Effect of dietary plant extracts on EpRE-dependent transcription

Master Thesis by

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Summary

Numerous epidemiological studies have indicated that a diet rich in plant based foods reduces the risk of several chronic diseases. The beneficial health effects of eating dietary plants have been proposed to be partly mediated by the phytochemicals found within these foods. Several dietary phytochemicals can induce EpRE-dependent transcription which is essential in up-regulation of cytoprotective proteins, and this has been suggested to be one of the mechanisms by which phytochemicals may provide protection against chronic diseases.

Dietary phytochemicals have been postulated to work in synergistic and additive manners, and thus this thesis investigated the effects of combinations of plant extracts on EpRE-dependent transcription *in vitro*. A potent inducer from the *in vitro* studies was furthermore tested *in vivo* in transgenic mice.

Of 11 extract combinations tested *in vitro*, 8 gave a statistically significant induction of EpRE-dependent transcription, and 7 of these were also statistically significantly higher than one or both of the separately added extracts. Most of the extract combinations appeared to exert additive effects, although some showed a trend toward a more synergistic or antagonistic effect. One of the combinations with the strongest EpRE induction and a trend towards synergistic effect was tested in a wider range of concentrations. In this extended experiment, trends towards antagonistic effects were observed.

A rosemary extract was used for an *in vivo* experiment with EpRE-LUC mice. For the mice given rosemary, the EpRE-dependent luciferase activity measured by *in vivo* imaging was significantly higher than the control mice 5h after gavage feeding. Furthermore, a trend towards increased EpRE activity in the intestine was found for the extract group compared to the control group.

Based on these results, further studies on effects of combinations of dietary plants on EpRE-dependent transcription and dietary effects of EpRE activity *in vivo* are warranted.

Contents

List of abbrevations

ABCC2	ATP-binding cassette, sub-family C, member 2
AMP	Adeninemonophosphate
ARE	Antioxidant responsive element
ATP	Adeninetriphosphate
AUC	Area Under Curve
BHA	2(3)-tert-butyl-4-hydroxyanisol
BTB	bric-a-brac, tramtrack, broad complex
bZIP	Basic Leuzine Zipper
CAT	Catalase
CBP	Cyclic AMP response element Binding Protein
CNC	Cap'n'Collar
CTR	Carboxyl terminal region
DAS	Diallyl sulphide
DC	Double glycine repeat (DGR) + Carboxyl terminal region (CTR)
DGR	Double glycine repeat
DMSO	Dimethylsulphoxide
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay
EpRE	Electrophile responsive element
ERK	Extracellular signal-regulated protein kinase
FRAP	Ferric Reducing Ability of Plasma
Gly	Glycine
GSH	Glutathione
GP	Glutathione Peroxidase
GR	Glutathione reductase
GST	Glutathione sulphotransferase
HCl	Hydrochloric Acid
HO-1	Heme oxygenase-1
$HO_2^{\cdot}$	Hydroperoxyl
H_2O_2	Hydrogen Peroxide
HNO_2	Nitrous Acid
HPLC	High performance liquid chromatography
IVR	Intervening region
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH associated protein-1
LB-medium	Luria-Bertany Broth - medium
LDH	Lactate Dehydrogenase
LUC	Luciferase
Maf	Musculoaponeurotic fibrosarcoma
MAPK	Mitogen Activated Protein Kinase
MEM	Minimum Essential Medium Eagle
MeOH	Methanol

mRNA	Messenger RNA
MQ	MilliQ
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
Neh	Nrf2-ECH homology
NF-κB	Nuclear Factor kappa B
$NO^{\cdot}$	Nitric Oxide
$NO_2^{\cdot}$	Nitric Dioxide
$NO_3^{\cdot}$	Nitrate Radical
NQO1	NADPH:quinine reductase
Nrf2	Nuclear factor E2 related protein-2
NTR	Amino terminal region
$OH^{\cdot}$	Hydroxyl Radical
O_2^{-}	Superoxide Anion
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate Buffered Saline
Phe	Phenylalanine
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	phorbol-12-myristate-13-acetate
POZ	Pox virus and Zink finger
Redox	Reduction/Oxidation
RLU	Relative Luminescence Unit
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen species
Rpm	Revolutions per minute
SEM	Standard error of the mean
SOD	Superoxide Dismutase
bBHQ	tert-butylhydroquinone
TEAC	Trolox Equivalent Antioxidant Capacity
TPTZ-Fe^{3+}	Ferric tripyrdyltriazine
Trp	Tryptophane
Trx	Thioredoxin
UGT	UDP-glucoronosyl transferase
UV	Ultra violet

1. Introduction

1.1 Diet and health

The diet is an important factor in the promotion and maintenance of a good health. Diet-related disorders range from neural tube defects in the unborn child, to age-related chronic diseases such as heart disease, cancer, hypertension, stroke, and type II diabetes (1;2). In the western world, classical deficiency symptoms caused exclusively by malnutrition occur rarely today (1;2). The prevalence and incidence of dietary related chronic diseases however, are increasing (2).

