Journal of Animal and Feed Sciences, Vol. 22, No. 1, 2013, 63–69
The Kielanowski Institute of Animal Physiology and Nutrition PAS Jabłonna

The influenc of hydrolyzed and non-hydrolyzed linden inflorescence (*Tilia cordata*) extract on metabolic and transcriptomic profile in rat liver

J. Wilczak[1], M. Łój, A. Prostek, D. Kamola, H. Kosińska, and M. Jank

Warsaw University of Life Sciences-SGGW, Faculty of Veterinary Medicine, Nowoursynowska 159, 02-776 Warsaw

KEY WORDS: flavonoids, quercetin, *Tilia cordata*, transcriptomic, antioxidant, liver, rats

ABSTRACT. The extract from linden inflorescence is one of the pharmacognostic resources which properties are associated with the presence of flavonoids (mainly: quercetin, kaempferol, acacetin glycosides and tiliroside). Flavonoids belong to polyphenols that may play a significant role in the dietoprophylaxis of civilization diseases. These compounds have many different mechanisms of action, but most important seem to be their antioxidant properties. The aim of the present study was to investigate the influence of linden extract on a) gene expression in rat liver and determine the difference in gene expression depending on the use of hydrolyzed or non-hydrolyzed linden extract, and b) antioxidant parameters of liver tissue. Rats were fed the diet containing hydrolyzed and non-hydrolyzed extract from linden inflorescence (*Tilia cordata*). The administration of hydrolyzed extract increased more than two-fold the level of quercetin in rats liver when compared to non-hydrolyzed extract. The transcriptomic study performed using microarray technology revealed 344 probes regulated by linden extract and 187 probes differentiating the action of hydrolyzed from nonhydrolyzed linden extracts. The most important molecular functions of regulated genes were as follows: defense and immunity, transporter, receptor, ion channel, oxidoreductase, cytoskeletal protein and cell adhesion molecule. Among most important biological processes identified were immunity and defense, transport, homeostasis and lipid, fatty acid and steroid metabolism. The analysis of oxidative status in rats liver together with the analysis of liver transcriptomic profile suggest that the antioxidant activity of hydrolyzed linden extract is higher that the non-hydrolyzed and occurs on the level of gene expression.

Received 5 October 2012
Revised 4 March 2013
Accepted 20 March 2013

[1] Corresponding author:
e-mail: jacek_wilczak@sggw.pl

Introduction

Extract from linden inflorescence (*Tilia cordata*) is one of the pharmacognostic resources which properties are associated with the presence of flavonoids. The main flavonoids present in linden flower are quercetin and kaempherol (Kohlmunzer 1933; Sadowska, 2003). Flavonoids belong to polyphenols which may play a significant role in the dietoprophylaxis of civilization diseases. These compounds have many different mechanisms of action, but most important seem to be their antioxidant properties (Hodek et al., 2002).

Flavonoon form which have lower molecular weight and hydrophobic properties. The aglycones of flavonoids can be transported across the

membranes of enterocytes viaapassive transport.

This is why hydrolysis of water linden extract results in better bioavailability of flavonoids when they are given orally (Aherne and O'Brien, 2002). Quercetin is a powerful antioxidant that is widely distributed in edible plants, mainly as glycosides such as rutin. The results of studies shown that rutin is absorbed more slowly than quercetin because it must be hydrolyzed by the cecal microflora, whereas quercetin is absorbed from the small intestine (Crespy et al., 2002; Manach et al., 1997). Dihal et al. (2006) revealed that quercetin, but not rutin inhibits azoxymethane-induced colorectal carcinogenesis in F344 rats. The aim of the present study was to investigate if rats which diets were enriched with hydrolyzed and non-hydrolyzed linden extracts have different blood parameters, liver transcriptomic profiles and some parameters of liver tissue antioxidant status.

Material and methods

Animals

Spraque-Dawley male rats at the age of 8 weeks were divided into three groups (number of rats in each group was 10). One was control group receiving standard diet (Labofeed D, Poland) (Table 1) whereas two experimental groups were receiving the diet enriched with 1% nonhydrolyzed water extract from linden inflorescence or 1% water extract from linden inflorescence hydrolyzed using HCl. Water and feed were provided *ad libitum*.

