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Medicinal Plants Used in the Treatment of Cardiovascular Disorders in the Czech Republic – Overview and Consumption Analysis in Years 2015 – 2019.

(Léčivé rostliny používané v terapii kardiovaskulárních onemocnění v České republice – přehled a analýza spotřeby v letech 2015 – 2019.)

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DECLARATION

I hereby declare that the work presented in this rigorous thesis is the result of my own investigations except where specifically stated in the text. I elaborated this work in the year 2020 with the use of the cited references.

Hradec Králové, 10.10.2020

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ABBREVIATION LIST

ABTS	2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACAT	acylCoA:cholesterol transferase
ACE	angiotensin converting enzyme
ADP	adenosine diphosphate
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AT1R	angiotensin type 1 receptor
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CO_2	carbon dioxide
CYP	cytochrome
DCFH	dichloro-dihydro-fluorescein
DOCA	deoxycorticosterone acetate
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDHF	endothelium-derived hyperpolarizing factor
EMA	European Medicines Agency
eNOS	endothelial nitric oxide synthase
FAS	fatty acid synthase
FDA	Food and Drug Agency
FMD	flow mediated dilatation
FRAP	ferric reducing antioxidant power
HDL	high-density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A reductase
HUA-EC(s)	human uterine arterial andothelial cell(s)

HUVEC(s)	human umbilical vein cell(s)
IC50	median inhibition concentration
ICAM-1	intercellular adhesion molecule-1
iNOS	inducible nitric oxide synthase
IP3	inosine triphosphate
LD50	median lethal dose
LDL	low-density lipoprotein
L-NAME	L-N$^{\omega}$-nitro-L-arginine methyl ester
LXRα	liver X receptor alpha
MMP	mitochondrial membrane potential
NADPH	nicotine amide dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
NrF2	nuclear factor-erythroid 2-related factor 2
NYHA	New York Heart Association
OTC	over the counter
PAF	platelet activating factor
PKA	protein kinase A
PPAR	peroxisome proliferator-activated factor
ROS	reactive oxygen species
Rx	prescription drug
SIRT 1	silent mating type information regulator 2 homolog 1
SREBP-1c	sterol regulatory element-binding protein 1c
SUKL	State Institute for Drug Control
TGF-β	transformation growth factor beta
TNF-α	tumor necrosis factor alpha
v/v	volume/volume

VCAM-1	vascular cell adhesion molecule-1
VLDL	very low-density lipoprotein
w/w	weight/weight

ABSTRACT

Medicinal plants still hold their strong position in the therapy of various human diseases, even in the modern era. Seven medicinal plants long known for their use in the treatment of cardiovascular disorders were chosen for this thesis, namely *Allium sativum* L. (garlic), *Crataegus* spp. (hawthorn), *Digitalis* spp. (foxglove), *Leonurus cardiaca* L. (motherwort), *Olea europaea* L. (olive), *Nigella sativa* L. (black cumin), and *Viscum album* L. (European mistletoe). Their beneficial effects on the cardiovascular system (antioxidant, cardioprotective, vasorelaxant, antiatherosclerotic, antihypertensive, and lipid-lowering actions) were reviewed with an emphasis on information concerning humans. When possible, the mechanism explanation, results of clinical trials, and established dosage range for the respective fields of action are presented.

The consumption analysis of the preparations containing the abovementioned medicinal plants was conducted with a total number of 33 over the counter herbal preparations and 2 prescription-restricted medicinal products. The products contain either the medicinal plant itself, its extract or active compound(s), and only those available on the Czech Republic pharmaceutical market were chosen for the consumption analysis. The years 2015 - 2019 were established as the reporting time period.

Data collected from participating pharmacies was categorized as big city (included pharmacies in cities with more than 50 thousand of inhabitants), small town (included pharmacies in towns under 50 thousand of inhabitants), and e-shop (pharmacies with home delivery regardless of the population size).

The majority of herbal preparations were represented by garlic herbal preparations, both in the number of the evaluated products and the consumption rate. High consumption rates were also observed for hawthorn and mistletoe preparations. The consumption of other medicinal plants preparations (motherwort, olive leaf, and black cumin) were remarkably lower than that of the aforementioned medicinal plants. The consumption of

garlic and hawthorn preparations showed a more or less stable trend with no significant fluctuations during the evaluated time period. Mistletoe and motherwort preparations exerted an increasing consumption trend, while olive leaf and black cumin preparations exhibited a decreasing consumption trend during the reporting time period.

The main issue with the OTCs is the lack of medicinal productss among them. Only two herbal teas are registered with SUKL, the remaining 31 preparations are categorized as food supplements. On the other hand, several of the food supplements were standardized for a certain amount of the active compounds and according to the available literature, some of these supplements may have a positive influence on human cardiovascular health.

The prescription-restricted medicinal preparations were represented by different-strenght digoxin tablets. While digoxin consumption during the reporting time period exerted a remarkable decreasing trend with the exception of 2018 in big city pharmacies, small town pharmacies exhibited a slower onset of the reduction in digoxin consumption and an increase in digoxin consumption in 2018. These differences were likely the consequence of two different impacts: the decrease in digoxin use in the heart failure treatment according to the newest guidelines that are applied more rapidly in big cities, and the outage of digoxin supplementation in 2018 due to the operational reasons of the producer.

ABSTRAKT

Léčivé rostliny jsou i v současné době nedílnou součástí terapie nejen lidských nemocí. Pro tuto práci bylo vybráno sedm léčivých rostlin s dlouhou tradicí léčby různých kardiovaskulárních onemocnění. Jedná se konkrétně o tyto rostliny: *Allium sativum* L. (česnek kuchyňský), *Crataegus* spp. (hloh), *Digitalis* spp. (náprstník), *Leonurus cardiaca* L. (srdečník obecný), *Olea europaea* L. (olivovník evropský), *Nigella sativa* L. (černucha setá) a *Viscum album* L. (jmelí bílé). Jejich příznivý účinek na kardiovaskulární systém (antioxidační, kardioprotektivní, vasorelaxační, antiaterosklerotické, antihypertenzivní, hypolipidemické) byl podrobně popsán s důrazem především na informace týkající se lidí. Pro jednotlivé oblasti byl popsán i mechanizmus účinku, výsledky klinických testů a doporučené dávkování, pokud byly tyto informace k dispozici.

Pro přípravky z vyjmenovaných léčivých rostlin byla provedena analýza spotřeby. Celkem bylo zahrnuto 33 volně prodejných přípravků a 2 přípravky vázané na recept. Tyto přípravky obsahují buď přímo rostlinnou drogu, nebo jednotlivé účinné látky nebo jejich kombinace. Pro tuto analýzu byly vybrány výhradně přípravky dostupné na farmaceutickém trhu v České republice. Rozhodné období pro pro hodnocení byly roky 2015 – 2019.

Data získaná ze zapojených lékáren byla rozdělena do třech kategorií: velká města (více než 50 tisíc obyvatel), malá města (méně než 50 tisíc obyvatel) a e-shop (lékárny doručující na domácí adresu bez ohledu na počet obyvtel daného města).

Přípravky s obsahem česneku představují největší objem v počtu prodaných balení. Současně mají největší spotřebu během celého hodnoceného období. Poměrně vysoká spotřeba byla zjištěna i u přípravků obsahujících hloh a jmelí. Spotřeba přípravků s obsahem ostatních hodnocených léčivých rostlin (srdečník, olivovník a černucha) byla výrazně nižší. Křivka spotřeby česneku a hlohu byla více-méně stabilní a nevykazovala žádné výrazné výkyvy. Spotřeba přípravků s obsahem srdečníku a jmelí v daném období rostla, zatímco spotřeba přípravků s obsahem olivovníku a černuchy klesala.

Největším problémem volně prodejných přípravků je nedostatek léčivých přípravků v této skupině. Pouze dva z nich mají registrační číslo SÚKLu a spadají do kategorie léčivý přípravek, zbylých 31 je v kategorii doplněk stravy. I přesto je významné procento přípravků standardizováno na určitý obsah účinných látek a podle dostupné literatury některé z nich by mohly vykazovat pozitivní vliv na lidský kardiovaskulární systém.

Léčivé přípravky vázané na recept, které byly zahrnuty do analýzy, představují tablety s digoxinem v různých sílách. Zatímco spotřeba digoxinu ve velkých městech během sledovaného období plynule klesala s výjimkou roku 2018, malá města vykazovala pomalejší nástup poklesu ve spotřebě a zvýšení této spotřeby v roce 2018. Příčina rozdílu je pravděpodobně dána poklesem v preskripci dle nejnovějších doporučení pro léčbu srdečního selhání a rychlejším uváděním nových postupů do praxe ve větších městech. Dále hrál roli i výpadek v distribuci digoxinu na trhu ve zmiňovaném roce 2018.

1. PREFACE

Since the rise of the first human civilization, medicinal plants have been widely used for the treatment of all the known diseases. For thousands of years people exploited a large variety of herbal species occurring around the world. From Eber's papyrus, through Dioscorides *De materia medica*, up to modern pharmacopoeias, herbal drugs and preparations have accompanied people on their journey to regain and keep their health.

Even now, in the age of advanced technologies, specialized medical care and an immense amount of various chemical drugs, medicinal plants firmly hold their significant position in human therapy. Current investigations in the field of phytochemistry and the therapeutic potential of medicinal plants are very intensive and numerous scientific papers dealing with these topics have been submitted in the last years. Recent requirements of an evidence-based approach in human medicine are also reflected in the research, and many clinical trials evaluating the efficacy and safety profiles of several medicinal plants have been successfully conducted, offering valuable knowledge.

It is well known that for people in developing countries medicinal plants sometimes still represent the only accessible treatment for the majority of their ailments. However, people of the Western civilizations also often choose herbal preparations as their first therapeutic option due to various reasons, be it the availability of the product or their inner belief in the efficacy of traditional systems of medicine.

And finally, regardless of the extraordinary headway in chemical sciences and biotechnologies, not all chemicals can be synthetized *de novo* in a laboratory because of their structural complexity. A brilliant representative of such compounds is digoxin, a cardenolide naturally occurring in foxglove, that has for more than 200 years been used in cardiology and that will be focused on in following chapters.

In the presented thesis I focused on the medicinal plants used in the treatment of various cardiovascular diseases with the emphasis on

cardioprotection, heart failure, and hypertension. Hypertension is a global health issue, some 600 million people worldwide have high blood pressure and nearly 3 million die every year as a direct result. And yet, according to the World Health Organization, seven out of ten people are not receiving adequate treatment.

Furthermore, hypertension is a component of the metabolic syndrome, a multifactorial metabolic disbalance with a high prevalence among the adult population. Although the definition of the metabolic syndrome has changed several times during the past two decades, the conditions of this syndrome represent a useful tool for identifying subjects at high risk of cardiovascular diseases. As dyslipidemia is another important component of the metabolic syndrome, I also focused on the action of selected medicinal plants on the lipid profile.

The medicinal plants mentioned in this thesis were chosen according to the availability of the herbal preparations on the Czech pharmaceutical market, and the consumption analysis of these products in the years 2015 – 2019 is also presented in this work.

AIMS OF THE PRESENT RESEARCH

1. To present a comprehensive overview of medicinal plants contemporary used in the Czech Republic in the treatment of various conditions of the human cardiovascular system, with the emphasis on cardioprotection, heart failure, and hypertension.

2. To evaluate the consumption of over the counter herbal preparations (both food supplements and medicinal products) in the Czech Republic in the years 2015 – 2019.

   Solved questions:
   - Are there enough OTC medicinal products containing selected medicinal plants on the pharmaceutical market of the Czech Republic?
   - Are there any preferences in the consumption of herbal preparations regarding the plant species and/or pharmacy category?
   - Are the recommended dosages in accordance with the information in scientific papers?

3. To evaluate the consumption of the foxglove cardenolide digoxin in the Czech Republic in the years 2015 – 2019.

   Solved questions:
   - Does the consumption of digoxin change in the reporting time period?
   - Are the any differences in digoxin consumption between big city and small town pharmacies?

2. INTRODUCTION

2.1. GARLIC – *ALLIUM SATIVUM* L. – FAM. *AMARYLLIDACEAE*

2.1.1. CHARACTERIZATION OF *ALLIUM SATIVUM* L. – A PHARMACOGNOSTIC CONTEXT

The genus *Allium* comprises more than 900 species, making it one of the largest monocotyledonous genera. Most likely the genus *Allium* originated during the late Eocene (approximately 40 millions of years ago) from central Asia and underwent different geographical pathways. It was firstly placed in the family *Lilliaceae*, then *Alliaceae* and after the revision of the classification system it now belongs to the family *Amaryllidaceae*. Many economically important species are included such as onion, leek, and garlic (Li *et al.*, 2016; Huo *et al.*, 2019; Xie *et al.*, 2020 and references therein).

Garlic, *Allium sativum* L. (Fig. 2.1.), is a perennial plant although often grown as annual. It is grown asexually from cloves. The cloves in a bulb are covered by a whitish or pinkish tunic named papery coat. Leaves (four to twelve) are long, sword-shaped, and attached to an underground stem. Flowers are greenish-white or pinkish, borne in a dense, spherical cluster on a spike up to 25 cm long. Seeds are not usually produced in the wild but have been produced under laboratory conditions. They have a black coat and are half the size of onion seeds (Wichtl, 2004; Alam, Hoq and Uddin, 2016).

For medicinal use (**Allii sativi bulbi pulvis**) the bulbs or separated cloves of the species *Allium sativum* L. are usually cut into 5 mm thick flakes and immediately either dried at a temperature not exceeding 65 °C or freeze-dried and then powdered in order to preserve the alliinase-allicin complex and to prevent, through dehydration, any further reaction of the already present allicin (see Ch. 2.1.2.). According to the European Pharmacopoeia, garlic powder must not contain less than 0.45% of allicin calculated with the reference to the dried drug. The European Pharmacopoeia also inlcudes a monograph on garlic for use in homeopathic medicinal products (**Allium**

sativum ad praeparationes homeopathicas; ESCOP, 2003; Wichtl, 2004; European Pharmacopoeia 10.0).

Fig. 2.1. *Allium sativum* L. Left – bulb and cloves, right – flowers. From thegarlicfarm.co.uk and pixers.fr.

2.1.2. Phytochemistry of Garlic

The genus *Allium* is rich in both non-volatile and volatile compounds. Non-volatile compounds are represented by sapogenins, steroidal saponins, and flavonoids. The first furostanol saponin isolated from garlic bulbs was proto-eruboside B, its spirostanol analogue, eruboside B, was obtained by its enzymatic hydrolysis. Several other furostanol and spirostanol saponins have since been identified in garlic bulbs. Garlic flavonoids include apigenin, quercetin, nobiletin, rutin, allixin, tangeretin, and myricetin (Matsuura *et al.*, 1988; Lanzotti, 2006; EMA/HPMC/7686/2013).

The volatile compounds are probably the most important active constituents of garlic. They are represented by **organosulfur compounds**, mainly thiosulfinates. Thiosulfinates are very unstable compounds and give rise to further rearrangements leading to a wide variety of derived sulfur compounds such as thiosulfonates, di- and tri-sulfur compounds, and ajoenes. At temperatures about 100 °C poly-sulfur compounds are formed (Lanzotti,

2006; Jangan and Badole, 2014). Some of the important garlic organosulfur compounds are depicted in Fig. 2.2.

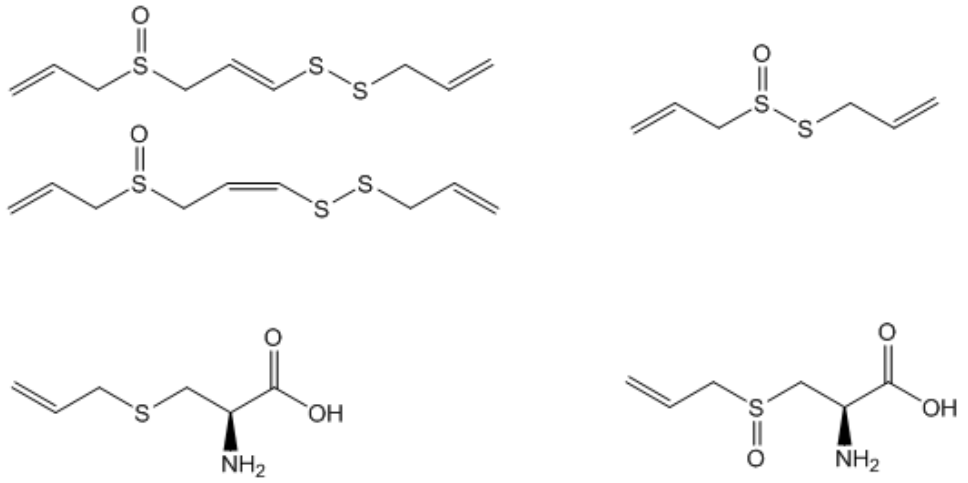

Fig. 2.2. Some of the *Allium sativum* L. active compounds. Upper left – ajoenes (*E* and *Z* isomeres), upper right – allicin, down left – S-allyl cysteine, down right – alliin. Courtesy of doc. Macáková.

The volatile compounds are responsible for both the pharmacological as well as the organoleptic properties of garlic. Their content is variable and it is strongly influenced by both pre- and post-harvest factors, such as genotype, irrigation, fertilization, and processing. Intact garlic has four major organosulfur compounds: S-allyl cysteine, alliin, S-methyl cysteine, and S-ethyl cysteine, although a total number of 85 sulfur containing compounds have been reported in garlic so far (Lanzotti, 2006; Martins, Petropoulous and Ferreira, 2016; Zeng *et al.*, 2017 and references therein; Abe, Hori and Myoda, 2020).

S-allyl cysteine is a water soluble compound, which has been identified as the major active compound in aged garlic. It has good bioavailability (more than 90%) and it is principally metabolized into the metabolite N-acetyl-S-allyl cysteine. **S-methyl cysteine** can also be found in aged garlic. Both S-methyl cysteine and **S-ethyl cysteine** are hydrophilic compounds (Amano *et al.*, 2015; Amano, Kazamori and Itoh, 2016; Kanamori *et al.*, 2020).

Alliin (S-allyl cysteine sulfoxide) is a hydrophilic and odorless non-proteinogenic amino acid and it represents the major odor precursor in garlic.

The biosynthesis of alliin takes place in the leaves, where alliin is accumulated at the earlier growth stages. Several biosynthetic pathways of alliin were proposed (involving two organosulfur compounds – either S-2-carboxypropyl glutathione or S-2-carboxypropyl cysteine) but the whole process still remains unclear. It is especially problematic determinig the origin of the allyl group and the double bond in alliin. Enzymes required for alliin biosynthesis are located partially in cytoplasm and partially in chloroplasts, it is therefore also unclear where the biosynthesis of alliin is located. After bulb formation is initiated, alliin is then translocated and stored in the bulbs (in cytoplasm or cytoplasmic vesicles), which represents approximately 85% of total alliin content. In the leaves remain about 12% of total alliin content. Application of sulfur during the growing season significantly increased the alliin content in bulbs (from 5.1 to 11.2 mg/g of dry weight), whereas high nitrogen rates had no significant effect (Bloem, Haneklaus and Schnug, 2010; Yamaguchi and Kumagai, 2019).

Allicin (diallyl thiosulfinate or S-(2-propenyl)-2-propene-1-sulfinothioate) is a bright yellow lipid-soluble sulfur-containing volatile compound. It is responsible for the pungent odor of fresh garlic and is considered to be the most biologically active organosulfur compound present in garlic. Allicin is formed when garlic cells are mechanically disrupted – by crushing or chopping of garlic cloves – from its precursor alliin and the enzyme alliinase (EC 4.4.1.4), which has optimum pH for activity 6.5. Alliin and alliinase are stored in different compartments (in cytoplasm and in vacuole, respectively) and if the cell is damaged they come into contact. Alliin is hydrolyzed by alliinase to allyl sulfenic acid, pyruvic acid, and ammonium. Two molecules of allyl sulfenic acid (a highly reactive intermediate) then condense spontaneously to form one molecule of allicin. Allicin represents about 70% of total thiosulfinates existing in the crushed garlic cloves. Allicin is not a very stable compound; it is further metabolized to form other compounds with the typically strong garlic odor, such as diallyl disulfide and diallyl trisulfide. It can be easily damaged by cooking and it is unstable even at room temperature. Its degradation products include ajoenes and vinyldithiins, which are more stable than allicin. Ajoenes are present as *(E)* and *(Z)* isomers, where *(E)*-ajoene is usually found in double amounts (Mazelis

and Crews, 1968; Borlinghaus *et al.*, 2014; Yamaguchi and Kumagai, 2019; El-Saber *et al.*, 2020 and references therein).

2.1.3. Medicinal Use of Garlic

Garlic is among the oldest of all cultivated plants. It has been used as a spice, food, and folklore medicine for over 4000 years, and is the most widely researched medicinal plant. As early as 3000 B.C., in ancient civilizations, including Egyptian, Phoenicians, Greek, Indian, Roman, Babylonian, Viking, and Chinese, garlic was used for the treatment of headache, heart conditions, arthritis, pulmonary complaints, respiratory infections, skin disease, wounds, symptoms of aging, diarrhea, bites, worms, abdominal growths (particularly uterine), ulcers, and tumors. Codex Ebers, an Egyptian medical papyrus dating to about 1550 B.C., includes 22 therapeutic formulations that mention garlic as an effective remedy for a variety of ailments (Block, 1985; Milner, 1996; Rahman, 2006).

According to EMA, garlic bulb as single herbal substance is authorized in Denmark, France, Germany, Hungary, Latvia, Poland, Spain, Sweden, and the United Kingdom. Several garlic preparations are present on the market: powder, liquid extraction preparation (extraction solvent refined rapeseed oil), and dry extract (extraction solvent ethanol 34%). Most of these preparations are marketed in Germany (EMA/HPMC/7686/2013). EMA lists two therapeutic indications of garlic – as an adjuvant for the prevention of atherosclerosis and the use for the relief of the symptoms of a cold (EMA/HPMC/7685/2013).

Lately, aged (or black) garlic preparations have become popular. Aged garlic is produced from fresh garlic bulbs under controlled high temperature and humidity. Changes in 38 compounds during the thermal processing of raw garlic were reported by Liang *et al.* (2015). Kodera *et al.* (2020) also focused on the changes in the chemistry of aged garlic and noted that the production mechanisms of several compounds identified in aged garlic have

yet to be elucidated. It is therefore to be expected that aged garlic can exert different biological properties from those of raw garlic.

Great attention is paid to the often problematic **standardization** of the active compounds content in garlic preparations, although the requirement for allicin content in garlic powder is clearly defined by the respective authorities. For example, Ghani (2010) determined the content of alliin and allicin in 7 garlic extracts (standard, Iraqi, Iranian, Lebanese, French, and Chinese), and the content varied significantly for both constituents. For alliin, the lowest content was in standard garlic (0.00025%), while Iraqi and Iranian garlic was alliin-rich (0.900% and 0.660%, respectively). The allicin content was again low in the standard garlic (0.00025%), while its highest values were detected in Iraqi and Lebanese garlic (1.290% and 0.330%, respectively). This particular problem was also noted by EMA in the assessment report on garlic: As very various extracts, quite often not standardized, are used in the experimental literature, it appears quasi impossible to generalize many of the results to a given herbal preparation (EMA/HPMC/7686/2013).

2.1.4. Effects of Garlic on Selected Cardiovascular Conditions

2.1.4.1. Antioxidant Effect of Garlic

Phelps and Harris (1993) reported a significant decrease (by 34%) in the *ex vivo* susceptibility of apolipoprotein B-containing lipoproteins to oxidation in healthy volunteers treated with garlic powder (600 mg) for 2 weeks. A similar trend of decreased susceptibility of lipoproteins to oxidation was also noted by Steiner and Lin (1998) who used aged garlic extract in moderately hypercholesterolemic men. Ahmadi *et al.* (2010) also reported a decrease in oxidized phospholipids in volunteers taking aged garlic extract for 12 months. Munday *et al.* (1999) found that *in vitro*, aged garlic extract has a stronger beneficial effect on the susceptibility of LDL particles to copper ions-mediated oxidation than raw garlic.

Dhawan and Jain (2004) investigated the effect of short-term garlic supplementation on the indices of oxidative stress in essential hypertensive patients. Twenty volunteers were administered with garlic pearls at a dose of 250 mg for 8 weeks. Garlic treatment led to a moderated increase of total antioxidant status and to a significant reduction in both urinary concentration of 8-iso-prostaglandin F2α (a biomarker of oxidative stress *in vivo*) and oxidized LDL cholesterol. In their subsequent study Dhawan and Jain (2005) further explored the antioxidant properties of garlic in hypertensive volunteers. Again, the dose of 250 mg of garlic pearls was used for 8 weeks. A significant reduction in lipid peroxidation and 8-hydroxy-2'-deoxyguanosine (a marker of oxidative stress-induced DNA damage) and a significant increase in total antioxidant status were observed at the end of the trial.

A remarkable decrease of lipid peroxidation products in volunteers with primary arterial hypertension taking 6 capsules of garlic preparations daily for 4 weeks was also reported by Duda, Sulibursкa and Pupek-Musialik (2008). The authors furthermore observed that treatment with garlic markedly increased vitamin E concentration in serum and led to a positive, although non-significant trend in increases in the levels of other antioxidant vitamins and glutathione peroxidase activity (Duda, Sulibursкa and Pupek-Musialik, 2008).

Aged garlic extract also exhibits a favorable antioxidant activity, partially due to S-allyl cysteine, the most abundant organosulfur compound present in the extract. The DPPH radical scavenging activity of S-allyl cysteine was higher than that of vitamin C and it furthermore showed good hydroxyl radical-scavenging properties. The antioxidant activity of S-allyl cysteine was concentration-dependent in both cases (Sun and Wang, 2016). S-allyl cysteine as well as other organosulfur compounds isolated from aged garlic were proven efficient in the inhibition of advanced glycation end-products (Wang, Sun and Chen, 2018). Intraperitoneal administration of either pure S-allyl cysteine (200 mg/kg) or aged garlic extract (1.2 mL/kg) every other day for 4 weeks in 5/6 nephrectomized rats resulted in an increase of superoxide dismutase activity (Cruz *et al.*, 2007).

Similarly, the positive effect of garlic on the activity of superoxide dismutase was observed after oral treatment of rats with either fresh garlic homogenate (125 or 250 mg/kg) or pure S-allyl cysteine sulfoxide (0.111 or 0.222 mg/kg) for 3 weeks. Both superoxide dismutase and catalase activities in the heart tissue were significantly increased (Asdaq and Inamdar, 2010).

The antioxidant activity of garlic is most likely dose-dependent. Pure allicin neutralized DPPH radical to a higher degree in higher concentrations, with the maximal inhibition being 90%. The transformation products of allicin (transformation was performed in methanol by using microwaves) had a lower antioxidant activity than the original compound (Ilić *et al.*, 2015). Li *et al.* (2017) also observed a concentration-dependent scavenging capacity of pure allicin, the maximal inhibition again being around 90%. And similarly, Oboh *et al.* (2019) stated that the aqueous garlic extract was able to scavenge the DPPH free radical and to reduce ferric ions in a dose-dependent manner. The hydroxyl radical-scavenging properties of heated and unheated garlic were evaluated by Prasad *et al.* (1996). Although heating of garlic to 100 °C decreased the ability to scavenge hydroxyl radicals generated by photolysis of hydrogen peroxide, approximately by 10%, the radical scavenging activity of garlic remained concentration-dependent in both cases. The unheated garlic was able to scavenge 30 – 100% of the exogenously produced radicals.

Aside from the organosulfur compounds, garlic protein and its hydrolysate obtained by the enzymatic activity of pepsin and trypsin also possess appreciable antioxidant activities and offered significant protective effects against hydrogen peroxide-induced oxidative damage in rats (Gao *et al.*, 2020).

2.1.4.2. Direct Effect of Garlic on Myocardium

The influence of garlic's active compounds on cardiomyocytes is mostly mediated via their antioxidant activity (see Ch. 2.1.4.1.). However, several reports of other direct effects of garlic on myocardium can be found in the available literature. For example, Martín *et al.* (1994) investigated the

anti-arrhythmic effect of garlic dialysate on dogs and isolated rat atria. Garlic suppressed premature ventricular contractions and ventricular tachycardia in dogs intoxicated with ouabain. Furthermore, garlic dialysate also subdued the ectopic rhythms induced by isoprenaline and aconitine on electrically driven left rat atria.

Pretreatment of rats with garlic powder added to standard chow (1%) for 10 weeks resulted in a significantly reduced incidence of ventricular tachycardia and ventricular fibrillation. Further, the time until occurrence in extrasystoles and ventricular tachycardia or fibrillation was prolonged in most cases, and the duration of arrhythmias was abbreviated, as observed on isolated rat hearts by Isensee, Rietz and Jacob (1993).

Sungnoon *et al.* (2008) tested on pigs if garlic could decrease the inducibility of ventricular arrhythmia. Although the intravenously administered garlic (standardized to contain 1.3% of allicin, used at doses of 20 or 40 mg/kg) did not alter the ventricular fibrillation threshold, it significantly decreased the upper limit of vulnerability (it is the stimulus strength above which electrical stimulation cannot induce ventricular fibrillation even when the stimulus occurs during the vulnerable period of the cardiac cycle).

The anti-arrhythmic effect of garlic is, however, limited to a certain concentration range. Higher doses of garlic aqueous extract were reported to cause arrhythmias in isolated rat hearts (Sharma *et al.*, 2012), or even bizarre EKG pattern in frog hearts treated with fresh garlic juice (Yadav and Verma, 2004).

A dose-dependent **depressant effect** of garlic dialysate on heart was observed by Martín *et al.* (1992) who reported a decrease in heart rate of anesthetized dogs. Preincubation of isolated rat left atria with garlic dialysate moreover partially antagonized the positive inotropic and chronotropic effects of isoproterenol. A decrease of positive inotropic and chronotropic effects of isoprenaline on isolated rat hearts was also influenced by garlic dialysate in a dose-dependent manner (Martín *et al.*, 1994).

Negative chronotropic and negative inotropic effects of fresh garlic juice applied direct on frog hearts were observed by Yadav and Verma (2004). Similarly, Radenković *et al.* (2010) reported negative inotropic and chronotropic effects of aqueous and ethanolic garlic extract on isolated rat atria. The ethanolic extract exhibited stronger negative inotropic properties (58.33%) than aqueous extract (43.66%). The difference in chronotropism between the two tested garlic extracts was very prominent – while the aqueous extract reduced the heart rate only slightly, the ethanolic extract reduced it by more than 40%. Intravenous administration of garlic ethanolic extract to normotensive rats also led to a significant decrease in heart rate (Branković *et al.*, 2011).

The administration of garlic dialysate with verapamil, diltiazem, or nifedipine (Ca^{2+} channel blockers) induced a concentration-dependent synergism on rat left atria. Based on their experiments with the garlic dialysate on isolated rat left atria, Martín *et al.* (1997) suggested that the negative inotropic effect of garlic is related to calcium availability.

In the ischemia/reperfusion myocardial injury, the **cardioprotective effect** of garlic was reported by several authors. Asdaq, Inamdar and Asad (2010) pretreated rats with an orally administered garlic homogenate (at doses of 125, 250, or 500 mg/kg) for 4 weeks and then induced ischemia/reperfusion injury on the isolated hearts. Garlic pretreatment at doses of 125 and 250 mg/kg provided significant protection to myocardium and offered a remarkable increase in levels of several antioxidant enzymes, such as superoxide dismutase and catalase. However, the highest dose of garlic failed to show any cardioprotection.

Similar results were obtained by Sharma *et al.* (2012) who evaluated different concentrations of garlic aqueous extract (ranging from 0.01% to 0.50%) on isolated rat hearts exposed to 20 minutes of ischemia followed by 40 minutes of reperfusion. Treatment with garlic significantly reduced the size of infarct area and lactate dehydrogenase release. The effective cardioprotective concentration of garlic aqueous extract was 0.05%. Lower garlic concentrations offered no protective effect, while higher concentrations were toxic, caused cardiodepression or even arrhythmias.

Administration of pure diallyl trisulfide (20 mg/kg), one of the major active garlic compounds, to rats undergoing myocardial ischemia/reperfusion surgery also resulted in a significantly improved heart function and a decrease in apoptosis of cardiomyocytes. Diallyl trisulfide (10 μmol/L) also attenuated the *in vitro* simulated ischemia/reperfusion injury in H9c2 cell culture. The molecular mechanism of diallyl trisulfide's cardioprotective effect included an increased phosphorylation of AMPK (Yu *et al.*, 2017).

A synthetic structural analog of S-allyl cysteine, known under the name ZYZ-802 (S-propargyl cysteine), reduced the infarct size area and partially recovered cardiac function in heart failure rat model, especially when linked with leonurine (see Ch. 2.4.4.2). This analog showed a more extensive cardioprotection in the ischemic rat heart model than S-allyl cysteine (Wen and Zhu, 2015).

Comparisons of the cardioprotective effects of raw garlic and aged garlic extract were performed by Czompa *et al.* (2018). Rats were treated with either raw garlic or aged garlic extract at a dose of 300 mg/kg for 4 weeks and then their isolated hearts were exposed to ischemia/reperfusion injury (30/120 minutes). While both garlic preparations had a significant protective effect on the cardiac functions, such as increased cardiac output, aortic flow, and stroke volume, no significant differences were observed between the effects of raw and aged garlic.

2.1.4.3. Effect of Garlic on Blood, Blood Cells, and Blood Vessels

Garlic was shown to be effective against **platelet aggregation** several decades ago. Bordia (1978) studied the effect of garlic essential oil on platelet aggregation *in vitro* and in healthy volunteers after 5 days of oral treatment with garlic essential oil at a daily dose of 0.5 mg. Garlic inhibited ADP-, epinephrine-, and collagen-induced platelet aggregation in a dose-dependent manner in both cases. Garlic aqueous extract in a dose-dependent manner also inhibited ADP-, epinephrine-, collagen, and arachidonate-induced platelet aggregation *in vitro* (Srivastava, 1984b).

Dried garlic powder at a daily dose of 900 mg in healthy volunteers significantly decreased platelet aggregation after one week and two weeks of administration. The ADP- and collagen-induced platelet aggregation was remarkably inhibited mere two hours after garlic ingestion (Lagnani *et al.*, 1993).

Kiesewetter *et al.* (1993) explored the effect of garlic in 60 volunteers with cerebrovascular risk factors and constantly increased platelet aggregation. Powdered garlic at a dose of 800 mg administered for 4 weeks significantly decreased both the pathologically increased value of circulating platelets and the spontaneous platelet aggregation. Similarly, Karagodin, Sobenin and Orekhov (2016) investigated the cardioprotective effects of time-released garlic pills. When administered to patients with cerebral atherosclerosis for 14 days, a decrease in ADP-induced platelet aggregation by 24% was observed. In their preliminary *in vitro* study, incubation of garlic with human plasma resulted in an even more pronounced decrease of ADP-induced platelet aggregation (by 36%; Karagodin, Sobenin and Orekhov, 2016).

The efficacy of garlic antiplatelet properties was also evaluated by some authors in the context of antiplatelet agents contemporarily used as a standard blood-thinning therapy in human patients. For example, the comparison of antiplatelet activity of garlic and acetylsalicylic acid *in vivo* was performed by El-Sabban and Radwan (1997). Mice were administered with different doses of garlic (12.2, 25, 50, and 100 mg/kg) and acetylsalicylic acid (25, 50, and 100 mg/kg) and then the time of appearance of the first platelet aggregate in pial arterioles was measured. A significant delay in platelet aggregation was observed at the dose of 100 mg/kg in the garlic group, while all doses of acetylsalicylic acid were able to postpone the platelet aggregation.

Fakhar and Hashemi (2012) conducted another comparative study with 36 healthy volunteers focused on the platelets anti-aggregatory effect of garlic and one of the standardly used blood thinners, Plavix (clopidogrel). After 3 weeks of treatment, ADP- and collagen-induced platelet aggregation was significantly decreased in volunteers taking 1200 mg or 2400 mg garlic and 75 mg Plavix. A low dose of garlic (600 mg) had no effect on platelet

aggregation except for the ristocetin-induced aggregation, which in turn was not affected by Plavix.

The **mechanism** of garlic's inhibitory effect on platelet aggregation was suggested by Vanderhoek, Makhej and Bailey (1980) who observed a pronounced decrease in the formation of both the thromboxane B_2 and the 12-hydroxyheptadecatrienoic acid from arachidonic acid. Garlic essential oil at intermediate concentrations (10 – 30 μg/mL) induced a redistribution of the lipooxygenase pathway products in platelets and at higher concentrations (30 – 60 μg/mL) the formation of all oxygenase products was completely suppressed. The alteration of the arachidonic acid metabolism (namely inhibition of biosynthesis of 6-keto prostaglandin F1α) by garlic aqueous extract was also reported by Srivastava (1984a).

Later, Mohammad and Woodwart (1986), in a series of experiments with aqueous garlic extract, identified the potent inhibitor of platelet aggregation as allicin. Lawson, Ransom and Hughes (1992) found that in platelet-rich plasma most of the anti-aggregatory activity of garlic clove homogenates was due to adenosine. However, in full blood all of the anti-aggregatory activity was due to allicin and other thiosulfinates. The authors also tested several commercially available garlic preparations and concluded that the best garlic powder tablets were equally active as clove homogenates, while steam-distilled oil and oil-macerates possessed a significantly lower activity against platelet aggregation (by 35% and 12%, respectively). A reduced inhibitory effect of boiled garlic extract on platelet aggregation was also reported by Ali, Bordia and Mustafa (1999).

Another garlic organosulfur compound derived from garlic through the conversion of alliin into allicin is ajoene (4,5,9-trithiadodeca-1,6,11-triene-9-oxide), which was also shown to possess antiplatelet activity. Ajoene strongly inhibited the fibrinogen-induced platelet aggregation in a dose-dependent manner, it had however no effect on ADP or epinephrine membrane receptors, even though it also inhibited ADP-, epinephrine-, and collagen-induced platelet aggregation (Apitz-Castro *et al.*, 1986a; Srivastava and Tyagi, 1993). Ajoene most likely interacted directly with the fibrinogen receptor (Apitz-Castro *et al.*, 1986b). Furthermore, ajoene inhibited the

formation of thromboxane A_2 and in higher concentrations it also inhibited the incorporation of arachidonic acid into platelet phospholipids (Srivastava and Tyagi, 1993). High concentrations of ajoene (50 μmol/L) or long periods of incubation of ajoene with platelets (10 minutes) led to a non-selective hyperphosphorylation of numerous proteins. Ajoene inhibited protein tyrosine phosphatase activity in platelets (Villar, Alvariño and Flores, 1997). The antiplatelet action of ajoene was synergistically increased with the presence of other physiologically and pharmacologically active antiplatelet agents, such as prostacyclin, indomethacine, and dipyridamol (Apitz-Castro *et al.*, 1986a).

The anti-aggregatory effect of aged garlic extract is probably mediated either by the suppression of the calcium ions influx by chelating calcium with platelet cytosol or by altering other intracellular second messengers within the platelets (Allison, Lowe and Rahman, 2006). In their latter study, Allison, Lowe and Rahman (2012) suggested that aged garlic extract inhibits platelet aggregation via inhibition of the GPIIb/IIIa receptor and an increase in cAMP and confirmed these findings four years later (Rahman, Lowe and Smith, 2016) with a new observation of a platelet shape change influenced by the aged garlic extract. Morihara and Hino (2017) observed a changed functional property of platelets exposed to aged garlic extract (namely a significantly increased amount of both the extracellular ATP, and the extra- and intracellular thromboxane B_2).

The effect of garlic on **erythrocytes** has been explored to a lesser extent. Harauma and Moriguchi (2006) noted that aged garlic extract treatment in spontaneously hypertensive rats for 10 weeks led to a decrease in the count of erythrocytes. Budoff (2006) also reported a lowered value of hematocrit in volunteers treated with aged garlic extract for one year. On the other hand, Moriguchi, Takasugi and Itakura (2001) added various concentrations of aged garlic extract to a rat erythrocyte suspension pretreated with *tert*-butyl hydroperoxide in order to induce lipid peroxidation. Aged garlic extract significantly lowered the increase in hemolysis caused by lipid peroxidation. Further, the oxidized erythrocytes became more rigid and lost their flexibility and deformability when exposed

to lipid peroxidation. The addition of aged garlic extract exhibited a dose-dependent significant prevention of the erythrocyte deformability loss. Moreover, aged garlic extract led to membrane stabilization of erythrocytes (Moriguchi, Takasugi and Itakura, 2001).

Kempaiah and Srinivasan (2002) also noted the positive effect of the addition of garlic powder (2.0%) to the cholesterol-enriched chow in rats. The erythrocytes of rats fed a high-cholesterol diet were markedly more osmotically fragile when compared to the control, while garlic appeared to correct this increased fragility of the erythrocytes. The structural changes in the membrane of erythrocytes in garlic powder-treated elderly volunteers (aged 70 years and over) included significantly increased mean membrane concentrations of phospholipids and cholesterol (an increase by 5.7% and 6.1%, respectively; Brosche, Platt and Dorner, 1990).

There are numerous investigations focused on the beneficial effect of garlic on **endothelial dysfunction**. For example, the effect of garlic extract on isolated rat pulmonary arteries was explored by Kim-Park and Ku (2000). Garlic extract at a concentration range 3 – 5 μg/mL produced a dose-dependent and nitric oxide-dependent vasorelaxation. In a hypoxic environment, the maximum garlic vasorelaxation was reduced but recovered after reoxygenation. Garlic further significantly inhibited endothelin-1-induced contractions of pulmonary artery in a dose-dependent manner (Kim-Park and Ku, 2000). Sun and Ku (2006) observed that only raw garlic with the active metabolite allicin elicited a potent, dose-dependent vasodilatation of rat isolated coronary arteries. Boiled garlic or aged garlic did not exhibit vasodilatatory activity, indicating that allicin is responsible for the vasodilatatory effect of raw garlic.

Baluchnejadmojarad *et al.* (2003) investigated the effect of intraperitoneally administered garlic aqueous extract in streptozocin-induced diabetic rats for 8 weeks. Streptozocin markedly increased contractile responses to phenylephrine and impaired endothelium-dependent relaxations to acetylcholine in aortic rings. Garlic treatment led to a remarkable improvement of the impaired endothelium-dependent relaxations and decreased the enhanced contractile response to

phenylephrine in the isolated rat aortic rings. The development of abnormal contractility was prevented with garlic treatment most likely through both endothelium-dependent and endothelium-independent mechanisms (Baluchnejadmorad and Roghani, 2003). A significant protection against high cholesterol diet-induced deterioration of aortic endothelium-dependent vasorelaxation to acetylcholine and an elevation in the intima/media ratio was also provided by the administration of pure allicin (10 mg/kg) for 4 weeks to rabbits (El-Sheakh *et al.*, 2016).

Contrary to the findings of Sun and Ku (2006), the beneficial effect of aged garlic extract on endothelium function was reported by Williams *et al.* (2005). They treated 15 volunteers with coronary artery disease with aged garlic extract for 2 weeks. Even this short-term garlic treatment led to a significantly increased flow-mediated endothelium-dependent dilatation of brachial artery (by 44%). Weiss *et al.* (2006) treated healthy volunteers with aged garlic extract during acute hyperhomocysteinemia induced with oral administration of methionine. The pretreatment with aged garlic extract for 6 weeks significantly diminished the adverse effects of an acute hyperhomocysteinemia (microvascular endothelial dysfunction indicated namely by a significant decrease in flow-mediated vasodilation of the brachial artery and a decrease in acetylcholine-stimulated skin perfusion).

Furthermore, aged garlic extract was reported to restore nitric oxide bioavailability in human endothelial cells, as observed by Weiss *et al.* (2013) in their *in vitro* study with human endothelial EA.hy 926 cells incubated with several different agents (hypoxanthine, aminopterin, thymidine, and methionine) in order to induce hyperhomocysteinemia leading to endothelial dysfunction. Aged garlic extract prevented the decline of nitric oxide output, increased cellular thiol antioxidant (cysteine and glutathione), and averted the oxidation of tetrahydrobiopterin.

Endothelial dysfunction is a risk factor for the development of **atherosclerosis**. Treatment with garlic may alleviate some of the negative changes leading to atherosclerosis. For example, Brändle *et al.* (1997) carried out an interesting investigation on the long-term effects of a dietary application of garlic (dried powder, 0.5% in weight of standard laboratory

chow) on the lifespan of spontaneously hypertensive rats. The dietary intervention started at the age of three weeks. Garlic treatment prolonged the life span significantly (453 days in garlic group vs. 434 days in control group). Moreover, neither signs of stroke nor arteriosclerotic plaques were detected in the rats treated with garlic.

The reduction in the progression of plaque formation was also reported by Budoff (2006) who treated volunteers on a standard statin and aspirin therapy with aged garlic extract for one year. In patients with the placebo (and aspirin and statin therapy), the arterial calcification progressed by 22.2% per year, while the addition of aged garlic extract reduced this progression to 7.5% per year. Szulińska *et al.* (2018) observed a reduced arterial stiffness index and improvement in endothelial function markers in obese patients treated with 400 mg of garlic extract for 12 weeks.

2.1.4.4. Antihypertensive Effect of Garlic

The antihypertensive effect of garlic has been studied by scientists for several decades and plentiful works focused on this topic were published. Among these studies on both animal models and human volunteers were submitted works with strong evidence for the beneficial action of garlic on blood pressure, as well as works where garlic offered no significant antihypertensive effect. Taking into account the quantity of the available works dealing with the blood pressure-lowering properties of garlic, it was impossible to mention all the meta-analyses or even primary trials conducted in this field of research, therefore only a selection of these studies will be presented in the following text.

The question of the **duration of the onset** of garlic hypotensive action was solved by several authors. In an animal model (spontaneously hypertensive rats), Foushee, Ruffin and Banerjee (1982) evaluated three different doses of garlic extract (0.1, 0.25, and 0.5 mL/kg) administered orally. A marked decrease in the systolic blood pressure occurred in each case within 30 minutes after the garlic ingestion. The lower doses of garlic extract

were able to sustain blood pressure within normal range for a very short time period (under two hours) but the dose of 0.5 mL/kg was efficient enough for 24 hours. Similar results were obtained in the rat two-kidney-one-clip Goldblatt model where rats were administered with 0.5 mL of garlic aqueous extract. A single dose exerted the maximum hypotensive effect 2 – 6 hours after administration and its residual effect continued for up to 24 hours (Al-Qattan, Alnaqeeb and Ali, 1999).

McMahon and Vargas (1993) administered a garlic preparation, standardized to contain 1.3% allicin, at a large dose of 2400 mg to human patients with rather severe hypertension (diastolic blood pressure ≥115 mm Hg). The peak effect of garlic was about 5 hours after the administration of the preparation with a significant decrease in blood pressure persisting for 5 – 14 hours.

Oral administration of a pure aged garlic extract constituent, S-1-propenyl cysteine, to spontaneously hypertensive rats resulted in a significantly decreased systolic blood pressure (by 10%), exerting the maximal effect 3 hours after administration. The values of blood pressure returned to the original levels within 24 hours after administration. The hypotensive effect of S-1-propenyl cysteine was dose-dependent, and the dose of 6.5 mg/kg was evaluated as the most effective (Ushijima *et al.*, 2018).

Several works focused on the **comparison of efficacy** of garlic treatment with the standardly used antihypertensive drugs, especially the blockers of the angiotensin converting enzyme (ACE inhibitors). When compared to cilazapril, the hypotensive effect of garlic in both unilaterally clipped and unclipped rats was significant (decrease from 196 mm Hg to 169 mm Hg) but lower than that of cilazapril (decrease to 137 mm Hg; Al-Qattan *et al.*, 2001). A similar trend of blood pressure reduction in fructose-induced hypertensive rats administered with either allicin or enalapril was also observed by Elkayam *et al.* (2001). In both cases the hypotensive effect was statistically significant but treatment with allicin (reduction by 11 mm Hg) resulted in a smaller decrease in blood pressure than treatment with enalapril (reduction by 30 mm Hg).

However, when allylmercaptocaptopril (a conjugate of allicin and captopril (53.5 mg/kg) and pure captopril at equimolar dose (40 mg/kg) were administered to spontaneously hypertensive, obese rats, the hypotensive effect of the conjugate was significantly pronounced when compared to pure captopril (reduction by 55 mm Hg and 37 mm Hg, respectively; Ernsberger *et al.*, 2007). The co-administration of pure allicin and captopril (4:1 ratio) for 3 weeks was also more effective in reducing systolic blood pressure in rats than treatment with allicin or captopril only (Asdaq and Inamar, 2010).

As to antihypertensive drugs other than ACE inhibitors, the diuretic agent hydrochlorothiazide (10 mg/kg) added with garlic homogenate at a dose of 250 mg/kg administered to hypertensive rats for 3 weeks exhibited synergistic hypotensive properties against fructose-induced hypertension (Asdaq and Inamar, 2011).

When compared to atenolol (a beta-blocker), garlic tablets at doses 900, 1200, and 1500 mg were more effective in both systolic and diastolic blood pressure reduction than atenolol at doses 50 or 100 mg in patients with essential hypertension during a treatment for 24 weeks (Ashraf *et al.*, 2013).

Silagy and Neil (1994) submitted a **meta-analysis** of the effect of garlic on blood pressure. They included only randomized controlled trials of garlic preparations with at least 4 weeks-duration on volunteers with mild hypertension. Out of the seven trials meeting the requirements, three showed a significant reduction in systolic blood pressure and four in diastolic blood pressure. A more comprehensive meta-analysis on the hypotensive effect of garlic was performed by Ried *et al.* (2008) who included 11 randomized controlled trials using various garlic preparations. The results (for systolic blood pressure a general mean decrease of 4.6 mm Hg, in the hypertensive subgroup the mean decrease was 8.4 mm Hg) suggest that garlic preparations are superior to placebo in reducing blood pressure in individuals with hypertension.

A similar conclusion of another meta-analysis of 10 trials was offered by Reinhart *et al.* (2008) who also included German language-written studies.

In patients with elevated blood pressure, the reduction of systolic and diastolic blood pressure was significant (reduction by 16.3 mm Hg and 9.3 mm Hg, respectively), however, garlic failed to exert any hypotensive action in patients without elevated blood pressure.

A deeply critical review regarding the quality of the existing trials focusing on the hypotensive effect of garlic was presented by Simons, Wollersheim and Thien (2009), who stated that previous meta-analyses have been based on trials with inadequate study designs, methodological deficiencies, and with too little information about the blood pressure measurements.

Stabler *et al.* (2012) provided another analysis of randomized, placebo-controlled trials of garlic preparations. The mean reduction of systolic and diastolic blood pressure was 10 – 12 mm Hg and 6 – 9 mm Hg, respectively. And similarly, another meta-analysis on the hypotensive effect of garlic that included nine trials with 482 individuals reported a mean decrease of systolic blood pressure by 9.1 mm Hg and of diastolic blood pressure by 3.8 mm Hg with high heterogenity (Rohner *et al.*, 2015).

Xiong *et al.* (2015a) in their meta-analysis offered even smaller changes in the mean values of systolic blood pressure (reduction by 6.7 mm Hg), although the mean decrease of diastolic blood pressure was higher (by 4.8 mm Hg). Ried (2016) in his updated meta-analysis on the garlic hypotensive effect included 20 trials with 970 participants and concluded a more significant blood pressure reduction in the hypertensive subgroup (a reduction of systolic blood pressure by 8.7 mm Hg and of diastolic blood pressure by 6.1 mm Hg).

However, all of the abovementioned works provided mean values of blood pressure reduction that fall within the known variability in blood pressure measurements, and thus this makes it difficult to determine the true impact of garlic on blood pressure lowering.

The antihypertensive **mechanism** of garlic has yet to be elucidated to a full extent. Several actions of garlic or its individual active compounds were proposed. Pedraza-Chaverrí *et al.* (1998) tested the effect of L-NAME (a

blocker of the nitric oxide synthase) on garlic-fed rats. While in control rats the administration of L-NAME induced hypertension, the garlic-fed rats had approximately the baseline values of nitric oxide stable end products (nitrite and nitrate, measured in urinary excretion) indicating that garlic activated nitric oxide synthase (Pedrraza-Chaverrí *et al.*, 1998). It was later shown that garlic-derived polysulfides stimulate the production of the vascular gasotransmitter hydrogen sulfide (H_2S) and enhance the regulation of endothelial nitric oxide, which subsequently induces smooth muscle relaxation, vasodilatation, and reduction of blood pressure (Ried and Falker, 2014).

