PhD thesis

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Health promoting effects of bioactive compounds in plants
Dissertation for the degree of Doctor of Philosophy
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English abstract

While type 2 diabetes is an increasing problem worldwide, there is still no cure and therefore search for the new insulin sensitizer continues. Plants are a natural source of bioactive compounds and have been used to improve human health and wellbeing for centuries. Today, several studies concentrate on screening plant extracts commonly used in folk medicine for pure compounds, exploiting promising results in treatment of, among others, type 2 diabetes. Another area of diabetes research, focused on the complex biology of adipose tissue and its influence on the development of insulin resistance. Moreover, the crosstalk between gut bacteria and insulin sensitive tissues like fat, pancreas and skeletal muscle has received much attention and all aspects are important in order to better understand the basics of this disease.

This PhD thesis is based on 5 scientific papers focusing on plant derived compounds and their influence on adipocyte differentiation, lipid storage, glucose uptake and gut microbiota.

Dansk resumé


Denne ph.d.-afhandling er baseret på 5 videnskabelige artikler, der fokuserer på stoffer baseret på plantetræk og deres indflydelse på fedtcelle differentiering, lipid opbevaring, glucose-optagelse og tarmflora.
List of papers included in the thesis


2. R. B. El-Houri, D. Kotowska, L. C. B. Olsen, S. Bhattacharya, L. P. Christensen, K. Grevsen, N. Oksbjerg, N. Færgeman, K. Kristiansen, K. B. Christensen, Screening for Bioactive Metabolites in Plant Extracts Modulating Glucose Uptake and Fat Accumulation, manuscript

3. C. Andersen, D. Kotowska, C. G. Tortzen, K. Kristiansen, J. Nielsen, R. K. Petersen, 2-(2-bromophenyl)-formononetin and 2-heptyl-formononetin are PPARγ partial agonists and reduce lipid accumulation in 3T3-L1 adipocytes, manuscript

4. D. Kotowska, J. Olesen, M. Hansen, R. S. Bienso, C. M. Kristensen, N. Brandt, S. A. B. Larsson, W. A. Al-Soud, L. Hansen, K. Kristiansen, H. Pilegaard, Resveratrol, exercise and gut microbiota, manuscript

5. D. Kotowska, R. B. El-Houri, K. B. Christensen, X. Frette, K. Grevesen, L. P. Christensen, K. Kristiansen, Novel PPARγ partial agonist isolated from Echinacea purpurea exploits insulin sensitizing effect in 3T3-L1 adipocytes, manuscript

Table of contents

English abstract .................................................................................................................. 3
Dansk resumé .................................................................................................................... 3
List of papers included in the thesis ................................................................................. 4
List of abbreviation ........................................................................................................ 6
1. Introduction .................................................................................................................. 7
  1.1 Obesity, insulin resistance and pharmacology ......................................................... 8
  1.2 Plant derived compounds ......................................................................................... 9
  1.3 Adipocytes and 3T3-L1 model ............................................................................... 18
  1.4 New player: microbiota ......................................................................................... 20
2. Discussion of the papers ............................................................................................ 22
  PAPER I .................................................................................................................... 23
  PAPER II .................................................................................................................. 24
  PAPER III ................................................................................................................ 25
  PAPER IV ............................................................................................................... 26
  PAPER V .................................................................................................................. 28
  OTHER PAPERS .................................................................................................... 29
  2.1 Concluding remarks ............................................................................................ 30
Literature ....................................................................................................................... 31
Annex ............................................................................................................................. 39
# List of abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TZDs</td>
<td>Thiazolidinediones</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome-proliferator activated receptor gamma</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin gallate</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated kinase</td>
</tr>
<tr>
<td>Irs</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>Glut</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Sirt2</td>
<td>NAD-dependent deacetylase sirtuin-2</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>2BrPhF</td>
<td>2-(2-bromophenyl)-formononetin</td>
</tr>
<tr>
<td>C7F</td>
<td>2-heptyl formononetin</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>aP2</td>
<td>Fatty acid binding protein 2</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phophatidylinositol 3-kinase</td>
</tr>
<tr>
<td>Fiaf</td>
<td>Fasting-induced adipocyte protein</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short chain fatty acid</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Tissue inhibitor of metalloproteinases 1</td>
</tr>
</tbody>
</table>
1. Introduction

Plants are the natural source of compounds and have been used to maintain human health and improve the quality of life for hundreds of years. Traditional medicine, using plant extracts, herbal mixtures and natural compounds isolated from various species of plants, is still used for primary health care, despite the growth of pharmaceutical industry (Egan, C.D., 2002). At the same time, WHO estimates, that approximately two thirds of modern drugs originate from plants.

Apart from being the great source of bioactive compounds and drugs, plants are also important sources of food. It is well known that intake of various types of fruits and vegetables has health promoting effect and may prevent modern life style associated diseases such as obesity, diabetes, atherosclerosis and metabolic syndrome. Indeed, thorough and careful exploration for novel, natural compounds has attracted renewed interest worldwide, especially in case of search for new insulin sensitizers and anti-obesity agents. The era of TZDs as potent insulin sensitizers and full PPARγ agonists seem to come to the end, mostly because TZDs’ unwanted side effects like weight gain, edema or fluid retention. Therefore new compounds, able to increase insulin sensitivity, prevent development of type 2 diabetes and avoid causing unwanted side effects are needed, and plants may be excellent sources of new, health promoting compounds.

First line of therapy for managing obesity and the related metabolic syndrome is to modify patient’s lifestyle. However, while a Mediterranean type of diet, rich in fruits and vegetables, is considered first line treatment to improve one’s health, change to more active lifestyle is also needed. It is known, that exercise has a beneficial effect in both animal models and human. Exercise promotes weight loss and improves insulin sensitivity and therefore physical activity may be considered an environmental factor influencing health and welfare together with a diet.

