardiac output, (b) cardiac work, (c) left ventricular end diastolic pressure (LVEDP). Results are means ± SEM (n = 8–10), analyzed by one-way ANOVA followed by Holm-Sidak *post hoc* test,*p<0.05 vs. Control, #p<0.05 vs. Diabetes.

Stellaria media treatment significantly improved cardiac output and cardiac work suggesting that *Stellaria media* tea may have beneficial effects on the heart in a diabetic state (Figure 11a,b). The diastolic function of the hearts was assessed by measurements of LVEDP, which inversely correlates with the function. LVEDP showed a tendency of elevation in the Diabetes group compared to the Control group; however, *Stellaria media* had no prominent effect on this parameter (Figure 11c).

There were no significant alterations in the other analyzed cardiac functional parameters among the three experimental groups (Table 2).

Parameter (unit)	**Control**	**Diabetes**	**Diabetes + *Stellaria m.***	**Significance**
Aortic flow (mL)	44.4 ± 2.6	27.3 ± 2.4*	34.4 ± 2.3*	p<0.05
Coronary flow (mL)	22.8 ± 0.7	21.8 ± 1.5	23.7 ± 1.0	ns
Max d*p*/d*t* (mmHg/s)	6323 ± 282	6260 ± 439	6431 ± 487	ns
Min d*p*/d*t* (mmHg/s)	– 4520 ± 188	– 4512 ± 397	– 4496 ± 374	ns
Aortic diastolic pressure (mmHg)	37.6 ± 0.5	37.9 ± 0.8	37.8 ± 1.1	ns
Aortic systolic pressure (mmHg)	114.8 ± 2.5	110.4 ± 3.3	114.9 ± 3.1	ns
LVDP (mmHg)	136.2 ± 4.6	130.0 ± 4.5	131.3 ± 5.2	ns
Heart rate (1/min)	240 ± 10	211 ± 16	231 ± 11	ns

Table 2: Parameters measured by working heart perfusion according to Neely. Results are means ± SEM (n = 8–10), analyzed by one-way ANOVA with Holm-Sidak *post hoc* test,*p<0.05 vs. Control. LVDP: left ventricular developed pressure; ns: non-significant.

6.3.4. Cardiac signaling pathways

Activation of STAT3 is proposed to play a role in diabetes-induced cardiac dysfunction. The phosphorylation of STAT3 was significantly elevated in the Diabetes group which was attenuated by *Stellaria media* treatment (Figure 12a,e), suggesting an association with the beneficial cardiac effect of *Stellaria media*. Akt and Erk proteins are key mediators of the Reperfusion Injury Salvage Kinase pathway, which is a known cardioprotective signaling pathway. Moreover, phosphorylation of Akt has been shown to influence the membrane expression of GLUT transporters[85], therefore being potentially relevant in diabetes. There were no significant differences among the three groups in the phosphorylation of Akt and Erk proteins (Figure 12b,c). Furthermore, since increased cardiac apoptosis represents greater risk for the development of diabetic cardiomyopathy[86-88], we examined apoptosis related proteins

in our study. In addition, the Bcl-2 protein family is not only a regulator of apoptosis: it plays important roles in normal cell physiology, calcium handling and mitochondrial dynamics as well[89], which processes are particularly crucial in the cardiac function. However, Diabetes and *Stellaria media* treatment had no effects on the proapoptotic Bax and antiapoptotic Bcl-XL proteins (Figure 12d,f).

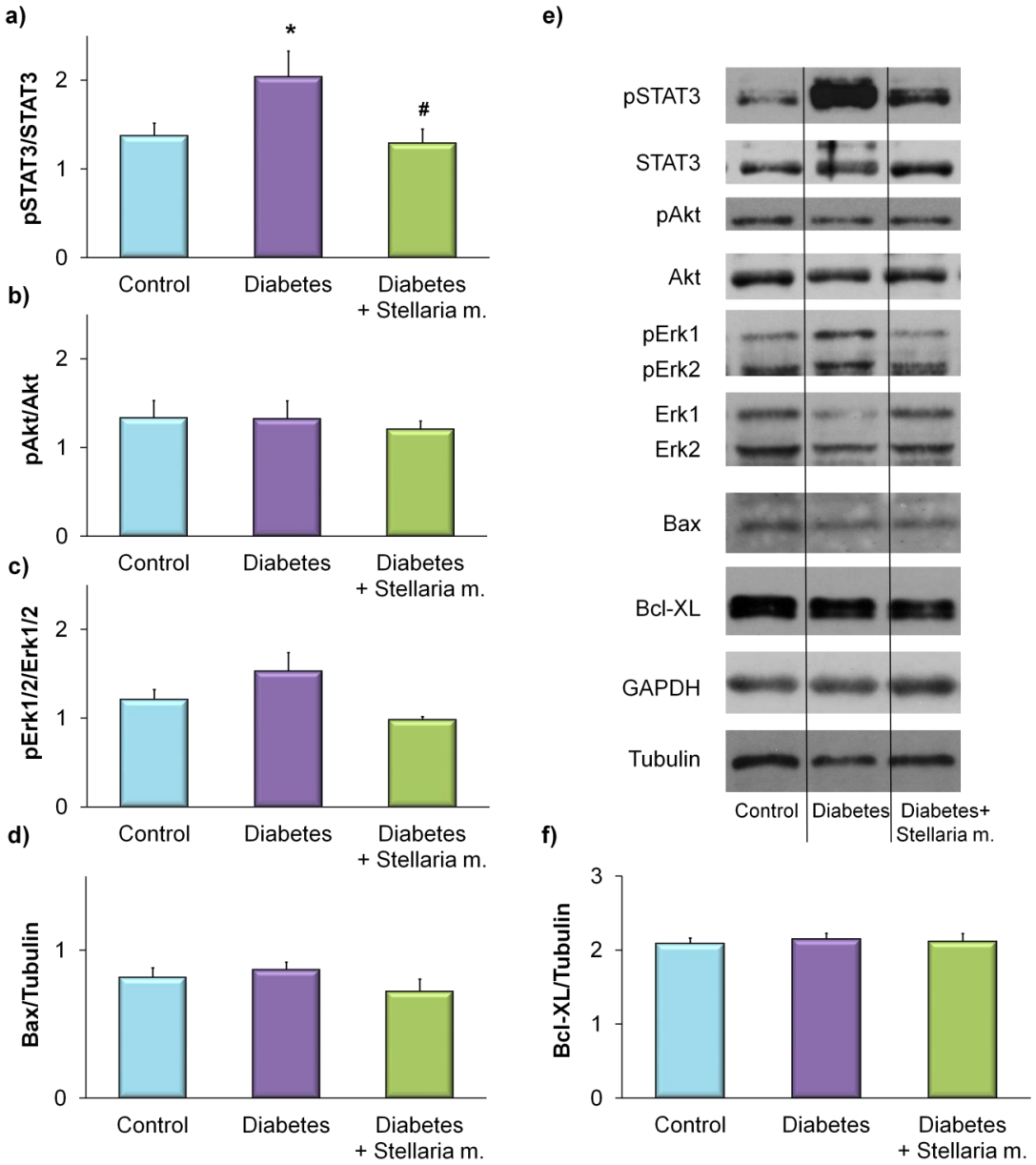

Figure 12: Western blot analysis of phosphorylation and activation of proteins: (a) signal transducer and activator of transcription 3 (STAT3), (b) protein kinase B (Akt), (c) extracellular signal-regulated kinase (Erk), (d) Bax, (e) representative bands, (f) Bcl-XL. Results are means ± SEM (n = 7), analyzed by one-way ANOVA followed by Fisher LSD *post hoc* test,*p<0.05 vs. Control, #p<0.05 vs. Diabetes.

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

6.4.1. Effects of kynurenic acid on cell viability and oxidative stress

Simulated I/R protocol induced a significant increase in cell death compared to the normoxic control group (Figure 13a). The protective effect of kynurenic acid in a concentration of 64 µM on cell viability was confirmed on H9c2 cells (Figure 13a). Compared to the normoxic control group, simulated I/R induced a significant increase in DHE fluorescence intensity normalized to cell viability, which is a marker of oxidative stress (Figure 13b). 64 µM kynurenic acid treatment attenuated the ratio of oxidative stress significantly (Figure 13b).

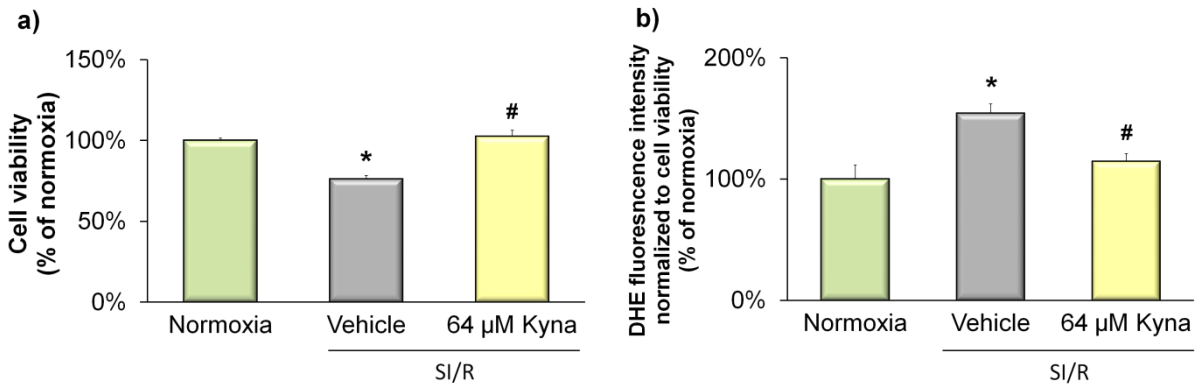

Figure 13: Effects of 64 µM kynureic acid (Kyna) in simulated ischemia/reperfusion (SI/R) on (a) cell viability (n = 7–14, from 4 separate experiments) and (b) oxidative stress measured by DHE fluorescence intensity (n = 7–14, from 5 separate experiments). Results are means ± SEM, analyzed by one-way ANOVA followed by Fisher LSD *post hoc* test,*p<0.05 vs. Normoxia, #p<0.05 vs. Vehicle.

6.4.2. Effects of NMDA treatment and its combination with antagonists on cell viability and oxidative stress

Based on literature, NMDA receptor antagonist property seemed to be a promising approach in the cardioprotective effect of kynurenic acid.

To demonstrate the presence of NMDA receptors in H9c2 cells and to confirm the antagonist effect of kynurenic acid in our model, cells were treated with the receptor agonist NMDA with or without the known antagonists kynurenic acid or MK-801. According to our results, NMDA receptor activation by NMDA treatment during simulated I/R significantly enhanced cell death in a dose dependent manner. 100, 200 and 400 µM NMDA treatment caused significant cell death elevation showing that the activation of NMDA receptor

exacerbate cell death in settings of simulated I/R (Figure 14a). Both antagonists were able to significantly decrease the NMDA-induced cell death in simulated I/R (Figure 14b).

In accordance with cell death, compared to the vehicle group, NMDA treatment increased the ratio of cell nuclei showing apoptotic morphological alterations, which was diminished with the combined treatment with antagonists (Figure 14c).

Interestingly, 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 (Figure 14d).

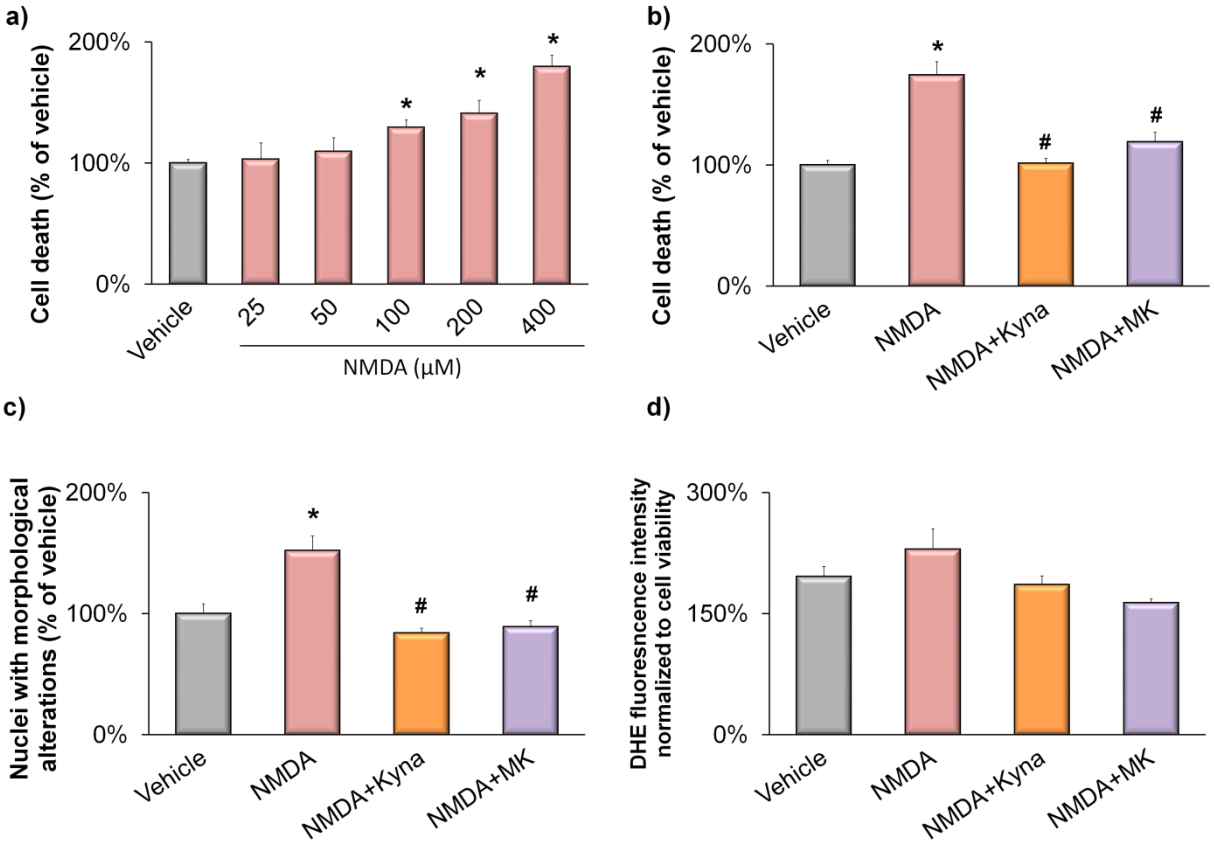

Figure 14: Effects of NMDA receptor activation in simulated ischemia/reperfusion: (a) Effect of 25-400 μM NMDA treatment on cell death (n = 7–14, Vehicle control: n = 14–28/experiment, from 9 separate experiments). (b) Effect of combination of NMDA (400 μM) and kynurenic acid (64 μM, Kyna) or MK-801 (7.5 μM) on cell death in simulated ischemia/reperfusion (n = 7–14, Vehicle control: n = 14–28/experiment from 7 separate experiments). (c) Effects of NMDA or combined treatments (NMDA+kynurenic acid or NMDA+MK-801) on apoptotic nuclear morphology by DAPI staining (n = 10 in each group). (d) Effect of NMDA, combination of NMDA (400 μM) and kynurenic acid (64 μM) or MK-801 (7.5 μM) on oxidative stress in in simulated ischemia/reperfusion (n = 7–14, Vehicle control: n = 14–28/experiment, from 3 separate experiments). Results are means ± SEM, analyzed by one-way ANOVA followed by Fisher LSD *post hoc* test,*$p<0.05$ vs. Vehicle, #$p<0.05$ vs. NMDA.