Mohamadi *et al.* (2000) also suggested the involvement of the nitric oxide system in the hypotensive effect of garlic, although based on their experiment with losartan, the renin-angiotensin system (RAS) is also involved. This hypothesis was further supported by several observations. Garlic has been shown to significantly down-regulate angiotensin II AT1 receptors to levels comparable with those of control animals in streptozocin-induced diabetic rats (Mansour *et al.*, 2013). The involvement of RAS in the hypotensive effect of garlic was also suggested by Oboh *et al.* (2019) who reported the inhibition of angiotensin-converting enzyme by garlic aqueous extract (IC50 = 0.59 mg/mL, the effect was significantly higher than that of purple or white onion). Similarly, appreciable angiotensin converting enzyme-inhibitory activity of garlic protein and its pepsin and trypsin hydrolysates were observed by Gao *et al.* (2020).

Furthermore, fresh garlic extract (final concentration 1%) and pure allicin decreased transmembrane currents of human epithelial sodium channels expressed in *Xenopus* oocytes in a dose-dependent manner within 10 minutes. These sodium channels represent a key factor in the transepithelial movement of sodium and consequently salt and water homeostasis in various organs. Dysregulated activity of these channels is associated with several diseases including hypertension. Contrary to allicin, the thiol non-reactive compounds alliin and diallyl sulfides had no effect on the epithelial sodium channels (Krumm *et al.*, 2012).

A metabolomic insight on the hypotensive mechanism of garlic was presented by Matsutomo *et al.* (2017). Treatment of spontaneously hypertensive rats with aged garlic extract led to changes in 30 endogenous metabolites and 7 of these metabolites were changed by S-1-propenyl cysteine, one of the major aged garlic extract constituents. The authors suggested that S-1-propenyl cysteine could exert its antihypertensive effect by affecting the metabolism of glycerophospholipids, threonine, tryptophan, glycine, and/or serine (Matsutomo *et al.*, 2017).

2.1.4.5. Effect of Garlic on Plasmatic Lipid Profile

The beneficial influence of garlic on the lipid profile is still the focus of detailed investigations because the existing evidence is a little controversial. However, without any doubts the antioxidant properties of garlic offer a strong protection of LDL cholesterol against oxidation (see Ch. 2.1.4.1.).

The **lipid-lowering effect** of garlic has been reported by many authors in both animal models as well as human volunteers treated with different garlic preparations. In animal models, for example, Ali *et al.* (2000) administered garlic powder containing 1.3% alliin (equivalent to 0.6% allicin) for 6 weeks to high cholesterol diet-fed rats. Significant reductions of serum cholesterol and triglyceride levels were observed at the end of the trial. A significant decrease in serum triglyceride level was also reported in high-fructose diet-fed rats obtaining synthetic allicin for 2 weeks (Elkayam *et al*., 2001). And similar results were reported by Elkayam *et al.* (2013) in their study with purified natural allicin administered at a dose of 80 mg/kg to rats for 6 weeks – the triglyceride levels were significantly decreased, while allicin had no effect on cholesterol.

Ernsberger *et al.* (2007) investigated if the conjugation of allicin to captopril (an angiotensin-converting enzyme inhibitor) may offer additional therapeutic actions in metabolic disease. Both captopril (40 mg/kg) and the conjugate allylmercaptocaptopril (53.5 mg/kg) administered to

spontaneously hypertensive, obese rats for 8 weeks were able to decrease the triglyceride values.

One of the first published investigations on the effect of garlic on blood lipids in humans was by Bordia *et al.* (1975) who treated healthy subjects with freshly extracted garlic juice of 50 g of garlic or ether-extracted essential oils after feeding them with 100 g of butter. Both garlic and its essential oil (mainly containing allylpropyl disulfide and diallyl disulfide) had a significant protective action against the fat-induced increases in serum cholesterol.

Auer *et al.* (1990) used garlic powder administered to volunteers for 12 weeks. The serum cholesterol and triglyceride levels were significantly lowered more rapid – only after 8 weeks of treatment. The reduction of triglycerides (by 15.9%) was also observed by Brosche, Platt and Dorner (1990) who treated 40 volunteers aged 70 years and over with 600 mg of garlic powder for 12 weeks. On the other hand, primigravidas with high risk of preeclampsia administered with 800 mg garlic tablets during the third trimester of pregnancy showed a significantly decreased total cholesterol, while garlic had no effect on the triglyceride level (Ziaei *et al.*, 2001). Similarly, Karagodin, Sobenin and Orekhov (2013) observed a decrease in LDL cholesterol (by 11.8%) and an increase in HDL cholesterol (by 11.5%) in mildly hypertensive men treated with garlic for 12 weeks.

Raw crushed garlic at daily dose of 100 mg/kg administered to 40 metabolic syndrome patients for 4 weeks was effective enough to significantly decrease the triglyceride levels and to significantly increase serum HDL cholesterol (Choudhary, Jani and Sharma, 2018).

Aged garlic extract also seems to have a positive influence on blood lipids, although higher doses are probably required to achieve a sufficient lipid-lowering performance. Steiner *et al.* (1996) administered aged garlic extract at a daily dose of 7.2 g to moderately hypercholesterolemic men for 24 weeks. At the end of the trial, total serum cholesterol was reduced by 7% and LDL cholesterol was reduced by 4%. A significant increase in HDL cholesterol after 12 weeks of treatment with a daily dose of 6 g of aged garlic extract with healthy volunteers was reported by Jung *et al.* (2014). A

markedly lower dose of aged garlic extract (only 1.2 g containing 1.2 mg of S-allyl cysteine) was used by Ried, Travica and Sali (2016) in volunteers for 12 weeks. Although the end-trial values were not statistically significant, trends in beneficial effects of aged garlic on the total cholesterol, LDL cholesterol, and apolipoproteins were still observed.

Contrary to the abovementioned works, several authors observed a **lack of efficacy** of garlic on the blood lipid parameters. Luley *et al.* (1986) tested two different doses of dried garlic (198 mg and 450 mg, three times daily) in hyperlipoproteinemic patients for 6 weeks. Neither dosage influenced total cholesterol, HDL cholesterol, LDL cholesterol, or triglyceride levels.

McCrindle, Helden and Conner (1998) evaluated the effect of 900 mg garlic extract in pediatric hypercholesterolemic patients (aged 8 to 18 years) for 8 weeks. No significant relative attributable effect on total cholesterol or LDL cholesterol was observed at the end of the trial. Superko and Krauss (2000) used the same dose of garlic extract (900 mg) in moderately hypercholesterolemic patients for 12 weeks with again no significant change in total, LDL, or HDL cholesterol. Overweight and smoking (more than 10 cigarettes/day) subjects treated with 2.1 g garlic powder for 12 weeks did not also exhibit any significant changes in the evaluated blood lipid parameters (total cholesterol, LDL cholesterol, and triglyceride levels; van Doorn *et al.*, 2006).

Even standardized garlic extract (1.12% allicin or 5.6 mg/tablet) administered to hypercholesterolemic volunteers for 12 weeks exhibited no significant changes in total, HDL or LDL cholesterol, or triglyceride levels (Satitvipawee *et al.*, 2003).

This **discrepancy** in results of various works may be the consequence of a different study design. A comprehensive meta-analysis on the effect of garlic on cholesterol was presented by Ried (2016) who included randomized controlled trials published between the years 1955 and 2013. The resulting total amount of 39 primary trials that involved 2300 adults treated for a minimum of 2 weeks was then analyzed. Garlic was suggested to be effective

in reducing total and LDL cholesterol by 10% if taken for more than 8 weeks by volunteers with slightly elevated concentrations. Thus, garlic may not offer any benefit regarding the blood lipid spectrum in volunteers with more pronounced hypercholesterolemia and/or treated for a too short time period.

Interestingly, Zhang *et al.* (2001) observed a remarkable difference in the effect of garlic oil between men and women for HDL cholesterol and total cholesterol values. The treatment with cyclodextrin-bound garlic oil for 11 weeks was more favorable for women than for men. It is therefore possible that the effect of garlic may also be dependent on gender.

The **mechanism** of the lipid-lowering effect of garlic is multifactorial. S-allyl cysteine, S-ethyl cysteine and S-propyl cysteine, which are abundantly present in aged garlic extract, were reported to decrease the incorporation of acetate into triglycerides in cultured rat hepatocytes. The inhibition was concentration-dependent, with S-allyl cysteine being the most effective compound (inhibition by 81%). Both S-allyl cysteine and S-propyl cysteine were also effective in decreasing the activity of fatty acid synthase in the rat hepatocytes culture (the activity was lowered by 32% and 27%, respectively; Liu and Yeh, 2001).

Nam *et al.* (2017) also tried to elucidate the mechanism of aged garlic extract on lipid metabolism. Treatment of mature 3T3-L1 adipocytes with aged garlic extract suppressed lipogenesis and induced lipolysis in a dose-dependent manner. At concentrations 2.5 and 5 mg/mL aged garlic extract significantly reduced protein expression of PPARγ. Similarly, Baek *et al.* (2019) reported a reduced expression of adipogenic genes (including *PPARγ*, *Adipsin,* and *Adipoq*) and inhibition of adipogenesis of 3T3-L1 preadipocyte cell culture treated with a β-carboline alkaloid isolated from garlic methanolic extract (namely (1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid).

As to inhibition of cholesterol synthesis, Yeh and Liu (2001) characterized the inhibitory potency of individual water-soluble and lipid-soluble compounds in garlic. While the lipid-soluble compounds exhibited at acceptable concentrations only a slight inhibitory effect on cholesterol

synthesis (by 10 – 15%), among the water-soluble organosulfur compounds especially S-propyl cysteine and γ-glutamyl-S-allyl cysteine offered a satisfactory inhibition activity of the cholesterol synthesis (by 40 – 60% and 20 – 35%, respectively). This inhibition was due to deactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase via enhanced phosphorylation but not via changing the levels of mRNA or the quantity of the enzyme (Liu and Yeh, 2002).

2.1.5. Safety Profile of Garlic Preparations

Garlic has been used as a spice and in traditional medicine for thousands of years and is therefore considered safe. The medicinal use of garlic has, however, its limitations. For example, EMA in the herbal monograph on *Allium sativum* L., bulbus stated that use of garlic in the treatment of a cold in children under 12 years of age has not been established due to the lack of adequate data. For the treatment of atherosclerosis, the age limit was set even higher; garlic is not to be used in children and adolescents under 18 years of age (EMA/HPMC/7685/2013).

A comprehensive review on garlic's **adverse effects** and drug interactions in humans was presented by Borrelli, Capasso and Izzo (2007). The well-documented adverse effects of garlic treatment were garlic odor, allergic reactions, photoallergy, cutaneous manifestations, coagulation alterations, and gastrointestinal adverse effects.

The allergenic potential of garlic is well recognized and the allergens have been identified as diallyl disulfide (which is considered to be the primary allergen), allylpropyl sulfide, and allicin (the latter may be an irritant). Contact dermatitis is the most common allergic reaction associated with garlic use. On the other hand, photoallergy occurs rarely, again originating from the organosulfur compound diallyl disulfide (Borreli, Capasso and Izzo, 2007 and references therein).

Studies on the possible impact of garlic on cytochrome P450 metabolic enzymes, and thus the potential of **pharmacokinetic interactions** indicate

consistently that neither single nor repeated administration of various garlic formulations up to 4 weeks may have the potential to exert notable alterations (inhibition or induction) of CYP1A2, CYP2D6, or CYP3A4 activities in humans, which are important metabolic pathways for many drugs likely to be coadministered with garlic supplements (Hermann and von Richter, 2012). On the other hand, garlic extract has been shown in human studies to decrease concentrations of drugs that are transported by the P-glycoprotein. These drugs (e.g. digoxin, quinidine, rosuvastatin, and verapamil) should therefore not be combined with garlic supplements (Asher *et al.*, 2017).

Due to the possible clinically significant pharmacokinetic interaction of garlic with some HIV protease inhibitors, EMA listed saquinavir/ritonavir therapy under the contraindications section for garlic use. The study on which this statement was based on was, however, criticized for its design and that it could be most conceivable that the study results merely mirrored saquinavir-mediated induction processes of its own metabolism and disposition rather than indicating a relevant garlic-mediated drug interaction (Herrman and von Richter, 2012; EMA/HPMC/7685/2013).

As to **pharmacodynamic interactions** of garlic, synergistic effect of garlic concomitantly administered with hydrochlorothiazide, diltiazem, and nifedipin was reported (Martín *et al.*, 1997; Asdaq and Inamar, 2011).

The greatest safety concern is about the influence of garlic on blood coagulation, especially the possible pronounced effect of garlic administered concomitantly with other blood thinning drugs because they may increase bleeding times. Seeing as the consequences could be severe, EMA recommends that garlic preparations should be used with caution in patients taking oral anticoagulation therapy and/or anti-platelet therapy. Garlic consumption should be avoided 7 days before surgery because of the post-operative bleeding risk (EMA/HPMC/7685/2013). However, Ge, Zhang and Zuo (2014) in their review, focused on updates on the clinically evidenced herb-warfarin interactions, concluded that although the interaction between warfarin and garlic is possible, serious interactions seem unlikely to happen. Aged garlic extract also seems to be safe for patients on warfarin therapy

(Macan *et al.*, 2006). In general, higher precaution in garlic use is recommended for older adults on warfarin therapy (Agbabiaka *et al.*, 2017).

2.2. HAWTHORN – *CRATAEGUS* SPP. FAM. *ROSACEAE*

2.2.1. CHARACTERIZATION OF THE *CRATAEGUS* GENUS – A PHARMACOGNOSTIC CONTEXT

Crataegus comprises a complex group of trees and shrubs. Shrubby hawthorn genera are mainly up to 3 m of height, while hawhorn tree species can grow up to 12 m. There are approximately 280 species in the genus around the world. Hawthorn is native to the Northern temperate zones, mostly between the latitudes of 30° and 50° N (North America, Europe, Asia) and various introducted hawthorn species can be found in temperate South America (including equatorial Andes), south Africa, New Zealand, and Australia. The plants are heliotropes varying sometimes to mesophylls. For hawthorns, the soil water regime is more important then the soil type. Hawthorns are generally considered as aggressive settlers, especially in their non-native environment (Kumar *et al.*, 2012; Phipps, 2020).

Short twigs and branches up to 2.5 mm in diameter in the beginning of flowering or leaves in full bloom are collected for medicinal use (**Crataegi folii cum flore**). The European Pharmacopoeia requires a minimum content of 1.5% of total flavonoids expressed as hyperoside, calculated with the reference to the dried drug. Chinese and European Pharmacopoeias both also recognize hawthorn fruits – **Crataegi fructus**. In Europe, plant material is collected from the hawthorn species *Crataegus monogyna* Jacq. (common hawthorn; Fig. 2.3.) and *C. laevigata* Poir. in Lam. (midland hawthorn) or their hybrids, less often from other *Crataegus* species. However, all the white-flowering hawthorn species are authorized for plant material collecting. In China, the species *C. pinnatifida* Bunge (Chinese hawthorn; Fig 2.3.) is prevalently used as a source of hawthorn plant material (Tomko *et al.*, 1989; EMA/HPMC/159075/2014; EMA/HPMC/159076/2014).

Fig. 2.3. *Crataegus monogyna* Jacq. Upper left – flowers, upper right – fruits. *Crataegus pinnatifida* Bunge. Down left – flowers, down right – fruits. From mailordertrees.co.uk, etsy.com, greenleafnurseries.co.nz, and pinterest.co.uk.

The European Pharmacopoeia further includes two monographs on hawthorn extracts (**Crataegi folii cum flore extractum fluidum quantificatum** and **Crataegi folii cum flore extractum siccum**). Extraction solvents permitted in hawthorn plant material processing are methanol, ethanol (both in various w/w ratio), water, or even sweet wine (EMA/HMPC/159075/2014; EMA/HPMC/159076/2014; European Pharmacopoeia 10.0).

Lately, alternative methods for obtaining biologically active compounds from hawthorn plant material have also been suggested. For example, Hu *et al.* (2019) established a simple, rapid, and sensitive method for extraction of phenolic compounds from hawthorn leaves using a microwave-assisted extraction. In order to avoid the use of organic solvents, aqueous solutions of solid reagents (sodium carbonate and sodium borate) were used. The optimal extraction procedure conditions included an extraction time of 10 minutes, a temperature set to 50 °C, and the pH adjusted to a value of 7.

2.2.2. PHYTOCHEMISTRY OF HAWTHORN

Nabavi *et al.* (2015) in their review on phytochemistry of *C. monogyna* Jacq. noted that the most common polyphenolic compounds present in this species are flavonoids, chlorogenic acids, and triterpenes. Among them, the monomeric flavan-3-ol compound (-)-epicatechin is abundantly present in the plant. The most important hawthorn **flavonoids** are represented by quercetin and apigenin glycosides: hyperoside (quercetin-3-O-glucoside), rutin (quercetin-3-O-rutinoside) and vitexin (apigenin-8-C-glucoside). These three flavonoids are depicted in Fig. 2.4. Vitexin was found to be the dominant compound in *C. monogyna* Jacq. leaves, while hyperoside was reported as one of the major components in the flowers, although other glycosides, such as rutin and spiraeoside, were also present. Cyanidin-3-O-galactoside (anthocyanin) was identified as the flower pigment in *C. monogyna* Jacq. (Raudonis *et al.*, 2009; Nabavi *et al.*, 2015 and references therein).

The **triterpenic fraction** isolated from aerial parts of hawthorn plants (including twigs, stems, and leaves) is represented by tetracyclic triterpenic alcohols butyrospermol and cycloartenol as well as by pentacyclic triterpenic acids arising from triterpenic alcohols by oxidation reactions. The most common hawthorn triterpenic acids are crataegolic (also known as maslinic), oleanic, and ursolic acid (Tomko *et al.*, 1989; García *et al.*, 1997; Froehlicher *et al.*, 2009; Nabavi *et al.*, 2015).

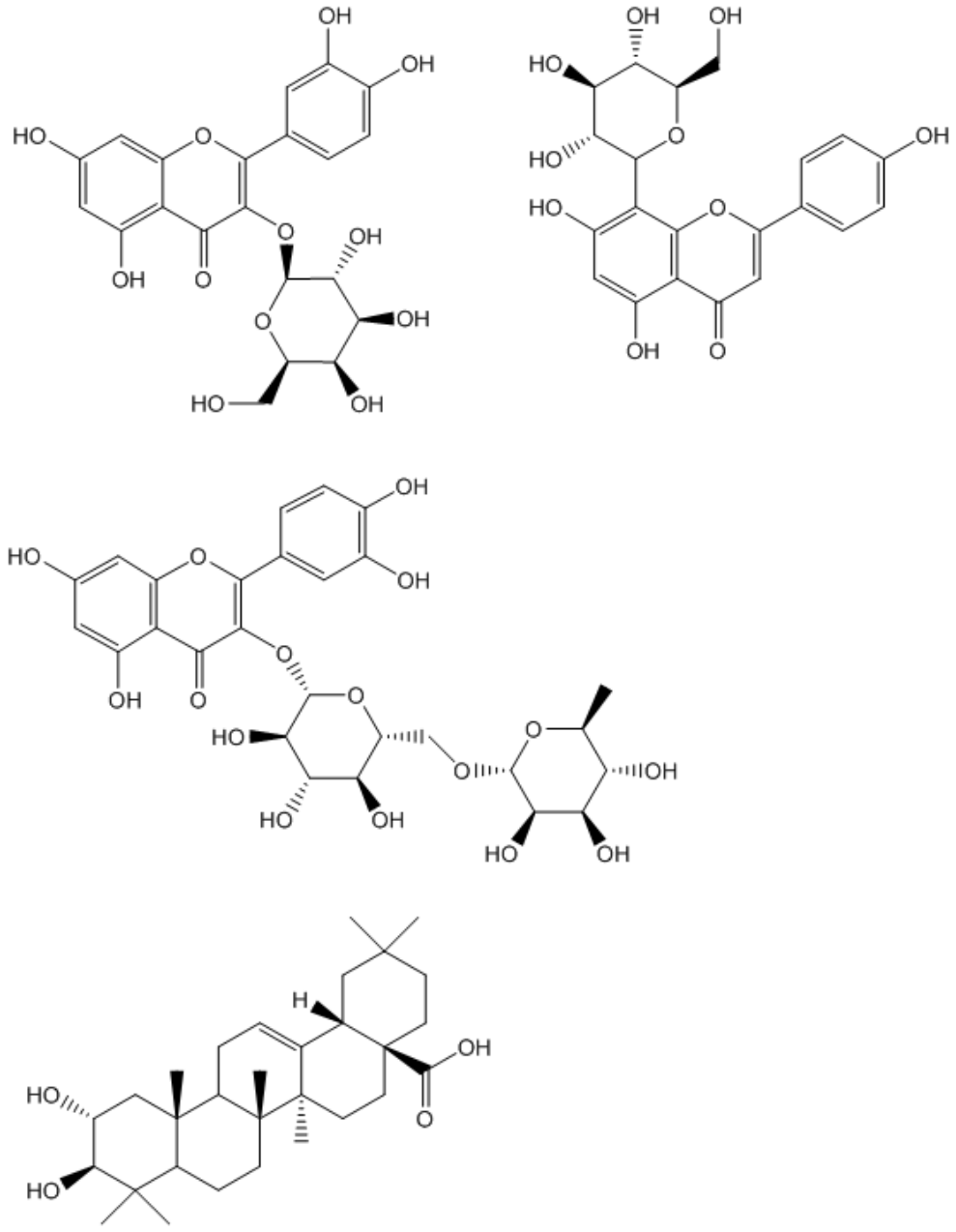

Fig. 2.4. Some of the *Crataegus* spp. active compounds. Upper left – hyperoside, upper right – vitexin, middle – rutin, down – crategolic acid. Courtesy of doc. Macáková.

The content and composition of phenolic compounds in hawthorn is influenced by a number of factors, such as genotype, plant organ, seasonal and geographical variation. *Crataegus* **species**, subspecies, and even varieties differ in their content of phenolic compounds, as observed by several authors. For example, Bahri-Sahloul *et al.* (2009) compared the total phenols content

of two *C. azarolus* L. varietes. The variety producing yellow fruits (*C. azarolus* var. *aronia* (Willd.) Batt.) was richer in phenolic compounds than the variety with red fruits (*C. azarolus* var. *eu-azarolus* Maire), with the contents being 16.4 mg of gallic acid/g of dry weight and 14.2 mg of gallic acid/g of dry weight, respectively. Belkhir *et al.* (2013) also noted that the yellow-fruited hawthorn species *C. azarolus* L. had significantly higher polyphenol content than the red-fruited species *C. monogyna* Jacq. Furthermore, while hyperoside was the main phenolic in yellow fruits, procyanidin B2 represented the major phenolic compound in red fruits.

Calişkan *et al.* (2012) analyzed the total phenolic content of five European hawthorn species and reported the highest phenolic content in *C. monogyna* subsp. *azarella* (Griseb.) Franco (55.2 mg of gallic acid equivalent/g of dry weight) and the lowest phenolic content in *C. aronia* subsp. *aronia* (L.) Rouy & E. G. Camus (35.7 mg of gallic acid equivalent/g of dry weight). Varieties of Chinese hawthorn species *C. pinnatifida* Bunge also differed in the phenolic content. Out of the three evaluated varieties, the shanlihong variety exhibited elevated levels of total phenolic and flavonoids content, including free and bond phenolics (Wen *et al.*, 2015).

The impact of the **plant organ** on the hawthorn phenolic content was also noted in some papers. For example, floral buds had higher total phenol, proanthocyanidin, and flavonoid content than opened flowers (Bahri-Sahloul *et al.*, 2009). Hawthorn flowers contained higher levels of hydroxycinnamic acids, vitexin derivates, and flavonols compared to fruits, while anthocyanins were present only in fruits (Issaadi *et al.*, 2020). Unripe hawthorn fruits were richer in phenolic compounds than ripened or over-ripened fruits, which in turn contained more sugars and saturated fatty acids (Barros, Carvalho and Ferreira, 2011). Hawthorn leaves contained more phenolic compounds than fruits (Šavikin *et al.*, 2017).

Seasonal variation of phenolic compounds in hawthorn *C. pinnatifida* Bunge was investigated by Luo *et al.* (2016) who found a positive correlation of the phenolic content with daytime light and temperature. While leaves, roots, and twigs underwent more or less significant fluctuations in phenolic content during the year (May – October), the phenolic content in the fruits

seemed to be quite consistent during the growth season. Seasonal changes are probably not affecting all types of phenolic compounds, as noted by Hellenbrandt *et al.* (2015) for procyanidins in *C. monogyna* Jacq. Their distribution during seasonal growth showed more or less constant contents with strong accumulation in the flowers and fruits.

Geographical variation of phenolics in the hawthorn species *C. monogyna* Jacq was also noted by Abuashwashi, Palomino and Gómez-Serranillos (2016). Aerial parts of hawthorn were collected from different locations in central Spain. The total polyphenol content in these samples ranged between 117.7 and 204.3 mg of gallic acid equivalent/g of extract with predominant content of kaempferol (flavonoid) and ursolic acid (phenolic acid). Similarly, Alirezalu *et al.* (2020) noted a difference in the phenolic content in hawthorn samples collected from different locations in Iran. The variations in values of phenolic compounds content could be, however, caused due to sampling of various hawthorn species.

2.2.3. Medicinal Use of Hawthorn

Hawthorn is commonly used in traditional medicine, it was mentioned in Dioscorides *De Materia Medica* and the traditional Chinese medicine uses the fruit of the hawthorn species *C. pinnatifida* Bunge under the name Shan-Zha. Hawthorn is considered to be a cardiotonic plant, it can also be used against sleep and stomach problems (Rigelski and Sweet, 2002; Nabavi *et al.*, 2015). According to EMA, hawthorn preparations are intended for use in two indications: (1) to relieve symptoms of temporary nervous cardiac complaints such as palpitations or perceived extra beat due to mild anxiety, or (2) for relief of mild symptoms of mental stress and aid to sleep. Hawthorn can be used in various preparation forms: comminuted or powdered herbal substance, dry extract, liquid extract, expressed juice from the fresh leaves and flowers, and tinctures (EMA/HMPC/159075/2014).

Two commercially available hawthorn preparations are the most often mentioned in the reviewed literature: WS 1442 and LI 132.

WS 1442 is a *Crataegus* special extract. It is a dry extract from hawthorn leaves with flowers (4 – 6.6:1) extracted with 45% ethanol (w/w) and then adjusted for certain oligomeric procyanidins (OPC) content. The extract usually contains 18.75% of OPCs, the range of OPCs can, however, vary between 17.3 and 20.1%. This extract also contains other active compounds, e.g. several flavonoids (including hyperoside, vitexin-rhamnoside, rutin, and vitexin), triterpenoids, and phenol carboxylic acids.

LI 132 is a hawthorn extract prepared from the leaves and flowers (extraction solvent 70% methanol), and standardized to contain 2.2% of flavonoids.

2.2.4. Effects of Hawthorn on Selected Cardiovascular Conditions

2.2.4.1. Antioxidant Effect of Hawthorn

The significant antioxidant activity of hawthorn has been known for a long time. Hawthorn is considered a plant with strong antioxidant properties. For example, Kiselova *et al.* (2006) compared the antioxidant activity of 23 Bulgarian herbs with that of mate; hawthorn and some other species exhibited higher antioxidant activity. Similarly, Hela *et al.* (2013) evaluated the antioxidant potential of 110 selected Egyptian plants. Out of them, the hawthorn species *C. sinaica* Boiss. exerted the third highest antioxidant activity. Furthermore, Jeong, Tulasi and Koyyalamudi (2016) investigated the antioxidant properties of 11 Chinese fruits, with hawthorn *C. pinnatifida* Bunge exerting the highest antioxidant activity. Hendrich *et al.* (2020) also reported excellent antioxidant properties of hawthorn in comparison with three other plant species known for their high phenolic content.

Several authors proposed that the antioxidant properties of hawthorn are mainly thanks to the content of phenolic compounds, especially the flavonoids. A strong positive **correlation** between the antioxidant capacity and hawthorn polyphenol, flavonoid, and/or phenolic content was reported, for example, by Kiselova *et al.* (2006), Luís, Domingues and Duarte (2011),

Abuashwashi, Palomino and Gómez-Serranillo (2016), and Issaadi *et al.* (2020). On the other hand, Masteiková *et al.* (2008) found no correlation between phenolics and antioxidant activity in hawthorn and motherwort tinctures, while Egea *et al.* (2010) and Aslam *et al.* (2017) reported only a weak correlation between antioxidant activity and the total phenolic and flavonoid contents.

Various **compounds** contribute to the antioxidant activity of hawthorn, mostly of the phenolic type. Masteiková *et al.* (2008) reported that epicatechin and hyperoside were present in great amounts in hawthorn tincure and also significantly contributed to the antioxidant properties. On the other hand, procyanidine B2, which was present in lower amount than rutin and quercetin, exerted higher antiperoxynitrite activity than the two aforementioned compounds. Bernatoniene *et al.* (2008) also noticed that in hawthorn aqueous and ethanolic extracts, epicatechin and catechin contribute to radical-scavenging properties more than the other compounds. Raudonis *et al.* (2009) observed that vitexin rhamnoside, the dominant compound of *C. monogyna* Jacq. leaves, was not a significant radical scavenger. In both hawthorn leaves and flowers, chlorogenic acid seemed to be the most active antioxidant compound.

In an ethyl acetate extract of another hawthorn species (*C. azarolus* L.), eight significant phenolic antioxidants were determined: chlorogenic acid, hyperoside, rutin, isoquercitrin, quercetin, procyanidin B2, (-)-epicatechin, and spiraeoside (Bahri-Sahloul *et al.*, 2009). Most of the newly isolated pinnatifidins C (dihydrobenzofuran neolignans) and pinnatifidaninside A – D (neolignan glycosides) from hawthorn seeds as well as proanthocyanidins from the hawthorn fruit stone also exerted potent antioxidant activity (Huang *et al.*, 2013; Huang *et al.*, 2014; Chai *et al.*, 2014). Benmalek *et al.* (2013) further noted that hawthorn flavonols presented high antioxidant activity when compared with anthocyanidins and standard antioxidants (ascorbic acid and quercetin).

The antioxidant activity of hawthorn is influenced by a variety of factors that have impact on the content of antioxidant compounds and the resulting antioxidant activity. Two of these factors are of great importance:

the **extraction solvent** and plant material used for the preparation of the extract. Bernatoniene *et al.* (2008) reported that ethanolic hawthorn fruit extract contained threefold more phenolic compounds than aqueous extract, with the antioxidant activity being 2.3 times higher. Similar results were obtained by Shao *et al.* (2016) who reported that the total phenols content in ethanolic hawthorn fruit extract was 3.9 times higher than that in aqueous extract and the ethanolic extract exerted stronger antioxidant activity.

The antioxidant activity of two hawthorn fruit extracts was also evaluated by Ebrahimzadeh and Bahramian (2009) who observed a higher antioxidant activity in aqueous rather than methanol extract. The determination of antioxidant activity of aqueous, methanolic, and ethyl acetate extracts from hawthorn leaves with flowers, fruit peel, and seeds revealed that the strongest antioxidant properties were exhibited by the ethyl acetate extract (approximately threefold higher than that of aqueous extract; Öztürk and Tunçel, 2011). Similarly, the ethyl acetate hawthorn extract was evaluated as being the most antioxidant active form by Wang *et al.* (2018).

Plant material is another crucial factor influencing the antioxidant properties of hawthorn extracts although the studies are not completely consistent in their findings. Barros, Carvalho and Ferreira (2011) performed a complete chemical and bioactive characterization of flower buds, flowers, unripe, ripened, and over-ripened hawthorn fruits. Their results suggested that unripe fruits have the highest phenolic levels and the most promising antioxidant properties (better than Trolox). Similarly, Keser *et al.* (2014) found the highest antioxidant activity in hawthorn fruits as compared to leaves and flowers.

On the other hand, Öztürk and Tunçel (2011) evaluated the antioxidant activity of hawthorn fruit peel, seed, and leaves with flowers and found the highest DPPH radical-scavenging activity in leaves with flowers. High antioxidant activity of hawthorn leaves was also confirmed by Belkhir *et al.* (2013) who furthermore observed that *C. azarolus L.* had higher antioxidant activity than *C. monogyna* Jacq. And similarly, Šavikin *et al.* (2017) reported that the leaf extract of the hawthorn species *C. orientalis* Pall. ex M. Bieb. was more effective as a DPPH radical scavenger than the fruit extract.

The beneficial antioxidant properties of hawthorn were also shown on cellular and whole organism **levels**. On the cellular level, a significant reduction of oxidative stress after incubation with hawthorn extract was observed, for example, in human blood lymphocytes (Hosseinimehr *et al.*, 2011), mouse lymphocytes (Cheng *et al.*, 2013), mouse lymphocytes and macrophages (Mustapha *et al.*, 2016b), PC12 cells (derived from pheochromocytoma of rat adrenal medulla; Chang, Lin and Lai, 2012), human myelogenous cells K562 (Mustapha *et al.*, 2014), HepG2 cells (human liver carcinoma cells; Krajka-Kuźniak *et al.*, 2014), or primary human keratocyte cells (Mustapha *et al.*, 2016a).

In various animal models, hawthorn extracts exerted hepatoprotective effects against both non-alcoholic (Al Humayed, 2017; Saeedi *et al.*, 2018) and alcoholic (Martínez-Rodríguez *et al.*, 2019) fatty liver disease, doxorubicin-induced (Mecheri *et al.*, 2019), carbon tetrachloride- or paracetamol-induced hepatotoxicity (Ganie *et al.*, 2014), neuroprotective (Gazdik *et al.*, 2008; Saoudi *et al.*, 2019), or even an anticataract effect (Wang *et al.*, 2011b). Furthermore, the protective effect of hawthorn against genotoxicity (Jalali, Hasanzadeh and Malekinejad, 2012; Shalizar Jalali and Hasanzadeh, 2013) and colitis (Malekinejad *et al.*, 2013) has been reported.

2.2.4.2. Direct Effect of Hawthorn on the Myocardium

Hawthorn extract has been shown to exert various beneficial properties regarding the cardiovascular system. The **positive inotropic effect** of hawthorn preparations has been repeatedly reported through the last three decades (e.g. Leukel, Frick and Hölze, 1986; Pöpping *et al.*, 1995; Schwinger *et al.*, 2000; Rodriguez *et al.*, 2008).

The cardioactive compounds responsible for the inotropic effect of hawthorn were identified by Leukel, Fricke and Hölze (1986) who fractionated a hawthorn aqueous extract into four fractions with two of them containing flavonoids and the other two proanthocyanidins. While all of the tested fractions exhibited a positive inotropic effect on isolated Guinea pig

hearts, fraction 4 (proanthocyanidins) was proven to be the most effective one followed by fraction 3 (proanthocyanidins). Both of the proanthocyanidins-containing fractions were effective in their inotropic actions at very low concentrations (0.5μg/mL and 50μg/mL, respectively). On the other hand, hawthorn flavonoids offered only slightly positive inotropic properties, mostly due to the presence of O-glycosides, such as luteolin-7-glucoside, hyperoside, and rutin. The flavonoid C-glycosides vitexin and vitexin-rhamnoside were even less effective (Schüssler, Hölzl and Fricke, 1995).

One of the first investigations of the mechanism underlying hawthorn inotropic action was provided by Pöpping *et al.* (1995) who used the hawthorn extract LI 132 on isolated cardiomyocytes from adult rats. The positive inotropic effect was observed in a certain concentration range and was accompanied by a moderate elevation of energy turnover for both mechanical and ionic processes. This intervention was very economical to myocytes when compared to other performed inotropic interventions, such as the application of isoprenaline, ouabain, and elevation of extracellular Ca^{2+} concentration.

Five years later Schwinger *et al.* (2000) in their study with another hawthorn extract (WS 1442) in the human myocardium from patients with congestive heart failure observed that both the force generation and the calcium transient were increased by WS 1442. However, while the extract in a concentration-dependent manner released the specifically bound 3H-ouabain from the Na^+/K^+ ATPase, the activity of adenylate cyclase was not affected and the authors proposed a cAMP-independent mechanism for the positive inotropic action of hawthorn glycosides.

Rodriguez *et al.* (2008) compared the positive inotropic effect of two commercially available hawthorn ethanolic extracts on adult rat cardiomyocytes. One extract was obtained from mixed plant material – leaves, flowers and fruits, the other only from fruits. According to their findings, the fruit extract appeared to be more appropriate for therapeutic use, as it did not cause calcium overload in the cardiomyocytes. On the other hand, the administration of the mixed plant material extract to cardiomyocytes resulted

in robust calcium transients and an eventual calcium overload. Aside from the impact of hawthorn on intracellular calcium levels, the involvement of Na^+/K^+ ATPase in the mechanism of hawthorn cardiac activity was again also confirmed (Rodriguez *et al.*, 2008).

Similarly, Willer *et al.* (2012) observed the impact of WS 1442 on the Na^+/K^+ ATPase. The authors further explained the increase in the intracellular calcium concentration after hawthorn extract administration as a result of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase inhibition and the consequential activation of the inositol 1,4,5-trisphosphate pathway. The WS 1442 extract did not induce the store-operated calcium entry, thus preventing the deleterious rise of Ca^{2+}.

Furthermore, **anti-arrhythmic activity** and **negative chronotropic effect** of hawthorn preparations have been reported. For example, Garjani *et al.* (2000) evaluated the effect of different *C. meyeri* A. Pojark extracts on arrhythmias induced by a period of myocardial ischemia in rats. Methanolic extract significantly reduced beats occurring as ventricular tachycardia, while chloroform extract reduced the occurrence of single extrasystoles. In both cases the effect led to a remarkable decrease in the total number of ventricular ectopic beats. Ethyl acetate and methanolic extracts further significantly reduced the time spent for ventricular fibrillation.

Long *et al*. (2006) noted that several commercial hawthorn extracts were able to induce rhythmicity in quiescent cultured cardiomyocytes. Hawthorn, unlike other cardioactive drugs, did not cause β-adrenergic receptor blockade at concentrations which normally cause negative chronotropic activities. Alp *et al.* (2015) even reported that hawthorn offered a strong activity against digoxin-induced arrhythmias in rats. The administration of *C. oxyacantha* L. fruit extract shortened the duration of both atrial and ventricular arrhythmias, and also led to a significant bradycardia.

A dose-dependent negative chronotropic effect of hawthorn leaf extract from *C. pentagyna* Waldst. & Kit ex Willd. was observed by Pahlavan *et al.* (2018) in their study with cardiomyocytes differentiated from healthy volunteers, long QT syndrome, and tachycardial patients-originating human

stem cells. This effect was further accompanied by a prolongation of the field potential durations in long QT syndrome patients-derived cardiomyocytes, and by a reduction of the beating frequencies and the occurrence of immature field potential, triggered by β-adrenergic stimulation in cardiomyocytes from tachycardial patients. The flavonoids isoquercetin and vitexin were especially efficient in the slowdown of isoproterenol-induced beating frequencies.

Hawthorn furthermore exerts a strong **cardioprotective effect.** Jayalakshmi, Thiruparasundari and Devaraj (2006) investigated the effect of hawthorn on mitochondrial function during experimentally induced myocardial infarction (isoproterenol, 85 mg/kg) in rats. Pretreatment with hawthorn at a dose of 5 mL/kg for 30 days resulted in the preservation of the mitochondrial antioxidant status, and prevented both the mitochondrial lipid peroxidative damage and the decrease in Kreb's cycle enzymes. A longer pretreatment with hawthorn (8 weeks) before the induction of a myocardial infarction with isoproterenol in a rat model was conducted by Vijayan, Thiruchenduran and Devaraj (2012). Again, hawthorn administration had a significant effect on the reversing of deterioration of biochemical factors caused by isoproterenol (creatine kinase and lactate dehydrogenase levels in serum, and lipid peroxidation) to nearly normal levels.

Swaminathan *et al.* (2010) also obsereved the beneficial effect of hawthorn on the myocardial ischemia/reperfusion injury (30 minutes/ 45 minutes) in isolated rat hearts. Hawthorn extract administered for 10 minutes during the reperfusion phase led to a significant recovery in cardiac contractile function, a reduction of infarct size, and a decrease in creatinine kinase and lactate dehydrogenase activities.

Cuevas-Durán *et al.* (2017) induced myocardial infarction in rats surgically by ligating the left anterior descending coronary artery. In this case, the pretreatment with hawthorn extract administered intraperitoneally for one week at a dose of 100 mg/kg prior to the onset of myocardial infarction also resulted in a significantly increased total antioxidant capacity, expression of superoxide dismutase and catalase, with the subsequent decline of malonyl dialdehyde level.

Hawthorn also offers a significant protection against carbon tetrachloride-induced heart tissue damage. Rats pretreated with hawthorn extract at a dose of 300 mg/kg for 15 days had significantly decreased serum creatinine and malonyl dialdehyde levels. Furthermore, hawthorn restored the levels of glutathione and antioxidant enzymes glutathione reductase, glutathione peroxidase, and glutathione transferase in the heart tissue (Ganie *et al.*, 2016).

The **mechanism** of hawthorn cardioprotective effect includes the reduction of oxidative stress and consequent apoptosis of cardiomyocytes (Vijayan, Thiruchenduran and Devaraj, 2012). A significant upregulation of the anti-apoptotic proteins Bcl-2 and Hsp70 with a simultaneous downregulation of the pro-apoptotic proteins cytochrome c and cleaved caspase-3 was also reported by Swaminathan *et al.* (2010).

Hawthorn exhibits several properties that may be highly beneficial in **heart failure** treatment, among them the anti-arrhythmic properties, anti-inflammatory, and antioxidant (see Ch. 2.2.4.1.) effects are of great interest. Schmidt *et al.* (1994) treated 78 chronic congestive heart failure patients (NYHA class II) with hawthorn extract LI 132 (600 mg per day) for 8 weeks. The working capacity and clinical symptoms were significantly better in the hawthorn treated group than in the placebo group.

The hawthorn extract LI 132 was also used in the study of Tauchert, Ploch and Hübner (1994). 132 stable heart failure patients (NYHA class II) were treated with either the hawthorn extract (900 mg per day) or the ACE inhibitor captopril (37.5 mg per day) for 8 weeks. In both groups the exercise tolerance increased significantly, and the incidence and severity of pursued typical symptoms were decreased by around 50%.

Zapfe (2001) used another commercially available hawthorn extract in his smaller study with 40 congestive heart failure (NYHA class II) patients. Patients were treated for 12 weeks either with WS 1442 extract or placebo. The exercise tolerance was higher in the hawthorn group than in the placebo group at the end of the trial.

In 2000 Holubarsch *et al.* presented the design of a large study on congestive heart failure patients (NYHA class II and III) that was also partially conducted in the Czech Republic. The Survival and Prognosis: Investigation of Crataegus Extract WS 1442 in congestive heart failure (SPICE) study included around 2300 patients. Patients were treated either with WS 1442 (900 mg per day) or placebo for 24 months. The results of this study were presented by the authors several years later (Holubarsch *et al.*, 2008). Among the final number of 2681 patients randomized to treatment, cardiac mortality in the hawthorn group as compared to placebo was reduced by 20%, with a significant reduction in sudden cardiac deaths by 39.7%.

A distinctively critical voice on hawthorn's effect in heart failure patients is represented by Zick, Gillespie and Aaronson (2008) and Zick *et al.* (2009) in their HERB CHF (Hawthorn Extract Randomized Blinded Chronic Heart Failure) trial which included 120 patients with heart failure (NYHA classes II or III) and a decreased left ventricular fraction ejection (≤40%) on a standard medical therapy (an ACE inhibitor or angiotensin receptor antagonist, a beta-blocker, and a diuretic). Patients were treated for 6 months with either hawthorn extract WS 1442 (450 mg twice daily) or placebo. Zick, Gillespie and Aaronson (2008) focused on the inhibition of progression in heart failure patients treated with hawthorn extract and concluded it did not decrease the risk of clinical heart failure progression compared to placebo. The outcomes of Zick *et al.* (2009) showed no effect of hawthorn extract on blood pressure, heart rate, oxidative stress, inflammation, or studied quality of life parameters such as the six minute walk distance and patient or physician global assessment. More favorable results were obtained for the parameter of the left ventricular ejection fraction, where a mild improvement was observed in the hawthorn-treated group.

However, the findings regarding hawthorn efficacy in chronic heart failure presented by the majority of investigators speak in favor of hawthorn use. Diehl (1998) found hawthorn extract to be useful in the management of symptoms of mild to moderate chronic heart failure. Rigelsky and Sweet (2002) in their clinical review on the therapeutic uses of hawthorn concluded that the limited data about hawthorn suggests it may be useful in the

treatment of chronic heart failure (NYHA class II) patients. Pittler, Guo and Ernst (2008) presented a comprehensive review on hawthorn extract for treating chronic heart failure. The results suggest that hawthorn extract (when compared with placebo) increases the maximal workload in patients with chronic heart failure. Koch and Malek (2011) also found hawthorn extract useful in chronic heart failure patients.

2.2.4.3. Effect of Hawthorn on Blood, Blood Cells, And Blood Vessels

The effect of hawthorn on **platelet aggregation** was investigated by several authors. Vibes *et al.* (1994) observed *in vitro* that *C. oxyacantha* L. flower extract caused inhibition of thromboxane A_2 biosynthesis by reducing the arachidonic acid metabolism and inhibiting the cyclooxygenase pathway. Shatoor *et al.* (2012) also reported a significant dose-dependent inhibition of platelet function in rats treated with hawthorn *C. aronia* Bosc non Decne extract at a concentration range 100 – 500 mg/kg. Hawthorn significantly altered the bleeding time, the closure time, and the thromboxane B_2 levels.

Similarly, Li *et al.* (2015) observed significant *in vitro* platelet aggregation-inhibitory properties of Chinese hawthorn (*C. pinnatifida* Bunge) leaf extract. All of the 15 evaluated monoterpenes and flavones exerted appreciable antiplatelet activity, with the (6S,7Z,9R)-roseoside, eriodictyol, and 2″-O-rhamnosyl vitexin being the most effective (inhibition of platelet aggregation in rat plasma by 87.18%, 72.92%, and 75.00%, respectively). Eriodictyol was further evaluated as the most active hawthorn extract compound exhibiting antithrombotic activity with significantly prolonged time to thrombus formation in zebrafish.

Contrary to all of the abovementioned works, Dalli *et al.* (2011) found no effect of *C. laevigata* Poir. in Lam. extract on platelet aggregation, closure time or thromboxane B_2 level after administration to healthy volunteers.

The hawthorn **vasodilatation effect** was also observed in several studies. Kim *et al.* (2000) observed relaxation of vascular tone and increased production of cGMP in isolated rat aorta in response to an extract with

flavonoids and procyanidins isolated from hawthorn species *C. oxyacantha L.* and *C. monogyna* Jacq. While the flavonoid fraction containing rutin, hyperoside, and vitexin did not affect the vascular tone, the procyanidine fraction caused an endothelium-dependent relaxation associated with the production of cGMP. Based on their experiments with various inhibitors, the authors proposed the activation of tetraethylammonium-sensitive K^+ channels as a possible mechanism of the endothelium-dependent NO-mediated vasorelaxation (Kim *et al.*, 2000).

Similar results were obtained by Brixius *et al.* (2006) who evaluated the hawthorn extract WS 1442 or three of its fractions (described as A, B, and C) on phenylephrine-precontracted rat aorta and human mammarian artery. No vasodilatation was observed in vessel models with mechanically disrupted endothelium. On the contrary, the most effective was vasodilatation with intact endothelium when fraction C (meaning hydrophilic, essentially flavonoid-free and rich in high molecular weight oligomeric procyanidins) was used. The vasorelaxation was induced by NO liberation from the endothelial cells. Endothelial NO synthase (eNOS) was activated by phosphorylation at serine 1177 (Brixius *et al.*, 2006). This eNOS phosphorylation-mechanism of vasorelaxation was further supported by Anselm *et al.* (2009) in their study on porcine coronary artery rings treated with hawthorn extract WS 1442.

More recently, Topal *et al*. (2013) studied the effect of *C. microphylla* K. Koch leaf extract on **endothelial dysfunction** in aortic rings of diabetic rats. Endothelium-dependent relaxations were much weaker in diabetic rats compared with control, but chronic treatment with low doses of hawthorn extract or pretreatment with a higher dose of hawthorn extract remarkably attenuated the impaired relaxation. The authors suggested a reduction in the formation of pro-inflammatory cytokines, the expression of inducible NO synthase (iNOS), and the prevention of lipid peroxidation as the prevalent effect of hawthorn extract.

Endothelial protection of hawthorn extract WS 1442 against endothelial barrier dysfunction was reported by Bubik *et al.* (2012). Hawthorn specifically interacted with endothelial permeability-regulating

systems by blocking or activating certain pathways (namely by blocking the Ca^{2+}/PKC/RhoA pathway and by activating the cAMP/Epac1/Rap1 pathway) that subsequently led to strong cortical actin rearrangements, which in turn were able to prevent the vascular leakage. The influence of hawthorn extract WS 1442 on protection against vascular barrier dysfunction was also studied by Willer *et al.* (2012) who reported that endothelial calcium signaling seemed to play a great role in this process.

Idris-Khodja *et al.* (2012) noted a beneficial effect of hawthorn extract WS 1442 on aging-related endothelial dysfunction in rats. Hawthorn treatment resulted in a remarkable improvement of both the aging-related endothelium-dependent relaxations and the induction of endothelium-dependent contractile responses, most likely by attenuating the increased oxidative stress as well as the overexpression of the pro-inflammatory enzymes cyclooxygenase COX-1 and COX-2.

An appreciable effect of hawthorn on the endothelial protection against atherosclerosis was also reported by Zhang *et al.* (2013) who found a reduced intima/media thickness ratio and a decrease in pathological changes in the arteries of rats administered with hawthorn for 12 weeks. And similarly, significantly decreased atherosclerotic lesions of aortic root in mice treated with hawthorn fruits were observed by Zhang *et al.* (2014b).

2.2.4.4. Antihypertensive Effect of Hawthorn

Antihypertensive effect of hawthorn is well documented, several clicnical trials focusing on the effect of hawthorn on hypertension have been conducted in the last years.

A significantly lower systolic blood pressure after 8 weeks of hawthorn extract LI 132 administration (600 mg/day) to patients with heart failure was reported by Schmidt *et al.* (1994). Walker *et al.* (2002) also found a very promising effect of hawthorn extract (500 mg/day) in patients with mild hypertension on diastolic blood pressure, even if it was not statistically significant. Four years later, in a randomized placebo-controlled trial, Walker

et al. (2006) treated the patients with higher doses of hawthorn extract (1200 mg/day) for 16 weeks. A significant mean diastolic blood pressure reduction in the hawthorn-treated group was observed at the end of the trial, indicating that the effect of hawthorn on blood pressure is both dose- and time-dependent.

Asgary *et al.* (2004) used mixed leaf and flower extract of an Iranian *Crataegus* species, namely *C. curvisepala* Lind, in a placebo-controlled clinical trial with 92 mildly hypertensive patients with no medication. Treatment with hawthorn for 12 weeks resulted in a significant decrease in both systolic and diastolic blood pressure.

Similarly, Al-Gareeb (2012) conducted his clinical study on 60 newly diagnosed patients with stage 1 hypertension with no medication. After 12 weeks of treatment either with WS 1442 extract (900 mg/day) or placebo, there were significant differences in blood pressure. Both the systolic and the diastolic blood pressure were significantly lower in patients treated with WS 1442 than in the placebo group after 4 weeks of treatment, and their systolic and diastolic blood pressure reached within the range of normal levels at the end of the trial.

The **mechanism** of hawthorn's antihypertensive effect is very likely multifactorial, seeing that there are many active compounds. Beside the vasorelaxing properties of hawthorn (see Ch. 2.2.4.3.) contributing to its hypotensive effect, other mechanisms come into consideration.

In 1987, Uchida *et al.* investigated the effect of condensed tannins from Rhei Rhizoma on angiotensin converting enzyme (ACE) and reported that procyanidins offered very good ACE-inhibitory activity. Lacaille-Dubois, Franck and Wagner (2001) later confirmed that flavonoids and proanthocyanidins from *C. oxyacantha* L. and *C. monogyna* Jacq. exhibit ACE-inhibitory activity, while phenolic acids offered no significant ACE inhibition. An ACE-inhibitory activity was also exerted by the extract from another hawthorn species, *C. microphylla* K. Koch (inhibition by ca 80%) as documented by Sharifi *et al.* (2013). Attard and Attard (2003) demonstrated the ACE-inhibitory activity of oleanolic acid in a hydroethanolic

C. monogyna Jacq. fruit extract, suggesting a minor role of triterpenic acids in the inhibition of ACE activity of hawthorn extract.