According to the World Health Organization (WHO), chronic diseases are the leading cause of death today, representing 60 % of all deaths (2). In 2005, 30 million people died from chronic diseases, and without any action the deaths from chronic diseases are expected to increase by 17 % over the next ten years (3). Due to the significant impact of the diet on the risk of developing chronic diseases, the diet is one of the most important modifiable factors in the globally increasing burden of chronic diseases. WHO has therefore stated that the strategies and policies that are applied in prevention of chronic diseases must fully recognize the essential role of the diet (2).

Dietary plants and health

Epidemiological studies have indicated that a high consumption of fruits, vegetables and whole grains is strongly associated with reduced risk of developing chronic diseases such as cancer, cardiovascular disease, diabetes, cataracts and Alzheimer disease (4-7). A diet rich in fruits, vegetables and whole grains may provide health benefits beyond basic nutrition, and a healthy diet seemingly depends on more than meeting the nutritional recommendations for macro-and micronutrients (8). A long with recommendations for a diet well balanced in macro- and micronutrients, a high consumption of fruit and vegetables has therefore been emphasized as a global priority in the prevention of chronic diseases (7).

In addition to important nutrients, such as vitamin C, vitamin E, folate, dietary fibre and different trace metals, dietary plants also contain several non-nutrient bioactive compounds such as phytochemicals (9). Several phytochemicals have been shown either in epidemiological or *in vitro* studies to have potential health benefits , and it is widely believed that phytochemicals are contributing to the beneficial health effects of a diet high in fruits and vegetables (7). A number of phytochemicals have been isolated and used as dietary supplements. It however appears as though the beneficial health effect observed for whole dietary plants can not be directly reproduced with one or a few of these pure compounds. Although observational studies suggests that people with a high intake of fruit and vegetables containing β-carotene have a lower risk of developing cancer and cardiovascular disease, randomized, double-blind and placebo controlled trials with β-carotene supplements have not supported these findings (10). It has therefore been suggested that several compounds are contributing simultaneously to the beneficial health effect of dietary plants. These beneficial effects can thus not be found when using only one, or a few, isolated compounds from dietary plants.

Despite the relatively consistent relationship between a high consumption of dietary plants and reduced risk of chronic diseases, the mechanisms behind the beneficial health effect of dietary plants are not fully elucidated. During the past decade, there has however been a rapid expansion of a number of scientific methods that are used to study molecular mechanisms by which dietary plant compounds may affect human physiology. This will contribute to extended knowledge of the mechanism behind the beneficial health effects of dietary plants, and possibly lead to more specific recommendations with respect to intake of dietary plant foods.

1.2 Dietary phytochemicals

Phytochemicals is a collective term for a variety of plant components found to have important functions in plants (7). Phytochemicals provides colour and flavour, and exert protection against herbivores, pathogens, ultra violet (UV) radiation in dietary plants (11). Numerous phytochemicals have been identified, several with strong antioxidant capacity, and a wide range of these compounds may be provided by one serving of vegetables (9).

Phytochemicals (other than the vitamins) are not essential to humans, and are thus generally defined as non-nutrient compounds (12). Due to their protective functions in plants however, phytochemicals have been linked to the beneficial health effect of a diet high in dietary plants (8;13).

1.2.1 Classification

The phytochemicals are classified based on to their chemical structure and functional characteristics (7). According to Liu, the main classes are carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulphur compounds **(Figure 1.1)** (14).

