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Abbreviations

[³ H]DAMGO	[D-Ala ² -N-Me-Phe ⁴ -Gly ⁵ -ol]enkephalin
6'-OH-ODMA	6'-hydroxy- <i>O</i> -desmethylangolensin
6-PN	6-prenylnaringenin
8-PN	8-prenylnaringenin
AhR	aryl hydrocarbon receptor
ANPU	apparent net protein utilization
ALP	alkaline phosphatase
ARNT	aryl nuclear translocase
BMI	body mass index
CHD	coronary heart disease
COX	cyclooxygenase
CRP	C-reactive protein
CVD	cardiovascular disease
CYP	cytochrome P450
DA	dopamine
DHD	dihydrodaidzein
DMBA	dimethylbenz[a]anthracene
DPPH	2,2-diphenyl-1-picrylhydrazyl
DRE	dioxin-response element
EMSA	electrophoretic-mobility shift assay
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ERE	estrogen response element
EROD	ethoxyresorufin O-deethylase
FCR	food conversion ratio
FSH	follicle-stimulating hormone
HRS	hormone-related supplement
HRT	hormone replacement therapy
HUVEC	human umbilical vein endothelial cell
IGFBP	IGF binding protein
IGF-I	Insulin-like growth factor I

i.p.	intraperitoneal
IX	isoxanthohumol
LC	liquid chromatography
LH	luteinizing hormone
LPO	lipid peroxidation
LPS	lipopolysaccharide
MRP	multidrug resistance protein
MS	mass spectrometry
NMR	nuclear magnetic resonance
NO	nitric oxide
NOAEL	no observed adverse effect level
NOEL	no observed effect level
O-DMA	<i>O</i> -desmethylangolensin
ORAC	oxygen radical absorbance capacity
OVX	ovariectomized controls
PCNA	proliferating cell nuclear antigen
PER	protein efficiency ratio
PVP	polyvinylpyrrolidone
RC	red clover
ROS	reactive oxygen species
SFE	supercritical fluid extraction
SGR	specific growth rate
SHBG	sex hormone binding globulin
TBARS	thiobarbituric acid reacting substances
TC	total cholesterol
TCDD	2,3,7,8 tetrachlorodibenzo-p-dioxin
TG	triglyceride
TNF- α	tumor necrosis factor- α
TXB ₂	thromboxane B ₂
VCAM-1	vascular cell adhesion molecule 1
WEC	whole embryo culture
XN	xanthohumol

1. Introduction

Red clover (*Trifolium pratense* L.) has been traditionally used as a medical herb in the treatment of several chronic and acute diseases (Booth et al. 2005). The plant contains various classes of chemical compounds: Carbohydrates, sugar acids, coumarins, proteins, essential oils, minerals and lipids. Saponins are also known compounds of red clover. Furthermore, a variety of polyphenol derivatives, such as isoflavonoids, procyanidins (Meagher et al. 2006) or pterocarpanes are found in red clover (RC). Phytoestrogens, including isoflavones and coumestans, became increasingly popular in research and the management of women's health (Thompson Coon et al. 2007). Isoflavones from red clover act as ligands for the estrogen receptors α and β , although the estrogenic effect is much lower than the one of 17- β -estradiol (Beck et al. 2005). RC is a high quality forage plant that can be grazed or used for hay. In addition, due to the mild estrogenic activity of isoflavones, the herb has gained increasing attention for its potential health benefits in the prevention of certain cancers, cardiovascular diseases, the treatment of climacteric symptoms and osteoporosis in postmenopausal women (reviewed in Booth et al. 2005, Wuttke et al. 2007).

For an overview over the huge body of scientific literature, a thorough compilation of the chemical, pharmacological and toxicological data of RC during 2004-2007 has been performed in this thesis.

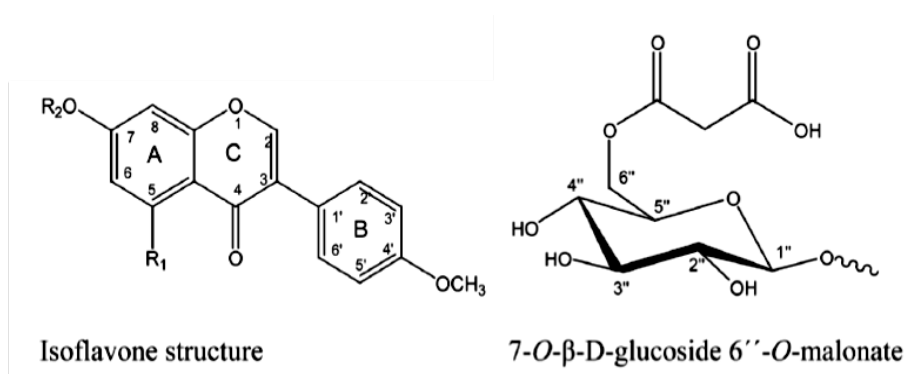
2. Chemistry

2.1. Chemical composition of red clover

The most important group of active compounds in the red clover is isoflavonoids. These substances are composed of a 3-phenyl-chromen-4-one system and therefore isomeric with the large group of flavones. Isoflavonoids can be further distinguished by the presence of various methoxy, hydroxyl, and/or glycoside substituents (Delmonte et al. 2006). Any differences in the chemical structure, such as an additional methoxy-group or the loss of a double bond, can affect the biological activity of the entire

molecule. The high structural diversity can be used for chemotaxonomic classifications of related species (Oleszek et al. 2007, Polasek et al. 2007). Red clover plants accumulate isoflavones usually as glycosylated and/or esterified glycosidic derivatives (Delmonte et al. 2006). 7-O- β -D-glycosides and especially the 6''-O-malonyl-7-O- β -D-glycoside derivatives appear to be the most prevalent derivatives.

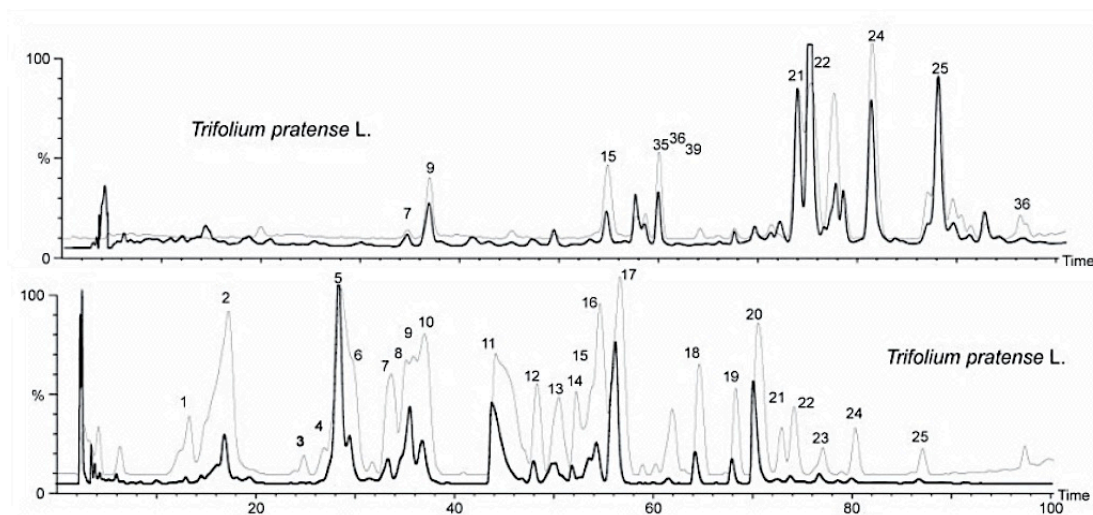
Nevertheless, isoflavones in red clover occur as aglycones, glycosides, malonyl-glycosides and acetyl-glycosides (Delmonte et al. 2006, Tsao et al. 2006). The most abundant isoflavone aglycones are formononetin and biochanin A, minor amounts occurring are daidzein, genistein, glycitein, pseudobaptigenin, calycosin, prunetin, irilone and pratensin (Heinonen et al. 2004, Klejdus et al. 2007). By use of reversed phase liquid chromatography (LC) coupled with mass spectrometry (MS) the profile of major isoflavones in RC was confirmed (**Fig.1, p.5**). The additional use of LC coupled with nuclear magnetic resonance (NMR) spectroscopy allowed the unambiguous structural identification of polyphenol isomers and their sugar conjugates, previously reported in *T. pratense* (de Rijke et al. 2004, Stürtz et al. 2006, Klejdus et al. 2007).



Name	R ₁	R ₂	R ₃	M _w
Daidzein	H	H	H	254
Daidzin	H	7- <i>O</i> -β-D-glc	H	416
Genistein	OH	H	H	270
Genistin	OH	7- <i>O</i> -β-D-glc	H	432
Formononetin	H	H	CH ₃	268
Ononin	H	7- <i>O</i> -β-D-glc	CH ₃	430
Formononetin-7- <i>O</i> -β-D-glucoside-6''- <i>O</i> -malonate	H	7- <i>O</i> -β-D-glc-6''- <i>O</i> -mal	CH ₃	516
Biochanin A	OH	H	CH ₃	284
Sissotrin	OH	glc	CH ₃	446
Biochanin A-7- <i>O</i> -β-D-glucoside-6''- <i>O</i> -malonate	OH	7- <i>O</i> -β-D-glc-6''- <i>O</i> -mal	CH ₃	532

Fig.1. Chemical structures of major isoflavone aglycones and their conjugated derivatives in red clover (modified from de Rijke et al. 2004).

In **Fig.2 (p.6)** a typical HPLC chromatogram (Polasek et al. 2007) of a broad spectrum of polyphenols after gradient elution is shown. In the dichloromethane extract primarily the presence of the most hydrophobic aglycones was demonstrated, whereas in the methanolic fraction the malonate glucosides were detected. More recently, micellar electrokinetic capillary chromatography was applied for the rapid determination of isoflavones (Zhang et al. 2007).



No.	R_t (min)	$[M + H]^+$ (m/z)	Fragment ions (m/z)	λ_{max} (nm)	Identification
1	13.1	360		290–322	<i>trans</i> -Clovamide
2	17.0	360		290–320	<i>cis</i> -Clovamide
3	27.7	463	301	260–290–340sh	Pratensein 7- <i>O</i> - β -D-glucoside
4	27.9	433	271	258–330sh	Genistin
5	28.5	465	303	256–354	Hyperoside
6	29.1	465	303	256–354	Isoquercitrin
7	33.4	445	283	250–290	Rothindin
8	34.7	449	287	264–290–344sh	Unknown isoflavonoid glycoside
9	35.6	431	269	250–305sh	Formononetin 7- <i>O</i> - β -D-glucoside
10	36.9	447	285	256–300sh	Glycitein 7- <i>O</i> - β -D-glucoside
11	43.9	551	303	256–354	Quercetin-3- <i>O</i> - β -D-glucoside 6''- <i>O</i> -malonate
12	48.1	461	299	270–325sh	Irlone 7- <i>O</i> - β -D-glucoside
13	50.3	535	287	261–290–335sh	Orobol 7- <i>O</i> - β -D-glucoside-6''- <i>O</i> -malonate
14	51.9	565	317	256–354	3-methylquercetin 7- <i>O</i> - β -D-glucoside-6''- <i>O</i> -malonate
15	53.4	447	285	260–320sh	Prunetin 4'- <i>O</i> - β -D-glucoside
16	54.4	533	283	256–285sh	Glycitein 7- <i>O</i> - β -D-glucoside 6''- <i>O</i> -malonate
17	56.4	517	269	260–310sh	Formononetin 7- <i>O</i> - β -D-glucoside 6''- <i>O</i> -malonate
18	64.3	547		270–305sh	Irlone 4'- <i>O</i> - β -D-glucoside-6''- <i>O</i> -malonate
19	68.0	533	285	260–325sh	Biochanin A 7- <i>O</i> - β -D-glucoside 6''- <i>O</i> -malonate
20	70.2	533	285	260–320sh	Unknown isomer of compound 19
21	72.6	283		250–292	Pseudobaptigenin
22	73.9	269		250–301sh	Formononetin
23	76.8	533	285	260–325sh	Prunetin 4'- <i>O</i> - β -D-glucoside 6''- <i>O</i> -malonate
24	80.1	299		270–325sh	Irlone
25	86.9	285		262–330sh	Prunetin
26	19.8	481	319	258–356	Unknown pentahydroxyflavonol glycoside
27	22.7	597	465/303	254–354	Quercetin-3-pentohexosyl
28	33.7	523	319	258–356	Unknown flavonoid glycoside acetate
29	62.0	287		266–285–344	Orobol
30	63.1	303		256–370	Quercetin
31	72.6	301		268–284–334	Pratensein
32	20.5	595	463	266–344	Unknown flavonoid-diglycoside
33	21.3	581	419	266–332	Unknown flavonoid diglycoside
34	38.4	449	287	266–336	Unknown flavonoid glycoside
35	59.3	473		260–300sh	Ononin 7-acetate
36	91.8	285		260–325sh	Biochanin A
37	72.5	301		284–330	3'-Methylorobol
38	88.5	283		250sh–300sh	Unknown methoxylated isoflavonoid
39	96.4	315		266–360	Unknown flavonoid
40	61.5	287		238–330	Unknown flavonoid
41	70.9	301		260–285–330	Unknown isoflavonoid (isomer 31)

Fig.2. HPLC/MS analysis of red clover extracts: (Upper panel) dichloromethane extract and (Lower panel) methanolic extract (modified from Polasek et al. 2007). HPLC-UV at 260 nm (—); HPLC-ESI/M total ion current (---)

In addition to a broad spectrum of isoflavonoids, also clovamides and other flavonoids were detected in RC (Polasek et al. 2007, **Fig.3**).

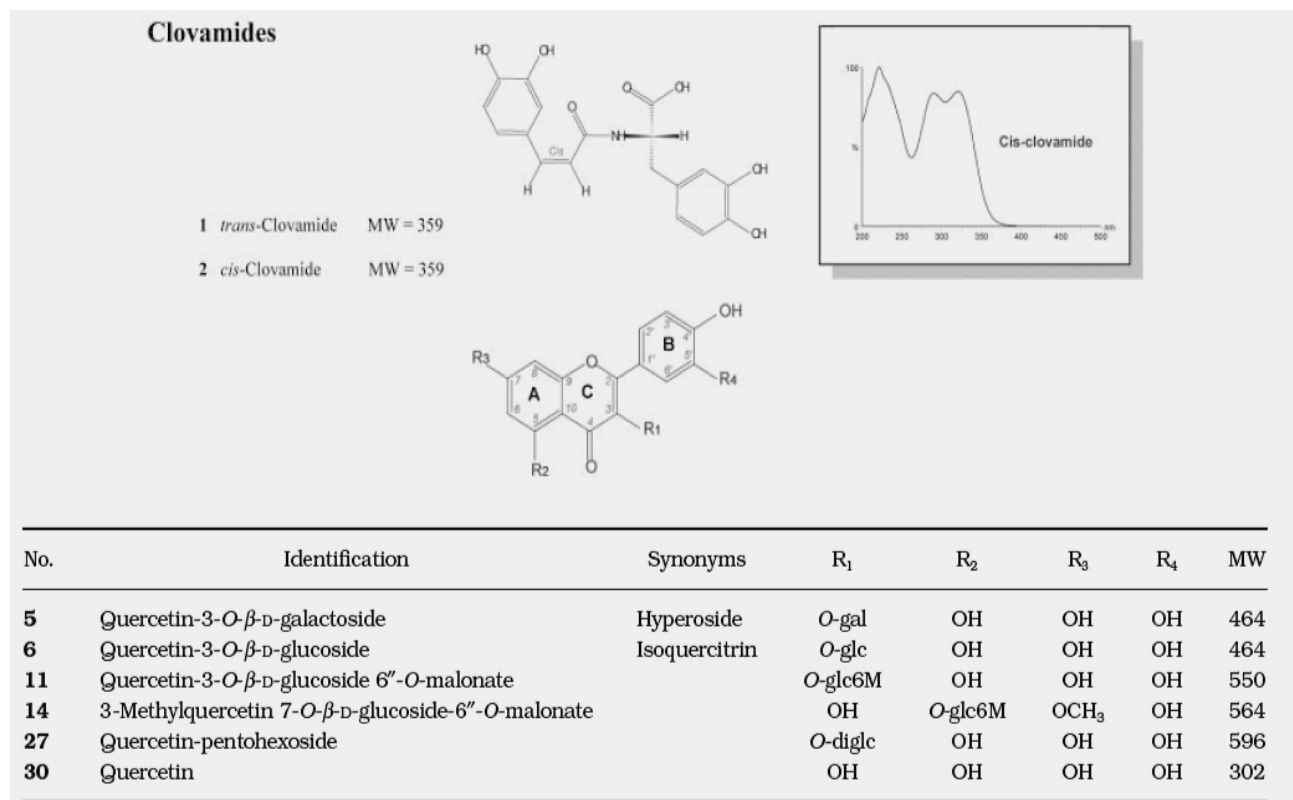


Fig.3. Structural overview of flavonoids, and clovamides detected in red clover species (adapted from Polasek et al. 2007).