Younis *et al.* (2020) also observed a significant ACE-inhibitory activity of hawthorn *C. songarica* K. Koch fruit extract. Normotensive rats were treated for one week with crude extract, aqueous soluble, and *n*-butanol soluble fractions. Remarkable diuretic and saluretic effects of hawthorn were observed especially in rats obtaining the aqueous soluble fraction. As the hypotensive and diuretic actions of this fraction were significantly reduced by atropine treatment, the involvement of the nitric oxide pathway activated by muscarinic receptors should also be taken into consideration (Younis *et al.*, 2020).

Similar conclusions (i.e. the NO pathway activated by muscarine receptors) were earlier stated by Shatoor (2013) in his study of *C. aronia* Bosc non Decne whole plant extract (stems, leaves, and fruits) administered parenterally to normotensive rats. In rats pretreated with a ganglionic blocker (hexamethonium) and an inhibitor of NO synthase (L-NAME), complete elimination of the hawthorn-induced hypotension was observed.

Haydari *et al.* (2017) studied the antihypertensive effect of *C. aronia* Bosc non Decne. fruit extract in rats. Treatment with hawthorn extract for 4 weeks resulted in a significantly lower systolic blood pressure and phenylephrine maximal response as well as significantly higher serum superoxide dismutase and serum glutathione reductase, indicating that the antihypertensive properties of the hawthorn fruit extract may involve both the antioxidant and the NO-releasing effects.

The nitric oxide-dependent hypotensive activity was further confirmed by Zheng *et al.* (2019) in their study on the effect of hawthorn fruit extract on high-salt hypertension. Hawthorn remarkably increased the NO concentration and decreased the concentration of hydrogen peroxide and malonyl aldehyde. This increase of NO synthase activity was especially noticeable in the renal medulla, indicating a possible renal-protective effect of hawthorn.

On the other hand, some studies doubt the nitric oxide pathway as the hypotensive effect of hawthorn. For example, Asher *et al.* (2012) conducted a randomized, controlled trial in prehypertensive and mildly hypertensive patients. Standardized hawthorn extract at higher doses (1000 mg, 1500 mg, and 2500 mg twice daily) was administered for 3 ½ days followed by brachial artery flow mediated dilatation (FMD), an indirect measure of NO release. No evidence of dose-response effect of hawthorn extract on FMD was found, leading authors to suggest that blood pressure lowering effect of hawthorn extract is likely to be mediated via an NO-independent mechanism.

2.2.4.5. Effect of Hawthorn on Plasmatic Lipid profile

Several studies focused on the impact of hawthorn on the plasmatic lipid spectrum and tried to illuminate the possible mechanism of action.

A comparison of the effects of aqueous and ethanolic (extraction solvent 70% ethanol) extracts of *C. pinnatifida* Bunge fruits on lipid spectrum of high-fat diet fed rats was performed by Shao *et al.* (2016). After 4 weeks of treatment, both hawthorn extracts exhibited a dose-dependent manner in reducing triglycerides, total cholesterol, and LDL cholesterol but the efficacy of the extracts was different, with ethanolic extract decreasing the lipid levels at lower doses than the aqueous extract. The difference was likely due to the different phenolic compounds content of both extracts: ethanolic extract and aqueous extract exhibited total phenols content of 1.99% and 0.51% in the same quality of hawthorn fruit, respectively.

The beneficial role of hawthorn triterpenic acids on lipid levels was demonstrated by Lin, Vermeer and Trautwein (2011) who treated hamsters either with hawthorn *C. pinnatifida* Bunge fruit extract, plant sterol esters (PSE), hawthorn plus PSE, oleanolic and ursolic acid combination, or control chow for 4 weeks. Non-HDL cholesterol (plasma VLDL and LDL fractions) was significantly lower in hamsters fed with hawthorn, hawthorn plus PSE, or PSE only than in the control group. The effect on triglycerides was similar, but it did not reach a statistically significant level. Moreover, only in the hawthorn

group a statistically non-significant trend of an increase of HDL cholesterol was observed. The most beneficial effect on cholesterol lowering was the combination of hawthorn extract and PSE.

Al-Gareeb (2012) obtained similar results in his clinical trial with mildly hypertensive adults treated with hawthorn extract WS 1442 (900 mg/day) for 12 weeks. In the hawthorn group, a significant reduction in mean total cholesterol and LDL cholesterol and a small non-significant decrease in serum triglyceride concentraction were observed. On the other hand, HDL cholesterol level was significantly higher in the hawthorn group.

Xu, Xu and Ryan (2009) compared the effect of hawthorn fruit extract and simvastatin on mice fed with a high-cholesterol diet after 8 weeks of treatment. In both hawthorn and simvastatin-treated groups a significant reduction in triglycerides and in the LDL cholesterol/serum cholesterol ratio were observed. However, the reduction of LDL cholesterol was evident only in the hawthorn treated group.

The hypolipidemic **mechanism** of hawthorn is multifactorial as both the cholesterol and triglycerides are influenced. Ye *et al.* (2010) isolated four compounds of ethanolic *C. pinnatifida* Bunge fruit extract with a high inhibitory activity against 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR-CoA), namely quercetin, hyperoside, rutin, and chlorogenic acid. Rutin and chlorogenic acid were predominant in the mixture, representing 1.45% and 0.95%, respectively of the extract content. The *in vivo* results demonstrated a more pronounced lipid-lowering activity of the hawthorn fruit extract than that of pure compounds. This indicates synergistic effects on HMGR-CoA inhibition and lipid-lowering among the active compounds.

Lin, Vermeer and Trautwein (2011) in their *in vitro* part of study incubated intestinal Caco-2 cell culture with increasing concentrations of *C. pinnatifida* Bunge fruit extracted with different solvents (dichloromethane, ethylacetate, acetone, ethanol, and heptane). The dichloromethane extract with the highest content of oleanolic and ursolic acid exerted the strongest inhibitory effects on acylCoA:cholesterol acyltransferase (ACAT) activity. When pure compounds were evaluated, ursolic acid had a slightly lower

ACAT-inhibitory effect than oleanolic acid. Both triterpenic acids had no effect on cell uptake on marked cholesterol or oleic acid from the medium, suggesting that the inhibitory effect is mediated via specific inhibition of the ACAT activity but not via inhibition of the transport of cholesterol esters precursors.

Another hypolipidemic mechanism of hawthorn was suggested by Niu *et al.* (2011) who analyzed the liver of high-fat diet-fed mice treated with hawthorn fruit extract (250 mg/kg) for one week. An increased expression of PPARα as well as of the PPARα-regulated β-oxidation-related enzymes in the liver were observed.

2.2.5. Safety Profile of the Hawthorn Preparations

Seeing as hawthorn fruits are being used as food (e.g. jam, jellies, wine) in many countries, it is reasonable to expect a low risk of medicinally used hawthorn extracts to have adverse effects. According to EMA, hawthorn preparations have no contraindications (with the exception of hypersensitivity to the active substance), drug interactions, or adverse effects (EMA/HPMC/159075/2014; EMA/HPMC/159076/2014).

A very comprehensive systematic review on the **adverse effects** of hawthorn human monopreparations (mainly the extracts WS 1442 and LI 132), which included data from 5577 patients, was presented by Daniele *et al.* (2006). Only 166 adverse events in total were reported, most of them being mild to moderate. The most frequently occurring adverse effects were dizziness, nausea, fall, erythematous rash, gastrointestinal hemorrhage, and circulation failure.

Since then, the situation had not changed much. Wang, Xiong and Feng (2013) in their review of evidence-based approach on hawthorn usage in cardiovascular disease prevention did not mention any new observed adverse effects or drug interactions. Most lately Holubarsch, Colucci and Eha (2018) provided a new evidence-based review on hawthorn extract WS 1442 and

concluded that WS 1442 has a very favorable safety profile even when administered as a part of a polydrug regimen.

As to **pharmacodynamic interactions** of hawthorn, some older studies mentioned in the Botanical Safety Handbook (Gardner and McGuffin, 2013) indicated that hawthorn extracts could potentiate the effects of digitalis glycosides. However, Tankanow *et al.* (2003) performed an interaction study on the coadministration of hawthorn extract and digoxin. Healthy volunteers were treated with both WS 1442 (900 mg/day) and digoxin for 3 weeks. No significant differences were observed in digoxin pharmacokinetic and pharmacodynamic parameters when coadministered with hawthorn.

Holubarsch, Colucci and Eha (2008) also found hawthorn safe for patients on digitalis therapy. Furthermore, no indications of possible drug interactions were observed in patients co-treated with hawthorn extract and ACE inhibitors, beta-blockers, spironolactone, and nitrates (Holubarsch, Colucci and Eha, 2008).

2.3. FOXGLOVE – *DIGITALIS* SPP. – FAM. *PLANTAGINACEAE*

2.3.1. CHARACTERIZATION OF THE GENUS *DIGITALIS* – A PHARMACOGNOSTIC CONTEXT

The genus *Digitalis* currently comprises 23 species including four species of the former genus *Isoplexis*, based on the molecular phylogeny of Bräuchler *et al.* (2004) and Herl *et al.* (2008). Formerly the genus *Digitalis* belonged into the family *Scrophulariaceae,* but since the family genera were regarded as a collection of unrelated lineages and the family was dismembered, *Digitalis* and some other genera of the former *Scrophulariaceae* family were integrated into the family *Plantaginaceae* (Kreis, 2017).

The genus *Digitalis* has two diversity centers, namely the Western species found on the Iberian Peninsula and in Northwestern Africa, and the Eastern species found on the Balkan Peninsula and in Asia Minor. However,

the species *D. purpurea* L. was introduced widely as an ornamental plant and it can readily be found along the West Coast of the USA and Canada as well as in New Zealand (Kreis, 2017).

The common foxglove, *D. purpurea* L. (Fig. 2.5., left), is an herbaceous biennial plant growing up to 150 cm of height. In the first year it forms a ground rosette of broad leaves and in the second year a simple leafy stem with terminal elongated inflorescence. The flowers are of outstanding purple-red color (although also pink, rose, yellow, and white-flowering plants are grown), tubular-shaped and heavily dark speckled on the inside surface. It mostly grows on soils with a calcium content higher than 1% (Tomko *et al.*, 1989).

Woolly foxglove, *D. lanata* Ehrh. (Fig. 2.5., right), is cultivated on peaty, sandy and clay soils. The plant is biennial or perennial, usually 40 – 80 cm tall with oblong-lanceolate leaves. The stem is bare, while in the inflorescence noticeable woolly (hence the name woolly foxglove). The flowers are short-stemmed, with crown of light yellow to whitish color with brown veins, arranged in a dense inflorescence (Mastenbroeck, 1985; Tomko *et al.*, 1989).

Fig. 2.5. *Digitalis purpurea* L. (left) and *Digitalis lanata* Ehrh. (right). From anniesannuals.com and osiva-semena.cz.

Foxgloves are commonly propagated by seeds, which are very small in size. The main sources for cardenolides used in therapy are *D. lanata* Ehrh. and *D. purpurea* L. Both species are cultivated for this purpose, however predominantly *D. lanata* Ehrh. is grown and used as the starting material for

cardenolide isolation and production. In Europe (Netherlands), the crop is sown in mid-April and the leaves are harvested from September to late November and subsequently dried at 50 – 60 °C. Foxglove plants are cultivated to offer a high yield of digoxin, the variety currently cultivated has digoxin content of dried leaves around 2.5 – 3.0‰, which is about 50% higher than the digoxin content of the original accession (Mastenbroeck, 1985; Kreis, 2017).

The Czechoslovak Pharmacopoeia recognized two drugs originating in foxglove species *D. purpurea* L. and *D. lanata* Ehrh. However, **Digitalis lanatae folium** is not monographed in the actual edition of the Czech Pharmacopoeia anymore and therefore only the monograph on **Digitalis purpureae folium** is listed in the current edition of the European Pharmacopoeia. Digitalis leaf consists of the dried leaf of *Digitalis purpurea* L. It contains not less than 0.3% of cardenolic glycosides, expressed as digitoxin, calculated with the reference of the dried drug. The drug has a faint but characteristic odor. The whole leaf is about 10 cm to 40 cm long and 4 cm to 15 cm wide, but being brittle, it often occurs broken. The upper surface is green and the lower surface is greyish-green. The leaf lamina is ovate lanceolate to broadly ovate. The winged petiole is from one quarter as long as to equal in length to the lamina (European Pharmacopoeia 10.0).

The current edition of the European Pharmacopoeia further includes a monograph on common foxglove for the use in homeopathic medicinal products (**Digitalis purpurea ad praeparationes homeopathicas**) and two monographs on the individual foxglove cardiac glycosides (**Digitoxinum** and **Digoxinum**).

2.3.2. PHYTOCHEMISTRY OF FOXGLOVE

Digitalis species offer a wide range of interesting phytochemicals. For example, digitanols, that are structurally resembling the cardioactive glycosides with their 14β-hydroxyl function and a sugar side chain, differ, however, significantly in their basic skeleton structure (Δ^5-pregnene).

Foxglove furthermore contains rare phytosterols, anthraquinone derivates (digiferruginol), and more than 40 flavonoids (among them the highly oxidized digicitrin). However, no secoiridoid glycosides, otherwise commonly occurring in the *Plantaginaceae* family, have been identified in the genus *Digitalis*. This blockage of iridoid biosynthesis was likely replaced by the biosynthesis of cardenolides (Taskova, Godfredsen and Jensen, 2005; Kreis, 2017).

The most important foxglove compounds are **cardioactive glycosides**, more than 100 different cardenolides have been isolated from various foxglove species. Some of the foxglove cardenolides are shown in Fig. 2.6A – C. In *D. lanata* Ehrh, a total of 17 cardenolides have been detected with lanatoside A, C, and E as major compounds, while in *D. purpurea* L., seven cardenolides were identified, including purpurea glycoside A, B, and E. Plants of the genus *Digitalis* produce cardenolides of the 5β-type. Primary glycosides have a sugar component consisting of several sugar moieties. They are stored in the vacuoles and in the case of a vacuole disruption they come into contact with enzymes catalyzing their conversion into secondary glycosides. In the dried material, which can be stored over long periods of time, enzymes capable of hydrolyzing cardenolides are inactive, therefore the conversion of primary to secondary glycosides is not yet completed at this stage (Kreis, 2017 and references therein; Ravia *et al.*, 2020).

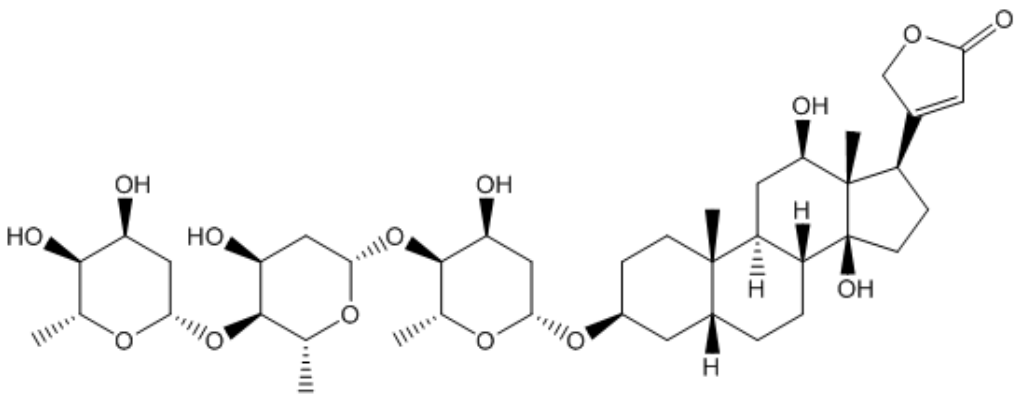

Fig. 2.6A. Some of the cardenolides occurring in the genus *Digitalis*. Digitoxin. Courtesy of doc. Macáková.

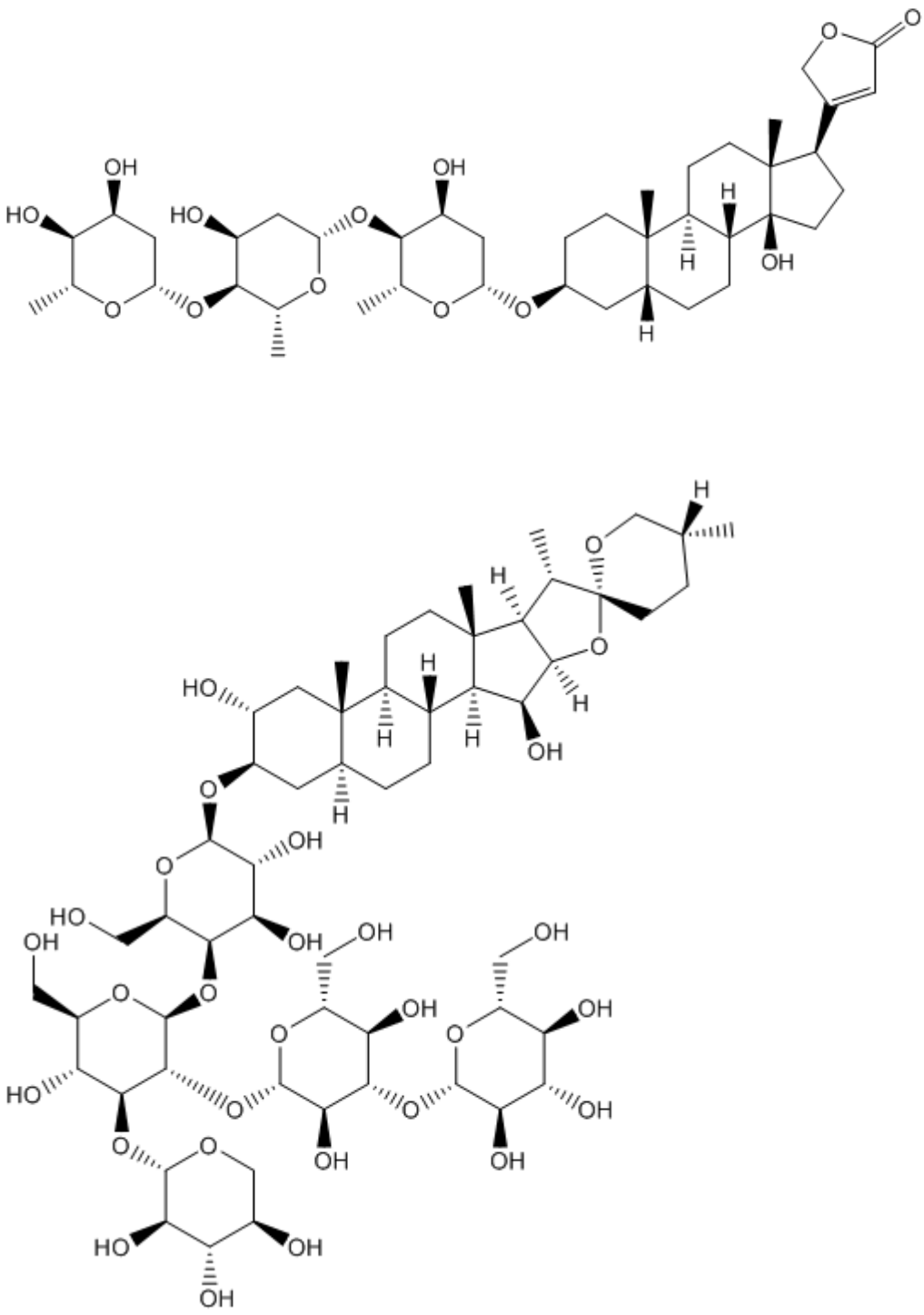

Fig. 2.6B. Some of the cardenolides occurring in the genus *Digitalis*. Up – digoxin, down – digitonin. Courtesy of doc. Macáková.

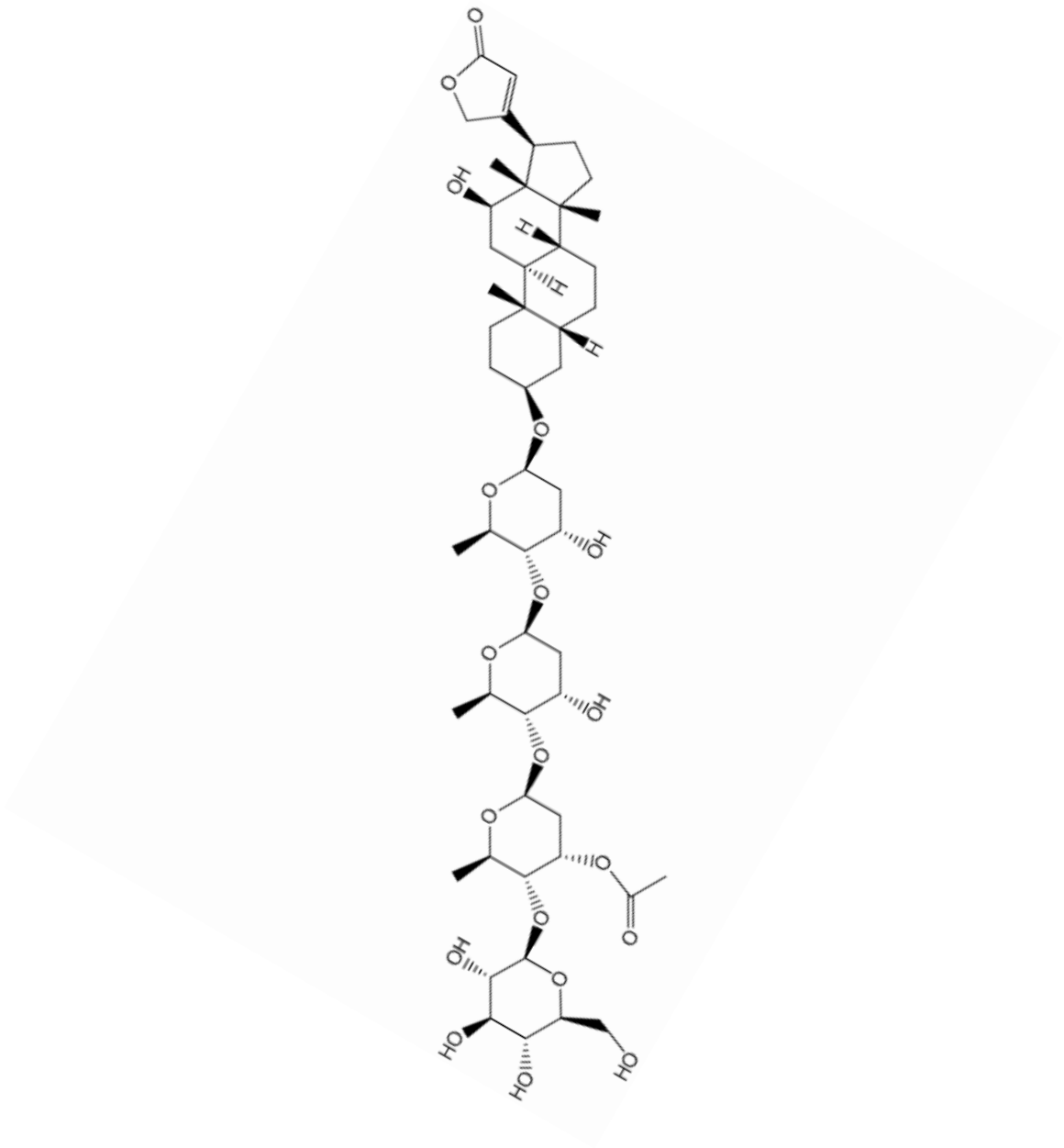

Fig. 2.6C. Some of the cardenolides occurring in the genus *Digitalis*. Lanatoside C.. Courtesy of doc. Macáková.

Among the cardenolides produced by various foxglove species, digoxin and digitoxin are of great economical and medical importance. However, digoxin is as a rule found in a mixture of glycosides with very similar chemical structure such as digitoxin and gitoxin, which makes it difficult to isolate the pure compounds (Novković *et al.*, 2014).

Cardenolide biosynthesis is a complicated process of aglycone synthesis to which then sugar moietes are connected. The **aglycone (genin)**

synthesis starts with precursors such as cholesterol, β-sitosterol, smilagenin, or sodium glycholate that are transformed to pregnenolone. This compound is converted into isoprogesterone that is subsequently isomerized to progesterone. Several specific enzymes catalyzing stereospecific reactions in order to determine that all foxglove cardenolides are β-configurated at carbon atoms C3, C5, C14, and C17 of the steroid skeleton are required during this process. An important enzyme (although not representing a major regulatory point of cardenolide biosynthesis) in the cardenolide biosynthetic pathway is the 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145), responsible for the isoprogesterone synthesis. It was suggested that this enzyme may also possess the Δ^5-Δ^4-ketosteroid isomerase activity needed for the isomerization of isoprogesterone to progesterone. This activity was later confirmed. The first committed step in the branch pathway leading to cardenolides is, however, the subsequential 5β-reduction of progesterone to 5β-pregnan-3,20-dione, a reaction catalyzed by the enzyme progesterone 5β-reductase (EC 1.3.1.3), which is considered to be a key enzyme in cardenolide biosynthesis. This enzyme is encoded by at least two genes (*P5βR* and *P5βR2*) in *D. purpurea* L., and while the expression of *P5βR* does not vary under stress conditions or chemical elicitors, the expression of *P5βR2* is highly responsive to these factors. Both of these enzymes were detected not only in cardenolide-accumulating foxglove leaves but also in cardenolide-free permanent cell suspension cultures initiated from foxglove leaf tissue (Seidel, Kreis and Reinhard, 1990; Finsterbusch *et al.*, 1999; Ernst *et al.*, 2010; Pérez-Bermúdez *et al.*, 2010; van Wietmarschen *et al.*, 2016 and references therein).

Sugar moietes (up to 5 units in the sugar side chain per cardenolide molecule) are subsequently attached at the C3 carbon atom of the aglycone steroid skeleton to form primary glycosides such as the lanatosides. The sugar components may be represented by commonly occurring sugars, such as D-glucose or L-rhamnose, but also by those more unusual and generally confined to the various *Digitalis* species, such as D-digitalose, D-digitoxicose, or D-diginose. Usually a molecule of D-glucose is attached as the terminal sugar unit. Drying or mechanical disruption of the leaf material is accompanied by partial hydrolysis of these primary glycosides. Both the terminal glucose and the acetyl group will be released and the secondary

glycosides such as digoxin are formed. The hydrolysis of primary glycosides is catalyzed enzymatically by glucohydrolases with different substrate specificity and furthermore varying between different foxglove species (for example, glucohydrolase II exhibits high specificity towards cardenolide disaccharides, especially to those with a 1-4-β-linked glucose-digitoxose moiety). The importance of the sugar side chain lies in the modulation of the pharmacological effect of the cardioactive glycoside. Contrary to the remarkably more stereochemically complicated aglycones, the sugar moieties have been successfully surveyed in the stereochemical field and synthetized *de novo*. Changes in the sugar side chain are associated with a fundamental shift in their pharmacological effect from cardioactive to another such as anticancer (Tomko *et al.*, 1989; Kreis and May, 1999; Hornberger *et al.*, 2000; Ganapaty *et al.*, 2009; Pérez-Bermúdez *et al.*, 2010; Wang *et al.*, 2011a; van Wietmarschen *et al.*, 2016; Kreis, 2017).

As cardenolides primarily act as a plant defense against pathogens and herbivores, their synthesis has been shown to be influenced by various **environmental factors**. For example, Stuhlfauth, Klug and Fock (1987) reported an increase of cardenolides content by 160% in foxgloves grown at higher concentrations of carbon dioxide (CO_2; 1000 ppm). CO_2 enrichment increased mainly the yield of the pharmacologically relevant major compound digoxin, whereas contents of two other cardenolides were decreased. The addition of exogenous hydrogen peroxide (0.5 and 1 mmol/L) directly to the medium of undifferentiated foxglove cell cultures also resulted in a stimulated cardenolide production, whereas the addition of catalase markedly reduced it (Paranhos, Fernández-Tárrago and Corchete, 1999). Further, mechanical wounding of foxglove leaves was reported to lead to a significant increase in cardenolides content in the plants. The highest content of cardenolides in the leaves was observed approximately 4 hours after wounding of the plant (Pérez-Bermúdez *et al.*, 2010).

The influence of naturally occurring **phytohormones** on cardenolide production in foxglove has also been investigated. Ethylene was shown to decrease the cardenolide synthesis (by 60%), whereas gibberelic acid had no significant effect on the accumulation of cardenolides in *in vitro* cultures of

different foxglove species (Berglund and Ohlsson, 1992; Gavidia, Segura and Pérez-Bermúdez, 1993).

Seasonal **variation** in the content of cardenolides and the expression of the gene encoding the progesterone 5β-reductase in 10 natural populations of foxglove species *D. obscura* L. was investigated by Roca-Pérez *et al.* (2004). Cardenolide contents exhibited more or less stable values in terms of their chemical composition regardless of the population studied or the degree of leaf maturity. They did, however, change in quantity terms during the growth season: the lowest production was recorded in May, followed by a fast cardenolide accumulation in summer, a decreasing phase in autumn, and a stationary phase in winter. The gene encoding progesterone 5β-reductase showed increased expression from February to July, while a reduction in its expression was observed toward autumn.

The importance of a sufficiently long light period for the cardenolide synthesis was also confirmed by Eisenbeiß, Kreis and Reinhard (1999) who reported a significant gradual decrease in cardenolide production in foxglove shoot cultures transferred to permanent darkness. After 12 weeks of this treatment, cardenolide content was non-detectable.

Variation of foxglove cardenolides during the growth of plants was reported by Balbaa, Hilal and Haggag (1970). The maximum yield of cardenolides was obtained from leaves collected from woolly foxglove plants in the stage just before flowering. Braga *et al.* (1997) also investigated the change of cardenolides during plant growth. Younger plants (12 months old) were shown to have higher total amount of cardenolides than older plants (18 months old). Interestingly, foxglove plants treated with cold shock (windows opened at the coldest time of the night) during the growth season had significantly higher digoxin content, while the content of gitoxin was significantly decreased (van Wietmarschen *et al.*, 2016).

Significant variation of cardenolide content was also observed within and among natural foxglove populations. The amounts of digitoxigenin and gitoxigenin obtained from foxglove leaves sampled from six different Sardinian locations varied considerably (11 – 241 mg/kg of fresh weight and

4 – 178 mg/kg of fresh weight, respectively; Nebauer, Del Castillo-Agudo and Segura, 1999; Usai, Matzei and Marchetti, 2007).

2.3.3. Medicinal Use of Foxglove

Foxglove was introduced to modern medicine by the botanist and physician William Withering who used it for the treatment of patients suffering from "dropsy" (congestive heart failure) and in 1785 published the clinical importance of *Digitalis*. In the homeopathic system of medicine, *Digitalis* was introduced in 1803 by Samuel Hahnemann. In 1954, the American Food and Drug Agency granted permission for the use of *Digitalis* cardioactive glycosides (Ganapaty *et al.*, 2009; Sharma and Purkait, 2012; Kreis, 2017). Contrary to all other medicinal plants reviewed in this work, no over the counter foxglove herbal preparations are on the market because of the nature of its active compounds.

As of 2020, in the Czech Republic two cardiac glycoside-containing medicinal products Digoxin Léčiva 0.125 mg tablety and Digoxin Léčiva 0.250 mg tablety are registered with SUKL for therapeutic use in adult patients and in children over 3 years of age with the following health conditions: (1) heart failure of hypertrophic and dilated heart with chronic pressure and volume overload, especially with a concomitant atrial fibrillation with rapid ventricular response, or (2) atrial fibrillation with rapid ventricular response, especially with a concomitant heart failure if cardioversion is not indicated (Digoxin Léčiva 0.250 mg SPC).

2.3.4. Effects of Foxglove on Selected Cardiovascular Conditions

2.3.4.1. Antioxidant Effect of Foxglove

The *in vitro* antioxidant properties of digoxin were observed by Rogozhin and Verkhoturov (1998). Digoxin was found to bind with the enzyme-substrate complex of horseradish peroxidase and *o*-dianisidine, and

inhibited the oxidation of *o*-dianisidine in the intermediate form of a stable semioxidized product. Katanić *et al.* (2017) investigated the antioxidant effect of methanolic extracts of two foxglove species, namely *D. ferruginea* L. var. *ferruginea* and an endemic Turkish species *D. lamarckii* Ivan. Both of them exhibited good antioxidant properties in all tests performed (DPPH, ABTS radical scavenging, reducing power, and β-carotene bleaching test). *D. lamarckii* Ivan extract offered stronger antioxidant properties, probably due to a higher flavonoid content.

Silva *et al.* (2017) investigated the differences of lipid membrane modulation and oxidative stress by digoxin and its semisynthetic derivate named BD-21 (21-benzylidene digoxin) and reported that lipid peroxidation and superoxide dismutase activity were decreased by digoxin and BD-21. Glutathione content and catalase activity were not affected by either of the tested cardiotonic steroids.

Similarly, de Souza Gonçalves *et al.* (2019) used low concentrations of digoxin and three of its semisynthetic derivates (namely BD-14, BD-15 and BD-16) in murine neuroblastoma cells neuro-2a, which were exposed to partial chemical sodium azide-induced ischemia followed by reperfusion. Cells pretreated with the evaluated compounds at a concentration of 150 nmol/L showed a decrease of the redox state, lipid peroxidation, hydrogen peroxide content, and glutathione peroxidase activity. The activity of superoxide dismutase remained higher than in control cells, and digoxin and its derivates did not have any influence on glutathione levels.

On the other hand, Shahouzehi, Nasri and Masoumi-Ardakani (2018) observed a deterioration of antioxidative parameters of heart tissues in iron-overloaded rats administered with digoxin at doses of 1 and 5 mg/kg for 4 weeks. The level of malonyl dialdehyde (a lipid peroxidation marker) was significantly elevated in digoxin treated rats. The elevation of malonyl dialdehyde was much higher in animals administered with higher dose of digoxin (5 mg/kg) when compared to 1 mg/kg of digoxin. Furthermore, digoxin treatment resulted in an increased superoxide dismutase and glutathione peroxidase quantity in heart tissue.

2.3.4.2. Direct Effect of Foxglove on Myocardium

Cardenolides possess **positive inotropic** and **negative chronotropic** properties, in other words, low concentrations of cardenolides are used to increase the contractile force of the heart and decrease its rate of contraction.

The main **mechanism** of action of cardenolides is the inhibition of the cellular Na^+/K^+ ATPase. Cardiac glycosides bind to the subunit α of the enzyme. Inhibition of the ATPase leads to an increase of sodium and a decrease in potassium concentration in cardiomyocytes, thus the membrane potential is reduced, and the formation of spontaneous action potentials is enabled. The increase of intracellular sodium concentration is accompanied by an increase of calcium influx and an increase in contractility. This inhibition of Na^+/K^+ ATPase is highly specific, no other ATPase has been found to be altered by physiologically active concentrations of cardenolides. However, cardenolides (namely digitoxin, digoxin, and ouabain) have been shown to also bind to other proteins – transcriptional regulator steroid receptor coactivators 3 and 1, causing inhibition of cell proliferation (Godfraind, 1984; Lincová and Farghali, 2007; Wang *et al.*, 2014; Askari, 2019).

The pharmacological activity of cardenolides is strongly dependent on their chemical structure. Choay, Corabeouf and Deroubaix (1978) suggested that the aglycone configuration in C5 and C17 positions is associated with different activity of two cardenolides, namely mitiphylline and holarosine B. In animal models (rat brain, cat left atrial strips) it was later shown that for the inotropic activity of foxglove cardenolides, the C17 side chain carbonyl oxygen distance of a given genin in relation to its position in the reference compound digitoxigenin was the primary determinant of its biological activity (Ahmed *et al.*, 1983; From *et al.*, 1984).

Due to their inotropic properties, cardenolides are mainly used in heart failure treatment. The use of cardenolides in the treatment of **arrhythmias** is limited nowadays. For example, Sethi *et al.* (2018) performed a large meta-analysis of clinical trials focused on treatment of atrial fibrillation and atrial flutter with digoxin. 28 trials with 2223 participants

were included. Based on their findings, the clinical effect of digoxin on all-cause mortality, quality of life, heart failure, and stroke remained unclear. The long-term effect of digoxin was also unclear, as all included trials offered only a short-term follow-up. In reducing of the heart rate, digoxin seemed to be superior to placebo but inferior to beta blockers.

Schupp *et al.* (2019) reported that in patients with an implantable cardioverter defibrillator suffering from atrial fibrillation and heart failure, digitalis treatment is associated with increased rates of recurrent ventricular tachyarrhythmias.

The efficacy of cardiac glycosides, regularly used in therapy of **heart failure** for their inotropic action for more than 200 years, still remains unclear. Aside from digitoxin and digoxin, the cardenolides digoxigenin, gitoxigenin, strophantidin, and ouabaigenin are also among the most prescribed commercial drugs for heart failure (El-Seedi *et al.*, 2019).

According to current guidelines for treatment of heart failure, cardiac glycosides (digoxin in case of the Czech Republic) are considered for treatment in patients with resistant symptoms even when on standard ACE and/or beta blockers therapy (Hradec, 2018). On an animal model (rats with heart failure and surgically induced myocardial infarction), dos Santos *et al.* (2013) showed that digitoxin treatment actually exerts more beneficial effects with the increasing severity of the disease. Digitoxin reduced mortality up to 43% in animals that showed parameters associated with a poor prognosis. On the other hand, in less severe cases, digitoxin led to an increased mortality of the animals (by approximately 20%).

Digoxin was shown to be a useful agent in heart failure patients. The PROVED trial results showed that even mild to moderate heart failure patients remaining on digoxin therapy exerted better achievements in the exercise duration test and demonstrated a lower percentage of treatment failures when compared to patients without digoxin therapy (Uretski *et al.*, 1993). Worsening of health condition was also observed in chronic heart failure patients (NYHA functional class II and III) treated with ACE inhibitors

when digoxin was switched to placebo during the 12 weeks-lasting RADIANCE trial (Packer *et al.*, 1993).

Hood *et al.* (2004) in their meta-analysis that included 13 clinical trials with 7896 congestive heart failure patients reported a positive effect of digoxin on the clinical status of symptomatic patients and reduction of hospitalization incidence, although no effect of digoxin on the long-term mortality was observed. The effect of digitalis treatment in patients with heart failure does not depend on gender, and digoxin also exerts clinical benefits in elderly heart failure patients (older than 65 years of age) with sinus rhythm (Carosella *et al.*, 1996; Domanski *et al.*, 2005).

Due to the lack of relevant data on the possible beneficial effect of digitoxin in heart failure treatment, Bavendiek *et al.* (2019) started a randomized, double-blind, placebo-controlled study, designed to demonstrate whether digitoxin on top of standard of care treatment improves mortality and morbidity in patients with heart failure and a reduced ejection fraction. This study was named DIGIT-HF (DIGitoxin to Improve ouTcomes in patients with advanced chronic Heart Failure). Only patients with chronic heart failure (NYHA functional class III-IV) and left ventricular ejection fraction ≤40% or patients in NYHA functional class II and left ventricular ejection fraction ≤30% were enrolled. A total number of 2190 patients in approximately 50 study centers in Germany and Austria were included. DIGIT-HF should provide important evidence, whether the cardiac glycoside digitoxin reduces the risk for all-cause mortality and/or hospital admission for worsening of heart failure in patients with advanced chronic heart failure with reduced ejection fraction, when concluded.

2.3.4.3. Effect of Foxglove on Blood, Blood Cells, and Blood Vessels

The influence of foxglove on **platelet aggregation** has yet to be fully understood and contradictory results regarding this topic have been reported throughout the time. For example, Ware *et al.* (1985) used blood samples obtained from healthy male volunteers before, and at 3, 5, and 7 weeks after

the beginning of digoxin therapy (0.375 mg/day). Digoxin treatment resulted in a significantly reduced total volume and mean size of platelet aggregates formed in response to ADP. The addition of nifedipine (a Ca^{2+} channel blocker), however, returned the parameters to baseline levels.

On the other hand, Pettersen *et al.* (2002) evaluated the impact of digitoxin on platelet function in healthy volunteers administered with descending concentrations of digitoxin (starting with 0.6 mg and then reduced to 0.1 mg) for 10 days. No significant differences were observed between volunteers obtaining digitoxin and placebo in all parameters determined (expression of activated fibrinogen receptor, von Willebrand's factor receptor, P-selectin, formation of platelet-platelet and platelet-leukocyte aggregates, and particle sizes).

And finally, Chirinos *et al.* (2005) investigated the effect of digoxin on platelet aggregation in patients with nonvalvar atrial fibrillation. Patients on digoxin therapy exerted increased levels of P-selectin expression in platelets when compared to patients without digoxin. Further, an increased endothelial activation was also observed in patients with digoxin. These findings were also confirmed by the results of large cohort studies that reported an increased risk of ischemic stroke in patients with atrial fibrillation on digoxin therapy. Digoxin patients exhibited a 1.4-fold increase in the risk of ischemic stroke (Chang *et al.*, 2013; Chao *et al.*, 2014). Lai *et al.* (2018a) reported an increased risk of ischemic stroke in patients on digoxin therapy when compared to patients obtaining amiodarone.

All of the abovementioned observations, with the exception of Ware *et al.* (1985), were surprisingly supported by Pastori *et al.* (2018), who performed the evaluation of digoxin's effect on platelet aggregation in healthy volunteers and in patients with atrial fibrillation both *in vitro* and *in vivo*. *In vitro*, platelets from healthy subjects and patients with atrial fibrillation were incubated with different digoxin concentrations in the range 0.6 – 2.4 ng/mL, with or without the prestimulation with collagen. In patients with atrial fibrillation, an increased basal platelet activation was observed when compared to healthy subjects. Digoxin, at the highest concentration used, led to calcium mobilization and platelet aggregation in patients with

atrial fibrillation but not in healthy subjects. When compared *in vivo*, patients with atrial fibrillation on digoxin therapy were shown to have higher values of the 11-dehydrothromboxane B2, a marker of platelet activation, in urine than healthy volunteers (Pastori *et al.*, 2018).

As to the **mechanism** by which foxglove affects the platelet aggregation, the information is scarce. Le Quan-Sang *et al.* (1987) investigated the impact of digoxin on changes in cytosolic Ca^{2+} concentration in platelets and erythrocytes obtained from healthy volunteers before and during treatment with digoxin (0.250 mg) for six days. While digoxin had no effect on the calcium concentration in erythrocytes, the value of platelet cytosolic Ca^{2+} concentration was significantly increased. This change in calcium concentration was not a consequence of a reduced membrane sodium gradient but reflected either the overload of intracellular Ca^{2+} stores or enhanced *in vivo* stimulation by hormones or neurotransmitters. The involvement of calcium in platelet aggregation influenced by digoxin was also reported by Pastori *et al.* (2018) who observed increased platelet aggregation induced by a supratherapeutic dose of digoxin. This aggregation was mediated via calcium-related phospholipase A2 phosphorylation.

2.3.4.4. Antihypertensive Effect of Foxglove

Currently, the use of foxglove cardioactive glycosides (namely digoxin) in the Czech Republic is limited only to specific cases of heart failure or atrial fibrillation. However, several reports of the antihypertensive properties of foxglove can be found. For example, Ayachi and Hall (1976) used digitoxin (0.5 mg/day) in unilaterally nephrectomized rats with bilateral adrenal compression for 2 weeks, starting on day 8 postoperative. Rats were also administered with sodium chloride solution in order to induce hypertension. In the digitoxin treated group, 80% of rats were hypertensive at the end of the trial vs. 100% in the adrenal compression only group, and 30% in the control group. Furthermore, digitoxin treatment not only delayed the onset of hypertension, it also attenuated the hypertension parameters, such as systolic blood pressure, incidence and severity of renal lesions, and heart weight.

Neither adrenal enlargement nor thymic atrophy occurred in the rats treated with digitoxin.

Digitoxin administered to rats at doses of 4-5 mg/kg for one week only also led to a decrease in systolic arterial pressure in spontaneously hypertensive rats, while this treatment had no effect of blood pressure of normotensive rats (Ayachi and Brown, 1980).

Manunta *et al.* (2000) treated rats with ouabain, ouabagenin, digoxin, and digitoxin in infusions (30 μg/kg/day) for 5 weeks. While rats treated with ouabain and ouabagenin had significantly increased mean arterial blood pressure, rats obtaining digitoxin exhibited a significant decrease in mean arterial blood pressure. A decreasing tendency in mean arterial blood pressure was also observed in rats treated with digoxin, the result was, however, not statistically significant. Moreover, when rats with ouabain-induced hypertension were administered digoxin, their values of mean arterial blood pressure decreased to control levels.

Diuretic effect probably contributes to antihypertensive properties of foxglove cardenolides only to a small extent. Nearly 90 years ago Kellum (1932) reported that digitalis administered to healthy volunteers in therapeutic doses seemed to exert only a slight diuretic effect. Similarly, Navarro *et al.* (2000) observed a slight diuretic and natriuretic effect in rabbits treated with methanolic extract of *D. purpurea* L. spp. *heywoodii*, containing mostly gitoxin derivates, at doses of 15 and 30 mg/kg.

In 1991, a cardiotonic steroid structurally identical with the cardenolide ouabain was isolated from human plasma (Hamlyn *et al.*, 1991). Since then, many works focusing on the structure and hormonal roles of these endogenous digitalis-like factors in hypertension were presented (e.g. Devynck *et al.*, 1983 or reviews by Bagrov and Shapiro, 2008; Paczula, Więcek and Piecha, 2016). However, in 2019 Askari in his distinctly critical review regarding digitalis questioned both the role of such substances as well as their characterization as hormones.

2.3.4.5. Effect of Foxglove on Lipid Profile

Foxglove is primarily not known for its beneficial effect on the serum lipid profile. When searching the literature on this topic, I found only one study dealing with foxglove. Ebaid *et al.* (2006) investigated the effect of digitonin on dyslipidemia induced by high sucrose intake in rats. After 4 weeks of treatment with 30% sucrose, rats were administered with a single intra-gastric dose of digitonin (15 mg/kg). Digitonin decreased the free fatty acids level, and reduced triglyceride and VLDL cholesterol concentrations to the values of control rats. Digitonin also increased the value of HDL cholesterol/triglycerides ratio.

A promising neuroprotective effect of digoxin and its semisynthetic derivates related to lipid contents was observed by de Souza Gonçalves *et al.* (2019) in their work focused on neural partial ischemia/reperfusion injury on a murine neuroblastoma cells neuro-2a model. When the cells were treated with sodium azide to induce ischemia, the content of phospholipids and cholesterol decreased. However, the cells pretreated with low concentrations of digoxin showed an increase in both phospholipids and cholesterol levels.

Much earlier than this, digitonin was reported to also exert an interesting influence on phospholipids. Tsao and Cornatzer (1967) observed that digitonin and digitoxin in a mammalian cell culture influenced the metabolism of phospholipids. Digitonin stimulated the incorporation of inorganic orthophosphate into the phospholipids. Further, digitonin caused a linear increase in the specific activity of phosphatidylinositol and diphosphatidylglycerol. Digitoxin stimulated the incorporation of inorganic orthophosphate into phosphatidylinositol and sphingomyelin fraction of human heart culture cells.

The effect of digitonin on Langmuir monolayers of phospholipids was investigated by Orczyk, Wojciechowski and Brezesinski (2017). Digitonin was able to adsorb at the air/water interface, both bare and covered with the uncompressed phospholipid monolayers. Digitonin reacted with the monolayers in a very selective way and did not cause any disruptive effects on the monolayers.

2.3.5. SAFETY PROFILE OF THE FOXGLOVE PREPARATIONS

According to Botanical Safety Handbook (Gardner and McGuffin, 2013), the genus *Digitalis* is considered to be in the safety class 3, meaning that the herb should be used only under the supervision of an expert qualified in the appropriate use of this substance.

The cardiac glycosides have many **adverse effects** that are largely dose related and require careful monitoring of drug levels. The most common side effects include dizziness, fatigue, headache, anxiety, gastrointestinal upset, change in taste, and blurred vision (photopsia was also reported). Severe side effects include seizures and coma, heart block, atrial and ventricular arrhythmias, and sudden cardiac death. Digoxin toxicity was reported to be significantly more frequent in patients over 80 years of age than in younger patients. On the other hand, cardiac glycosides have not been reported to cause any clinically significant liver injury (Taylor, 1985; Carosella *et al.*, 1996; Oishi *et al.*, 2006; Ramlakhan and Fletcher, 2007; LiverTox, 2018).

A large study (17897 patients) on the effect of digoxin on mortality in patients with atrial fibrillation concluded that the risk of death and sudden death was significantly higher in new digoxin users. Patients with serum digoxin concentration ≥1.2 ng/mL also had a 56% increased hazard of mortality compared with those not on digoxin. For patients both with and without heart failure, for each 0.5 ng/mL increase in serum digoxin concentration, the risk of death was 19% higher compared to patients without digoxin therapy (Lopes *et al.*, 2018). An increased risk of all-cause death in patients with non-valvular atrial fibrillation on digitalis therapy was also reported by Kodani *et al.* (2019). Significant risk factors in digitalis treated patients were older age, male sex, heart failure, coronary arterial disease, and lower body mass index.

Needless to say, Gonzalez-Loyola *et al.* (2018) in their cohort study, which included 13334 heart failure patients, found no association of digoxin or diuretics with higher mortality in these patients, which is a contradictory observation to the abovementioned studies.

The high **interaction potential** of *Digitalis* and digoxin has been recognized in several works. For example, Botanical Safety Handbook classified the genus *Digitalis* as herb belonging to the interaction class C meaning herbs with clinically relevant interactions. Digoxin has a narrow therapeutic index, its serum concentrations should be within the range of 0.5 – 0.8 ng/mL. Supratherapeutic concentrations of digoxin may cause severe adverse effects (Lincová and Farghali, 2007; Gardner and McGuffin, 2013). The requirement of an experienced prescribing physician where digoxin therapy is considered is also mentioned by Ewy (2015) who noticed that digitalis toxicity was and is almost always iatrogenic.

On the **pharmacokinetic level**, the mechanism of digoxin interactions with other drugs is mainly due to the interference with the P-glycoprotein transporter. Inhibitors of P-glycoprotein can enhance oral bioavailability of digoxin by decreasing its efflux from the enterocytes into the lumen of the intestine and decrease its active tubular secretion into the urine in the kidney. Well-known P-glycoprotein inhibitors are, for example, verapamil and amidarone used in cardiology, or macrolide antibiotics. Concomitantly administered verapamil and digoxin were shown to lead to an increase of serum digoxin concentration by 70% (Marcus, 1985; Mladěnka *et al.*, 2018).

Pharmacodynamic interactions of digoxin with other drugs have an impact on the pharmacological performance of digoxin by increasing or decreasing its therapeutic effect. For example, the additive negative chronotropic effect of beta blockers combined with digoxin can result in bradycardia or even an atrioventricular block (partial or complete interruption of impulse transmission from the heart atria to the ventricles), digoxin administered concomitantly with amiodarone may cause dysrhythmias. Antikaliuretic drugs amiloride and triamterene were reported to increase the inotropic actions of cardenolides. Interestingly, licorice was also reported to be able to increase digoxin toxicity. On the other hand, concomitant administration of digoxin with herbal preparations of another cardiotonic herb, hawthorn, was shown to be safe for the patients (Seller *et al.*, 1975; Tachijan, Maria and Jahangir, 2010; Holubarsch, Colucci and Eha, 2018; Mladěnka *et al.*, 2018).

2.4. MOTHERWORT – *LEONURUS* SPP. – FAM. *LAMIACEAE*

2.4.1. CHARACTERIZATION OF THE *LEONURUS* GENUS – A PHARMACOGNOSTIC CONTEXT

According to the Assessment report on *Leonurus cardiaca* L., herba (EMA/HPMC/127430/2010), the *Leonurus* genus comprises 24 species, which are divided in 3 sections and in 5 sub-sections. The taxonomy of *Leonurus* is complicated, several different methods for identifying the individual *Leonurus* species have been suggested (Yang *et al.*, 2011 – molecular analysis based on ITS sequences; Marciniuk *et al.*, 2013 – molecular analysis based on RAPD markers; Pitschmann *et al.*, 2017 – anatomical characteristics, such as corolla shape and trichomes; Garran *et al.*, 2019 – discriminating metabolite isomerism strategy).