6.4.3. Effect of the NMDA receptor antagonist, MK-801 on cell viability

To elucidate the involvement of NMDA receptors in the protective effects of kynurenic acid, cardiac cells were treated with MK-801, a potent, selective, and non-competitive NMDA receptor antagonist. Surprisingly, 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 (Figure 15), suggesting that the cytoprotection elicited by kynurenic acid may be NMDA receptor independent.

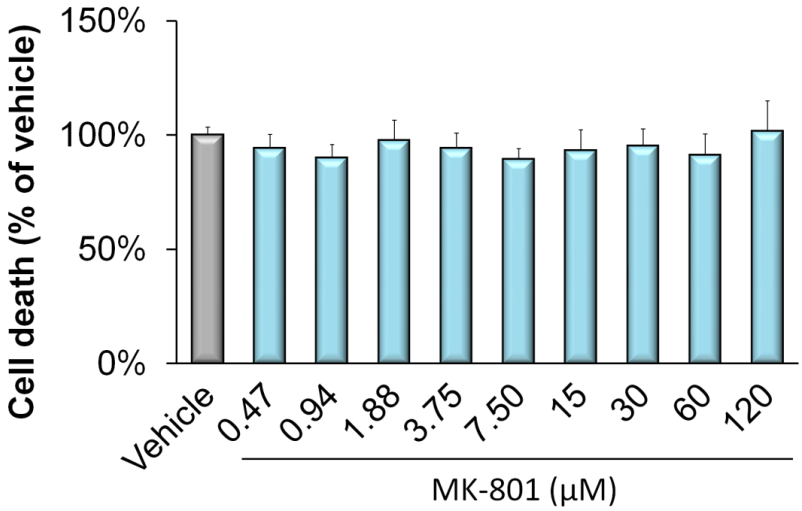

Figure 15: Effect of the NMDA receptor antagonist MK-801 (0.47-120 μM) treatment on cell death in simulated ischemia/reperfusion (n = 7–14, Vehicle control: n = 14–28/experiment, from 11 separate experiments). Results are means ± SEM, analyzed by one-way ANOVA.

7. Discussion

In the present 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 or by inducing cytoprotective effects. We tested the potential therapeutic efficacy of *Stellaria media* tea on the severity of two modifiable risk factors, i.e., hypercholesterolemia and diabetes, and assessed the impact of the tea on cardiac function in these metabolic disorders. We also elucidated the direct cardiocytoprotective effect of kynurenic acid and the involvement of NMDA receptors in this effect on I/R injury.

7.1. 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.

7.2. Management of modifiable metabolic cardiovascular risk factors

In prevention of ischemic heart disease including acute myocardial infarction, targeting modifiable risk factors, particularly hypercholesterolemia and diabetes have great relevance. Nowadays, natural substances and medicinal herbs are gaining popularity in prevention and treatment of these metabolic diseases in comparison to standard pharmacotherapy. In folk medicine, *Stellaria media* is mostly consumed as tea and is believed to decrease blood cholesterol and glucose levels[33-35]. In accordance, several tea products are available with these claims or indications. Nevertheless, the cholesterol- and glucose-lowering effects of *Stellaria media* tea have not been investigated previously. In our studies, we intended to model the human use of chickweed as close as possible. For this reason, according to folk medicinal practice, only above-ground parts of *Stellaria media* were used. The extract was prepared as

tea infusion like in human use, and the dosage was calculated according to the typical human dose. Since we were primarily interested in the effects of *Stellaria media* tea on the severity of hypercholesterolemia and diabetes, as well as on diabetes-induced cardiac consequences, we applied experimental rat models of diet-induced hypercholesterolemia and experimental diabetes achieved by fructose-enriched diet supplemented with a single streptozotocin injection.

Based on our findings, we did not confirm the cholesterol-lowering effect of *Stellaria media* tea, since no alterations in blood lipid profile (i.e., total cholesterol, triacylglycerol, LDL and HDL cholesterol) were observed compared to the untreated hypercholesterolemic group. Moreover, our transthoracic echocardiographic measurements showed that *Stellaria media* treatment did not affect cardiac morphology and parameters related to cardiac function in diet-induced hypercholesterolemia.

In the literature, the antiobesity effect of *Stellaria media* was examined using various rodent obesity models[90-92]. Only one of these studies demonstrated a cholesterol-lowering effect of *Stellaria media* administered as 900 mg/kg body weight lyophilized juice in a high-fat diet-induced obesity model in male Swiss albino mice[92]. In the same study, the lyophilized juice of *Stellaria media* also reduced the high-fat diet-induced increase in triacylglycerol level and body weight. Nevertheless, lyophilized juice at 400 mg/kg body weight had no beneficial effects. The contradictions of these findings with our results are likely due to significant differences in the experimental setups. The major difference is the type and dose of *Stellaria media* extracts used in the studies. We treated animals with *Stellaria media* tea lyophilizate at a dose of 100 mg/kg body weight, while the other research group used lyophilized herb juice at an effective dose of 900 mg/kg body weight. The different extraction methods likely resulted in qualitative and quantitative differences in the active substances of the extracts, and the dose of the lyophilized juice of *Stellaria media* seems to be unrealistically high. In two other publications, ethanolic and methanolic extracts of *Stellaria media* were tested in progesterone-induced obesity[91] or in cafeteria diet-induced obesity models in female rats or mice[90]. Administration of 400 mg/kg body weight methanolic extract decreased triacylglycerol levels in both obesity models, but it did not affect total cholesterol levels. The ethanolic extract had no beneficial effects in these studies. It is worth mentioning that the administration of methanolic extract has no relevance in human use and the assessment of the ethnopharmacological application of chickweed.

According to our results, *Stellaria media* tea appeared to have beneficial effects on cardiac dysfunction induced by diabetes, since the treatment improved the impaired cardiac

output and cardiac work and preserved cardiac STAT3 phosphorylation. However, this effect seems to be independent of the modulation of diabetes severity as the application of the tea treatment did not influence fasting hyperglycemia or glucose intolerance.

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[93]. In that study, *Stellaria media* treatment attenuated fasting blood glucose levels, decreased hemoglobin A1c levels and inhibited pancreatic α-amylase and β-glucosidase enzyme activities[93]. 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[90, 91]. However, they also found that 200 and 400 mg/kg methanolic extract significantly attenuated serum glucose levels in these models[90, 91]. These controversial results may be explained by the differences in (i) the composition of the extracts, (ii) delivery time, administration and dose of *Stellaria media*, (iii) the applied animal models and strains. It should be also 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, showing that this experimental diabetes model has some adverse effects on the heart. *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. To the best of our knowledge, in the literature currently there is no other experimental data concerning the effects of *Stellaria media* on cardiac function or cardiomyopathy. Various mechanisms may play a role in the development of cardiac dysfunction in diabetes, e.g., oxidative stress, diffuse apoptosis of cardiomyocytes, dysregulation of cardiac signaling pathways, mitochondrial dysfunction, fibrosis or hypertrophy[87, 94]. Interestingly, there are more than 50 medicinal herbs, which have beneficial effects on experimental diabetic cardiomyopathy[94]. These plants have been suggested to exert antioxidant properties that may attenuate oxidative stress or inflammation, and to reduce apoptosis, and cardiac remodeling[94]. In the literature, there are some experimental data suggesting similar antioxidant and anti-inflammatory properties of *Stellaria media*, which can be associated with the improvement of certain cardiac parameters in our present study[36].

Nevertheless, the *in vitro* antioxidant capacity of *Stellaria media* aqueous and methanolic extracts was found to be rather weak[95].

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[36]. The flavonoid content of *Stellaria media* has been discussed by several papers[95-97]. In a phytochemical study, the flavonoid content was determined not less than 1.2% in raw plant material[97]. In an experiment, the total flavonoid content (determined by HPLC) of a lyophilized juice was 25.6 mg/g, and in an ethanolic extract 63.9 mg/g[95]. Our extract was prepared with hot water (in accordance with the human use) and this might explain the lower flavonoid content. UPLC 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 improve left ventricular dysfunction in STZ-induced diabetes[98, 99] and in high-carbohydrate, high-fat diet models[100]. LC-MS/MS analysis of the extract we used in our study afforded identification of various glycosylated apigenin-derivatives, which finding is in accordance with literature data[95]. 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[101], 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[102] and to exert protective effects against cardiac dysfunction in myocardial infarction in diabetic rats[103], suggesting that apigenin-derivatives may contribute to the beneficial cardiac effects of *Stellaria media* tea.

We were interested whether signaling pathways are involved in the cardiac effects of chickweed tea. Based on literature data, STAT3 is proposed as a key mediator of diabetes-induced cardiac dysfunction[104-106]. In conjunction with other studies[107-109], we showed that diabetes increased cardiac STAT3 phosphorylation. Moreover, *Stellaria media* tea treatment prevented diabetes-induced cardiac STAT3 dysregulation, suggesting a protective role. Indeed, some studies demonstrated that attenuation of the enhanced cardiac STAT3 activation may be beneficial in diabetes. In a high-glucose, high-fat diet and STZ injection-induced diabetic rat model, losartan attenuated the cardiac STAT3 phosphorylation and improved heart function[109]. In STZ-induced diabetic mice hearts, inhibition of diabetes-induced activation of EGFR-STAT3 signaling was associated with restoring cardiac fibrosis and hypertrophy related factors, and prevented diabetes-induced cardiac dysfunction[106].

Interestingly, the possible presence of rutin in *Stellaria media* tea may be a feasible explanation for the observed effects in our study, as rutin was suggested to exert cytoprotection by inhibiting STAT3 phosphorylation[110]. Similarly, apigenin and its derivatives has been shown to attenuate STAT3 activation in tumor cells[111, 112], which is in agreement with our present findings observed in cardiac tissue and may be related to the beneficial effect of *Stellaria media* tea on diabetic cardiomyopathy.

7.3. Inducing cytoprotection: effects of kynurenic acid and NMDA receptor modulation

Apart from attenuating the severity of modifiable risk factors and preventing their harmful cardiac consequences, another therapeutical approach is to induce cytoprotection with pharmacological agents to ameliorate cell death in I/R injury. Therefore, we elucidated the therapeutic potential of a natural substance in inducing cytoprotection; we tested the effects of kynurenic acid and NMDA receptor modulation in an *in vitro* simulated I/R model.

According to our results, cardioprotective effect of kynurenic acid was confirmed on H9c2 cells. We were interested in the underlying molecular mechanisms and started to examine the involvement of NMDA receptors in the cardioprotective effect of kynurenic acid, because in the nervous tissue kynurenic acid is known to act as an antagonist on NMDA receptors. According to our results, NMDA receptor activation by NMDA treatment during simulated I/R increased cell death in a dose-dependent manner, but kynurenic acid and another potent receptor antagonist, MK-801 were able to ameliorate this effect. 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[61]. Moreover, activation of the receptors facilitated atrial fibrillation in rats[113].

Furthermore, according to literature data, NMDA receptor antagonism has been shown to have protective effects in the heart. The NMDA receptor antagonist memantine reduced infarct size and improved cardiac performance in *ex vivo* ischemia/reperfusion experiments in rat hearts[114]. In *in vivo* isoprotenerol-induced infarction model[114] and heart failure[115], pretreatment with memantine attenuated cardiac remodeling, lipid peroxidation and neutrophil recruitment. 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 modulation, one may speculate about other possible receptors and 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[54].

Activation of GPR35 has been shown to inhibit pro-inflammatory signals, such as lipopolysaccharide-induced tumor necrosis factor-α secretion[116]. The receptor has been implicated to be involved in cardiovascular diseases as well[117-119]. In response to hypoxia and hypoxia-inducible factor 1 activation, mRNA and protein levels of GPR35 increased in neonatal mouse cardiomyocytes[119]. In accordance with this finding, myocardial GPR35 expression was induced in *in vivo* mouse models in response to hypoxia due to acute myocardial infarction and chronic pressure overload[119]. Moreover, in heart failure patients, myocardial GPR35 expression was significantly upregulated compared to healthy controls[118]. These literature data suggest that GPR35 may be a potential marker or therapeutic target in heart failure, hypertension and hypoxia-related cardiovascular diseases.