Proanthocyanidins (condensed tannins) are present in flower heads of different clover species but missing in herbal parts. Floral procyanidins could contribute to the formation of polyphenols. The terminal parts consist mainly of catechin, whereas epicatechin serves as extension unit (Meagher et al. 2006).

Booth et al. (2006a) confirmed the pterocarpan maackianin in a raw red clover extract, which was used in a clinical trial. Pterocarpan belongs to the subgroup of the flavanols and are related to the isoflavones. Moreover, 11.1% flavonoids (quercetin, naringenin, kaempferol), <0.03% coumarins (scopoletin, fraxidin, xanthotoxol) as well as 0.03%

tyramine were identified in the preformulated clover extract (**Fig.4**). Tyramine was identified merely in the aqueous fraction and might be a degradation product from decomposition of amino acids (Booth et al. 2006a).

Tapia et al. (2007) used supercritical fluid extraction (SFE) to investigate the profiles of volatiles in red clover roots. Following analysis of the root extracts by GC-MS, they identified the volatile compounds as butyl acetate, E-2-hexenal, α -pinene, benzaldehyde, 6-methyl-5-hepten-2-one, limonene, acetophenone, methyl benzoate, nonanal, octanoic acid and decanal.

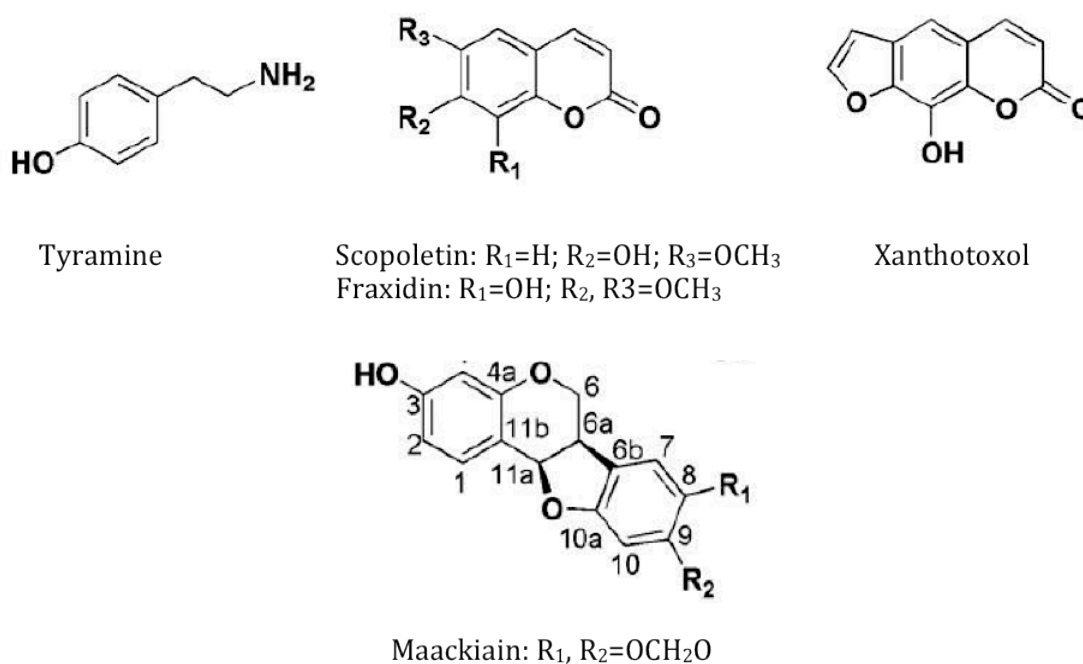


Fig.4. Chemical structures of coumarins, pterocarpan and tyramine identified in a red clover extract (modified from Booth et al. 2006a).

2.2. Distribution of isoflavones in red clover

There is convincing evidence in the literature that the composition and concentration of single isoflavones in plant material strongly depend on several factors such as plant part and maturity, environmental conditions, cultivar and preservation methods (Sivesind et al. 2005, Swinny et al. 2005, Tsao et al. 2006).

It is well established that environmental stress to plants such as long-term exposure to UV_B radiation (Swinny et al. 2005), water stress (de Rijke et al. 2005) and application of certain elicitors (Sivesind et al. 2006) result in an upregulation of secondary metabolites arising from the phenylpropanoid pathway. Isoflavonoids exert anti-microbial and/or anti-fungal effects but mediate interactions between plants and symbiotic nitrogen-fixation bacteria as well (Carlsson et al. 2006, de Rijke et al. 2005, Klejdus et al. 2005).

For the two principal isoflavones in red clover, biochanin A and formononetin, and the minor aglycones, the highest concentrations were located in growing leaves followed by the stems (Sivesind et al. 2005, Tsao et al. 2006). In leaves and flowers, formononetin and biochanin A are the main compounds equally, as opposed to stems and petioles where formononetin is the leading component (Sivesind et al. 2005). The aerial parts contain generally more total isoflavones than flowers (Booth et al. 2006b, **Fig.5, p.10**). During flower development, the total isoflavone concentration in the above ground parts decreased continuously. The maximum average temperature correlates with the maximum concentrations in flower heads. On the other hand, maximum rainfall correlates rather with the maximum isoflavone concentrations stored in the whole plants (Booth et al. 2006b). Long-term exposure to high UV_B radiation led to an overall increase in formononetin and biochanin A concentration (Swinny et al. 2005).

Leaves at a late flowering state contained higher concentrations (about 30%) of isoflavones than those collected before blooming (**Fig.5, p.10**), whereas in stems the total isoflavone content was reduced (Tsao et al. 2006). Conclusively, concentrations of

isoflavones appear to vary significantly with harvest times reflecting different stages of maturity (Sivesind et al. 2005, Booth et al. 2006b).

	Plant material	Formononetin	Biochanin A	Genistein	Daidzein
Booth et al.b 2006	aerial parts	0.21-0.59	0.07-0.33	0.014-0.068	0.013-0.033
	flower	0.047-0.12	0.07-0.14	0.018-0.019	0.003-0.008
Tsao et al. 2006	flower	0.047-0.11	0.061-0.12	0.002-0.012	n.d.
	leaves (EB)	0.607-1.12	0.574-1.14	0.049-0.089	0.040-0.094
	leaves (LF)	0.802-1.33	0.759-1.79	<0.007-0.033	<0.001-0.021

Fig.5. Isoflavone content of red clover plant preparations expressed as percent found per dry weight of plant [(mg isoflavone/mg dry plant) × 100]; EB: early bud stage, LF: late flowering stage, n.d. / not detected.

It is noteworthy that at least two studies have reported higher isoflavone content in red clover flower heads as compared with leaves (Booth et al.b 2006, Tsao et al. 2006). Contradictory results also exist with regard to studies on the seasonal variation of isoflavones in red clover. This is partly due to fluctuations in chemical content according to geographic location, differing photoperiods of sunlight, weather, soil fertility, and partly because of differences in the sensitivity and specificity of older analytical chemical methods used to quantify isoflavones. Earlier analytical methods, based on thin-layer chromatography and subsequent fluorimetric detection, had been less sensitive and produced a poor chromatographic separation of formononetin from biochanin A (Booth et al.b 2006b).

In addition, the isoflavone concentration is likely to be subject to genetic regulation (Sivesind et al. 2005). Tsao et al. (2006) showed that the isoflavone composition among 13 cultivars was akin; however the total and the single isoflavone concentrations varied significantly among the cultivars.

Studies analyzing isoflavone profiles in crude red clover extracts or in dietary supplements by HPLC often found high amounts of aglycones, indicating deconjugation of isoflavones (Booth et al. 2006a, Toebe et al. 2005, Tsao et al. 2006). Isoflavone malonated derivatives are unstable, thermolabile and easily degraded during extraction and processing by endogenous β -glucosidases into their corresponding glucosides or aglycones (Xu et al. 2005). The amount and activity of glucosidases vary for different plant parts and among species (Tsao et al. 2006, Delmonte et al. 2006, Toebe et al. 2005). Tris was identified as a suitable inhibitor for glucosidases in red clover and resulted in concentrations of malonate glycosides in fresh leaves 13 to 24 times higher than without the use of Tris (Toebe et al. 2005).

Alkaline extraction produced mostly 7-O-glycosides, whereas an acidic extraction led to a better persistence of malonylglucosides, slightly more aglycones and a low rate in isoflavone glycosides (Toebe et al. 2005). This study verified clearly that post-harvest plant management as well as extraction procedures diminished the levels of malonate glycosides in leaves by approximately 90%. The malonylated glycosidic forms of formononetin and biochanin A in flowers were reduced by 38% and 55%, in roots by 54% and 65%, respectively (Toebe et al. 2005). Supercritical fluid extraction (SFE) with carbon dioxide provides a milder extraction method that might attenuate the decomposition (Klejdus et al. 2005).

"Aglycones can undergo further thermal degradation above 150 °C dependent on their structural features. Heating periods of up to 7 h at a pH value of 3.1 showed pronounced differences in aglycone stability and content determined by HPLC/DAD. Genistein and formononetin appeared to be more stable after 5h at 150 °C than daidzein and biochanin A. Aglycones with 4'-methoxysubstitution (formononetin) revealed an improved stability as compared with 4'-hydroxy derivatives (daidzein). On the other hand, in 5,7,4'-substituted isoflavones (genistein) a 4'-OH group instead of a 4'-methoxy group (biochanin A) led to a slightly higher heat tolerance. Daidzein was more vulnerable to heat than genistein, suggesting that additional OH-substitution at the A ring might enhance stability (Stintzing et al. 2006)." At all time points (3, 5 and 7 hours) daidzein and biochanin A were more sensitive than formononetin and genistein.

Swinny et al. (2005) found that vacuum drying degraded isoflavones nearly quantitatively into their parent aglycones, whereas freeze-drying preserved the glycosylated forms. Aglycones were present in freeze-dried material less than 1% of the total isoflavone yield (**Fig.6**).

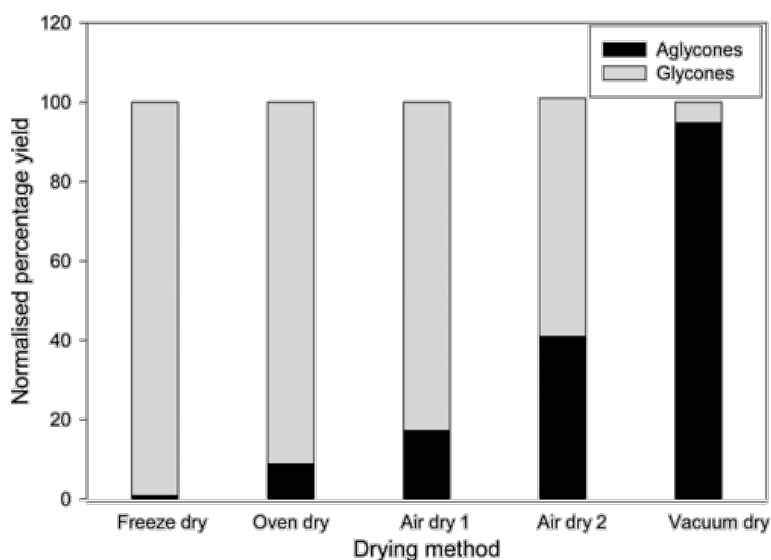
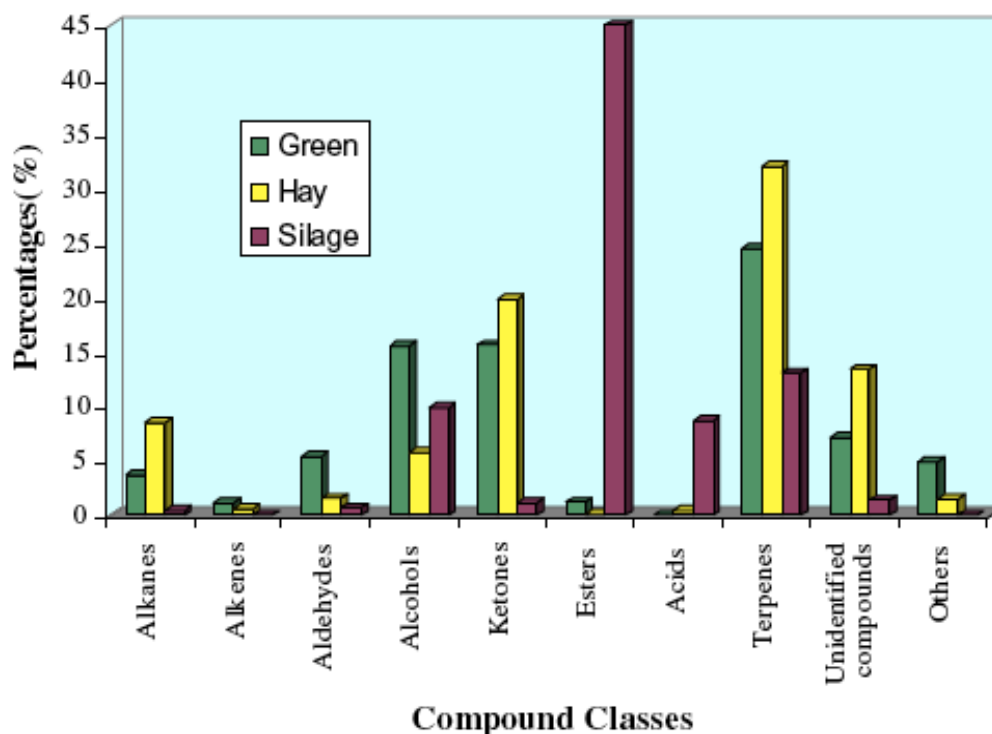


Fig.6. Effect of the drying method on isoflavone glycoside/aglycone ratio (Swinny et al. 2005).

Postharvest processing influences the chemical composition of RC as well. Fresh herb contains higher formononetin and total isoflavone concentrations (>18%) than silage and hay (Sivesind et al. 2005). Besides the isoflavones, also volatile compounds are influenced (**Fig.7, p.13**). In fresh material, terpenes (as β -caryophyllene, β -farnesene) as well as 3-octanol, 3-methyl-1-butanol, 3-octanone, benzaldehyde and 6,10,14-trimethyl-2-pentadecanone (phytol degradation product) are predominant (Figueiredo et al. 2007). During the process of ensiling, the concentration of isoflavones especially of formononetin decreased, while so-called “inducible volatile organic” compounds increased. The volatile compounds were analyzed by GC and GC/MS.

In hay the percentage of alcohols and aldehydes were diminished. Instead, the amount of β -farnesene and a phytol degradation product increased significantly. The alcohol 1-octen-3-ol, a metabolite from the fatty acid metabolism, was found exclusively in hay.

In silage, high amounts of esters (mainly ethyl and methyl esters) and free fatty acids, but no alkenes were detected. Intriguingly, phenol and 4-ethyl-2-methoxyphenol could only be detected in silage (Figueiredo et al. 2007).