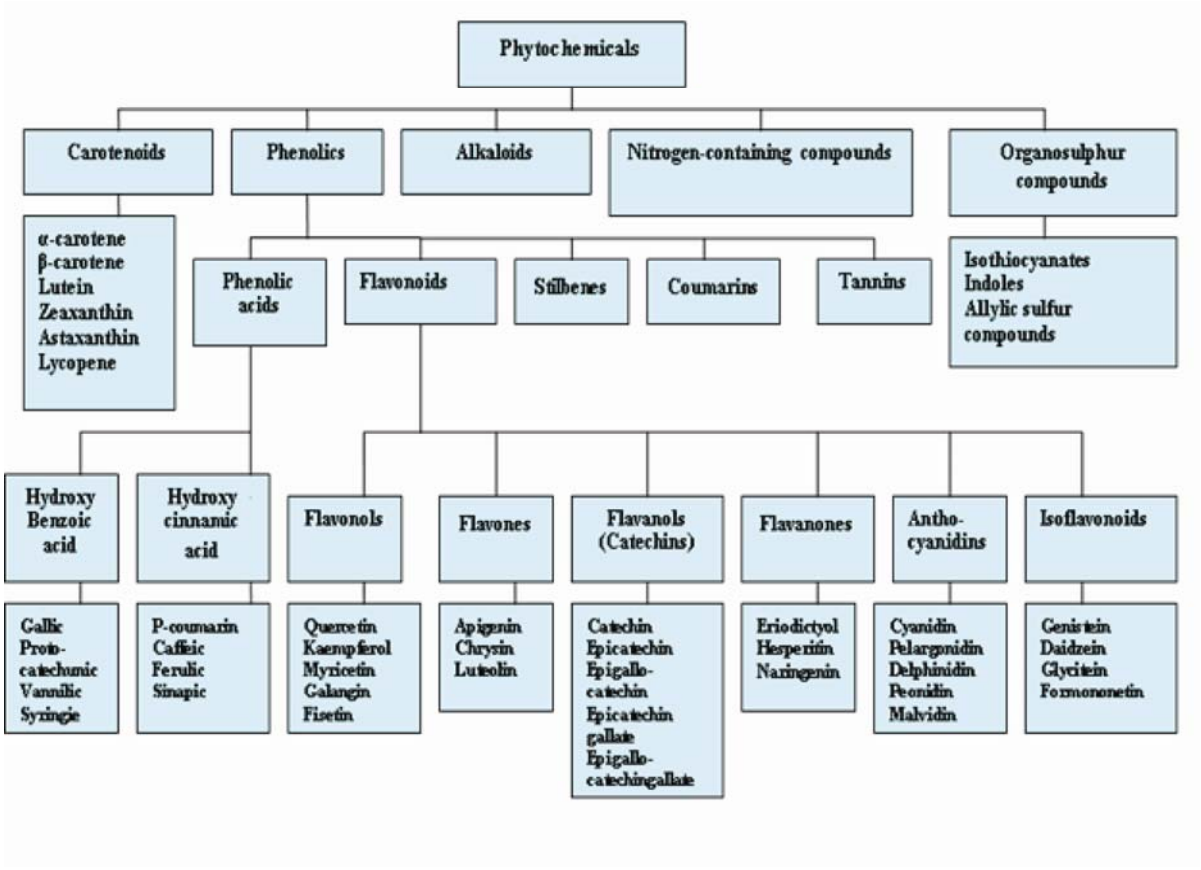


Figure 1.1 Overview of the phytochemical subdivisions (adapted from Liu (14))

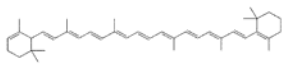
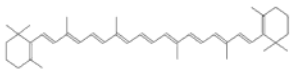
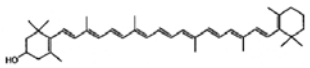
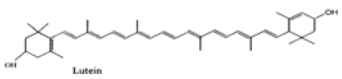
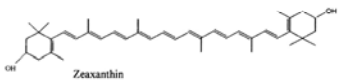
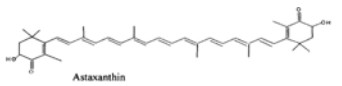
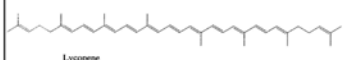
Carotenoids

The carotenoids are naturally fat-soluble pigments, which have received great attention because of their pro-vitamin A and antioxidant function (14). The subclasses, chemical structure, and sources of the most common carotenoids are presented in **Table 1.1**. The α-carotene, β-carotene, and β-cryptoxanthin can function as pro-vitamin A. Lutein and zeaxanthin are the major carotenoids in the macular region of the retina in humans (14), while astaxanthin is the pigment providing certain fish and crustaceans, *e.g.* salmon and lobsters, with their reddish color (15).