Aryl-hydrocarbon receptor pathway has been shown to play an important role in the development and function of the cardiovascular system. In aryl-hydrocarbon receptor knockout mice, cardiac hypertrophy, vascular remodeling and hypertension were observed[120]. Activation of aryl-hydrocarbon receptors have been reported to mediate cardioprotective effects against doxorubicin-induced cardiotoxicity[121]. 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[120].

Based on literature data, involvement of GPR35 or aryl-hydrocarbon receptors 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.

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 DHE fluorescence intensity in our study. This is in accordance with literature data, where kynurenic acid has been reported to exert antioxidant properties[50]. It exerted hydroxyl and superoxide radical scavenging activity *in vitro* in cerebellum and forebrain homogenates which seems to be independent of its antagonist activity on NMDA receptors[50].

Another interesting approach can be modulation of apoptosis, programmed cell death, which is reported to be a relevant process in development of myocardial I/R-induced cell

death. In I/R injury, hypoxia activates both extrinsic and intrinsic apoptosis pathways, activating the caspase cascade and changing mitochondrial membrane integrity resulting in cell death via proteolysis[122]. In the literature, pretreatment with kynurenic acid has been reported to attenuate MPP(+)-induced neuronal cell death via inhibiting mitochondrial apoptotic processes, such as increased proapoptotic Bax protein expression and caspase activity, and collapse of mitochondrial membrane potential[123]. Moreover, intravenous administration of kynurenic acid attenuated hypothalamic neuronal degeneration and apoptosis, furthermore the treatment ameliorated spleen, kidney, liver, and lung apoptosis in a rat model of heatstroke[124]. These data suggest that attenuation of myocardial apoptosis may be a promising approach behind the cardioprotective effect of kynurenic acid; nevertheless, further studies are needed to elucidate these processes.

Besides apoptosis, autophagy also plays another major role in the development of myocardial I/R injury[122]. Autophagy is a programmed intracellular process for degradation of damaged macromolecules and organelles in cytoplasmic lysosomal membrane vesicles. During I/R injury, autophagy is enhanced both in ischemia and in reperfusion, in response to energy crisis and oxidative stress[125]. It can act as a doubled-edged sword in the pathology of I/R injury, in exerts both beneficial and detrimental effects[125]. On the one hand, it can antagonize negative cardiac processes with restoration of ATP generation, mitophagy and proteostasis, while it can also contribute to further cell damage with excessive protein deletion and its association with apoptosis[125]. In the literature, kynurenic acid has been reported to enhance autophagy markers (LC3 conversion, p62 degradation, autophagosome formation) in hepatocytes contributing to attenuation of nonalcoholic fatty liver disease[126]. However, further studies are needed to investigate the involvement of autophagy in the cardioprotective effect of kynurenic acid in simulated I/R.

7.4. Limitations of our studies and future perspectives

Although we provide interesting data on the effects of *Stellaria media* in hypercholesterolemia and diabetes as well as on the involvement of NMDA receptors in the cardioprotective effect of kynurenic acid, as always, there may be some possible limitations of our studies.

The animals received *Stellaria media* tea from the onset of the experiments, together with the high cholesterol- or fructose-enriched diet. It would be worthwhile testing its effects 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 also be considered as a possible limitation. Nevertheless, a better understanding of the molecular mechanism underlying the proposed beneficial cardiac effects of *Stellaria media* tea would require additional research in the future. 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. Testing the effects of the tea in female rats could reveal sex differences in the effectiveness of the extract. 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.

We tested the effects of kynurenic acid and NMDA receptor modulation in *in vitro* experiments. 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.

8. Conclusions

In this thesis, we focused on the role of natural substances (a medicinal herb and an endogenous molecule) in prevention or treatment of ischemic heart disease including acute myocardial infarction using two different approaches: improving the severity of modifiable metabolic cardiovascular risk factors and their cardiac consequences, or inducing cytoprotection in order to decrease cell damage induced by I/R.

Our research group demonstrated for the first time that the tea made of a known medicinal herb, *Stellaria media* does not alter blood lipid profile in experimental hypercholesterolemia and does not improve glucose homeostasis in diabetes. These findings do not support the rationale for using chickweed tea in order to lower cholesterol or glucose levels. However, more importantly, the tea may protect against diabetes-induced cardiac dysfunction; although, this effect seems to be independent of the modulation of fasting hyperglycemia or glucose intolerance in rats. We revealed that *Stellaria media* prevents diabetes-induced STAT3 phosphorylation in the heart, which may play a role in the beneficial cardiac effect. Nevertheless, additional studies are needed to further investigate the beneficial effects and the molecular mechanisms of *Stellaria media* tea in diabetic conditions.

Moreover, 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 I/R injury. However, in the protective effect of kynurenic acid in simulated I/R, NMDA receptor antagonism may not be the main mechanism, since treatment with the synthetic antagonist MK-801 does not influence the developing cell death significantly. Nevertheless, further studies are needed to address the exact molecular mechanisms underlying the proposed cardioprotective effect of kynurenic acid in simulated I/R.

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.

9. Acknowledgements

We acknowledge the support of grants from the Hungarian National Research, Development and Innovation Office (OTKA K115990), Ministry of Human Capacities, Hungary (20391-3/2018/FEKUSTRAT). This work was prepared in the frame of the Economic Development and Innovation Operative Programme, GINOP-2.3.2-15-2016-00034 and GINOP-2.3.2-15-2016-00006 projects.

I would like to express my sincere acknowledgement to Prof. László Dux for providing me with the opportunity to work at the Doctoral School of Multidisciplinary Medical Sciences, as well as for widening my knowledge in biochemistry.

I owe my gratitude to my supervisor and friend, Tamás Csont. He supported, guided and encouraged me throughout my undergraduate and PhD student years.

I am grateful to Renáta Gáspár for her teaching and encouragement in the early years.

I am particularly thankful for our collaborators Dezső Csupor and Tivadar Kiss for introducing us to the field of medicinal herbs and providing valuable help in the preparation of *Stellaria media* tea, as well as in the flavonoid content, flavonoid screening and antioxidant capacity measurements.

I wish to thank the co-authors their valuable help in performing the experiments: Gergő Szűcs in working heart perfusion and Márta Sárközy in transthoracic echocardiography measurements; Andrea Siska and Imre Földesi in the analysis of various serum parameters; Zsuzsanna Darula and Roland Tengölics in the LC/MS-MS analysis; Dóra Halmi and Renáta Gáspár in the kynurenic acid viability and oxidative stress measurements.

I am thankful for all the members of the research group: Csaba Csonka, Márton Pipicz, Renáta Gáspár, Gergő Szűcs, Márta Sárközy, Andrea Sója, Márton Szabó, Petra Diószegi, Zsuzsanna Kovács, Mónika Kovács, Dóra Halmi, Róbert Soltész, Dóra Csóré, Réka Somogyi, Atina Čolić and Éva Plechl; and all the members of the Department of Biochemistry, particularly the PhD students of the ‘first lab’: Kitti Szabó, Zoltán Köhler and Dániel Becsky for their friendship and encouragement. I received generous technical support and assistance from Zita Felhő Makráné, Erzsébet Rádi, Ildikó Engi, Tünde Bodnár and Imre Ocsovszki.

Above all, I am deeply thankful for the support of my loving family and close friends. Without their help this thesis would not have materialized. My husband, Márton Pipicz provided me a stable background and never-ending support throughout the years to overcome the difficulties. I would like to dedicate this thesis to them.

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11. Annex

I.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2020, Article ID 5109328, 7 pages
https://doi.org/10.1155/2020/5109328

Research Article

Effect of *Stellaria media* Tea on Lipid Profile in Rats

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Received 16 August 2019; Revised 21 December 2019; Accepted 3 January 2020; Published 25 January 2020

Academic Editor: Sokcheon Pak

Background. In folk medicine, common chickweed (*Stellaria media*) has traditionally been applied for the treatment of hypercholesterolemia; however, there is no firm experimental proof to support the rationale of this practice. Therefore, we aimed to assess the efficacy and safety of *Stellaria media* tea in hypercholesterolemic rats. *Materials and Methods*. Adult male Wistar rats were divided into 3 groups. The (i) control group received standard laboratory chow, the (ii) hypercholesterolemic group received cholesterol-enriched diet, and the (iii) chickweed-treated hypercholesterolemic group received cholesterol-enriched diet and 100 mg/kg body weight *Stellaria media* tea lyophilizate for 8 weeks. Blood samples were collected to determine serum lipid profile as well as liver and kidney function, and echocardiography was performed to assess cardiac morphology and function. *Results*. Cholesterol-enriched diet significantly increased serum total cholesterol, LDL- and HDL-cholesterol levels, but did not affect triacylglycerol concentrations. The addition of chickweed to the diet did not cause any significant change in serum lipid profile or body weight increase. Liver and kidney functions were unaltered and cardiac morphology and function were not changed due to *Stellaria media* tea lyophilizate. *Conclusion*. Although chickweed does not seem to be toxic, our results do not support the rationale of its use in the treatment of hypercholesterolemia.

1. Introduction

The wide ecological tolerance and short-term vegetative reproduction period make chickweed (*Stellaria media* (L.) Vill., Caryophyllaceae) a common and widespread species. In cool, moist, and moderately shaded environment, huge territories are covered by this plant; thus, its presence in cultivated fields is a serious agricultural problem.

Although chickweed has been consumed as salad and has been applied in folk medicine as tea [1, 2], its safety and efficacy have not been investigated in clinical trials [3]. Moderate interest has been shown toward this plant from the middle of the last century. Because of the potential biological benefits and its application in cosmetics, phytochemical and pharmacological studies have started to focus on species of *Stellaria* genus. These studies are based mainly on *in vitro* or *in vivo* animal experiments. According to these studies, several *Stellaria* species have noteworthy pharmacological activities (e.g., antibacterial, anti-inflammatory, and anti-allergic effects) [4].

Nowadays, *Stellaria media*, mostly consumed as tea, is gaining popularity as a remedy to lose weight [5] and it is widely used for its believed beneficial effects on blood lipid profile [6]. According to a popular Hungarian traditional healer, the infusion of 2.5 g chickweed two times daily normalizes increased cholesterol level within some weeks [7]. Moreover, consumption of chickweed tea for cholesterol lowering is recommended by several websites dealing with health and lifestyle issues [8, 9]. Since there is no clinical evidence supporting this hypothesis and the designs of the

available animal experiments differ from the human use, the aim of the present work was the investigation of chickweed tea on rats to gather additional data on efficacy and safety.

2. Methods and Materials

2.1. Animals. The experiment conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (National Institutes of Health publication 85–23, revised 1996), 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).

Altogether 24 adult (8-week old) male Wistar rats were used in this study (purchased from Charles River Laboratories, Göttingen, Germany), weighing 270–324 g at the onset of the experiments. Using only male rats in the study was intentional as the hormonal changes during the menstrual cycle of females have been shown to influence serum lipids [10, 11]. Rats were kept under standard climatic conditions ($22 \pm 2^{\circ}$C room temperature, 12 h light/dark cycles), in pairs, in individually ventilated cages (Sealsafe IVC system, Buguggiate, Italy) and had *ad libitum* access to tap water and laboratory rat chow.

2.2. Experimental Setup. After one week of acclimatization, the rats were randomized into three groups: control (Cont), hypercholesterolemia (HChol), and hypercholesterolemia + *Stellaria media* treatment (HChol + SM). Rats in the control group ($n = 8$) received standard laboratory rat chow. The other 16 rats were fed a special cholesterol-enriched diet, i.e., a standard laboratory rat chow (Innovo Ltd., Isaszeg, Hungary) supplemented with 2% (w/w) cholesterol (Hungaropharma, Budapest, Hungary) and 0.25% (w/w) sodium-cholate-hydrate (Sigma-Aldrich, St. Louis, USA) for 8 weeks to induce experimental hypercholesterolemia as described earlier [12–14]. We have chosen this cost-effective model of experimental hypercholesterolemia because our research group has previously accumulated extensive experience regarding the use of this model [12–14] and the lipoprotein profile of the cholesterol-fed rats (LDL/HDL ratio) is quite similar to that of humans. The diet of 8 animals receiving cholesterol-enriched chow was further supplemented with *Stellaria media* tea lyophilizate mixed into cookie balls (HChol + SM) in order to examine the potential cholesterol-lowering effect of *Stellaria media*. On the eighth week, rats were anesthetized with sodium pentobarbital (Euthasol, 50 mg/kg body weight, ip., Produlab Pharma b.v., Raamsdonksveer, The Netherlands), the abdominal cavity was opened, and blood samples were taken from the abdominal aorta. Collected blood was allowed to clot and was centrifuged ($2000 \times$ g, 20 min, 4°C); then serum was separated for analysis of various serum parameters to evaluate the efficacy and safety of *Stellaria media* treatment, including lipid profile and parameters representing liver and kidney function. Before termination, echocardiography was performed in order to evaluate the effects of experimental hypercholesterolemia and *Stellaria media* on cardiac morphology and function.

2.3. Preparation of Stellaria media Tea Lyophilizate. Stellaria media was harvested in Algyő (Hungary) by "Ezerjófű" Association in 2017. Voucher specimen (no: 882) was deposited in the herbarium of the University of Szeged, Faculty of Pharmacy, Department of Pharmacognosy. The drug was dried and stored at room temperature.

The dried and grounded drug was extracted with boiling water (1 : 10 w/v ratio) for 15 minutes by ultrasonication. The highly dense extract was separated from solid particles by mechanical press, and the water extract was lyophilized. Approximately, 1.5 g lyophilizate was obtained from 10.0 g dried drug.