	Red Clover fresh material	Red clover hay	Red clover silage
Total compounds (C ₇ -C ₂₀)	79.20%	83.70%	81.10%
Alkanes (%)	3.70%	8.54%	0.44%
Alkenes (%)	1.12%	0.66%	0.00%
Aldehydes (%)	5.38%	1.54%	0.71%
Alcholes (%)	15.60%	5.73%	9.82%
Ketones (%)	15.70%	19.90%	1.09%
Esters (%)	1.20%	0.12%	46.00%
Acids (%)	0.00%	0.44%	8.64%
Terpenes (%)	24.50%	32.00%	13.10%

Fig.7. Compound classes in fresh material, hay and silage of red clover (modified from Figueiredo et al. 2007).

3. Pharmacological effects of isoflavones in red clover

A vast body of publications reported biological effects of red clover extracts and standardized preparations. Numerous effects have been tested in various in-vitro assays and clinical trials. Isoflavones exert beside their well-known estrogenic effects, anti-oxidative and anti-carcinogenic activities and are able to interact with diverse transcription factors (Beck et al. 2005).

3.1. In-vitro data

3.1.1. Antioxidant activity

Many cellular reactions produce free radicals and reactive oxygen species (ROS), by-products of the normal metabolism. ROS are highly reactive but immediately eliminated by antioxidants. An imbalance between ROS production and antioxidative defense leads to ROS accumulation (reviewed in Blomhoff 2005), which subsequently alters function and structure of macromolecules (DNA, RNA, lipoproteins, cellular proteins). Polyunsaturated fatty acids, components of biomembranes and membrane-rich organelles exert a high sensitivity to oxidants owing to their double bonds.

In a study of Georgetti et al. 2007, the commercial product Isoflavin Beta® and a dry red clover extract were analyzed for their antioxidative activity (**Fig.8, p.15**). Isoflavin Beta® contained a higher content of total isoflavones and proteins compared to the dry extract. Both red clover formulations, however, exhibited antioxidant activity in a concentration-dependent manner as determined by the lipid peroxidation (LPO) assay. Isoflavin Beta® and the dry extract inhibited Fe²⁺-induced lipid peroxidation with IC₅₀ values of 21.03 µg/ml and 10.34 µg/ml, respectively.

A potent scavenging capacity was also measured by inhibition of malonylaldehyde (MDA) originated from the degradation of desoxyribose. Higher concentrations of isoflavones showed a stronger scavenging activity. The IC₅₀ values were 33.5 ng/ml, and 32.1 ng/ml for Isoflavin Beta® and the dry extracts, respectively.

Extracts	Total flavonoids (mg/g)	Isoflavonoids (mg/g)		Total polyphenol (mg/g)	Total protein (mg/g)
		Daidzein	Genistein		
Isoflavin Beta®	6.8	196.0	87.0	75.0	136.0
Red clover	8.4	5.4	2.9	45.0	40.0

Table 1. Chemical characteristics of the Isoflavin Beta® and red clover.

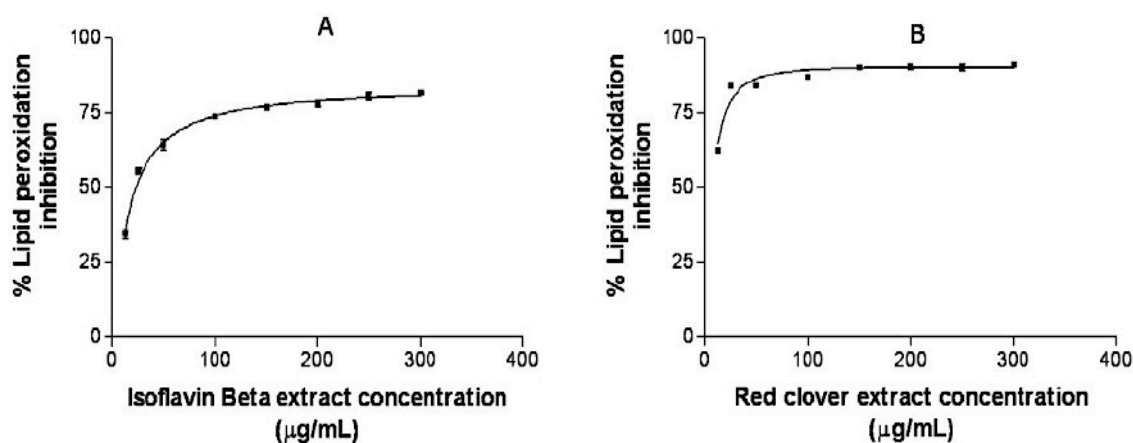


Fig.8. Composition and effect of two red clover preparations on anti-oxidant activity. (A) Isoflavin Beta® and (B) red clover dry extract inhibited LPO formation (adapted from Georgetti et al. 2007).

In DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, Isoflavin Beta® was less effective than the dry extract; IC₅₀ values were 161.8 µg/ml and 30.27 µg/ml, respectively (Georgetti et al. 2007). Kroyer (2004) confirmed a DPPH radical scavenging capacity for an ethanolic red clover extract (EC₅₀=0.32 mg/ml) and the red clover preparation Menoflavon® (EC₅₀=0.50 mg/ml) as compared to a soy preparation (EC₅₀=1.2 mg/ml).

Likewise, Booth et al. (2006a) showed for a raw extract of a phase II clinical trial that the DPPH scavenging activity was associated with flavones as quercetin, naringenin, and kaempferol (**Fig.9, p.16**). The IC₅₀ for the extract was 143.1±8.2 µg/ml.

Component	DPPH radical scavenging (IC ₅₀ , µM)
Daidzein	>200
Genistein	>200
Formononetin	>200
Biochanin A	>200
Quercetin	49.9±8.3
Fisetin	41.6 ±2.7
Kaempferol	64.9±2.8

Fig.9. Radical scavenging activity of the red clover components (from Booth et al. 2006a).

In the study of Rüfer et al. (2006), the oxygen radical absorbance capacity (ORAC) and the in-vitro oxidation of low density lipoproteins and the thiobarbituric acid reacting substances (TBARS) formation as end points were used for the determination of radical scavenging and antioxidant effects of different isoflavones and their metabolites. The oxidative metabolites of daidzein and genistein as well as equol showed the highest antioxidative activities in all of these three assays, even higher than the positive controls quercetin and ascorbic acid. In contrast, formononetin showed the lowest anti-oxidant activity.

In the oxygen radical absorbance capacity (ORAC) assay, isoflavones seem to show stronger effects on scavenging peroxy radicals than on chelating metal ions (Rüfer et al. 2006). A structure/antioxidant relationship could be established from this study. A 2,3 double bond together with the 4-oxo group and the 3'-hydroxyl group in the C ring, furthermore the 5,7-hydroxyl groups in the A ring, and ortho dihydroxyl group in the B ring appear to be essential for the antioxidative activity. In agreement, genistein was more effective than daidzein, which lacks the 5-OH group. Methylation of hydroxyl groups as in biochanin A and formononetin abolished their antioxidant capacity.

Interestingly, equol showed a high antioxidant activity despite the lack of structural consistencies and warrants further evaluation (Rüfer et al. 2006).

3.1.2. Receptor binding assays

3.1.2.1 Estrogen receptor binding assays

17- β -estradiol is predominantly synthesized in the ovaries via estrogen receptor (ER) of which two subtypes have been reported so far (Wuttke et al. 2007).

The two major subtypes ER α and ER β show a notable variability in their C-terminal ligand-binding domain and in the N-terminal transactivation domain. However, according to other nuclear receptors, ER α and ER β share a core structure with five functional domains A/B, C, D, E and F. Domain A/B is part of protein-protein interactions and transcription of target genes. Domain C plays a role for DNA binding and ER dimerization. Domain D contains the nuclear localization signal and connects domain C and the ligand-binding domain E. Domain F contains further necessary co-factor recruitment zones (Beck et al. 2005).

ER α and ER β have an overlapping but not identical tissue distribution and expression pattern, suggesting distinct biological roles. ER α shows an expression profile with presence in the uterus, vagina, ovaries, mammary gland and hypothalamus. ER β is distributed in the prostate and ovaries, with smaller number in the lungs, brain and bones. ER α is mainly responsible for tissue proliferation and an aberrant proliferation may contribute to malignant transformation. Knowledge is mainly based on knockout mice. Knockout animal models demonstrate the essential role of both ERs in the reproductive tract, bone, cardiovascular system, hematopoiesis and the nervous system. In certain tissues, ER β seems to be involved in differentiation of proliferating tissue; in others it appears to have even antiproliferative, i.e. ER α -antagonistic effects (Wuttke et al. 2007).

The molecular structures of isoflavones as genistein and 17- β -estradiol show some similarity which could explain their estrogenic effects. As shown in **Fig.10**, the intramolecular distances between the hydroxyl groups in these flat molecules are comparable. Both molecules contain aromatic rings with the OH-groups in 3-position and an OH group in similar distance at the other end. This structure enables the binding of isoflavones to the ligand-binding pocket of the ERs (Wuttke et al. 2007).

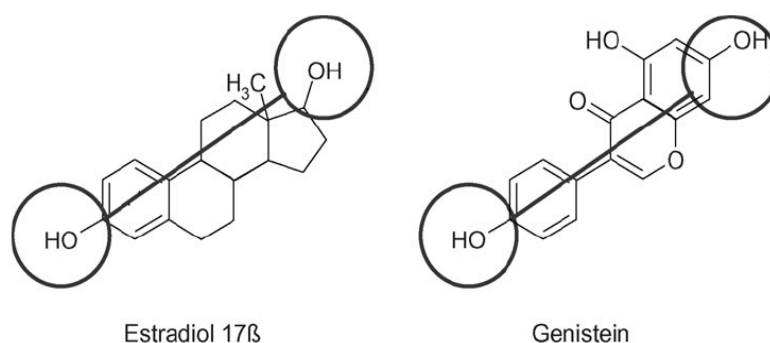


Fig.10. Structure relationships between genistein and 17- β -estradiol (adapted from Wuttke et al. 2007).

Booth et al. (2006) studied the ER binding of a commercial red clover extract (30% isoflavones defined as total content of all four main isoflavone aglycones) and its unformulated crude extract without excipients. The IC_{50} value to ER α was 18.4 ± 4.9 $\mu\text{g/ml}$ and 32.6 ± 9.0 $\mu\text{g/ml}$, respectively. For ER β the IC_{50} values were 1.9 ± 0.8 $\mu\text{g/ml}$ and 3.4 ± 0.7 $\mu\text{g/ml}$, respectively. This data confirmed stronger binding affinity of isoflavones to the ER β than ER α receptor (**Fig.11, p.19**). Due to this ER β dominance, isoflavones should affect predominantly tissues expressing ER β . Therefore isoflavones are often called natural selective estrogen receptor modulators (SERM).

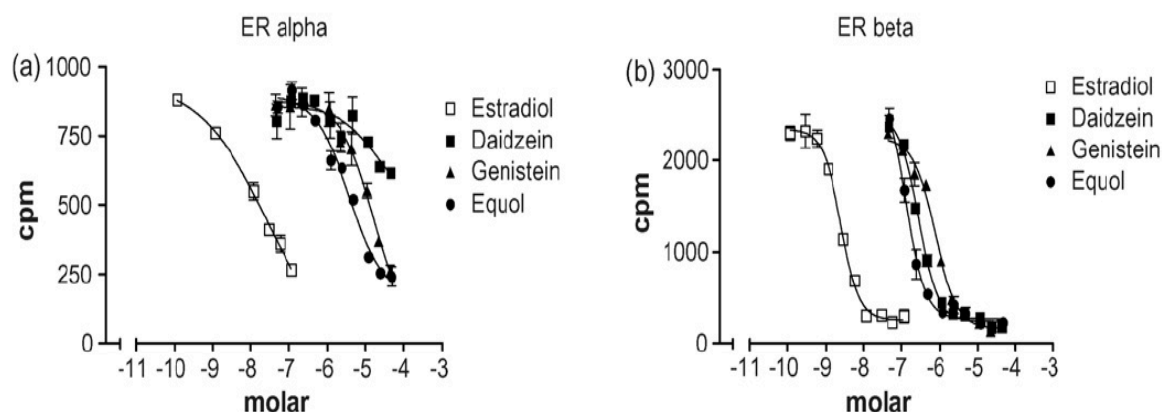


Fig.11. Differences in binding affinity of isoflavones and estradiol to ERα and ERβ (adapted from Wuttke et al. 2007).

Binding assays resulted in the identification of daidzein, formononetin, biochanin A and genistein as estrogenic constituents affecting primarily ERβ (Booth et al. 2006a, Booth et al. 2006b, Beck et al. 2005). In another study, Overk et al. (2005) compared the estrogenicity of hops and red clover extracts. The results showed similar ERα activity between hops and red clover with IC₅₀ values of 15 and 18 µg/ml, respectively. However, both extracts varied in their ERβ IC₅₀ values of 27 and 2 µg/ml. The binding affinities for ERα of isolated single isoflavones were in the order: genistein = 8-PN (8—prenylnaringenin) > daidzein > biochanin A > formononetin > IX (isoxanthohumol).

The relative binding affinities for ERβ were genistein > 8-PN = daidzein > biochanin A > formononetin = IX. Genistein and 8-PN had similar affinities for ERα with IC₅₀ values of 0.51 and 0.3 µM, respectively, whereas genistein was more selective for ERβ as indicated by the IC₅₀ values of 0.020 and 1.7 µM, respectively.

Moreover, Overk et al. (2004) measured the ERE luciferase expression as a sign for estrogenicity. Ishikawa and MCF-7 cells were transiently cotransfected with ERE-luciferase and treated with 2 µg/ml of a hops or red clover extract as well as 100 nM of single compounds to assess the formation of a functional ER-ERE complex. In Ishikawa cells the hop and red clover extracts exerted significant 3 and 7.2 fold induction, respectively. Genistein and 8-PN showed both an increase of 3.5 fold. None of the other compounds showed significant effects on ERE luciferase activity. In MCF-7 cells, hops and red clover extracts activated ERE to a 4.2 and 6.3 fold increase, respectively.

Biochanin A, genistein and 8-PN led approximately to 4-fold induction of ERE luciferase.

3.1.2.2 Opiate receptor binding assay

Because of their phytoestrogen contents (including formononetin, biochanin A, daidzein and genistein), red clover extracts were initially thought to exert their effects exclusively through sex-hormone receptor systems. Mounting data from *in vitro* and *in vivo* studies are indicating a dynamic link between female sex hormones, menopause and the opiate receptor system in the central nervous system through several mechanisms. Recent studies suggest that the opioid system, especially μ - and δ -receptors, is essential for temperature and hormone homeostasis (Nissan et al. 2007).

Nissan et al. (2007) assessed an extract (30% total isoflavone aglycones) of red clover for the binding affinity at the μ - and δ -opiate receptors. Red clover extract concentrations of 0.3, 1, 10, 30, 100 and 300 $\mu\text{g/ml}$ were used. The extract was able to displace competitively the tritium-labeled enkephalin [^3H]DAMGO ([D-Ala²-N-Me-Phe⁴-Gly⁵-ol]enkephalin), a selective μ -opiate receptor agonist, in a dose-dependent manner from the receptor expressed in CHO-hMor cells (containing human μ -opiate receptors). The IC_{50} (25.6 ± 4.2 $\mu\text{g/ml}$) and K_i (9.7 ± 1.6 $\mu\text{g/ml}$) values revealed a high affinity of components in the extract to the μ -opiate receptor. At the highest concentration, the extract was able to replace the [^3H]DMAGO from the binding pocket completely.

Tritium-labeled deltorphin, an agonist for δ -receptors expressed in CHO-hDOR cells (containing the human δ -receptor) showed comparable results with IC_{50} and K_i values of 30.9 ± 4.6 , and 15.9 ± 2.4 $\mu\text{g/ml}$, respectively (Nissan et al. 2007).

3.1.3. Cell-based assays

3.1.3.1 Tests on breast cancer cell lines

The estrogen-sensitive breast cancer cell line MCF-1 was used to evaluate estrogen agonistic and antagonistic activities of red clover preparations. MCF-1 cells show an increasing proliferation rate in response to estrogen or estrogen-like substances. A red clover leaf preparation containing 40 mg isoflavones per tablet induced a significant increase in the MCF-1 proliferation rates, indicating an estrogen-agonistic activity in the absence of estradiol. The preparation was tested at dilutions from 10^{-2} to 10^{-7} and exerted significant proliferative effects at dilutions from 10^{-4} to 10^{-6} , whereas higher concentrations remained without effects (Bodinet et al. 2004).