Nearly a thousand naturally-occurring carotenoid pigments have been identified, occurring in plants, microorganisms and animals (16). Their structure contains conjugated double bonds, which enable them to function as pigments (14). The conjugated structure also enables the carotenoids to quench singlet oxygen by transferring the excitation energy from $^1O_2$ to a carotenoid, leaving the oxygen at a

ground state and dissipating thermal energy to the surroundings to restore the ground state carotenoid (17). This provides photosynthetic systems with protection against oxidative damage, and it has further been suggested that carotenoids may also exert protection against damage by UV-light in the humans (18;19). The structure also enables the carotenoids to be incorporated within lipid membranes and proteins (20), and carotenoids may thus modify the fluidity and reduce oxidative degeneration of these (16). Carotenoids may however also function as pro-oxidants under certain conditions, and the effect of carotenoids as antioxidants/pro-oxidants needs further investigation (21). Specific carotenoids have further been suggested from epidemiological studies to have a cancer protective role, such as lycopene indicated to reduce the risk of prostate cancer (22).

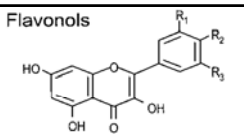
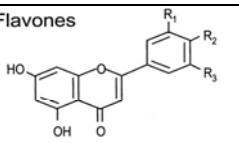
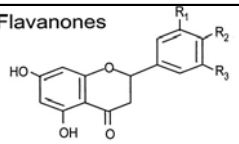
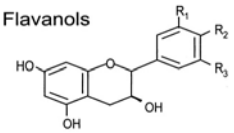
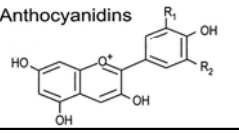
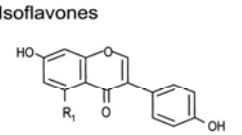
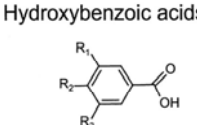
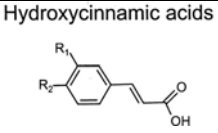
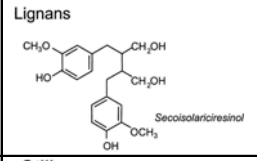
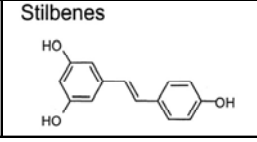
Table 1.1 Carotenoids (14;15;23;24)

Carotenoids		
Compound	**Chemical structure**	**Dietary Sources**
α- Carotene		Carrots, red pepper, banana, green pepper, apricot, avocado
β- Carotene		Orange vegetables and fruits (carrots, sweet potatoes, winter squash, pumpkin, papaya, mango, cantaloupe)
β- Cryptoxanthin		Papaya, red pepper, yellow watermelon, mango, guava, watermelon, oranges
Lutein		Pea, kale, parsley, spinach, red pepper, cress, basil, endive, lettuce, leek, broccoli
Zeaxanthin		Red pepper, cornflakes, green pepper
Astaxanthin		Algae, yeast, crustacean byproducts, farmed fish feeds (synthetic)
Lycopene		Tomatoes and tomato products, red watermelon, papaya, guava, mango, pineapple, banana, pink grapefruits, apricots

Phenolics

Phenolics are products of secondary metabolism in plants, in which they appear to act as defense mechanisms and contribute to the color (14;25). The phenolics comprise a wide variety of complex molecules with both monophenol and polyphenol structure, generally categorized according to the number of phenol rings and the structural elements that bind these rings together (14) **(Table 1.2).**

Table 1.2 Phenolics (14)

Phenolics				
Class	**Group**	**Chemical structure**	**Examples (See also figure 1.1)**	**Dietary Sources**
Flavonoids	Flavonols		Quercetin Kaempherol Myricetin Galangin Fisetin	Onion, blue berries broccoli, tea, red wine
	Flavones		Apigenin Chrysin Luteolin	Parsley, celery, cereals, citrus fruit skin
	Flavanones		Eriodictyol Hesperitin Naringenin	Citrus fruits, tomatoes, mint plant
	Flavanols		Catechins	Green tea, chocolate, fruits (apricots especially), wine
	Anthocyanidins		Cyanidin Pelargonoidin Delphinidin Malvidin	Berries, red wine, red cabbage, red onions, radishes
	Isoflavones		Genistein Daidzein Glycitein Formononetin	Leguminous plants; soy products
Phenolic acid	Hydroxybenzoic acid		Gallic acid	Red fruits, black radish, onions, tea
	Hydrocinnamic acid		*p*-coumaric acid Sinapic acid Cafeic acid Ferulic acid	Coffee, blueberry, kiwi, cherry, plum, apple, grains
Lignans			Matairesinol Secoisolariciresinol	Linseed, cereals, grains, fruits (pears, prunes), vegetables (garlic, aspargeous, carrots)
Stilbenes			Resveratrol	Grapes, red wine.