2.4. Stellaria media Administration. Rats in the HChol + SM group received 100 mg/kg body weight lyophilized *Stellaria media* tea mixed into cookie balls once a day. The recipe of cookie dough included 55% plain flour, 20% caster sugar, and 25% water [15]. All animals received 2 g cookie dough/kg body weight per day. We have found in a pilot study that administration of 2 g/kg body weight cookie dough for 7 days in control rats did not cause significant changes in levels of blood cholesterol, triacylglycerol, or glucose (data not shown). The dough was prepared once a week and kept at 4°C until use. The 100 mg/kg body weight dose of lyophilized *Stellaria media* tea was considered as equal to human daily dose, calculated according to Nair and Jacob [16]. Individual portions of lyophilized *Stellaria media* tea were freshly mixed with the cookie balls right before administration. 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. We always made sure that the whole cookie ball was eaten, and the success rate of this technique was 100% during the experiment. Cookie balls were preferred instead of the traditional gavage technique in order to cause less daily stress to the animals and to model human exposure the most objectively.

2.5. Measurements of Serum Lipid Levels. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol, triacylglycerol levels, and pancreatic lipase enzyme activities were analyzed by using Roche Cobas 8000 analyzer system in the Department of Laboratory Medicine using enzymatic colorimetric assays from Roche (Mannheim, Germany). Low-density lipoprotein (LDL) cholesterol levels were measured using a kit from Diagnosticum, Budapest, Hungary, adopted to a plate reader (FLUOstar Optima, BMG), as described earlier [17].

2.6. Measurements of Serum Parameters Representing Liver and Kidney Function. Several other serum parameters were measured using Roche Cobas 8000 analyzer system to monitor the effect of diet-induced hypercholesterolemia as well as *Stellaria media* treatment on liver and kidney functions. Total protein, albumin, and creatinine concentrations as

well as alkaline phosphatase (ALP) enzyme activities were analyzed by colorimetric assays from Roche (Mannheim, Germany). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzyme activities and carbamide levels were measured with Roche UV assays (Mannheim, Germany), as described earlier [14].

2.7. Transthoracic Echocardiography. Cardiac morphology and function were assessed by transthoracic echocardiography at week 8 as described previously [18–20]. Briefly, rats were anesthetized with sodium pentobarbital (Euthasol, 50 mg/kg body weight ip.). Then, the chest was shaved and the rat was placed in a supine position onto a heating pad. Two-dimensional and M-mode echocardiographic examinations were performed by the criteria of the American Society of Echocardiography with a Vivid IQ ultrasound system (General Electric Medical Systems, Boston, USA) using a phased array 5.0–11 MHz transducer (GE 12S-RS probe). Data of three consecutive heart cycles were analyzed (EchoPac Dimension software; General Electric Medical Systems, Boston, USA) by an experienced investigator in a blinded manner. The mean values of three measurements were calculated and used for statistical evaluation.

2.8. Statistical Analysis. Statistical analysis was performed by using SigmaPlot 12.0 for Windows (Systat Software Inc). 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 the Tukey *post hoc* test.

3. Results

3.1. Body Weight. Body weight showed a continuous increase from 305 ± 4 g at the onset of the experiment to 505 ± 13 g at week 8 in the control group fed a normal diet (Figure 1(a)). Neither cholesterol-enriched diet nor *Stellaria media* treatment affected body weight significantly at any time points (Figure 1(a)). Weight gain during the 8-week feeding protocol was also not affected significantly by any of the treatments (Figure 1(b)).

3.2. Serum Lipid Parameters. Lipid levels were measured from serum in order to validate the development of diet-induced hypercholesterolemia by the end of an 8-week feeding protocol. Total cholesterol concentration was significantly elevated in the HChol and the HChol + SM groups compared to the control group; however, there was no significant difference between HChol and HChol + SM values (Figure 2(a)). Triacylglycerol levels showed no significant difference due to cholesterol-enriched diet or *Stellaria media* treatment (Figure 2(b)). Similarly to total cholesterol, serum LDL cholesterol concentration was significantly higher in the HChol group, which was not affected by *Stellaria media* tea lyophilizate (Figure 2(c)). Serum HDL cholesterol level was significantly higher in the HChol group compared to control values; however, *Stellaria media* treatment did not affect significantly HDL cholesterol level (Figure 2(d)). Serum pancreatic lipase enzyme activities were not statistically different among the three experimental groups (Cont: 5.5 ± 0.27 U/L, HChol: 7.25 ± 1.07 U/L, and HChol + SM: 6.13 ± 0.35 U/L). These results suggest that *Stellaria media* tea lyophilizate does not have triacylglycerol- and cholesterol-lowering effect.

3.3. Liver Weight and Function. Diet-induced hypercholesterolemia caused marked alterations in some liver parameters. Liver weight, serum total protein, and albumin concentrations, as well as ALP activity were significantly higher in the HChol group compared to the control group (Figures 3(a)–3(d)). ALT and AST activities were not altered due to diet-induced hypercholesterolemia (Figures 3(e) and 3(f)). *Stellaria media* treatment did not influence significantly the hypercholesterolemia-induced alterations (Figure 3). Interestingly, AST enzyme activities were significantly higher in the HChol + SM group compared with the control group.

3.4. Kidney Function. Diet-induced hypercholesterolemia and *Stellaria media* treatment did not influence the serum parameters representing kidney function since there was no significant difference among the experimental groups in serum carbamide and creatinine levels (Figure 4).

3.5. Transthoracic Echocardiography. Transthoracic echocardiographic measurements performed at the end of the feeding protocol showed that diet-induced hypercholesterolemia and *Stellaria media* treatment did not affect cardiac morphology as there were no significant differences in systolic and diastolic wall thickness parameters (Table 1). Parameters related to cardiac function including left ventricular end-diastolic and end-systolic volume, stroke volume, ejection fraction, and heart rate were also not influenced significantly by diet-induced hypercholesterolemia and *Stellaria media* treatment (Table 1).

4. Discussion

In folk medicine, *Stellaria media* is mostly consumed as tea and is believed to decrease blood cholesterol level. In accordance, several tea products are available with this claim or indication. Nevertheless, the cholesterol-lowering effect of *Stellaria media* tea has not been investigated previously.

In our present study, we intended to model the human use of chickweed as close as possible. For this reason, according to folk medicinal practice, only above-ground parts of *Stellaria media* were used. The extract was prepared as tea infusion like in human use, and the dosage was calculated according to the typical human dose. Since we were primarily interested in the potential cholesterol-lowering effect of *Stellaria media*, we applied an experimental model of diet-induced hypercholesterolemia that has been previously characterised and extensively used in our laboratory [12–14]. In our present study, we did not confirm the

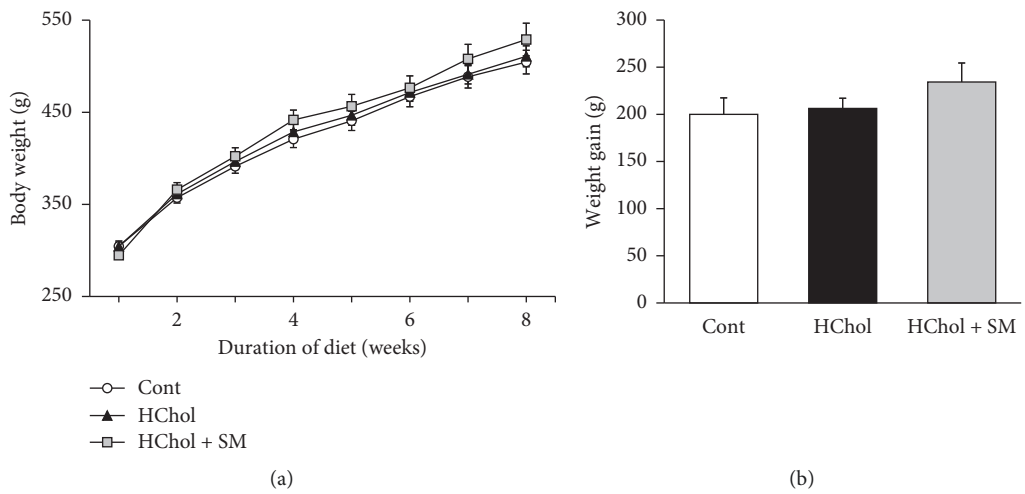

Figure 1: Body weight (a) and weight gain after 8 weeks (b) in the control group (white spheres) and rats fed with cholesterol-enriched diet (black triangles) or cholesterol-enriched diet with *Stellaria media* extract (grey squares). Results are means ± SEM ($n = 8$ in each group), analyzed by one-way ANOVA with Tukey *post hoc* test.

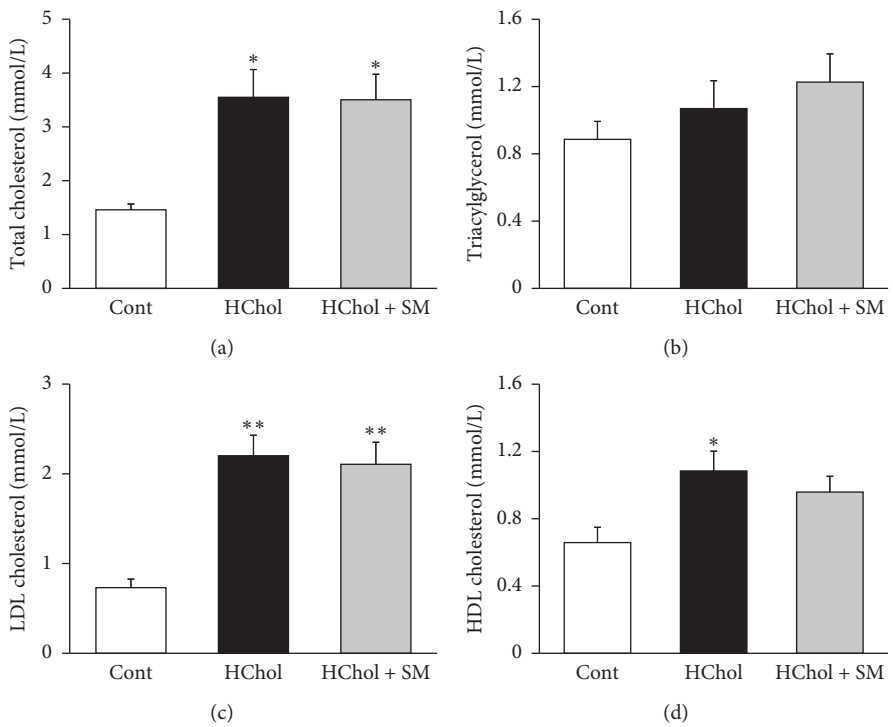

Figure 2: Serum total cholesterol (a), serum triacylglycerol (b), LDL cholesterol (c), and HDL cholesterol (d) levels in rats fed either cholesterol-enriched (HChol), cholesterol-enriched + *Stellaria media* (HChol + SM), or normal diet (Cont). Results are means ± SEM ($n = 8$ in each group), analyzed by one-way ANOVA with the Tukey *post hoc* test. $^{*}p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control.

cholesterol-lowering effect of *Stellaria media* tea. No alterations in lipid metabolism were observed since pancreatic lipase activity was not inhibited, and blood lipid profile (i.e., total cholesterol, triacylglycerol, LDL, and HDL cholesterol) was not significantly different compared to the untreated hypercholesterolemic group.

In the literature, the antiobesity effect of *Stellaria media* was examined using various rodent obesity models [21–23]. Only one of these studies demonstrated a cholesterol-lowering effect of *Stellaria media* administered as 900 mg/kg body weight lyophilized juice in a high-fat diet-induced obesity model in male Swiss albino mice [23]. In the same study, the lyophilized juice of *Stellaria media* also reduced the high-fat diet-induced increase in triacylglycerol level and body weight. Nevertheless, lyophilized juice at 400 mg/kg body weight had no beneficial effects. The contradictions of these findings with our results are likely due to significant differences in the experimental setups. The major difference is the type and

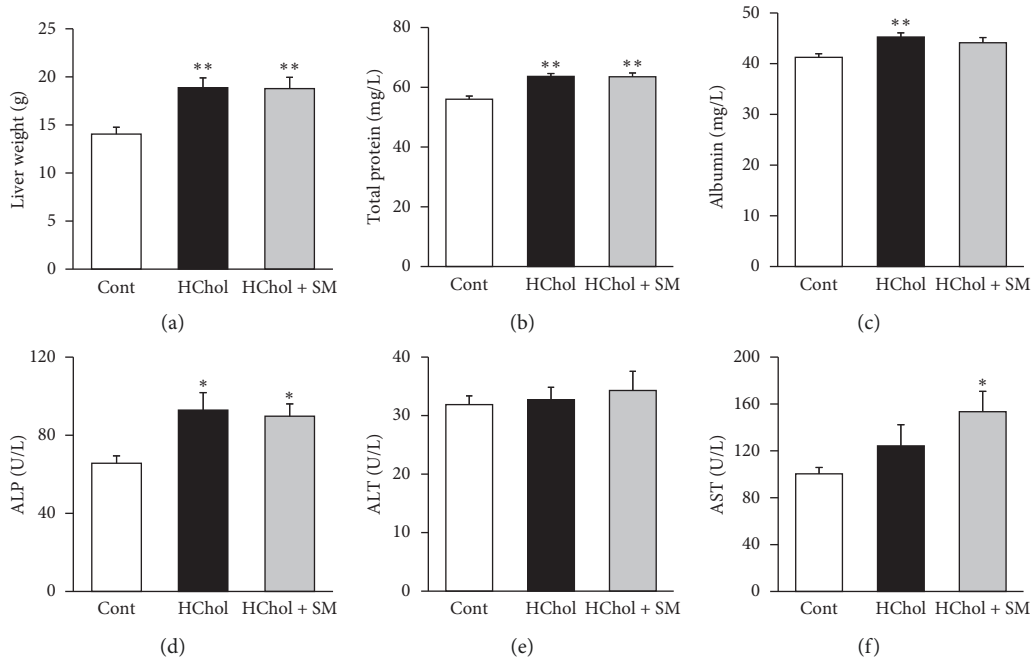

Figure 3: Liver weight (a), serum total protein levels (b), albumin levels (c), alkaline phosphatase (ALP) activity (d), alanine aminotransferase (ALT) activity (e), and aspartate aminotransferase (AST) activity (f) in rats fed either cholesterol-enriched (HChol), cholesterol-enriched + *Stellaria media* (HChol + SM), or normal diet (Cont). Results are means ± SEM ($n = 8$ in each group), analyzed by one-way ANOVA with the Tukey *post hoc* test. $^{*}p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control.