The work presented in this thesis was initiated as part of collaboration between three Danish universities with a goal of identifying new plant compounds exhibiting health promoting effects. In order to address this broad topic we focused our attention on insulin resistance and the development of adipocytes based on established cell culture model. By using unique rational combination of screening modalities we were able to identify two polyacetylenes (falcarinol and falcarindiol) isolated from Daucus carota and dodeca-2E,4E,8Z,10E/Z tetraenoic acid- 2- methylbutylamides- novel compound isolated from Echinacea purpurea. Both polyacetylenes and Echinacea alkamide show promising insulin sensitizing effects and may serve as leads for the development of the next generation of pharmaceuticals targeting obesity and obesity related disorders.
1.1 Obesity, insulin resistance and pharmacology

Obesity is now considered major problem for public health in developing and developed countries. According to WHO over four hundred million adults are considered obese and approximately 1,6 billion are overweight (http://www.who.int). Corresponding with an increased number of obese individuals is an increasing number of people suffering from obesity related disorders such as type 2 diabetes (T2D), cancer and hypertension (Rappange, D. R., et al., 2009). This rapid growth in the number of obese individuals is most probably connected to altered dietary intake versus energy expenditure on top of a genetic makeup responsible for adipose tissue development.

Adipocytes play important role in metabolic regulation by storing lipids (and releasing fatty acids) and secreting adipokines, which regulate insulin sensitivity and therefore regulate systemic energy balance. In the normal healthy person, energy intake is balanced by energy expenditure. Excess energy is stored in adipocytes in form of fat droplets and released as fatty acids when energy intake is scarce. However, when energy intake continuously exceed energy expenditure adipocyte tissue expands first due to hypertrophy and later due to hyperplasia. Hypertrophied adipocytes secrete large amounts of adipokines, which are known to not only interfere with insulin signaling pathways but also recruit macrophages. In turn, recruited macrophages secrete pro-inflammatory cytokines, inducing an inflamed state in the tissue and further increase the level of insulin resistance in the adipocytes. Moreover, secreted cytokines cause a decrease in the net flux of fatty acids into the adipocytes and, as a result, increase deposition of fatty acids in other tissues such as liver, muscle and pancreas (Siersbaek, R. et al., 2010). The ectopic fatty acid deposition leads to insulin resistance in these tissues and further, to systemic insulin resistance and type 2 diabetes, if need for insulin exceed the secretory capacity of the beta cells (Lehrke, M. et al., 2005).

Before insulin therapy in 1922, starvation and traditional, herbal medicine were the only treatments for diabetic patients but as soon as insulin was introduced to broad use, traditional treatments were forgotten. However, insulin is a life- saver, but it is not a cure and therefore other kind of drugs was needed. In the late 1990s TZDs (glitazones) were introduced as a new class of anti-diabetic, insulin sensitizers. Thiazolidinediones are selective agonists of peroxisome proliferated- receptor gamma. When PPARγ is activated by TZDs, differentiation of non- visceral adipose depots is initiated. Fatty acid storage in peripheral tissues is increased, creating “lipid steal” phenomenon, which in turn leads to lower levels of circulating fatty acids and reduced concentration of triglycerides in muscles and liver. At the same time thiazolidinediones acutely reduce insulin levels and arrest the decline in beta-cell function in the long run.

Promotion of peripheral tissue adipogenesis is only one of the ways that TZDs are working. Adipocytes per se are endocrine organ secreting adipokines (including leptins, adiponectin, resistin and TNFα) and TZDs, via PPARγ pathway, increase the production of adiponectin and reduce secretion of TNFα and resistin, responsible for impaired insulin actions (Gurnell, M. et al., 2003, Mudaliar, S. et al., 2001, Greenfield, J. R., 2004, Tang, W. et al., 2011, Cariou, B., 2012).
Despite their therapeutic abilities, exploit of TZDs was challenged by observed in clinical use side effects, which include severe weight gain, edema, fluid retention, cardiac failure and bone fractures. Recently, two drugs, rosiglitazone and troglitazone were withdrawn from the market due to observed hepatotoxicity and a remaining drug, pioglitazone, has been reassessed in light of increased risk of bladder cancer (Cariou, B. et al., 2012, Kahn, B. B. et al., 2010).

Besides TZDs, other anti-obesity and anti-diabetes therapies are introduced, but there is still no ideal drug on the market. The only drug approved in Europe is orlistat (Baretic, M., 2012), working as pancreatic lipase inhibitor, which results in secretion of undigested fatty acids through feces. Until recently sibutramine, an appetite suppressant, working via inhibition of reuptake of serotonin and norepinephrine, was an alternative to orlistat, but was withdrawn due to cardiovascular side effects (James, W. P. et al., 2010).

Taken together, novel compounds able to increase insulin sensitivity without causing unwanted side effects are needed. As mentioned before, plants have long history of preventing diseases and improving human health and they are usually considered less toxic and elucidate less side effects compared to synthetic drugs (Yeh, G. Y. et al., 2003) therefore can be considered excellent source of bioactive agents.

1.2 Plant derived compounds

Phytochemicals are reported to have beneficial effect on prevention of cardiovascular diseases, inflammation, glucose intolerance and obesity and plant extracts and purified plant compounds are gaining more and more interest since they are components of the everyday diet. in vivo, they work via different pathways causing, among others, inhibition of nutrient absorption, appetite suppression, increase of energy expenditure and modulation of fat storage. Below, there are few examples of plants derived compounds and extracts, showing promising effects in case of treating obesity and type 2 diabetes.

Catechins are main compounds of green tea, which is one of the most popular beverages in the world (right after water). Green tea contains five major catechins, including: catechin, epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate (EGCG), and the anti-obesity effect is mostly attributed to EGCG. The mechanism of action of EGCG involves inhibition of adipocyte differentiation and proliferation, inhibition of fat absorption from gut and inhibition of fatty acids oxidation in brown adipose tissue (Hursel, R. et al., 2010, Lin, J. K., 2006, Ikeda, I., 2005). In 3T3-L1 cells, EGCG inhibited adipocyte proliferation by decreasing levels of phosphorylated ERK1/2 and inducing apoptosis in mature cells (Lin, J., et al., 2005) and increasing phosphorylation of AMPK (Murase, T., et al., 2009).

Green tea is only one of the extracts exhibiting beneficial effects in anti-obesity treatment. Another example is an extract of chokeberry (Aronia melanocarpa), rich in anthocyanins. In rats, consumption of chokeberry extract together with high fructose diet, improved insulin sensitivity in epididymal adipose tissue via Irs1 and Irs2 pathway and increased expression of Glut1 and Glut4 (Qin, B., et al., 2011). Therefore, it was
concluded that chokeberry extract may be promising diet supplement, but human studies are still needed to assess the beneficial effects vs. risk factors.