For evaluation of antiestrogenic effects the test was extended by addition of estradiol at 10^{-7} M to the test solutions. The antagonist tamoxifen (10^{-5} M) served as the positive control. Tests of antagonistic effects demonstrated that the tested red clover preparation was not synergistic to estradiol and reduced the estrogen-induced proliferation at 10^{-2} M (Bodinet et al. 2004).

Han et al. (2006) used MCF-1 cells to address the question whether isoflavones interact with members of the CYP 450 family. In MCF-1 cells pre-treated with 1 μ M 7,12-dimethylbenz[a]anthracene (DMBA) for 18 h induced CYP1A1 activity was measured by the EROD assay. Treating MCF-1 cells with biochanin A alone caused likewise an accumulation of CYP1A1 mRNA (3.5-fold increase) and CYP1A1 specific 7-ethoxyresorufin O-deethylase (EROD) activity in a dose-dependent manner from 10 to 50 μ M.

In addition, the biochanin A treatment in the absence of DMBA facilitates the DNA-binding of the aryl hydrocarbon receptor (AhR) to the dioxin-response element (DRE) of CYP1A1, assessed in the electrophoretic-mobility shift assay (EMSA). AhR is a cytosolic protein that binds environmental toxins (polycyclic aromatic hydrocarbons, DMBA, benzpyrene). Upon binding, the AhR translocates into nucleus where it binds the aryl nuclear translocase (ARNT).

Heterodimerization of the transcription factor leads as a result to induction of CYP1A1 gene which is able to catalyze the degradation of aromatic polycyclic aromatic hydrocarbons and other genotoxic compounds.

Biochanin A was not only able to reduce the 7,12-dimethylbenz[a]anthracene-(DMBA) induced 7-ethoxyresorufin O-deethylase (EROD) activity but also able to alter the CYPs by binding to the AhR and competing with the tritium-labeled ligand 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) for binding sites at the AhR. Treatment of cells with biochanin A during DMBA exposure resulted in the dose dependent inhibition of CYP1A1 activity. Moreover, biochanin A affected DMBA-DNA adduct formation in MCF-7 cells. MCF-7 cells were treated with tritium-labeled DMBA or DMBA plus biochanin A (10 μ M-50 μ M). After an incubation for 16 h, DMBA-DNA lesions were determined by scintillation counting. Cells incubated with biochanin A demonstrated a significant decreased formation of the DMBA-DNA adducts in a dose dependent manner (Han et al. 2006).

3.1.3.2 Tests on human endothelial cell lines

Endothelial cell-derived nitric oxide (NO) plays a critical role in cardiovascular physiology and disease. It is well known that endothelial nitric oxide synthase (eNOS) is a target of estrogens. Estrogen induces rapid eNOS activation through nongenomic recruitment of mitogen-activated protein kinase and phosphatidylinositol-3 kinase (Simoncini et al. 2005). Simoncini et al. (2005) tested the effects of the RC isoflavones genistein, daidzein, formononetin and biochanin A on NO synthesis and eNOS activity in serum-starved steroid-deprived human umbilical vein endothelial cells (HUVECs).

HUVECs were treated with increasing concentrations of the isoflavones, starting from the dose detectable in plasma after absorption of a standardized red clover extracts preparation (Menoflavon® containing 0.6 mg genistein, 9.6 mg biochanin A, 2.3 mg daidzein and 27.5 mg formononetin per tablet). After 30 minutes, neither an increase in NO synthesis nor any induction of eNOS activity was observed. Because estrogens are able to enhance NO synthesis through putative transcriptional induction, steroid-

deprived HUVECS were exposed for 48 hours to increasing concentrations of the compounds. Administration of genistein and daidzein showed a concentration-dependent increase in NO release in the cell culture medium with concurrent activation of eNOS.

In the same study, the effects of the coadministration of the isoflavones at the steady state concentration found in plasma of postmenopausal women receiving Menoflavon (Mix plasma, **Fig. 12, p.24**), as well as of a 10-fold higher concentration of the same ratio of isoflavones (10×Mix plasma, **Fig. 12, p.24**) were investigated. It was shown that the effect of the pool of isoflavones is stronger than the effect of the single compounds. In addition, a comparable amount of the same isoflavones in the ratio as in Menoflavon® tablets (Mix tablet, **Fig. 12, p.24**) before absorption and intestinal metabolism, showed a much lower effect than that of the Mix plasma (**Fig. 12, p.24**) indicating that the pool enriched in genistein and daidzein (as after intestinal absorption) is more effective than the original combination of isoflavones in red clover.

In an additional setting, the possible interferences of isoflavones with estradiol-dependent signaling to eNOS were tested. In this regard, the single isoflavones as found in plasma (Mix plasma) or before intestinal absorption (Mix tablet) were coadministered with follicular phase levels (100 pg/ml) or lower, menopausal levels (10pg/ml) of 17β-estradiol. It was shown that single isoflavones do not alter the effect of follicular phase level-estradiol. However, the combination of the isoflavones with estradiol caused a synergic augmentation of the estrogen's effect. This synergic effect was not observed with the Mix tablet.

	Formononetin ng/ml	Biochanin A ng/ml	Daidzein ng/ml	Genistein ng/ml
Mix plasma	20	20	100	40
10× Mix plasma	200	200	1000	400
Mix tablet	125	42.5	10	2.5

Fig.12. Compositions of the treatments (modified Simoncini et al. 2005).

Mix Plasm: combination of amounts of isoflavones after absorption of 1 tablet Menoflavon

10× Mix Plasma: 10-fold higher concentrations Mix Plasma

Mix tablet: combination of the amounts of isoflavones provided in the ratio found in Menoflavon before absorption and metabolism.

In parallel experiments using a postmenopausal concentration of estradiol, a significant augmentation of the effect of estradiol was observed when genistein and daidzein were coadministered. This effect, however, was not detected with formononetin and biochanin A. The enhancement of estradiol-induced NO synthesis and eNOS activation was proportionally higher with the "plasmatic pool" of isoflavones, whereas the "preabsorption" pool had a much lower action and was only able to significantly augment the NO synthesis.

In the same investigation and in order to assess whether the recruitment of estrogen receptors is required for the activation of eNOS by isoflavones, parallel 48-hour treatments with bioequivalent concentrations of 17 β -estradiol (10 pg/ml), genistein (400 ng/ml) or daidzein (1000 ng/ml) in the presence or absence of the pure ER antagonist, ICI 182,780 (10⁻⁶ M) were performed. ICI 182,780 effectively reversed the activation of eNOS and NO synthesis induced by estradiol, genistein or daidzein indicating that isoflavones signal to eNOS through functional estrogen receptors.

On the other hand, selective activation of ER α or ER β by their respective selective agonists PPT (4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol or DPN (2,3-bis(4-hydroxyphenyl)-propionitrile) showed a similar enhancement of NO synthesis as well as of eNOS activity that were effectively reversed by ICI 182,780 demonstrating the

contribution of both receptors in the transcriptional activation of eNOS expression (Simonici et al. 2005).

3.1.3.3 Tests on endometrial cells

In a study performed by Overk et al. (2005), extracts of red clover and hops and their individual constituents including daidzein, formononetin, biochanin A and genistein from red clover and 8-PN (8-prenylnaringenin), 6-PN (6-prenylnaringenin), IX (isoxanthohumol) and XN (xanthohumol) from hops were tested using the alkaline phosphatase (AP) induction assay in the Ishikawa cell line. The Ishikawa cell line is an ER α positive endometrial adenocarcinoma cell line that could respond to both estrogens and antiestrogens at near physiological levels. AP induction is an important feature of an estrogenic response whereas competition with estrogens and prevention of AP expression suggests an antiestrogenic response (Overk et al. 2005, Beck et al. 2005).

The Ishikawa cells were treated with a hop or red clover extract or individual isoflavones. All of the samples were tested at non-cytotoxic concentrations for the cell line. In this assay, the EC₅₀ values for the hop and red clover extracts were comparable, i.e. 1.1 and 1.9 $\mu\text{g/ml}$ respectively. The isolated hop compound 6-PN, however, was not active in this assay even when tested at 20 $\mu\text{g/ml}$. The relative EC₅₀ order for all tested compounds was as follows: 8-PN >> genistein $\approx$ daidzein > IX > biochanin A. None of the samples had antiestrogenic activity.

Likewise, Booth et al. (2006a) tested the same red clover extract in an AP induction assay. The preformulated extract was composed of 35.54% isoflavones, 1.11% flavonoids, 0.06% pterocarpanes, $\leq 0.03\%$ coumarins and $\leq 0.03\%$ tyramine. Daidzein, genistein, formononetin, biochanin A, coumestrol and naringenin were estrogenic in the AP assay and all of these, except formononetin, bound to one or both ERs. Based on the percentage and relative activity, the principal estrogenic components of the extract were the isoflavones, daidzein, genistein, formononetin, and biochanin A. The red clover extract exhibited an EC₅₀ value of 2.2 $\mu\text{g/ml}$ in the AP estrogenicity assay and an

IC₅₀ of 32.6 µg/ml and 3.4 µg/ml in the ERα and ERβ binding assays, respectively (Booth et al. 2006a).

3.1.3.4 Tests on neuronal cells

Microglia are the resident immune cells in the brain. They contribute to immune surveillance and host defense against infectious agents under normal condition. Nevertheless, in response to injuries, infections or inflammation, microglia become readily activated and produce different proinflammatory factors, including cytokines e.g. tumor necrosis factor-α (TNF-α) and the free radicals nitric oxide (NO) and superoxide. Accumulation of these factors is detrimental to neurons. Dopaminergic neurons in the substantia nigra, due to their reduced antioxidant capacity and potential defect in mitochondrial function are especially vulnerable to oxidative stress (Chen et al. 2007).

Chen et al. (2007) investigated the protective effects of biochanin A on the lipopolysaccharide (LPS)-mediated damage to dopaminergic neurons. For this purpose, mesencephalic neuron-glia cultures were pretreated with vehicle or 0.25–2.5 µM biochanin A for 30 min before treatment with 10 ng/ml LPS. Seven days later, dopamine (DA) uptake assays indicated that LPS treatment reduced the uptake capacity to 36.7% of that of vehicle-treated control culture. Biochanin A significantly attenuated the LPS-induced decrease in DA uptake (**Fig.13, p.27**) in a dose-dependent manner and attenuated the release of proinflammatory mediators as TNFα, NO and superoxide.

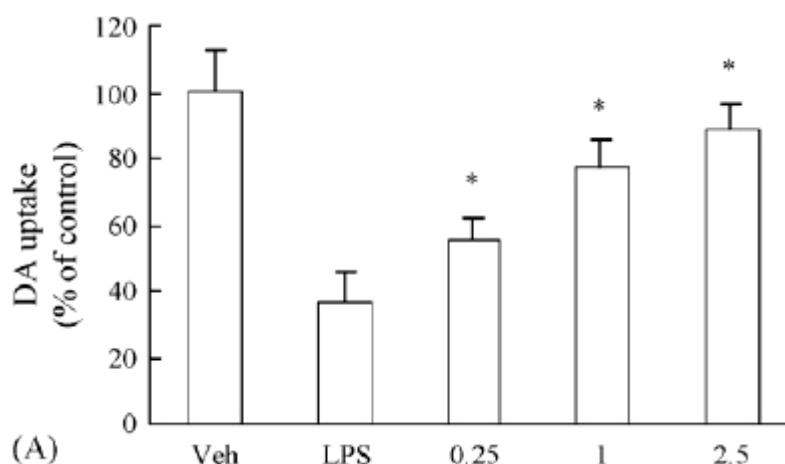


Fig.13. Effect of biochanin A on DA uptake in primary mesencephalic neuronglia cultures; Biochanin A (0.25 μ M, 1.0 μ M and 2.5 μ M) attenuated LPS-induced decrease in DA uptake in primary mesencephalic neuron-glia cultures (adapted from Chen et al. 2007).

3.1.3.5 Tests on immune cells

Lam et al. (2004) examined the ability of the isoflavones in red clover to inhibit cyclooxygenase (COX) enzyme activity in both the murine macrophage cell line RAW 264.7 and human monocytes.

The mouse macrophage cell line RAW 264.7 was treated with concentrations 1, 10 and 40 μ M of the isoflavones genistein, biochanin A and formononetin. Compared to vehicle-treated cells, formononetin, biochanin and genistein inhibited dose-dependently the synthesis of PGE₂ in RAW264.7 cells after stimulation with LPS (**Fig.14, p.28**). Genistein was the most potent inhibitor and even at 1 μ M significantly inhibited PGE₂ synthesis by 62%. Formononetin significantly reduced PGE₂ production at 10 μ M by 60%, but not at 1 μ M. Biochanin A significantly inhibited PGE₂ production at 10 μ M by 75%, but not at 1 μ M. Daidzein was the weakest inhibitor with significant effect only at 40 μ M.

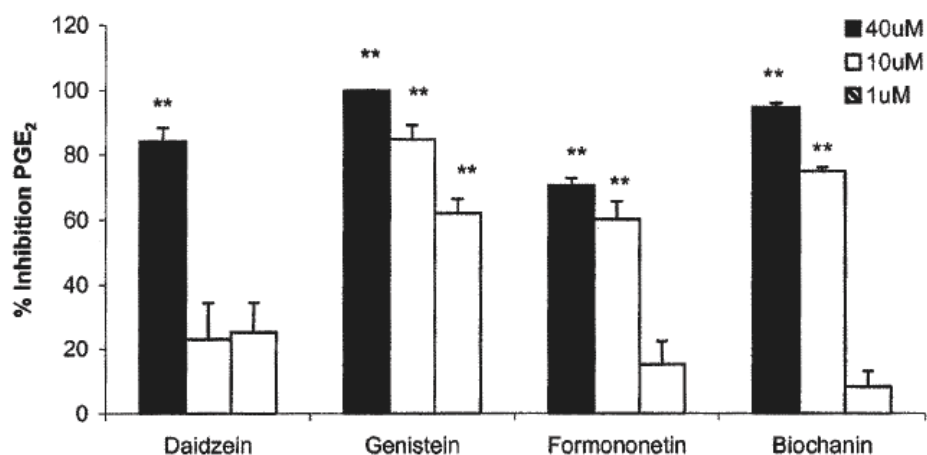


Fig.14. Inhibition of LPS-induced prostaglandin E_2 synthesis by isoflavones in RAW264.7 cells.
**P < 0.05, **P < 0.001, statistically significant reduction compared with vehicle control values.*
(adapted from Lam et al. 2004).

In the assay using human monocytes stimulated with LPS, daidzein, genistein and formononetin inhibited the synthesis of PGE_2 significantly at 100 μM . At a concentration of 10 μM , the inhibition was significant for formononetin and biochanin but not for genistein. Genistein, formononetin and biochanin were able to inhibit thromboxane B_2 (TXB_2) at 100 μM , only formononetin significantly inhibited TXB_2 at 10 μM .

3.2. In-vivo data

A large number of different in vivo experiments in a variety of models for assessing the different effects and modes of action of phytoestrogens and the safety of dietary isoflavone supplements have been performed.

3.2.1. Animal studies

3.2.1.1 Effects of isoflavones on growth performance

Turan et al. (2007) investigated the effects of dietary red clover on growth, body composition and survival rate of common carp *Cyprinus carpio*. The fish were fed with diets supplemented with three concentrations of red clover extract (50, 100 and 200 mg/kg Menoflavon®) and a control diet for 12 weeks. Survival rate was higher than 88% in all dietary groups (**Fig.15**). The growth rate significantly increased in fish fed with red clover-supplemented diets in comparison with the control groups. Weight gain and specific growth rate (SGR) of the 100 mg/kg red clover diet group were significantly higher than for control and other groups. Food conversion ratio (FCR), protein efficiency ratio (PER) and apparent net protein utilization (ANPU) were significantly higher in 100mg/kg group than in the other groups. The findings of this study suggest that 100 mg/kg supplementation of red clover improves growth rate, feed utilization and survival of common carp (**Fig.15**).