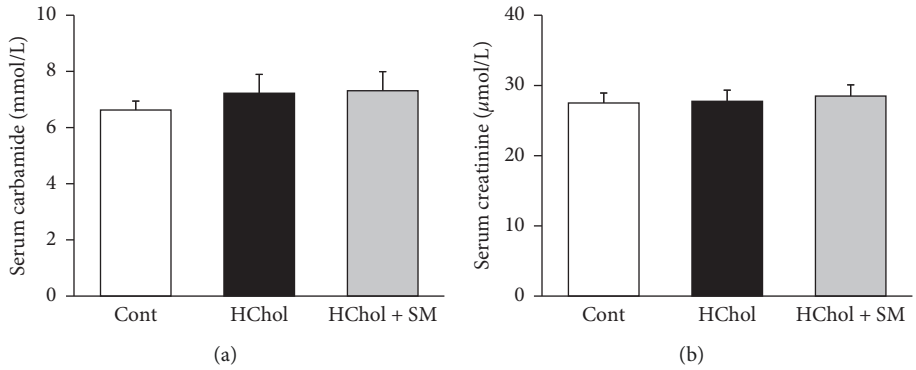

Figure 4: Serum carbamide (a) and creatinine (b) levels in rats fed either cholesterol-enriched (HChol), cholesterol-enriched + *Stellaria media* (HChol + SM), or normal diet (Cont). Results are means ± SEM ($n = 8$ in each group), analyzed by one-way ANOVA with the Tukey *post hoc* test.

dose of *Stellaria media* extracts used in the studies. We treated animals with *Stellaria media* tea lyophilizate at a dose of 100 mg/kg body weight, while the other research group used lyophilized herb juice at an effective dose of 900 mg/kg body weight. The different extraction methods likely resulted in qualitative and quantitative differences in the active substances of the extracts, and the dose of the lyophilized juice of *Stellaria media* seems to be unrealistically high.

In two other publications, ethanolic and methanolic extracts of *Stellaria media* were tested in progesterone-induced obesity [22] or in cafeteria diet-induced obesity models in female rats or mice [21]. Administration of 400 mg/kg body weight methanolic extract decreased triacylglycerol levels in both obesity models, but it did not affect total cholesterol levels. The ethanolic extract had no beneficial effects in these studies. It is worth mentioning that the administration of methanolic extract has no relevance in human use and the assessment of the ethnopharmacological application of chickweed. Unfortunately, credibility of the data in these latter two papers is rather questionable because there are numerous contradictions between the reported data in the tables and the description and interpretation of the findings in the text. Furthermore, the results are not comparable with the findings of other reports since the poorly described extraction method is unclear.

We have also investigated some safety issues, and based on our results, SM treatment does not seem to have a severe toxic effect on the liver or the kidneys, since several liver

Table 1: Effects of *Stellaria media* on left ventricular morphological and functional parameters. Transthoracic echocardiographic measurement values in rats fed either cholesterol-enriched (HChol), cholesterol-enriched + *Stellaria media* (HChol + SM), or normal diet (Cont). Results are means ± SEM ($n = 8$ in each group), analyzed by one-way ANOVA with the Tukey *post hoc* test. 2D: two dimensional; 4CH: four chambers view; MM: M (motion) Mode; ns: not significant.

Parameter (unit)	View/mode	Week 8			Significance
		Cont	HChol	HChol + SM	
Anterior wall thickness-systolic (mm)	Short axis/MM	3.57 ± 0.11	3.41 ± 0.17	3.10 ± 0.12	ns
Anterior wall thickness-diastolic (mm)	Short axis/MM	2.20 ± 0.11	2.22 ± 0.17	2.16 ± 0.11	ns
Inferior wall thickness-systolic (mm)	Short axis/MM	3.85 ± 0.11	3.46 ± 0.15	3.69 ± 0.09	ns
Inferior wall thickness-diastolic (mm)	Short axis/MM	2.14 ± 0.09	2.13 ± 0.14	2.19 ± 0.13	ns
Posterior wall thickness-systolic (mm)	Long axis/MM	3.82 ± 0.08	3.68 ± 0.12	3.62 ± 0.09	ns
Posterior wall thickness-diastolic (mm)	Long axis/MM	2.19 ± 0.03	2.39 ± 0.13	2.52 ± 0.19	ns
Septal wall thickness-systolic (mm)	Long axis/MM	3.79 ± 0.06	3.62 ± 0.12	3.54 ± 0.17	ns
Septal wall thickness-diastolic (mm)	Long axis/MM	2.50 ± 0.11	2.24 ± 0.10	2.35 ± 0.11	ns
Left ventricular end-diastolic volume (μl)	4CH/2D	127 ± 15	129 ± 13	105 ± 13	ns
Left ventricular end-systolic volume (μl)	4CH/2D	49 ± 8	53 ± 5	46 ± 6	ns
Stroke volume (μl)	4CH/2D	79 ± 8	76 ± 8	59 ± 8	ns
Ejection fraction (%)	4CH/2D	63 ± 2	59 ± 1	56 ± 3	ns
Heart rate (1/min)	4CH/2D	343 ± 14	367 ± 8	370 ± 11	ns

marker enzymes were elevated in the HChol group without being affected by SM treatment (total protein, albumin, and ALP). Although AST enzyme activities were elevated in the HChol + SM group compared with the control group, the AST activity in the HChol + SM group did not differ from the values measured in the HChol group. Elevated AST level in the blood is often considered as a sign of liver damage; however, AST is not specific for the liver and may be also increased due to injuries of the heart, muscle, pancreas, kidney, or red blood cells [24]. Interestingly, SM treatment actually was found to be hepatoprotective in a liver toxicity model [25]. Overall these data suggest that *Stellaria media* treatment has no deleterious effect on liver function; however, a possible limitation of our study is the lack of a group receiving herbal treatment without cholesterol-enriched chow.

5. Conclusions

In our current study, we have also investigated some safety issues and found that *Stellaria media* was neither toxic nor caused alterations in liver or kidney functions and cardiac morphology compared with hypercholesterolemic rats. This suggests a safe use of *Stellaria media* tea. The human use of chickweed tea for lowering blood cholesterol level was examined *in vivo* in rats, using an experimental design to mimic the human use of the herb. Since the body weight and blood lipid profile were not significantly altered in the group treated with *Stellaria media* compared with the group fed with cholesterol-enriched diet only, our experiment does not support the rationale for using chickweed tea in order to lower cholesterol level.

Abbreviations

2D: Two dimensional
4CH: Four-chamber view
ALP: Alkaline phosphatase
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
Cont: Control
HChol: Hypercholesterolemia
HChol + SM: Hypercholesterolemia + *Stellaria media*
HDL: High-density lipoprotein cholesterol
LDL: Low-density lipoprotein cholesterol
MM: M (motion) mode.

Data Availability

The data used to support the findings of this study are provided in this paper. Any further data are available from the corresponding author upon request.

Disclosure

Virág Demján and Tivadar Kiss are co-first authors of this study. Dezső Csupor and Tamás Csont are co-corresponding authors of this paper. The results of this research were partly presented as a poster entitled "*In vivo* examination of antihyperlipidaemic effect of *Stellaria media* in rats" at the 67th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (1–5 September 2019, Innsbruck).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Virág Demján, Tivadar Kiss, Dezső Csupor, and Tamás Csont contributed equally to this manuscript. DC and TC conceived the study. DC, TC, VD, and TK designed the experiment. TK identified the plant material. VD, TK, AS, IF, and MS performed experiments and generated data. VD, TC, and MRS analyzed the data. VD, TK, DC, and TC wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the Economic Development and Innovation Operative Programme (GINOP-2.3.2-15-2016-00012 and GINOP-2.3.2-15-2016-00006) and EFOP-3.6.2-16-2017-00006 and by grants from the National Research, Development and Innovation Office (K115990 and K115796), the Ministry of Human Capacities, Hungary, (20391-3/2018/FEKUSTRAT), and the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. MS, VD, and MRS were supported by the New National Excellence Program of the Ministry of Human Capacities (UNKP-18-4-SZTE-63, UNKP-19-3-SZTE-47, and UNKP-19-3-SZTE-269, respectively).

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Journal of Traditional and Complementary Medicine xxx (xxxx) xxx

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Stellaria media tea protects against diabetes-induced cardiac dysfunction in rats without affecting glucose tolerance

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ARTICLE INFO

Article history:
Received 29 April 2021
Received in revised form
4 August 2021
Accepted 5 August 2021
Available online xxx

Keywords:
Diabetic co-morbidity
Prevention
Medicinal herb
Flavonoid
Signal transducer and activator of transcription 3

ABSTRACT

Background and aim: Common chickweed (*Stellaria media*) tea has traditionally been applied for treatment of various metabolic diseases including diabetes in folk medicine; however, experimental evidence to support this practice is lacking. Therefore, we aimed to assess the effect of *Stellaria media* tea on glucose homeostasis and cardiac performance in a rat model of diabetes.
Experimental procedure: Hot water extract of *Stellaria media* herb were analyzed and used in this study, where diabetes was induced by fructose-enriched diet supplemented with a single injection of streptozotocin. Half of the animals received *Stellaria media* tea (100 mg/kg) by oral gavage. At the end of the 20-week experimental period, blood samples were collected and isolated working heart perfusions were performed.
Results and conclusion: Compared to the animals receiving standard chow, serum fasting glucose level was increased and glucose tolerance was diminished in diabetic rats. *Stellaria media* tea did not affect significantly fasting hyperglycemia and glucose intolerance; however, it attenuated diabetes-induced deterioration of cardiac output and cardiac work. Analysis of the chemical composition of *Stellaria media* tea suggested the presence of rutin and various apigenin glycosides which have been reported to alleviate diabetic cardiomyopathy. Moreover, *Stellaria media* prevented diabetes-induced increase in cardiac STAT3 phosphorylation. We demonstrated for the first time that *Stellaria media* tea may beneficially affect cardiac dysfunction induced by diabetes without improvement of glucose homeostasis.

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

https://doi.org/10.1016/j.jtcme.2021.08.003

Please cite this article as: V. Demján, A. Sója, T. Kiss *et al.*, *Stellaria media* tea protects against diabetes-induced cardiac dysfunction in rats without affecting glucose tolerance, Journal of Traditional and Complementary Medicine, https://doi.org/10.1016/j.jtcme.2021.08.003

Rutin and/or apigenin glycosides as well as modulation of STAT3 signaling may be implicated in the protection of *Stellaria media* tea against diabetic cardiomyopathy.

List of abbreviations

Akt	protein kinase B
ALP	alkaline phosphatase
ALAT	alanine aminotransferase
ASAT	aspartate aminotransferase
AUC	area under the curve
CK	creatine kinase
CK-MB	creatine kinase – myocardial band
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
ELISA	enzyme-linked immunosorbent assay
Erk	extracellular signal-regulated kinase
HbA1c	haemoglobin A1c
HDL	high-density lipoprotein cholesterol
HPLC	high performance liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LDH	lactate dehydrogenase
LVDP	left ventricular developed pressure
LVEDP	left ventricular end-diastolic pressure
OGTT	oral glucose tolerance test
ORAC	oxygen radical absorbance capacity
QE	quercetin equivalent
Stellaria m.	*Stellaria media*
STAT3	signal transducer and activator of transcription 3
STZ	streptozotocin
UPLC	ultra-performance liquid chromatography

1. Introduction

Diabetes is a common metabolic disease characterized by elevated blood glucose level and impaired glucose tolerance. The prevalence of diabetes for adults was approximately 171 million patients worldwide in 2000 and according to predictions, this number is expected to be doubled by 2030.[1] Due to the continuously increasing trend in diabetes morbidity, investigation of harmful consequences of the disease and potential therapeutic interventions is a relevant current research area.[1] In the absence of proper therapy diabetes may lead to development of various complications including cardiomyopathy, cataract, kidney failure, as well as neuronal damage. Diabetic cardiomyopathy is characterized by diastolic and/or systolic dysfunction which can lead to heart failure without the presence of classic risk factors such as dyslipidaemia, hypertension, or coronary artery disease.[2,3]

Nowadays, medicinal plants are gaining popularity in prevention and treatment of various diseases including diabetes. There are some herbs with a well-described and scientifically proven *anti*-hyperglycaemic properties, for instance aqueous leaf extract of stinging nettle (*Urtica dioica*)[4,5] or powdered fenugreek seeds (*Trigonella foenum graecum*).[6,7] In folk medicine, *Stellaria media* is believed to be a remedy to lose weight[8] and it is suggested to be used for its believed beneficial effects on blood lipid profile.[9] According to a popular Hungarian traditional healer, *Stellaria media* tea improves general metabolism, lowers blood glucose level, making it an adjuvant therapy for diabetic patients.[10] Moreover, consumption of chickweed tea for lowering blood glucose level is recommended by some websites dealing with medicinal plants and health issues.[11,12] Although, an animal experiment proposed an *anti*-hyperglycaemic effect of intraperitoneally administered alcoholic *Stellaria media* extract,[13] the clinical translation of this design is limited since *Stellaria media* is mostly consumed as tea. Nevertheless, potential effects of *Stellaria media* tea on diabetes-induced cardiac dysfunction has never been investigated.