Table 1. The composition of standard diet fed for 6 weeks to 8-week-old rats. The diets were enriched with 1% nonhydrolyzed or hydrolyzed water extract from linden inflorescence, depending on the group

Ingredients	Content, %
Protein	22.5
Lysine	1.2
Crude fat	5
Crude fibre	4.5
Crude ash	6.5
Calcium	0.9
Phosphorus	0.7
Vitamin A	15.000 IE · kg^{-1}
Vitamin D_3	600 IE · kg^{-1}
Vitamin E	75 mg · kg^{-1}
Copper	5 mg · kg^{-1}

The study lasted 6 weeks. After this period of time animals were euthanized with isoflurane inhalation, then livers were collected during autopsy.

Microarrays

Liver samples were frozen with liquid nitrogen immediately after collection and stored at –80°C until extraction. Total RNA was extracted with Total RNA kit (A&A BIOTECHNOLOGY, Poland) following the manufacturer's recommended protocol. Subsequently, potential genomic DNA contamination was eliminated using Deoxyribonuclease I Amplification Grade (Sigma, USA) and RNeasyMinElute Cleanup Kit (Qiagen, Germany). RNA quantity and quality was measured using NanoDrop ND1000 (NanoDrop Technologies, USA) and Bioanalyzer (Agilent Technologies, USA).

Afterwards, samples were pooled as follows: 1) combined RNA extracted from 5 rats which received diet enriched with non-hydrolyzed *Tilia cordata* extract, and 2) combined RNA extracted from 5 rats which received diet enriched with hydrolyzed *Tilia cordata* extract.

Preparation of cDNA was carried out using SuperScript Plus Indirect cDNA Labeling Kit (Invitrogen, USA). cDNA was labeled with Alexa555 and Alexa647. Every pool was labeled 6 times (3 times with Alexa555 and with Alexa 647 – dye swap quality control). Samples were analyzed on cDNA microarrays Array-Ready Oligo Set™ for the Rat Genome Version 3.0 (Operon, Germany). Hybridization was carried with an automated hybridization system HybArray12 (PerkinElmer, USA). Microarrays were scanned using DNA ScanArray HT (PerkinElmer, USA).

A statistical analysis was performed using linear model that was described by Smyth (2004). Student's t-test was used to find genes with altered expression between research groups.

The antioxidant parameters

The quercetin concentration in liver homogenates we assayed using HPLC method with electrochemical detection. To express liver tissue antioxidant status the activity of superoxide dismutase (SOD) and total antioxidant status (TAS) were measured using Randox test. To estimate the ability of lipids in hepatocytes cell membranes to oxidation the sum of tiobarbituric acid reactive substances (TBARS) were measured, based on the following method: a final reaction mix containing liver tissue homogenates, 0.8% SDS, 10% acetic acid, and 0.17% thiobarbituric acid were incubated for 1 h at 100°C, centrifuged, and then absorbance values were measured at 530 nm. Values were referred to a calibration curve of 1,1,3,3-tetraethoxypropane.

Statistical evaluation

The results were statistically evaluated using GraphPadPrism™ version 5.00 software (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between two groups were evaluated using Student's t-test. A probability: $P \leq 0.05$ was regarded as significant.

Results

The transcriptomic profile of livers from rats receiving hydrolyzed and non-hydrolyzed linden extract

The statistical analysis revealed 532 differentially regulated genes ($P < 0.05$) in the livers of rats receiving hydrolyzed and non-hydrolyzed linden extract groups. Among them there were 43 genes regulated at the highest level of significance ($P < 0.01$) (data not shown).

The analysis of regulated genes ontology and function conducted using Pathway Studio Software showed that among differentially expressed genes there were 27 ones regulating the generation of reactive oxygen species (ROS) (Table 2). This analysis revealed also that the regulated genes differentiating the action of hydrolyzed and non-hydrolyzed linden extract groups regulate the expression of phase II detoxifying enzymes such as NQO1 (Figure 1) and HO^{-1} (Figure 2).