In this work, all of the names of the motherwort species are used as presented by the authors of the respective studies, however, the official botanical names are as follows:

- *Leonurus cardiaca* L. – motherwort cardiaca
- *Leonurus japonicus* Houtt. (synonyms: *Leonurus heterophyllus* Sweet, *Leounurus artemisia* (Lour) S.Y. Hu) – Chinese motherwort
- *Leonurus cardiaca* L. var. villosus Desf. (synonyms: *Leonurus quinquelobatus* Desf.)

L. japonicus Houtt. and *L. cardiaca* L. (Fig. 2.7.) are the typical species in Eastern Asia and in Europe, respectively. *L. cardiaca* L. is a perennial herb widespread in Europe, commonly found in rural areas throughout the lowlands and foothills. The plants are sparsely pubescent, reaching the height of 50 – 200 cm, pink blooming in June and July. Motherwort is even recognized as one of the most valuable melliferous plants (Wojtyniak, Szymański and Matławska, 2013; Sermukhamedova *et al.*, 2017; Zhang *et al.*, 2018).

Fig. 2.7. *Leonurus cardiaca* L. upper left – whole plant, upper right – closeup of flowers. *Leonurus japonicus* Houtt. down left – whole plant, down right – closeup of flowers. From botany.cz, minnesotawildflowers.info, botany.hawaii.edu, alchetron.com.

For medicinal use (**Leonuri cardiacae herba**), the blooming tops of the herb up to 40 cm are used only. They are collected at the beginning of the flowering. The thickness of the stems must not exceed 4 mm in diameter. The plant material can be whole or cut; it should be dried at a temperature of 35 °C. The drying temperature seems to be important especially for the storing of labdane diterpenes, as they were reported to decompose as low as 40 °C (Fuchino *et al.*, 2013).

For the quality control of the herb, different chemical compounds are applied as standards for various *Leonurus* species. For the Chinese **Herba leonuri**, the alkaloids leonurine (> 0.05%) and stachydrine, and for the

European **Leonuri cardiacae herba**, a minimum of 0.2% of flavonoids, expressed as hyperoside; these are calculated with reference to the dried drug and respectively used as quality control markers (according to the relevant authorities: National Pharmacopoeia Committee, 2015 and European Pharmacopeia 10.0).

2.4.2. PHYTOCHEMISTRY OF MOTHERWORT

The characteristic compounds of the *Leonurus* genus are alkaloids, diterpenes, triterpenes, iridoide glycosides, flavonoids, phenolic compounds, and sterols (Fig. 2.8.). **Essential oils** are also present in traces (about 0.03%). In the essential oil from the leaves of *L. cardiaca* L., mainly caryophylene (39.8%) and *α*-humulene (34.8%) occur. The essential oil of the aerial parts of *L. panzeroides* Popov contains mainly eugenol (30.93%) and *p*-vinyl-guaiacol (15.77%), while that of *L. turkestanicus* V. I. Krecz & Kuprian is rich in thymol (40.10%) and octen-3-ol (13.07%; Mamadalieva *et al.*, 2017; Sermukhamedova *et al.*, 2017; Fierascu *et al.*, 2019 and references therein).

The *Leonurus* **alkaloids**, especially leonurine and stachydrine (Fig. 2.8.), are of great interest because of their pharmacological properties, as will be described in detail in the latter chapters. The guanidine alkaloid leonurine is more typical for the Chinese motherwort than for motherwort cardiaca: the respective content of leonurine was determined for the aerial parts of *L. japonicus* Houtt. within the range of 0.001% – 0.102% compared to a value of 0.0068% or not even detected for aerial parts of *L. cardiaca* L. (EMA/HPMC/127430/2010; Kuchta, Ortwein and Rauwald, 2012; Zhang *et al.*, 2018). The process of synthesizing of artificial leonurine with the same pharmacological properties as the naturally occurring alkaloid was also reported (Cheng *et al.*, 1979), and this synthetic leonurine or its derivates were tested in several studies (see Ch. 2.4.4.2.).

Stachydrine represents alkaloids with a quaternary ammonium cation. It is another important pharmacologically active compound found not only in the *Leonurus* genus but also in several other species within the family

Lamiaceae, or even belonging into other plant families. The content of stachydrine in the plant is higher than that of leonurine; in the aerial parts of *L. cardiaca* L. it was reported within the range of 0.6 – 1.5%, and within the range 0.2 – 1.0% for the aerial parts of *L. japonicus* Houtt. (Kuchta, Volk and Rauwald, 2013; Zhang *et al.*, 2018).

Bitterly tasting **diterpenes** of the *Leonurus* genus usually belong to the labdane type. So far, around 147 labdane diterpenes were isolated from various *Leonurus* species; more than 90 of them can be found in *L. japonicus* Houtt. (Zhang *et al.*, 2018; Miao *et al.*, 2019). Knöss and Zapp (1998) investigated the accumulation of furanic labdane diterpenes in *L. cardiaca* L. The labdane diterpenes were produced and accumulated only in the aerial parts, especially in the leaves and flowers. The highest content of labdane diterpenes was found in the young leaves and buds. Labdane diterpenes are at least partially stored in the peltate glandular trichomes.

According to Zhang *et al.* (2018), anti-inflammatory properties are the main action of the labdane diterpenes. However, in the last years a fair number of new labdane diterpenes were isolated from various *Leonurus* species, and several of them exhibit beneficial pharmacological actions, such as cytoprotection, anti-aggregation, antioxidant properties, and inhibition of acetylcholinesterase (Satoh *et al.*, 2003; Boalino *et al.*, 2004; Giang *et al.*, 2005; Cai *et al.*, 2006; Romero-Gonzáles *et al.*, 2006; Agnihotri *et al.*, 2008; Fuchino *et al.*, 2013; Xiong *et al.*, 2015b; Lai *et al.*, 2018b; Jiang *et al.*, 2019).

Several **flavonoids** were isolated from the *Leonurus* genus, including rutin, genkwanin, quercetin, and kaempferol. Hyperoside is used as the quality control of Leonuri cardiacae herba (Zhang *et al.*, 2018, European Pharmacopoeia 10.0).

Out of the other **phenolic compounds** present in the *Leonurus* genus, verbascoside (Fig. 2.8.) and lavandulifolioside are of great importance. Lavandulifolioside in fact represents the arabinoside of verbascoside, and the content of it in the aerial parts of *L. cardiaca* L. was determined to be 0.1%. On the other hand, verbascoside, also known under the name acteoside, can be found both in the aerial parts and the roots of motherwort, and in

approximately 200 other plant species belonging to 23 plant families (Miłkowska-Leyck, Filipek and Strzelecka, 2002; Alipieva *et al.*, 2014).

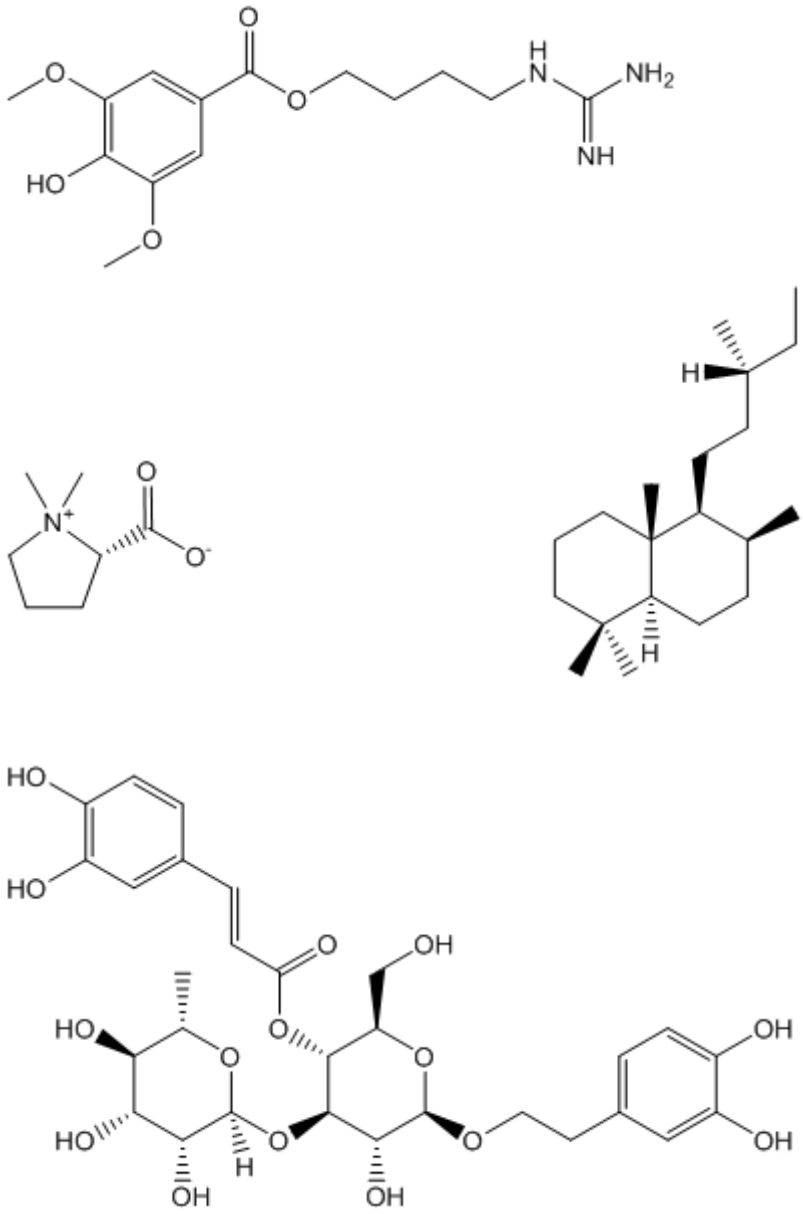

Fig. 2.8. Some of the active compounds of the *Leonurus* genus. Up – leonurine, middle left – stachydrine, middle right – labdane-type diterpene skeleton, down – verbascoside. Courtesy of doc. Macáková.

The phytochemistry of motherwort is fairly interesting – new compounds with different pharmacological properties have been isolated in the last few years, several of them for the first time in the *Leonurus* genus. Apart from the abovementioned labdane diterpenes, for example, iridoid

glycosides with antimicrobial activity (Tasdemir *et al.*, 1999), phenylethanoid glycosides with hepatoprotective activity (Li *et al.*, 2012), sesquiterpenoids and their glycosides (Li *et al.*, 2012; Xiong *et al.*, 2013), and glucarid acids with hepatoprotective activity (Jiang *et al.*, 2015) were discovered. Similarly, as reported by Garran *et al.* (2019), a total of 257 hydrophilic compounds were identified in the chemical profile of *L. japonicus* Houtt. and *L. cardiaca* L., using their developed discriminating metabolite isomerism strategy. Out of them, 212 compounds were found for the first time in the *Leonurus* genus.

Moreover, geographical and seasonal differences in motherwort's active constituents have been reported. Knöss and Zapp (1998) found that no labdane diterpenes were detected in *L. cardiaca* L. plantlets during the first four to five weeks following the germination. Tan *et al.* (2020) studied samples of *L. japonicus* Houtt. from 11 different producing regions in China and reported that the different geographical position could affect the accumulation of the active compounds.

2.4.3. Medicinal Use of Motherwort

According to the EMA monograph on *Leonurus cardiaca* L. herba, the traditional use is for relieving symptoms of nervous tension and/or for relieving symptoms of nervous cardiac complaints such as palpitations, after serious conditions have been excluded by a medical doctor (EMA/HMPC/127428/2010). However, several *Leonurus* species were proven to exhibit a beneficial effect in more areas, including gynecological disorders, neuroprotection, anti-inflammatory, and antimicrobial activity (Wojtyniak, Szymański and Matławska, 2013; Sayed *et al.*, 2016; Zhang *et al.*, 2018).

Motherwort can be used as a comminuted or powdered herbal substance, tincture, or liquid extract. In traditional Chinese medicine, Zhixion capsules are also used. They contain an equal mixture of extracts from four plants (including *L. japonicus* Houtt.) and leech extract, and are used against

inflammation, hyperlipidemia (their effect is comparable with that of simvastatin), or blood stasis (EMA/HMPC/127428/2010; Zhai *et al.*, 2019).

2.4.4. Effects of Motherwort on Selected Cardiovascular Conditions

2.4.4.1. Antioxidant Effect of Motherwort

Matkowski and Piotrowska (2006) performed several assays with methanolic extracts obtained from six wild European species belonging to the family *Lamiaceae* (including *L. cardiaca* L.) in order to assess their antioxidant effects. In the free radical-scavenging activity (DPPH) test, the motherwort extract was evaluated as the strongest one. In the lipid peroxidation assay, the maximum inhibition reached was 78% (*Salvia officinalis* L., *Marrubium vulgare* L.), *L. cardiaca* L. slightly exceeded 70%. These results indicate a great antioxidant potential in *L. cardiaca* L.

The high antioxidant activity of a *L. cardiaca* L. fluid extract was also confirmed by Bernatoniene *et al.* (2009). In the free radical-scavenging activity test, motherwort extract was assessed as the strongest one of the extracts tested (inhibition by nearly 85%). An antioxidant activity about 15-fold lower than the antioxidant activity of ascorbic acid was observed in polyphenol-rich extract from *L. cardiaca* L. (Sadowska *et al.*, 2017), while Pereira *et al.* (2019) reported the antioxidant activity of a hydroethanolic extract from *L. cardiaca* L. to be approximately three times higher than that of ascorbic acid. This extract also showed an antioxidant activity in a cell culture previously exposed to potassium dichromate in order to cause a massive increase in reactive oxygen species. This effect could be countered by the extract from *L. cardiaca* L.

In the free radical-scavenging test, ethanolic extract from *L. sibiricus* L. exhibited a weak antioxidant effect, as reported by Park *et al.* (2016). However, Oliveira *et al.* (2017) investigated the antioxidant, anti-nociceptive, and local anti-inflammatory effect of the ethanolic extract from *L. sibiricus* L. (total phenolic and flavonoid contents 60.1 mg of gallic acid and 15.4 mg of

catechin equivalent/g extract, respectively). In the *in vitro* antioxidant assays this extract displayed strong antioxidant properties (including the inhibition of a spontaneous or a ferrous sulphate-induced lipid peroxidation).

All of these partially contradicting results may be the consequence of the variation in active compounds in the plants (see Ch. 2.4.2), and the various solvents used resulting in different chemical compositions and subsequent antioxidant activity of the tested extracts. For example, Jafari *et al.* (2010) used the aerial parts of *L. cardiaca* L. for the determining the total phenolics and flavonoids, and their antioxidant activity in different solvents. The primary extract was obtained by using methanol 80% (v/v), then it was dried and the dry extract was fractioned using chloroform, ethyl acetate, *n*-butanol, and methanol 50% (v/v). The highest inhibition in the extensively used DPPH assay was obtained by the *n*-butanolic extract (86.2%), although this extract did not contain the highest amounts of either phenolics or flavonoids.

Similarly, Chua and Aminah (2013) tested the antioxidant activity of *L. sibiricus* L. leaves extracted by two different solvents in three bioassay systems (auto-oxidation of linoleic acid lipid system, xanthine oxidase superoxide scavenging activity, and DPPH radical scavenging activity). In all the bioassays performed, the aqueous leaf extract antioxidant activity was marked as high (72.2 – 78.2%), while the antioxidant activity of the ethanolic leaf extract was high in only one assay (71.6%) and moderate in two of the assays (69.7% and 48.8%, respectively). The most significant difference (29.4%) in the determined antioxidant activity of the two extracts was in the most used DPPH assay. Therefore, a standardization of the tested extracts would be very helpful.

Triterpenoids of the ursane class isolated from ethanolic extract of *L. cardiaca* L. (ursolic acid, ilelatifol D, corosolic acid, and euscaphic acid) were tested for their superoxide scavenging activity both in a cellular and a cell-free system by Ali *et al.* (2007). Ursolic acid did not exhibit any scavenging activity in the cell-free system (xanthine/xanthine oxidase system), while in the respiratory bursts activity with activated neutrophils it was the most effective compound tested (nearly 75% inhibition). Other

triterpenoids were only mildly active in the respiratory burst assay (11 - 16% inhibition) and also showed a low superoxide scavenging activity.

Other terpenic compounds occurring in the *Leonurus* genus also exhibit antioxidant properties, as reported by Lai *et al.* (2018b) in their screening of new diterpenes from *L. japonicus* Houtt.

The alkaloid stachydrine was reported to decrease the number of ROS positive myocardial cells in a myocardial cell hypertrophy model (Guo *et al.*, 2012). The beneficial effect of stachydrine in the cardiac hypertrophy model was also observed by Cao *et al.* (2017; a reduced generation of ROS) and Zhao *et al.* (2017b; an increased activity of superoxide dismutase and decreased malonyl dialdehyde levels in the serum of rats with an isoproterenol-induced cardiac hypertrophy).

Stachydrine likely also has a protective role in an anoxia/reperfusion model of endothelial cell injury. Stachydrine treatment of human umbilical vein endothelial cell culture restored the declined concentrations of superoxide dismutase and glutathione peroxidase after anoxia/reoxygenation (Yin *et al.*, 2010).

The antioxidative properties of another *Leonurus* alkaloid, leonurine, were also reported in cardiomyocytes (Liu *et al.*, 2010a, 2010b, 2013; Wang *et al.*, 2019), endothelial cells (Liu *et al.*, 2012a, 2012b; Zhang *et al.*, 2012), podocytes (Liu *et al.*, 2018), or brain tissue (Liu *et al.*, 2012). In all the abovementioned studies, treatment of the respective tissue with leonurine led to a decreased ROS production and to the induction of various antioxidant enzymes, such as superoxide dismutase or catalase.

2.4.4.2. Direct Effect of Motherwort on Myocardium

Miłkowska-Leyck, Filipek and Strzelecka (2002) reported that both the *n*-butanolic extract and lavandulifolioside, a phenylpropanoid glycoside isolated from *L. cardiaca* var. *vulgaris* [Moench] Briquet aerial parts, exhibited negative **chronotropic properties**. Lavandulifolioside administered to the

isolated rat heart at doses of 1 – 100 μg non-significantly decreased heart rate (by 4 – 19%), while higher doses (200 – 2000 μg) managed to decrease the heart rate significantly (by 23 – 29%). The butanolic motherwort extract demonstrated its negative chronotropic action at an even lower dose than pure lavandulifolioside (50 μg). At higher doses, both lavandulifolioside and the butanolic motherwort extract also caused several significant EKG-changes (namely the prolongation of the P-Q interval, Q-T interval, and QRS complex).

On the other hand, another phenolic compound from *Leonurus* genus, the phenylethanoid glycoside verbascoside, was reported to exhibit positive chronotropic and inotropic properties. Pennachio *et al.* (1996) observed a significant increase in the heart rate (by 42%) and an increase in the contractile force (by 13%) after administration of verbascoside at the concentration of 1 mmol/L into isolated rat hearts. The positive chronotropic effect lasted for several minutes, while the positive inotropic effect diminished within two minutes after the onset (Pennachio, 1997). These effects coincided with remarkable increases in intracellular levels of cAMP (an increase by 1733%; Pennachio *et al.*, 1996). Seeing as the verbascoside content in the aerial parts of motherwort is lower than the content of lavandulifolioside (91.1 μg/mL and 169.1 μg/mL, verbascoside and lavandulofolioside, respectively in a butanolic extract from *L. sibiricus* L.; Pitschmann *et al.*, 2016), the predominant chronotropic effect is likely to be that of lavandulifolioside.

Sun *et al.* (2005) observed a **cardioprotective effect** from an aqueous extract of *L. heterophyllus* Sweet aerial parts on rats exposed to a myocardial injury (after a surgical procedure the developed infarct sizes ranged between 40% and 60% of the left ventricular mass). The extract containing mainly stachydrine, quercetin, kaempferol, leonurine, and apigenin exhibited strong antioxidant activity *in vitro*, and helped, especially in the acute phase, to attenuate the damage to rat hearts caused by the myocardial infarction via restoring the activity of the cardiac antioxidant enzymes superoxide dismutase and glutathione peroxidase.

The protective effect of *L. japonicus* Houtt on a myocardial remodeling induced by isoproterenol was reported by Zhang *et al.* (2011). The authors

concluded that treatment with motherwort had a beneficial effect on both systolic and diastolic function, and downregulated the expression of collagen.

Subsequently, several studies with two pure compounds isolated from the *Leonurus* genus – the alkaloids stachydrine and leonurine – were performed on the model of a myocardial ischemia and/or fibrosis (remodeling), both *in vitro* and *in vivo*. Unfortunately, all of the studies that will be mentioned in the following text used only one of the abovementioned alkaloids (i.e. either stachydrine or leonurine), thus not enabling to provide information about the presence or absence of a possible synergistic effect of these two cardioprotective compounds.

The effect of **stachydrine** on the noradrenaline-induced hypertrophy of cardiomyocytes *in vitro* was studied by Zhang *et al.* (2014a). Ventricular myocytes were isolated and cultured with noradrenaline in the presence or absence of stachydrine for 72 hours. The hypertrophy was characterized by increased cell surface area, protein synthesis, and changes in the intracellular calcium transients (an increased Ca^{2+} amplitude, an accelerated decay of the Ca^{2+} transient). In correlation to increased norepinephrine, the activation of β1-adrenergic system (an increased intracellular cAMP level leading to the overactivation of protein kinase A – PKA) was also observed. All of these changes were significantly inhibited with the addition of 10 μmol/L stachydrine to the cultivation medium.

Zhao *et al.* (2017b) generated an overload-induced cardiac hypertrophy and heart failure in rats by performing an aortic constriction surgery. Animals were treated either with telmisartan (selective AT1R antagonist as the positive control) or stachydrine (8 mg/kg) for 12 weeks. Stachydrine treatment significantly attenuated the hypertrophy of the heart, characterized by increased weight of the heart, heart weight/body weight ratio, and left ventricle wall thickening. The hemodynamic parameters were also improved by the stachydrine treatment – the ejection fraction was enhanced; the cardiac output, the stroke work, and the potential energy were normalized. Moreover, rats treated with stachydrine showed considerably less fibrous tissue, suggesting that stachydrine was able to attenuate the myocardial fibrosis. Based on the Western blotting analysis of the heart tissue

protein lysates, increased levels of the two main receptors of TGF-β (transformation growth factor β1), TGF-βR1 and TGF-βR2, were found in the hypertrophic hearts. The TGF-β is an important fibrotic mediator and effector in the fibrotic process and the regulation of the extracellular matrix in general. The decrease of TGF-βR2 after stachydrine administration thus may represent a part of the antifibrotic effect of stachydrine (Zhao e*t al.*, 2017b and references therein).

Confirmation of the antifibrotic effect of stachydrine in another animal heart failure model was presented by Liu *et al.* (2019,) who also tried to further elucidate the molecular mechanism of such an effect. The authors used mice with transverse aorta constriction surgery in order to generate the cardiac hypertrophy and heart failure. Animals were treated with either telmisartan as a positive control or stachydrine (a higher dose was used than in the previous study, 12 mg/kg) for 4 weeks. The cardiac hypertrophy was characterized firstly by increased heart weight/body weight ratio, which was significantly decreased with telmisartan treatment and showed a trend of such reduction in the stachydrine treated group, and secondly by the thickness of left ventricular wall – this parameter was also partially alleviated with stachydrine. Again, stachydrine treatment suppressed the markers of the myocardial fibrosis (including collagen I and III, and elevated transcriptional levels of ACE, AT1R, and TGFβ1). The molecular mechanism of the antifibrotic effect of stachydrine includes the suppression of the fibrotic axis regulated by angiotensin II and TGFβ1 via certain signaling pathways that finally enhance the transcriptional control of fibrosis-related genes.

The cardioprotective effect of **leonurine** was investigated by Liu *et al.* (2009) on isolated neonatal rat cardiomyocytes and on infarcted rat hearts. The cardiomyocytes were treated with leonurine at 1 mmol/L concentration 8 hours prior to exposure to the hypoxia and serum deprivation (in order to induce ischemia). Leonurine treatment led to the induction of the antioxidant enzymes superoxide dismutase and catalase, and to a significantly increased cell viability. In the leonurine treated cells, the gene expression levels of pro-apoptotic genes (*Bax*, *Fas*) were significantly downregulated, while the anti-apoptotic *Bcl-2* gene expression was upregulated. In the whole-heart model,

rats were administered leonurine intraperitoneally one week prior to left coronary artery ligation, which induced ischemia. Leonurine treatment led to a significantly decreased infarct size in the hearts.

In their following studies, Liu and his colleagues again confirmed the anti-apoptotic action of both naturally occurring leonurine (Liu *et al.*, 2010a) and synthetic leonurine (SCM-198; Liu *et al.*, 2010b; 2010c) on rat hearts and cardiomyocytes. The downregulation of the pro-apoptotic gene *Bax* and the upregulation of anti-apoptotic gene *Bcl-2* were observed in the isolated rat hearts and in an H9c2 cardiac myocyte model (rat embryonic heart-derived cells). Furthermore, leonurine treatment led to a decrease in the infarct size of the ischemic rat hearts (Liu *et al.*, 2010a) and it also significantly improved the myocardial function (Liu *et al.*, 2010b). The H9c2 cells exposed to hydrogen peroxide, with a leonurine pretreatment for mere 4 hours, led to a concentration-dependent increase in cell viability and to the attenuation of apparent apoptotic characteristics (including the fragmentation of DNA, the translocation of the Bax protein to mitochondria, the loss of mitochondrial membrane potential, and the activation of caspase 3; Liu *et al.*, 2010c).

Similarly, Xu *et al.* (2018) reported on the anti-apoptotic properties of leonurine treatment in rats with a surgically induced myocardial infarction (ligation of the left coronary artery). Leonurine (15 mg/kg) was administered for 4 weeks. This treatment resulted in a significant downregulation of the pro-apoptotic gene *Bax* expression and a significant increase in the anti-apoptotic gene *Bcl-2* expression. Moreover, a significant reduction of the infarct size and a remarkable decrease in the collagen content was observed in the leonurine treated group.

Based on their studies, Liu, Gu and Zhu (2010) even proposed a synthetic leonurine conjugate as a new cardioprotective agent. The conjugate, namely 3,5-dimethoxy-4-(2-amino-3-prop-2-ynylsulfanyl-propionyl)-benzoic acid 4-guanidino-butyl ester, was designed with an improved pharmacology efficiency by combining leonurine with S-propargyl-L-cysteine via a phenolic hydroxyl ester bond, which could be readily hydrolyzed. This conjugate was effective at a lower molar concentration than pure leonurine.

2.4.4.3. Effect of Motherwort on Blood, Blood Cells, and Blood Vessels

A significant improvement in rheological properties of human blood (a decrease of **platelet aggregation**, blood viscosity, fibrinogen volume, and an increase of the red blood cells deformability) was observed by Zou *et al.* (1989) in their clinical study with 105 patients who were administered with motherwort extract (*L. heterophyllus* Sweet) for 15 days.

The inhibition of platelet aggregation by *L. heterophyllus* Sweet was again confirmed two years later by Lee *et al.* (1991). The authors had isolated prehispanolone, a labdane diterpene, from the herb and tested its activity on both rabbit platelet membranes and intact rabbit platelets. Prehispanolone inhibited the specific platelet activating factor (PAF) from binding to rabbit platelet membranes. The inhibitory potency of prehispanolone was increased in the presence of sodium chloride in a certain concentration range. In a concentration-dependent manner, prehispanolone also inhibited the PAF binding to intact rabbit platelets. The authors also tested prehispanolone binding to some other types of receptors, such as calcium channels, benzodiazepine receptors, nucleoside transporter, and A_2 adenosine receptors, but it showed no binding activity whatsoever. With the structural changes in the molecule of prehispanolone the PAF-binding activity also differed: opening up of the tetrahydrofuran ring resulted in the loss of such activity, while the replacement of the keto moiety with a methoxy or hydroxyl moiety led to a significantly increased PAF-binding activity. Based on these findings, it was suggested that prehispanolone interacts with the PAF receptor as an antagonist (Lee *et al.*, 1991).

Xiong *et al.* (2013) reported a strong platelet aggregation inhibition of rat platelets induced by ADP with a sesquiterpenic compound (namely (2*S*,5*S*)-2-hydroxy-2,6,10,10-tetramethyl-1-oxaspiro[4.5]dec-6-en-8-one) that was isolated from the ethyl acetate fraction of an ethanolic extract of *L. japonicus* Houtt. Yang *et al.* (2014) also observed the *in vitro* inhibition of platelet aggregation of platelets induced by ADP in the presence of isogosferal and murrayone, two coumarins isolated from *L. japonicus* Houtt.

Similarly, a *L. cardiaca* L. polyphenols-rich extract with a concentration of 100 µg/L significantly reduced platelet aggregation, but only in the presence of arachidonic acid. No significant effect was found for either ADP- or collagen-induced platelets, regardless of the motherwort extract concentration. A significant reduction was found in the PAF secretion by the cells treated with 50 and 100 µg/L of motherwort extract for 4 hours as well as 24 hours (Sadowska *et al.*, 2017).

A possible explanation of the mechanism of platelet aggregation inhibition by *L. cardiaca* L. extract was proposed two years later by Sadowska *et al.* (2019). The authors again used a polyphenols-rich extract (with hydroxycinnamic acid derivates as the predominant phenolic compounds) on human platelets obtained from healthy volunteers. The motherwort extract significantly reduced both the adhesion of platelets to fibrinogen and the expression of the receptor GPIIB/IIa on the ADP-activated platelets. Moreover, the highest concentration of motherwort extract used (350 µg/L) also decreased the expression of the adhesion molecule P-selectin. These findings led the authors to the conclusion that the disruption of the platelet-fibrinogen interactions may explain the antiplatelet effect of the motherwort extract.

The motherwort alkaloid leonurine had been reported to induce **vasorelaxation**. Chen and Kwan (2001) observed a concentration-dependent and endothelium-independent vasorelaxation of rat aortic arterial rings pretreated with phenylephrine in the presence of leonurine. The authors tested the activity of leonurine in several mediums leading them to the suggestion that leonurine is an effective inhibitor of vascular smooth tone, most likely acting by inhibiting the Ca^{2+} influx and the release of intracellular Ca^{2+}.

The more abundantly occurring alkaloid stachydrine also contributes to the vasorelaxation, as observed by Xie *et al.* (2018). Rat thoracic aortas, mesenteric arteries, and renal arteries were incubated with homocysteine or a control medium for one hour before measuring the acetylcholine-induced endothelium-dependent relaxation. Homocysteine significantly impaired the vasorelaxation in all three types of arteries but stachydrine effectively

restored the vascular relaxation in response to acetylcholine. As observed in tests with an inhibitor of the nitric oxide synthase (L-NAME), the stachydrine-promoted vasorelaxation was dependent on nitric oxide signaling.

Active compounds from a phenolic-rich *L. sibiricus* L. extract were reported to have a beneficial influence on the **endothelial dysfunction**, as demonstrated by Lee *et al.* (2010). Human umbilical vein endothelial cells were incubated with various concentrations of *L. sibiricus* L. extract for one hour before the addition of TNF-α, and then incubated for another 12 hours. The expression of adhesion molecules (namely the vascular cell adhesion molecule-1 (VCAM-1) and the intercellular adhesion molecule-1 (ICAM-1)) was significantly lower in the cells treated with motherwort extract than in the control. The reduction of VCAM-1 was up to 94% with the mothewort extract concentration of 400 μg/L.

The protective effect of the alkaloid stachydrine on the endothelial function was observed by Xie *et al.* (2018) who used bovine aorta endothelial cells coincubated with homocysteine. In these cells, the nitric oxide production was significantly reduced, but 12 hours of pretreatment with stachydrine effectively attenuated the effect of homocysteine on NO production in the endothelial cell culture. The authors also reported that homocysteine decreased the production of BH4, an essential cofactor for endothelial nitric oxide synthase to completely couple the NADPH oxidation to the NO production. However, the stachydrine pretreatment effectively restored the BH4 level in the endothelial cell culture. More specifically, stachydrine can induce the phosphorylation of the endothelial nitric oxide synthase and the subsequent vasorelaxation by the activation of AMP-activated protein kinase and protein kinase B/Akt (Xie *et al.*, 2019).

Liu *et al.* (2012a; 2012b) in their *in vitro* studies reported that the pretreatment of human umbilical vein endothelial cells, activated with lipopolysaccharide (2012a) or with TNF-α (2012b), with the alkaloid leonurine led to a significantly decreased expression of the adhesion molecules (ICAM-1, VCAM-1).

A decrease in the levels of the serum soluble adhesion molecules (sICAM-1, sVCAM-1) was also observed in an *in vivo* study with rabbits fed a high-cholesterol diet and treated with synthetic leonurine (SCM-198) for 8 weeks. In the aortas of these rabbits, a decrease in the expression of platelet-endothelial cell adhesion molecule-1 and the mRNA levels of VCAM-1 was also observed. Leonurine treatment significantly alleviated the development of atherosclerosis in a dose-dependent manner: the atherosclerotic lesions were smaller, the elasticity of the arteries and the hemodynamic status improved, and a decrease of the smooth muscle cell migration, and macrophage infiltration was observed (Zhang *et al.*, 2012).

2.4.4.4. Antihypertensive Effect of Motherwort

Pure lavandulifolioside isolated from *L. cardiaca* L. administered intravenously at a dose of 77 mg/kg to normotensive rats produced a significant decrease in both systolic (by 13 – 15%) and diastolic (by 16 – 19%) blood pressure. The duration of the action was for more than one hour (Miłkowska-Leyck, Filipek and Strzelecka, 2002).

Shikov *et al.* (2011) used *L. cardiaca* L. extracted with soybean oil (1:10, w/v) to ensure extraction of non-polar and middle-polarity compounds, such as iridoids. The extract was administered to 50 volunteers with stage 1 or 2 hypertension, accompanied with anxiety and sleep disorders (600 mg oil extract twice daily) for 4 weeks. In the patients with stage 1 hypertension, the statistically significant onset of the hypotensive effect was reported a week earlier than in patients with stage 2 hypertension (21 days and 28 days, respectively). In addition, a positive improvement in the psycho-emotional status of the patients (anxiety, emotional liability, headache, and sleep disorders) was observed.

Wojtyniak, Szymański and Matławska (2013) in their review on *L. cardiaca* L. mentioned old studies carried out by Rossyjskij (1949) who treated patients suffering from nervous cardiovascular conditions, hyperthyroidism, atherosclerosis, heart diseases, and hypertension with

various motherwort preparations. In the patients with essential hypertension, a decrease in blood pressure (8 – 20 mm Hg) was observed after 8 – 10 days of the treatment.

Interestingly, the alkaloid stachydrine was found to be the second most influential metabolite for discriminating between the DASH and control dietary patterns, as reported by Rebholz *et al.* (2018). The DASH dietary pattern (Dietary Approaches to Stop Hypertension) is rich in fruit, vegetables, and low-fat dairy products, while moderate in meat, fish, poultry, nuts, and beans. It leads to a reduction in systolic blood pressure (a reduction about 5 mm Hg), and epidemiologic studies have shown that higher adherence to the DASH diet was associated with a multitude of favorable health outcomes, including a reduced risk of hypertension, cardiovascular disease, kidney disease, and mortality (Rebholz *et al.*, 2018 and references therein).

2.4.4.5. Effect of Motherwort on Plasmatic Lipid Profile

The beneficial effect of *L. sibiricus* L. extract on the plasma lipid levels of mice fed an atherogenic diet was observed by Lee *et al.* (2010). The animals were administered with motherwort extract at a dose of 0.01 g of extract/20 g body weight for 14 weeks. Treatment with motherwort extract resulted in significantly decreased plasma total cholesterol levels, plasma triglyceride level (this level was even lower than in the control group fed with a normal diet), and atherogenic index, while the level of HDL cholesterol was significantly increased when compared to all other groups. The liver weight of the mice fed an atherogenic diet was higher than that of the mice on a standard diet (which may be due to the fatty liver), while the liver weight of mice with motherwort extract treatment recovered, and was comparable to that of the control group.

Rabbits treated with high doses of synthetic leonurine (SCM-198) for 8 weeks also exhibited some changes in the lipid parameters: a decrease in triglycerides and an increase in HDL cholesterol were observed (Zhang *et al.*, 2012).

Pure leonurine treatment (administered intragastrically, 10 mg/kg) resulted in decreased levels of total cholesterol and triglyceride levels in the serum of mice treated for 8 weeks. A detailed assessment of the plasma lipoproteins showed a decrease in LDL-cholesterol and an increase in HDL cholesterol in the leonurine-treated animals. Moreover, the atherosclerotic lesion sizes in aortic roots of leonurine-treated mice were remarkably diminished when compared with the control group (Jiang *et al.*, 2017).

Lee, Park and Ma (2017) investigated the effect of an ethanolic motherwort extract from *L. japonicus* Houtt. on the fat deposition and serum lipid levels of high-fat diet fed mice. The 14 weeks motherwort treatment led to a significantly reduced body weight gain, retroperitoneal and mesenteric visceral white adipose tissue, and to a significant decrease in serum triglycerides and LDL cholesterol.

The effect of synthetic leonurine (SCM-198) on dyslipidemia in three mammal species (mice, rabbits, Rhesus monkeys) was studied by Suguro *et al.* (2018). Leonurine, especially at higher doses, decreased total cholesterol and triglyceride levels, and its effect was comparable to that of atorvastatin. In leonurine treated mice and monkeys, a reduction in LDL cholesterol was also observed, while in rabbits, leonurine failed to decrease the LDL cholesterol level.

An ethanolic motherwort extract from *L. sibiricus* L. was used by Park *et al.* (2016) in their *in vitro* study with 3T3-L1 cells. These preadipocyte stadium cells were treated with motherwort extract (10 and 50 μg/mL) and a differentiation medium for 4 days. The higher concentration of motherwort extract inhibited the levels of intracellular lipid accumulation by 75%.

Similarly, such a pronounced diminution of the lipid accumulation in human THP-1 macrophage-derived foam cells was observed by Jiang *et al.* (2017) who investigated the possible antiatherogenic effect of the pure alkaloid leonurine in the *in vitro* part of their work. THP-1 cells fully differentiated into foam cells were incubated with several concentrations of leonurine (5, 10, 20, 40, and 80 μmol/L) for 24 hours. All of the tested

leonurine concentrations significantly decreased the cellular cholesterol content (including total cholesterol, free cholesterol, and cholesteryl ester) and increased the apo-A-I-mediated or HDL-mediated cholesterol efflux. The decrease of the total cellular cholesterol at a leonurine concentration of 80 μmol/L was 67%. Based on the performed assays, the proposed molecular **mechanism** of the leonurine effect on lipid metabolism in foam cells was the increase in certain cholesterol transporters of the cell membrane (namely ABCA1 and ABCG1). The expression of these cholesterol transporters is modulated by LXRα (liver X receptor alpha), and a common upstream factor of LXRα-induced signaling is the nuclear receptor PPARγ, which plays critical roles in lipid homeostasis. The authors demonstrated that leonurine does activate the PPARγ/LXRα pathway in a dose-dependent manner, and thus enables the increased cholesterol efflux. Furthermore, Jiang *et al.* (2017) tested this hypothesis on an animal model (mice) and found that the protein levels of PPARγ, LXRα, ABCA1, and ABG1 in the tissue homogenate of aortic roots of mice treated with leonurine were significantly increased when compared with the control group.

The downregulation of markers of the lipogenesis by an ethanolic motherwort extract (from *L. japonicus* Houtt.) in hepatic HepG2 cells was observed by Lee, Park and Ma (2018) in the *in vitro* part of their study. HepG2 cells were exposed to free fatty acids to induce hepatic steatosis, and then incubated for 24 hours with different concentrations of motherwort extract (0, 250, 500, 750, and 1000 μg/mL). Free fatty acids-induced HepG2 cells showed significant increases in lipid droplets, the production of which was significantly inhibited by motherwort extract at the two highest concentrations used. The motherwort extract treatment in a concentration-dependent manner also decreased the protein levels of lipogenic markers (namely SREBP-1c, FAS, SCD-1, and CD36), as showed by further analysis.

Suguro *et al.* (2018) also reported the decreased protein levels of FAS and SCD-1 in mice treated with synthetic leonurine, which led to reduced fatty acid synthesis in the liver, thus contributing to the reduction of blood lipid levels.

2.4.5. Safety Profile of the Motherwort Preparations

EMA and the Botanical Safety Handbook both find *L. cardiaca* L. to be safe, except for pregnant women. *L. japonicus* Houtt. is also considered to be safe, with the same exception of its usage during pregnancy. No interactions with other medicinal products have been reported so far (EMA/HMPC/127428/2010; Gardner and McGuffin, 2013).

On the cellular level in an *in vitro* study, a polyphenol-rich *L. cardiaca* L. extract (total polyphenol content ca 137 mg/g) exhibited no cyto**toxicity** up to a concentration of 4500 µg/L, when added to an endothelial cell culture (Sadowska *et al.*, 2017).

A large toxicity assessment of a motherwort aqueous extract (Herba leonuri) was performed by Han *et al.* (2013) on one animal model. The extract was administered orally once daily at doses 0 – 2000 mg/kg for 13 weeks to male and female rats. The only alteration was observed at the highest dose in the forestomach, where it caused squamous cell hyperplasia. Based on the results of the performed tests, the authors determined the acceptable daily value intake to be 10 mg/kg per day.

Treatment of Rhesus monkey with synthetic leonurine (10 mg/kg) for 178 days had no significant effects on the hepatic enzymes (ALT, ATS), hematologic parameters, or biochemical examinations (Suguro *et al.*, 2018).

Data on motherwort **adverse effects** in humans is sparse. Shikov *et al.* (2011) in their study with motherwort oil extract stated that all volunteers estimated the tolerability of motherwort oil extract as excellent. Meng *et al.* (2019) investigated the efficacy and safety of motherwort injection for the prevention of post-partum blood loss and observed that there were no statistical significant differences in the incidence of adverse events between the treatment and the control group.

As for possible **pharmacodynamic interactions** with other active compounds, Tachijan, Maria and Jahangir (2010) mentioned that motherwort potentiates antithrombotic and antiplatelet effects, and increases the risk of

bleeding. Taken with benzodiazepines, motherwort can also have a synergistic sedative effect.

2.5. Black cumin – *Nigella sativa* L. - fam. *Ranunculaceae*

2.5.1. Characterization of the Genus *Nigella* – a Pharmacognostic Context

The genus *Nigella* includes only about 18 species, all of which are therophytes – annual plants that complete their life cycle in a short favorable period and survive the inhospitable rest of the year as seeds. The most widely known and pharmaceutically most interesting is the species *Nigella sativa* L. (Fig. 2.9.), also known as black cumin, black seed, or (more contemporary and in the Unites States preferred) nigella. Black cumin is an annual herb with pale blue flowers, it can grow up to 45 cm. Black cumin is native to Southern Europe, North Africa, as well as South and Southwest Asia (Rajsekhar and Kuldeep, 2011; Engels and Brinckmann, 2017).

Fig. 2.9. *Nigella sativa* L. Left – flowers, right – seeds. From territorialseeds.com and britannica.com.

For the medicinal use, mostly seeds or seed oil are exploited. The **seeds** are black or dark grey on the outside and white inside, with angular, ovoid or obpyramidal shape, and small in size (Fig. 2.9., right). The length of

the black cumin seed ranges from 2.5 to 3.5 mm, the width from 1.5 to 2 mm, and the weight of the seed is about 1 to 5 mg. The seeds have a slightly aromatic odor and are bitter to taste due to the presence of the protein nigellin (El-Tahir and Bakeet, 2006; Rajsekhar and Kuldeep, 2011; Abedi *et al.*, 2017; Kalidasu *et al.*, 2017). The stability study of black cumin seeds stored under different conditions was presented by Bustaman *et al.* (2017).

The **seed oil**, also known as the essential oil, is cold-pressed from the seeds. The black cumin essential oil is a yellow to dark amber liquid and does not show fluorescence. The fixed oil is produced by the hydraulic expression of the black cumin seeds. And finally, the volatile oil is obtained by steam distillation of the black cumin seeds. It has a yellowish brown color and an unpleasant odor (Dajani, Shahwan and Dajani, 2016).

Abedi *et al.* (2017) suggested a microwave-assisted extraction of black cumin essential oil. This extraction method is thought to be energy-saving and effective: the extraction yields for 30 minutes were higher than the extraction yields of conventional hydrodistallation. Moreover, the content of the most active compound thymoquinone was pronouncedly higher in the microwave-assisted extraction (20.41%) when compared to hydrodistallation (3.71%).

The influence of various extraction methods of black cumin seeds on the composition of essential oils was investigated by Kokoška *et al.* (2008). The black cumin seeds were extracted with four methods: hydrodistillation, dry steam distillation, steam distillation of crude oils obtained by solvent extraction, and supercritical fluid extraction. The thymoquinone content was the highest in the oil obtained by the supercritical fluid extraction (76.7% vs. 0.5% by hydrodistillation), while the contents of *p*-cymene and *α*-thujene were dramatically decreased in the oil obtained by the supercritical fluid extraction (8.6% and 0.3% vs. 56.2% and 15.1% by hydrodistillation).

The different extraction methods have significant influence on both quantitative and qualitative composition of the black cumin seed oil; this was also demonstrated by Mohammed *et al.* (2016). The analysis of black cumin oil extracted by supercritical fluid or cold press showed that while the content of thymoquinone was approximately the same (16.21% and 16.80%,

respectively), the cold press method was evidently more suitable for the extraction of *α*-pinene, *γ*-terpinene, and limonene (not detected in the supercritical fluid assay and 7.10%, 2.82%, and 0.41%, respectively using the cold press method). Carvacrol and longifolene also had higher yields using the cold press method than by the supercritical fluid method (3.90% vs. 1.82% and 4.49% vs. 3.50%, respectively). On the other hand, total phenolic content was higher in the oil obtained with the supercritical fluid extraction (Mohammed *et al.*, 2016).

In order to increase the content of the most active black cumin compound thymoquinone in the essential oil, controlled heating of the black cumin seeds for 10 minutes at a temperature between 50 to 150 °C is recommended. Heating to temperatures of 200 °C or higher led to a significantly decreased thymoquinone content, while heating at 25 °C did not show any effect on the thymoquinone content (Agbaria *et al.*, 2015). For the exact quantifying of the black cumin active constituents thymoquinone, dithymoquinone, thymodihydroqiunone, and thymol in the seed oil, Ghosheh, Houdi and Crooks (1999) developed an HPLC method.

2.5.2. Phytochemistry of Black Cumin

N. sativa L. seeds contain 30 – 40% fat, 30 – 35% carbohydrates, and around 20% proteins. Aside from the oil, also saponins (α-hederin), terpenes (nigellamines), and alkaloids (nigelicine, nigellimine, and nigellidine) are also present in the seed (Morikawa *et al.*, 2004a, b; El-Tahir and Bakeet, 2006; Mohammed *et al*., 2016 and references therein).

Black cumin seed fixed oil is primarily composed of unsaturated fatty acids (linoleic acid and oleic acid, 45 – 57% and 21 – 25%, respectively). The most abundant saturated acid in the fixed oil is palmitic acid (12 – 14%). Sterols constitute only about 0.5% of the total seed oil, and the major sterol forms are the steryl glucosides, although free sterols, steryl esters, and acylated steryl glucosides were also isolated from *N. sativa* L. Phospholipids are represented mainly by phosphatidylcholine (46 – 48%), followed by

phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine (Menounos, Staphylakis and Gegiou, 1986; Ramadan, Kroh and Mörsel, 2003; El-Tahir and Bakeet, 2016).

The chemical composition of black cumin volatile oil undergoes significant changes during the six stages of **seed maturation** (75 days), as reported by Botnick *et al.* (2012). Dramatical changes were observed especially in monoterpene levels and their composition. While in the flower stage, mainly *2E*-hexenal and *2E*-hexenol were detected. There were no accumulations of these compounds in the mature seed volatile oil. The first monoterpene detected in developing seeds was *γ*-terpinene (day 30). The accumulation of other important monoterpenes started even later (thymoquinone and carvacrol at day 50, *p*-cymene at day 55). It is possible that *γ*-terpinene serves as a precursor of *p*-cymene in the black cumin seed, as a strong decrease in *γ*-terpinene levels was observed at day 55 in parallel to a significant increase in *p*-cymene (Botnick *et al.*, 2012). Some of the black cumin monoterepenes are depicted in Fig. 2.10.

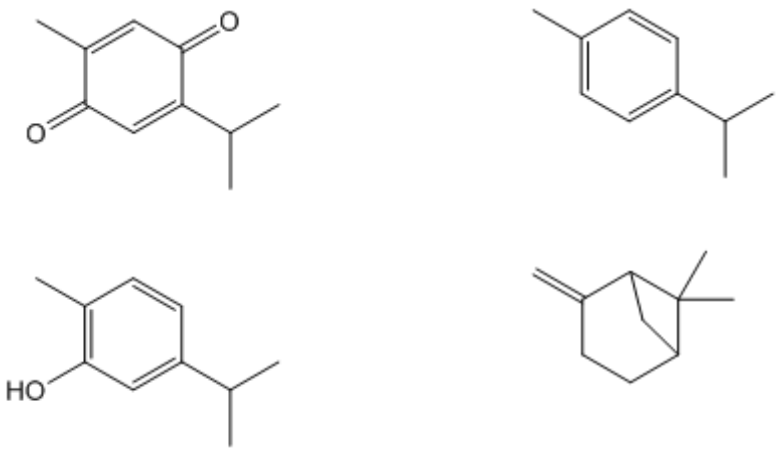

Fig. 2.10. Some of the *Nigella* genus monoterpenes. Upper left – thymoquinone, upper right – *p*-cymene, down left – carvacrol, down right – *β*-pinene. Courtesy of doc. Macáková.

The volatile terpenes of black cumin were only detected in the seed coat, while in the inner endosperm, embryo, leaves, shoot, or roots there were none. According to a fluorescence study, it appears that the volatiles accumulate in the subepidermal layer of the seed coat, in the form of droplets distributed evenly throughout this layer (Botnick *et al.*, 2012).

Tab. 2.1. The composition of black cumin essential oil with different geographical origin. Values are expressed as percentages. T – traces, ND – not detected. Sources: India – Singh *et al.* (2014), India 2 – Kalidasu *et al.* (2017), Iran – Nickavar *et al.* (2013), Tunisia – Bourgou *et al.* (2010), Turkey – Kokoška *et al.* (2008)

compound	India	India 2	Iran	Tunisia	Turkey
α-thujene	5.6	5.1	2.4	6.9	15.1
β-pinene	1.7	1.3	1.3	2.4	4.0
γ-terpinene	0.2	ND	0.5	3.5	1.2
p-cymene	31.4	27.8	14.8	60.5	56.2
trans-anethole	ND	ND	38.3	ND	ND
carvacrol	1.4	2.4	1.6	2.4	0.8
carvone	T	ND	4.0	ND	1.1
limonene	1.0	ND	4.3	ND	2.9
longifolene	2.0	3.2	0.7	0.9	2.6
thymohydroquinone	3.4	2.4	ND	0.4	T
thymol	0.2	ND	ND	ND	ND
thymoquinone	37.6	28.7	0.6	3.0	0.5

The quantitative and qualitative composition of the black cumin seed essential oil may also be different due to the **geographical origin** of the plant. For example, Tunisian black cumin essential oil was rich in the monoterpene *p*-cymene (60.5%), *α*-thujene (6.9%), *γ*-terpinene (3.5%), carvacrol (2.4%), and *β*-pinene (2.4%). The content of thymoquinone was only 3.0% (Bourgou *et al.*, 2010). Indian essential oil contained predominantly thymoquinone (37.6%), *p*-cymene (31.4%), *α*-thujene (5.6%), and thymohydroquinone (3.4%), while the content of carvacrol, *β*-pinene, and *γ*-terpinene was low (1.4%, 1.7%, and 0.2%, respectively; Singh *et al*., 2014; and very similar amounts were provided by Kalidasu *et al.*, 2017). Iranian essential oil differed significantly, with the major compound being *trans*-anethole (38.3%). Further it contained *p*-cymene (14.8%), limonene (4.3%), and carvone (4.0%), while the content of thymoquinone, carvacrol, *α*-thujene, *β*-pinene, and *γ*-terpinene were low (0.6%, 1.6%, 2.4%, 1.3%, and 0.5%, respectively; Nickavar *et al.*, 2003). And finally, Turkish volatile oil is rich in *p*-cymene (56.2%), *α*-thujene (15.1%), *β*-pinene (4.0%), and limonene (2.9%; Kokoška *et al.*, 2008) For a

more detailed overview of the composition of the black cumin essential oil, see Tab. 2.1.