Taken together, there is no firm experimental or clinical evidence supporting the anti-diabetic effect of *Stellaria media* tea. Therefore, the aim of the present study was to investigate the potential therapeutic efficacy of *Stellaria media* tea on the severity of diabetes and on harmful cardiac consequences induced by diabetes.

2. Materials and methods

2.1. Animals

The experiment conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health [National Institutes of Health publication 85–23, revised 1996] 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).

Altogether 30 adult (9-week old) male Wistar rats were used in this study (purchased from Charles River Laboratories, Göttingen, Germany), weighing 292–420 g at the onset of the experiments. Rats were kept under standard climatic conditions (22 ± 2 °C room temperature, 12h light/dark cycles) in pairs in individually ventilated cages (Sealsafe IVC system, Buguggiate, Italy) and had *ad libitum* access to tap water and rat chow.

2.2. Experimental setup

After one week of acclimatization, the rats were randomized into three groups: Control, Diabetes, Diabetes + *Stellaria media* treatment. Rats in the control group (n = 10) received standard laboratory rat chow for 20 weeks. The other 20 rats were fed a special fructose-enriched diet, i.e. a standard laboratory rat chow (Innovo Ltd., Isaszeg, Hungary) supplemented with 60% (w/w) fructose (Floravita Ltd., Soltvadkert, Hungary) for 20 weeks[14] and were treated with a low-dose streptozotocin (STZ) injection (20 mg/kg body weight, ip.) on the 17th week. Combination of fructose-enriched diet/drinking water and low-dose STZ treatment has been reported to be an alternative approach to achieve experimental type 2 diabetes[15–17] (Fig. 1A). In order to prevent STZ-induced hypoglycaemia, drinking water containing 10% glucose

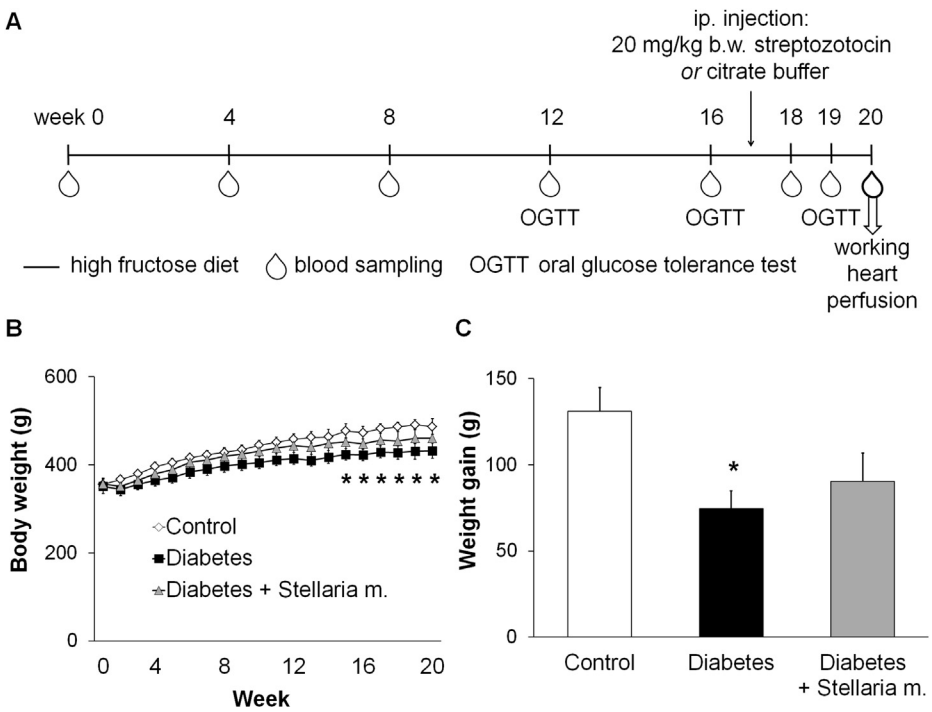

Fig. 1. Experimental protocol: rats were divided into Control, Diabetes and Diabetes + *Stellaria media* groups receiving either a standard chow or a chow supplemented with 60% fructose, respectively, for 20 weeks (A). Fasting blood glucose measurement was performed every four weeks until week 16. Oral glucose tolerance test (OGTT) was performed on week 12, 16 and 19. At week 17, rats in the Diabetes and Diabetes + *Stellaria media* groups were injected with a low-dose streptozotocin (20 mg/kg body weight) intraperitoneally. Fasting blood glucose was measured on week 18 and 19 to monitor the effect of streptozotocin injection. On week 20, the animals were anaesthetised and sacrificed. Blood samples were collected from the abdominal aorta, hearts were isolated and subjected to working heart perfusion according to Neely (A). Body weight (B) and weight gain (C) during 20 weeks in the Control group (circles), Diabetes group (squares) and Diabetes + *Stellaria media* group (triangles). Results are means ± SEM ($n = 8–10$), analyzed by repeated measures two-way ANOVA or one-way ANOVA with Holm-Sidak *post hoc* test,*$p < 0.05$ Diabetes vs. Control.

was given to the animals after STZ injection. The diet of 10 animals receiving fructose-enriched chow was further supplemented with *Stellaria media* tea administered by oral gavage (Diabetes + *Stellaria media*) in order to examine the potential effects of *Stellaria media* tea on the glucose homeostasis and cardiac function, while the other rats received equal amount of distilled water. One animal in the Control group and two animals in the Diabetes group were excluded due to technical problems occurring during the treatments.

Fasting blood glucose level measurements were performed every 4 weeks, accompanied by oral glucose tolerance (OGTT) tests at weeks 12, 16 and 19 (Fig. 1A). On the 20th week, rats were anaesthetised with sodium pentobarbital (Euthasol, 50 mg/kg body weight, ip., Produlab Pharma b.v., Raamsdonksveer, The Netherlands), the abdominal cavity was opened, and blood samples were taken from the abdominal aorta. Collected blood was allowed to clot and was centrifuged (2000 g, 20 min, 4 °C), then serum was separated for analysis of various serum parameters. Then the rats were given 500 U kg^{-1} heparin intravenously into the *vena cava inferior*. Isolated hearts were subjected to working heart perfusion according to Neely in order to evaluate cardiac function.

2.3. Preparation of Stellaria media tea

Stellaria media was harvested in Algyő (Hungary) by 'Ezerjófű' Association in 2017. Voucher specimen (No: 882) was deposited in Herbarium of the University of Szeged, Faculty of Pharmacy, Department of Pharmacognosy. The drug was dried and stored at room temperature. The dried and grounded drug was extracted with boiling water (1:10 w/v ratio) for 15 min by ultrasonication. The highly dense extract was separated from solid particles by mechanical press. The aqueous extract was dry-freezed. 1.5 g of dark-green lyophilized powder was equal to 10 g of dried drug. The lyophilized powder was dissolved in distilled water to achieve a final concentration of 100 mg/ml. The extract and the vehicle (distilled water) were then stored at 4 °C and were brought to room temperature before administration.

2.4. Stellaria media administration

Rats in the Diabetes + *Stellaria media* group received 100 mg/kg body weight *Stellaria media* tea with traditional oral gavage technique once a day since the onset of the experiment. The Control and Diabetes groups received equal amount of distilled water. The treatment was carried out at the same 2-h time range every day to minimalize the possible influence of circadian rhythm. The 100 mg/kg body weight dose of *Stellaria media* tea was considered as equal to the recommended human daily dose, that is 2 cups of tea, prepared with 3 g dried herb per cup, calculated according to Nair and Jacob considering the differences in body surface area.[18]

2.5. Working heart perfusion

Cardiac performance and function was assessed in isolated Neely working hearts, as described earlier.[19,20] Cardiac functional parameters including 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. Cardiac work was calculated by multiplying cardiac output and maximum pressure. For more information, see Supplementary Material.

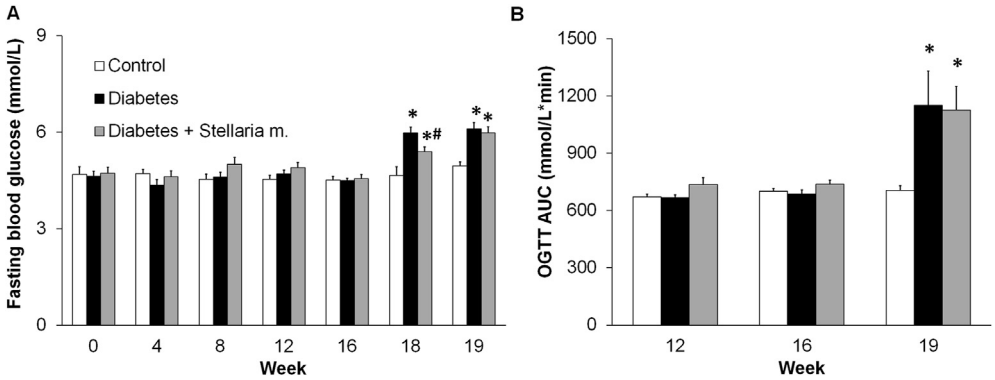

Fig. 2. Fasting blood glucose levels (A) and area under the curve (AUC) (B) values of oral glucose tolerance tests (OGTT) measurements. Results are means ± SEM (n = 8–10), analyzed by one-way ANOVA followed by Holm-Sidak *post hoc* test,*p < 0.05 vs. Control, #p < 0.05 vs. Diabetes.

2.6. Measurements of serum parameters

Several serum parameters describing pancreas, liver and kidney function, cardiac markers, lipid panel and electrolytes were analyzed by Roche Cobas 8000 Analyzer System using enzymatic colorimetric assays from Roche (Mannheim, Germany). For further details, please see Supplementary Material. Haemoglobin A1c (HbA1c) levels were analyzed by DCA Vantage Analyzer System (Siemens) provided by Diagnosticum Ltd. (Budapest, Hungary).

2.7. Measurement of serum insulin levels

Serum insulin levels were determined by enzyme-linked immunosorbent assay (ELISA) technique (Mercodia, Ultrasensitive Rat Insulin ELISA) according to the instructions of the manufacturer.[21]

2.8. Total flavonoid content and screening for flavonoids

The total flavonoid content was determined as quercetin equivalent (QE) using the aluminum chloride colorimetric method. The lyophilizate powder was dissolved in methanol to get a solution with a concentration of 1 mg/mL. For calibration curve 5, 10, 25, 50 μg/mL methanolic solutions of quercetin were prepared. As reagent, 2% aluminum chloride methanolic solution was used. Reaction mixtures were prepared by mixing 2 mL of solution and 2 mL of aluminum chloride, respectively. After 60 min of incubation at room temperature, the absorbance was measured against blank by applying UV-VIS spectrophotometer (Helios β ThermoSpectronic) at 420 nm. The total flavonoid concentration was calculated using a calibration plot ($R^2 = 0.9999$). The measurements were carried out in triplicate.

The flavonoid content of the lyophilized *Stellaria media* aqueous extract was screened by ultra-performance liquid chromatography (UPLC). For this experiment 1 mg/mL methanolic solution was prepared from the lyophilizate. The presence of ubiquitous flavonoids in plants, namely apigenin, apigenin-7-glucoside, kaempferol, luteolin, quercetin, and rutin was screened by UPLC. Experiments were carried out on a Shimadzu Nexera X2 UHPLC liquid chromatography system. For more information, see Supplementary Material.

2.9. Liquid chromatography-tandem mass spectrometry analysis

In order to gain more information about the chemical composition, the aqueous extract was filtered through a 0.22 μm membrane filter and diluted tenfold with 0.1% formic acid and analyzed by data-dependent liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using a Waters MClass nanoUPLC system online coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer. MS2 data were subjected to a spectral library search against the MzCloud database using the Compound Discovery software. For more information, see Supplementary Material.

2.10. Measurement of the antioxidant activity

The measurement of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging activity was carried out according to the method of Fukumoto et al. with some modifications.[22] The Oxygen Radical Absorbance Capacity (ORAC) assay was carried out according to the method of Mielnik et al.[23] For further details, please see Supplementary Material.

2.11. Western blot analysis

Phosphorylation of key proteins of well-known cardioprotective signaling pathways, signal transducer and activator of transcription 3 (STAT3), protein kinase B (Akt) and extracellular signal-regulated kinase (Erk) proteins and activation of apoptosis-related Bax and Bcl-XL proteins and GLUT4 glucose transporter were detected by Western blot technique as described earlier.[24] For more information, see Supplementary Material.

2.12. Statistical analysis

All values are presented as mean ± SEM. Repeated measures two-way ANOVA was applied for analysis of time-dependent 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 minimalize the effect of extremities.