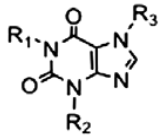
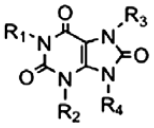
A wide range of phenolics are found within dietary plants, and the phenolic content of several food items have been published and reviewed (26;27). The main sources of polyphenols are fruit and beverages such as tea, coffee and red wine. Whereas some phenolics are specific to particular foods (*e.g.* isoflavones in leguminous fruit), others are found in many types of plant products (quercetin in juice, vegetables, cereals etc.) (28). It has been estimated that two thirds of consumed phenolics are provided by the flavonoids, while the remaining one third is provided by phenolic acids (14).

Epidemiological studies have indicated that phenolics may protect against major chronic diseases, such as cardiovascular disease (24), diabetes type II (29), cancer of the gastrointestinal tract (30), breast, and prostate (31), in addition to improving brain function (32).

Alkaloids

The alkaloids are nitrogen-containing secondary metabolites derived from amino acids, which are assumed to contribute to plants defence against herbivores and pathogens (33). Alkaloids occur in 20 % of plant species (34;35), and have been investigated as pharmaceuticals, stimulants, narcotics and poisons (33). Some of the most studied alkaloids are the purine alkaloids methylxanthines and methyluric acids, found in tea, coffee and other non-alcoholic beverages (36) **(Table 1.3)**.

Table 1.3 Alkaloids (36)

Alkaloids				
Class	**Group**	**Chemical structure**	**Compound**	**Dietary Sources**
Purine alkaloids	Methylxanthines		Theophyllin	Tea, maté
			Paraxanthin	Coffee
			Theobromine	Cacao, tea, coffee, maté
			Caffeine	Coffee, guarana, tea, mate, citrus fruit
	Uric acids and methyluric acids		Theacrine	Chinese tea (kucha)
			Liberine	Coffee, tea
			Methylliberine	Coffee

Caffeine, a central nervous stimulant (37), has been offered special research attention. Many diseases have been associated with caffeine and coffee consumption in previous epidemiological studies (*e.g.* psychiatric diseases, coronary heart disease, and pancreatic cancer) (37). Recent studies however, have suggested that previous studies may have been confounded, and data on potential health benefits of coffee in cancer-prevention have increased during the last decades (38). To what extent caffeine may contribute to this effect is however not fully elucidated.

Nitrogen-containing compounds

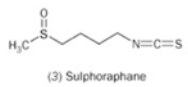
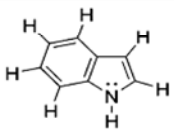
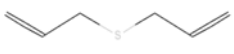
The nitrogen-containing compounds within foods have been relatively poorly investigated. Such compounds have been extracted from different plant species used in herbal medicine, and are considered by some to be the bioactive compounds behind the potential health effect of these medicines (39). Nitrogen-containing compounds such as indole-3-carbinol, can be found in cruciferous vegetables, a vegetable group associated with a decreased incidence of cancer in humans (40) **(Table 1.4)**.

Organosulphur compounds

Organosulphur compounds are naturally occurring sulphur containing compounds, found in foods such as garlic, mustard and cruciferous vegetables (41;42). Some of the organosulphur compounds have a sharp taste which provides the characteristic taste of their respective vegetables **(Table 1.4)**.

Epidemiologic studies have indicated a beneficial health effect of the allium family of vegetables containing garlic and onion, and this has been coupled to their organosulphur compounds (43). The organosulphur compounds within garlic have been found to have beneficial effects *in vitro* as inhibitors of platelet aggregation (41) and tumour cell proliferation (42). Isothiocyanates, the dominating sulphur-containing components of mustard, horse raddish and some cruciferous vegetables, have also been proposed and investigated as potentially chemopreventive compounds (reviewed in (44)).