2.5.3. Medicinal Use of Black Cumin

The European Medicines Agency does not recognize black cumin as medicinal plant. *N. sativa* L. is mentioned in two final assessment reports (*Pimpinella anisum* L., fructus and aetheroleum and *Tilia cordata* Miller, *Tilia platyphyllos* Scop., *Tilia* x *vulgaris* Heyne or their mixtures, flos) – as a component of a herbal mixture tested for its inhibitory effect on histamine released from rat peritoneal mast cells, and as a herbal product used by diabetic patients in Jordan, respectively (EMA/HPMC/321181/2012; EMA/HPMC/337067/2011).

On the other hand, black cumin is widely used in the African and Asian traditional medicine: Ayurvedic, Siddha, and Unani systems of medicine in India, traditional Arabic and Islamic medicine, Iranian traditional medicine, and Sudanese medicine among other systems of medicine (Engels and Brinckmann, 2017).

Black cumin is used in several diseases including cough, jaundice, skin diseases, conjunctivitis, abdominal disorders, dyspepsia, flatulence, diarrhea, and fever. *N. sativa* L. is also used traditionally as a part of a polyherbal formulation for the treatment of metabolic disorders like diabetes and hyperlipidemia in Pakistan. This formulation further contains *Cichorium intybus* L., *Trigonella foenum-graecum* L., and *Gymnena sylvestre* (Retz.) R. Br. ex Schult. All of the components are in equal proportion (Rajsekhar *et al.*, 2011 and references therein; Malik *et al.*, 2017a; b).

2.5.4. Effects of Black Cumin on Selected Cardiovascular Conditions

2.5.4.1. Antioxidant Effect of Black Cumin

The antioxidant potential of black cumin was established quite early. For example, Houghton *et al.* (1995) tested fixed oils from different sources of *N. sativa* L. seeds and pure thymoquinone as possible inhibitors of eicosanoid generation and membrane lipid peroxidation. While both substances inhibited the non-enzymatic peroxidation in ox brain phospholipid liposomes, thymoquinone was about ten times more potent. The authors also observed that the inhibition of lipid peroxidation by the black cumin fixed oil was greater than is expected from its thymoquinone content, thus it is likely that other components of the fixed oil also possess antioxidant properties (Houghton *et al.*, 1995).

Burits and Bucar (2000) proved that in the black cumin essential oil several other compounds beside thymoquinone also demonstrated respectable radical scavenging activity, namely carvacrol, *trans*-anethole, and 4-terpineol. All compounds were effective hydroxyl radical scavenging agents in the assay for non-enzymatic lipid peroxidation but their antioxidant activity varied in the DPPH test. Probably no significant antioxidant activity can be expected from *p*-cymene or *α*-pinene, as these compounds, unlike thymoquinone, did not ameliorate the oxidative stress caused by carbon tetrachloride in mice (Mansour *et al.*, 2001). This finding was confirmed several years later by Bourgou *et al.* (2010) who reported that monoterpene hydrocarbons *p*-cymene, *γ*-terpinene, and *β*-pinene from black cumin seed were inactive in the DCFH assay for antioxidant and pro-oxidant properties. Interestingly, the *ex vivo* antioxidant activity of phenolic carvacrol was also very low (Bourgou *et al.*, 2010).

Ramadan, Kroh and Mörsel (2003) investigated the radical scavenging activity of the black cumin crude seed oil and its fractions. The crude seed oil was extracted with *n*-hexane and then further fractionated into neutral lipids, glycolipids, and phospholipids that were tested for their radical scavenging activity toward the stable galvinoxyl radical and the DPPH radical. The

phospholipids, represented prevalently by phosphatidylethanolamine and phosphatidylcholine, exhibited the greatest radical scavenging activity, followed by glycolipids and neutral lipids. The inhibition of the DPPH radical was relatively low, 28.6% for phospholipids, 26.5% for glycolipids, and 16.2% for neutral lipids. On the other hand, phospholipids quenched 83.7% of the galvinoxyl radical.

A high antioxidant activity of black cumin seed essential oil was also reported in DCFH assay by Bourgou *et al.* (2010) and by Singh *et al.* (2013) who determined the radical scavenging activity of the essential oil in the DPPH test (95.4%). Furthermore, Abdel-Wahhab and Aly (2005) investigated the ability of black cumin volatile oil to scavenge free radicals generated during aflatoxicosis in rats. Treatment with black cumin in rats fed an aflatoxin-contaminated diet resulted in a significant protection against aflatoxicosis, indicating good free radicals scavenging activity of *N. sativa* L.

Kökdil *et al.* (2006) also observed a significant increase in the total antioxidant status in rats treated with black cumin fixed oil at a dose of 1 mL/kg for 4 weeks. Similarly, El-Gindy *et al.* (2019) reported a significant increase in blood total antioxidant capacity and a significant decrease in malonyl aldehyde values in rabbits treated with black cumin seeds at doses of 300 or 600 mg/kg for 8 weeks. An *in vitro* evaluation of antioxidant effects of black cumin extract and black cumin oil showed that the highest antioxidant activity (80%) was observed at the concentrations of 10 and 20 mg/mL that were equal to the concentration 200 mg/mL of vitamin E (Soleimani *et al.*, 2008).

Fixed and essential oils from *N. sativa* L. seeds probably improve the antioxidant status through modulation of antioxidant enzymes. Sultan *et al.* (2014) found that both the fixed and essential black cumin oils fed to rats enhanced the expression of hepatic enzymes. Their results also indicated that the antioxidant potential is positively correlated with the increase of hepatic enzyme levels. In their following study, the investigators confirmed that the black cumin fixed and essential oils positively modulated the activities of several antioxidant enzymes, such as catalase, superoxide dismutase, glutathione transferase, glutathione reductase, and glutathione peroxidase.

Furthermore, markers of the antioxidant status like tocopherols and glutathione were in a linear correlation with the levels of glutathione peroxidase, glutathione reductase, and glutathione transferase (Sultan *et al.*, 2015).

As black cumin seeds, extracts, fixed, or volatile oils exhibit strong antioxidant properties, they thus also exhibit the ability to protect cells from oxidative stress, as demonstrated in a fair amount of studies focused on different cells and organs, such as erythrocytes (Suboh, Bilto and Aburjai, 2004; Kanter, Coskun and Gurel, 2005), fibroblasts (Ashraf *et al.*, 2011), macrophages (Tripathi, Chaturvedi and Pandey, 2012), pre-adipocytes (Bordoni *et al.*, 2019), brain (Hamdy and Taha, 2009; Abdel-Zaher, Abdel-Rahman and Elwasei, 2010; Erşahin *et al.*, 2011; Hassan *et al.*, 2016), heart (Seif, 2013), intestines (Terzi *et al.*, 2010), kidney (Bayrak *et al.*, 2008, Yildiz *et al.*, 2010; Attia *et al.*, 2011; Hassan *et al.*, 2016; Norouzi *et al.*, 2017), liver (Al-Ghamdi, 2003; Kanter *et al.*, 2003; Kanter, Coskun and Budancamanak, 2005; Yildiz *et al.*, 2008; Attia *et al.*, 2011; Hassan *et al.*, 2016), and lung (Zaki, 2019).

Similarly, the antioxidant potential of pure **thymoquinone** was demonstrated in several studies. Badary *et al.* (2003) performed various assays with thymoquinone (namely microsomal lipid peroxidation, DPPH, hydroxyl radical, and superoxide anion scavenging test) and concluded that thymoquinone is acting mainly as a potent superoxide anion scavenger with IC50 3.35 μmol/L.

Treatment with thymoquinone was reported to attenuate the oxidative stress and injury in various tissues including: brain (Hamdy and Taha, 2009), brown adipose tissue (Mahmoudi *et al.*, 2018), kidney (Badary *et al.*, 2000; Khattab and Nagi, 2007; Nader, El-Agamy and Suddek, 2010), heart (Hamdy and Taha, 2009; Nagi *et al.*, 2011; Xiao *et al.*, 2018), liver (Mansour *et al.*, 2001; Zafeer *et al.*, 2012), lung (Pourgholamhossein *et al.*, 2016), skeletal muscle (Hosseinzadeh, Taiari and Nassiri-Asl, 2012), and smooth muscle (aorta and pulmonary artery – Nader, El-Agamy and Suddek, 2010). The main protective effect of thymoquinone was the restoration of the antioxidant capacity in the respective tissue.

However, thymoquinone, especially at higher doses, might be metabolized to reactive species and increase the oxidative stress, as observed in hepatocytes by Khader, Bresgen and Eckl (2009; see also Ch. 2.5.5.). On the other hand, thymoquinone treatment in hypercholesterolemic rats led to significantly increased liver antioxidant enzyme levels (including superoxide dismutase 1 and glutathione peroxidase) and to the upregulation of the superoxide dismutase 1, catalase, and glutathione peroxidase genes (Ismail, Al-Naqeep and Chan, 2010).

2.5.4.2. Direct Effect of Black Cumin on Myocardium

Shafei, Boskabady and Parsaee (2005) investigated the effect of an aqueous extract from aerial parts of *N. sativa* L. (the plant ingredient concentration was 10 g/100 ml) and diltiazem on isolated guinea pig hearts. Four different concentrations of black cumin extract (0.5, 1, 2, and 5 mg) and diltiazem (0.1, 1, 10, and 100 μmol/L) were used on hearts perfused with either a standard or a calcium free Krebs solution. Both diltiazem and the black cumin extract significantly reduced heart rate and contractility of the isolated hearts. However, the **negative chronotropic** and **negative inotropic effects** were more pronounced in the hearts perfused with the calcium free Krebs solution. This greater effect of black cumin extract on the heart rate in the calcium free Krebs solution and differences in the slope of concentration-response curves between extract and diltiazem may indicate an additional effect for aqueous extracts other than the calcium channel-inhibitory effect. The authors suggested that the additional effect could be a potassium opening effect (Shafei, Boskabady and Parsaee, 2005).

Interestingly, all other works presented in the available literature and that will be mentioned in the following text, reported **positive inotropic** and **chronotropic effect** of either black cumin seed oil or pure thymoquinone. This apparent discrepancy may be due to the fact that the aqueous *N. sativa* L. extract was prepared from whole aerial parts of the plant that may contain other compounds than black cumin seeds.

Oral supplementation of *N. sativa* L. seeds in rats at a dose of 800 mg/kg for 8 weeks led to a significantly increased myocardial flow rate, baseline heart rate, baseline peak tension, and baseline maximum rate of tension development. Furthermore, the black cumin seeds administration induced a moderate **homogenous cardiac hypertrophy**, evident by significant increases in the left ventricular and whole heart weights, and the relative heart weight/body weight ratio, as observed by El-Bahai *et al.* (2009).

This homogenous heart hypertrophy, very similar to the hypertrophy induced by exercise-training in rats treated with black cumin (800 mg/kg) for 8 weeks, was reported by Al-Asoom *et al.* (2014a; b). The ventricular myocytes were normal, with no signs of excessive extracellular collagen fibrosis but with significantly increased diameter. The heart weight/body weight and left ventricle weight/body weight ratios were also significantly higher in rats treated with black cumin and/or rats with physical training (running on treadmill for 2 hours daily). Rats with both treatments exhibited even a significant reduction in the heart rate, which may indicate that black cumin supplementation has an additive effect that helps to potentiate the adaptive response of exercise training (Al-Asoom *et al.*, 2014a). Moreover, an elevation of the total serum antioxidant activity was also observed in rats obtaining both treatments (Al-Asoom *et al.*, 2014b).

Al-Asoom and Al-Hariri (2019) in another study of theirs observed a positive inotropic effect of pure thymoquinone administered orally at a dose of 10 mg/kg to rats for 8 weeks. This effect was demonstrated by a significantly higher peak tension in the thymoquinone treated group when compared to the control. On the other hand, no hypertrophy was noted, with comparable heart and left ventricle weights, and the heart weight/body weight, left ventricle weight/ body weight, and left ventricle weight/heart weight ratios between the two studied groups. The authors concluded that either thymoquinone is not responsible for the cardiac hypertrophy or it needs another compound(s) for the induction of the cardiac hypertrophy (Al-Asoom and Al-Hariri, 2019).

The **cardioprotective effect** of a *N. sativa* L. hydroalcoholic extract administered daily at a dose of 100 mg/kg to rats with inflammation-induced

myocardial fibrosis was reported by Norouzi *et al.* (2017). Treatment with black cumin reduced cardiac fibrosis, collagen deposition, and inflammatory cell infiltrates that were induced by lipopolysaccharide administration.

The dose-dependent reduction of cardiac fibrosis and heart permeability after a chronic exposure to lipopolysacharide (1 mg/kg intraperitoneally for 3 weeks) in rats treated with pure thymoquinone (2, 5, and 10 mg/kg) was also observed by Asghardeh *et al.* (2018). Treatment with thymoquinone improved pathological changes in the heart tissue, such as infiltration of inflammatory cells, disarrangement of myofibers, and collagen deposition in myocardium. The highest tested dose of thymoquinone reversed the cardiac permeability to the control level.

Furthermore, thymoquinone pretreatment (oral administration of 12.5, 25, and 50 mg/kg for 7 days) in rats before the onset of an isoproterenol-induced myocardial injury attenuated the effect of isoproterenol. In the heart tissue, the injury induced by isoproterenol was represented by patchy areas of mild necrosis with the infiltration of polymorphonuclear cells, while thymoquinone-pretreated hearts had the histological appearance of the passive control hearts. On the biochemical level, isoproterenol significantly (up to 5 times) increased the activity of glutathione reductase and lipid peroxidation, while it significantly decreased the activity of superoxide dismutase. These changes were reversed by thymoquinone pretreatment. At the highest dose of thymoquinone, almost all of the studied parameters were even reversed back to normal levels (Randhawa, Alghamdi and Maulik, 2013).

A prolonged thymoquinone pretreatment (for 21 days) before isoproterenol administration was performed by Ojha *et al.* (2015). Thymoquinone at a dose of 20 mg/kg decreased the infarct area size, as observed by the presence of less necrotic tissues. Furthermore, thymoquinone had a significant effect on the myocardial histoarchitecture negatively influenced by isoproterenol: only very mild degree of myonecrosis, edema, and infiltration of inflammatory cells was observed. The lipid peroxidation and the decrease in superoxide dismutase activity induced by isoproterenol were also attenuated by thymoquinone.

Similarly, a cardioprotective effect of pure thymoquinone on the ischemia/reperfusion cardiac injury in rats was reported by Gonca and Kurt (2015). Thymoquinone at a dose of 10 mg/kg was administered intraperitoneally 20 minutes before the ischemic period induced by the left coronary artery ligation. Thymoquinone reduced the infarct area (by 54%) and decreased the incidence of arrhythmias (ventricular tachycardia and ventricular fibrillation) during the reperfusion period. Xiao *et al.* (2018) also observed that thymoquinone pretreatment led to a reduction of the infarct size in isolated rat hearts after an ischemia/reperfusion injury. The authors furthermore reported a decrease of the oxidative stress and a suppression of apoptosis in hearts with thymoquinone pretreatment.

The supposed cardioprotective **mechanism** of thymoquinone on the myocardial ischemia/reperfusion injury was suggested by Lu *et al.* (2018). Based on their experimental protocol with isolated rat cardiomyocytes, thymoquinone at low doses (i.e. up to 8 μmol/L) increased cell viability of injured cardiomyocytes. This effect was likely due to the activation of the silent mating type information regulator 2 homolog 1 (SIRT 1; an NAD^+-dependent histone deacetylase, which plays a critical role in many cellular pathways, including apoptosis). Upregulation of SIRT 1 can attenuate the mitochondrial oxidative stress and maintain mitochondrial function which is crucial in averting the apoptotic cell death. The upregulation of SIRT 1 further leads to the suppression of p53 acetylation. Thymoquinone treatment also led to an upregulation of the anti-apoptotic protein Bcl-2 expression, and to a downregulation of expression of the pro-apoptotic proteins Bax and cleaved-caspase 3. All of the abovementioned changes in regulatory proteins most likely prevent the death of cardiomyocytes.

2.5.4.3. Effect of Black Cumin on Blood, Blood Cells, and Blood Vessels

The effect of methanolic extract of black cumin seeds on **platelet aggregation** and blood coagulation was investigated by Enomoto *et al.* (2001). The methanol soluble part was further fractioned by silica gel column chromatography to give ten fractions, some of them active in the assays.

Namely, fractions II and IV showed a high inhibitory activity on arachidonic acid-induced platelet aggregation (inhibition by 92% and 88%, respectively). Further, fractions II and IX exhibited a strong inhibitory effect on blood coagulation. All ten fractions displayed only a low level of inhibitory effects on ADP-induced platelet aggregation. Purification of the active fractions led to the identification of active compounds. A platelet aggregation-inhibitory effect had two known compounds (carvacrol and thymol), and a new compound, namely 2-(2-methoxypropyl)-5-methyl-1,4-benzendiol.

The influence of black cumin on the **platelet count** is according to the available literature a little confusing. Firstly, Zaoui *et al.* (2002a) observed some significant changes in rat blood parameters after 12 weeks of treatment with black cumin seed fixed oil. The counts of leukocytes and platelets decreased (by 35% and 32%, respectively), while the hemoglobin level increased (by 17.4%). On the other hand, Al-Jishi and Abuo Hozaifa (2003) reported that black cumin treatment (for 1, 2, or 4 weeks) did not change the platelet count at any dose tested (90, 180, 360, and 540 mg/kg of powdered seeds). And finally, Asgary *et al.* (2012) observed that rabbits fed a normal diet in combination with black cumin for 8 weeks had significantly increased platelet count, with the risk of enhanced coagulation. The only other available investigation of black cumin effect on platelet count was conducted by Saadia *et al.* (2017). The authors used an aqueous extract of black cumin seeds in rats administered with chloroquine phosphate which induced thrombocytopenia along with a significant decrease of antioxidants and trace metals. Treatment with the black cumin aqueous extract at a dose of 15.25 mg/kg for 12 days increased the platelet count 1.59 fold and elevated the serum levels of catalase. The aim of the study was to investigate the effect of black cumin extract in alleviating the severity of dengue fever that is accompanied with very a low platelet count (7.62 fold lower than control patients).

Awat and Binder (2005) studied the impact of black cumin oil on the fibrinolytic system of the human umbilical vein endothelial cells (HUVECs) and human uterine arterial endothelial cells (HUA-ECs). Both cells cultures exhibited a concentration-dependent increase in the tissue-type plasmogen

activator. The maximum effect for both HUVECs and HUA-ECs was achieved at a black cumin oil concentration of 100 μg/mL.

A positive effect of black cumin on **erythrocytes** was reported by Suboh, Bilto and Aburjai (2004). Preincubation of human erythrocytes with black cumin before the exposure to hydrogen peroxide protected the erythrocytes against the oxidative stress influence, including protein degradation, loss of deformability, and increased osmotic fragility. Similarly, Kanter, Coskun and Gurel (2005) in their investigation of the protective effect of black cumin in cadmium-induced oxidative stress in rats, found that treatment with black cumin reduced the membrane destruction remarkably, as well reducing hemolytic changes of erythrocytes. Further, black cumin significantly decreased the elevated malonyl aldehyde level in erythrocytes (Kanter, Coskun and Gurel, 2005).

The decrease of malonyl aldehyde formation in both intact erythrocytes and an erythrocyte homolysate was also reported by Ahmad and Beg (2013) who used the thymoquinone-rich fraction of black cumin extract in rats fed an atherogenic diet for 4 weeks.

Endothelial dysfunction is also positively influenced by black cumin. For example, Nader, El-Agamy and Suddek (2010) investigated the possible protective effect of thymoquinone on development of atherosclerosis in rabbits fed a high-cholesterol diet. Treatment with thymoquinone for 4 weeks attenuated the negative impact of high-cholesterol diet, such as endothelial damage and thickened foam cell formation. Similar results were obtained when black cumin either powdered (1000 mg/kg) or in oil (500 mg/kg) was administered to rabbits fed an atherogenic diet for 8 weeks. Both black cumin forms were efficient in the inhibition of the plaque formation and in the reduction of the intima/media ratio (Al-Naqeep *et al.*, 2011).

Moreover, pure thymoquinone was also shown to improve the aging-related endothelial dysfunction characterized by blunted nitric oxide- and endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation in arteries. Middle-aged rats were treated with thymoquinone at a dose of 10 mg/kg for 2 weeks. This treatment resulted in the restoration of both

nitric oxide- and EDHF-mediated relaxations of the mesenteric artery. Furthermore, thymoquinone normalized the oxidative stress and the expression level of endothelial nitric oxide synthase (eNOS) and components of the angiotensin system (Idris-Khodja and Schini-Kerth, 2012).

The regulation of eNOS level is most likely a part of the endothelium-protecting mechanism. Black cumin was reported to significantly increase eNOS both in mRNA level and function in rats treated with a seed hydroalcoholic extract at different doses (100, 200, and 400 mg/kg) for 6 weeks. Meanwhile, the expression of vascular cell adhesion molecule-1 and receptor for oxidized low-density lipoprotein (markers of endothelial dysfunction) was decreased in the vascular cells of aortic tissue in rats treated with black cumin (Abbasnezhad *et al.*, 2019). The authors furthermore observed markedly improved **vasorelaxant responses** of the aortic rings to acetylcholine in rats in the black cumin group.

Similar results were obtained in streptozocin-induced diabetic rats treated with a hydroalcoholic black cumin extract (100, 200, or 400 mg/kg) for 6 weeks. Both the contractile and dilatation responses of isolated aortic rings of black cumin treated rats were improved when evaluated with the administration of different agents (phenylephrine, acetylcholine, potassium chloride, and sodium nitroprusside; Abbasnezhad *et al.*, 2016).

The vasorelaxant **mechanism** of black cumin was in part elucidated by Suddek (2010) who investigated the effect of pure thymoquinone on isolated rat pulmonary arterial rings precontracted with phenylephrine. Thymoquinone decreased the tension of the precontracted arterial rings in a concentration-dependent manner. The vasorelaxant effect of thymoqiunone was partially abolished by pretreatment of the arterial rings with a non-selective blocker of the ATP-sensitive K^+ channels (glibenclamide), and significantly potentiated by an endothelin receptors ET_A and ET_B antagonist (bosentan). Hence, the vasorelaxant effect of thymoquinone is at least partially mediated by the activation of ATP-sensitive potassium channels and probably by the non-competitive blocking of endothelin receptors (Suddek, 2010).

The involvement of the ATP-dependent K^+ channels in vasorelaxation caused by black cumin was also confirmed by Niazmand *et al.* (2014). The authors used a hydroethanolic extract (ethanol 50%) of black cumin seeds on isolated intact or endothelium-denuded rat aorta precontracted by potassium chloride and phenyleprhine. The black cumin extract exhibited a concentration-dependent relaxation of the aorta, independent on the endothelium presence. Furthermore, this vasorelaxation was significantly reduced by diltiazem (a Ca^{2+} channel blocker) and heparin (an IP_3 receptor blocker. The IP_3 receptors act as intracellular Ca^{2+} channels), which indicates that the vasorelaxation was mediated through the inhibition of the calcium flow. These observations suggest that thymoquinone is not the only vasorelaxant active compound present in black cumin.

2.5.4.4. Antihypertensive Effect of Black Cumin

A dose-dependent decrease of arterial blood pressure and heart rate in rats after intravenous administration of black cumin volatile oil (4 – 32 μmL/kg) and pure thymoquinone (0.2 – 1.6 mg/kg) was observed by El-Tahir, Ashour and Al-Harbi (1993). Similarly, Hebi *et al.* (2016) observed a dose-dependent reduction in the mean arterial blood pressure accompanied by a significant fall in heart rate after an intravenous application of black cumin aqueous extract (50, 100, or 200 mg/kg) in normotensive rats.

Zaoui *et al.* (2000) orally administered a dichloromethane extract of *N. sativa* L. seeds at a dose of 0.6 mL/kg/day to spontaneously hypertensive rats for 15 days. Black cumin extract treatment led to a significantly increased diuresis (by 16%) and decreased mean arterial pressure (by 22%). The diuretic effect of black cumin was also reported by Asif *et al.* (2015) who used a crude aqueous extract. Rats were administered with either furosemide (10 mg/kg) or black cumin extract (10, 30, or 50 mg/kg) intraperitoneally. The black cumin extract had a dose-dependent diuretic, kaliuretic, and natriuretic effect. At the highest concentration tested, the black cumin extract showed a 46% diuretic activity compared with furosemide.

Similarly, Toma *et al.* (2015) observed the diuretic effect of *N. sativa* L. in rats treated with 100 mg/kg of ethanolic extract. Black cumin extract did exhibit a higher natriuretic than kaliuretic effect. Interestingly, when the investigators tested another *Nigella* species for the presence of the diuretic effect, *N. damascena* L. ethanolic extract did not exhibit a diuretic effect.

The hypotensive effect of a traditionally used polyherbal composition containing equal portions of *N. sativa* L., *Cichorium intybus* L., *Trigonella foenum-graecum* L., and *Gymnena sylvestre* (Retz.) R. Br. ex Schult and its individual components were evaluated by Malik *et al.* (2017a, b) in rats treated for 7 weeks. The intervention with this polyherbal mixture resulted in a significant decrease of mean arterial pressure (Malik *et al.*, 2017b) and systolic blood pressure (Malik *et al.*, 2017a). Administration of particular components demonstrated that black cumin exhibited the most effective hypotensive action of the polyherbal composition (Malik *et al.*, 2017b).

The beneficial effect of *N. sativa* L. on blood pressure in humans has also been reported in literature. For example, Dehkordi and Kamkhah (2008) treated patients with mild hypertension, with black cumin seed extract orally (100 or 200 mg, twice daily) for 8 weeks. The values of diastolic blood pressure were found to be significantly reduced after 8 weeks in a dose-dependent manner. Similarly, Fallah Huseini *et al.* (2013) observed a significant decrease in both systolic and diastolic blood pressure in healthy volunteers aged 34 to 63 years treated with 2.5 mL of black cumin oil for 8 weeks.

Badar *et al.* (2017) provided results of a one-year long trial with 114 poorly controlled type 2-diabetes patients who were treated with 2 g/day of powdered black cumin seeds. Both mean arterial blood pressure and diastolic blood pressure were significantly lower in the black cumin group at the end of the trial, and the systolic blood pressure value also exhibited a decrease, although it was statistically non-significant. Moreover, after three months of black cumin treatment, the heart rate showed a significant decrease that was even more pronounced at the end of the trial.

Qidwai *et al.* (2009) also observed a tendency of blood pressure lowering after 6 weeks of administration of *N. sativa* L. powdered seeds (1000 mg twice daily). The changes were not significant due to the small sample size – only 39 patients out of 64 in the treatment group completed the study.

The **mechanism** of hypotensive action of active constituents from *N. sativa* L. was investigated by several scientists and it appears to be the combination of various actions that contribute to the complex, including the aforementioned diuretic effect.

Based on their experiments with reserpine, atropine, and cyproheptadine, El-Tahir, Ashour and Al-Harbi (1993) concluded that the cardiovascular depressant effects of black cumin volatile oil and thymoquinone in rats were mediated mainly centrally, via indirect and direct mechanisms that involved both muscarinic and 5-hydroxytryptaminergic mechanisms. The direct mechanism may be due to the presence of thymoquinone in the volatile oil, because the cardiovascular depressant effect of thymoquinone was not antagonized by reserpine.

A peroral daily treatment of rats with pure thymoquinone (0.5 mg/kg) after chronic inhibition of nitric oxide synthesis with L-NAME attenuated the increase of systolic blood pressure induced by L-NAME in a dose-dependent manner. Thymoquinone treatment also increased glutathione to normal levels (Khattab and Nagi, 2007). Similar results were obtained by Jaarin *et al.* (2015) in L-NAME-induced hypertensive rats treated with black cumin oil (2.5 mg/kg) for 8 weeks. The blood pressure reduction was associated with a reduction in cardiac lipid peroxidation product, NAPDH oxidase, angiotensin converting enzyme activity, and plasma nitric oxide.

Furthermore, Mahmmoud and Christensen (2011) identified a modulator of the Na^+/K^+ ATPase in black cumin seed oil as a mixture of oleic and linoleic acid that are found in the seed oil in high amounts (see Ch. 2.5.2.). This mixture strongly modified the K^+-dependent reactions in pig kidney ATPase. The authors moreover observed that oleic and palmitic acid stabilized a pump conformation that binds ouabain with high affinity. For the

modulation of the pump sensitivity to cardiac glycosides, the dynamic changes in plasma levels of oleic and linoleic acids are important.

The involvement of calcium flow in the antihypertensive mechanism of black cumin was suggested by Malik *et al.* (2017b) who proposed the mediation through the inhibition of Ca^{2+} influx via membranaceous Ca^{2+} channels and α-adrenergic receptor operated pathways. This suggestion was based on the results of a dose-dependent decline of mean arterial blood pressure caused by black cumin aqueous extract that was similar to the effect of verapamil (a Ca^{2+} channel blocker).

2.5.4.5. Effect of Black Cumin on Plasmatic Lipid Profile

The favorable effect of black cumin on lipid profile is well documented in both animal models and humans. Mollazadeh, Mahdian and Hosseinzadeh (2019) in their review of medicinal plants used for treatment of hypertriglyceridemia even classified black cumin as one of the five herbs with the best triglyceride lowering effect.

Among the first studies of hypolipidemic effect of black cumin conducted on animal models, El-Dakhakhny, Mady and Halim (2000) observed a significant decrease in serum total cholesterol, LDL cholesterol, triglycerides, and a significant elevation of serum HDL cholesterol in rats treated with black cumin oil at a dose of 800 mg/kg for 4 weeks.

A similar result was obtained by Zaoui *et al.* (2002a, b) who used an oral dose of 1 mL/kg of black cumin fixed oil in rats for 12 weeks. This treatment caused a significant decrease in serum total cholesterol and triglyceride levels (by 15.5 and 22%, respectively). Le *et al.* (2004) also reported that rats treated with petroleum ether extract of *N. sativa* L. seeds by intragastric gavage for 4 weeks had lower plasma levels of triglycerides and higher HDL cholesterol. The effect of black cumin on serum lipid levels in rats after a more prolonged administration (20 weeks) was investigated by Dahri *et al.* (2005). And again, a significant decrease in serum LDL cholesterol (by 33%) and a significant increase in HDL cholesterol (by 100%) were observed.

El-Gindy *et al.* (2019) used two doses of black cumin seeds (300 or 600 mg) in rabbits for 8 weeks. The supplementation of black cumin resulted in a significant decrease of serum total lipids, triglycerides, and LDL cholesterol, while it significantly increased the HDL cholesterol level.

The hypolipidemic effect of black cumin is most likely both dose- and duration-dependent, as observed by Kocyigit, Atamer and Uysal (2009). They used four doses of black cumin powdered seeds (100, 200, 400, and 600 mg/kg) and the treatment of rats with black cumin seeds lasted for 1, 2, or 4 weeks. After one week, a significant increase in the HDL cholesterol level was observed with the 400 mg dose. LDL cholesterol levels decreased significantly after one week for 400 and 600 mg doses and after 2 weeks for all doses. After 2 weeks for 400 mg dose and after 4 weeks for all doses, a significant decline in triglyceride levels were noticed. The last to decline was the total cholesterol level that needed 4 weeks of treatment to decrease significantly.

As to investigations of black cumin hypolipidemic effect on human volunteers, for example, Dehkordi and Kamkhah (2008) accomplished a randomized, double-blind, placebo controlled trial to evaluate the efficacy of treatment with an oral *N. sativa* L. seed extract supplement in patients with mild hypertension. After 8 weeks of administration of either 100 or 200 mg of black cumin extract twice daily, a significant decline in total cholesterol and LDL cholesterol levels were observed.

Najmi *et al.* (2008) used black cumin oil (2.5 mL twice daily) in combination with atorvastatin (10 mg) and metformin (500 mg twice daily) for 6 weeks in a total of 60 patients. A significant reduction of total cholesterol and LDL cholesterol was observed in the black cumin treated group compared with the control receiving only atorvastatin and metformin. A favorable effect of black cumin seeds (500 mg twice daily) on serum lipid levels was also reported by Quidway *et al.* (2009), however, because of the small sample size (only 39 patients in the treatment group completed the study), the results were not statistically significant.

Kaatabi *et al.* (2012) recruited 94 patients who were treated with three doses of black cumin seeds (1, 2, or 3 g/day) for 12 weeks. The only statistically significant change in lipid parameters at the lowest dose was the increase in HDL cholesterol after 4 weeks of treatment. The dose of 2 g of black cumin led to a significant decrease in total cholesterol, triglyceride, and LDL cholesterol levels and to a significant increase in HDL cholesterol levels. The highest dose did not exhibit more hypolipidemic effect than the dose of 2 g/day.

Furthermore, the coadministration of black cumin with simvastatin was evaluated as more effective than simvastatin alone in the correction of dyslipidemia in patients treated for 8 weeks (Ahmad Alobaidi, 2014). When compared, treatment with black cumin or simvastatin in rats fed a high-cholesterol diet, both substances showed a significant improvement in the lipid profile. However, in the black cumin treated group, no significant elevation of the alanine amino trasferase level was observed as in the simvastatin treated group, thus indicating a hepatoprotective effect of black cumin (Muneera, Majeed and Naveed, 2015).

Also, black cumin treatment combined with a low-calorie diet or aerobic training is more effective in the normalization of the lipid profile in overweight women than the diet or training alone. The combination of black cumin and aerobic exercise even has a synergistic effect in the improvement of the lipid parameters (Farzaneh *et al.*, 2014; Mahdavi *et al.*, 2015). The hypolipidemic effect of black cumin, however, lasted for only 2 months after cessation of the black cumin supplement in menopausal women, and then they tended to move towards the pre-treatment levels (Ibrahim *et al.*, 2014).

Interestingly, black cumin has a favorable effect on the serum lipid profile disturbed not only by a high-cholesterol diet, but also in cases where the hyperlipidemic state is induced by various chemicals. For instance, lindane, a highly persistent organochlorine insecticide, causes oxidative stress in cells and tissues, and has a very negative influence on different serum biochemical parameters. However, pretreatment with black cumin seed oil alleviated the harmful effects of lindane on rat lipid profiles, as noticed by Attia *et al.* (2011). Black cumin oil administered for 20 days prior to a

sublethal dose of lindane significantly reduced total lipids, total cholesterol, and triglyceride levels and at the same time significantly increased the HDL cholesterol level in rats. The most marked reduction was in the triglyceride level where black cumin pretreatment reversed the effect of lindane almost to a normal level. A decrease in LDL cholesterol was also observed but it was not statistically significant (Attia *et al.*, 2011).

Treatment with pure **thymoquinone** also seems to improve the serum lipid spectrum. Badary *et al.* (2000) administered thymoquinone at a dose of 10 mg/kg to rats with a doxorubicin-induced hyperlipidemic nephropathy. Thymoquinone supplementation with drinking water 5 days prior to the doxorubicin application and then daily thereafter led to a significant decrease in serum total cholesterol and triglycerides.

Ragheb *et al.* (2008) investigated the effect of two different doses of thymoquinone (10 and 20 mg/kg) in rabbits fed a hypercholesterolemic diet. After 8 weeks of treatment, thymoquinone at lower dose decreased serum total cholesterol (by 26%) and LDL cholesterol (by 29%). Thymoquinone at higher dose further decreased serum triglycerides. Nader, El-Agamy and Suddek (2010) also used the rabbit model for investigating the effect of thymoquinone on the development of atherosclerosis. The administration of thymoquinone with a high-cholesterol diet led to significantly decreased levels of total cholesterol, LDL cholesterol, and triglycerides, while it significantly elevated the level of HDL cholesterol.

The proposed hypolipidemic **mechanism** of black cumin was presented by Al-Naqeep, Ismail and Allaudin (2009). They investigated the effect of pure thymoquinone (2 μg/mL) or thymoquinone-rich fraction obtained by the supercritical fluid extraction from black cumin seeds (80 μg/mL; thymoquinone content 2.77% w/w) on two genes involved in the cholesterol metabolism (LDL receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMG-CoA reductase) in a cell culture of HepG2 cells. Both tested substances significantly upregulated the LDL receptor mRNA level (thymoquinone-rich fraction 7-fold and pure thymoquinone 2-fold) and significantly downregulated the mRNA level of HMG-CoA reductase (thymoquinone-rich fraction by 71% and pure thymoquinone by 12%). These

results indicate that black cumin contains more compounds with hypolipidemic properties. Furthermore, black cumin and thymoquinone influence the cholesterol metabolism on two levels: the uptake of LDL cholesterol (which they increase) and the synthesis of cholesterol (which they decrease).

Consistent with these findings are the results obtained by Ahmad and Beg (2013) who used a methanolic extract (100 mg) and the volatile oil (20 mg) extracted from black cumin seeds in rats fed an atherogenic diet. Both tested fractions significantly decreased the hepatic HMG-CoA reductase activity (by 44% and 42%, respectively). The methanolic extract containig high amount of linolenic acid (an ω-6 unsaturated fatty acid; 75%) along with palmitic acid (3%) was more effective than the volatile oil. The linolenic acid shows hypocholesterolemic activity in the presence of palmitic acid in the ratio of 27:1 (Ahmad and Beg, 2013).

Furthermore, Haas *et al.* (2014) demonstrated a significant elevation of apolipoprotein A-I (apo A-I), as well as apo A-I mRNA and gene promoter activity in hepatocytes (a HepG2 cell culture) and intestinal cells (a Caco-2 cell culture) treated with black cumin seed extract. As apo A-I is the major structural and functional protein component of HDL cholesterol, the increase in its expression may explain the heightened levels of serum HDL cholesterol after black cumin treatment.

Some of the dolabellane-type diterpene alkaloids also contribute to the hypolipidemic effect of black cumin. Morikawa *et al.* (2004a, b) isolated several nigellamines from *N. sativa* L. and examined their effect on stored triglycerides in primary cultured mouse hepatocytes. A potent reduction of the triglyceride levels exhibited mainly nigellamines A_1, A_5, B_1, and B_2 with their activities being equivalent to that of clofibrate (a hypolipidemic agent acting as a PPARα agonist). For example, the activity of nigellamine A_5 represented a 64% inhibition of triglyceride levels in the hepatocytes (Morikawa *et al.*, 2004b).

2.5.5. Safety Profile of the Black Cumin Preparations

Black cumin has been used for hundreds of years as a spice or in traditional medicine. It can therefore be considered safe for use in humans. For example, the American Food and Drug Administration agency categorized black cumin as one of the "substances generally recognized as safe" and it is listed in §182.10 Spices and other natural seasonings and flavorings.

There are several works mentioning *N. sativa* L. seeds or seeds oil **toxicity** investigations. On the cellular level, for example, Le *et al.* (2004) used isolated rat hepatocytes. The addition of a petroleum ether extract of black cumin seeds at a concentration of 500 μg/mL and subsequential incubation for 2, 4, or 6 hours did not significantly affect the cell viability.

The safety of *N. sativa* L. was also tested in various animal models. For instance, black cumin oil administered to rats orally at a daily dose of 800 mg/kg for 4 weeks did not adversely affect the serum transaminases (ALT and AST), alkaline phosphatase, or serum bilirubin, indicating low subchronic toxicity to liver tissues (Al-Dakhakhny, Mady and Halim, 2000).

Similarly, the safety profile of *N. sativa* L. seed fixed oil was investigated by Zaoui *et al.* (2002b) on two animal models. The acute toxicity was tested in mice. Black cumin seed fixed oil was administered both orally and peritoneally. The found LD50 values were 28.8 mL/kg for oral administration and 2.06 mL/kg for peritoneal administration. In their another study with *N. sativa* L. seed fixed oil, no mortality was observed in mice after an oral administration of 10 mL/kg of oil during the 15 day period after the administration (Zaoui *et al.*, 2002a). The chronic toxicity of black cumin seed fixed oil was studied in rats treated daily with an oral dose of 2 mL/kg for 12 weeks. After this period, there were no changes in key hepatic enzymes (transaminases AST, ALT, and GGT) and histopathological modifications (heart, liver, kidneys, and pancreas).

Le *et al.* (2004) also found no physicial or behavioral signs of toxicity, such as lethargy, hyperactivity, restlessness, respiratory distress, or

convulsions, in rats treated with a black cumin seed petroleum ether extract at a dose equivalent to 2 g/kg of the original seed powder for 4 weeks.

In 136 patients with chronic kidney disease, black cumin oil (2.5 mL perorally, once daily) was used as an add-on therapy for 12 weeks. Most of the observed **adverse effects** were mild (no hospitalization, no change of therapy, no additional treatment needed). All of the adverse effects were latent or subacute in onset, no case belonged to the category of acute onset (within 60 minutes). Mild nausea in the beginning of the trial was reported, but it did disappear during the second week of the duration of the study (Alam *et al.*, 2020).

Ibrahim *et al.* (2008) even observed a hepatoprotective effect of the crude *N. sativa* L. seed oil in rats with a tetrachloride carbon-induced downregulation of cytochrome P450 isozymes. Oral pretreatment with 1 mL/kg of black cumin oil for 7 days before the intraperitoneal application of carbon tetrachloride alleviated the suppression of CYP2B, CYP3A2, CYP2C11, and CYP1A2 enzymes. The role of black cumin and its bioactive constituents (namely thymoquinone, thymol, and α-hederin) as hepatoprotectant agents was in detail reviewed by Tabassum, Ahmad and Ahmad (2018). The abovementioned compounds protect from liver injury mainly via their antioxidant and anti-inflammatory activities.

As to the toxicity of pure compounds contained in black cumin, the situation is more complicated. Unlike the complex *N. sativa* L. seed oil, the most active compound, **thymoquinone**, is evidently less safe. Mansour *et al.* (2001) stated that higher doses of thymoquinone were found to induce oxidative stress leading to a hepatic injury. The LD50 for thymoquinone in mice was 90.3 mg/kg.

The same unfavorable effect was observed on the cellular level by Khader, Bresgen and Eckl (2009). They used isolated rat hepatocytes incubated for 48 hours with different concentrations of thymoquinone ranging only from 1 to 20 μmol/L, because higher concentrations induced severe cell death within minutes to hours of treatment. At the concentration of 1 mmol/L, thymoquinone induced immediate cytotoxicity, as evidenced by

nuclear shrinkage and plasma membrane blebs within minutes of application. The concentrations of 20 and 10 μmol/L also showed cytotoxic effects by inducing high levels of cell necroses. The lower concentrations of thymoquinone were less toxic but induced adverse effects, such as increased levels of necroses, frequencies of chromosomal aberrations, and formation of micronuclei (Khader, Bresgen and Eckl, 2009).

Notable decreased cell viability after treatment of rat cardiomyocytes with thymoquinone at concentrations of 16 and 32 μmol/L was also reported by Lu *et al.* (2018). However, lower concentrations of thymoquinone (1, 2, 4, and 8 μmol/L, respectively) did not negatively affect the cell viability of cardiomyocytes (Lu *et al.*, 2018). A similar concentration of thymoquinone was found to be safe for hepatocytes HepG2 cell culture by Al-Naqeep, Ismail and Allaudin (2009). They reported that incubation of the cells with 2 μg/mL (equals to approximately 12 μmol/L) did not alter the proliferation rate of HepG2 and most cells (87%) were found to be in the stage of viable cells.

Llana-Ruiz-Cabello *et al.* (2015) again showed that oxidative stress plays a role in the damage induced to intestinal cells (a Caco-2 cell culture) by carvacrol and its mixture with thymol at high concentrations (460 μmol/L for carvacrol and 300/30 μmol/L for the mixture). However, at lower concentrations both compounds and their mixture protected cells against the damage induced by hydrogen peroxide.

2.6. Olive – *Olea europaea* L. – fam. *Oleaceae*

2.6.1. Characterization of *Olea europaea* L. – a Pharmacognostic Context

Olea europaea L. (Fig. 2.11.) is a subspecies of the subgenus *Olea*, one of the three subgenera of the genus *Olea*. The whole genus *Olea* includes approximately 40 taxa of shrubs and trees. Olive is a small evergreen tree, most likely native to parts of southern Europe and Asia Minor. It is found mostly in tropical and subtropical areas (e.g. the forests of Mediterranean, the Sahara, Australia, and China). The olive tree can, however, survive in a wide

range of environments. Olive oil and its fruit are edible (Ray *et al.*, 2009 and references therein).

For medicinal use, usually fresh olive leaves are collected (**Oleae folium**). The leaves are of elliptical shape, thick and leathery, whole-marginated. The upper surface of the leaf is bright green with a particular shine to it, while the bottom surface of the leaves is silvery, slightly haired. The content of secoiridoids must not be less than 5.0%, expressed as oleuropein, calculated with reference to the dried drug. The European Pharmacopoeia furthermore includes a monograph on the olive leaf extract (**Oleae folium extractum siccum**) and two monographs on olive oil (**Olivae oleum raffinatum** and **Olivae oleum virginale**; Rahfeld, 2017; European Pharmacopoeia 10.0).

Fig. 2.11. *Olea europaea* L. A closeup of a tree branch with unripe fruits. From shutterstock.com.

Fresh leaves are further processed by extraction with different solvents. The extraction of olive leaves with 80% ethanol leads to a high phenolic and flavonoid content, as demonstrated by Lee *et al.* (2009), and it is commercially used in the production of olive leaf extract EFLA 943 (see Ch. 2.6.3.), although the extraction with butanol or ethylacetate also yields high amounts of these compounds.

Lately, alternative methods for obtaining phenolics from olive tree plant material with the emphasis on the eco-friendly and sustainable extraction procedure were also reported. For example, Japón-Lujan and Luque de Castro (2007) proposed a simple and rapid (under 10 minutes) extraction process of biophenols from the small branches of olive trees, accelerated by microwave, as an alternative form of olive leaf extraction. Olive pomace, which is produced in great amounts as a waste product in the olive oil industry, also seems to be a promising source of several bioactive compounds. A multi-frequency multimode modulated ultrasonic technique for the extraction of phenolic compounds from olive pomace with a high yield of these compounds was presented by Nunes *et al.* (2018). Further, Paze *et al.* (2019) optimalized the pressurized liquid extraction of phenolics from olive pomace without organic solvents. The yields of secoiridoids and flavonoids in this optimalized pressurized liquid extraction were three to four times higher than in the conventional extracts.

2.6.2. Phytochemistry of Olive Leaf

The primary olive leaf compounds are secoiridoids (oleuropein and its derivates), hydroxytyrosol, polyphenols (verbascoside, apigenin-7-glucoside, luteolin-7-glucoside), triterpenes (including oleanolic acid), and flavonoids (rutin and diosmin). Oleuropein content is significantly higher in the leaf than in other parts of the plant (Olive Leaf Monograph, 2009).

Up to 36 distinct phenolic compounds can be found in olive fruit. A defining structural component of the **secoiridoid group** of olive phenolic compounds is elenolic acid. The molecule of elenolic acid is incorporated within the most important phenolics, such as oleuropein, ligstroside, and elenolic acid-linked hydroxytyrosol and tyrosol (Ray *et al.*, 2009). Some of the olive leaf phenolics are depicted in Fig. 2.12.

Oleuropein is generally the most prominent phenolic compound in olive cultivars. Its molecule consists of three structural subunits: a polyphenol (hydroxytyrosol), a secoiridoid (elenolic acid), and a glucose molecule. The

biosynthesis of oleuropein proceeds via a branching in the mevalonic acid pathway from the secondary metabolism, forming the oleosides from which secoiridoids are derived (Omar, 2010a, b).

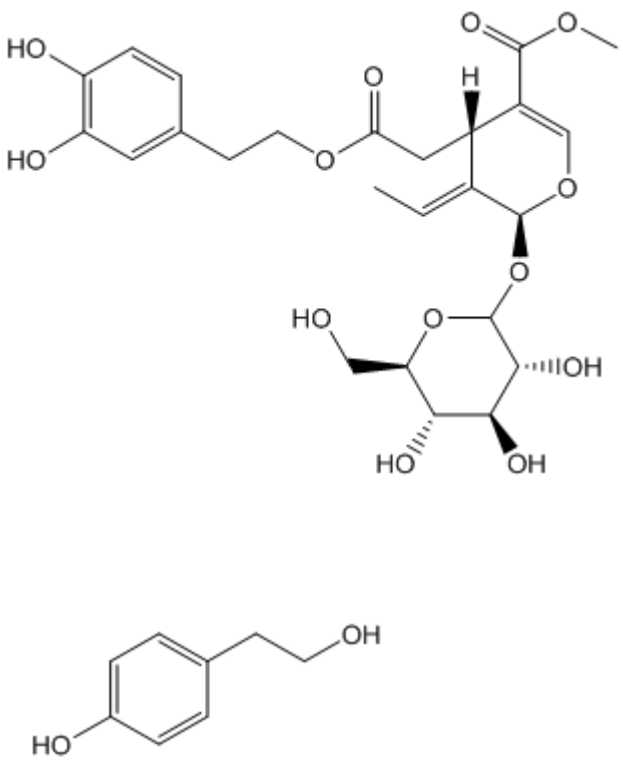

Fig. 2.12. Some of the *Olea europaea* L. phenolics. Up – oleuropein, down – tyrosol. Courtesy of doc. Macáková.

The content of oleuropein and other active compounds in olive leaves may vary considerably depending on several factors, such as genotype or season of the year. The genotype variation in oleuropein content was reported in seven Italian cultivars (Ranalli *et al.*, 2006) and in nine Turkish olive cultivars (Orak *et al.*, 2019). A comprehensive chemical analysis of Iranian olive cultivars (Ghasemi *et al.*, 2018) demonstrated that oleuropein was even not detected in four out of the seventeen cultivars tested.

The seasonal variation of phenolic content in olive trees was studied by Xu *et al.* (2018), who reported that the oleuropein content was high in spring and winter months (the highest oleuropein content was detected in

March and November), while low oleuropein content was observed in the summer and autumn months (May and September, respectively).

On the other hand, the developmental stage of the leaf does not seem to play any role in the oleuropein content which stays fairly constant from immature leaf stadium throughout the full expansion of the leaf (Malik and Bradford, 2006). Contrary to this observation, oleuropein content in the olive fruit varies significantly. The highest oleuropein content was reported in the growth phase. Later, in the green maturation phase and in the black maturation phase of the fruit, oleuropein content consequently decreases (Omar, 2010a and references therein).

2.6.3. Medicinal Use of Olive Leaf

According to the EMA monograph on *Olea europaea* L., folium, olive leaf is a traditional herbal product used to promote the renal elimination of water in mild cases of water retention after serious conditions have been excluded by a medical doctor. It can be used as an herbal tea (prepared of fresh or dried leaves) or in the form of dried leaves (comminuted or powdered; EMA/HMPC/359238/2016). However, most of the recent studies have focused on the other beneficial influences of olive leaf on human health with an emphasis on the antioxidant, antiatherogenic, antihypertensive, lipid-lowering, and even anti-cancer properties of the active compounds.

In several of studies mentioned in the following text, a commercially available olive leaf extract EFLA 943 was used. This extract is obtained through the extraction of olive leaves with 80% ethanol (w/w), and the resultant product is purified by a patented procedure to remove the undesired contaminants and residues. Then the solvent is removed, resulting in a free flowing powder containing 18 – 26% oleuropein (w/w).

2.6.4. Effects of Olive Leaf on Selected Cardiovascular Conditions

2.6.4.1. Antioxidant Effect of Olive Leaf

There are numerous studies documenting the antioxidant activity of olive fruit, olive leaf, or olive oil extracts or their individual active compounds. Nearly all of the studies presenting the influence of olive leaf extract or individual olive compounds on the hypertension or the lipidic profile also mentioned the antioxidant activity of olive phenolics. Some of the works were, however, focused solely on the assessment of the antioxidant capacity of olive leaf extract and/or pure phenolics.

For example, Visioli, Bellomo and Galli (1998) focused on the scavenging activity of oleuropein and hydroxytyrosol, and in their work demonstrated that both studied phenolics are potent scavengers of superoxide radicals and inhibitors of neutrophils' respiratory bursts. Kruk *et al.* (2005) performed a complex study on the antioxidant activity of oleuropein (and genistein, respectively) and confirmed the strong antioxidant activity of oleuropein against superoxide radicals. Furthermore, oleuropein also reacted with peroxyl radicals (60% inhibition) and hydroxyl radicals (up to 90% inhibition).