	Red clover (mg kg ⁻¹)			
	0	50	100	200
Weight gain (g)	12.08±0.10 ^{ab}	12.33±0.36 ^b	14.60±0.20 ^c	11.33±0.27 ^a
SGR	2.17±0.05 ^{ab}	2.19±0.03 ^b	2.35±0.07 ^c	2.10±0.02 ^a
FCR	1.49±0.01 ^b	1.46±0.04 ^b	1.23±0.01 ^a	1.59±0.03 ^c
PER	2.40±0.02 ^{ab}	2.45±0.07 ^b	2.90±0.03 ^c	2.25±0.05 ^a
ANPU (%)	52.06±3.95 ^{ab}	52.03±4.03 ^{ab}	63.08±3.33 ^b	50.30±2.03 ^a
Survival (%)	88.89±2.22 ^a	93.33±2.10 ^a	93.33±3.85 ^a	88.90±2.22 ^a

*Values (mean±S.E. of triplicate) with different superscripts in each line indicate significant differences (p<0.001) (Water temperature 26±1°C)

Fig.15. Growth performance, feed utilization efficiencies and survival rate of common carp fed with different concentrations of dietary red clover for 12 weeks (adapted from Turan et al. 2007).

Moorby et al. (2004) examined the effect of the concentration of formononetin in red clover on the growth rate and carcass characteristics of lambs, which were grazed on three different forages: red clover with a high formononetin (HF) concentration (4.7 g/kg dry matter), red clover with a low formononetin (LF) concentration (3.3 g/kg dry matter), and a perennial ryegrass without formononetin as control.

Half of all animals were slaughtered immediately after reaching score 3L (score 1=thin, score 5=fat), while the other half were moved to a common ryegrass plot for 3 weeks as a 'withdrawal' period. Lambs grazing the HF clover gained 40 g/day live weight more than other groups. Following the 3-week withdrawal period on ryegrass, there were significant residual effects of previous grazed forage on carcass weight, with HF lambs producing heavier carcasses than other lambs.

Plasma concentrations of total proteins, globulins, glucose, and β -hydroxybutyrate were unaffected by dietary treatment, in both sexes. Clover-fed animals had significantly higher plasma albumin and plasma urea concentrations than grass-fed animals. The plasma urea concentrations were significantly different among all three treatment groups with the lambs grazing the HF clover sward having higher plasma urea concentration than LF lambs. The Forage type had significant effects on growth hormone and IGF-1 concentrations. Plasma growth hormone concentrations were higher in clover-fed lambs than grass-fed lambs, and were higher in lambs grazing the HF clover sward compared with lambs grazing the LF clover sward. Similarly, plasma IGF-1 concentrations were significantly higher in clover-fed lambs compared with grass-fed lambs, and also tended to be affected by sex.

"Plasma insulin concentrations were not significantly affected by forage type, although there was a significant difference between the two sexes." No significant differences in plasma estradiol concentrations were detected, although male animals grazing the ryegrass sward showed lower estradiol concentrations (Moorby et al. 2004).

3.2.1.2 Effects of isoflavones on reproductive organs

Rimoldi et al. (2007) supplemented the diet of ovariectomized rats with two doses of genistein daily [5.4 (low) or 54 (high) mg/kg body weight] orally over 3 months and compared the effects with a treatment with two doses of 17 β -estradiol [E₂; 0.17(low) and 0.7 (high) mg/kg body weight]. After 3 months the uteri, vaginae and mammary glands were removed to determine the weight and perform morphological and immunohistochemical examinations.

The vaginae of the E₂ animals (both doses) displayed a typical squamous multilayered epithelium. All animals developed around 10–15 cell layers with cornification. No clear differences were found between controls and animals treated with genistein. With genistein, epithelium thickness seemed slightly augmented in some areas, the number of cell layers did not differ from controls and no cornification was seen. Genistein at the higher dose increased epithelial thickness and also the number of cell layers (5–10 layers). Cornification was detected in 1 animal of this group, and cytoplasmatic vacuolization in all samples.

The uterine weight of ovariectomized animals was very low. "In animals treated with either E₂ dose, uterine weight was increased to the range of that of intact animals." The high genistein dose led to increased uterine weight, whereas it remained unchanged at the low genistein dose.

"With high E₂ treatment, all uterine structures were hypertrophic and hyperplastic. Low genistein treatment induced negligible effects. In animals receiving the high dosage of genistein, endometrial cells were stimulated but no pathologic signs were detected."

"Mammary gland tissue was atrophic in the ovariectomized control rats, and the few detectable terminal structures were collapsed. E₂ at the low dose induced luminal formation but no secretion, whereas in the mammary gland of animals at E₂ high dose, ample tissue and signs of secretion were observed. Genistein did not have marked

effects on mammary gland morphology; half of the animals in each genistein dose group had an incipient luminal formation."

"Proliferating cell nuclear antigen (PCNA) is a nuclear protein expressed in proliferating cells during the S phase of the cell cycle and used as a tool in mammary cancer prognosis research. It can be visualized by immunohistochemistry which allows the estimation of the percentage of proliferating cells in relation to the total number of cells. In deep terminal mammary structures, epithelial proliferation was increased by both E₂ doses. PCNA analysis also indicated that genistein has a stimulatory effect on the mammary gland epithelial cells. Stimulation of mammary cell proliferation is an increased risk factor for the development of cancer" (Rimoldi et al. 2007).

3.2.1.3 Effects of isoflavones on skin

In a study performed by Circosta et al. (2006), preventive effects of red clover isoflavones on the progress of degenerative skin changes induced by ovariectomy in rats were investigated. "Four independent methods were used to detect changes in the skin connective tissue, namely the physical measurement of skin thickness, the assessment of total collagen by determining hydroxyproline, the determination of the skin capacitance by measuring the water content and the histological examination of the distribution and morphology of collagen bundles, elastic fibers, glands and vascularity by histology."

The skin of the animals treated with a commercial red clover extract (receiving 20 and 40 mg of total isoflavones daily for 14 weeks, respectively) looked well organized with a normal epidermis with uniform thickness and regular keratinization. Collagen bundles and elastic fibers were well developed. "The amount of collagen significantly increased in the treated group in comparison with the control group." The red clover extract significantly increased the thickness of the skin and the collagen content, as measured by skin hydroxyproline, in ovariectomized rats (**Fig.16, p.33**).

Group	Skin thickness (mm)	Hydroxyproline ($\mu\text{g}/\text{mg}$ of frozen skin)	Collagen content ($\mu\text{g}/\text{mg}$ of frozen skin)
I (sham animals)	1.80 ± 0.07	54.00 ± 4.20	425.1 ± 8.32
II (OVX control)	1.28 ± 0.04^a	30.50 ± 2.22^a	240.1 ± 9.40^a
III (OVX treated) 20 mg/day	1.62 ± 0.06^b	43.06 ± 2.80^b	339.0 ± 7.25^b
IV (OVX treated) 40 mg/day	1.70 ± 0.09^b	45.04 ± 2.55^b	354.6 ± 8.00^b

Mean $\pm$ SEM of $n = 5/\text{group}$.

^a $p < 0.05$ group II compared with group I.

^b $p < 0.01$ group III or IV compared with group II.

Fig.16. Effect of red clover extract on physical and biochemical cutaneous parameters in ovariectomized rats (adapted from Circosta et al. 2006).

3.2.1.4 Effects of isoflavones on cardiovascular system

Asgary et al. (2007) evaluated the effects of red clover on the development of atherosclerosis in male hyperlipidemic rabbits. Of twenty rabbits in four groups, two groups received either normal diet or normal diet supplemented with dried red clover (8%). Two other groups received similar diets to both of which 1% cholesterol was added which is considered to be a high cholesterol diet.

At the end of the treatment after 14 weeks, no significant differences between the weights of animals were observed. However, the use of dietary red clover in hyperlipidemic rabbits significantly decreased C-reactive protein (CRP), cholesterol, triglyceride and low-density lipoprotein, whereas this diet significantly increased high-density lipoprotein (**Fig.17, p.34**).

The scores for the formation of fatty streaks in aorta, left and right coronary arteries of hypercholesterolemic rabbits were increased to values of 1.51 ± 1 , 1.54 ± 1.33 and 2.2 ± 1.78 , respectively. In the hypercholesterolemic animals treated with red clover, the same scores were reduced to the values 0.62 ± 0.33 , 0 ± 0 and 0.26 ± 0.06 , respectively. After supplementation with red clover, the decreased fatty streak formation was confirmed histologically (Asgary et al. 2007).

Blood factor (mg/dL)	High Chol/High Chol + RC (n = 5)	p	Normal/Normal + RC (n = 5)	p	High Chol/ Normal (n = 5)	p
FBS	27.4 ± 5.8	0.213	6.4 ± 3.2	0.757	-31.6 ± 4.3	0.178
CRP	2.00 ± 0.2	0.029 ^a	-1.80 ± 0.3	6.094	-2.4 ± 0.2	0.029 ^a
HDL-C	-31.6 ± 19.1	0.0163 ^a	6.4 ± 10.2	0.524	-74.4 ± 14.2	0.003 ^a
LDL-C	171.0 ± 11.4	0.001 ^a	-13.6 ± 9.8	0.652	-552.0 ± 12.3	0.000 ^a
TG	96.0 ± 15.2	0.052 ^a	-55.2 ± 12.3	0.438	-187.8 ± 7.6	0.001 ^a
Chol	168.00 ± 8.7	0.001 ^a	-6.6 ± 7.6	0.530	-650.8 ± 12.4	0.000 ^a

High Chol, standard diet supplemented with 1% cholesterol; High Chol + RC, standard diet supplemented with 1% cholesterol as well as 8% dried red clover; Normal, normal standard diet; Normal + RC, normal standard diet supplemented with 8% dried red clover; RC, red clover; FBS, fasting blood sugar; CRP, C-reactive protein; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; TG, triglyceride; Chol, cholesterol.

^a Significant at $p < 0.05$.

Fig.17. Comparing mean differences of blood factors between groups before and after the treatment (adapted from Asgary et al. 2007).

Haines et al. (2006) compared the protective effect of a red clover extract to that of estradiol using an animal model of atheroma formation and cholesterol accumulation in the aorta. The preparation used in this study contained 25 mg formononetin and 2 mg biochanin A per dosage (1 tablet of 500 mg).

Rabbits were ovariectomized before starting the treatment period. The control group 1 received isoflavone-free chow, the control group 2 1% cholesterol-enriched isoflavone-free chow. Group 3 was fed with 1% cholesterol-enriched isoflavone-free chow plus one 500 mg red clover tablet and the fourth group with 1% cholesterol-enriched isoflavone-free diet plus one 0.5 mg tablet of 17- β -estradiol.

At baseline no significant differences in the concentrations of total cholesterol, non-HDL-cholesterol or triglycerides between the four groups were detected. After 12 weeks of treatment, serum concentrations of total cholesterol and non-HDL-cholesterol were significantly higher in groups 2, 3 and 4 (receiving a cholesterol-enriched diet) compared to group 1. No significant changes in concentrations of triglycerides were detected in any of the groups throughout the treatment period. Moreover, measurement of lipids and total antioxidant activity (based on ferric acid-reducing ability of plasma) showed a significant increase in all four groups compared with their corresponding baseline values. The differences in the control group were the smallest. Significantly higher cholesterol accumulation in the aorta and higher percentage of staining of atheroma was observed in all groups as compared to control.

Nevertheless, in group 4 the increase was significantly lower than in group 2 and 3.

3.2.1.5 Effects of isoflavones on bone loss

"Estrogen-deficiency in postmenopausal women is at least in part responsible for the decrease in bone mass and the increase in the incidence of fractures. There is evidence that diets with high levels of phytoestrogenic isoflavones are associated with a low incidence of osteoporosis and menopausal symptoms" (Occhiuto et al. 2007).

Occhiuto et al. (2007) evaluated the preventive effect of red clover on the rapid bone loss after ovariectomy in female rats. "Twenty Wistar rats were randomized into four groups of five animals each. The rats from all groups underwent bilateral ovariectomization except for group 1 that served as sham operated controls (Sham). Rats from group 2 received the vehicle and were defined as ovariectomized controls (OVX). The groups 3 and 4 were treated daily with an oral dose of 20 and 40 mg of isoflavones, respectively. Treatment started 7 days after ovariectomy and continued for 14 weeks." The red clover extract was standardized to a minimum of 11% isoflavones (w/w) consisting of 0.18% genistein, 0.62% daidzein, 2.62% biochanin A and 7.53% formononetin.

The ovariectomized rats developed bone changes "indicated by a decrease in femur weight, density, bone mineral content, mechanical strength of the tibia and by an increase of bone specific alkaline phosphatase and of the number of osteoclasts in the femur sections. Treatment with isoflavones significantly increased bone mineral content, biomechanical strength of the tibia, femoral weight and femoral density" (**Fig.18, p.36**). Additionally the increase of serum alkaline phosphatase levels compared with ovariectomized control rats was reduced and the elevated number of osteoclasts was significantly decreased in isoflavone-treated rats (groups 3 and 4).

Parameter	Sham	OVX	ISO 20 mg	ISO 40 mg
Thickness (mm)	5.70 ± 0.15	5.65 ± 0.12	5.75 ± 0.10	5.72 ± 0.18
Length (mm)	27.55 ± 0.24	27.35 ± 0.25	27.60 ± 0.18	27.45 ± 0.16
Weight (g)	0.58 ± 0.015	0.52 ± 0.020 ^a	0.56 ± 0.010 ^b	0.57 ± 0.012 ^b
Volume (mL)	0.40 ± 0.011	0.41 ± 0.013	0.39 ± 0.012	0.40 ± 0.010
Density (g/mL)	1.45 ± 0.018	1.26 ± 0.015 ^a	1.43 ± 0.014 ^b	1.42 ± 0.012 ^b
Total ash (g)	0.35 ± 0.010	0.32 ± 0.016 ^a	0.33 ± 0.018	0.34 ± 0.011 ^b
Calcium content (mg/g)	198.20 ± 5.30	181.40 ± 3.70 ^a	193.45 ± 4.65 ^b	195.38 ± 3.50 ^b

All values are expressed as mean ± SEM.

^a $p < 0.01$ compared with Sham group.

^b $p < 0.01$ compared with OVX group.

Fig.18. Effects of red clover on physical features of the femur (adapted from Occhiuto et al. 2007).

The authors deduced from the results that red clover isoflavones "are effective in reducing bone loss induced by ovariectomy, probably due to the inhibition of bone resorption as evidenced by the lower number of osteoclasts compared with OVX controls" (Occhiuto et al. 2007)

3.2.2. Clinical trials

Menopause is defined as the point in time following 12 consecutive months of amenorrhea and occurs in response to normal physiological changes in the hypothalamic-pituitary-ovarian axis (reviewed in Doyle et al. 2007).

Due to the decline in estrogen and progesterone production and the increase in follicle-stimulating hormone (FSH) and luteinizing hormone (LH), women experience vasomotor symptoms (hot flashes and sweating) or other symptoms such as mood swings, depression, vaginal dryness, sleep disorders and joint pain. Moreover, the incidence of chronic diseases e.g. cardiovascular disease, osteoporosis, urogenital atrophy, incontinence and dementia increases dramatically (Beck et al. 2005, Doyle et al. 2007).

Menopausal disorders are conventionally treated with synthetic estrogens. However, conventional hormone replacement therapy (HRT) is suspected to increase the risk of

breast cancer and to cause other undesirable side effects (Beck et al. 2005, Pitkin 2004). A retrospective analysis performed by Ness et al. (2006) demonstrated that the cessation of HRT use in postmenopausal women led to the development of menopausal symptoms in more than 40%. Thus, after termination of HRT an alternative for symptom management is needed in a significant proportion of postmenopausal women. Vasomotor symptoms and urogenital complaints were the most frequent symptoms, with the former particularly among younger postmenopausal women and the latter predominating among older patients (over 65 years).