Pathway Studio software allows also the identification the factors (or proteins) which are responsible for the regulation of identified genes expression (so called expression targets). In our study the following proteins were identified as factors which regulate the expression of highest numbers (over 10) of genes considered as regulated ones: prostaglandin-endoperoxide synthase 2 (*PTGS2*), vascular endothelial growth factor A (*VEGFA*), tumor necrosis factor (*TNF* superfamily, member 2) (*TNF*), interleukin 6 (interferon, beta 2) (*Il6*), cyclin D1 (*CCND1*), nitric oxide synthase 2, inducible (*NOS2*), interleukin 8 (*Il8*), matrix metallopeptidase 9 (*MMP9*), interleukin 2 (*Il2*), chemokine (C-C motif) ligand 2 (*CCL2*), matrix metallopeptidase 1 (*MMP1*), B-cell CLL/lymphoma 2 (*BCL2*), *BCL2*-associated X protein (*BAX*),

Table 2. The list of differentially expressed genes involved in reactive oxygen species generation in liver of rats fed with the diets enriched with 1% hydrolyzed or 1% non-hydrolyzed linden extract for 6 weeks weeks

Gene symbol	RefSeq	Description	logFC	P–value
PTK2	NM_013081.1	PTK2 protein tyrosine kinase 2	–0.514	0.028
PRL	NM_012629.1	Prolactin	–0.659	0.015
MYC	NM_012603.2	v-myc myelocytomatosis viral oncogene homolog (avian)	–0.57	0.029
MAP3K5	XM_218780.2	Mitogen-activated protein kinase kinase kinase 5	0.56	0.023
EDNRA	NM_012550.1	Endothelin receptor type A	0.654	0.014
CCL20	NM_019233.1	Chemokine (C-C motif) ligand 20	–0.623	0.017
SERPINE1	NM_012620.1	Serpin peptidase inhibitor. clade E (plasminogen activator inhibitor type 1)	–0.713	0.029
PAK1	NM_017198.1	p21 protein (Cdc42/Rac)-activated kinase 1	–0.412	0.027
MTOR	NM_019906.1	Mechanistic target of rapamycin (serine/threonine kinase)	–0.762	0.011
ATM	XM_236275.3	Ataxia telangiectasia mutated	–0.409	0.048
TRPV1	NM_031982.1	Transient receptor potential cation channe, subfamily V, member 1	–0.471	0.034
CXCL3	NM_138522.1	Chemokine (C-X-C motif) ligand 3	–0.448	0.031
SYK	NM_012758.1	Spleen tyrosine kinase	0.488	0.044
SEMA3A	NM_017310.1	serpin peptidase inhibitor, clade E (nexin. plasminogen activator inhibitor type 1), member 1	0.494	0.037
NR3C2	NM_013131.1	Suclear receptor subfamily 3, group C, member 2	–0.546	0.047
TRIB3	NM_144755.1	Tribbles homolog 3 (Drosophila)	–0.471	0.041
CP	NM_001270961.1	Ceruloplasmin (ferroxidase)	0.541	0.033
CKMT1B	NM_001012738.1	Creatine kinase, mitochondrial 1B	–0.605	0.016
ADA	NM_130399.2	Adenosine deaminase	–0.659	0.049
HP	NM_012582.2	Haptoglobin	0.519	0.043
MAP3K7	XM_232855.3	Mitogen-activated protein kinase kinase kinase 7	–0.543	0.048
FDX1	NM_017126.1	Ferredoxin 1	–0.541	0.032
GGPS1	NM_001007626.1	Geranylgeranyl diphosphate synthase 1	–0.465	0.026
HSP90AB1	NM_001004082.2	Heat shock protein 90kDa alpha (cytosolic), class B member 1	0.564	0.028
ACP1	NM_021262.2	Acid phosphatase 1. soluble	–0.679	0.041
CYP2A13	NM_012692.1	Cytochrome P450, family 2, subfamily A, polypeptide 13	–0.695	0.036
CTRL	NM_054009.1	Chymotrypsin-like	–0.807	0.024

FBJ murine osteosarcoma viral oncogene homolog (*FOS*), interferon, gamma (*IFNG*), tumor protein *p53* (*TP53*), cyclin-dependent kinase inhibitor 1B (*p27*, *Kip1*) (*CDKN1B*), interleukin 10 (*Il10*), bone gamma-carboxyglutamate (gla) protein (*BGLAP*).