Roche *et al.* (2005) tested the antioxidant activity of several olive phenolics in various models. The highest number of radicals trapped per antioxidant molecule was attributed to dihydrocaffeic acid. Oleuropein, hydroxytyrosol, and caffeic acid on the other hand had the highest rates of transfer of the most labile H-atoms of the antioxidant.

As to the **mechanism** of antioxidant action, Stupans *et al.* (2002) tested the potency of selected natural antioxidants including oleuropein and hydroxytyrosol. Their results suggest that complex phenols, such as hydroxytyrosol, both inhibit the radical generation and act as free radical scavengers. Roche *et al.* (2005) described in detail the proposed mechanism of peroxyl radical scavenging by olive phenolics. This process includes the

oligomerization and a specific fragmentation of the formed dimers and trimers.

Oleuropein in its chemical structure contains an ortho-diphenolic moiety, which is able to scavenge ROS through hydrogen donation, and to stabilize oxygen radicals with an intramolecular hydrogen bond. Tyrosol, on the other hand, has only a single hydroxyl substitution providing none of the antioxidant properties (Nediani *et al.*, 2019 and references therein).

2.6.4.2. Direct Effect of Olive Leaf on Myocardium

Petkov and Manolov (1972) tested pure oleuropein obtained from olive leaves on isolated rabbit hearts. Their results show the activity of oleuropein against a barium chloride-induced **arrhythmia** in rabbits, and against a calcium-induced arrhythmia in cats. Esmailidehaj *et al.* (2012b) measured the effect of oleuropein treatment in rats on arrhythmia induced by ischemia and reperfusion. The administration of oleuropein at a dose of 100 mg/kg one hour or three hours before the ischemia resulted in a significantly decreased reperfusion arrhythmia in various forms, such as ventricular ectopic beats, ventricular tachycardia, and ventricular fibrillation.

In their another study with oleuropein on rats (Esmailidehaj *et al.* 2012a), the authors tested a lower dose of oleuropein (20 mg/kg) administered one to 48 hours before an intravenous infusion of aconitine. This single dose did not offer any preconditioning effect against the aconitine-induced arrhythmia. However, a prolonged administration of oleuropein (4 weeks) prior to the aconitine infusion led to a significant increase of the mean initiation time of both arrhythmia and ventricular tachycardia, to a significant increase of reversible ventricular fibrillation, and to a significant increase in the mean death time.

Olive leaf extract EFLA 943 was further proven to have negative inotropic activity in isolated guinea pig hearts both when applied alone and in a mixture with *Hibiscus sabdariffa* flower extract. Moreover, the mixture showed negative chronotropic efficacy (Micucci *et al.*, 2015; 2016).

The **cardioprotective effect** of olive leaf in an ischemic myocardium was reported by several authors in the last fifteen years. The authors used either isolated hearts or whole animals of several species and different pretreatment regimes with oleuropein in various concentrations, but the cardioprotective effect was observed in every work mentioned in the following text. The cardioprotective effect is evidently dose-dependent and time-limited. Esmilidehaj *et al.* (2012) estimated that the duration of the cardioprotective effect of a single dose of oleuropein (100 mg/kg) to be 6 hours.

In isolated heart experiments, the dosage of olive leaf extract is usually in the range of 10 to 50 μg per g of heart weight. For example, Manna *et al.* (2004) used isolated rat hearts pretreated with oleuropein (20 μg/g heart) and then subjected them to 30 minutes of global ischemia and following reperfusion. The release of oxidized glutathione, a sensitive marker of the heart's exposure to an oxidative stress, was significantly reduced in the hearts pretreated with oleuropein.

Very similar results were presented by Esmailidehaj *et al.* (2016), who demonstrated the cardioprotective effect of oleuropein (10 and 50 μg/g heart) in isolated rat hearts 5 minutes before the induction of 30 minutes of global ischemia or at the beginning of 90 minutes of reperfusion. Pretreatment with oleuropein resulted in a significant improvement of both the coronary outflow and the decrease in the size of the infarct area.

A higher dose of oleuropein (tenths to hundreds mg/kg of body weight per day) is used in the animal model studies. Janahmadi *et al.* (2015) treated rats with pure oleuropein (10, 20 or 30 mg/kg/day, respectively) for 7 days before coronary ligation, a surgically-induced model of myocardial infarction. This pretreatment with oleuropein prevented the impairment of cardial functions, such as left ventricular developed and systolic pressures, stroke volume, and ejection fraction and resulted in a decrease of the infarct area size.

Andreadou *et al.* (2015) also used the oleuropein pretreatment (20 mg/kg/day) in rabbits for 6 weeks before subjecting the animals to

30 minutes of myocardial ischemia followed by 10 minutes of reperfusion. Myocardial biopsies were then taken from the ischemic areas. In the oleuropein treated rabbits, oxidative biomarkers (namely malonyl dialdehyde and nitrotyrosine) were significantly reduced.

Xu *et al.* (2018) found that olive leaf extract can even ameliorate the effects of an isoprenaline-induced, acrolein-aggravated myocardial infarction in rats, a situation simulating the air pollution in big cities. In this case, the olive leaf extract pretreatment (200 and 400 mg/kg/day, respectively) again resulted in the reduction of myocardial tissue damage and in a decrease in the infiltration of inflammatory cells. Furthermore, the olive leaf extract pretreatment reversed the values of the parameters of cardiac systolic function, such as ejection fraction and fractional shortening.

The **mechanism** of the cardioprotective effect of olive leaf extract or individual compounds may be partially explained by the inhibition of the apoptosis in the cardiomyocytes. Bali *et al.* (2014) tested the effect of olive leaf extract and pure compounds (namely oleuropein, hydroxytyrosol, and quercetin) on chemically induced oxidative damage to rat cardiomyocytes. The apoptosis induced by 4-hydroxynonenal was inhibited by both methanol and ethanol olive leaf extracts as well as by all tested individual compounds. The ethanolic extract had more protecting ability with a higher content of oleuropein, hydroxytyrosol, and quercetin. Olive leaf extract or pure compounds attenuated all studied parameters, including the reactive oxygen species production, the activation of the pro-apoptotic enzyme caspase-3, the phosphorylation of stress-induced transcription factors, and furthermore enhanced cell viability and mitochondrial function.

Zhang *et al.* (2017) simulated an ischemia/reperfusion-induced cardiomyocyte injury *in vitro*. The addition of oleuropein to neonatal rat cardiomyocytes led to a significantly reduced intracellular reactive oxygen species generation as well as the stabilization of mitochondrial membrane potential (MMP). MMP is an important early marker of the mitochondrial apoptotic pathway. Further analysis of some apoptosis-related proteins in oleuropein-treated cells revealed the attenuation of the expression of

pro-apoptotic enzymes caspase-3 and caspase-9, and changes in the Bcl-2/Bax ratio (high values of this ratio indicate a pro-apoptotic status).

Similarly, Xu *et al.* (2018) examined critical molecules in the endoplasmic reticulum stress pathway (namely GRP78 and CHOP) and apoptosis-related molecules (Bcl-2/Bax ratio) in the infarct tissue of rats with an isoprenaline-induced, acrolein-aggravated myocardial infarction. All of the mentioned molecules were increased at the protein and mRNA levels more obviously with the acrolein treatment. However, the altered expression of these molecules was significantly reversed by olive leaf extract pretreatment. Based on their *in vitro* part of the study with cardiomyocyte H9c2 cells, the pretreatment with pure oleuropein or hydroxytyrosol can protect the cells from the acrolein-induced apoptosis, with hydroxytyrosol being the more efficient compound.

The assay of apoptosis-related proteins from rat heart tissue exposed to a simulated ischemia/reperfusion injury showed that oleuropein treatment led to an attenuated caspase-3 activity and p-53 protein expression, which again indicates the anti-apoptotic effect of oleuropein (Jin *et al.*, 2018).

2.6.4.3. Effect of Olive Leaf on Blood, and Blood Vessels

The effect of a decoction of olive leaves on smooth vascular muscle was studied by Zarzuelo *et al.* (1991). The lyophilized decoction caused **relaxation** of isolated rat aorta both in the presence and in the absence of endothelium, indicating that the vasorelaxant activity of olive extract is independent of the integrity of endothelium.

The vasorelaxant effect of virgin olive oil phenolic extract on isolated rat aorta was also reported by Benkhalti *et al.* (2003). The effect persisted even when the activity of nitric oxide synthase was inhibited. On the other hand, pure oleuropein did not exhibit any vasorelaxant activity, indicating another role of this compound. Further, the vasorelaxant potency of olive leaf extract on isolated guinea pig smooth muscle was noticed by Micucci *et al.* (2015). The potassium-induced contraction of vascular smooth muscle was

reduced by olive leaf extract in a dose-dependent manner. And similarly, a chronic administration of olive leaf extract in rats led to a reversion of the impaired aortic endothelium-dependent relaxation to acetylcholine in spontaneously hypertensive rats. Olive leaf extract restored the aortic endothelial nitric oxide synthase (eNOS) phosphorylation and increased the eNOS activity (Romero *et al.*, 2016).

The role of oleuropein may be the **inhibition of endothelial activation** (as a part of the antiatherogenic effect), as reported by Carluccio *et al.* (2003). The authors incubated several phytochemicals from olive oil and red wine including oleuropein, tyrosol, and hydroxytyrosol with human umbilical vein endothelial cells for 30 minutes, and then they attempted to trigger the adhesion molecule expression by the co-incubation with a bacterial lipopolysaccharide or cytokines. At nutritionally relevant concentrations, both oleuropein and hydroxytyrosol did reduce the activation of endothelium, measured as the monocytoid cell adhesion to the stimulated endothelium and the amount of the vascular cell adhesion molecule-1 mRNA.

Similarly, Turner *et al.* (2005) investigated the activity of olive oil phenolics oleuropein, tyrosol, hydroxytyrosol, and homovanillic alcohol in cell cultures. Their results show that these tested phenolics lead to a significant decrease of the secretion of the intercellular adhesion molecule-1 and the vascular cell adhesion molecule-1.

A polyphenol extract from olive pomace was also reported to have a beneficial effect on the endothelial function impaired by anoxia. Palmieri *et al.* (2012) exposed human endothelial cells to the anoxic stress in the presence or the absence of the polyphenol extract or pure oleuropein and tyrosol. The polyphenol extract was more effective in preventing endothelial dysfunction (measured as the expression of genes involved in proteolysis, angiogenesis, and inflammation) than pure oleuropein or tyrosol. The polyphenol extract moreover prevented the proliferation and migration associated with anoxia in the endothelial cells.

The inhibition of **platelet aggregation** in the presence of the phenolic compounds of olive oil was reported by Petroni *et al.* (1995). The inhibition of

aggregation of platelet-rich plasma was more effective when either phenol-enriched extract obtained from aqueous waste from olive oil, or a specific component of olive oil (namely DHPE: 2-(3,4-dihydroxyphenyl)-ethanol) was used. Oleuropein and some selected flavonoids (luteolin, apigenin, and quercetin) were found to be much less effective in the inhibition of platelet aggregation. In cell cultures, oleuropein was even shown to not exert any platelet aggregation-inhibitory properties (Turner *et al.*, 2005).

Singh *et al.* (2008) incubated full human blood from healthy volunteers with five different concentrations of commercially available olive leaf extract (standardized to contain 5.40 mg/mL of oleuropein) for 6 minutes. The platelet aggregation was strongly inhibited at the two highest olive leaf extract concentrations, thus indicating that the anti-aggregant activity of oleuropein is dose-dependent.

Similarly, Dell'Agli *et al.* (2008) observed the ability of oleuropein to inhibit the platelet aggregation. The authors used human washed platelets stimulated with thrombin and subsequently treated with olive oil phenols (extract or pure compounds including oleuropein aglycone, tyrosol, hydroxytyrosol, and homovanillic alcohol) for the aggregation assay. Of all individual compounds tested, oleuropein aglycone exhibited the strongest inhibitory effect on the platelet aggregation.

Zbidi *et al.* (2009) again confirmed the platelet aggregation-inhibitory activity of oleuropein and cycloolivil, another polyphenolic compound isolated from an ethyl acetate extract of olive tree wood. Both compounds reduced the ability of thrombin to stimulate platelet aggregation, and furthermore reduced the thrombin-evoked calcium release and entry. Interestingly, this effect was greater in the platelets from diabetes mellitus type 2-patients than in those from healthy individuals when compared to the control.

2.6.4.4. Antihypertensive Effect of Olive Leaf

The research on the antihypertensive effect of olive extract (leaf or fruit) clearly shows that treatments with these extracts have a beneficial influence on blood pressure in both animal models and humans. The animal model for the majority of the studies is a spontaneously hypertensive rat model, although Khayyal *et al.* (2002), for example, treated L-NAME-induced hypertensive rats with olive leaf extract EFLA 943 (100 mg/kg) for 6 weeks. The blood pressure of the animals, which had risen after the L-NAME treatment, started to drop progressively over the course of the experiment to reach a normalized blood pressure at the end of the trial.

Romero *et al.* (2016) treated rats with oleuropein-enriched olive leaf extract (containing 15% of oleuropein; 30 mg/kg) for 5 weeks and observed a significant reduction of the systolic blood pressure and heart rate at the end of the experiment. Furthermore, this treatment led to a reduction of the cardiac and renal hypertrophy.

The influence of olive leaf extract EFLA 943 and pure oleuropein on systemic hemodynamic parameters in spontaneously hypertensive rats was studied by Ivanov *et al.* (2018). A bolus dose of olive leaf extract EFLA 943 (5, 25, or 50 mg/kg) was administered to the animals directly before the measurement. After the application of both higher doses of EFLA 943, systolic pressure, diastolic pressure, and mean arterial pressure were all significantly decreased when compared to the values before treatment. Furthermore, the highest administered dose of EFLA 943 led to a significantly decreased heart rate and cardiac output.

The hypotensive effect of olive leaf extract was also tested in human volunteers with untreated suboptimal blood pressure. Treatment with 1000 mg of olive leaf extract EFLA 943 for 8 weeks led to a significantly decreased systolic blood pressure (Perrinjacquet-Moccetti *et al.*, 2008).

A comparative study on the efficacy of olive leaf extract EFLA 943 versus captopril (an ACE inhibitor) in mildly hypertensive patients was presented by Susalit *et al.* (2011). The volunteers were given either olive leaf

extract (500 mg twice daily) or captopril (12.5 mg twice daily) for 8 weeks. The olive leaf extract demonstrated a comparable blood pressure lowering effect to that shown by captopril. Furthermore, the olive leaf extract was found to significantly reduce both the total cholesterol and the triglyceride levels, while in the captopril group no such beneficial effect occurred.

The **mechanism** of the hypotensive activity seems to be multifactorial, according to various studies conducted through the last twenty years. Hansen *et al.* (1996) observed that *in vitro*, an aqueous extract of olive leaves (namely from the species *O. europaea* L. and *O. lancea* Lam.) exhibited an inhibitory effect on the angiotensin converting enzyme. The main inhibitory compound was identified as the secoiridoid oleacin. The authors further tested selected secoiridoid glycosides (including oleuropein) for the ACE-inhibitory activity. While the glycosides showed no significant ACE inhibition, after the process of enzymatic hydrolysis, inhibitory activity of the corresponding compounds rapidly increased. Oleacin, a dialdehyde derivate of oleuropein, was identified as a strong ACE inhibitor.

The ACE-inhibitory activity of oleuropein was also observed *in vivo* in rats pretreated with oleuropein (20 or 40 mg/kg) for 7 days and then intoxicated with isoproterenol to induce a myocardial injury. While the application of isoproterenol resulted in a 78% increase of ACE activity, treatment with oleuropein induced a remarkable decrease of ACE activity by 33 and 40%, respectively (Mnafgui *et al.*, 2015). Sun *et al.* (2017) used a higher dose of oleuropein (60 mg/kg) and a prolonged treatment (8 weeks), and again confirmed the significantly reduced expression of the components of the renin-angiotensin system in spontaneously hypertensive rats.

Gilani *et al.* (2005) tested an olive fruit extract on animal models and observed a fall in systolic, diastolic, and mean arterial blood pressure in normotensive rats. Pretreatment with atropine, a competitive blocker of acetylcholine at muscarinic receptors, did not change the hypotensive effect of olive fruit extract, thus leading to the suggestion that the olive extract mediates its hypotensive action through mechanisms independent of the muscarinic receptor activation. The existence of the calcium antagonistic activity was confirmed when the olive extract caused a dose-dependent

rightward shift in the Ca^{2+} dose-response curves, similar to verapamil (a standard Ca^{2+} channel blocker). Three years later Scheffler *et al.* (2008) used voltage clamp experiments in cultured rat cardiomyocytes to demonstrate that olive leaf extract suppresses the L-type calcium channel directly and reversibly.

Non-phenolic compounds also contribute to the hypotensive effect of olive leaf extract, as demonstrated by Somova *et al.* (2003) who used only triterpenoids, isolated from the olive leaves of three cultivars (from Greece, South Africa, and a wild African olive). All three extracts were administered at a dose of 60 mg/kg to salt-sensitive insulin-resistant rats for 6 weeks and prevented the development of severe hypertension and atherosclerosis.

2.6.4.5. Effect of Olive Leaf on Plasmatic Lipid Profile

The antioxidant properties of olive phenolics on the **oxidation of low-density lipoprotein** (LDL) have been extensively studied. In 1994 Visioli and Galli tested the protective role of oleuropein against the copper sulfate-induced LDL oxidation. Oleuropein effectively inhibited the LDL oxidation even at low concentrations (10^{-5} mol/L). Caruso *et al.* (1999) evaluated the potency of virgin olive oil phenolics, individual compounds tyrosol and oleuropein, and a widely used synthetic antioxidant probucol during the photo-induced LDL oxidation. Their results clearly demonstrated that olive phenolics had a significantly stronger influence on preventing of the cholesterol oxide formation and apoproteic moiety modification than the individual compounds, and even more than probucol.

Coni *et al.* (2000) in their *in vivo* study with rabbits fed either a standard diet, a diet with addition of 10% extra virgin olive oil, or a diet with an addition of oleuropein (7 mg/kg) observed that the addition of oleuropein not only increased the ability of LDL to resist oxidation (evaluated as the less conjugated diene formation) but also decreased the plasmatic levels of total, free, and ester cholesterol.

Leenen *et al.* (2002) incubated blood plasma with individual compounds or olive oil extracts, and then LDL was isolated and exposed to oxidation. The results show that ortho-dihydroxy phenols (oleuropein aglycone and hydroxytyrosol) are more efficient than mono-hydroxy phenols (tyrosol and ligstroside aglycone) in increasing the resistance of LDL to oxidation.

A fairly complex insight on the effect of various olive oil constituents on inhibition of the copper sulfate-induced LDL oxidation was presented by Andrikopoulos *et al*. (2002). The authors examined polyphenolic and non-phenolic compounds at various doses. All of the tested compounds had a higher LDL mean protection activity than tocopherol, with the only exception for the phytosterol stigmasterol. The highest LDL mean protection activity was found in triterpenoid ursolic acid, flavonoid luteolin, and phenolic oleuropein at the highest used concentration.

Benkhalti *et al.* (2003) and Turner *et al.* (2005) again confirmed the inhibitory effect of oleuropein on LDL oxidation. A new insight on this effect was presented by Briante, Febbrario and Nucci (2004) who used individual compounds (oleuropein, hydroxytyrosol) or complex mixtures obtained by biotransformation of olive leaf extracts in their study on the copper ion-induced LDL oxidation. The authors reported that when the concentration of phenols is higher than that of copper ions, the LDL oxidation is inhibited by oleuropein and hydroxytyrosol in the initiation phase of the reaction. However, at a lower concentration both phenols anticipated the initiation process of the LDL oxidation, thus exerting pro-oxidant capacities. The pro-oxidant behavior of oleuropein was also reported by Mazziotti *et al.* (2006) in a Fenton-like *in vitro* experiment with ferrous ions. Needless to say, the effect of both oleuropein and hydroxytyrosol on the copper ion-induced LDL peroxidation is indeed determined by a balance of their pro-oxidant and antioxidant capacities. Furthermore, a synergistic effect among the phenolic compounds enhanced their antioxidant capacities and in this manner they avoided the pro-oxidant effects (Briante, Febbrario and Nucci, 2005).

Olive leaf extract moreover has a beneficial influence on serum **triglyceride** and **cholesterol levels**, as observed by several authors on both

animal models and with human volunteers. For example, Ivanov *et al.* (2018) reported significantly decreased values of total cholesterol and triglycerides in the plasma of rats treated with olive leaf extract EFLA 943. Treatment with pure oleuropein at a dose of 10 mg/kg had no effect on total cholesterol concentration, but it lowered the concentration of triglycerides in the plasma.

Similar results were obtained by Malliou *et al.* (2018) who tested the effect of oleuropein supplementation at a dose of 100 mg/kg on mice. After 6 weeks, a significant reduction of serum triglyceride levels and liver triglyceride content as well as total cholesterol concentration was observed, indicating that a higher dose of oleuropein is needed for the total cholesterol reduction.

Jemai *et al.* (2008) tested the effect of oleuropein-rich olive leaf extracts and their enzymatic and acid hydrolysates, respectively rich in oleuropein aglycone and hydroxytyrosol, on rats fed a high-cholesterol diet for 16 weeks. Administration of the olive leaf extracts significantly lowered the serum levels of total cholesterol, LDL cholesterol, and triglycerides and increased the level of HDL cholesterol.

Similarly, Fki *et al.* (2020) administered hydroxytyrosol-rich and oleuropein-rich olive leaf extracts to rats fed a high-fat diet for 8 weeks. Both oleuropein and hydroxytyrosol treatment resulted in a decrease of total cholesterol, LDL cholesterol and triglyceride levels, while the HDL levels increased. Based on the data provided by the authors, hydroxytyrosol seems to be more effective in the alteration of the lipid profile than oleuropein.

The beneficial effect of olive leaf extract on the lipid profile in human volunteers was demonstrated by Perrinjacquet-Moccetti *et al.* (2008) who used olive leaf extract EFLA 943 in their study with 40 pairs of monozygotic twins. The volunteers were provided either with placebo or 500 mg EFLA 943 for 8 weeks, and subsequently either with 500 mg or 1000 mg EFLA 943 for another 8 weeks. Absolute cholesterol levels showed a decrease during the course of experiment in all treatment groups. A statistically significant decrease was observed for the total cholesterol and LDL cholesterol concentrations in volunteers treated with 1000 mg EFLA 943.

Araki *et al.* (2019) focused on the effect of olive leaf tea and low-concentration olive leaf tea on non-obese and non-diabetic volunteers (330 mL of the respective beverage, 3 times daily). After 12 weeks, a significant decrease in serum levels of LDL cholesterol and triglycerides was observed, this decrease being more pronounced in the olive leaf tea group.

The **mechanism** of the hypolipidemic effect of olive active compounds is multifactorial and most likely involves the cessation of both adipocyte differentiation and the process of adipogenesis. Either way, transcription factors of the PPAR family (peroxisome proliferator-activated receptor; they play essential roles in the control of lipid metabolism and energy homeostasis) represent a key component.

Svobodová *et al.* (2014) investigated the effect of oleuropein on an *in vitro* 3T3-L1 adipocyte cell culture and reported that oleuropein inhibited the differentiation of most adipocytes at the concentrations of 200 and 400 μM, respectively. Furthermore, starting at a 10μM concentration, oleuropein in a dose-dependent manner reduced the expression of the genes for the transcription factor PPARγ and several others that are involved in the process of adipogenesis. The activity of PPARγ was inhibited by 30 – 50% by oleuropein at a concentration of 200μM.

Four years later, Malliou *et al.* (2018), in the *in vitro* part of their study with a HepG2 hepatocyte culture, studied the role of oleuropein on the serum triglyceride reduction. Based on the structural characteristics of oleuropein, the transcription factor PPARα, and on the theoretical models, the authors assumed and later by several assays confirmed, that oleuropein acts as a PPARα ligand in the function of an agonist. On the contrary, hydroxytyrosol, a metabolite of oleuropein, does not posses any structural similarities to bind to PPARα.

An *in vivo* study with adult Medaka fish (Torró-Montell *et al.*, 2019) focused on the influence of olive extracts in the expression of genes involved in lipid metabolism. Fish were fed a diet rich in carbohydrates and various olive extracts for five days. Then the hepatic DNA for three lipolytic and three lipogenic enzymes was quantified. The expression of genes for the lipolytic

molecules (including PPARα) was significantly decreased and the olive extracts did not reverse the situation. However, the extracts did prevalently contain hydroxytyrosol, while the content of oleuropein was fairly low, and based on the knowledge of Malliou *et al.* (2018; see text above), any significant activation of PPARα could not be expected. The two lowest relative expression of PPARα were observed in the fish fed with the lowest oleuropein content, which is again in accordance with Malliou *et al.* (2018). On the other hand, the increased expression of lipogenic genes could be reversed by treatment with olive extracts, thus indicating that other enzymes involved in the lipid metabolism are also influenced by olive phenolics.

2.6.5. Safety Profile of the Olive Leaf Preparations

Phenols from olive leaf are absorbed and to some extent metabolized in humans, as observed by Vissers *et al.* (2002) in their study with healthy volunteers, both with a colon and ileostomy subjects. Based on the analyses of collected ileostomy effluent and urine, the authors estimated that the apparent absorption of phenols was at least 55 – 66% of the ingested dose. The olive leaf phenols are extensively modified in the human body and excreted mainly in urine in the form of hydroxytyrosol. Simple olive phenols, such as tyrosol and hydroxytyrosol, are found to be excreted as glucuronide conjugates (Visioli *et al.*, 2000) or undergo the action of catechol-o-methyl transferase, and are excreted as homovanillic alcohol and homovanillic acid (Caruso *et al.*, 2001). Various interactions with other drugs and preparations thus may be possible.

However, EMA finds olive leaf to be safe for human health. No undesirable effects are known, nor interactions with other medicinal products, or other forms of interaction, have been reported. The only contraindication listed is the hypersensitivity to the active substance EMA/HPMC/359238/2016). *O. europaea* L. is even not mentioned in the Botanical Safety Handbook (Gardner and McGuffin, 2013).

The safety profile of olive leaf extract in animal models was in the focus of some studies. For example, Farag, El-Baroty and Basuny (2003) evaluated the effect of phenolic compounds extracted from olive fruits and leaves on the liver and kidney function and the lipid profile of rats after 7 weeks of treatment. Only the highest tested concentration of olive phenolics (1600 ppm) caused a significant increase of liver transaminases and serum total lipids, as well as severe damage to the tissues of the kidney and liver.

A comprehensive safety assessment of a commercially available water-soluble olive leaf extract was performed by Clewell *et al.* (2016). The olive leaf extract was tested on mutagenicity (mammalian chromosomal aberration test), genotoxicity (mouse micronucleus test), mortality, and toxic effect (oral toxicity study). No adverse effect level was observed after administration of 1000 mg/kg in rats in a 12 weeks study.

A safety assessment of ethanolic extract of olive leaves on a rat animal model was provided by Guex *et al.* (2018). Acute toxicity was assessed using a single dose of 2000 mg/kg and subacute toxicity was tested by administration of different doses (100, 200, and 400 mg/kg) during 4 weeks. No signs of toxicity were observed after exposure to single or repeated doses of ethanolic olive leaf extract.

Concerning the safety of olive leaf extract in humans, the data is scarcer. Since the olive fruit is used as a food and the olive leaf as medicine for several hundreds of years, it can be assumed that the use of olive leaf extract would be safe for human use. Susalit *et al.* (2011) in their study with 262 volunteers also reported only tolerable mild **adverse effects** from olive leaf extract EFLA 943, such as cough, vertigo, muscle discomfort, and headache, which had resolved by the end of the study. The olive leaf extract also did not affect liver and renal functions, electrolyte balance, and hematological parameters of the participants, and therefore it was declared to be safe and tolerable for patients with stage-1 hypertension.

As to the **pharmacokinetic interactions** of olive leaf active compounds, oleuropein was found to be a relatively weak inhibitor of CYP1A2-mediated 7-methoxyresorufin-o-deethylation (24% inhibition at

100 µmol/L oleuropein; Stupans *et al.*, 2001). Further, pinoresinol, one of the three main olive oil phenols, was reported to inhibit vitamin D uptake both *in vitro* (intestinal Caco-2 cells) and *in vivo* (rats). However, oleuropein and hydroxytyrosol did not exhibit such an activity (Goncalves *et al.*, 2016).

A potentially significant interaction of olive leaf extract with some antihypertensive drugs was reported by Mmopele *et al.* (2018). The authors used an extract from wild African olive in an *in vitro* model (an intestinal Caco-2 cell culture). The olive leaf extract was found to increase the permeability of propranolol in the absorptive direction, and enhanced the permeation of diltiazem in both the absorptive and secretory direction. This suggests a potential increase in the blood levels of these drugs when administered simultaneously with the olive leaf extract.

There is also a remarkable lack of data on the **pharmacodynamic interactions** of olive leaf active compounds. In fact, I have found only one work dealing with this topic in the available literature. Lim *et al.* (2016) investigated the antimicrobial properties of olive leaf extract and five of its phenolic compounds (including oleuropein and hydroxytyrosol) against the bacterial species *Escherichia coli* and *Staphylococcus aureus*. Their activity as stand-alone antimicrobial agents was weak, but when co-administered with ampicillin, a synergistic interaction between ampicillin and hydroxytyrosol was observed. Oleuropein, verbascoside, caffeic acid, and the whole olive leaf extract showed additive effects.

2.7. Mistletoe – *Viscum album* L. – fam. *Loranthaceae*

2.7.1. Characterization of *Viscum album* L. – a Pharmacognostic Context

The genus *Viscum* comprises hemi-parasitic species capable of nourishing off a large number of host species – Barney, Hawksworth and Geils (1998) identified as many as 452 host species of *Viscum album* L. The plants are dioecious small woody shrubs with a tendency to form globular shapes on

the host plant, extending up to 1 m in diameter (Fig. 2.13.). The branches are usually 15 – 80 cm long with opposite pairs of narrowly obovate, dark green leathery leaves capable of photosynthesis. Waxy white to yellowish colored fruits, berries, contain only one seed (Vicaş, Rugină and Socaciu, 2011).

The taxonomy of the genus *Viscum* is partially confusing. Firstly, even the taxonomical placement of the whole genus is challenging. Some authors place the genus in the family *Viscaceae*, some state that, according to the genetic research provided by The Angiosperm Phylogeny Group, the genus should be in the family *Santalaceae*. According to EMA, the genus *Viscum* is currently placed into the family *Loranthaceae* (EMA/HPMC/246778/2009; Vicaş, Rugină and Socaciu, 2011).

Fig. 2.13. *Viscum album* L. Left – whole plant, right – a closeup of a branch with fruits. From union.ces.ncsu.edu and flickr.com.

Furthermore, the nomenclature of the individual species and subspecies is also troublesome. Therefore, to prevent misunderstanding, in the presented work I will use the term "European mistletoe" as a synonym for *Viscum album* L. subsp. *album* and "Korean mistletoe" for *Viscum album* subsp. *coloratum* Kom., regardless of the nomenclature used by the original authors.

Visci albi herba are dried aerial parts of the mistletoe species *Viscum album* L. The thickness of the collected stems should not exceed 5 mm in diameter. The sessile leaves are 2 – 6 cm long, green, stiff, and leathery. The

aerial parts are collected with the berries containing one seed and a sticky juice (Tomko *et al.*, 1989; Wichtl, 2003).

2.7.2. Phytochemistry of Mistletoe

It is necessary to remind that mistletoe as a hemi-parasitic plant nourishes to a certain extent on the host plant. Therefore, the phytochemistry of mistletoe is strongly affected by the host.

The main active compounds of mistletoe are the proteins lectins (mistletoe lectins I – III, glycoproteins binding with N-acetyl-D-galactosamine, D-galactose, and cell surfaces), polypeptides (especially viscotoxins A_1 – B composed of 46 amino acids), oligosaccharides (mostly galactose and glucose), polysaccharides (prevalently galacturonans and arabinogalactans), sugar alcohols (mainly inositol), and alkaloids (Ochocka and Piotrowski, 2002; Arda *et al.*, 2003; Vicaş and Socaciu, 2007; EMA/HPMC/246778/2009 and references therein). The mistletoe alkaloids remained long unidentified because of their claimed extreme lability, until Amer *et al.* (2012) isolated and characterized two aminoalkaloids (trihydroxy and tetraydroxy derivates of iminodibenzoic acid) that even define a novel group of aminoalkaloids.

However, for the cardiovascular action of mistletoe (see Ch. 2.7.4.), phenylpropanoid compounds, flavonoids, and lignans are of greater importance (Nazaruk and Orlikowski, 2016). Becker and Exner (1980) isolated eight flavonoids with flavonol structure from European mistletoe; all of them were derivates of quercetin with a different degree and position of methylation. Lignans with various structures, such as syringoresinol and ligalbumosides A – E, were also reported in European mistletoe leaves and stems (Nhiem *et al.*, 2012). Some of the mistletoe's active compounds are depicted in Fig. 2.14.

The content of individual compounds in mistletoe is very strongly influenced by the **host tree**. For example, Schaller *et al.* (1998) reported different content of viscotoxins isolated from European mistletoe grown on eight different hosts, with the highest total viscotoxin levels in acacia- and

poplar-grown mistletoe, while pine-grown mistletoe offered the lowest level of total viscotoxins. Further, Deliorman *et al.* (1999) observed differences in the amounts of phenylpropanoids isolated from mistletoe grown on three different hosts. The highest amounts of syringin, coniferin, and kalopanaxin D were found in mistletoe grown on apricot tree, while they were detected only in trace amounts in mistletoe grown on pine trees.

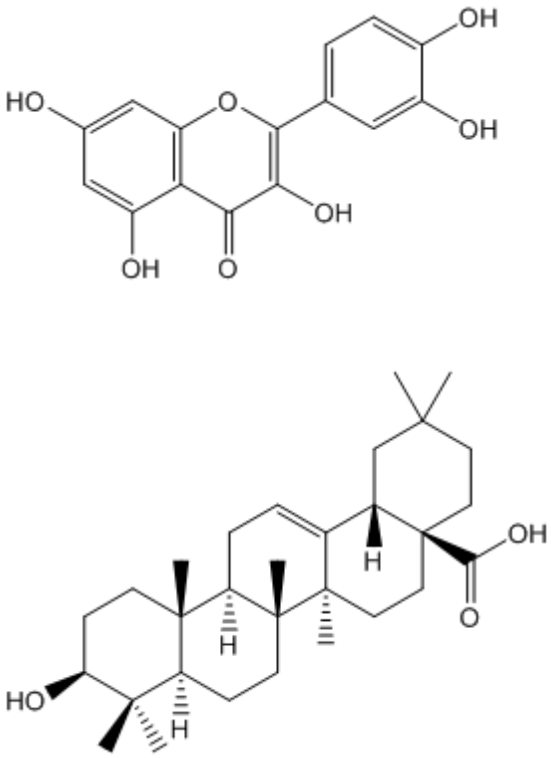

Fig. 2.14. Some of the *Viscum album* L. active compounds. Up – quercetin, down – oleanolic acid. Courtesy of doc. Macáková.

Likewise, phenolic acids composition isolated from European mistletoe grown on six hosts (rowan, apple, pear, poplar, plane, and oak tree) exhibited significant variations. Mistletoe hosted by oak contained 15 phenolic compounds with *o*-coumaric acid as a typical compound; digallic acid was specific for plane tree-grown mistletoe, and vanillic acid for mistletoe gathered from apple and pear tree. The authors noticed that the phenolic acids composition showed similarities in mistletoe grown on apple and pear trees (Łuckiewicz *et al.*, 2001). Arda *et al.* (2003) even reported differences in monosaccharides (glucose 9% vs. 29%, galactose 17% vs. 44%)

and polyols (inositol 58% vs. 21%, xylitol 8% vs. 1.5%) in mistletoes grown either on lime trees or on fir trees.

Deliorman Orhan and Orhan (2006) noticed differences in fatty acid composition of European mistletoe. Apricot tree-grown mistletoe had the highest fatty acids content (36.15% of the lipophilic extract; it was especially rich in palmitic acid – 11.47%), followed by pear-grown mistletoe (19.49%). The lowest fatty acids content was found in quince-grown mistletoe (10.10%).

Similarly, Vicaş *et al.* (2009) demonstrated the different content of phenolic compounds and carotenoids obtained from European mistletoe grown on five hosts (ash, poplar, acacia, apple, and maple tree). Phenolic content ranged from 0.44 to 0.65 mg of gallic acid equivalent/g of fresh weight, the highest being from mistletoe grown on maple tree. Carotenoid content ranged from 2.47 to 7.00 mg of β-carotene/g of fresh weight, the highest again originating from mistletoe grown on maple tree.

Wójciak-Kosior *et al.* (2017) also observed differences in the amount of oleanolic and betulinic acids in European mistletoe grown on seven different hosts (acacia, rowan, birch, lime, apple, poplar, and pine tree). In general, oleanolic acid was the prevalent triterpenic acid in mistletoe with its content about 10-fold higher than that of betulinic acid. The highest content of oleanolic acid was found in pine-grown mistletoe, followed by poplar- and apple-tree-grown mistletoe.

Furthermore, **seasonal changes** were reported to have an impact on the content of the individual compounds of mistletoe. Escher *et al.* (2003) mentioned the seasonal differences in reduced sulfur containing compounds in poplar- and fir-grown mistletoe. High levels of the sulfur-containing compounds were detected in spring and autumn, and low levels in summer. Seasonal variation of amino acids concentrations in the xylem sap of European mistletoe grown on poplar and pine trees was observed by Escher *et al.* (2004a). Poplar-grown mistletoe exhibited significant changes, especially in the glutamine and asparagine content, with the total amino acids content generally being the highest in the spring and the lowest in the

autumn. Fir-grown mistletoe rather than seasonal changes in amino acids content exhibited significant changes between years. A seasonal variation in the amino acid arginine in European mistletoe was also reported. The increase in arginine during late autumn and winter time in mistletoe grown on deciduous trees suggested that continued transpiration within mistletoe after the shedding of the host leaves caused a displacement of the solutes from the host xylem into the mistletoe leaves (Urech, 1997).

And finally, Escher *et al.* (2004b) noticed changes in the sugar levels in the xylem sap of European mistletoe grown on poplar- and fir-trees. In this case, however, the authors again suggested the changes are due to the physiology of the deciduous (high sugar concentrations in spring, low in other seasons) and coniferous trees (intermediate sugar concentrations throughout the year), rather than a seasonal variation in the respective mistletoes.

Wójciak-Kosior *et al.* (2017) evaluated the levels of triterpenic acids in European mistletoe collected during four seasons (spring, summer, autumn, and winter). Mistletoes grown on deciduous trees exhibited the highest levels of oleanolic and betulinic acid in the summer, with a declining tendency during the autumn and winter, and the lowest levels of both acids being detected in the spring. On the contrary, mistletoe grown on a coniferous tree (pine) had the highest amounts of triterpenic acids in the winter, with a significant decrease in the summer.

The **developmental stadium** may also play a role in the qualitative and quantitative content of the individual compounds found in mistletoe. The changes in the composition of European mistletoe epicuticular waxes were investigated by Wollenweber, Wieland and Haas (2000) who reported that the predominant component in the epicuticular waxes is the triterpenoid oleanolic acid, with a 70% content in the young leaves and it may rise up to 80% in the second vegetation period. On the contrary, flavonol aglycones (quercetin and kaempferol 5-methoxy derivates) accompanying the aliphatic compounds in the mistletoe epicuticular waxes reach their maximum amount in the young developing leaves, while in the second vegetation period they are found only in small amounts.

Similarly, Vicaş, Rugină and Socaciu (2008) observed a higher antioxidant activity in young European mistletoe plants (collected in May, regardless of host plant) than in older ones, indicating important changes in the phytochemical composition.

The active compounds of mistletoe are not distributed evenly in the different **plant organs**, as observed by several investigators. For example, the content of phenolic compounds in leaves was up to 30% higher and carotenoids even up to 100% higher than that of stems in European mistletoe regardless of the host tree (Vicaş *et al.*, 2009).

2.7.3. Medicinal Use of Mistletoe

According to the Assessment report on *Viscum album* L. herba, European mistletoe has been used in the treatment of a variety of cardiovascular disorders and in the oncology. Mistletoe extracts for the use for cancer therapy were introduced around 1920, while the first written notice of mistletoe for cardiovascular action dates back several hundreds of years earlier, in 1564 (EMA/HPMC/246778/2009). The history of the medicinal use of mistletoe for the treatment of cardiovascular diseases was intriguingly written by Inci Bowman (Bowman, 1990).

For the cardiovascular use of mistletoe in Europe, herbal preparations in solid or liquid dosage forms for oral use are intended. The mistletoe herbal preparations include tea, infusion in cold water, syrup, expressed juice, ethanolic extracts, wine extracts, aqueous extracts, tinctures, and dry extracts (EMA/HPMC/246778/2009).

2.7.4. Effects of Mistletoe on Selected Cardiovascular Conditions

2.7.4.1. Antioxidant Effect of Mistletoe

The antioxidant properties of mistletoe are strongly dependent on plant material, extractant, and host plant. Good antioxidant properties are attributed to phenolic compounds (and that including phenolic acids, flavonoids, and lignans), terpenoids, and carotenoids. In general, mistletoe offers respectable antioxidant qualities, as reported by Alali *et al.* (2007) who investigated the antioxidant activity of 95 Jordanian plants. Both aqueous and methanolic extracts from the mistletoe species *V. cruciatum* Sieb. exerted the third highest activity of all the evaluated plants.

Leu *et al.* (2006) investigated the ability of several individual compounds isolated from Korean mistletoe to scavenge the superoxide anion in human neutrophils. The most potent activities were exhibited by viscolin (a 1,3-diphenylpropanone), oleanolic acid (a triterpenic acid), and pinocembrin (a flavanone). And similarly, all three flavanone glycosides obtained from a Korean mistletoe **ethanolic extract** (namely eriodictyol glucopyranoside/ diglucopyranoside, homoeriodictyol glucopyranoside/diglucopyranoside, and naringenin glucopyranoside) exhibited significant scavenging effects on both hydroxyl and superoxide anion radical, although naringenin glucopyranoside was less active than the other two compounds (Yao *et al.*, 2006).

The ethanolic extract of European mistletoe isolated by Papuc *et al.* (2010) also offered a good hydroxyl radical-scavenging activity (inhibition by 34.44%), although the activity against the superoxide anion was less pronounced (inhibition by 16.66%). This extract further exhibited slight nitric oxide-scavenging activity (inhibition by 28.43%; Papuc *et al.*, 2010).

Mistletoe **methanolic extract**s also possess strong antioxidant properties. Önay-Uçar *et al.* (2006) determined the antioxidant capacity of methanolic extracts obtained from acacia-, lime-, and maple-grown European mistletoe. The lime-grown mistletoe extract exerted the highest scavenging activity (95.12%) in the DPPH test and in the ferric thiocyanate assay, while

all tested extracts offered approximately the same inhibition of lipid peroxidation. Janakat and Al-Thnaibat (2007) reported a strong inhibition of lipid peroxidation of three olive-grown European mistletoe extracts (methanolic, non-boiled aqueous, and boiled aqueous), with the methanolic extract exerting the highest activity (the inhibition of lipid peroxidation by 98.7% for leaves, by 90.5% for stems, and by 89.9% for fruits). Not surprisingly, the methanolic extract from mistletoe leaves offered the highest antioxidant capacity of all plants evaluated, even higher than that of *Rosmarinus officinalis* L., which was used as a control (Janakat and Al-Thnaibat, 2007).

High antioxidant activity and total phenolic content of a Turkish European mistletoe methanolic extract were reported by Sengul *et al.* (2009). In the β-carotene-linoleic assay, mistletoe had the highest antioxidant capacity of all the plant extracts tested (82.23%), while the phenolic content was the second highest (19.43 mg of gallic acid equivalent/g of dry weight). Likewise, a high total phenolic content and antioxidant activity of methanolic extracts obtained from cocoa- and cashew-grown European mistletoe was demonstrated by Ademiluyi and Oboh (2008). The extracts contained 182 mg/100 g (cocoa tree) and 160 mg/100 g (cashew tree) of total phenolics.

And similarly, flavanone and diphenylpropanone glycosides isolated from another Asian *Viscum* species, namely *V. articulatum* Burm., exhibited a strong antioxidant activity in the DPPH assay (Kuo *et al.*, 2010).

Vicaş *et al.* (2009) determined the hydrophilic and lipophilic antioxidant activities of the European mistletoe grown on five different host trees (maple, apple, ash, poplar, and acacia tree). The hydrophilic antioxidant activity of European mistletoe was about 100-fold higher than the lipophilic antioxidant activity, as determined by the FRAP method. The stems exhibited slightly higher antioxidant capacity than the leaves. The highest antioxidant activity was exhibited by the extract of mistletoe grown on apple trees.

Petrus (2011) also investigated the antioxidant properties of both hydrophilic and lipophilic fractions of another mistletoe species, namely

V. capitellatum Smith, growing as an obligate hyperparasite on another parasitic mistletoe. Again, the majority of the total antioxidant capacity of this mistletoe extract was concentrated in the more polar methanolic fraction (67.4%), while the less polar acetone fraction contributed to the antioxidant activity only with 18.4%, and the lipophilic dichloromethane fraction only with about 11.0%. In the hydrophilic fraction, especially phenolic acids (chlorogenic acid, caffeic acid, and *p*-coumaric acid) were identified as the most antioxidant-active compounds. In the lipophilic fraction, there were mainly triterpenic acids (oleanolic acid and betulinic acid), terpenes (lupeol and betulin), and carotenoids that acted as antioxidants (Petrus, 2011).

Mistletoe's antioxidant properties were also assessed in several ***in vivo* studies**. For instance, Shi *et al.* (2006) investigated the antioxidant and free radical scavenger properties of Korean mistletoe alkali in rats treated with carbon tetrachloride. The 4 weeks administration of mistletoe led to a normalization or even to an increase of the antioxidant enzyme values (superoxide dismutase, glutathione reductase, and glutathione peroxidase), and to the protection of liver and kidney against the oxidative damage. Very similar antioxidative and protective properties were observed in carbon tetrachloride-intoxicated rats treated with different fractions of European mistletoe (Cebovic and Popovic, 2006).

Shahaboddin *et al.* (2011) treated alloxan-administered rats with an aqueous extract from mistletoe grown on oak tree at a dose of 500 or 1000 mg/kg for 24, 48, or 72 hours. The serum antioxidant capacity was significantly higher in both treatment groups than in the control group after 72 hours, but the onset of the restoration of antioxidant capacity was earlier at the higher dose: 24 hours vs. 48 hours at the lower dose after the application of alloxan injection.

2.7.4.2. Direct Effect of Mistletoe on Myocardium

Older works regarding mistletoe's influence on the myocardium were focused on the effects of the polypeptidic viscotoxins. For instance, Rosell and

Samuelsson (1966) reported that viscotoxin A_3 exerted **negative inotropic effect** on the heart. Similarly, Andersson and Jóhannsson (1973) investigated the effects of mistletoe viscotoxins on a rabbit heart. The application of viscotoxin B at low concentrations (1 – 10 μg/mL) led to a contraction and progressive depolarization of the heart muscle that could be reversed by increasing the extracellular calcium concentration. The negative inotropic effect of dodonein, a compound isolated from an African mistletoe species (*Agelanthus dodoneifolius* (DC.) Polhill & Wiens, fam. *Loranthaceae*), on rat heart was also observed by Carré *et al.* (2014). The authors suggested that this effect of dodonein was associated with the hypotensive property and the blockade of the L-type calcium channels.

Wu *et al.* (1994) investigated the effect of Korean mistletoe flavonoids on the fast response action potentials of hearts (precisely, in canine Purkinje fibers and guinea pig ventricular papillary muscles). Mistletoe extract at a concentration of 100 μg/mL accelerated the depolarization of the fast response action potentials and increased the effective refractory period/action potential duration, thus indicating an **antiarrhythmic effect**. Chu *et al.* (2006) used Korean mistletoe flavonoid glycosides (15 and 75 mg/kg) in rats with an aconitine-induced arrhythmia. Mistletoe flavonoid glycosides significantly shortened the action potential duration that was prolonged by aconitine via influencing the Ca^{2+} channels in cardiomyocytes. Khusmatov, Makhmudov and Mavlyanov (2015) confirmed the involvement of calcium channels in the antiarrhythmic mechanism of flavonoids in the aconitine-induced arrhythmias and reported that out of all tested flavonoids, quercetin exerted the strongest antiarrhythmic effect.

During the course of the last ten years, mistletoe's **cardioprotective properties** have been demonstrated by several authors. The protective effect of a methanolic extract of pear-grown European mistletoe and pure quercetin against the cyclophosphamide-induced cardiotoxicity in mice was investigated by Şekeroğlu, Aydın and Şekeroğlu (2001). Cyclophosphamide significantly decreased the activity of the antioxidant enzymes catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase, while the pretreatment with the mistletoe extract (250 mg/kg) and quercetin

(50 mg/kg) returned them to almost normal values, with quercetin being more effective than mistletoe extract in this task. Both mistletoe and quercetin also ameliorated the cyclophosphamide-induced lipid peroxidation.

Bachhav and his colleagues treated L-NAME-induced hypertensive rats with either a methanolic extract (200 and 400 mg/kg; Bachhav *et al.*, 2012) or pure oleanolic acid (60 mg/kg; Bachhav *et al.*, 2015), isolated from the mistletoe species *V. articulatum* Burm., for 4 weeks. The administration of both methanolic extract and oleanolic acid was able to partially prevent the morphological abnormalities in the heart tissue induced by L-NAME, such as moderate diffuse myocardial degeneration and necrosis with inflammatory infiltration.

Histopathological changes of an isoproterenol-induced heart injury improved by mistletoe treatment were also observed by Karagöz *et al.* (2016). Rats were administered with an aqueous extract obtained from pear-grown European mistletoe (250 mg/kg) for 24 days. This treatment remarkably attenuated the isoproterenol-induced increase in heart weight, left ventricular wall thickness, and the presence of the brain natriuretic peptide (an indicator of heart failure) in cardiomyocytes (80% vs. 99% in the isoproterenol-only group). The left ejection ventricular fraction was also improved and the heart rate partially decreased to almost normal values by mistletoe treatment.

Moreover, mistletoe seems to have a beneficial effect on the ischemia/reperfusion heart injuries. Chu *et al.* (2008) and Li *et al.* (2009) investigated the effect of flavonoid glycosides of Korean mistletoe in rats with an acute myocardial infarction. Treatment with 15 or 75 mg/kg of mistletoe flavonoid glycosides significantly decreased the infarct size and recovered the activity of superoxide dismutase. Suveren *et al.* (2017) used European mistletoe extracts (methanolic and aqueous) in an ischemia/reperfusion injury model on isolated rat hearts. The hearts were perfused with different concentrations of the methanolic (5, 10, or 20 mg/L) and aqueous (1, 10, or 50 mg/L) mistletoe extracts from 10 minutes prior to the onset of the coronary occlusion until 10 minutes after reperfusion. Both extracts significantly reduced the infarct size, although the best results were obtained

by applying the methanolic extract at a dose of 5 mg/L. Interestingly, a high dose of the aqueous extract (50 mg/L) exerted negative inotropy, decreased the contractile force, and subsequently abolished the heart function. This may be due to the presence of viscotoxins, where negative inotropic properties were reported (Rosell and Samuelsson, 1966).

The **mechanism** of mistletoe-mediated cardioprotection most probably involves the restoration of the antioxidant capacity, the blockage of the ATP-sensitive potassium channels (Suveren *et al*., 2017), the activation of NO-mediated vasodilatation by overexpression of NOS-2 and NOS-3 (Tenorio-López *et al*., 2006), and by blocking of the platelet activating factor-induced Ca^{2+} overload in cardiomyocytes (Chu *et al.*, 2008; Li *et al.*, 2009). Taking into account that quercetin and its derivates are abundant in mistletoe, the cardioprotective effect of mistletoe most likely also involves the enhancement of the contraction and calcium transients of cardiomyocytes mediated by both quercetin itself as well as by its metabolite tamarixetin (Hayamizu *et al.*, 2018).

2.7.4.3. Effect of Mistletoe on Blood, Blood Cells, and Blood Vessels

Mistletoe **lectins** have long been known for their ability to aggregate human blood cells. Franz, Ziska and Kindt (1981) reported that all three lectins isolated from European mistletoe reacted with human erythrocytes without any specificity for the A, B, and 0 blood groups. Timoshenko, Cherenkevich and Gabius (1995) even specified the order in which the galactose-specific mistletoe lectin aggregated blood cells: neutrophils, mononuclear cells, thrombocytes, and erythrocytes. The galactose-specific mistletoe lectin was further found to induce the aggregation of human platelets in a dose- and sugar-dependent manner. The platelet aggregation was completely reversed with the addition of lactose (Samal, Gabius and Timoshenko, 1995).