3. Results

3.1. Time course of body weight, fasting blood glucose and OGTT values

Body weight was measured every 3 days in order to calculate *Stellaria media* tea dosage and to monitor weight gain. It showed a continuous increase from 356 ± 7 g at the onset of the experiment to 482 ± 16 g at week 20 in the Control group fed with standard

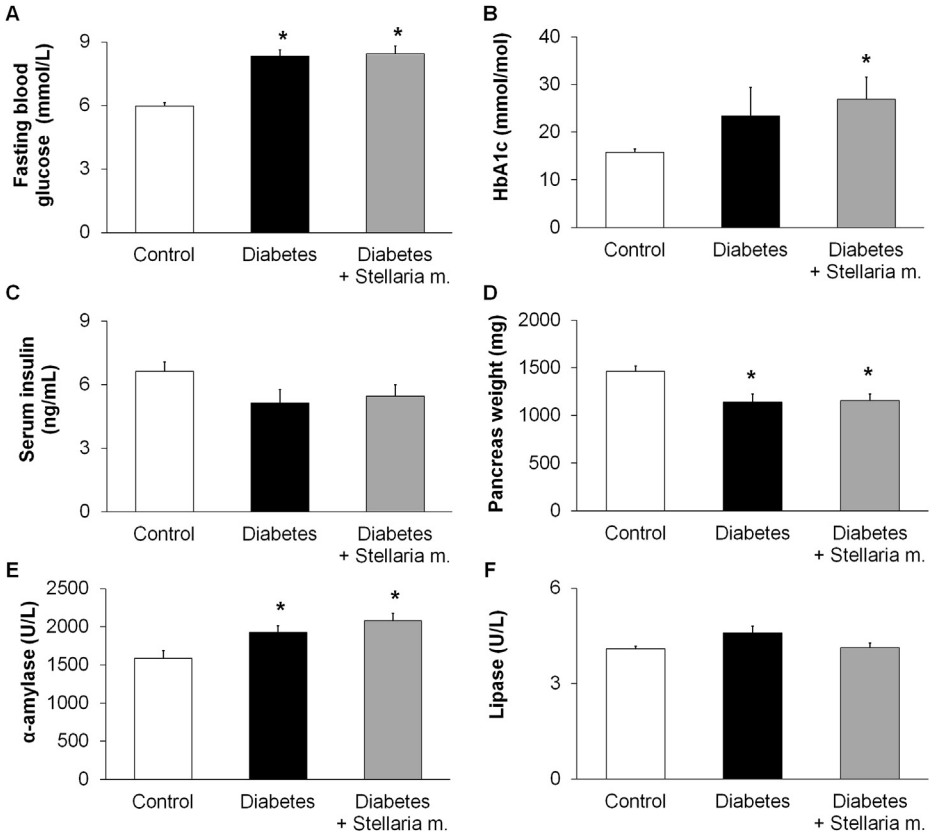

Fig. 3. Parameters representing pancreatic function at week 20: fasting blood glucose at termination (A), HbA1c levels (B), serum insulin levels (C), pancreas weight (D), enzyme activities of α-amylase (E) and lipase (F). Results are means ± SEM (n = 8–10 except for serum insulin measurement where $n = 6–8$), analyzed by one-way ANOVA followed by Holm-Sidak *post hoc* test,*$p < 0.05$ vs. Control.

laboratory rat chow (Fig. 1B). Compared to the controls, fructose-enriched diet plus STZ injection reduced body weight increase in the last 6 weeks in the Diabetes group, however, this effect was not significant in the Diabetes + *Stellaria media* group (Fig. 1B). Weight gain during the feeding protocol was significantly lower in the Diabetes group compared to the Control group (Fig. 1C). Similarly, there was a tendency of decrease in the Diabetes + *Stellaria media* group compared to the controls; however, it did not reach the level of statistical significance (Fig. 1C).

Fasting blood glucose levels were measured every 4 weeks since the onset of the experiment, accompanied by OGTT at weeks 12, 16 and 19. In fasting blood glucose levels and glucose tolerance, there was no significant difference among the three groups until week 16 (Fig. 2A and B). On the 17th week, the rats in the Diabetes and Diabetes + *Stellaria media* groups were injected with a low dose of STZ injection (20 mg/kg body weight, ip.), while the Control group was treated with equal amount of vehicle (citrate buffer). Following the STZ injection a significant elevation in fasting blood glucose levels could be observed in the Diabetes group compared to the Control group, achieving an experimental diabetes state (Fig. 2A). Although on week 18 the blood glucose elevation in the Diabetes + *Stellaria media* group was significantly lower compared to the Diabetes group, this difference was faded away by week 19 (Fig. 2A). According to our OGTT results, there was a significant elevation in the area under the curve (AUC) levels in the Diabetes group compared to the Control group on week 19 (Fig. 2B) representing an impairment of glucose tolerance. *Stellaria media* treatment did not affect glucose intolerance (Fig. 2B).

Table 1
Serum parameters representing liver and kidney function, cardiac markers, lipid panel and electrolytes.

	Control	Diabetes	Diabetes + *Stellaria m.*	*p*
Liver function:				
ALAT (U/L)	34.7 ± 2.0	23.9 ± 2.2*	29.1 ± 2.5	<0.05
ASAT (U/L)	65.1 ± 2.5	50.4 ± 1.8*	55.6 ± 1.9*	<0.05
ALP (U/L)	46.9 ± 1.9	86.5 ± 12.7*	101.4 ± 10.4*	<0.05
Albumin (g/L)	40.8 ± 0.8	41.8 ± 0.6	42.0 ± 0.4	ns
Total protein (g/L)	54.0 ± 1.5	55.9 ± 0.4	57.3 ± 0.7*	<0.05
Kidney function:				
Urea (mmol/L)	6.3 ± 0.3	3.8 ± 0.7*	3.8 ± 0.3*	<0.05
Creatinine (μmol/L)	32.1 ± 1.3	35.3 ± 1.8	35.2 ± 1.6	ns
Cardiac markers:				
CK (U/L)	276.9 ± 25.7	235.1 ± 19.4	289.7 ± 29.8	ns
CK-MB (U/L)	438.6 ± 29.1	433.8 ± 37.9	524.8 ± 55.6	ns
LDH (U/L)	281.7 ± 26.0	298.4 ± 23.3	359.3 ± 42.9	ns
Lipid panel:				
Cholesterol (mmol/L)	1.58 ± 0.05	1.74 ± 0.16	1.78 ± 0.11	ns
HDL-Cholesterol (mmol/L)	0.95 ± 0.04	1.03 ± 0.08	1.08 ± 0.10	ns
Electrolytes:				
Sodium (mmol/L)	142.1 ± 0.9	141.5 ± 0.9	140.2 ± 1.1	ns
Potassium (mmol/L)	4.8 ± 0.1	5.1 ± 0.1	5.1 ± 0.2	ns
Chloride (mmol/L)	101.9 ± 0.5	101.3 ± 0.8	101.0 ± 0.5	ns

Results are means ± SEM (n = 8–10), analyzed by one-way ANOVA followed by Holm-Sidak *post hoc* test,*$p < 0.05$ vs. Control. ALP alkaline phosphatase, ALAT alanine aminotransferase, ASAT aspartate aminotransferase, CK creatine kinase, CK-MB creatine kinase – myocardial band, HDL high-density lipoprotein cholesterol, LDH lactate dehydrogenase, *ns* non-significant.

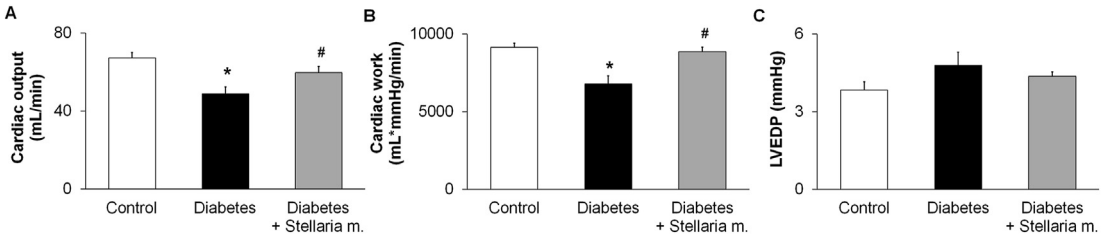

Fig. 4. Cardiac function in isolated hearts subjected to working perfusion according to Neely: cardiac output (A), cardiac work (B), left ventricular end diastolic pressure (LVEDP) (C). Results are means ± SEM (n = 8–10), analyzed by one-way ANOVA followed by Holm-Sidak *post hoc* test,*p < 0.05 vs. Control, #p < 0.05 vs. Diabetes.

Table 2
Parameters measured by working heart perfusion according to Neely.

	Control	Diabetes	Diabetes + *Stellaria m.*	*p*
Aortic flow (mL)	44.4 ± 2.6	27.3 ± 2.4*	34.4 ± 2.3*	p < 0.05
Coronary flow (mL)	22.8 ± 0.7	21.8 ± 1.5	23.7 ± 1.0	ns
Max dp/dt (mmHg/s)	6323 ± 282	6260 ± 439	6431 ± 487	ns
Min dp/dt (mmHg/s)	− 4520 ± 188	− 4512 ± 397	− 4496 ± 374	ns
Aortic diastolic pressure (mmHg)	37.6 ± 0.5	37.9 ± 0.8	37.8 ± 1.1	ns
Aortic systolic pressure (mmHg)	114.8 ± 2.5	110.4 ± 3.3	114.9 ± 3.1	ns
LVDP (mmHg)	136.2 ± 4.6	130.0 ± 4.5	131.3 ± 5.2	ns
Heart rate (1/min)	240 ± 10	211 ± 16	231 ± 11	ns

Results are means ± SEM (n = 8–10), analyzed by one-way ANOVA with Holm-Sidak *post hoc* test,*p < 0.05 vs. Control. LVDP left ventricular developed pressure, *ns* non-significant.

3.2. Parameters reflecting endocrine and exocrine function of the pancreas

At the end of the 20-week experiment, elevated fasting blood glucose, non-significantly increased HbA1c (3.6 ± 0.1% in Control and 4.3 ± 0.5% in Diabetes, respectively) and serum insulin levels decreased by approximately 20% indicated impaired endocrine pancreatic function (Fig. 3A–C) in the Diabetes group. *Stellaria media* tea failed to improve these parameters (Fig. 3A–C), and HbA1c was 4.6 ± 0.4% in this group. Pancreas weight was significantly lower in the Diabetes group compared to the Control group (Fig. 3D), showing that there might have been pancreatic damage due to the fructose-enriched diet plus STZ injection. *Stellaria media* did not affect this alteration. The serum activity of α-amylase was significantly elevated both in the Diabetes and Diabetes + *Stellaria media* groups (Fig. 3E), while there was no difference among the three groups in the activity of lipase enzyme (Fig. 3F).

3.3. Serum parameters of liver and kidney function, cardiac markers, lipid panel and electrolytes

At termination of the animals, blood samples were collected from the abdominal aorta and several serum parameters were measured. Markers of liver function, such as alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and alkaline phosphatase (ALP) enzyme activities, as well as albumin and total protein concentrations were measured. Diabetes caused a significant decrease in ALAT and ASAT enzyme activities and an elevation in ALP enzyme activity without affecting albumin or total protein concentration indicating lack of considerable liver damage (Table 1). Serum parameters describing kidney function were also measured. Urea levels were significantly lower in the Diabetes group compared to the Control group (Table 1). There was no difference in the creatinine levels. *Stellaria media* tea did not affect liver and kidney function compared to the Diabetes group (Table 1). There was no significant difference among the three groups in creatine kinase (CK), Creatine kinase – myocardial band (CK-MB) and lactate dehydrogenase (LDH) activities suggesting the lack of significant cellular injury in the heart and other tissues (Table 1). Cholesterol, high-density lipoprotein (HDL) cholesterol, as well as relevant electrolytes, such as sodium, potassium and chloride levels were not changed significantly among the groups (Table 1).

3.4. Ex vivo working heart perfusion

Cardiac dysfunction is a frequent consequence of diabetes, therefore we assessed cardiac performance in hearts subjected to working heart perfusion. Aortic flow, cardiac output and cardiac work, reflecting systolic heart function, were significantly impaired in the Diabetes group compared to the Control group (Fig. 4A and B, Table 2), indicating diabetic adverse effects on the heart. *Stellaria media* treatment significantly improved cardiac output and cardiac work suggesting that *Stellaria media* tea may have beneficial effects on the heart in a diabetic state (Fig. 4A and B). The diastolic function of the hearts was assessed by measurements of LVEDP, which inversely correlates with the function. LVEDP showed a tendency of elevation in the Diabetes group compared to the Control group, however, *Stellaria media* had no prominent effect on this parameter (Fig. 4C). There were no significant alterations in the other analyzed cardiac functional parameters among the three experimental groups (Table 2).

3.5. Total flavonoid content, LC-MS/MS analysis and antioxidant activity of Stellaria media tea

Flavonoids are active compounds in several medicinal herbs and are related to anti-oxidative properties, therefore we analyzed the flavonoid content of the *Stellaria media* tea. The total flavonoid content determined by means of UV-VIS absorbance was 9.88 ± 0.10 mg quercetin equivalent/gram (QE/g). The flavonoid screening by means of UPLC indicated rutin being a possible component, based on comparison of its retention time and UV spectrum with a reference standard.