Moderate red wine intake was positively correlated with lower occurrence of cardiovascular and metabolic diseases, including obesity and type 2 diabetes (Zoechling, A., et al., 2011). A grape skin ethanol extract was also found to decrease adipogenic transcription factor gene expression and therefore inhibit triglyceride accumulation in 3T3-L1 adipocytes mostly via PPARγ signaling pathway (Jeong, Y. S., et al., 2012), and grape seed extract was reported to lower triglyceride and total cholesterol levels in the plasma of rats fed high-fat diet (Charrad, K., et al., 2011). These studies correlate with reports of the pure compound, resveratrol, found in grapes, pistachios, red wine and berries. Resveratrol is well-studied polyphenol and has been suggested to have beneficial effect in preventing several diseases, including cancer, cardiovascular disease, obesity and diabetes (Hung, L. M. et al., 2000, Atten, M. J., et al., 2005, van der Spuy, W. J. et al., 2009). In rodents fed high fat diet supplemented with resveratrol, significant reduction of body fat depots was observed (Ahn, J. et al., 2008, Macarulla, M. T., et al., 2009, Shang, J., et al., 2008, Lagouge, M., et al., 2006, Baile, C. A., et al., 2011). Also, our own study confirms these findings. In our setup, mice fed high fat diet supplemented with resveratrol at dose 4g/kg food at libitum feeding were protected against diet induced obesity and their body weight was not significantly different from body weight of mice from control group fed with standard, chow diet (PAPER IV).

Resveratrol’s beneficial effects on obesity are reported to be due to not only AMPK phosphorylation but also Akt and Sirt1 signaling pathway (Zang, M., et al., 2006, Ahn, J., et al., 2008, Floyd, Z. E., et al., 2008). All these studies, including our own, suggest that grape derived compound resveratrol contributes to prevention of obesity through multiple pathways. However, thorough clinical trials on healthy and obese subjects are needed to support data obtained in the cell cultures and animals.

Another compound exhibiting anti-obesity properties is berberine isolated from *Cortidis rhizoma*. *Cortidis rhizoma* extract is known in traditional Chinese medicine as an anti-bacterial drug and in the recent studies of rat islets pretreated with this extract, a retained insulin-secretion capacity was found. Berberine was shown to inhibit adipocyte proliferation and differentiation and decrease lipid accumulation in 3T3-L1 cells. In high fat diet fed animals, berberine was shown to reduce serum cholesterol, triglycerides and LDL-cholesterol. Reduction of LDL-cholesterol was also observed in hypercholesterolemic patients and in diabetic animal models hypoglycemic action of insulin was enhanced (Kwon, K. B., et al., 2005).

Berberine, resveratrol and EGCG were reported to activate multiple pathways, including PPARγ, AMPK, Akt, Sir1 and ERK1/2 (Huang. C., et al., 2006, Kong, W., et al., 2004, Brusq, J. M., et al., 2006, Ko, B. S., et al., 2005). It has been suggested that natural compounds, in contrast to synthetic ones, may work through many pathways, activating them simultaneously. Resveratrol, berberine and EGCG but also: curcumin (isolated from turmeric, activating AMPK and PPARγ pathway), kaempferol and quercetin (main compounds in *Euonymus alatus*, activating PPARγ and IκB), artepillin C (isolated from Brazilian propolis, working via PPARγ and PKC pathways) and piperine (component of black pepper, activating PPARγ and LXRα) (Table 1) are the examples of plant derived

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plant of origin</th>
<th>Activated pathways</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>turmeric</td>
<td>AMPK, PPARγ</td>
<td>Ejaz, A. et al., 2009</td>
</tr>
<tr>
<td>Artepillin C</td>
<td>Brazilian propolis</td>
<td>PPARγ, PKC</td>
<td>Choi, S.-S. et al., 2011</td>
</tr>
<tr>
<td>Piperline</td>
<td>Black pepper</td>
<td>PPARγ, LXRα</td>
<td>Park, U.-H., et al., 2012</td>
</tr>
<tr>
<td>Berberine</td>
<td><em>Cortidis rhizoma</em></td>
<td>PPARγ, AMPK</td>
<td>Kwon, K. B., et al., 2005</td>
</tr>
<tr>
<td>EGCG</td>
<td><em>Camellia sinensis</em></td>
<td>AMPK, ERK1/2</td>
<td>Murase, T., 2009</td>
</tr>
</tbody>
</table>

Table 1. Examples of plant derived compounds activating multiple signaling pathways.

Isolation and identification of new, bioactive compounds from plant extracts can be performed in two ways: 1) plant extracts showing promising properties are scanned and one or two major compounds are isolated and tested further, or 2) extracts are fractionated and fractions are next submitted to bioassay guided fractionations. Scanning for major bioactive compounds is more commonly used, as it is faster and cheaper to perform. However, major phytochemicals are not always the ones responsible for beneficial effects of the plant. Moreover, synergistic effects of two or more compounds may occur and will be missed if bioassay guided fractionation is not performed.
Bioassay guided fractionation was used by Shang, N., et al. in search for adipogenic constituents from bark of *Larix laricina* du Roi for the treatment of type 2 diabetes symptoms. Starting from ethanol extract of *Larix laricina* sixteen primary fractions was obtained from silica gel column chromatography. These fractions were tested alongside with crude extract for ability to increase triglyceride accumulation in 3T3-L1 adipocytes. Six of the tested fractions were found to significantly increase triglyceride content and pure compounds were isolated from these fractions, which resulted in discovery of new cycloartane triterpentoid (23-oxo-3alpha-hydroxycycloart-24-en-26-oid acid) among the other 9 compounds. Interestingly, 23-oxo-3alpha-hydroxycycloart-24-en-26-oid acid was not the main component of the extract and still seemed to be responsible for adipogenic activity of the fraction it was isolated from (Shang, N., et al., 2012).