Phytoestrogens exert estrogenic activity and are used for the treatment of menopausal disorders. Positive effects of these compounds on menopausal disorders such as osteoporosis, cardiac risk factors or breast cancer have been reported. Dietary supplements with isoflavones from soy and red clover have been two of the most commonly utilized preparations for peri- and postmenopausal women (Geller et al. 2006, Pitkin 2004, Barentsen 2004, Albertazzi et al. 2004). Due to these facts several clinical studies with red clover preparations have been performed in the investigated period.

3.2.2.1 Isoflavones and climacteric complaints

Hot flashes are one of the common symptoms of climacteric and comprise the most common reason for climacteric women seeking medical advice. "Approximately two thirds of women who reach menopause develop menopausal symptoms, primarily hot flashes" (Geller et al. 2005), which primarily affect women in the transition to menopause or who have become menopausal. Most epidemiological studies from North America and Europa reported the highest incidence of hot flashes in the peri-menopausal years ranging from 58 to 93% after menopause and from 28 to 65% in pre-menopausal women (Haimov-Kochman et al. 2005).

The effects of isoflavones against vasomotor menopausal symptoms have been investigated in numerous clinical trials and have been analysed in reviews and meta-

analyses (Howes et al. 2006, Nelson et al. 2006, Williamson-Hughes et al. 2006, Tempfer et al. 2007).

A meta-analysis (Howes et al. 2006) of randomized, controlled, parallel group studies comparing isoflavone therapy with soy or red clover products to non-isoflavone, non-estrogenic therapies suggests that dietary isoflavone supplementation may result in a small to modest reduction in the number of hot flushes with the highest benefit when the number of daily flushes experienced is high. The results also showed the large placebo response in the treatment of menopausal flushes (Howes et al. 2006).

In a review by Nelson et al. (2006), the efficacy and adverse effects of nonhormonal therapies for menopausal hot flashes were compared. Six placebo-controlled trials with 2 types of red clover preparations provided data for the meta-analysis, including Promensil® (40 mg/d, 80-82 mg/d, 160 mg/d isoflavones) and Rimostil® (57 mg/d isoflavones). "All trials used Promensil®, which contained a higher proportion of biochanin and genistein, and in one trial Rimostil® was studied as well which contained a higher proportion of formononetin and daidzein." Only one fair-quality trial slightly improved hot flash frequency with Promensil® compared with placebo. Reduction in hot flashes was significantly faster with Promensil® than with placebo in one trial. However, hot flash frequency was not reduced when the results of all trials of red clover isoflavone extracts were combined (Nelson et al. 2006).

Krebs et al. (2004) systematically reviewed 22 randomized placebo-controlled trials to assess the effect of phytoestrogens on the severity and frequency of hot flushes and menopausal symptom scores. Five of these trials (n=400) used red clover supplementation, and compared the effects of red clover extract with that of placebo. All of these trials tested the preparation Promensil®. One trial included a third arm with Rimostil®. Overall, red clover extract did not improve menopausal symptoms compared with placebo. Only in the 2 smallest trials (n=30) reported a significant improvement in hot flush frequency (Krebs et al. 2004).

Another meta-analysis performed by Thompson Coon et al. (2007) evaluated 5 randomized clinical trials.

All trials used Promensil® at recommended doses of 40–80 mg/day and assessed hot flush frequency via patient diaries. In one trial a dosage of 160 mg was included as well. Supplementation of women experiencing five to nine hot flushes per day for 12–16 weeks reduced hot flush frequency by approximately one hot flush per day. "Meta-analysis of all trials indicated a difference of borderline statistical significance between women receiving active treatment and those receiving placebo" (Thompson Coon et al. 2007).

3.2.2.2 Isoflavones and breast cancer

Phytoestrogens are widely believed to attenuate menopausal symptoms, yet their relationship with breast cancer risk has not been fully investigated. Rebbeck et al. (2007) tried to evaluate in a population-based case-control study of women in the Philadelphia region (949 breast cancer cases and 1,524 controls) to assess the relationship between breast cancer risk and commonly used hormone-related supplements (HRS). Between 10 and 20% of women reported the use of any HRS. The proportion varied by race, with African American women being more likely than European American women to use any HRS (19.2% vs. 14.7%).

Among the used preparations, African American women used significantly more black cohosh than European American women (5.4% vs. 2.0%), ginseng (12.5% vs. 7.9%) and red clover (4.7% vs. 0.6%). Among the supplements, only black cohosh had a significant breast cancer protective effect (adjusted odds ratio 0.39, 95% CI: 0.22–0.70) (Rebbeck et al. 2007).

Atkinson et al. (2004b) conducted a randomized, placebo-controlled trial to determine the effects of a red clover supplement on mammographic breast density. 205 women (from 49–65 years) were assigned to receive either a red clover preparation containing 26 mg biochanin A, 16 mg formononetin, 1 mg genistein and 0.5 mg daidzein per tablet or placebo. Tested parameters were mammographic breast density, the assessment of serum estradiol, FSH, LH, lymphocyte tyrosine kinase activity and menopausal symptoms from baseline to 12 months.

This isoflavone supplement did not increase mammographic breast density which decreased to a similar extent in both treatment groups. A strong interaction between the isoflavone-treated group and estrogen receptor polymorphism was determined. Moreover, there were no statistically significant effects on estradiol, FSH, LH (assessed only in postmenopausal women), on lymphocyte tyrosine kinase activity or menopausal symptoms (Atkinson et al. 2004b).

Parvu (2004) collected case reports from her homeopathic practice with cystic mastosis or breast cancer patients. The authors concluded that women with estrogen receptor positive breast cancer, cystic mastosis or with ovarian cysts might benefit from herbal red clover medicines but more evidence-based trials are still needed.

3.2.2.3 Isoflavones and colon cancer

Prospective epidemiological studies indicate that higher circulating concentrations of insulin-like growth factor I (IGF-I) are associated with increased colorectal cancer risk. As estrogenic substances are associated with decreased colon cancer risk, isoflavones due to their weak estrogenic activity were studied for their potential to induce an estrogen-like reduction of IGF-I in the circulation. For this purpose, the effect of a 2-month supplementation with a red clover preparation (84 mg isoflavones/d) on serum concentrations of IGF and various IGF binding proteins (IGFBP) in males at increased risk of colorectal cancer was investigated in a randomized, placebo-controlled, double-blind crossover study (Vrieling et al. 2007).

The study duration was 6 months, consisting of two 8-week intervention periods separated by an 8-week washout period. The participants were treated with isoflavone tablets (Promensil®, Novogen) in the first period and placebo tablets for the second or vice versa. At the end of each treatment, serum concentrations of total IGF-I, free IGF-I, IGF binding proteins 1, 2 and 3 (IGFBP1, IGFBP2, IGFBP3) were measured. The isoflavone treatment did not change total serum levels of IGF-I, nor did it alter other parameters.

The change in serum IGF-I concentration did not correlate with serum genistein concentrations, but was negatively correlated with serum equol concentrations after verum. For 8 of 9 (out of a study population of 37) men, classified as equol producers, serum IGF-I decreased after isoflavone intake compared with placebo. In conclusion, this study suggests "that isoflavones might have an IGF-I lowering effect in equol producers only" (Vrieling et al. 2007).

3.2.2.4 Isoflavones and reproductive system

In a randomized double-blind crossover study, Imhof et al. (2006) assessed the effects of a red clover-derived preparation (80 mg/day of a standardized red clover extract, MF11RCE) on sex hormones [testosterone, estradiol, FSH, LH, sex hormone binding globulin (SHBG)] and endometrial thickness. 109 postmenopausal women (40 years or older; amenorrhea>12 months; Kupperman index $\geq$ 15) were enrolled. The study groups received either MF11RCE or placebo per day for 90 days. After a 7 day washout period, subjects switched to receive the opposite treatment for another 90 days.

This study demonstrated a significant effect of 80 mg per day red clover-derived isoflavones on testosterone levels (appr. 22% increase) and endometrial thickness (appr. 15% reduction) as compared to baseline (**Fig.19, p.42**). The plasma testosterone levels increased also significantly compared with placebo while endometrial thickness decreased. No effect on E₂, FSH and SHBG was observed compared to baseline as well as compared to placebo. LH was significantly decreased under placebo but not under verum.

The reduction of endometrial thickness is a positive outcome for the treatment of menopausal disorders, particularly in terms of suspected adverse effects of estrogen replacement therapy.

	Before MF11RCE	After MF11RCE	Change (%)
T (ng/ml)	0.54 ± 0.26	0.66 ± 0.29	22.12
Endometrium (mm)	3.8 ± 1.9	3.2 ± 1.5	-14.69

Fig.19. Effects of isoflavones on endometrial thickness and testosterone level (adapted from Imhof et al. 2006).

Using the same red clover extract (MF11RCE), Chedraui et al. (2006) evaluated in a randomized placebo-controlled study the effect of MF11RCE supplementation on vaginal and sexual health of 53 postmenopausal women. Inclusion criteria were over 40 years with amenorrhea of >12 months, basal FSH levels >30 mIU/ml, no hormone therapy and a basal Kupperman index >15. The enrolled women were treated either with 80 mg MF11RCE for 90 days or placebo. "After a 7-day washout period, the medication was crossed-over for another 90 days. Body mass index and vaginal/sexual health related indicators during menopause (dryness, decreased libido, dyspareunia) were assessed at baseline, 90 and 180 days."

MF11RCE supplementation resulted in a positive effect on the vagina which was characterized by a significant improvement of the karyopyknotic, cornification and basal cell maturation index, correlated with a decrease in the rate of women suffering from dyspareunia, vaginal dryness and decreased libido (**Fig.20**).

	Baseline (n=53)	After MF11RCE	After Placebo
Karyopyknotic index (%)	6.1 ± 11.7	45.6 ± 27.4	3.6 ± 7.6
Mean change ‡		(-39.5; 26.5; -46.8 ~ -32.2) ^{a,*}	(42; 28.2; 34.3 ~ 49.8) ^{b,*}
Cornification index (%)	6.1 ± 11.6	45.7 ± 26	3.9 ± 8.8
Mean change		(-39.6; 25.3; -46.6 ~ -32.7) ^{a,*}	(41.3; 27.1; 34.3 ~ 49.3) ^{b,*}
Parabasal cells (%)	68.4 ± 36.7	1.9 ± 12.7	65.2 ± 37.4
Mean change		(66.4; 37.7; 56 ~ 76.9) ^{a,*}	(-63.2; 37.8; -73.6 ~ -52.8) ^{b,*}
Intermediate cells (%)	27.4 ± 32.5	39.6 ± 25.4	30.2 ± 31.9
Mean change		(-12.2; 38.7; -22.9 ~ -1.5) ^{a,*}	(9.4; 35.8; -0.5 ~ 19.3) ^{b,*}
Superficial cells (%)	4.2 ± 7.8	57.9 ± 25.9	4.7 ± 9.8
Mean change		(-53.8; 24.7; -60.6 ~ -47) ^{a,*}	(53.2; 28.5; 45.4 ~ 61) ^{b,*}
Vaginal dryness	47 (88.7%)	25 (47.2%) ^{a,*}	44 (83%) ^{b,*}
Moderate-severe score	33 (62.3%)	4 (7.5%) ^{a,*}	20 (37.7%) ^{b,*}
Dyspareunia ¹	41/52 (78.8%)	18/52 (34.6%) ^{a,*}	36/52 (69.2%) ^{b,*}
Moderate-severe score	22/52 (42.3%)	5/52 (9.6%) ^{a,*}	15/52 (28.9%) ^{b,*}
Decrease of libido	50 (94.3%)	28 (52.8%) ^{a,*}	39 (73.6%) ^{a, b,*}
Moderate-severe score	38 (71.7%)	13 (24.5%) ^{a,*}	20 (37.7%)

* Mean change or percentage was significantly different: ^a vs. baseline, ^b vs. red clover phase, $p < 0.05$; ‡ Values in parenthesis are: mean difference; standard deviation; 95% confidence interval; ¹ One subject was not considered for calculation as she was not sexually active.

Fig.20. Effects of MF11RCE supplementation on postmenopausal vaginal and sexual health (adapted from Chedraui et al. 2006).

The outcome of the same study on blood pressure and Kupperman index was published in a second paper (Hildalgo et al. 2005).

After completing either trial phase, no significant effect on baseline body mass index (BMI), weight and blood pressure was seen. Menopausal symptoms assessed with the Kupperman index decreased significantly after red clover supplementation and returned to the baseline values after completion of the placebo phase (Hildalgo et al. 2005).

In a population-based epidemiological case-control study, Heald et al. (2007) did not observe any association with prostate cancer risk for either serum isoflavones or isoflavone intake. On the contrary, another population-based case-control study demonstrated "that high overall intake of foods high in phytoestrogens lowers the risk of prostate cancer. Inconsistencies in results between studies could arise due to differences in measurement tools for evaluating dietary intake, the questionnaire design to assess relevant food items for phytoestrogen intake and/or the phytoestrogen content of food", etc. (Hedelin et al. 2006).

3.2.2.5 Isoflavones and osteoporosis

Ovarian hormone deficiency is one of the most important risk factors for osteoporosis (Atkinson et al. 2004c). "Women experience accelerated bone loss at a rate of 3% to 5% per year for about 5 to 7 years following menopause. Plant-derived estrogens may exert agonist or antagonist effects on various estrogen target tissues as selective estrogen receptor modulators" (Zhang et al. 2005).

In a randomized, double-blind placebo-controlled trial performed by Knudson et al. (2004), the effects of two red clover supplements on bone turnover markers in symptomatic menopausal women were evaluated. Compared with placebo, Promensil® (82 mg total isoflavones) and Rimostil® (57.2 mg total isoflavones) marginally changed serum osteocalcin (a marker of bone formation) and urinary N-telopeptide (a marker of bone resorption) (Knudson et al. 2004).

In another double-blind, randomized placebo-controlled trial, Atkinson et al. (2004c) recruited 205 women (aged 49-65) and randomly assigned them to receive daily either a red clover-derived isoflavone tablet (Promensil®; containing 26 mg biochanin A, 16 mg formononetin, 1 mg genistein and 0.5 mg daidzein) or a placebo. After 12 months the bone mineral content, bone turnover markers and bone density of especially the lumbar spine were evaluated.

The results showed a lower progression of bone loss in lumbar spine and increased bone formation markers in the isoflavone-treated group. Trends for the bone mineral content and density of the hip were similar to those seen in the lumbar spine, however differences between treatments were not significant (Atkinson et al. 2004c).

3.2.2.6 Isoflavones and cardiovascular system

"Cardiovascular disease, particularly coronary heart disease (CHD) is the main cause of death among women in developed countries. Women are four to eight times more likely to die of CHD than of any other disease. Natural menopause alters the lipoprotein profile of women" (Knudson et al. 2004). "Estrogen loss at the menopause has been associated with increased risk of cardiovascular diseases which is partly attributable to an adverse lipoprotein profile and arterial dysfunction." To date, it is uncertain whether isoflavones can, like estrogen, exert beneficial effects on lipoprotein profile (Vafeiadou et al. 2006).

In a trial performed by Knudson et al. (2004), the effects of two red clover supplements Promensil® (82 mg total isoflavones) and Rimostil® (57.2 mg total isoflavones) on blood lipids in symptomatic menopausal women were evaluated. Compared with placebo, the red clover preparations increased HDL-cholesterol slightly. A significant decrease in triglyceride levels was observed after verum compared to placebo, primarily in women with elevated triglyceride levels at baseline. No differences in total cholesterol, LDL-cholesterol, or the ratio of HDL- to LDL-cholesterol were detected among treatment groups (Knudson et al. 2004).

Hidalgo et al. (2005) evaluated, in addition to the effects on the vaginal cytology (see page 44), the effect of a red clover preparation on the lipid profile in postmenopausal women. No significant change in body weight or blood pressure was observed during the treatments. In this study, red clover supplementation significantly decreased the basal total cholesterol, LDL cholesterol and triglycerides levels. However, only the decrease in triglyceride levels was significant as compared with levels after the placebo phase.