In the LC-MS/MS analysis, several components have been

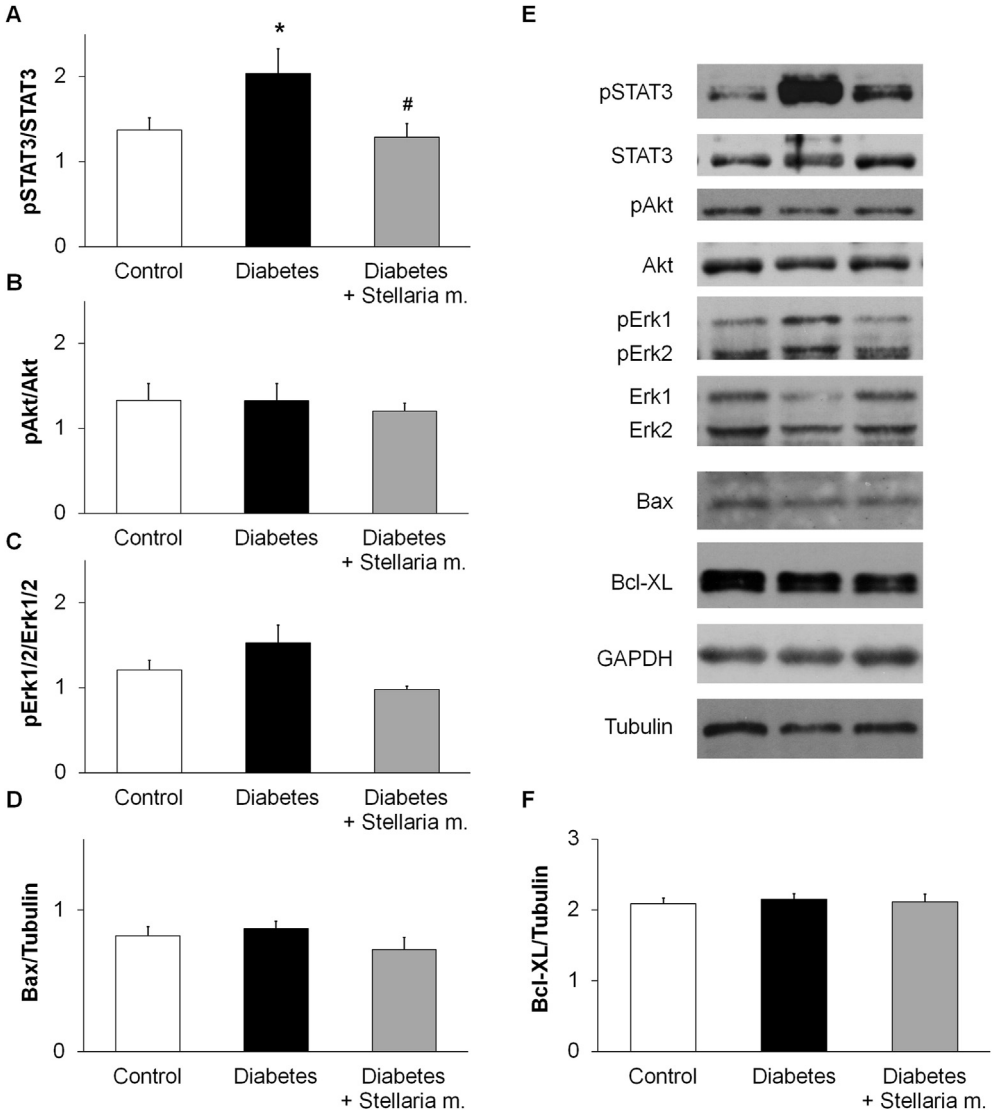

Fig. 5. Western blot analysis of phosphorylation of proteins: signal transducer and activator of transcription 3 (STAT3) (A), protein kinase B (Akt) (B), extracellular signal-regulated kinase (Erk) (C), proapoptotic Bax (D), representative bands (E), antiapoptotic Bcl-XL (F). Results are means ± SEM (n = 7), analyzed by one-way ANOVA followed by Fisher LSD *post hoc* test,*$p < 0.05$ vs. Control, #$p < 0.05$ vs. Diabetes.

detected. These comparisons revealed altogether five possible components out of the ten most intense sample components, which appeared to be various glycosylated apigenin-derivatives. MS2 spectrum of *m*/*z* 595.1660 displayed reasonable resemblance (MzCloud best match: 62.9) to MzCloud spectral library entries to two isomeric compounds, 6-arabinosyl-8-galactosylapigenin (corymboside) and 6-β-D-glucopyranosyl-8-β-D-ribopyranosyla pigenin (schaftoside), while MS2 spectrum of *m*/*z* 595.1660 showed good agreement (MzCloud best match: 82.8) with apigenin-6,8-di-*C*-glucoside (vicenin). Two further components, *m*/*z* 757.2195 and 933.2657 showed similarity to MS2 data acquired on 2″-*O*-α-L-rhamnopyranosyl-isovitexin, a compound with the same apigenin base structure. The molecular mass of these two components were 178.1 and 354.1 Da higher compared to the database entry. Six further components (*m*/*z* 274.2737, 535.1448, 679.2974, 677.2817, 611.1608 and 381.0793) did not show any resemblance to MzCloud entries indicating that these components were not included with an MS2 spectrum in the spectral library.

Moreover, the lyophilized powder was evaluated for antioxidant activity using DPPH and ORAC assays. The lyophilized aqueous extract of *Stellaria media* exerted low direct antioxidant capacity in both assays: EC_{50} 168.30 ± 11.06 μg/L in the DPPH assay and 0.97 ± 0.16 Trolox Equivalent mmol/g in the ORAC study.

3.6. Cardiac signaling pathways

Activation of STAT3 is proposed to play a role in diabetes-induced cardiac dysfunction. The phosphorylation of STAT3 was significantly elevated in the Diabetes group which was attenuated by *Stellaria media* treatment (Fig. 5A), suggesting an association with the beneficial cardiac effect of *Stellaria media*. There were no significant differences among the three groups in the phosphorylation of Akt and Erk proteins (Fig. 5B and C). Since increased cardiac apoptosis represents greater risk for the development of diabetic cardiomyopathy,[25–27] we examined apoptosis related proteins in our study. Diabetes and *Stellaria media* treatment had no effects on the proapoptotic Bax and antiapoptotic Bcl-XL proteins (Fig. 5D and F). We also investigated GLUT4 transporter expression

as GLUT4-mediated glucose uptake may play a relevant role in diabetes. Diabetes and *Stellaria media* treatment did not influence GLUT4 protein expression (data not shown).

4. Discussion

In the present study, we tested the effects of *Stellaria media* tea on the severity of diabetes as well as on diabetes-induced cardiac consequences in an *in vivo* rat model. Based on our findings, *Stellaria media* tea appears to have beneficial effects on cardiac dysfunction induced by diabetes, since the treatment ameliorated the impaired cardiac output and cardiac work. However, this effect seems to be independent of the modulation of diabetes severity as the application of the tea treatment did not influence fasting hyperglycaemia or glucose intolerance.

In folk medicine *Stellaria media* is consumed mainly as tea. To the best of our knowledge, this is the first study investigating the hot water extract of chickweed in the settings of diabetes. In our study, *Stellaria media* was not effective in lowering blood glucose level or improving glucose tolerance in diabetic rats. In the literature only one other study examined specifically the effect of *Stellaria media* on the severity of diabetes. Ethanolic leaf extract of *Stellaria media* in a dose of 100–400 mg/kg/day administered by intraperitoneal injection has been shown to attenuate hyperglycaemia in a 21-day alloxan-induced diabetic rat model.[13] In that study, *Stellaria media* treatment attenuated fasting blood glucose levels, decreased haemoglobin A1c levels and inhibited pancreatic α-amylase and β-glucosidase enzyme activities.[13] There are also some studies using various models where glucose homeostasis was estimated among other metabolic parameters. For instance, ethanolic radix extract of another member of the *Stellaria* genus (*Stellaria dichotoma*) improved glucose homeostasis in high-fat-diet fed mice.[28] In contrast, Chidrawar et al. found that ethanolic extract of *Stellaria media* was ineffective to decrease hyperglycaemia in both cafeteria-diet- and progesterone-induced obesity models.[29,30] However, they also found that 200 and 400 mg/kg methanolic extract significantly attenuated serum glucose levels in these models.[29,30] These controversial results may be explained by the differences in (i) the composition of the extracts, (ii) delivery time, administration and dose of *Stellaria media,* (iii) the applied animal models and strains. It should be also 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, showing that this experimental diabetes model has some adverse effects on the heart. *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. To the best of our knowledge, in the literature currently there is no other experimental data concerning the effects of *Stellaria media* on cardiac function or cardiomyopathy. Our research group tested the effects of *Stellaria media* tea lyophilizate in another chronic metabolic disease, i.e. hypercholesterolaemia, where we demonstrated that the treatment has no blood cholesterol lowering effect in diet-induced hypercholesterolaemia in rats.[31] In the same study, we investigated some safety issues of the *Stellaria media* treatment on the heart. Our transthoracic echocardiographic measurements showed that *Stellaria media* treatment did not affect cardiac morphology and parameters related to cardiac function in diet-induced hypercholesterolaemia.[31]

Various mechanisms may play a role in the development of cardiac dysfunction in diabetes, e.g. oxidative stress, diffuse apoptosis of cardiomyocytes, dysregulation of cardiac signaling pathways, mitochondrial dysfunction, fibrosis or hypertrophy.[26,32] Interestingly, there are more than 50 medicinal herbs, such as sesame,[33] which have beneficial effects on experimental diabetic cardiomyopathy (for review please see Refs. 32,34). These plants have been suggested to exert antioxidant properties that may attenuate oxidative stress or inflammation, and to reduce apoptosis, and cardiac remodelling.[32] In the literature, there are some experimental data suggesting similar antioxidant and anti-inflammatory properties of *Stellaria media*, which can be associated with the improvement of certain cardiac parameters in our present study.[35] Therefore, we tested the *in vitro* antioxidant capacity of *Stellaria media* tea, and we found a low antioxidant activity. This phenomenon was observed by another study too, where the antioxidant activity of the aqueous and methanolic extract was weak.[36] *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.[35] We determined the total flavonoid content of *Stellaria media* tea and it was 9.88 ± 0.10 mg QE/g. The flavonoid content of *Stellaria media* has been discussed by several papers.[36–38] In a phytochemical study, the flavonoid content was determined not less than 1.2% in raw plant material.[38] In an experiment, the total flavonoid content (determined by HPLC) of a lyophilized juice was 25.6 mg/g, and in an ethanolic extract 63.9 mg/g[36]. Our extract was prepared with hot water (in accordance with the human use) and this might explain the lower flavonoid content. UPLC 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 proposed. Some studies demonstrated that rutin alleviates diabetic cardiomyopathy and improve left ventricular dysfunction in STZ-induced diabetes[39–42] and in high-carbohydrate, high-fat diet models.[43] Rutin was also shown to exert neuroprotective effects which may be beneficial in diabetes as well.[44] LC-MS/MS analysis of the extract we used in our study afforded identification of various glycosylated apigenin-derivatives, which finding is in accordance with literature data.[36] 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,[45] 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[46] and to exert protective effects against cardiac dysfunction in myocardial infarction in diabetic rats,[47] suggesting that apigenin-derivatives may contribute to the beneficial cardiac effects of *Stellaria media* tea.

The limited antioxidant capacity of the water extract raised the question whether signaling pathways are involved in the cardiac effects. Based on literature data, STAT3 is proposed as a key mediator of diabetes-induced cardiac dysfunction.[48–50] In conjunction with other studies[51–53] (for specific review please see Ref. 54), we showed that diabetes increased cardiac STAT3 phosphorylation. Moreover, *Stellaria media* tea treatment prevented diabetes-induced cardiac STAT3 dysregulation, suggesting a protective role. Indeed, some studies demonstrated that attenuation of the enhanced cardiac STAT3 activation may be beneficial in diabetes. In a high-glucose, high-fat diet and STZ injection-induced diabetic rat model, losartan attenuated the cardiac STAT3 phosphorylation and improved heart function.[53] In STZ-induced diabetic mice hearts, inhibition of diabetes-induced activation of EGFR-STAT3 signaling was associated with restoring cardiac fibrosis and hypertrophy related factors, and prevented diabetes-induced cardiac dysfunction.[50] Interestingly, the possible presence of rutin in *Stellaria media*

tea may be a feasible explanation for the observed effects in our study, as rutin was suggested to exert cytoprotection by inhibiting STAT3 phosphorylation.[55,56] Similarly, apigenin and its derivatives has been shown to attenuate STAT3 activation in tumor cells,[57,58] which is in agreement with our present findings observed in cardiac tissue and may be related to the beneficial effect of *Stellaria media* tea on diabetic cardiomyopathy.

Nevertheless, a better understanding of the molecular mechanism underlying the proposed beneficial cardiac effects of *Stellaria media* tea would require additional research in the future. 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. Testing the effects of the tea in female rats could reveal sex differences in the effectiveness of the extract. It would be worth studying the effects of *Stellaria media* tea not only in a rat model, but in humans as well whether we can observe functional cardiac improvement in diabetic patients who drink chickweed tea regularly.

Although we provide interesting data on the effects of *Stellaria media* in diabetes, as always, there may be some possible limitations of our present study. The animals received *Stellaria media* tea from the onset of the experiments, together with the fructose-enriched diet. It would be worthwhile testing its effects in a developed diabetic state, which may have greater clinical relevance. Moreover, elucidation of the causal relation between the tea's beneficial effect and STAT3 phosphorylation with state-of-the-art experiments or further analysis of downstream targets in diabetic cardiomyopathy would be straightforward in the future. We tested the effects in a rat model, which might differ from the human metabolism. Moreover, the lack of a group receiving only herbal treatment without fructose-enriched diet can also be considered as a possible limitation.

In conclusion, the tea made of *Stellaria media* (i.e. common chickweed) may protect against diabetes-induced cardiac dysfunction; however, this effect seems to be independent of the modulation of fasting hyperglycemia or glucose tolerance in rats. *Stellaria media* prevented diabetes induced STAT3 phosphorylation in the heart, which may play a role in the beneficial cardiac effect. Nevertheless, further studies are needed to reveal the exact molecular mechanisms underlying the proposed cardioprotective effect of *Stellaria media* in diabetic conditions.

Declaration of competing interest

None.

Acknowledgements

We would like to thank Dr. Márta Sárközy and Enikő Páger for their help in the *Stellaria media* administration, Ágota Berek and Tibor Kiss for providing the plant material.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2021.08.003.

Funding

This work was supported by the Economic Development and Innovation Operative Programme [GINOP-2.3.2-15-2016-00012 and GINOP-2.3.2-15-2016-00006] and EFOP-3.6.2-16-2017-00006, and by grants from the National Research, Development and Innovation Office [K115990, K115796 and PD128271] the Ministry of Human Capacities, Hungary [20391–3/2018/FEKUSTRAT] and the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. HCEMM has received funding from the EU's Horizon 2020 research and innovation program under grant agreement No. 739593. V.D., A.F. and F.D.G. were supported by the New National Excellence Program of the Ministry of Human Capacities, respectively [UNKP-19-3-SZTE-47, UNKP-20-2–SZTE-60, UNKP-20-2–SZTE-61].

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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

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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. Gáspár Renáta Dr. Halmi Dóra

Szeged, 2021.10.05.