Similar work was performed while searching for anti-adipogenic activities of two extracts isolated from *Alnus incana* and *Populus balsamifera* barks respectively (Fig.1a, b). However no new compounds were discovered during this study, the isolated phytochemicals responsible for anti-adipogenic activity (oregonin from *Alnus incana* and salicortin from *Populus balsamifera*) were not major components of the crude extracts, similar to the previously described studies (Martineau, L. C., et al., 2010).
Fig. 1a. From Martineau, L. C., et al., 2010; Outline for bioassay guided fractionation of methanol extract of *Alnus incana* and chemical structure of isolated compound: oregonin.
Fig. 1b. From Martineau, L. C., et al., 2010; Outline for bioassay guided fractionation of methanol extract of *Populus balsamifera* and chemical structure of isolated compound: salicortin.
In 2009 Christensen K. B. et al., published a bioassay guided screening platform (Fig.2) for identification of plant extracts with potential antidiabetic properties (Christensen K. B., et al., 2009). The screening platform used in this study served as a base for our own studies, in which we performed bioassays guided fractionations of two dichloromethane extracts from roots of *Daucus carota* (carrot) and *Echinacea purpurea* (Fig.3).

![Diagram](image)

Fig.2. Christensen K. B. et al., 2008, Outline for the analytical screening platform applied in the paper.
Fig. 3. Outline for bioassays guided fractionation and compound purification used on dichloromethane extracts of *Daucus carota* and *Echinacea purpurea*.
Bioassay guided fractionation of carrot extract led us to two pure compounds: falcarinol and falcarindiol, which chemically belong to the class of polyacetylenes and are one of the most important compounds found in carrot (Killeen, D. P., et al., 2013). Therefore, this study stays in contradiction to classical bioassay guided research. However, polyacetylenes are wildly researched group of natural compounds, mostly in anti-cancer, anti-inflammatory, anti-bacterial and immune stimulation studies (Brandt, K., et al., 2004, Hansen, S. L., et al., 2003, Metzeger, B. T., et al., 2009), so there came no surprise, the same compounds were able to stimulate glucose uptake, induce lipolysis and not promote adipogenesis in 3T3-L1 adipocytes.

Our second study concentrated on bioassays guided fractionations of extract of well-studied plant: *Echinacea purpurea*. *Echinacea* extracts from three different species of this flower (*E. pallida, E. augustifolia and E. purpurea*) are used commonly for treatment and prevention of upper respiratory tract infections and preparations are made from both roots and aerial parts. Main bioactive metabolites of *Echinacea* are polysaccharides, caffeic acid derivatives and alkamides (Spelman, K., et al., 2009, Starvaggi Cucuzza, L., et al., 2008, Christensen, K. B., et al., 2009, Goey, A. K. L., et al., 2012). In our study, isolated and purified compound responsible for insulin sensitizing effect in 3T3-L1 adipocytes belongs to the group of alkamides, therefore one of the major bioactive metabolites in *Echinacea*. However, this compound (dodeca-2E,4E,8Z,10E/Z tetraenoic acid-2-methylbutylamides), according to our knowledge, was never purified before from *Echinacea* and it’s structure was never published and therefore it is a novel compound working as insulin sensitizer, inductor of adipogenesis and partial agonist of PPARγ (Fig.4)

![Fig.4. Chemical structure of falcarinol and falcarindiol (polyacetylenes, isolated from carrot) and dodeca-2E,4E,8Z,10E/Z tetraenoic acid-2-methylbutylamides (alkamide, isolated from *Echinacea*)](image-url)
Isolated plant compounds, can be used in their naïve form or can be further modified and introduced modifications can modulate their potency. In our study, we have used formononetin (4'-O-methylidaidzein, isoflavon found in red clover) main structure and modified it by adding 2-bromophenyl or 2-heptyl groups to the formononetin scaffold. 2-(2-bromophenyl)-formononetin (2BrPhF) and 2-heptyl formononetin (C7F) decreased lipid accumulation in 3T3-L1 adipocytes and were acting as partial agonists of PPARγ, while formononetin in its native form increased triglyceride accumulation and did not bind to PPARγ. C7F, but not 2BrPhF and formononetin, also increased glycerol release and the upregulation of lipolytic genes was observed when cells were treated with this compound, therefore suggesting, that small modifications can have big impact on compounds potency and molecular mechanisms (Fig.5) (PAPER III).

![Chemical structure of native structure of formononetin and its two derivatives: 2-(2-bromophenyl)-formononetin (2BrPhF) and 2-heptyl formononetin (C7F)](image)

**Fig.5.** Chemical structure of native structure of formononetin and its two derivatives: 2-(2-bromophenyl)-formononetin (2BrPhF) and 2-heptyl formononetin (C7F)

### 1.3 Adipocytes and 3T3-L1 model

Obesity is one of the most challenging and common health dilemma throughout the world and its complex etiology is an obstacle for development of the new treatment. The obese state is mainly caused by an imbalance between energy intake and expenditure, which results in accumulation of lipids and triglycerides in adipose tissue. Adipocytes are no longer believed to be “storage organ”; on the contrary, they play very important role in regulation of lipid homeostasis, glucose uptake and endocrine communication. Under the conditions of excess energy, adipose tissue increases the volume first due to hypertrophy and later due to hyperplasia. These fully functional adipocytes, together with osteoblasts, chondrocytes and myoblasts originate from mesenchymal stem cells (MSC). Due to differentiation process they become first committed preadipocytes, then growth arrested preadipocytes, which go through 2 or 3 cycles of mitotic clonal expansion and terminal differentiation to become lipid-laden and insulin-responsive mature adipocytes. The whole adipogenesis process is well organized and involves cascade of transcription factors such as peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding proteins (C/EBPs) and sterol
regulatory element binding protein (SREBP) family (Rosen, E. D., et al., 2000). PPARγ and C/EBPα represent early markers of adipogenesis and are essential for activation of downstream, adipocyte specific genes, such as: fatty acid binding protein 2 (aP2), glucose transporter type 4 (Glut4), leptin, insulin receptor (IR), fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) (Lehrke, M., et al., 2005). SREBP-1c enhances the expression of genes related to lipid and cholesterol metabolism (Hsu, J. M., et al., 2003).

Adipocytes, together with muscle and liver are responsible for regulation of glucose homeostasis and insulin sensitivity.