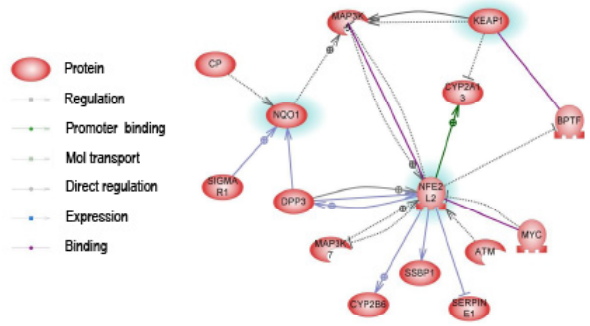

Figure 1. The relations between Nrf2 (NFE2L2), Keap1, NQO1 proteins and proteins encoded by genes differentially regulated in livers of rats fed for 6 weeks with the diets enriched with 1% hydrolyzed or 1% non-hyrolyzed linden extracts

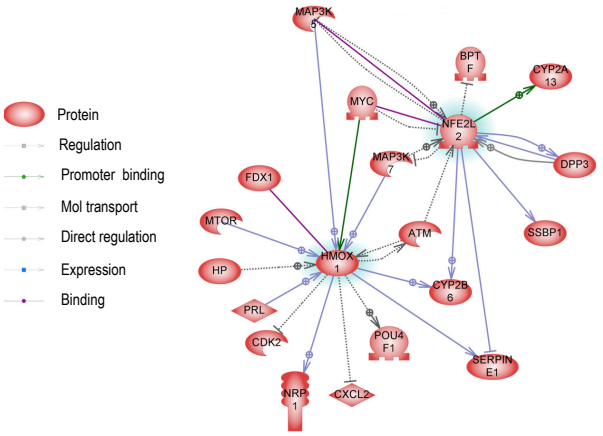

Figure 2. The relations between Nrf2 (NFE2L2), HO-1 proteins and proteins encoded by genes differentially regulated in livers of rats fed for 6 weeks with the diets enriched with 1% hydrolyzed or 1% non-hydrolyzed linden extracts

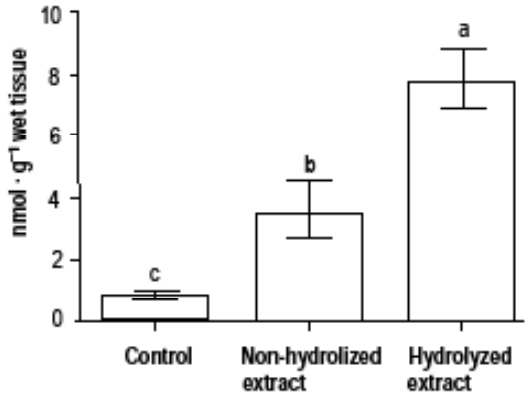

Figure 3. Quercetin content in liver tissue of rats fed for 6 weeks with standard diet and diets enriched with 1% hydrolyzed or 1% non-hydrolyzed linden extracts; bars with different letters represents the level of significant P≤0.05

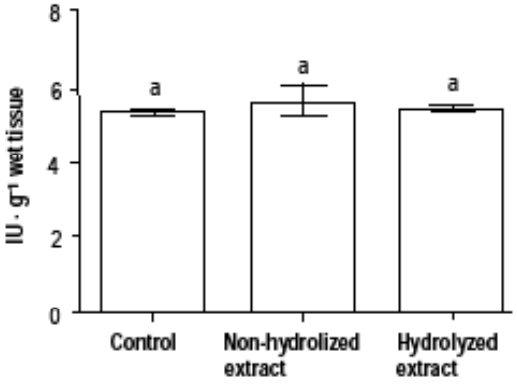

Figure 4. Activity of superoxide dismutase (SOD) in liver tissue of rats fed for 6 weeks with standard diet and diets enriched with 1% hydrolyzed or 1% non-hydrolyzed linden extracts; bars with different letters represents the level of significant P ≤ 0.05