Aside from lectins, other mistletoe active compounds exhibit an inhibitory effect on **platelet aggregation**. Panossian *et al.* (1998) assessed

the effect of phenylpropanoids isolated from pear-grown European mistletoe. The *n*-butanol-soluble fraction of the ethanolic mistletoe extract contained four major compounds: syringenin glucoside, syringin, coniferyl alcohol glucoside, and coniferin. All of these compounds inhibited the ADP-induced platelet aggregation at the concentration range of 0.001 to 1.0 µmol/L. Similarly, the inhibition of both ADP- and adrenaline-induced human platelet aggregation by the *V. cruciatum* Sieber crude extract at concentrations 0.3 – 1.2 mg/mL was reported by Gilani *et al.* (2009). The mistletoe extract was more effective against the ADP-mediated than the noradrenaline-mediated aggregation. The authors suggested a possible intrinsic calcium-antagonistic effect of the mistletoe extract (Gilani *et al.*, 2009).

The **vasorelaxant effect** of four extracts (aqueous, ethanolic, petroleum ether, and *n*-butanolic) of European mistletoe grown on 12 different hosts was investigated by Ergun *et al.* (1995). The most effective vasorelaxants were the ethanolic extracts, especially those obtained from pear-, cherry-, quince-, wild pear-, and acacia-grown mistletoes. On the other hand, no vasorelaxant activity on the noradrenaline-precontracted rat aortic rings was exhibited by the aqueous extracts. The extract obtained from plum-grown mistletoe was even slightly vasoconstrict active. Vasoconstriction was also reported in the vessels in the skin and skeletal muscle (Rosell and Samuelsson, 1966), and rabbit aortic strips (Anderson and Jóhannsson, 1973) treated with mistletoe viscotoxin A_3, especially in high doses.

Deliorman *et al.* (2000) further fractioned the *n*-butanolic extract of apricot-grown European mistletoe into 38 subfractions, and these were screened for their vasorelaxant effect on noradrenaline-precontracted rat aortic rings. In the more polar subfractions, contractile responses were observed in a dose-dependent manner. The most vasorelaxant effects observed were that of the subfractions 18-20 and the subfractions 13-15 (23.4% and 20.2%, respectively). Isolated phenolic compounds exhibited mostly slight vasoconstrictive actions (syringin 3.8%, coniferin 2.0%), with the exception of kalopanaxin D (1.0% relaxation).

Tenorio-López *et al.* (2005) investigated the effect of an aqueous mistletoe leaf extract on isolated guinea pig hearts from normotensive

animals. A continuous infusion of mistletoe extract at a dose of 450 mg significantly decreased the coronary vascular resistance. This effect was blocked by L-NAME (an inhibitor of the nitric oxide synthase), gadolinium chloride (an inhibitor of the inducible nitric oxide synthase), and by the compound 1*H*-[1,2,4]oxadiazolo[4,2-*a*]quinoxalin-1-one (a selective inhibitor of the NO-sensitive guanylate cyclase), which indicated that the vasorelaxant effect of mistletoe was mediated through the NO release.

The vasorelaxant effect of mistletoe may be mediated partially by viscolin, a diphenylpropane derivate isolated from Korean mistletoe. Hwang *et al.* (2006) observed that viscolin induced a substantial increase in cAMP levels that occurred through the inhibition of the phosphodiesterase (PDE) activity. PDE inhibitors demonstrated various pharmacological properties including vasodilator and antihrombic (Rahimi *et al.*, 2010).

Mojiminiyi *et al.* (2008) investigated the vasorelaxant effect of oil palm tree-grown European mistletoe aqueous extract at a concentration range 2 – 16 mg/mL on potassium chloride- and noradrenaline-precontracted rat aortic rings, and found that the noradrenaline precontraction seemed to be more susceptible to mistletoe extract than the KCl precontraction. Based on other experiments of theirs, the authors suggested that the vasorelaxant effect of mistletoe aqueous extract is mediated via the antagonistic action on the Ca^{2+} influx and via the inhibition of the mobilization of intracellular Ca^{2+}. The presence of Ca^{2+} entry-blocking constituents in European mistletoe fruit's crude and ethyl acetate extracts was also confirmed by Khan *et al.* (2016) who used an isolated rabbit thoracic aorta precontracted with phenylephrine.

Shen *et al.* (2018) reported that the flavanone compound homoeridictyol, previously isolated from Korean mistletoe, has the ability to **protect endothelial cells** from damage caused by ROS, namely hydrogen peroxide. The authors in their *in vitro* study with HUVEC cells demonstrated that homoeridictyol acts as an inducer of the nuclear factor-erythroid 2 -related factor 2, an important intracellular defense system protecting cells against the oxidative injury caused by ROS, thus alleviating the hydrogen peroxide-induced loss of the mitochondrial membrane potential. Furthermore, homoeriodictyol inhibited deleterious changes in

several apoptosis-related proteins, such as Bcl-2, caspase-3, and caspase-9, induced by hydrogen peroxide.

2.7.4.4. Antihypertensive Effect of Mistletoe

Eno *et al.* (2004) examined the antihypertensive effect of a crude extract of citrus-grown European mistletoe in both normotensive and hypertensive rats. The intravenous administration of the crude extract at a concentration range 5 – 160 mg/kg led to a significant blood pressure reduction in a dose-dependent manner. In normotensive rats, the blood pressure reduction ranged from 8.99% to 54.1%, while in the DOCA-induced hypertensive rats, the blood pressure reduction was 4.8% – 43.9%. The duration of the hypotensive effect was also dose-dependent. Very similar results were obtained in their following work (Ofem *et al.*, 2007), again conducted with crude mistletoe extract.

On the other hand, Radenković *et al.* (2009) investigated the effects of different extracts (ethanolic, ether, and ethyl acetate) of European mistletoe stems on the arterial blood pressure in rats. The ethanolic extract exerted the strongest hypotensive action with a significantly lowered arterial blood pressure even at the lowest dose, while the ether extract significantly decreased the arterial blood pressure only at the highest administered dose.

A methanolic extract administered at doses of 200 and 400 mg/kg obtained from mistletoe *V. articulatum* Burm. grown on *Cordia macleodii* (Grift) Hook and Thoms. (an endangered ethnomedical tree belonging to the family *Boraginaceae*) was used by Bachhav *et al.* (2012) to treat rats with the L-NAME-induced hypertension for 4 weeks. Mistletoe remarkably decreased the systolic blood pressure that had been increased by the L-NAME administration.

In their consequent study, Bachhav *et al.* (2015) used pure oleanolic acid isolated from epicuticular wax of *V. articulatum* Burm. in order to investigate its antihypertensive activity in relation to the NO system. Rats were administered with L-NAME to induce hypertension and oleanolic acid

(60 mg/kg), L-arginine, or enapril (15 mg/kg, an ACE inhibitor as the positive control) for 4 weeks. Significant decreases of both systolic blood pressure and mean arterial pressure were observed in rats treated with oleanolic acid.

Unfortunately, there is a lack of information on the antihypertensive effect of mistletoe in humans. In fact, the only work published in the last twenty years that I was able to find was that of Poruthukaren *et al.* (2014). The authors evaluated the efficacy of a *V. album* L. mother tincture as an antihypertensive agent in 41 volunteers in prehypertension stage, as well as stage 1 and stage 2 hypertension, all of whom were untreated with any form of antihypertensive therapy. The volunteers had to take 10 drops of the tincture 3 times daily for 12 consecutive weeks. Treatment with the mistletoe tincture resulted in a significant decrease of both systolic and diastolic blood pressure.

The antihypertensive **mechanism** of mistletoe is most likely multifactorial. Firstly, Wagner *et al.* (1986) isolated two phenylpropanes (syringin and its derivate) and two lignans (eleutheroside E and a mono-glucoside syringaresinol derivate) from leaves and stems of European mistletoe. Butanolic mistletoe extract containing these compounds showed a significant cAMP-phosphodiesterase inhibitory activity, indicating cardiotonic and vasodilator properties of mistletoe.

Eno *et al.* (2004), in concordance with their examinations, proposed that the crude mistletoe extract does not utilize the adrenergic or cholinergic system, and the moderation of the calcium availability to cardiomyocytes plays an important role in the antihypertensive properties of mistletoe.

Then Radenković *et al.* (2009), based on their examination of European mistletoe extracts coadministered with atropine and hexocycline (both compounds are blockers of muscarine receptors), suggested that the mistletoe extracts achieve their hypotensive effect mainly via the muscarine receptors.

Furthermore, Jadhav *et al.* (2010) reported a strong and dose-dependent diuretic and natriuretic activity from the methanolic extract of mistletoe *V. artriculatum* Burm., administered at doses of 100 – 400 mg/kg to

rats. Although the mistletoe extract partially mimicked the furosemide pattern (i.e. a significant potassium loss attributed to oleanolic acid), polyphenolic compounds present in the extract exhibited a potassium-saving effect, thus the resultant diuretic effect of mistletoe was more favorable than that of furosemide. The natriuretic effect of oleanolic acid isolated from *V. articulatum* Burm. was also confirmed by Bachhav *et al.* (2015), coupled with a nephroprotective activity, although the oleanolic acid failed to prevent the decrease in serum NOx.

2.7.4.5. Effect of Mistletoe on Plasmatic Lipid Profile

Ben *et al.* (2006) treated rats with a crude extract of European mistletoe grown on citrus at a dose of 200 mg/kg for 10 weeks. A significant increase in both HDL cholesterol and total cholesterol, and a non-significant (by 6%) decrease in LDL cholesterol were observed. Both crude aqueous and ethanolic extracts of European mistletoe (100 mg/kg) were used by Avcı *et al.* (2006) in mice fed a hypercholesterolemic diet for 4 weeks. While the ethanolic extract significantly decreased total cholesterol, LDL cholesterol, and triglyceride levels and increased the HDL cholesterol level, the aqueous extract only significantly influenced the triglyceride level.

The beneficial effect of European mistletoe on hyperlipidemia in diabetic rats was reported by Adaramoye *et al.* (2012). Rats received a methanolic extract obtained from mistletoe grown on kola tree at a dose of 100 mg/kg for 3 weeks. Mistletoe significantly decreased both total cholesterol and triglyceride levels, while it significantly increased the HDL cholesterol level.

Mistletoe also has a positive influence on human serum lipid profiles, as demonstrated by Poruthukaren *et al.* (2014) who used an European mistletoe mother tincture in volunteers in prehypertension to stage 2 hypertension for 12 weeks. The triglyceride level was significantly reduced and the HDL cholesterol level exerted an increasing tendency at the end of the trial.

The hypolidpidemic **mechanism** of mistletoe has yet to be fully elucidated. Wang *et al.* (2008) compared the ability of parasitic mistletoes belonging to two families – *Loranthaceae* and *Viscaceae* – in the terms of their ability to irreversibly inhibit the fatty acids synthase. The genus *Viscum* was represented by two species, namely by *V. articulatum* Burm. and *V. liquilambaricola* Hayata. The inhibitory potential of the genus *Viscum* was, however, very low when compared with the plants belonging to family *Loranthaceae*, especially with the genus *Taxillus* Tiegh. The FAS-inhibitory activity of these mistletoes was 400 times higher than that of *Viscum* spp.

On the other hand, Jung *et al.* (2013) used a preadipocyte 3T3-L1 cell culture incubated with a crude aqueous extract of oak-grown Korean mistletoe at a dose of 6 μg/mL in order to examine the effects of Korean mistletoe extract on adipogenic factors. The addition of mistletoe led to a remarkable decrease in the expression levels of the transcription factors PPAR-γ, C/EBP-α, and SREBP-1c (by 64%, 60%, and 32%, respectively) in 3T3-L1 cells. Furthermore, the expression levels of adipogenic enzymes, such as fatty acid synthase, acyl-coenzyme A synthase, and acyl-coenzyme A carboxylase, was reduced (by 69%, 55%, and 22%, respectively) in the cell culture treated with mistletoe. All of these transcription factors and enzymes are crucial for adipogenesis and adipocyte differentiation.

Kim *et al.* (2015) also examined the effect of a Korean mistletoe aqueous extract on dyslipidemia in rats. The hepatic triglyceride content was lowered with this extract by increasing the expression of carnitine palmitoyltransferase-1 and by decreasing the expression of SREBP-1c, which supports the findings of Jung *et al.* (2013). Furthermore, Kim *et al.* (2020) suggested that the main bioactive compound of Korean mistletoe responsible for the hypolipidemic action is viscothionin, a heat-stable 6 kDa polypeptide. In their former study, Kim *et al.* (2014) discovered a connection between viscothionin and the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway, which is involved in lipid metabolism. The phosphorylation of AMPK and acetyl-coenzyme A carboxylase was increased by viscothionin treatment in a hepatic HepG2 cell culture. Moreover,

viscothionin-treated 3T3-L1 preadipocyte cells decreased the level of the expression of SREBP-1c and fatty acid synthase (Kim *et al.*, 2020).

2.7.5. Safety Profile of the Mistletoe Preparations

According to EMAgency, European mistletoe can be considered as non-toxic following oral ingestion. In Jordan, the mistletoe species *V. cruciatum* Sieb. is even used as an edible plant. The LD50 values of mistletoe extracts, lectins, viscotoxins, and polysaccharides can be found in the Assessment report on *Viscum album* L., herba (Janakat and Al-Thnaibat 2007; EMA/HPMC/264778/2009).

The toxicity of European mistletoe is mostly due to the lectins and viscumin, a protein with a cytotoxic activity. Lectins react with human blood cells (Franz, Ziska and Kindt, 1981), and both lectins and viscumin were found to inhibit protein synthesis (Olsnes *et al.*, 1982). However, in the parenterally administered mistletoe preparations, used as a complementary cancer treatment, no severe **adverse effects** were noted even in immunocompromised patients (HIV positive volunteers). During the gradual dose escalation (0.01 – 10.0 mg), more adverse effects occurred at the lower dose range. Registered drug-related adverse events were flu-like symptoms and transient exacerbations of gingivitis, fever, and eosinophilia (Gorter *et al.*, 1999).

A comprehensive review on the safety of higher dosages of European mistletoe in both animals and humans was provided by Kienle, Grugel and Kiene (2011). They included 69 clinical studies and 48 animal experiments with a dosage >1 mg in humans, corresponding to >0.02 mg/kg in animals. Adverse effects consisted mainly of dose-dependent flu-like symptoms, fever, and local reactions at the injection site. Occasionally, allergic reactions were reported. The authors concluded that mistletoe extract seemed to exhibit a low risk.

Flavonoids isolated from an European mistletoe ethyl acetate fraction (flavanone and chalcone derivates) were even shown to possess a remarkable

anti-nociceptive activity without inducing any apparent acute toxicity or gastric damage when administered orally at doses of 30 mg/kg for pure compounds or 250 mg/kg for ethyl acetate fraction to mice (Orhan *et al.*, 2006).

The impact of mistletoe on the cytochrome P450 was also investigated. Engdal and Nilsen (2009) reported that although mistletoe inhibited the CYP3A4 metabolism *in vitro*, any clinically relevant systemic or intestinal **pharmacokinetic interactions** with CYP3A4 were considered unlikely due to the high IC50 value (3594 μg/mL). Doehmer and Eisenbraun (2012) assessed three commercially available mistletoe extracts for their potential to interfere with the cytochrome P450 metabolism. For all three extracts none or minor induction or inhibition was observed, thus indicating that the risk of herb-drug interactions is low. Other commercially available European mistletoe preparations were assessed by Schink and Dehus (2017). Again, no major inhibition occurred in any of the CYP marker reactions. Furthermore, no induction capacity was found, thus a clinically relevant herb-drug interaction is not expected.

Most of the information regarding **pharmacodynamic interactions** of mistletoe with other drugs is coming from the oncological use of mistletoe, thus conferring prevalently to lectins and viscotoxins. For example, a synergism was observed when mistletoe lectin ML-I was administered with a clinically important anticancer drug, paclitaxel, in cultured human hepatocarcinoma SK-Hep1 cells (Pae *et al.*, 2001). Galm *et al.* (2002) also reported the synergistic effects of mistletoe viscumin and anticancer drugs vincristine, mafosfamide, idarubicin, and cisplatin in the human leukemia cell lines K562 and KG1a.

2.8. Legal Requirements on Herbal Preparations

According to the Collection of Laws of the Czech Republic, all of the herbal preparations evaluated in the consumption analysis in this rigorous thesis fall into two categories: medicinal products or food supplements. These

two categories differ significantly in the legal requirements imposed on the respective herbal preparation.

2.8.1. Medicinal Products

Medicinal products have to meet the requirements of the Act No 378/2007 on medicines and amending certain related acts, issued by the Parliament of the Czech Republic. They are registered with the SUKL and have a registration number. In principle, only medicinal products may be prescribed by the physicians or specialists as products intended for use in human (or animal) patients in the treatment or prevention of a disease.

Furthermore, medicinal products have a clearly defined qualitative as well as quantitative composition regarding both the active compound(s) as well as excipient(s). The production, distribution, and expedition of medicinal products have to mandatory follow the guidelines of Good Manufacturing Practice, Good Distribution Practice, and Good Pharmacy Practice. Medicinal products may be (with the exception of a few selected medicinal products) sold only via pharmacies.

Medicinal products containing medicinal plants, which are traditionally used in Europe, belong mostly to one of the following two categories: traditional herbal medicinal products or human homeopathic medicinal products. Both of these categories of medicinal products have a simplified registration procedure, and no preclinical or clinical trials are required.

The use of **human homeopathic medicinal products**, based on a remarkably wide range of medicinal plant species, is also reflected in the European Pharmacopoeia: it has, for example, listed the monographs on garlic and foxglove for the use in homeopathic medicinal products (Allium sativum ad praeparationes homeopathicas and Digitalis purpurea ad praeparationes homeopathicas; European Pharmacopoeia 10.0).

Traditional herbal medicinal products* have to fulfill several legal conditions. Particularly important is the requirement on bibliographic or scientific evidence in order to demonstrate the therapeutic use of the medicinal product or an equivalent product for at least 30 years, including at least 15 years of the use in the European Union.

Furthermore, the existence of sufficient data on the traditional use of such a medicinal product is mandatory – it shall be demonstrated that the product is not harmful under the conditions of use and that the pharmacological effects or efficacy of this medicinal product are apparent from the long-term use and experience.

The package and any advertisements on a traditional herbal medicinal product have to include the information that "The use of this traditional herbal medicinal product is based exclusively on the long-term experience."

*Note: According to the Act No 378/2007, the traditional herbal medicinal products may consist of plant substances or herbal products or a combination thereof. Herbal products in this context means products obtained by processing a herbal substance or substances by means such as extraction, distillation, pressing, fractionation, purification, concentration, or fermentation. Herbal products include crushed or powdered herbal substances, tinctures, extracts, essential oils, pressed juices, and processed secretions (Act No 378/2007). In the present work I use the term "herbal product" more widely, including both medicinal products and food supplements.

2.8.2. Food Supplements

Food supplements have to meet the requirements of the Regulation No 58/2018 on food supplements and food composition, issued by the Ministry of Agriculture. The most important difference between food supplements and medicinal products is the fact that food supplements are legally considered as foods, therefore no therapeutic or preventive effect can be attributed to them. The producer does not have to follow any of the Good Practice guidelines, and food supplements may be sold also outside the pharmacy network.

There are, however, several legal requirements on these herbal preparations regarding the labeling of the supplement, permitted and prohibited substances and, in particular cases, also the maximal allowed amount of these compounds in a daily dose. For example, according to this regulation, the content of dried hawthorn fruit, leaves, or flowers must not exceed the dose of 1000 mg per day (Regulation No 58/2018).

Furthermore, appropriate **nutrition and health claims** may be used for food supplements. There are several types of health claims and their use is strictly regulated, in the European Union namely by the regulation (EC) No 1924/2006 of the European Parliament (on nutrition and health claims made on foods).

As of June 2020, the EU register on nutrition and health claims comprises a total number of 2338 claims, out of them only 261 were authorized, and thus they may be used in the specific written form. The main reason for rejecting an assessed health claim (and subsequently categorizing it as a non-authorized) was the non-compliance with the regulation (EC) No 1924/2006, more particularly: "because on the basis of the scientific evidence assessed, this claimed effect for this food has not been substantiated."

Health claims regarding the medicinal plants reviewed in the present thesis are scarce. My search in the EU register on nutrition and health claims disclosed neither authorized nor non-authorized health claims on garlic, hawthorn, motherwort, black cumin, or mistletoe or their active compounds, with the exception of flavonoids. The assessed flavonoids (either as a mixture of individual compounds or simply defined as "bioflavonoids") were mostly of the *Citrus* spp. or *Vitis vinifera* L. origin, and none of the 21 assessed health claims were authorized. An individually assessed flavonoid was quercetin, the claim "For cardiovascular health" was, however, non-authorized for the intended cardiovascular use.

For the olive tree, a total amount of 17claims is listed in this register, with only one of these claims authorized. The authorized claim on olive oil polyphenols is as follows: "Olive oil polyphenols contribute to the protection

of blood lipids from oxidative stress." This claim may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivates per 20 g of olive oil. Other claims on the beneficial effects of olive on HDL cholesterol, LDL cholesterol, triglycerides, and blood sugar levels were rejected by the assessment committee.

3. Materials and Methods

3.1. Review Part

For the review part of the present work, mostly PubMed and Elsevier databases were searched for relevant scientific papers. In order to provide the most actual data on the chosen topic, works submitted after the year 2000 were preferred when possible. Primary sources of information, such as research articles and clinical trials were preferred when possible. Prevalently English language written papers were exploited, however, some German and Czech language written works were also used.

To include a plant species in the review, the following conditions had to be fulfilled: (1) the plant is traditionally used in the treatment of one or more cardiovascular disease(s), (2) the availability of OTC or Rx products containing either the drug itself or pure compounds or their mixture obtained from the plant material in an authorized dosage form on the Czech pharmaceutical market between the years 2015 – 2019, (3) sufficient quantity and quality of scientific papers focused on the plant genus or species.

3.2. Medicinal Products And Food Supplements Used for the Consumption Analysis

Herbal preparations included for the consumption analysis were chosen according to their availability in pharmaceutical distribution. Only preparations marketed through pharmaceutical wholesaler companies (PHOENIX and ViaPharma) in the evaluated time period were included. Some of the preparations are multi-component, therefore they were included in the analysis for each of the medicinal plants contained in the respective product.

3.2.1. List of Over the Counter Herbal Preparations Included in the Consumption Analysis

The analysis of herbal preparations consumption in years 2015 – 2019 included the following preparations:

Herbal preparations containing **garlic** included in the consumption analysis are as follows (listed under their respective trade name in alphabetical order):

1. Allnature Česnek Premium 1500 mg 100 tbl

Contains: *Allium sativum* L. extract standardized to contain 3.75 mg allicin in each tablet

Dosage: 1 tablet once to thrice daily

Special warning(s): Not intended for use in children, pregnant and breast-feeding women.

Producer: Allnature s.r.o., Březhradská 148/3, 503 32 Hradec Králové, Czech Republic

2. Allivictus Tinktura 25 mL

Contains: garlic extract containing 11.75 mg sulfur compounds/ 100 mL

Dosage: 1 drop/ kg of body weight, to be taken on an empty stomach

Special warning(s): Not intended for use in children under 3 years of age.

Producer: Allivictus s.r.o., Podhradní 437, 552 03 Česká Skalice, Czech Republic

3. Allivictus Tinktura 3x25 mL

Contains: garlic extract containing 11.75 mg sulfur compounds/ 100 mL

Dosage: 1 drop/ kg of body weight, to be taken on an empty stomach

Special warning(s): Not intended for use in children under 3 years of age.

Producer: Allivictus s.r.o., Podhradní 437, 552 03 Česká Skalice, Czech Republic

4. Bioaktivní Česnek 60 tbl

Contains: dried garlic powder 300 mg containing 4.7 mg of alliin

Dosage: 1 tablet once daily, to be taken on an empty stomach

Special warning(s): Not intended for use in children.

Producer: Pharma Nord Praha s.r.o., Valčíkova 1149/14, 182 00 Praha 8, Czech Republic

5. Česnek extra strong 1500 mg 100 tbl

Contains: extract of *Allium sativum* L. containing min. 3% of allicin (125 mg)

Dosage: 1 tablet once to thrice daily

Special warning(s): Not intended for use in children, pregnant and breast-feeding women. Suitable for vegans.

Producer: Nutritius s.r.o., Dopravní 500/9, 104 00 Praha 10 , Czech Republic

6. Dr. MAX Česnek UlLTRA 120 cps

Contains: Garlicin extract 10 mg (equivalent to 1000 mg of fresh garlic)

Dosage: 1 capsule twice to thrice daily

Special warning(s): Not intended for use in children under 3 years of age.

Producer: Dr. Max Pharma s.r.o., Na Florenci 2116/15, 110 00 Praha 1, Czech Republic

7. Dr. Popov Kapky bylinné Tlak – srdce 50 mL

Contains: Herba visci albi, Crataegi fructus, Melissae herba, Flos tiliae, Leonuri cardiacae herba, Bulbi allii sativi. Each 100 g of the preparation contain extract of 3.32 g mistletoe, 3.32 g hawthorn, 2.9 g motherwort, and 0.41 g garlic.

Dosage: 20 drops thrice daily

Special warning(s): Not intended for use in children, pregnant and breast-feeding women.

Producer: Dr. Popov s.r.o., Plzeňská 857, 348 15 Planá, Czech Republic

8. Jamieson Česnek bez zápachu 500 mg 100 cps

Contains: garlic powder extract 100:1 5 mg

Dosage: 2 capsules daily, to be taken with meal

Special warning(s): Not intended for use in children. Consultation with your physician is recommended if your symptomps or problems persist or worsen. Especially pregnant women, diabetics, people on blood thinners or protease inhibitors therapy should consult a physician about taking this preparation.

Producer: Jamieson Laboratories Ltd., 4025 Rhodes Drive Windsor, ON N8W 5B5, Canada

9. Jamieson Česnek bez zápachu 500 mg 300 cps

Contains: garlic powder extract 100:1 5 mg

Dosage: 2 capsules daily, to be taken with meal

Special warning(s): Not intended for use in children. Consultation with your physician is recommended if your symptoms or problems persist or worsen. Especially pregnant women, diabetics, people on blood thinners or protease inhibitor therapy should consult a physician about taking this preparation.

Producer: Jamieson Laboratories Ltd., 4025 Rhodes Drive Windsor, ON N8W 5B5, Canada

10. Liftea Hloh Jmelí Česnek 30 cps

Contains: *Allium sativum* extract 150 mg, *Crataegus* spp. extract 60 mg, *Viscum album* extract 60 mg

Dosage: 1 – 2 capsules per day

Special warning(s): Not intended for use in children under 3 years of age.

Producer: SWISS CAPS AG, Husenstrasse 35, CH-9533 Kirchberg, Switzerland

11. MedPharma Česnek 1500 mg 107 cps

Contains: garlic oil: *Allium sativum* extract 500:1 3 mg (equivalent to 1500 mg of fresh garlic)

Dosage: 1 capsule once daily

Special warning(s): Not intended for use in children under 3 years of age.

Producer: MedPharma s.r.o., Sivice 510, 664 07 Pozořice, Czech Republic

12. Nature's Bounty Garlic Oil 1000 mg 100 cps

Contains: garlic oil 2 mg (equivalent to 1000 mg of fresh garlic)

Dosage:1 capsule once daily

Special warning(s): Not intended for use in children under 3 years of age.

Producer: Nature's Bounty, 2100 Smithtown Avenue, 11714 NY, USA

13. Naturvita Allicor česnek 60 tbl

Contains: garlic powder 150 mg

Dosage: 1 tablet once to twice daily

Special warning(s): Not intended for use in children under 3 years of age.

Producer: Naturvita a.s., Veselá 227, 763 15 Slušovice, Czech Republic

14. Naturvita Allicor Forte 60 tbl

Contains: garlic powder 150 mg, beta carotene 1 mg, vitamin C 15 mg, vitamin E 10 mg, rutin 4 mg

Dosage: 1 tablet once to twice daily

Special warning(s): Not intended for use in children under 3 years of age.

Producer: Naturvita a.s., Veselá 227, 763 15 Slušovice, Czech Republic

15. Naturvita Allicor Super 60tbl

Contains: garlic powder 150 mg, beta carotene 0.4 mg, thiamin 0.7 mg, riboflavin 0.8 mg, niacin 9 mg, pantothenic acid 3 mg, pyridoxin 1 mg, cyanocobalamine 0.5 µg, folic acid 0.1 mg, biotin 75 µg, vitamin D3 2.5 µg, vitamin C 15 mg, vitamin E 5 mg, rutin 2.5 mg

Dosage: 1 tablet once to twice daily

Special warning(s): Not intended for use in children under 3 years of age.

Producer: Naturvita a.s., Veselá 227, 763 15 Slušovice, Czech Republic

16. Terezia Černý česnek 60 cps

Contains: fermented garlic powder 500 mg

Dosage: 1 capsule once daily, to be taken before meal

Special warning(s): Not intended for use in children under 3 years of age. Suitable for celiacs.

Producer: TEREZIA COMPANY s.r.o., Na Návrší 997/14, 141 00 Praha 4 – Michle, Czech Republic

Herbal preparations containing **hawthorn** included in the consumption analysis are as follows (listed under their respective trade name in alphabetical order):

1. Arkokapsle Hloh 60 cps

Contains: powder of hawthorn (*Crataegus monogyna* JACQ., *Crataegus laevigata* (POIR (DC.) leaves and flowers 350 mg in 1 capsule with a guaranteed minimum content of 1.5% flavonoids expressed as hyperoside).

Dosage: 2 capsules daily, to be taken before dinner or before sleep

Special warning(s): Intended for use in adults only. Not suitable for people with heart problems.

Producer: Laboratoires Arkopharma, Zone Ind 1ere Avenue 9eme Rue Carros, 06511 France

2. Čaj bylináře Krevní tlak 40x1.6 g

Contains (in 1 infusion bag): Crataegi flos 800 mg, Filipendulae ulmariae herba 480 mg, Melissae herba 040 mg, Leonuri cardiacae herba 80 mg

Dosage: 1 cup daily

Special warning(s): Not intended for use in children.

Producer: MEDIATE s.r.o., Dolní Libchavy 325, 561 16 Libchavy, Czech Republic

3. Dr. Popov Kapky bylinné Hloh obecný 50 mL

Contains: Crataegi fructus extract

Dosage: 20 drops thrice daily (contains extract of 174 mg of hawthorn)

Special warning(s): Not intended for use in children, pregnant and breast-feeding women.

Producer: Dr. Popov s.r.o., Plzeňská 857, 348 15 Planá, Czech Republic

4. Dr. Popov Kapky bylinné Tlak – srdce 50 mL

Contains: Herba visci albi, Crataegi fructus, Melissae herba, Flos tiliae, Leonuri cardiacae herba, Bulbi allii sativi. Each 100 g of the preparation contain extract of 3.32 g mistletoe, 3.32 g hawthorn, 2.9 g motherwort, and 0.41 g garlic.

Dosage: 20 drops thrice daily

Special warning(s): Not intended for use in children, pregnant and breast-feeding women.

Producer: Dr. Popov s.r.o., Plzeňská 857, 348 15 Planá, Czech Republic

5. Fytos Kapky pro optimální tlak 50 mL

Contains: hawthorn 0.5 g, mistletoe 5 g, lemon balm 5 g, motherwort 5 g, valerian root 1 g, horsetail 5 g

Dosage: adults: 7 drops twice daily, children over 4 years of age: 5 drops once daily

Special warning(s): Not intended for use in children under 3 years of age, pregnant and breast-feeding women.

Producer: FIPO SOBOTKA s.r.o., Na Benešově 737, 507 43 Sobotka, Czech Republic

6. Leros Alvisan Neo 1x100 g

Contains: Visci albi herba 40.0 g; Hyperici herba 20.0 g; Crataegi folium cum flore 16.5 g; Crataegi fructus 10.0 g; Equiseti herba 7.5 g; Menthae piperitae herba 2.0 g; Melissae herba 2.0 g, Matricariae flos 2.0 g

Dosage: 1 tablespoon of tea mixture, to be poured over with 250 mL of boiling water, twice daily

Special warning(s): Not intended for use in children and adolescents under 18 years of age, pregnant and breast-feeding women. When using products containing St. John's wort, intense UV radiation must be avoided.

Registration holder: Leros s.r.o., U Národní galerie 470, 156 00 Praha 5 – Zbraslav, Czech Republic

7. Leros Alvisan Neo 20x1.5 g

Contains (in 1 infusion bag): Visci albi herba 0.600 g, Hyperici herba 0.300 g, Crataegi folium cum flore 0.247 g, Crataegi fructus 0.150 g, Equiseti herba 0.113 g, Menthae piperitae herba 0.030 g, Melissae herba 0.030 g, Matricariae flos 0.030 g

Dosage: 1 cup twice daily

Special warning(s): Not intended for use in children and adolescents under 18 years of age, pregnant and breast-feeding women. When using products containing St. John's wort, intense UV radiation must be avoided.

Registration holder: Leros s.r.o., U Národní galerie 470, 156 00 Praha 5 – Zbraslav, Czech Republic

8. Liftea Hloh Jmelí Česnek 30 cps

Contains: *Allium sativum* extract 150 mg, *Crataegus* spp. extract 60 mg, *Viscum album* extract 60 mg

Dosage: 1 – 2 capsules per day

Special warning(s): Not intended for use in children under 3 years of age.

Producer: SWISS CAPS AG, Husenstrasse 35, CH-9533 Kirchberg, Switzerland

9. MaxiMag Cardio 375 mg + B6 + hloh + srdečník 30 tbl

Contains: magnesium 375 mg, *Crataegus monogyna* extract 100 mg, *Leonurus cardiaca* extract 50 mg, vitamin B6 1.4 mg

Dosage: 1 tablet daily

Special warning(s): none

Producer: Naturprodukt CZ s.r.o, Na Viničkách 638, 250 92 Šestajovice, Czech Republic

10. Megafyt Čaj z hlohu 20x1.5 g

Contains (in 1 infusion bag): Crataegi folium cum flore 1.5 g

Dosage: 1 cup thrice daily

Special warning(s): Intended for use in adults only. Consulting a physician during use is recommended.

Producer: MEGAFYT PHARMA s.r.o., U Elektrárny 516, 252 46 Vrané nad Vltavou, Czech Republic

11. Naturvita Jmelí – hloh plus Hořčík 60 cps

Contains: mistletoe 1000 mg (equals to 250 mg of extract 4:1), hawthorn 100 mg (equals to 25 mg of extract 4:1), magnesium 40 mg

Dosage: 1 capsule twice daily

Special warning(s): Not intended for use in children, pregnant and breast-feeding women.

Producer: Naturvita a.s., Veselá 227, 763 15 Slušovice, Czech Republic

Herbal preparations containing **motherwort** included in the consumption analysis are as follows (listed under their respective trade name in alphabetical order):

1. Anti-stress 20 tbl

Contains: Valerianae radix 170 mg, Melissae herba 50 mg, Lupuli flos 50 mg, Leonuri cardiacae herba 50 mg

Dosage: 1 tablet thrice daily

Special warning(s): Not intended for use in children.

Producer: Laboratorium Farmaceutyczne Labofarm sp. z o.o. Sp. k, Lubichowska 176B, 83 250 Starograd Gdański, Poland

2. Anti-stress 60 tbl

Contains: Valerianae radix 170 mg, Melissae folium 50 mg, Lupuli flos 50 mg, Leonuri cardiacae herba 50 mg

Dosage: 1 tablet thrice daily

Special warning(s): Not intended for use in children.

Producer: Laboratorium Farmaceutyczne Labofarm sp. z o.o. Sp. k, Lubichowska 176B, 83 250 Starograd Gdański, Poland

3. Čaj bylináře Krevní tlak 40x1.6 g

Contains (in 1 infusion bag): Crataegi flos 800 mg, Filipendulae ulmariae herba 480 mg, Melissae herba 040 mg, Leonuri cardiacae herba 80 mg

Dosage: 1 cup daily

Special warning(s): Not intended for use in children.

Producer: MEDIATE s.r.o., Dolní Libchavy 325, 561 16 Libchavy, Czech Republic

4. Dr. Popov Kapky bylinné Tlak – srdce 50 mL

Contains: Herba visci albi, Crataegi fructus, Melissae herba, Flos tiliae, Leonuri cardiacae herba, Bulbi allii sativi. Each 100 g of the preparation contain extract of 3.32 g mistletoe, 3.32 g hawthorn, 2.9 g motherwort, and 0.41 g garlic.

Dosage: 20 drops thrice daily

Special warning(s): Not intended for use in children, pregnant and breast-feeding women.

Producer: Dr. Popov s.r.o., Plzeňská 857, 348 15 Planá, Czech Republic

5. Fytopharma Bylinný čaj na vysoký tlak 20x1.5 g

Contains: Herba visci albi, Leonuri cardiacae herba, Millefolii herba, Melissae herba

Dosage: 1 cup twice to thrice daily

Special warning(s): Not intended for use in children, pregnant and breast-feeding women.

Producer: FYTOPHARMA a.s., Duklianskych hrdinov 47/651, 901 27 Malacky, Slovak Republic

6. Fytos Kapky pro optimální tlak 50 mL

Contains: hawthorn 0.5 g, mistletoe 5 g, lemon balm 5 g, motherwort 5 g, valerian root 1 g, horsetail 5 g

Dosage: adults: 7 drops twice daily, children over 4 years of age: 5 drops once daily

Special warning(s): Not intended for use in children under 3 years of age, pregnant and breast-feeding women.

Producer: FIPO SOBOTKA s.r.o., Na Benešově 737, 507 43 Sobotka, Czech Republic

7. MaxiMag Cardio 375 mg + B6 + hloh + srdečník 30 tbl

Contains: magnesium 375 mg, *Crataegus monogyna* extract 100 mg, *Leonurus cardiaca* extract 50 mg, vitamin B6 1.4 mg

Dosage: 1 tablet daily

Special warning(s): none

Producer: Naturprodukt CZ s.r.o, Na Viničkách 638, 250 92 Šestajovice, Czech Republic

Herbal preparations containing **black cumin** included in the consumption analysis are as follows (listed under their respective trade name in alphabetical order):

1. Černý kmín olej 100% 50 mL

Contains: cold-pressed black cumin oil

Dosage: 1 teaspoon thrice daily

Special warning(s): Not intended for use in children under 3 years of age.

Producer: VIRDE s.r.o., Štemplovec 10, 747 74 Holasovice, Czech Republic

Herbal preparations containing **olive leaf** included in the consumption analysis are as follows (listed under their respective trade name in alphabetical order):

1. OptiTensin 30 tbl

Contains: olive leaf extract 5:1 350 mg containing min. oleuropein 16%

Dosage: 1 tablet once to twice daily, to be taken after meal

Special warning(s): Not intended for use in children, pregnant and breast-feeding women. Not suitable for people with low blood pressure.

Producer: WALMARK a.s., Oldřichovice 44, 739 61 Třinec, Czech Republic

2. OptiTensin 60 tbl

Contains: olive leaf extract 5:1 350 mg containing min. 16% of oleuropein

Dosage: 1 tablet once to twice daily, to be taken after meal

Special warning(s): Not intended for use in children, pregnant and breast-feeding women. Not suitable for people with low blood pressure.

Producer: WALMARK a.s., Oldřichovice 44, 739 61 Třinec, Czech Republic

Herbal preparations containing **mistletoe** included in the consumption analysis are as follows (listed under their respective trade name in alphabetical order):

1. Aromatica TlakTEA 20x1.5 g

Contains: Herba bursae pastoris, Solidaginis herba, Equiseti herba, Millefolii herba, Herba cardui benedicti, Herba visci albi, Taraxaci officinalis folium

Dosage: 1 cup twice daily

Special warning(s): Intended for use in children over 3 years of age and adults.

Producer: AROMATICA CZ s.r.o., Masarykovo náměstí 101/3, 664 51 Šlapanice, Czech Republic

2. Dr. Popov Kapky bylinné Jmelí bílé 50 mL

Contains: Herba visci albi extract

Dosage: 20 drops twice daily (equal to extract of 116 mg of mistletoe)

Special warning(s): Not intended for use in children, pregnant and breast-feeding women.

Producer: Dr. Popov s.r.o., Plzeňská 857, 348 15 Planá, Czech Republic

3. Dr. Popov Kapky bylinné Tlak – srdce 50 mL

Contains: Herba visci albi, Crataegi fructus, Melissae herba, Flos tiliae, Leonuri cardiacae herba, Bulbi allii sativi. Each 100 g of the preparation contain extract of 3.32 g mistletoe, 3.32 g hawthorn, 2.9 g motherwort, and 0.41 g garlic.

Dosage: 20 drops thrice daily

Special warning(s): Not intended for use in children, pregnant and breast-feeding women.

Producer: Dr. Popov s.r.o., Plzeňská 857, 348 15 Planá, Czech Republic

4. Fytopharma Bylinný čaj na vysoký tlak 20x1.5 g

Contains: Herba visci albi, Leonuri cardiacae herba, Millefolii herba, Melissae herba

Dosage: 1 cup twice to thrice daily

Special warning(s): Not intended for use in children, pregnant and breast-feeding women.

Producer: FYTOPHARMA a.s., Duklianskych hrdinov 47/651, 901 27 Malacky, Slovak Republic

5. Fytos Kapky pro optimální tlak 50 mL

Contains: hawthorn 0.5 g, mistletoe 5 g, lemon balm 5 g, motherwort 5 g, valerian root 1 g, horsetail 5 g

Dosage: adults: 7 drops twice daily, children over 4 years of age: 5 drops once daily

Special warning(s): Not intended for use in children under 3 years of age, pregnant and breast-feeding women.

Producer: FIPO SOBOTKA s.r.o., Na Benešově 737, 507 43 Sobotka, Czech Republic

6. Leros Alvisan Neo 1x100 g

Contains: Visci albi herba 40.0 g; Hyperici herba 20.0 g; Crataegi folium cum flore 16.5 g; Crataegi fructus 10.0 g; Equiseti herba 7.5 g; Menthae piperitae herba 2.0 g; Melissae herba 2.0 g, Matricariae flos 2.0 g

Dosage: 1 tablespoon of tea mixture, to be poured over with 250 mL of boiling water, twice daily

Special warning(s): Not intended for use in children and adolescents under 18 years of age, pregnant and breast-feeding women. When using products containing St. John's wort, exposure to intense UV radiation must be avoided.

Registration holder: Leros s.r.o., U Národní galerie 470, 156 00 Praha 5 – Zbraslav, Czech Republic

7. Leros Alvisan Neo 20x1.5 g

Contains (in 1 infusion bag): Visci albi herba 0.600 g, Hyperici herba 0.300 g, Crataegi folium cum flore 0.247 g, Crataegi fructus 0.150 g, Equiseti herba 0.113 g, Menthae piperitae herba 0.030 g, Melissae herba 0.030 g, Matricariae flos 0.030 g

Dosage: 1 cup twice daily

Special warning(s): Not intended for use in children and adolescents under 18 years of age, pregnant and breast-feeding women. When using products containing St. John's wort, exposure to intense UV radiation must be avoided.

Registration holder: Leros s.r.o., U Národní galerie 470, 156 00 Praha 5 – Zbraslav, Czech Republic

8. Liftea Hloh Jmelí Česnek 30 cps

Contains: *Allium sativum* extract 150 mg, *Crataegus* spp. extract 60 mg, *Viscum album* extract 60 mg

Dosage: 1 – 2 capsules per day

Special warning(s): Not intended for use in children under 3 years of age.

Producer: SWISS CAPS AG, Husenstrasse 35, CH-9533 Kirchberg, Switzerland

9. Naturvita Jmelí – hloh plus Hořčík 60 cps

Contains: mistletoe 1000 mg (equals to 250 mg of extract 4:1), hawthorn 100 mg (equals to 25 mg of extract 4:1), magnesium 40 mg

Dosage: 1 capsule twice daily

Special warning(s): Not intended for use in children, pregnant and breast-feeding women.

Producer: Naturvita a.s., Veselá 227, 763 15 Slušovice, Czech Republic

3.2.2. List of Prescription Restricted Medicinal Products Included in the Consumption Analysis

Medicinal products containing **foxglove cardenolides** included in the consumption analysis are as follows (listed under their respective trade name in alphabetical order):

1. Digoxin Léčiva 0.125 mg tablety

Contains: digoxinum 0.125 mg

Dosage: individual

Special warning(s): see SPC for more details

Registration holder: Zentiva, k. s., U Kabelovny 130, 102 37 Praha 10, Czech Republic

2. Digoxin Léčiva 0.250 mg tablety

Contains: digoxinum 0.250 mg

Dosage: individual

Special warning(s): see SPC for more details

Registration holder: Zentiva, k. s., U Kabelovny 130, 102 37 Praha 10, Czech Republic

3.3. Statistical Analysis

Data from participating pharmacies was either collected personally or, due to the epidemiological situation regarding the COVID-19, some of the participating pharmacies sent data electronically. For operational reasons, the data from e-shop pharmacies was sent only by electronic means.

For the statistical analysis data was divided into three categories: big city, small town, and e-shop. The category "big city" included pharmacies in cities with at least 50 thousand of inhabitants, "small town" included pharmacies in towns under 50 thousand of inhabitants, and "e-shop" included pharmacies offering delivery service regardless of the number of inhabitants.

The evaluation period for the consumption analysis is represented by the years 2015 – 2019. Data on sales of the selected herbal food supplements and medicinal products (see Ch. 3.2.1. and Ch. 3.2.2.) was collected as the total amount of packages sold per year. For all products, the number of daily doses of each package size was calculated according to the respective package leaflet. Daily dose for a product with a dosage range was calculated as the mean value of this range. In case of a dosage calculated with the reference to body weight, the body weight of 70 kg was used. For medicinal products containing digoxin, the defined daily dose as established by World Health Organization was used as a reference for the calculation of the number of daily doses.

Data was then processed to obtain percentile values calculated for each of the evaluated medicinal plants with the exception of digoxin-containing medicinal products. In specific cases the number of respective daily doses of the chosen medicinal plant preparations was directly compared with the total number of daily doses – for example in order to establish the

trends of different dosage form sales (see Ch. 4.1.2., Ch. 4.1.3., and Ch. 4.1.6. for more details).

To avoid the bias caused by differences in the amount of sold daily doses, which was strongly affected by the pharmacy category, firstly the percentage of daily doses of the respective plant was calculated for each pharmacy, and then these percentile values were compared between the pharmacy categories. The consumption of the respective medicinal plant was then expressed as the mean value of these percentages.

During the data processing, a large disparity was discovered between the sale of garlic preparationss and other medicinal plants preparations. Therefore an additional data modification was applied – garlic preparations correction. In this case, the data on garlic preparations was omitted and respective percentages were calculated as described above with the total number of daily doses without garlic preparations.

For digoxin, the total number of defined daily doses (DDD) of each included year was divided by the number of inhabitants of the respective city/town, and the result for statistical purposes was expressed as number of DDD per thousand of inhabitants. The use of DDD for drug consumption analysis is widely accepted as a value not affected by the price or package size (Kořístková and Grundmann, 2006). In this case the more frequently used value DDD/ 100 hospital days was not applicable.

4. RESULTS

4.1. OVER THE COUNTER HERBAL PREPARATIONS

A total number of 33 over the counter herbal preparations was included in the consumption analysis. Most of these OTCs are solid dosage form-products (20), 6 products are herbal teas, and the rest is marketed in a liquid dosage form (drops, tincture, or oil). Two of these products meet the legal requirements on a medicinal product and are registered with SUKL, while the majority of the evaluated herbal products are categorized as food supplements. For further details see the respective chapter on medicinal plant products consumption.

4.1.1. GARLIC HERBAL PREPARATIONS

Generally, the garlic-containing preparations represent the majority of the evaluated products. With the exception of three products (namely Allivictus tinktura in two package sizes and Dr. Popov Kapky bylinné Tlak – srdce), they are produced in solid dosage forms, i.e. tablets or capsules. No herbal tea containing garlic is marketed in the Czech Republic.

Most garlic preparations (10) contain various garlic extracts, only 6 preparations contain powdered garlic cloves. Six preparations were standardized for a certain garlic active compounds, namely alliin, allicin, total sulfur compounds, garlic oil, or a special extract marketed under the trade name garlicin.

Garlic represents the majority of the herbal preparations bought by clients in big city, small town, and e-shop pharmacies, with the percentages of these products ranging from 72% to 86%. No distinctive fluctuation in garlic consumption during the evaluation time period occurred for the respective pharmacy category. A single interesting feature of the consumption curve is a rapid increase in garlic consumption in 2019 for e-shop pharmacies,

accompanied by a less pronounced decrease in garlic consumption in the same year for small town pharmacies (Fig. 4.1.).

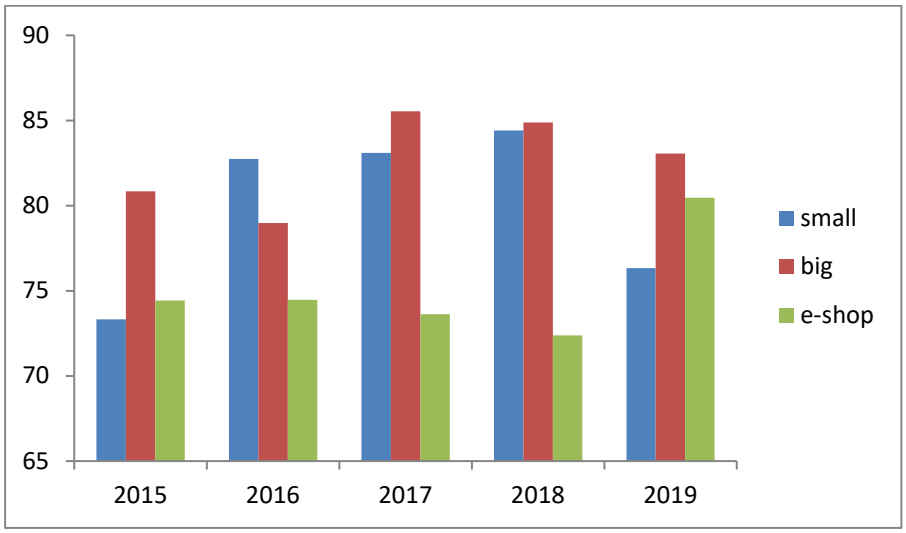

Fig. 4.1. Garlic preparations consumption in the years 2015 – 2019. Values are expressed as percentages. Small – small town pharmacies, big – big city pharmacies, e-shop – e-shop pharmacies.

4.1.2. Hawthorn Herbal Preparations

Hawthorn-containing preparations are (together with mistletoe) the second most bought by clients both in pharmacies and e-shops. Out of the 11 evaluated hawthorn preparations only one contains a hawthorn extract, and three of them contain Crategi folium cum flore, meeting the requirements of European Pharmacopeia on the amount of active compounds. Four preparations are herbal teas, three preparations are in a liquid (drops), and four are produced in a solid dosage form. The comparison of consumption of the hawthorn tea and non-tea preparations in total amount of daily doses regardless of the pharmacy category during the reporting time period, expressed as percentages, is shown in Fig. 4.2.

The consumption of hawthorn preparations in the evaluated time period for big city, small town, and e-shop pharmacies exhibits a more or less balanced trend, not exceeding 12% of total daily doses of herbal preparations. This stability of the hawthorn consumption trend is even more highlighted when the garlic preparations correction was applied (Fig. 4.3. and Fig. 4.4.).