Insulin is the most potent anabolic hormone known. It promotes storage of triglycerides in adipocytes by multiple mechanisms, including increasing the differentiation of preadipocytes to mature adipocytes, synthesis of triglycerides inhibiting lipolysis and stimulating glucose uptake (Taniguchi, C. M., et al., 2006). In adipose tissue, two glucose transporters were identified: Glut4 and Glut1. While Glut4 is insulin-dependent glucose transporter and its movement from cell surface to within cell is stimulated via PI3K pathway, Glut1 is responsible for basal glucose uptake required for maintaining respiration in cells and its expression levels in cell membrane are increased by reduced glucose levels (Karnieli, E., et al., 2008). Simplified schematic of insulin signaling pathways is presented on Fig.6.

Most of the knowledge about signaling pathways and molecular mechanisms is obtained primarily from in vitro studies. In the subject of obesity and insulin sensitivity, most of the in vitro assays are performed in cell culture in 3T3-L1 cell line, which we also used in our experiments. 3T3-L1 cell line was established in late seventies and, developed through clonal expansion and immortalization, contains only one single cell type (Green, H., et al., 1974, Green, H., et al., 1976, Fernyhough, M. E., et al., 2005). However very widely used in laboratories all over the world, 3T3-L1 cells are not identical to primary cells or in vivo adipocytes. They follow different developmental patters and their gene expression, despite a lot of similarities, is not identical, therefore the results obtained from cell cultures experiments must be confirmed in in vivo studies (Poulos, S. P., et al., 2010).
1.4 New player: microbiota

Plant derived phytochemicals are reported to influence the development of adipose tissue and connected with it glucose intolerance, obesity and type 2 diabetes. As components of every-day diet they also have an impact on gut microbiota: an active organ within the host body.

The gut microbiota has been considered forgotten organ within the body and until recently, its role in nutrition and metabolism was neglected. Accumulating evidence indicates however that the gut microbiota is associated with the etiology or development of obesity and diabetes (Backhed, F., et al., 2004, Turnbaugh, P. J., et al., 2006). Diet and gut microbiota are strictly connected: gut bacteria help to digest otherwise indigestible food components such as plant polysaccharides or increase the capacity of energy harvest from a diet. Due to mutualistic host- bacteria relationship, harvested energy can be stored in adipocytes through pathway, that involves microbial regulation of the intestinal epithelial expression of fasting-induced adipocyte protein (Fiaf). Bacterial suppression of Fiaf results in reduced levels of circulating inhibitor of lipoprotein lipase (LPL) which in turn increases the LPL activity in adipocytes and
enhance storage of liver-delivered triglycerides in fat tissue (Ley, R. E., et al., 2005), therefore influencing the host energy balance.

Gut bacteria not only influence food processing, they are also influenced by the dietary intake. It has been shown, the high fat diet promotes changes in bacterial ecology within the gut, mostly by changing the ratio of different taxa. Also selective modulation of the structure or activity of intestinal microbiome by using prebiotics or probiotics has been demonstrated in both human and animal research to confer beneficial effects. Therefore, gut microbiota can be considered potential target of therapeutic drugs or nutritional interventions (Jia, W., et al., 2008, Zhao, L., et al., 2010).

Plant enrichment of diet had been shown to have beneficial effect in case of obesity and diabetes, and plant compounds, isolated from the dietary-plants had been shown to work via multiple pathways. On top of that findings, gut bacteria may also contribute to the health-promoting effects of phytochemicals by digesting them or by structural changes in the ecology of gut, promoted by the certain compound. Schematic interconnection between gut microbiota, diet, inflammation markers and body fat is shown on Fig.7.

![Diagram](image)

Fig.7. From Ravussin, Y., et al., 2012; modified. Schematic depicting possible interactions between diet composition, body fat, gut microbiota, circulating adipokines, mucin production, Fiaf and inflammatory markers.

One of the examples of combined action of gut bacteria and plant derived compound is berberine. As mentioned before, berberine was reported to reduce serum cholesterol, triglycerides and LDL-cholesterol levels in high fat diet fed animals and
inhibit differentiation of 3T3-L1 adipocytes. Further studies suggest, berberine also modulates gut microbiota by inhibiting wide range of intestinal bacteria, which alleviates systemic inflammation and enriching short chain fatty acid (SCFA) producers and therefore increasing SCFA levels in the intestine, which in turn contributes to the beneficial effects of berberine against insulin resistance and obesity (Zhang, X. et al., 2012).

Also resveratrol was shown to interfere with bacterial ecology in mice fed with high fat diet. As described previously: rodents fed with resveratrol supplementation in the high fat diet had significantly reduced body fat depots, what was confirmed in our own study, too. Gut bacteria seem to contribute in heath promoting effect of resveratrol, by converting it into dihydroresveratrol, 3,4'-dihydroxy-trans-stilbene and lunarin, which are absorbed from the gut (Bode, L. M., et al., 2013). From our study we can conclude, resveratrol supplementation shifts the physiology of high fat diet fed animals towards mice on standard chow diet and this study stays in agreement with Baur, J. A., et al., research (Baur J. A., et al., 2006). A the same time, we were not able to observe any significant changes in bacterial composition in the gut of mice provided with high fat diet and resveratrol, which may indicate, that resveratrol does not interfere with ecology of the intestine (PAPER IV).

2. Discussion of the papers

Maintaining a proper balance between energy intake and energy expenditure and therefore between storage of lipids and their mobilization is of great importance. When this balance is disturbed and energy intake exceed energy expenditure, obesity and related with obesity disorders are occurring. Change of diet and lifestyle is the first line of prevention and treatment but not always successful, therefore drugs and diet supplements must be introduced. However, there is still no ideal anti-obesity drug available and search for novel, bioactive compounds is continued.

Also, for obesity related disorders, such as T2D, no perfect drug exists and much research is concentrating on finding new compounds, which can work as insulin sensitizers and not cause the patients gaining severe weight.

Plant extracts seem to be ideal for searching for novel anti-obesity and anti-diabetic compounds. Plants have been used for this purposes for hundreds of years in folk medicine, therefore there is a strong argument that plant kingdom may hold a cure for these two disorders. However, careful and thorough research is needed, before any conclusions can be draw.