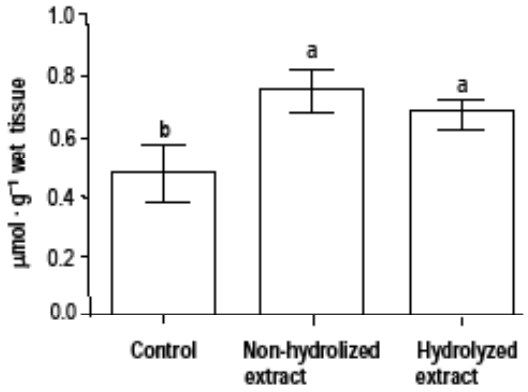

Figure 5. Level of total antioxidant status (TAS) in liver tissue of rats fed for 6 weeks with standard diet and diets enriched with 1% hydrolyzed or 1% non-hydrolyzed linden extracts; bars with different letters represents the level of significant P ≤ 0.05

Antioxidant action of hydrolyzed and non-hydrolyzed linden extract

The 6 weeks of the rat diet supplementation with hydrolyzed and non-hydrolyzed linden extract resulted in the highest concentration of quercetin in the liver of rats receiving the hydrolyzed one ($P < 0,05$). We were also able to identify some traces of quercetin in the liver of control rats which could be a result of its presence in the ingredients used for rat feed production, mainly grains. The acid hydrolysis of linden inflorescence did not influence significantly the activity of superoxide dismutase (SOD). The Total Antioxidant Status (TAS) describes the total antioxidant properties of the tissue (in this case liver) and it was increased by the two investigated extracts, when compared to control rats. The TBARS value is a parameter describing the level of the fatty acid oxidation and it was the highest in the control, non-supplemented group. The lowest TBARS were observed in the group of rats receiving hydrolyzed linden extract (Figure 6).

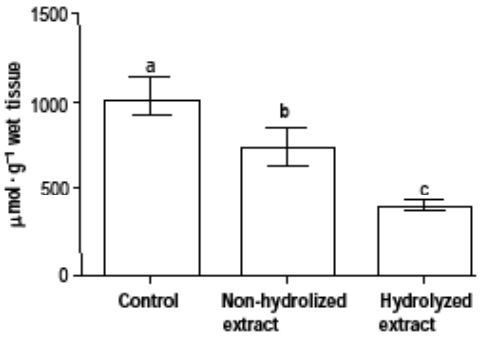

Figure 6. Level of the sum of tiobarbituric acid reactive substances (TBARS) in liver tissue of rats fed for 6 weeks with standard diet and diets enriched with 1% hydrolyzed or 1% non-hydrolyzed linden extracts; bars with different letters represents the level of significant $P \leq 0.05$

Discussion

The results of the present study revealed that genes which were differentially regulated in livers of rats receiving the diet with hydrolyzed or non-hydrolyzed water linden flower extracts are related with generation of reactive oxygen species (ROS) (Table 2). One of the main components of this extract is quercetin that occurs in plants mainly as a glycoside – rutoside. Zerina et al. (2012) have suggested that quercetin may be used to minimize oxidative stress via reducing the generation of ROS. This effect was due to modulating expression of antioxidant genes in A549 cells. Kaempferol is another component of linden flower which also has strong antioxidant properties. Hong et al. (2009) shown that pretreatment of PC12 cells with kaempferol reduced intracellular generation of reactive oxygen species (ROS) in response to H_2O_2 and strongly elevated cell viability.