"In a pan- European study (ISOHEART), the effects of isoflavone supplementation on the risk markers of cardiovascular diseases (CVD) in a large group of healthy post-menopausal women were investigated" (Vafeiadou et al. 2006). "The response to isoflavones may vary with respect to genotype and equol-production status. The effects of isoflavone doses (50 mg/day) on cardiovascular risk parameters, including markers of lipid and glucose metabolism and inflammatory biomarkers were investigated in a placebo-controlled double blind 2×8-week randomized cross-over study in 117 healthy post-menopausal women."

"The ISOHEART study has shown that isoflavones have no beneficial effects on plasma lipids, including total cholesterol, HDL-C, LDL-cholesterol or triacylglycerol in healthy post-menopausal women. Additionally, it was shown that isoflavones have no effect on on plasma insulin and glucose." However, isoflavones intake showed a beneficial effect on the C-reactive protein (CRP) concentrations.

"Most importantly, it was demonstrated that certain subgroups may respond more beneficially to isoflavone supplementation, as already demonstrated by the decrease in plasma VCAM-1 concentrations in one of the genotypes of the ER β *AluI* polymorphism and an increase in HDL-C in one of the genotypes of the ER β cx *Tsp509I* polymorphism" (Vafeiadou et al. 2006).

3.2.3. Toxicology and adverse effects

"Concerns about the adverse effects of estrogen have led to increased interest in alternative therapies for improving menopausal symptoms. Women with breast cancer

who cannot take estrogens are also seeking alternatives" (Nelson et al. 2006). Adverse effects of isoflavones and their metabolites are assumed due to their structural similarity to the estrogens and their interference with the hormonal balance. They are described as rhinitis, skin tension, allergies, local edema, itching, flushing, exanthema, nausea, dizziness, abdominal pain, skin rash, tachycardia, fertility disorders, exhaustion, coughing, sweating, anxiety, thorax pain, paraesthesia and precordial pain (BfR Expert Opinion 2007).

No serious adverse effects have been reported in clinical trials or in the medical literature. But because red clover extracts contain semipurified isoflavones, the question of safety in hormone-sensitive tissue has been raised, especially when high doses over prolonged periods are used (Low Dog 2005). Toxicological studies showed that single isolated isoflavones at very high doses could impair or change the functions of hormone-sensitive tissues as thyroid gland and mammary gland tissue. In treatment of menopausal woman these estrogen-like effects could promote the development of breast cancer, hence the hormone safety is an important concern (BfR Expert Opinion 2007).

Red clover extract reduced the activity of various CYP enzymes. Biochanin A may be involved in the chemopreventive properties by reducing the formation of carcinogens through inhibition of enzymes such as CYP1A1, an enzyme known to be involved in carcinogen activation. Red clover can further inhibit diverse members of the CYP 450 family (CYP1A1, CYP1B1, CYP2C9) (Low Dog 2005, Unger et al. 2004, Han et al. 2006).

Subchronic and chronic safety studies with genistein (orally at 0, 50, 150 or 500 mg/kg/day) in dogs for a period of 4 and 52 weeks, respectively, did not result in significant systemic toxicity despite the use of very high dose levels. Effects of genistein on the reproductive tract at very high doses were functional in nature and expectable for a compound with weak estrogenic activity.

"In this study, the "no observed effect level" of genistein (NOEL) was considered to be 150 mg/kg/day due to the observation of functional hormone-related changes in the uterine weight observed in female dogs treated with 500 mg/kg/day and the absence

of these effects at 150 mg/kg/day. The "no observed adverse effect level" (NOAEL) for the 52-week study was determined >500 mg/kg/day based on the transient functional hormone-related effects in the reproductive tract of male dogs and the absence of any adverse histopathological changes in the tissues examined" (McClain et al. 2005).

In Wistar rats, the NOEL of genistein administered orally with the diet was 5 mg/kg/day based on the hormonally induced functional changes due to the expected endocrine effects at high doses. The NOAEL was determined as 50 mg/kg/day based on the observation of mild hepatic effects at 500 mg/kg/day. Almost all of these findings were expected due to the estrogenic properties of genistein. "The hormonally related changes were considered to be functional in nature and thus not adverse effects." Furthermore, most of the findings were limited to a dose of 500 mg/kg/day. Few signs observed at 50 mg/kg/day were minor and also not classified as adverse effects. "The increased incidence of minimal bile duct proliferation and slightly increased gamma glutamyl transferase are indicative of a mild hepatic effect at the high dose of 500 mg/kg/day" (McClain et al. 2006b).

In another study, genistein was evaluated for its *in vitro* and *in vivo* mutagenic and clastogenic activity. This study found "no evidence for a mutagenic effect in the *in vitro* *S. typhimurium* assay without metabolic activation. In the *in vitro* mouse lymphoma assay, genistein increased resistant mutants with and without metabolic activation." Although both small and large colonies were increased, genistein induced predominantly small colonies indicating clastogenic activity.

"Of the three *in vivo* micronucleus assays performed with genistein, one was conducted in mice at dose levels of 0, 0.2, 2 and 20 mg/kg/day for 14 days in which there was no evidence for a treatment related increase in micronuclei." In two further micronucleus studies with acute treatment in RAIf and Wistar rats at very high doses (up to 2000 mg/kg/day) also no treatment related increase in micronuclei occurred (McClain et al. 2006a).

In reproductive safety studies performed in Wistar rats, genistein had no teratogenic effect *in vivo* at very high doses (up to 1000 mg/kg/day by gavage) in the embryo-fetal

toxicity study or up to 500 mg/kg/day by administration via the diet in the definitive prenatal developmental study, "even though these doses were maternally toxic and fetal-toxic. *In vitro*, genistein had teratogenic potential at high concentrations in the Whole Embryo Culture screening (WEC) assay. In the *in vitro* whole embryo culture screening assay anomalies were observed at doses of 10 or 30 µg/mL in the absence of general embryo or yolk sac toxicity, genistein is considered to exhibit a teratogenic potential in this assay at concentrations of ≥ 10 µg/mL (37 µmol/L)." However the *in vitro* findings were not predictive of the *in vivo* effects.

"On the basis of the prenatal developmental study, the NOAEL for maternal toxicity and adverse effects on embryonic and fetal development was considered to be 100 mg/kg/day when administered orally by dietary admix" (McClain et al. 2007).

"There are no human data available on developmental or reproductive toxicity of purified genistein. Available experimental data are sufficient to conclude that purified genistein can produce reproductive or developmental toxicity in rats and mice. The most highly reported exposed human population is Japanese adults with ingestion of approximately 0.43 mg/kg/day. However, adverse effects in rodent studies were not observed at levels below 35–44 mg/kg/day. Therefore, it is plausible that adults would be unlikely to consume sufficient daily levels of genistein to cause adverse reproductive or developmental effects under current exposure conditions" (Rozman et al. 2006).

4. Metabolism and pharmacokinetics of isoflavones

Jia et al. (2004) investigated the absorption of formononetin and biochanin A alone or in a red clover extract using the four-site intestinal perfusion model. They showed that absorption was rapid and site-dependent regardless of whether the isoflavones were administered alone or in an extract with minimal effects on water flux. But differences between amounts absorbed for these two compounds were observed in the different regions of the intestine. "For formononetin, the amount absorbed was the highest in the colon. For biochanin A, amounts absorbed in duodenum (47.7 nmol/30 min or 81% of perfused amounts) and colon (50.8 nmol/30 min or 89%) were higher than that in jejunum (31.5 nmol/30 min or 49%) and terminal ileum (30.5 nmol/30 min or 54%)."

Moon et al. (2006) reported a rapid absorption and biexponential decline in plasma biochanin A concentrations after i.v. and oral administration in rats (**Fig.23, p.56**). The i.v. studies indicated "a high clearance and a high volume of distribution for biochanin A. The high clearance was explained the rapid metabolism. Less than 3% of unchanged biochanin A was detected in the urine and bile. The genistein concentration in the plasma and bile was low, suggesting that biochanin A after rapid *O*-demethylation to genistein is rapidly conjugated. When the plasma samples were treated with the glucuronidase and sulfatase, the plasma concentrations of biochanin A and genistein increased ~10- and 50-fold, respectively. They also showed that "genistein has a high clearance and a high volume of distribution in rats after i.v. administration. High concentrations of genistein conjugates were observed in the plasma, indicating that genistein is rapidly conjugated (glucuronidated and/or sulfated)" not only after intravenous but also after oral delivery. The oral bioavailability of genistein and biochanin A was poor (3.4% at 10 mg/kg and 2.6% at 5 mg/kg, respectively). The metabolite formation profile after an intraperitoneal (i.p.) dose of biochanin A was similar to after i.v. or oral administration. The C_{max} after an IP dose was ~80-fold higher than that after an oral dose when biochanin A was administered at 50 mg/kg.

The main part of isoflavones in red clover is conjugated to sugar residues occurring as β -glycosides (genistin, daidzin, ononin, sissotrin). To these glycosides malonyl or acetyl

residues may be attached. For the full performance of the biological activities of phytoestrogens, isoflavones have to be present in a chemical form available to the organism. In the gut, intestinal bacteria convert conjugated isoflavones to their respective aglycones and the methylated isoflavones to their demethylated forms which are readily absorbed from the intestine. When plants are processed, malonyl as well as acetyl glycosides are converted to the more stable β -glycosides or to the aglycones (Beck et al. 2005, Booth 2005).

Because of their limited occurrence in the human diet, the metabolism of formononetin and biochanin A in humans has not been studied in detail. Once ingested, these isoflavones are readily demethylated by intestinal microflora to daidzein and genistein, respectively. Prunetin, a minor isoflavone may also be converted to genistein (**Fig.21, p.52**) (Beck et al. 2005, Booth et al. 2005, Heinonen et al. 2004). Daidzein is converted to dihydrodaidzein, which can be further metabolized to both equol and *O*-desmethylangolensin. Genistein is reduced by gut bacteria to dihydrogenistein followed by a cleavage of the C-ring to form 6'-hydroxy-*O* desmethylangolensin which can be further degraded to 4-hydroxyphenyl-2-propionic acid. Subsequent decarboxylation can lead to the putative metabolic end product 4-ethylphenol (Rüfer et al. 2006).

Isoflavones and their metabolites are absorbed, and they appear in blood plasma mainly as glucuronide and/or sulfate conjugates and to a small extent as unconjugated compounds. The conjugates are excreted in both urine and bile, and they undergo enterohepatic circulation (Heinonen et al. 2004, Rüfer et al. 2004). A study by Heinonen et al. (2004) analyzed the metabolic profile of formononetin and biochanin A in seven healthy women (aged 20-60 years) after consumption of 4 commercially available red clover supplements. The main isoflavones of the tablet were formononetin and biochanin A, besides small amounts of daidzein, genistein, pseudobaptigenin, prunetin, and calycosin. The ingested amounts of isoflavones were 51.7 mg of formononetin, 84.0 mg of biochanin A, 3.2 mg of daidzein and 5.2 mg of genistein. Three of the seven participants were able to produce equol. Additionally new metabolites, angolensin, 6'-OH-angolensin, dihydroformononetin, and dihydrobiochanin A, and previously tentatively suggested 4'-*O*-Me-equol were

identified in human urine for the first time (Heinonen et al. 2004).

The main step in the biotransformation of formononetin and biochanin A is the demethylation of the methoxy group at the 4'-position, yielding high urinary and plasma levels of daidzein and genistein. The demethylation of the methoxy group at ring B represents the easier metabolic reaction than the demethylation at ring A (Heinonen et al. 2004).

The end products of the genistein and daidzein biodegradation are carboxylic acids and alkyl phenols. Genistein degradation culminates into 4-ethylphenol and 4-hydroxypropionic acid (Rüfer et al. 2004). Conversion of genistein and daidzein to their oxidative metabolites 3'-OH-genistein and 3', 6-, and 8-hydroxy-daidzein as well as to the bacterial metabolite equol enhances their antioxidant properties (Rüfer et al. 2006).

As mentioned above, "isoflavone aglycons are taken up by enterocytes and converted to glucuronide and/or sulfate conjugates by phase II enzymes before their delivery into the circulation. Enterocytes also express cytochrome P450 (CYP) 3A4 abundantly, as well as CYP2C9 and CYP2C19 at moderate levels and lower or variable amounts of the inducible isoforms CYP1A1, CYP1B1 and CYP2E1. Previous *in vitro* studies have shown that human liver microsomal enzymes catalyze isoflavone 4'-O-demethylation. However, the major hepatic CYP450 isoform, CYP3A4, does not catalyze biochanin A or formononetin demethylation, and recombinant human CYP2C9 and CYP2C19 are only weakly competent to catalyze this transformation. These findings suggest that other CYP450 isoforms, namely the CYP1 family, catalyze the conversion of formononetin and biochanin A to daidzein and genistein, respectively. In fact, human CYP1A2 is the primary hepatic CYP450 isoform responsible for this activity provoking the hypothesis that extrahepatic CYP1 isoforms are also important for isoflavone metabolism" (Roberts et al. 2004).

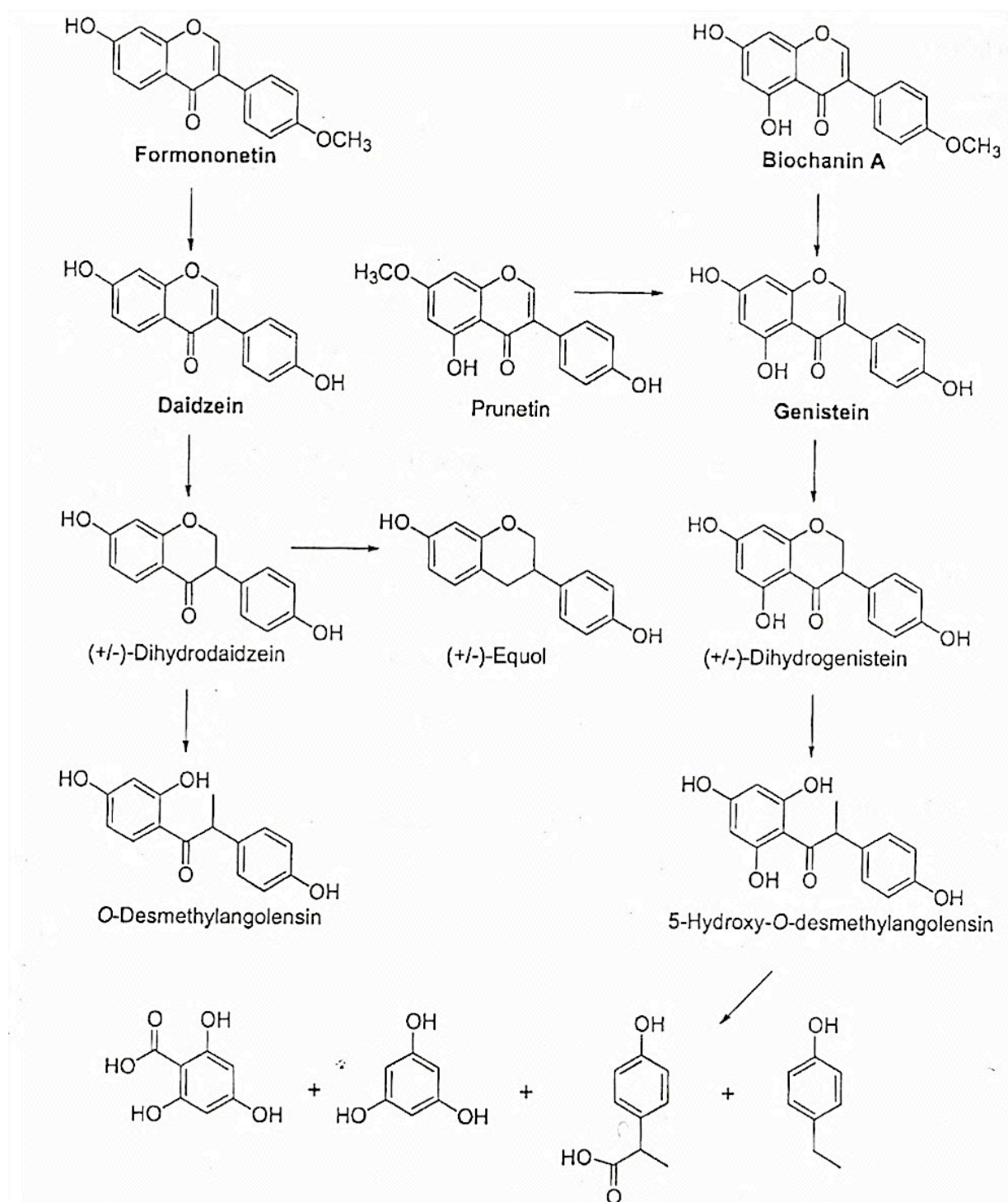


Fig.21. Phase II biotransformation of the main isoflavones in red clover (adapted from Booth et al. 2005).