The work presented here revolved around the topic of plants being a source of bioactive compounds exploiting promising features for activation of nuclear receptor PPARγ, increasing glucose uptake and insulin sensitivity, promotion of adipogenesis and lipolysis in in vitro assays and changing gut bacterial composition in mice. First paper (PAPER I) presented here concentrated on PPARγ activation by metabolites of sage, native to the Mediterranean region herb, while PAPER V concentrates on novel
compound isolated from *Echinacea purpurea* and it’s insulin sensitizing effect in 3T3-L1 adipocytes. Second paper (PAPER II) takes the advantage of the whole plant extracts and concentrates not only on PPARγ activation but also modulation of glucose uptake and lipid accumulation in adipocytes, myotubes and *C. elegans*.

Isolated plant compounds, can be used in their naïve form or can be further modified and introduced modifications can modulate their potency, and that was a main focus of third paper (PAPER III). Using formononetin (4′-O-methyl DAidzein, isoflavon found in red clover) as a scaffold, 2-(2-bromophenyl)-formononetin (2BRPHF) and 2-heptyl-formononetin (C7F) analogues were synthetized and tested together with native formononetin in 3T3-L1 cells. Finally, in fourth paper (PAPER IV) we investigate plant derived compound resveratrol and its influence on mouse physiology and ecology of gut microbiota in mice.

**PAPER I**


Nuclear receptors are a class of proteins with the ability to bind directly to DNA and regulate expression of specific genes, when specific ligand is present and therefore, they constitute potential therapeutic targets for many disorders.

In this paper we focused on PPARγ (peroxisome proliferator-activated receptor gamma), a nuclear receptor belonging to the superfamily of ligand-dependent transcription factors. PPARγ is predominantly present in adipose tissue and has been shown to be a master regulator of adipocyte differentiation as well as glucose homeostasis. PPARγ, when activated by ligand, binds to DNA and promotes transcription of genes involved in lipid and glucose metabolism. Ligands for PPARγ include fatty acids and eicosanoids and despite much research, PPARγ endogenous ligand(s) is/are still unknown. Therefore, it is speculated that PPARγ regulates gene expression based on total concentration of fatty acids.

TZDs are synthetic full PPARγ agonists, binding to the receptor pocket with very high affinity. Glitazones were introduced as potent insulin-sensitizing drugs for the treatment of type 2 diabetes. However, TZDs may cause unwanted side effects, such as: weight gain, edema, heart enlargement and hepatotoxicity. Therefore, it was hypothesized, that PPARγ ligand, acting as partial (instead of full) agonist might not cause unwanted side effects while maintaining insulin-sensitizing activity.

In this paper we investigate the known traditional medicinal plant: *Salvia officinalis* L. (Lamiaceae). Sage is a widely used culinary and medicinal herb, especially in the Mediterranean region. Leaves are used as spices and preparations of sage leaves have been used traditionally as a remedy towards diabetes in Iran and Morocco. However, compounds responsible for anti-diabetic features of sage extracts are still unknown, despite several studies reporting that aqueous and ethanol extracts of *Salvia*
officinalis are decreasing blood glucose in laboratory animals (Alarcon- Aguilar, F. J., et al., 2002, Eidi, M., et al., 2005). As PPARγ plays significant role in regulation of insulin sensitivity, phytochemicals isolated from sage extract and activating PPARγ might, at least partially, explain the glucose lowering effect of *Salvia officinalis*.

In contrast to previous research, we prepared dichloromethane (DCM) extracts in place of aqueous or ethanol extracts. Solvent and extraction method are crucial parameters for chemical behavior of lipophilic and hydrophilic compounds. Choosing DCM as extraction solvent over ethanol or water and dissolving isolated compounds in DMSO we could shift our spectra of compounds towards lipophilic rather than hydrophilic phytochemicals. As a result of this choice, we were able to isolate and identify compounds different from those reported in previous studies (for example, viridiflorol, α-linoleic acid, oleanolic acid and manool). Also, 20-hydroxyferruginol was first time isolated from *Salvia officinalis* in this study and epiosmanol ester of 12-O-methyl carnosic acid was new. However whereas none of these new compounds activated PPARγ, we found two known metabolites able to significantly activate PPARγ in transfected cell cultures: α-linoleic acid and 12-O-methyl carnosic acid, which may suggest that the anti-diabetic activity of sage extract may, to some extend, be related to PPARγ activation pathway.

**PAPER II**

R. B. El-Houri, **D. Kotowska**, L. C. B. Olsen, S. Bhattacharya, L. P. Christensen, K. Grevsen, N. Oksbjerg, N. Færgeman, K. Kristiansen, K. B. Christensen, Screening for Bioactive Metabolites in Plant Extracts Modulating Glucose Uptake and Fat Accumulation, manuscript

Adipose tissue, systemic insulin resistance and skeletal muscle are closely related. Impaired insulin action and insulin resistance occur first in adipose tissue before the systemic glucose intolerance develops, therefore adipose tissue is recognized as the primary site of the type two diabetes origin (Hotamisligil, G. S., 2000). However, also skeletal muscle from obese patients exhibits insulin resistance in vivo and insulin resistance effect is not present when muscle cells obtained from obese subjects are cultured in vitro. Therefore, it was hypothesized, that negative crosstalk between excess of adipose tissue and skeletal muscle results in first, altered insulin signaling and later in insulin resistance in skeletal muscle. The communication between adipose tissue and muscle occurs through metabolic mediators, including free fatty acids and adipokines, such as TNFα, IL-6, leptin, TIMP-1 and adiponectin. Thus, insulin resistance in skeletal muscle is likely to result from a combination of the endocrine effect of adipokines on muscle cells and the metabolic effect of free fatty acids on both tissues (Sell, H., et al., 2006).

In the light of this research, the screening for novel insulin sensitizers could be developed based on, not only adipocyte cells, but also myocytes and therefore gives broader idea about their mechanisms of action.
In vitro studies are very useful but they never mimic the whole organism environment and therefore the results obtained from cell cultures must be confirm by in vivo studies. However, screening in search for novel insulin sensitizers requires fast and efficient way of testing new compounds in vivo. Caenorhabditis elegans is perfect model for this purpose: it is a multicellular organism, with simple but well organized anatomy and large (up to 78%) degree of homology to human genes and cell signaling pathways. Many studies, including investigation of therapeutic drugs and their possible side effects, development of neurons and lipid accumulation were performed in worms as confirmation of cell culture studies or novel, exploratory research.