The use of advanced gene ontology analysis tools (Pathway studio) gives the unique possibility of identification of factors (proteins) which regulate the expression of genes identified transcriptomic studies. In the present study we were able to identify proteins for which our regulated genes are the expression targets. The analysis revealed that many our differentially regulated genes were expression targets of proteins involved in inflammation process. Among them were tumor necrosis factor (TNF superfamily, member 2) (TNF) and nitric oxide synthase 2 (NOS2). It is known that quercetin has anti-inflammatory properties through its ability to inhibit nitric oxide production and iNOS expression (Ortega et al., 2010). Also other expression targets such as Il6, Il8, Il2, IL10, TNF are important for the inflammation process. It is known that phase II detoxifying enzymes, such as NAD(P)H:quinone oxidoreductase (NQO1), glutathione S-transferase (GST), heme oxygenase (HO-1) and nitric oxide synthase (iNOS), contain specific nucleotide sequences – in particular, an antioxidant-responsive element/electrophile-responsive element (ARE/EpRE) – in their gene promoters that contribute to the protection of cells against carcinogens and oxidative stress (Chen and Kong, 2004). There are several molecules proposed as potential modulators of ARE/EpRE. Among them nuclear factor-E2-related factor 2 (Nrf2) has been repeatedly proven to be a strong activator of ARE-mediated gene expression. The study conducted by Weng et al. (2011) shown that quercetin could be beneficial for the prevention of environmental oxidant-induced liver damage. One of the proposed mechanisms of this action was enhancing Nrf2 DNA-binding activity. Another author revealed that quercetin activated Nrf2-ARE-mediated NQO1 transactivation not only through enhanced the steady-state level of Nrf2 at both transcriptional and posttranslational levels, but also reduced the steady-state level of Keap1 through 26S proteasome-independent degradation (Tanigawa et al., 2007). The Figure 1 presents relations between Nrf2 (NFE2L2), Keap1, NQO1 proteins and proteins encoded by genes regulated in our study. The experiments conducted by Granado-Serrano et al. (2012), Tanigawa et al. (2007) and Weng et al. (2011) were performed in human hepatoma cells (HepG2). Our study was performed *in vivo* and did not check the influence flavonoids in aglycone forms (like above mentioned) but was focused on the differences between flavonoids given in aglycone and glycoside forms. This may be the reason why our results did not show direct changes in expression of Nrf2 (NFE2L2), Keap1 and NQO1, but indicate only changed expression of genes regulated by these three proteins. In our study we were able to identify the 12 genes which were expression targets of NQO1 (Table 3). It may be that the regulation of some genes in our study results from the changed activity of Nrf2 (NFE2L2), Keap1 and NQO1 on protein level and not their changed expression on gene level.

The next important phase II detoxifying enzyme is heme oxygenase (HO-1). Growing data indicate that HO-1 plays a key role in defense mechanisms against oxidative damages (Nakahira et al., 2003). HO-1 is one of the target genes of transcription factor – Nrf2. Many studies show that changes in expression of HO-1 are related with action of flavonoids, often quercetin and kaempferol. Liu et al. (2012) have suggested that quercetin attenuates ethanol-induced oxidative stress through a pathway which HO-1 upregulation. The second most abundant active substance in linden inflorescence,

Table 3. The list of differentially regulated genes in liver of rats fed for 6 weeks with the diets enriched with 1% hydrolyzed or 1% non-hydrolyzed linden extracts being expression targets of NQO1

Gene symbol	RefSeq	Description	logFC	P–value
MAP3K5	XM_218780.2	Mitogen-activated protein kinase kinase kinase 5	0.56	0.023
MYC	NM_012603.2	v-myc myelocytomatosis viral oncogene homolog (avian)	–0.57	0.029
MAP3K7	XM_232855.3	Mitogen-activated protein kinase ki nase kinase 7	–0.543	0.048
ATM	XM_236275.3	Ataxia telangiectasia mutated	–0.409	0.048
CYP2B6	NM_198733.1	cytochrome P450. family 2, subfamily B, polypeptide 6	–0.448	0.048
SERPINE1	NM_012620.1	Serpin peptidase inhibitor, clade E (plasminogen activator inhibitor type 1)	–0.713	0.029
CP	NM_001270961.1	Ceruloplasmin (ferroxidase)	0.541	0.033
SIGMAR1	NM_030996.1	Sigma non-opioid intracellular receptor 1	–0.481	0.019
BPTF	XM_003750948.1	Bromodomain PHD finger transcription factor	0.385	0.05
CYP2A13	NM_012692.1	Cytochrome P450. family 2, subfamily A, polypeptide 13	–0.695	0.036
SSBP1	NM_183328.2	Single-stranded DNA binding protein 1	–0.488	0.019
DPP3	NM_053748.1	Dipeptidyl-peptidase 3	–0.414	0.048