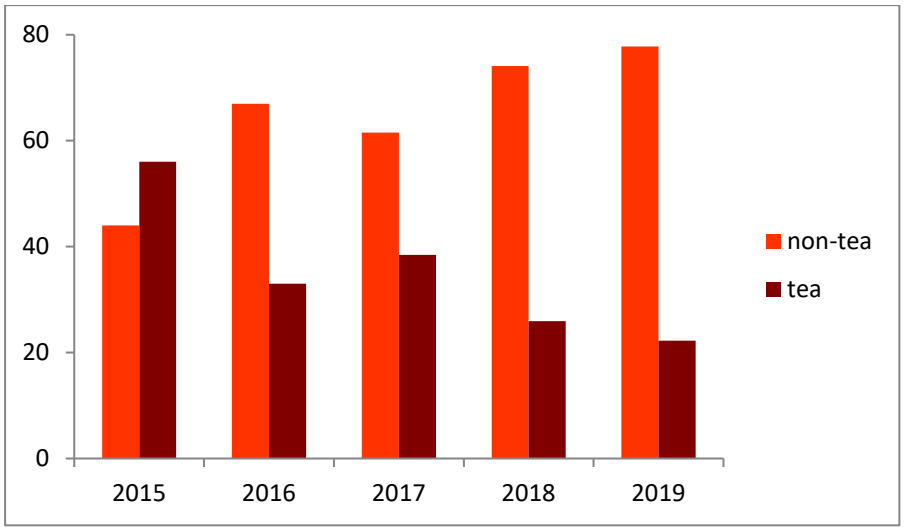

Fig. 4.2. Hawthorn tea and non-tea preparations consumption in the years 2015 – 2019. Values are expressed as percentages of hawthorn daily doses. Non-tea – preparations in all dosage forms with the exception of herbal tea, tea – herbal tea preparations.

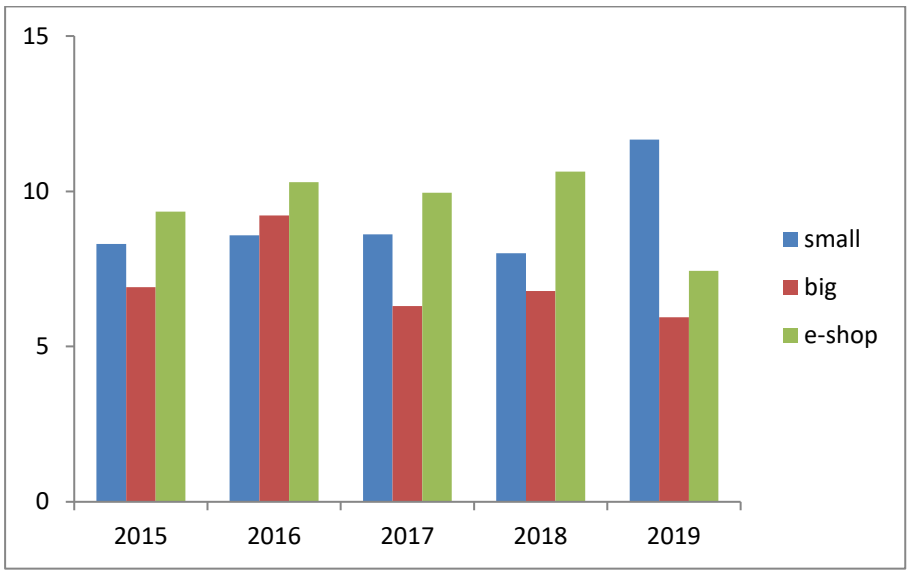

Fig. 4.3. Hawthorn preparations consumption in the years 2015 – 2019 without the garlic preparations correction of data. Values are expressed as percentages. Small – small town pharmacies, big – big city pharmacies, e-shop – e-shop pharmacies.

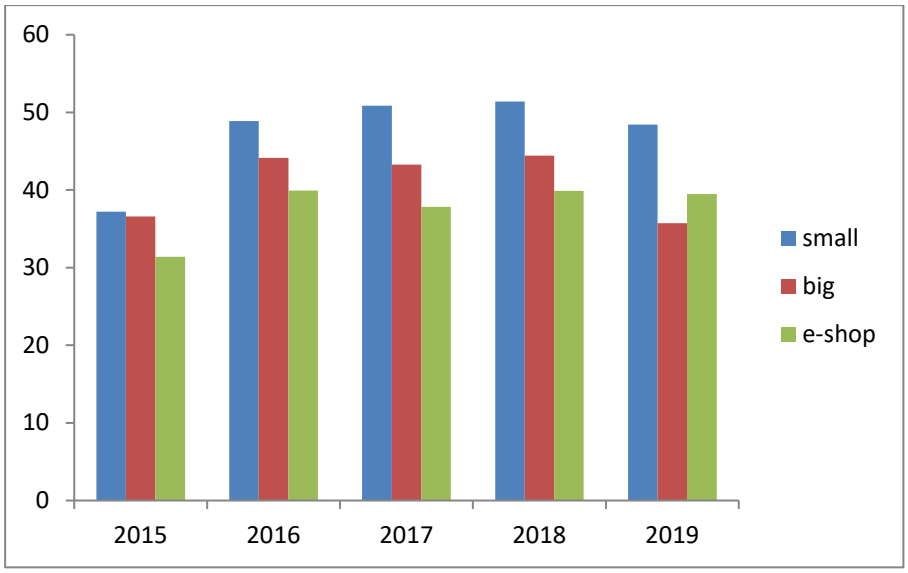

Fig. 4.4. Hawthorn preparations consumption in the years 2015 – 2019 with the garlic preparations correction of data. Values are expressed as mean percentages for each pharmacy category. Small – small town pharmacies, big – big city pharmacies, e-shop – e-shop pharmacies.

4.1.3. Motherwort Herbal Preparations

Motherwort preparations marketed in the Czech Republic are very scarce, and only five of the seven evaluated preparations are primarily intended in cardiovascular use (see Ch. 5.4. for more details). The two other preparations (namely Anti-stress 20 tbl and Anti-stress 60 tbl) are mainly used in relieving of mild stress and anxiety conditions, often related with heart palpitations. The comparison of the consumption of motherwort sedative and non-sedative products in the total amount of daily doses regardless of the pharmacy category during the reporting time period, expressed as percentage, is shown in Fig. 4.5.

Out of the 7 marketed motherwort preparations, none of them meets the requirement for a medicinal product and they are all categorized as food supplements. Three preparations are in a solid dosage form, two are offered as tinctures, and the remaining two preparations are represented by the herbal tea form.

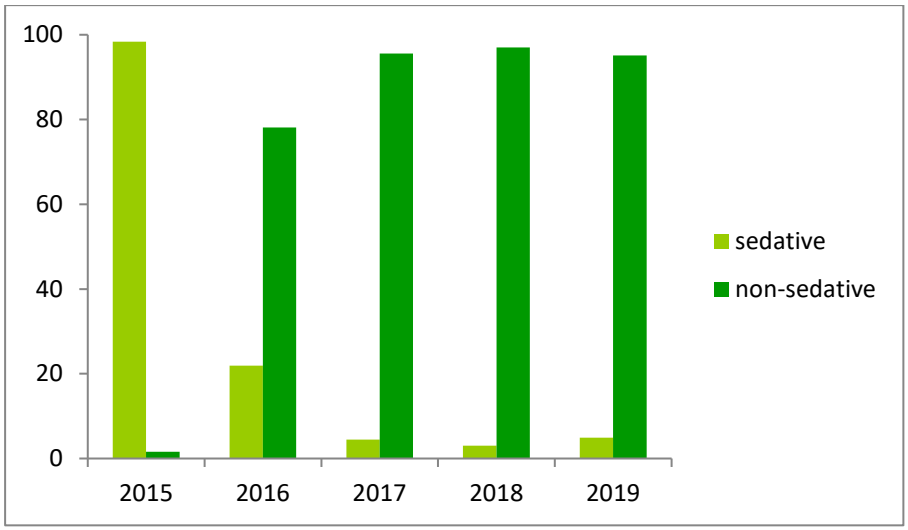

Fig. 4.5. Motherwort sedative and non-sedative preparations consumption in the years 2015 – 2019. Values are expressed as percentages of motherwort daily doses regardless of pharmacy category. Sedative – preparations primarily used in treatment of stress and anxiety, non-sedative – preparations with primary cardiovascular use.

The consumption of motherwort preparations is fairly exiguous, ranging in minor percent units, not exceeding 4% of total herbal preparations during the whole evaluation time period. Interestingly, while in 2015 the majority of motherwort daily doses was sold in big city and small town pharmacies, there is a rapid drop in sales of motherwort preparations in 2016 for these categories, and sales through e-shop pharmacies gained in importance for the remaining years of the reporting period (Fig. 4.6.).

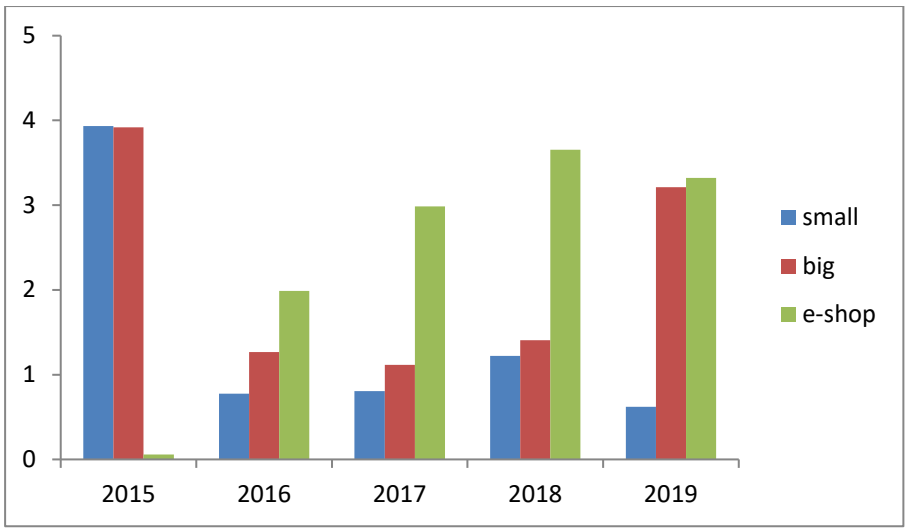

Fig. 4.6. Motherwort preparations consumption in the years 2015 – 2019 without the garlic preparations correction of data. Values are expressed as percentages. Small – small town pharmacies, big – big city pharmacies, e-shop – e-shop pharmacies

The fluctuations of motherwort preparation sales throughout the evaluated time period are more emphasized when the garlic preparations correction was applied, with markedly increases for the e-shop pharmacies, and an accentuated decrease in 2016 for big city pharmacies (Fig. 4.7.).

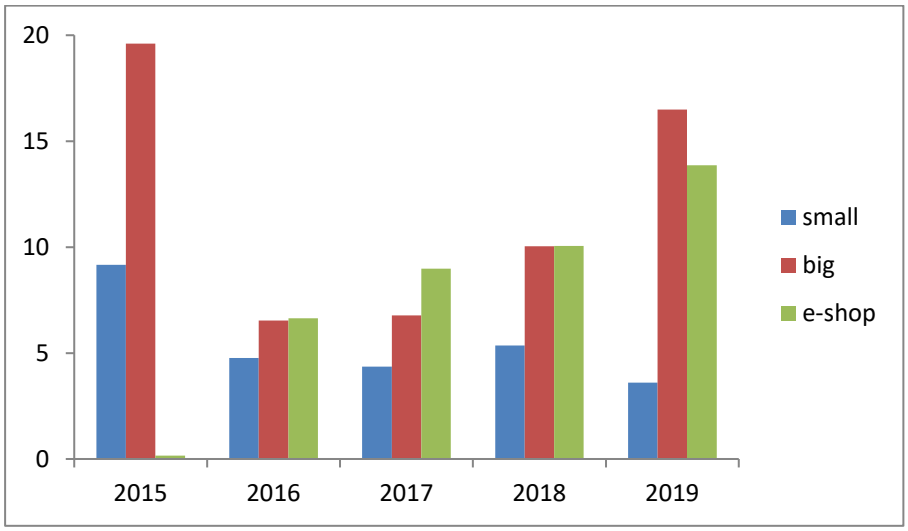

Fig. 4.7. Motherwort preparations consumption in the years 2015 – 2019 with the garlic preparations correction of data. Values are expressed as mean percentages for each pharmacy category. Small – small town pharmacies, big – big city pharmacies, e-shop – e-shop pharmacies.

4.1.4. Black Cumin Herbal Preparations

Black cumin is marketed in the Czech Republic only in a liquid dosage form (oil) and this category is represented by a single food supplement available in one package size only. It contains pure cold-pressed seed oil with no standardization of active compounds.

In the evaluated time period this product was bought predominantly via e-shop pharmacies. The consumption of black cumin shows a more or less linear decreasing trend with a pronounced reduction in 2016, in general not exceeding 2% of the total daily doses of herbal preparations. Note the emphasized linearity of the decreasing consumption curve for black cumin products when the sales for small town and e-shop pharmacies in 2016 were united (Fig. 4.8., Fig. 4.9., and Fig. 4.10.).

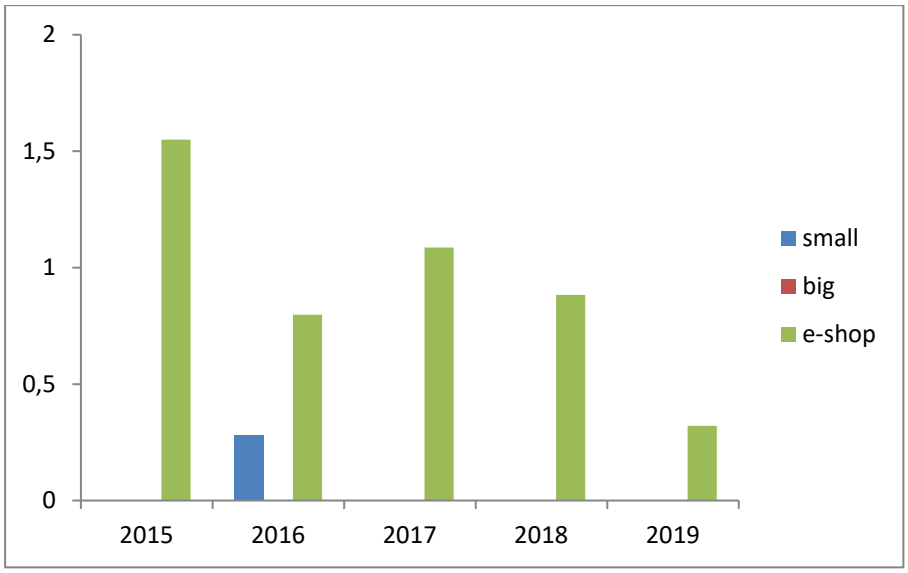

Fig. 4.8. Black cumin preparations consumption in the years 2015 – 2019 without the garlic preparations correction of data. Values are expressed as percentages. Small – small town pharmacies, big – big city pharmacies, e-shop – e-shop pharmacies.

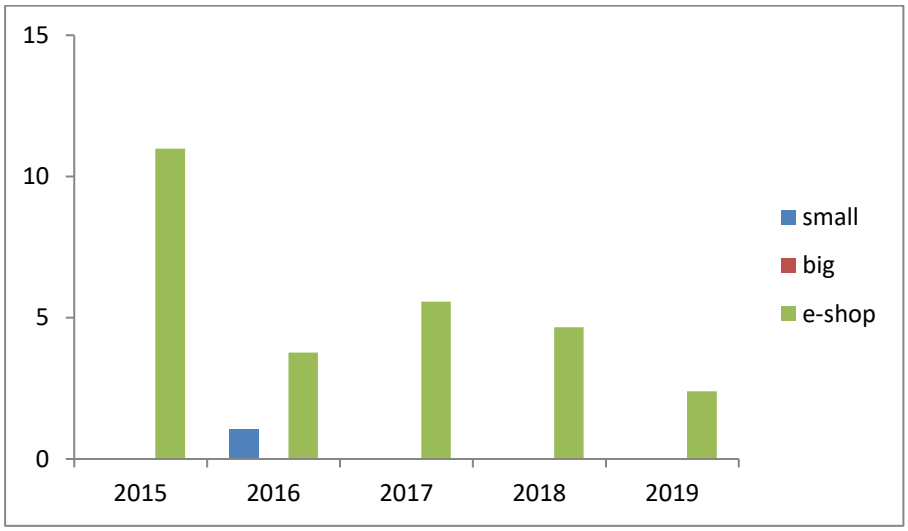

Fig. 4.9. Black cumin preparations consumption in the years 2015 – 2019 with the garlic preparations correction of data. Values are expressed as mean percentages for each pharmacy category. Small – small town pharmacies, big – big city pharmacies, e-shop – e-shop pharmacies.

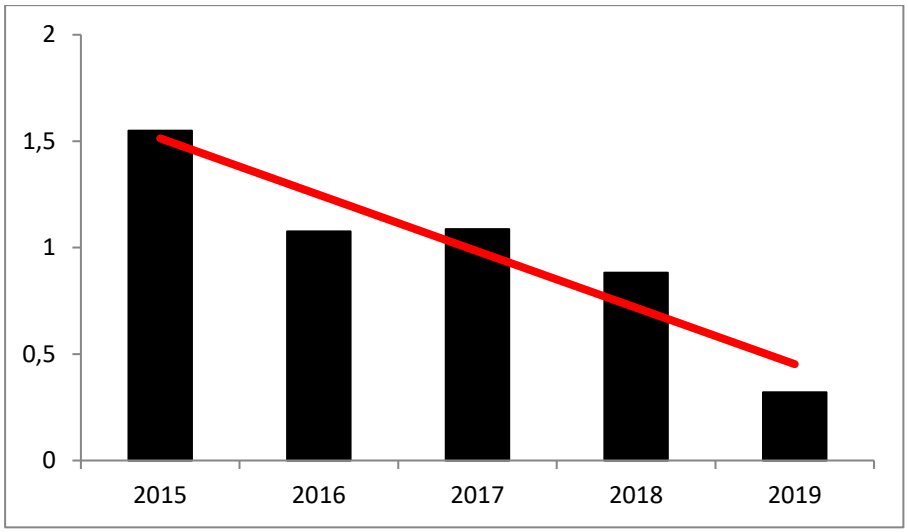

Fig. 4.10. Black cumin preparations consumption in the years 2015 – 2019 regardless of pharmacy category. Values are expressed as percentages. Note the strongly linear decreasing trend in the consumption with the coefficient of determination R^2=0.8908.

4.1.5. Olive Leaf Herbal Preparations

Olive leaf preparations marketed in the Czech Republic are represented by a single herbal preparation, categorized as food supplement, and available in different package sizes. This supplement is produced in a solid dosage form (tablets), and it is standardized to contain a minimum 16% of oleuropein.

The consumption of this olive leaf food supplement shows a decreasing trend in the evaluated time period, this trend being especially notable for the e-shop pharmacies, and partially also for the small town pharmacies. On the other hand, big city pharmacies exert a more or less non-fluctuating trend of the olive leaf preparations sales with the exception of the year 2018. In general, the consumption of olive leaf preparations did not exceed 10% of total daily doses of herbal preparations except for e-shop pharmacies in 2015 where it reached 13% (Fig. 4.11. and Fig 4.12.).

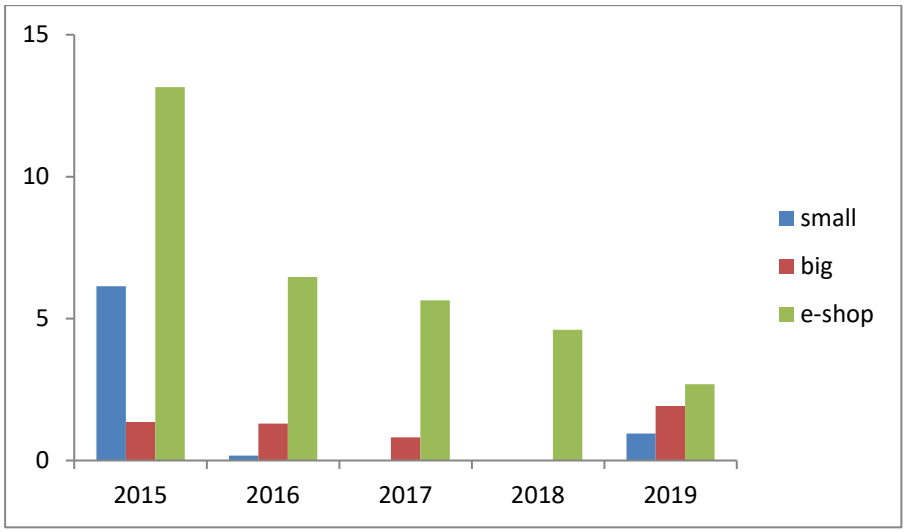

Fig. 4.11. Olive leaf preparations consumption in the years 2015 – 2019 without the garlic preparations correction of data. Values are expressed as percentages. Small – small town pharmacies, big – big city pharmacies, e-shop – e-shop pharmacies.

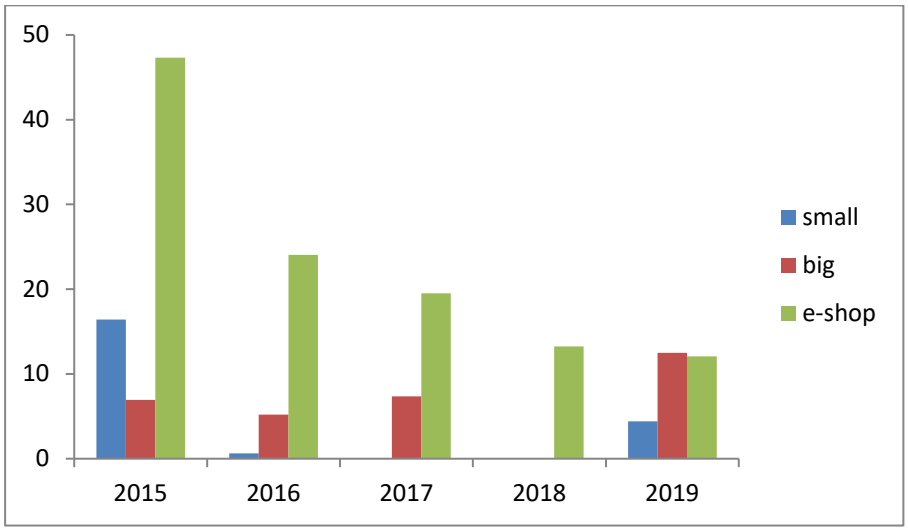

Fig. 4.12. Olive leaf preparations consumption in the years 2015 – 2019 with the garlic preparations correction of data. Values are expressed as mean percentages for each pharmacy category. Small – small town pharmacies, big – big city pharmacies, e-shop – e-shop pharmacies.

4.1.6. Mistletoe Herbal Preparations

Mistletoe preparations marketed in the Czech Republic are prevalently represented by herbal teas (4 preparations) and liquid dosage form (drops;

3 preparations). The two remaining preparations are available in a solid dosage form. One preparation contains a mistletoe extract, two products meet the requirements on Visci albi herba. Two of these herbal products are registered with SUKL, thus fulfilling the legal conditions of a medicinal product.

The comparison of the consumption of the mistletoe tea and non-tea preparations in the total amount of daily doses sold through the e-shop pharmacies during the reporting time period, expressed as percentages, is in Fig. 4.13.

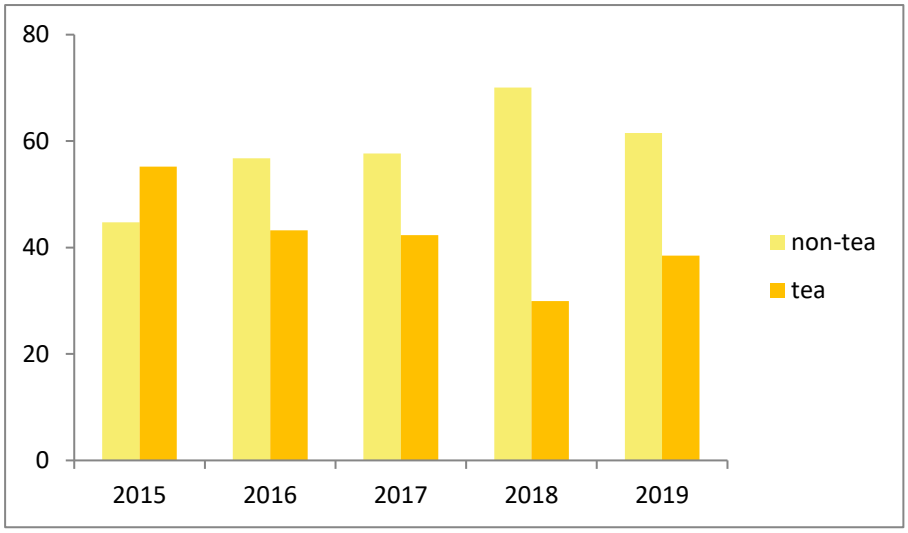

Fig. 4.13. Mistletoe tea and non-tea preparations consumption in the years 2015 – 2019 in the e-shop pharmacies. Values are expressed as percentages of mistletoe daily doses. Non-tea – preparations in all dosage forms with the exception of herbal tea, tea – herbal tea preparations.

The consumption of mistletoe herbal preparations during the evaluated time period shows a stable trend for small town and big city pharmacies with a minor decrease in 2019 for big city pharmacies; it is, however, increased remarkably for e-shop pharmacies. This increase has become more emphasized when the garlic preparations correction was applied (Fig. 4.14. and Fig. 4.15.).

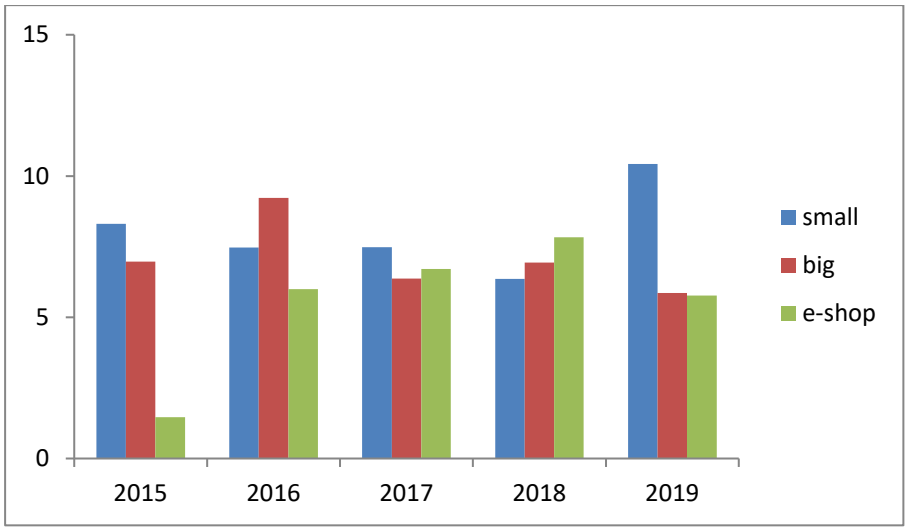

Fig. 4.14. Mistletoe preparations consumption in the years 2015 – 2019 without the garlic preparations correction of data. Values are expressed as percentages. Small – small town pharmacies, big – big city pharmacies, e-shop – e-shop pharmacies.

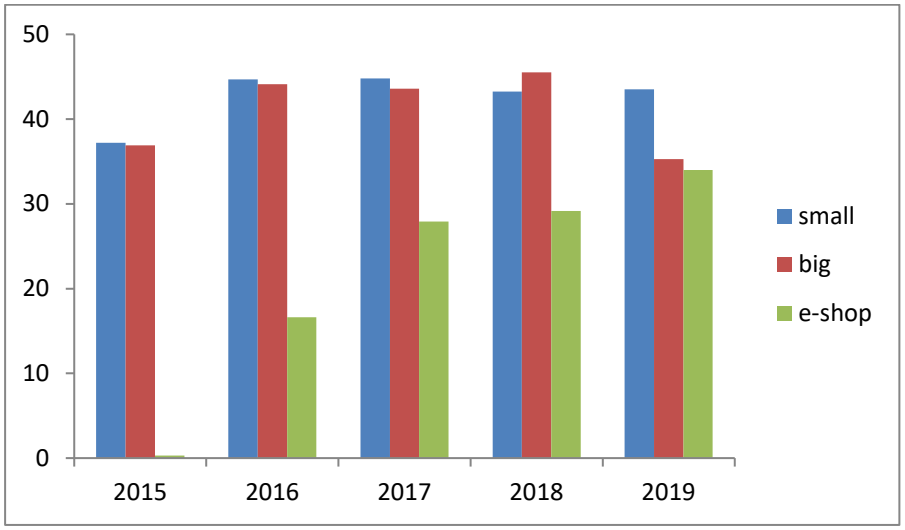

Fig. 4.15. Mistletoe preparations consumption in the years 2015 – 2019 with the garlic preparations correction of data. Values are expressed as mean percentages for each pharmacy category. Small – small town pharmacies, big – big city pharmacies, e-shop – e-shop pharmacies.

4.2. Prescription-Restricted Medicinal Products

Only two of the evaluated medicinal plant preparations fall within this category, both of them contain the foxglove cardenolide digoxin. These

medicinal products are registered with SUKL and prescription-restricted due to their indications.

4.2.1. FOXGLOVE – DIGOXIN

Digoxin consumption in years 2015 – 2019 is shown in Fig. 4.16. and Fig. 4.17. for big city and small town pharmacies, respectively. For the big city pharmacies, a decreasing trend in digoxin consumption can be seen with a pronounced reduction in 2018 followed by a slight increase in 2019 (Fig. 4.16.).

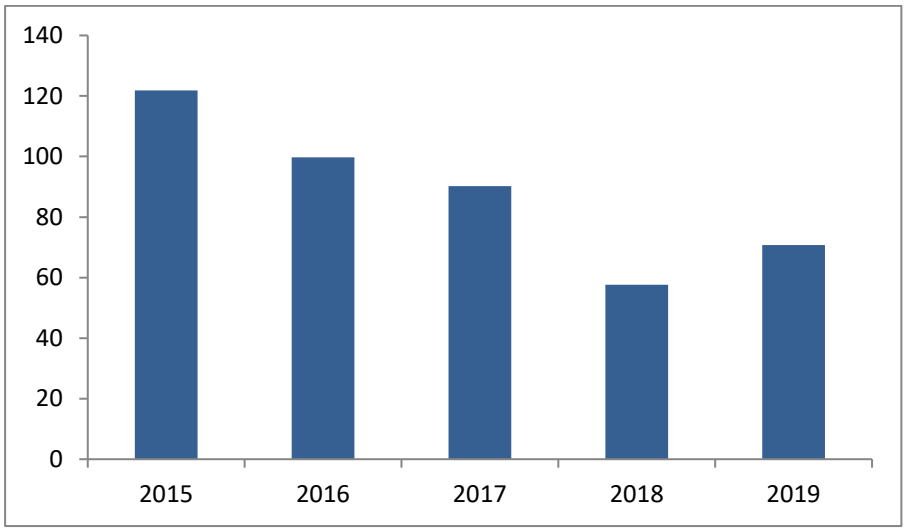

Fig. 4.16. Digoxin consumption in the years 2015 – 2019 for big city pharmacies. Values are expressed as DDD/1000 inhabitants.

For the small town pharmacies, the decreasing trend in consumption was also observed, however, it had a prolonged onset (2017) and an emphasized change. Furthermore, for small town pharmacies in 2018 a slight increase and in 2019 a slight reduction to the values of the year 2017 of digoxin consumption was noted (Fig. 4.17.).

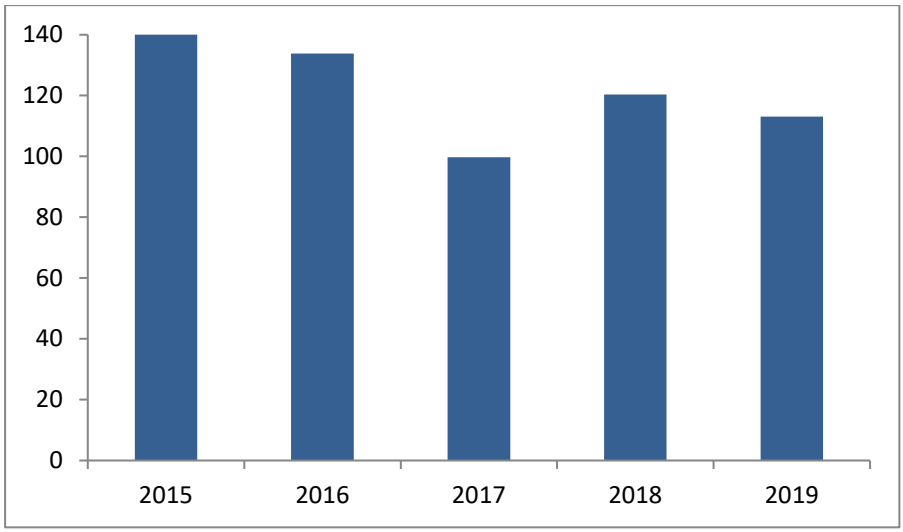

Fig. 4.17. Digoxin consumption in the years 2015 – 2019 for small town pharmacies. Values are expressed as DDD/1000 inhabitants.

5. DISCUSSION

5.1. GENERAL REMARKS ON THE EVALUATED HERBAL PREPARATIONS

For the consumption analysis, a total of 35 herbal preparations containing garlic, foxglove cardenolides, hawthorn, motherwort, black cumin, olive leaf, or mistletoe were chosen. With the exception of the products Leros Alvisan Neo 1x 100 g, Leros Alvisan Neo 20x1.5 g, Digoxin Léčiva 0.125 mg tablety, and Digoxin Léčiva 0.250 mg tablety, none of the evaluated herbal preparations have the status of a medicinal product, and they are categorized as food supplements. In fact, there is no medicinal product in a solid dosage form (i.e. tablet, capsule) on the pharmaceutical market of the Czech Republic containing garlic, hawthorn, motherwort, black cumin, olive leaf, or mistletoe.

Medicinal products containing hawthorn and mistletoe are marketed in the Czech Republic only in the form of herbal teas with defined contents. Visci albi herba and Crategi folium cum flore have to contain strictly defined amounts of the active compounds, according to the requirements of EMA or European Pharmacopoeia 10.0.

Food supplements evaluated in the consumption analysis have to follow the requirements of the national regulation No 58/2018 (on food supplements and food composition), as well as the requirements of the (EC) regulation No 1924/2006 (on nutrition and health claims made on foods). According to these regulations, herbal preparations containing hawthorn have to follow the maximum allowed amount of the leaves and flowers extract (1000 mg daily). No health claims (see Ch. 2.8.2.) were authorized for any of the evaluated herbal preparations, which, in fact, makes it harder to offer as an alternative for treatment of different cardiovascular conditions.

In the following chapters, the potential positive effects on human cardiovascular system are discussed separately for each of the selected medicinal plants. It is, however, necessary to emphasize that several of the evaluated preparations are multi-component, and thus the possibility of

interactions between the individual components needs to be considered. The interaction potential of each plant was also reviewed in the theoretical part of this thesis, see the relevant chapters for more details. Additive effects of medcinal plant combinations on cardiovascular health conditions were reported by several authors (e.g. Brändle *et al.*, 1997; Malik *et al.*, 2017a, b).

5.2. Garlic Consumption in the Years 2015 – 2019

Garlic preparations represent the most sold herbal peparations of all the OTC preparations evaluated in all of the pharmacies – being it in the small town, big city, or e-shop category, which is in concordance with the fact that garlic is one of the oldest medicinal herbs, and has been grown for centuries in this geographical area. Notably, the e-shop sales were usually lower than those of brick-and-mortar pharmacies. This discrepancy is probably due to the fact that garlic preparations are popular with the clients, and smaller pharmacies also regularly offer these preparations, so there is no need to place a special order (unlike, for example, black cumin preparations).

The lower consumption of garlic preparations in small town pharmacies compared to big city pharmacies may also be due to the fact that people in smaller towns and villages are more likely to grow their own garlic, as they are often living in houses with gardens. Also, the purchasing power of people living in big cities is usually higher than those of small town people, allowing them to pay for such "luxury goods" as garlic processed to capsules and tablets.

The fundamental issue with garlic herbal preparations is the lack of any medicinal products among them (see Ch. 5.1.). Although most of the marketed garlic preparations are standardized (10 out of 16), the majority use standardizations that, in fact, offer no valuable information on the active compounds content: sulfur compounds, garlicin, and garlic oil are just general and non-descriptive labels. Only three preparations are standardized to contain a particular active compound (namely allicin or alliin) in a specific amount.

High doses (2400 mg) of garlic preparations standardized to contain 1.3% allicin were shown to significantly decrease blood pressure even in patients with a rather severe hypertension (diastolic blood pressure ≥115 mm Hg), with the hypotensive effect persisting up to 14 hours (McMahon and Varga, 1993). When compared to the preparation standardized to contain allicin, the abovementioned work used a minimum of 31.2 mg allicin – that is about four times as much as the allicin content in the recommended daily dose of the evaluated garlic supplement. Thus, the hypotensive performance of the evaluated preparation most likely will not be that significant, but this supplement could be considered for people in a prehypertensive stage, or in patients with a mild hypertension. Garlic as an add-on therapeutic to several standard antihypertensive drugs was observed to increase the hypotensive effect (Asdaq and Inamar, 2010; Ashraf *et al.*, 2013), in some cases even exhibiting synergistic effect with the antihypertensive drug (Asdaq and Inamar, 2011).

As to hypolipidemic effect of garlic, it has been shown that patients with mild dyslipidemia treated for a minimum of 8 weeks may benefit from garlic treatment (Ried, 2016). Reduction of triglyceride and/or cholesterol levels was reported in volunteers using 600 – 800 mg of garlic powder. Aged garlic extract needs remarkably higher doses (over 7 g daily) to exert its hypolipidemic properties (Steiner *et al.*, 1996). None of the evaluated garlic preparations offer a daily dose that high, the recommended dosage for most preparations is about three to four times lower.

5.3. Hawthorn Consumption in the Years 2015 – 2019

Hawthorn herbal preparations are fairly popular with people and their consumption in the evaluation time period is more or less stable, with no remarkable changes, especially when the correction on garlic preparations was applied (see Ch. 3.3. and Ch. 4.1.2.).

Interestingly, while in small town and big city pharmacies mostly hawthorn-containing herbal teas are preferred, a gradual but steady increase of the non-tea at the expense of tea hawthorn preparations can be observed in e-shop pharmacies (Fig. 4.2.). Partially due to limited selection of hawthorn herbal preparations offered by most pharmacies – mainly teas – and partially due to the fact that predominantly younger people are placing e-shop orders. Based on my ten years of experience, mostly the elderly prefer herbal teas to solid dosage forms.

Similarly to garlic preparations, the main issue with hawthorn herbal preparations is the content of active compounds. Crataegi folium cum flore should meet the requirement of a minimum 1.5% of flavonoids expressed as hyperoside, calculated with the reference to the dried drug (European Pharmacopoeia 10.0). This content is guaranteed for the two medicinal products in this category.

According to the available literature, high doses of hawthorn extracts are needed for the onset of the blood pressure lowering effect (900 mg/day or higher; e.g. Walker *et al.*, 2006 and Al-Gareeb *et al.*, 2012). Lower doses of hawthorn extract effective in reduction of the blood pressure were reported by Schmidt *et al.* (1994) for the commercially available hawthorn extract LI 132. The extract LI 132 at the dose of 600 mg/kg exerts a statistically significant reduction of the systolic blood pressure in humans. This extract is standardized to contain at least 2.2% of flavonoids, therefore the daily dose of Crataegi folium cum flore should be 880 mg. Each teabag of the medicinal tea contains exactly 494 mg of the drug, thus with two teas recommended per day, the amount of active compounds should be sufficient enough. However, the LI 132 is extracted with 70% methanol, while teabags are extracted with boiling water. Therefore, the active compounds of these two extracts may differ significantly.

As to the lipid-lowering effect of hawthorn, a positive effect of hawthorn on the human lipid profile was observed at high doses of WS 1442 extract (900 mg twice daily; Al-Gareeb, 2012). While none of the evaluated products offer such a high dosage, the WS 1442 extract is standardized for a certain content of proanthocyanidins not flavonoids (see Ch. 2.2.3.), therefore

the dosage used in this clinical trial and the hawthorn supplement marketed in the Czech Republic are not entirely comparable.

On the other hand, the flavonoid hyperoside was reported to exert a strong inhibitory activity against the key enzyme in cholesterol synthesis (HMG Co-A; Ye *et al.*, 2010), thus a prolonged use of hawthorn products may be taken into consideration in mildly hypercholesterolemic patients.

5.4. Motherwort Consumption in the Years 2015 – 2019

The consumption of motherwort herbal preparations in the evaluated time period is fairly interesting: with a simple declining trend in small town pharmacies and an increasing trend in e-shop pharmacies, with a rapid reduction in 2016 and rapid increase in 2019 for big city pharmacies, it is quite difficult to estimate the background of such changes.

Two main issues arise with motherwort products. Firstly, two of the solid dosage form preparations are primarily intented for the use as a mild sedative, offering further a mild anxiolytic action in people with pronounced psychic tension, mostly caused by various stress situations. In these preparations, 50 mg of motherwort herb is accompanied by valerian root, lime balm herb, and hop cone. Thus, in this case it is not likely that people would buy such a product with the intention of a cardiovascular use. Note, however, the rapid shift in the sold daily doses of these sedative versus non-sedative motherwort preparations, and the switch toward the non-sedative motherwort preparationss in the evaluated time period (Fig. 4.5.).

On the other hand, all the five remaining preparations are marketed as food supplements for the cardiovascular use. Here is the second main issue of these products particularly noticeable: the uncertain content of active compounds. Motherwort content in these food supplements varies from 50 mg to 370 mg in a daily dose.

According to European Pharmacopoeia, Leonuri cardiacae herba should contain at least 0.2% of flavonoids expressed as hyperoside, calculated

with the reference to the dried drug (European Pharmacopoeia 10.0), thus the motherwort products contain flavonoids at least within the range of 0.1 mg to 0.75 mg. It is, however, quite complicated to estimate the hypotensive effect of such doses, especially with the significant lack of information on this topic in the available literature.

Namely, the hypotensive action of motherwort extract in human patients with stage 1 and stage 2 hypertension was reported by Shikov *et al.* (2011) who used higher doses of motherwort extract (600 mg) twice daily. Furthermore, the extract used in this study was obtained from extraction of motherwort with soybean oil in order to obtain the non-polar active compounds, which clearly is not the case of hyperoside. Therefore it is safe to presume that the chemical composition of this particular extract and that of evaluated motherwort preparations differ too much to draw any conclusions on their hypotensive actions.

It is also difficult to evaluate a possible positive effect of motherwort preparations marketed in the Czech Republic on human lipid profile, as relevant information is scarce. I was not able to find any trials regarding the lipid-lowering motherwort activity with human volunteers. The closest animal species was Rhesus monkey. In this case, however, the synthetic motherwort alkaloid leonurine (SCM-198) was used (Suguro *et al.*, 2018). Leonurine is a compound more characteristic for Chinese motherwort than for motherwort cardiaca where its content is very low (under 1 per mille, see Ch. 2.4.2.), and thus no significant effect can be expected in this field of action.

5.5. Black Cumin Consumption in Years 2015 – 2019

Similarly to olive leaf herbal preparations, the consumption of black cumin products exerts a decreasing trend in the evaluated time period (Fig. 4.9.). Only one black cumin preparation is marketed on the pharmaceutical market of the Czech Republic, although several black cumin oils are available mostly via specialized food stores. Also, the majority of this black cumin preparation was sold in e-shop pharmacies which is mostly due

to the unavailability of this products in brick-and-mortar pharmacies, be it in small town or big city.

The marketed black cumin oil is a pure, cold-pressed seed oil. According to the producer, the oil origin country is Egypt or Ukraine (personal communication). Since it has been reported that the black cumin active compounds differ significantly due to the origin of the plant material (Nickavar *et al.*, 2003; Kokoška *et al.*, 2008; Bourgou *et al.*, 2010; Singh *et al.*, 2014; Kalidasu *et al.*, 2017), it is necessary to know the geographical region where plants were grown. Tunisian and Turkish black cumin cultivars, that are most likely to be grown in the abovementioned countries, are characterized by a high content of *p*-cymene (about 60%) and a fairly high content of thymoquinone (about 3% for the Tunisian oil).

Fallah Huseini *et al.* (2013) reported a significant decrease of both systolic and diastolic pressure in volunteers treated with 2.5 mL of black cumin oil twice daily for 8 weeks. The dosage of the marketed product is 5 mL thrice daily, which should be sufficient enough. Furthermore, the abovementioned trial was conducted in Iran, using Iranian black cumin oil that was reported to be rich in *trans*-anethole and low in thymoquinone (under 1%; Nickavar *et al.*, 2003; see also Tab. 2.1.). Thus, compared to the marketed black cumin oil, the dose of thymoquinone is likely higher in the evaluated preparation and in the recommended dosage it is expected to have a positive influence on the blood pressure reduction.

As to the impact of black cumin on the lipid profile, Najmi *et al.* (2008) and Ahmad Alobaidi (2014) both observed a pronounced hypolidpidemic action of statins (atorvastatin and simvastatin, respectively) coadministered with black cumin oil in dyslipidemic patients treated with black cumin. Najmi *et al.* (2008) used the dose of 2.5 mL of black cumin oil twice daily for eight weeks. Therefore, adding black cumin oil as an adjuvant therapy in hyperlipidemic patients should be taken into consideration.

Moreover, Amini *et al.* (2011) even reported a positive effect of black cumin oil administered at a dose of 2.5 mL twice daily in healthy volunteers. All of the trials were performed with Iranian black cumin oil (low

thymoquinone content), thus the evaluated black cumin preparation is most likely to exert a pronounced hypolipidemic activity because of its higher thymoquinone content.

5.6. Olive Leaf Consumption in the Years 2015 – 2019

The consumption analysis of olive herbal preparations clearly shows a decreasing trend in both the amount of daily doses as well as percentage of sales of these products in the evaluated time period (Fig. 4.11. and Fig. 4.12.). As this trend is notable in all categories of pharmacies, there is clearly a reduction of interest from the clients.

Olive leaf herbal preparations marketed in the Czech Republic are only in solid dosage form, containing olive leaf extract standardized for a minimum of 16% of oleuropein. This amount is fairly high, as the European Pharmacopoeia requires a minimum of 5% of secoiridoids, expressed as oleuropein (European Pharmacopoeia 10.0). The commercially available olive leaf extract EFLA 943 is standardized for oleuropein content within the range 18 – 26 %.

Perrinjacquet-Moccetti *et al.* (2008) and Susalit *et al.* (2011) both reported a significantly decreased blood pressure in volunteers taking 1000 mg of EFLA 943 daily, divided in two doses. This two-dose pattern is recognized also by the producer of the olive leaf extract food supplement, however, the dose is lower, only 350 mg in each tablet, thus the daily dose contains about 112 mg of pure oleuropein. Contrary to that, the reported hypotensive effect of olive leaf was achieved with at least 180 mg of pure oleuropein. At this dose, the antihypertensive effect of oleuropein was comparable to that of 25 mg of captopril (Susalit *et al.*, 2011). The difference in daily dose of oleuropein is about 30%, which is quite significant, on the other hand, this marketed olive leaf supplement is one of a few preparations at least nearing the requirements for an effective treatment of hypertension.

As to the effect of olive leaf on lipid profile, Perrinjacquet-Moccetti *et al.* (2008) observed a statistically significant reduction in volunteers taking 1000 mg of olive leaf extract EFLA 943. Again, this dose contains at least 180 mg of oleuropein which is higher than 112 mg of oleuropein in the evaluated product. The authors, however, also observed a positive trend in the cholesterol reduction with the dose of 500 mg of EFLA 943 (i.e. 90 mg of oleuropein). Thus, in this case the dose of olive leaf preparation could be considered for clients with only slightly elevated cholesterol levels. Araki *et al.* (2019) also reported a positive effect of olive leaf tea on triglycerides and LDL cholesterol in volunteers drinking olive leaf tea, which surely contains less oleuropein than the evaluated supplement.

5.7. Mistletoe Consumption in Years 2015 – 2019

The consumption of mistletoe herbal preparations during the evaluated time period shows a stable trend for small town and big city pharmacies, however, it increased remarkably for e-shop pharmacies. This increase is noticeable especially when the garlic preparations correction was applied (Fig. 4.14. and Fig. 4.15.).

The mentioned non-fluctuation trend in big city and small town pharmacies is mostly due to the fact that mistletoe is predominantly sold as herbal tea, often bought by elder people. The increase in e-shop sales of mistletoe preparations is on the other hand strongly influenced by the increase of the non-tea mistletoe preparations (Fig. 4.13.). Again, as with hawthorn non-tea preparations, mostly people of younger age prefer these dosage forms, and often place their orders via e-shop pharmacies.

Out of the nine evaluated mistletoe preparations, only two are medicinal products registered with SUKL, both of them being herbal teas. All the remaining preparations, including those in a solid-dosage form, are categorized as food supplements.

As the information on the dosage for an effective hypotensive action of mistletoe is scarce, I am not able to compare the effectivity of the evaluated mistletoe preparations. The only study giving a dosage for mistletoe in prehypertensive to stage 2 hypertension patients (Poruthukaren *et al.*, 2014) administered 10 drops of a mistletoe tincture thrice daily. Two preparations marketed in this dosage form in the Czech Republic have a higher recommended daily dose, therefore it is likely to expect a similar beneficial effect in mildy hypertensive patients as that reported by Poruthukaren *et al.* (2014).

The lack of information on an effective mistletoe dosage for hyperlipidemia treatment makes it again almost impossible to evaluate the marketed preparations. The abovementioned study of Poruthukaren *et al.* (2014) also noted a significant reduction in triglycerides, and an increasing trend for the HDL cholesterol in patients treated with the mistletoe tincture. Thus, the two aforementioned mistletoe preparations should exert a similar hypolipidemic effect as the tincture used in the trial of Poruthukaren *et al.* (2014).

Furthermore, Ben *et al.* (2006) noted that aqueous mistletoe extract was less effective in the hypolipidemic action in rats than the ethanolic extract. It is therefore possible that the marketed herbal teas (be it food supplements or medicinal products) will not exhibit any positive effect on the lipid profile.

5.8. Digoxin Consumption in the Years 2015 – 2019

The different trends of digoxin consumption between small towns and big cities in the years 2015 – 2019 (Fig. 4.16. and Fig. 4.17.) are likely due to several factors. Firstly, the consumption of prescription-restricted drugs is closely tied to the prescription of such a drug by the physicians. According to the guidelines for treatment of heart failure in the Czech Republic, digoxin should be considered in specific cases only (Hradec, 2018), therefore the

prescription and consequently the consumption of digoxin should decrease in time.

This decreasing trend of digoxin can be observed in big cities (Fig. 4.16.), with a constant decrease throughout the evaluated time period. For digoxin consumption in small towns (Fig. 4.17.), the decrease in time is also evident, though the decreasing trend is less pronounced when compared to big city. Physicians in small towns probably tend to hold on on their usual prescription pattern but the changes were seen in the following years, most notably in 2017.

Secondly, the digoxin consumption in the year 2018 and partially also in the beginning of the 2019 was strongly influenced by the unavailability of digoxin on the Czech pharmaceutical market. The producer stated production reasons for digoxin supply disruption. This digoxin outage lasted for about three months according to SUKL, and was longer for Digoxin 0.250 (October 15th – January 15th) than for Digoxin 0.125 (December 10th – January 16th). The pronounced reduction of digoxin consumption in big cities is logically countered by the increase of digoxin consumption in small towns – when the resources in big cities were depleted, they still remained in small town pharmacies, thus increasing the consumption. The year 2019 represents a return to the original trend of digoxin consumption for both big city and small town pharmacies.

CONCLUSIONS

- The overview includes seven medicinal plants. For each plant its phytochemistry, medicinal use, effects on the cardiovascular system (including antioxidant, cardioprotective, vasorelaxing, antithrombotic, antiatherogenic, antihypertensive, and lipid-lowering actions) and safety profile of herbal preparations were reviewed with the emphasis on works published within the last twenty years.
- Over the counter herbal preparations containing selected medicinal plants are predominantly categorized as dietary supplements (31). Only two medicinal preparations are marketed in the Czech Republic, both of these preparations are herbal teas.
- The majority of herbal preparations are represented by garlic supplements. Their consumption exhibited no significant fluctuations during the reporting time period. Garlic supplements are the most sold herbal preparations in all pharmacy categories. Hawthorn preparations consumption exhibited a similarly non-fluctuating trend. The consumption of mistletoe and motherwort preparations increased in the evaluated time period, while the consumption of black cumin and olive supplements decreased in the evaluated time period.
- Recommended dosages of the evaluated herbal preparations are in general lower than the dosages reported in the available literature.
- Digoxin consumption in general exerts a decreasing trend during the reporting time period.
- Digoxin consumption is different between big city and small town pharmacies, these differences are remarkable both in the time frame and number of digoxin DDD/1000 inhabitants.

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1. **EMA/HPMC/246778/2009** Assessment report on *Viscum album* L., herba
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