In this study we concentrated on sixteen extracts from seven plants (spices or sources of food) exhibiting promising results in treatment of obesity and insulin insensitivity. Plant extracts in this study were tested in three different setups: in adipocytes, porcine muscle cells and C. elegans. This combined approach led us to two DCM extracts (golden root and thyme DCM extract), which weakly activated PPARγ and did not induce adipocyte differentiation in 3T3-L1 cells. Thyme significantly stimulated insulin dependent glucose uptake in both adipocyte cells and myotubes, while golden root significantly increased glucose uptake in myotubes but not adipocytes. However, both extracts did not increase fat accumulation in C. elegans. Therefore we speculated, that golden root and thyme DCM extracts might contain bioactive compounds, exhibiting anti-obesity and insulin-sensitizing activity. However, more detailed study will be needed to confirm our findings.

PAPER III
C. Andersen*, D. Kotowska*, C. G. Tortzen, K. Kristiansen, J. Nielsen, R. K. Petersen, 2-(2-bromophenyl)-formononetin and 2-heptyl-formononetin are PPARγ partial agonists and reduce lipid accumulation in 3T3-L1 adipocytes, manuscript
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Isoflavones are bioactive compounds occurring naturally, that have been reported to have beneficial health promoting effects, including anti-obesity and anti-diabetic effects.

Isoflavones are reported to be PPARγ agonists (Shen, P., et al., 2006, Chacko, B. K., et al., 2007, Mueller, M. M., et al., 2008) and several of them was shown to act as partial agonists and compete with full agonist (if present) for receptor occupancy and therefore produce a net decrease in PPARγ activation. Moreover, despite partial efficacy, they still retain anti-diabetic action. Isoflavones have been reported to also have other potencies like: osteogenic and antioxidant activity, modulation of enzymes in steroid biosynthesis (Barnes, S., 2010), and modulation of lipid metabolism.

Bioactive phytochemicals, like isoflavones, can serve as starting point for creation of new drugs or diet supplements. Small modifications introduced to, derived from natural source, scaffold can alter the biological effects of the naïve compound. One of the examples is an acetylation of salicylic acid derived from willow extracts. This
Modification increased pain-killing ability of the native compound and thus a new drug was created (today commonly known as aspirin) (Mahdi, J. G., et al., 2006).

Apigenin (4',5,7-trihydroxyflavone) belongs to flavone class is present in many plants, like chamomile, apples, celery, oregano, basil and parsley. Apigenin has been reported to exhibit anti-inflammatory, anti-tumor and anti-bacterial activity (Kim, H.P., et al., 1998, Basile, A., et al., 1999). However, compared to already existing drugs, apigenin does not exhibit strong enough activities. Introduced small modifications (aminomethyl groups substitutions on the C-8 position) significantly improved antiproliferative, antibacterial, and antioxidant activities compared with the parent apigenin (Liu R., et al., 2012).

![Chemical structures](image)

**Fig. 8.** Chemical structure of native apigenin (on the left) and formononetin (on the right)

Apigenin and formononetin share similar core structure. Moreover, small modifications of formononetin scaffold (made by substitutions of bromophenyl and heptyl groups at the 2-position), similarly to apigenin, significantly improved parent compound bioactive abilities. In our study, 2-(2-bromophenyl)-formononetin (2BrPhF) and 2-heptyl-formononetin (C7F) decreased lipid accumulation in 3T3-L1 adipocytes and were acting as PPARγ partial agonists, while parent formononetin did not bind to PPARγ and increased triglyceride accumulation. Analogue of formononetin with 2-heptyl substitution (C7F) significantly increased glycerol release and upregulated lipolytic genes in 3T3-L1 cells while 2BrPhF and parent formononetin did not exploit these features, therefore suggesting that substitution of different type of groups can have different impact on bioactive features.

**PAPER IV**

D. Kotowska*, J. Olesen*, M. Hansen, W. A. Al-Soud, R. S. Bienso, C. M. Kristensen, N. Brandt, S. A. B. Larsson, L. Hansen, K. Kristiansen, H. Pilegaard, Resveratrol, exercise and gut microbiota, manuscript

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Microbial research was dependent on culture-based methods for decades, which, however useful, were limited to cultivable microbes. Recent development of molecular techniques independent of culturing provided novel insight into complex
microbial ecology. Especially high-throughput sequencing technologies, like pyrosequencing allowed simultaneous assessments and in-depth analysis of microbial communities and emerging biological patterns within these communities.

Gut bacteria and their hosts co-evolved together and influence each other throughout the life of the host. Human intestine is sterile at birth and colonized immediately after, first with bacteria from the mother and later with bacteria present in the environment. Early colonization is very quick and seems to be chaotic but there are few patterns emerging in the recent research. For example: children born by caesarean section had been found to have higher risk of developing obesity compared to children born through vaginal canal and this tendency was associated with early colonization of gut by different groups of bacteria (Isolauri, E., 2012, Backhed, F., 2011). Throughout the life of the host, composition and complexity of gut bacteria increases until adulthood and can be modified by different dietary products.

Gut bacteria influence their host in many different ways. They have profound effects on host gene expression in the enterohepatic system, including genes involved in immunity, metabolism and inflammation. Gut bacteria, thanks to their unique sets of enzymes, can also digest components of the diet such as plant polysaccharides otherwise unavailable for the host organism. Intestinal microbiota ecology can be influenced by food components as well, for example: mice fed high fat diet have completely changed bacterial composition compared to chow fed animals. Moreover, this change is reversible by modulations of the diet. (Turnbaugh, P. J., et al., 2008).

In this paper we focused on changes in gut bacteria ecology in mice fed HFD with addition of resveratrol (polyphenolic compound found in wine, pistachios, grapes and berries) and trained- exercising. Resveratrol is well-studied compound and its anti-obesity properties have been described (Baur, J. A., et al., 2006). Also, the contribution of bacteria in digestion of resveratrol was reported by Bode, L. M. in 2013. However, little is known about the impact of resveratrol on the gut microbiota and therefore it is difficult to correlate observed physiological shifts of mice fed HFD with supplementation of resveratrol towards physiology of lean mice fed standard chow diet. However, in agreement with Baur, J. A. (Baur, J. A., et al., 2006), we did not observe any significant changes in intestinal microbiota composition and therefore we speculated, that resveratrol does not interfere with bacterial ecology of the intestine in mice.