Table 4. The list of differentially regulated genes in liver of rats fed for 6 weeks with the diets enriched with 1% hydrolyzed or 1% non-hydrolyzed linden extracts being expression targets of HO-1

Gene symbol	RefSeq	Description	logFC	P – value
PRL	NM_012629.1	Prolactin	–0.659	0.015
MYC	NM_012603.2	v-myc myelocytomatosis viral oncogene homolog (avian)	0.57	0.029
MAP3K5	XM_218780.2	Mitogen-activated protein kinase kinase kinase 5	0.56	0.023
CDK2	NM_199501.1	Cyclin-dependent kinase 2	–0.501	0.017
SERPINE1	NM_012620.1	Serpin peptidase inhibitor, clade E (plasminogen activator inhibitor type 1)	–0.713	0.029
MTOR	NM_019906.1	Mechanistic target of rapamycin (serine/threonine kinase)	–0.762	0.011
ATM	XM_236275.3	Ataxia telangiectasia mutated	–0.409	0.048
CXCL3	NM_138522.1	Chemokine (C-X-C motif) ligand 3	–0.448	0.031
NRP1	NM_145098.2	Neuropilin 1	–0.591	0.01
HP	NM_012582.2	Haptoglobin	0.519	0.043
MAP3K7	XM_232855.3	Mitogen-activated protein kinase kinase kinase 7	–0.543	0.048
FDX1	NM_017126.1	Ferredoxin 1	–0.541	0.032
BPTF	XM_003750948.1	Bromodomain PHD finger transcription factor	0.385	0.05
CYP2A13	NM_012692.1	Cytochrome P450, family 2, subfamily A, polypeptide 13	–0.695	0.036
POU4F1	XM_341372.2	POU class 4 homeobox 1	–0.362	0.044
SSBP1	NM_183328.2	Single-stranded DNA binding protein 1	–0.488	0.019
CYP2B6	NM_198733.1	Cytochrome P450, family 2, subfamily B, polypeptide 6	–0.448	0.048
DPP3	NM_053748.1	Dipeptidyl-peptidase 3	–0.414	0.048

namely kaempferol, inhibits nitric oxide production and nitric oxide synthase protein expression via HO-1-mediated ROS reduction in murine macrophages (Choi et al., 2012). Although in our study the administration of hydrolyzed and non-hydrolyzed linden extract did not influenced the HO-1 gene expression we were able to identify the 18 genes which were expression targets of HO-1 (Table 4). The Figure 2 presents genes differentially regulated in our study and their relations with HO-1, Nrf2 (NFE2L2) proteins.

The analysis of antioxidant parameters of liver tissue suggests that acid hydrolysis of water extract from linden inflorescence increases the accumulation of quercetin in livers of rats receiving the diet enriched in this extract. The level ofquercetin in this case is more than two-fold higher when compared to rats receiving the diet enriched with non-hydrolyzed extract. This proves the theory that flavonoids in aglycone form and not in glycoside form are preferentially absorbed in small intestine, although the it is glycosides which naturally occur in raw materials of plant origin (McAnlis et al., 1999; Zhao et al., 2011). The increased accumulation of quercetin in the livers of rats receiving hydrolyzed extract resulted in the increase of total antioxidant status of the liver and consequently the decreased fatty acids oxidation in the hepatocytes cell membranes. These results are in accordance with many other studies, which indicated the possibilities of the

use of biologically active substances of plant origin as a effective strategy for the prevention from the damaging activity of free radicals in intracellular fluids and in cell membranes (Matsukawa et al., 2009; Egert et al., 2008).

Also transcriptomic data clearly show that the non-hydrolyzed linden extract regulates the expression of genes involved in the prevention from free radical oxidative damage and the regeneration after oxidative damage.

Conclusions

The analysis of oxidative status in rats liver together with the analysis of liver transcriptomic profile suggest that the antioxidant activity of hydrolyzed linden extract is higher that the non-hydrolyzed and occurs on the level of gene expression. However, genes regulated by hydrolyzed linden extract are not only involved in antioxidant status of rat livers but also in immunity and defense, transport, homeostasis and lipid, fatty acid and steroid metabolism.

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