One of the most important metabolites of red clover, produced by the action of the microflora of the gastrointestinal tract in mammals, is equol. "Several *in vitro* or animal studies indicated that equol is biologically more active than the parent compounds daidzein and formononetin. Only 30–50% of the human population is capable of

converting daidzein to equol". Mustonen et al. (2006) detected, in chiral HPLC analysis of the serum of ewes, solely (*S*)-equol after consumption of red clover silage (Mustonen et al. 2006).

"Despite their reported biological activities in vitro, in several animal models and in limited human studies, isoflavones have poor bioavailability (generally in the range of less than 5%)" (Jia et al. 2004). "After oral intake, free isoflavones (aglucones) are absorbed more quickly than the glycosylated forms which must first be cleaved in the lower small intestine by β -glucosidases. The bioavailability of free isoflavones seems, however, to be slightly lower than that of glycosylated isoflavones. The level of absorption is said to be around 20-55%. The bioavailability of isoflavones, measured as percentage excretion in faeces and urine, varies from individual to individual and is dependant on various variables like gender, processing of the food, food matrix, intestinal flora, passage time and type and amount of dietary fibre ingested" (BfR Expert Opinion 2007).

"Bioavailability of the ingredients in various food supplements containing isolated isoflavones varies considerably. Furthermore, daidzein in isolated form has higher bioavailability than in the natural matrix as soy flour" (BfR Expert Opinion 2007).

Blakesmith et al. (2005) investigated the interaction between macronutrient intake and urinary isoflavonoid excretion in premenopausal women. They found that isoflavonoid excretion was associated with the relative intakes of protein and dietary fibre. Whether manipulation of these macronutrients could enhance efficacy of isoflavone supplements remains to be determined.

In contrast to their low bioavailability, isoflavones have half-lives of three to seven hours. Wang et al. (2006) proposed a hypothesis of a duo recycling scheme, where coupling of efflux transporters and conjugating enzymes (or "enzyme-transporter coupling") in the gut enables enteric recycling. In this study "a rat intestinal perfusion model together with rat intestinal and liver microsomes was used. In the intestinal perfusion model, significant absorption and excretion differences were observed between isoflavones and their corresponding glucuronides" (**Fig.22, p.55**). Prunetin

showed the fastest absorption compared to the other aglycones. Formononetin glucuronides showed the highest excretion rates in the small intestine followed by biochanin A and formononetin. In contrast, the aglycones biochanin A and formononetin displayed the highest absorption in the colon and to a lesser extent in the other three intestine parts.

Moreover, the pharmacokinetics of methoxylated isoflavones demonstrated distinct differences depending on the position of the methoxy group in the molecule. The results of this study suggested that absorption favors the methoxylated derivatives. Genistein and daidzein without methoxy groups are absorbed at the smallest rates in colon and duodenum or are in the group with the smallest amount of absorption in jejunum and ileum. The excretion in the colon was generally postponed compared to the other three regions, and excretion of formononetin glucuronide was the slowest among the six isoflavones in the colon. Analysis showed that the amount of excreted conjugates also relied significantly on chemical structure between these isoflavone types.

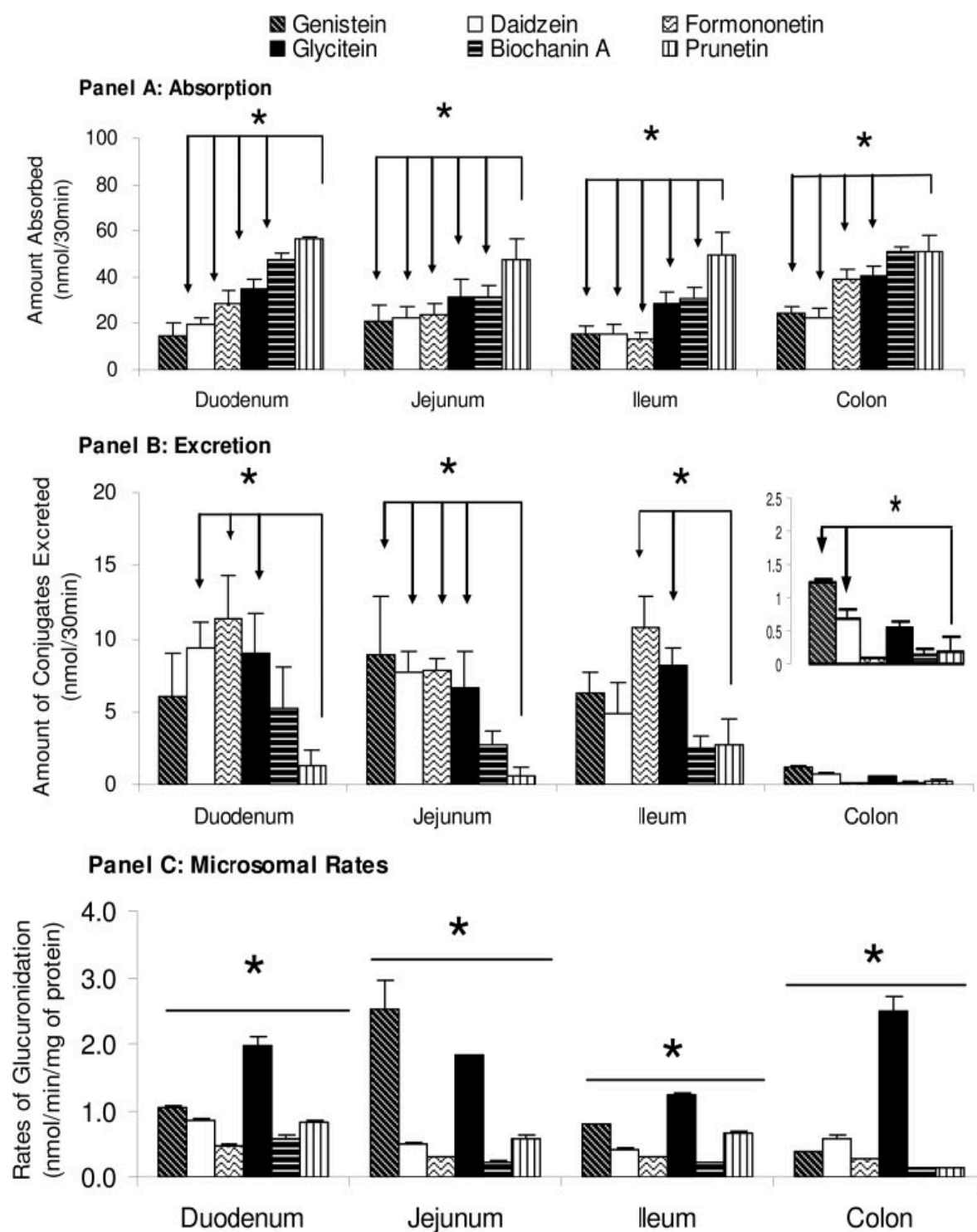


Fig.22. Absorption and metabolism rates of different isoflavones in a rat perfusion model (adapted from Wang et al. 2006).

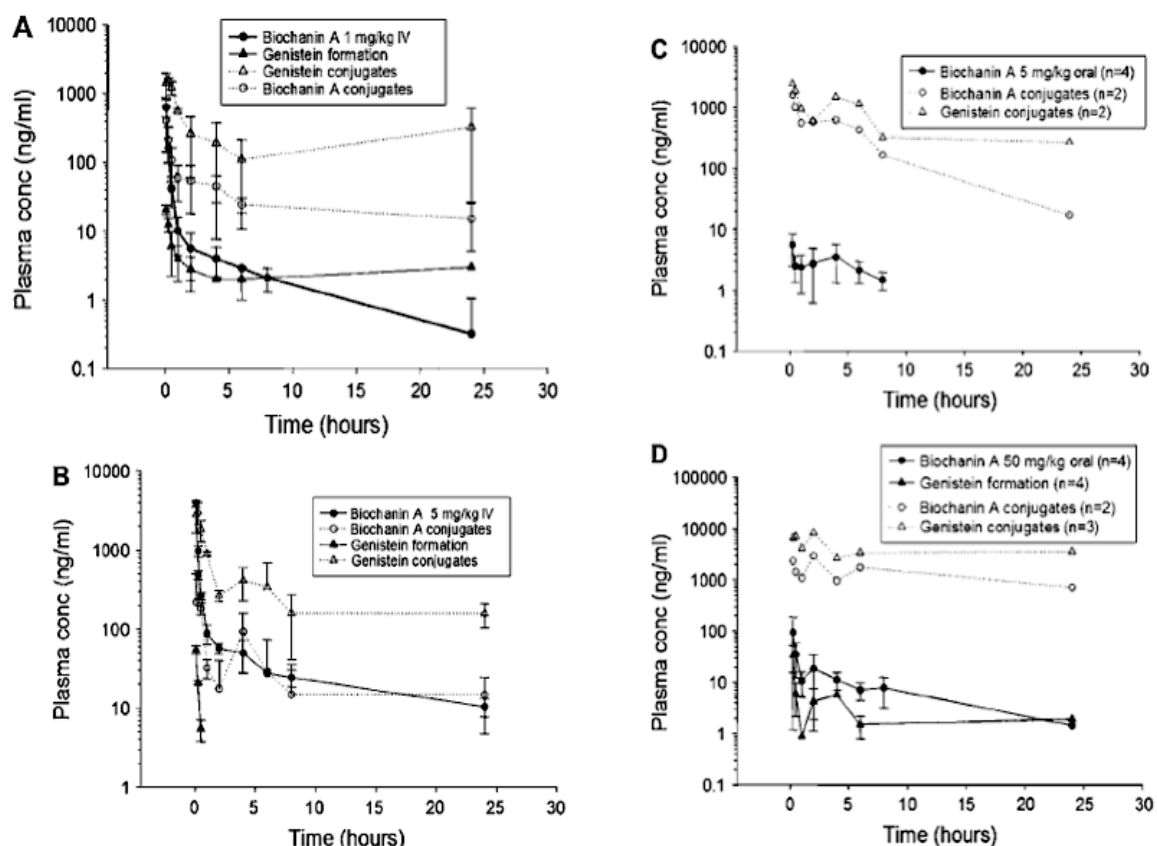


Fig.23. Pharmacokinetic profiles of biochanin A and its metabolites after the i.v. and oral administration. (A) 1 mg/kg i.v., (B) 5 mg/kg i.v., (C) 5 mg/kg oral and (D) 50 mg/kg oral biochanin A administration (modified from Moon et al. 2006).

None or only a slight reentry peak after consumption of 5mg/kg biochanin A was observed after i.v. administration. The reentry peak after intraperitoneal administration of 5mg/kg biochanin A indicated, however, that both the absorption and the enterohepatic recirculation plays a role in biochanin A metabolism. A reentry peak of genistein was also observed after 5 mg/kg i.v. and after 10 mg/kg oral application.

The half-life of genistein of approximately 6-7 hours was comparable with results from other studies. Pharmacokinetic parameters obtained in this study are summarized in **Fig.24 (p.57)** (Moon et al. 2006).

BCA	CL (L/h/kg)	Vd _{ss} (L/kg)	t _{1/2} (h)	C _{max} (ng/mL)	AUC _{0-t} (ng·h/mL)
1 mg/kg IV	4.9 ± 2.3	10 ± 6.5	5.5 ± 3.2	651 ± 112 [†]	258 ± 55 [†]
5 mg/kg IV	2.8 ± 0.4	11 ± 2.5	6.8 ± 1.3	3910 ± 355 [†]	1730 ± 204 [†]
5 mg/kg oral			7.2 ± 4.1	13 ± 16	26 ± 22 [‡]
50 mg/kg oral			7.1 ± 1.4	96 ± 89	151 ± 96 [‡]
GEN	CL (L/h/kg)	Vd _{ss} (L/kg)	t _{1/2} (h)	C _{max} (ng/mL)	AUC _{0-t} (ng·h/mL)
5 mg/kg IV	3.6 ± 1.1	8.2 ± 5.5	5.8 ± 3.0		1450 ± 417
10 mg/kg oral			7.6 ± 7.5	130 ± 47	142 ± 46

*CL indicates total systemic plasma clearance; Vd_{ss}, volume of distribution at steady state; t_{1/2}, elimination half-life; C_{max}, peak plasma concentration; AUC_{0-t} (ng·h/mL), area under the plasma concentration-time curve from time 0 to the last point; IV, intravenous; GEN, genistein.

[†]P < .001

[‡]P < .05

Fig. 24. Pharmacokinetic parameters of biochanin A and genistein obtained by Noncompartmental Analysis (adapted from Moon et al. 2006).

5. Summary

Red clover preparations have gained public interest as a “soft” alternative to conventional synthetic estrogens. A lot of *in vitro* studies as well as clinical trials reported various biological effects of isoflavones in estrogen-sensitive tissues. Moreover, protective antioxidant activities were identified for red clover extracts. The exact mechanism of action of isoflavones is not yet fully understood. During the observation period, a small amount of data focused primarily on safety and efficiency issues of red clover. Long-term safety data, however, are still missing.

In order to perform comparable studies, standardized isoflavone preparations would be a prerequisite. At present, red clover preparations vary considerably regarding isoflavone content and aglyca/glucoside ratio, which might explain partly the variations among different studies. Most of the red clover supplements currently on the market, as opposed to soy preparations, contain mainly aglycones which show faster adsorption in the gut (Beck et al. 2005). Recent studies could demonstrate significant differences between content declared by the manufacturers and study analysis.

Conclusively, red clover preparations might attenuate menopausal symptoms such as hot flushes and vasomotor symptoms but seem to be more effective in combination with other drugs. Undoubtedly, more examinations are still needed to gain a deeper knowledge into their mechanism of action and safe use.

6. Zusammenfassung

Rotklee-Präparate haben als „sanfte“ Alternative zu konventionellen synthetischen Estrogenen öffentliches Interesse geweckt. Sowohl zahlreiche In-vitro-Studien als auch klinische Studien berichten über diverse biologische Wirkungen von Isoflavonen auf estrogen-empfindliches Gewebe. Darüber hinaus wird eine schützende antioxidative Aktivität von Rotklee-Extrakten entdeckt. Der genaue Wirkmechanismus von Isoflavonen ist jedoch noch nicht vollständig erfasst. Während des Beobachtungszeitraums konzentriert sich ein kleiner Teil der Daten hauptsächlich auf die Sicherheits- und Effizienz-Aspekte von Rotklee. Langzeit-Sicherheitsdaten fehlen jedoch noch.

Um Vergleichsstudien durchzuführen zu können, sind standardisierte Isoflavon-Präparate Voraussetzung. Gegenwärtig variieren Rotklee-Präparate stark in ihrem Isoflavon-Gehalt und Aglyka/Glucosid-Verhältnis, was zum Teil die Unterschiede zwischen den verschiedenen Studien erklären könnte. Die meisten Rotklee-Präparate auf dem derzeitigen Markt enthalten im Gegensatz zu den Soja-Präparaten hauptsächlich Aglyka, die eine erhöhte Adsorption im Darm zeigen (Beck et al. 2005). Jüngste Studien konnten wesentliche Unterschiede zwischen den Gehaltsangaben der Hersteller und den Studienanalysen demonstrieren.

Abschließend kann man sagen, dass Rotklee-Präparate menopausale Symptome wie Hitzewallungen und vasomotorische Symptome abmildern könnten, jedoch in Kombination mit anderen Wirkstoffen effektiver zu sein scheinen.

Es ist unbestritten, dass zusätzliche Untersuchungen notwendig sind, um genaue Erkenntnisse über den genauen Wirkmechanismus und die sichere Anwendung gewinnen zu können.

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