Not only diet, but also physical activity is a key environmental contributor that should be taken under consideration when trying to understand the link between gut microbiota and obesity (Kallus, S. J., et al., 2012). Exercise training has been shown to promote weight loss even when animals were fed HFD (Bradley, R. L., et al., 2007; Yan, L., et al., 2012). However, role of gut microbiota in interconnecting health beneficial effect of exercise and host physiology is not well elucidated.

In our study, mice fed HFD and trained- exercise did not lose weight compared to sedentary, HFD fed animals. Therefore, our study stays in contradiction to Bradley, R. L. (Bradley, R. L., et al., 2007) research. However, to our surprise, there was a visible and clear shift in ecology of trained- exercised animals’ gut bacteria and this shift was towards bacteria of chow fed mice. Therefore we concluded, that diet and physical activity determined the composition of murine microbiome independently of obese
state and the changes may be observed on the level of genus and class. However, exact link between changes in bacterial flora and exercise training cannot be established based on the present findings and future work is required to elucidate this.

**PAPER V**

D. Kotowska*, R. B. El-Houri*, K. B. Christensen, X. Frette, K. Grevsen, L. P. Christensen, K. Kristiansen, Novel PPARγ partial agonist isolated from *Echinacea purpurea* exploits insulin sensitizing effect in 3T3-L1 adipocytes, manuscript

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In search for novel, bioactive phytochemicals, extracts of plants commonly used in folk medicine are examined. However, whereas screening for major phytochemicals is useful and fast, the major phytochemicals are not always the ones responsible for the beneficial effects of extract. Bioassay guided fractionations- based screening platforms have an advantage of focusing on active fractions and compounds, which are not necessarily overrepresented in crude extracts. Moreover, synergistic effects of two or more compounds may occur and will be missed if simple screening for major compounds is performed.

A bioassay guided fractionation-based platform was developed for a study, researching adipogenic constituents from the bark of *Larix laricina* du Roi and in the study researching the anti-adipogenic activities of two extracts isolated from *Alnus incana* and *Populus balsamifera* barks (Shang, N., et al., 2012, Martineau, L. C., et al., 2010). Use of this platform, when crude extract, its fractions and purified from them compounds were tested alongside in different assays, led to discovery of new cycloartane triterpentoid (23-oxo-3alpha-hydroxycycloart-24-en-26-oid acid) among the other 9 compounds. Interestingly, 23-oxo-3alpha-hydroxycycloart-24-en-26-oid acid was not the main component of the extract and still seemed to be responsible for adipogenic activity of the fraction it was isolated from (Shang, N., et al., 2012).

Bioassay screening platform, which served as a base, for the platform used in our study, was used by Christensen, K. B., et al., (Christensen, K. B., et al., 2009) to screen plant extracts for their anti-diabetic and anti-obesity properties. Among others, *Echinacea purpurea* DCM extract was found to activate PPARγ and increase insulin dependent glucose uptake while not promoting adipogenesis in Christensen’s study, which stays in agreement with the study presented in this paper.

*Echinacea* extracts from three different species of this plant (*E. pallida*, *E. augustifolia* and *E. purpurea*) are used commonly for treatment and prevention of upper respiratory track infections and preparations are made from both roots and aerial parts. Main bioactive metabolites of *Echinacea* are polysaccharides, caffeic acid derivatives and alkalamides (Spelman, K., et al., 2009, Starvaggi Cucuzza, L., et al., 2008, Christensen, K. B., et al., 2009, Goey, A. K. L., et al., 2012). However, little is known about *Echinacea* and *Echinacea* derived compounds in treatment of obesity and diabetes. In this study, using bioassay guided fractionations, we decided to investigate an effect of *Echinacea*
*purpurea* DCM extract, fractions and isolated compound on adipocyte differentiation and glucose uptake in 3T3-L1 adipocytes and try to reveal mechanism of action of purified compound. Our approach led us to the discovery of new compound, belonging to the group of alkamides: dodeca-2E,4E,8Z,10E/Z tetraenoic acid-2-methylbutylamides (Compound X). Compound X significantly increased basal and insulin dependent glucose uptake in 3T3-L1 adipocytes in dose dependent manner and promoted adipocyte differentiation in maturing adipocytes, acting as partial PPARγ agonist. Therefore, we speculated, that Compound X exploits anti-diabetic and insulin sensitizing effect *in vitro* and future study should follow into testing *in vivo* in laboratory animals.

**OTHER PAPERS**
2.1 Concluding remarks

The treatment of obesity and related disorders usually begins with dietary and behavioral modification with the aim of reducing caloric intake and increase energy expenditure. However, in the light of research presented in this thesis, diet (especially Mediterranean type, rich in fruits and vegetables) can not only be used as a way to reduce energy intake, but also as a source of health promoting compounds. Bioactive compounds from dietary products may influence adipocytes through several mechanisms, including: suppression of adipocyte differentiation and proliferation or induction of apoptosis in mature adipocytes, inhibition/induction of fat absorption/release of triglycerides or induction/suppression of glucose uptake and therefore facilitate weight loss or gain.

However well the idea of therapeutic food sounds, it is not easy to achieve. Food derived compounds are first digested by host organism, before being processed by bacteria living in the guts. Microbiota, with their unique sets of enzymes, are able to cleavage the chemical bonds, animals cannot cut and make available for host organism complex phytochemicals, which otherwise cannot be absorbed to bloodstream. Bacterial actions also introduce changes to plant derived compounds structures, which can increase health-promoting features of compounds if only by making them available for the host.

Dietary intake and bacteria are interacting with each other: bacteria are able to modify delivered compounds and compounds are able to influence microbial ecology, mostly by promoting growth of selected taxa over the others and therefore modulate the quality (and amounts) of energy intake.

The net of processes and interplay between phytochemicals, bacteria and host is very complex and still not fully understood. According to today’s knowledge it is clear, that compounds mechanism of action and their fate within the body are as important as identification of novel phytochemicals. The work presented in this thesis focuses on initial steps of compound identification and elucidate probable molecular mechanisms underlying the beneficial effects of investigated plants and therefore tries to give a new meaning to common adage: “You are what you eat”.

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Annex