Table 1.4 Organosulphur and nitrogen-containing compounds (14;41;42)

Organosulphur and nitrogen-containing compounds			
Class	**Chemical structure**	**Compound**	**Dietary Sources**
Isothiocyanates	 (3) Sulphoraphane	Phenethyl isothiocyanate	Cruciferous vegetables
		Sulforaphane	Cruciferous vegetables
Indoles		Indole-3-carbinol	Cabbage, kale, brussels sprouts, rutabaga, mustard greens
Allylic sulphur compounds	 **Allyl sulfide**	Diallyl sulfide	Garlic, onions, leeks, chives, shallots
		Diallyl disulfide	Garlic
		Diallyl trisulfide	Garlic
		Ajoene	Garlic
		S-allyl cysteine	Garlic, onions
		S-ethyl cysteine	Garlic, onions
		S-propyl cysteine	Garlic, onions

1.2.2 Bioavailability of phytochemicals

Bioavailability describes the amount of an ingested compound that reaches the systemic circulation (12) **(Figure 1.2)**. Bioavailability is therefore an essential aspect when studying potential health effects of dietary phytochemicals.

Many factors affect the bioavailability, and these factors can be divided into two distinct categories. The first category includes exogenous factors, such as the complexity of the food matrix, the chemical composition of the food, and other compounds consumed at the same time. The second category includes endogenous factors such as the rate of gastric emptying, intestinal transit time, amount of enzymes in the intestine, and the metabolism of the compounds (12).

A prerequisite for any compound to be bioavailable is that they are bioaccessible from the intestinal lumen (12). Bioaccessibility is defined as the amount of the substance that is potentially absorbable from the intestine, and can be highly influenced by the food matrix, co-ingested compounds, food processing and gastric and luminal digestion. The bioaccessibility of carotenoids for example have been shown to be greatly improved by cooking and pureeing (45), and, due to their fat-soluble characteristic, by addition of oil to the meal (46). Phytochemicals further occur in a large number of structurally different compounds, of which several need to be modified before absorption (12). Phenolics are commonly present in food as esters, glycosides, or polymers, and need to be hydrolyzed by food processing, intestinal enzymes, or the colonic microflora to be absorbed.

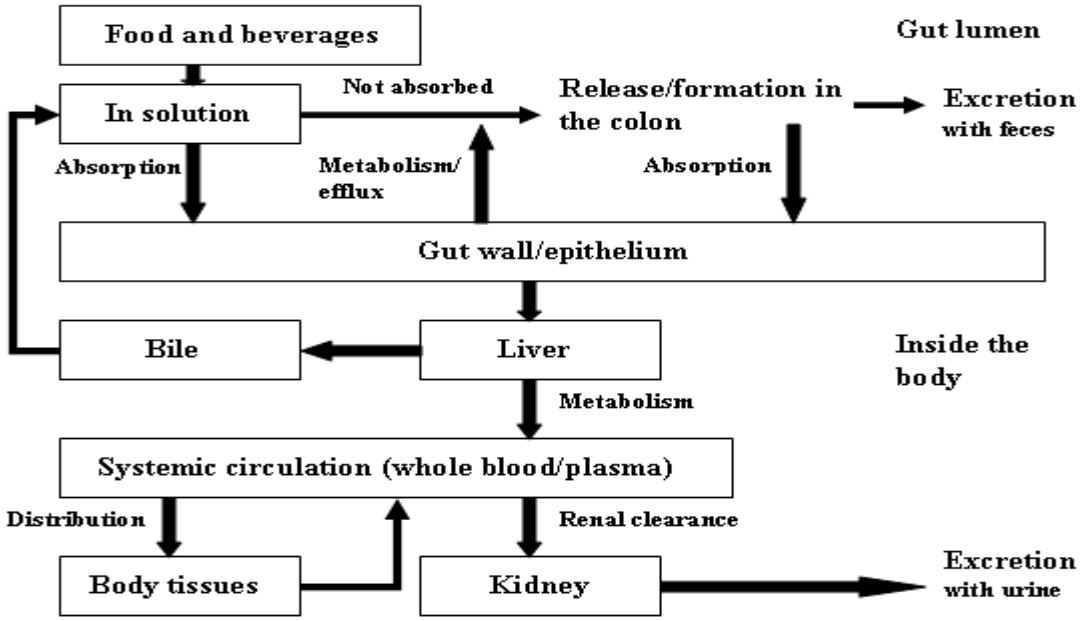


Figure 1.2 Model of the absorption and distribution of foods (modified from Holst and Williamson (12)).

Compared to most nutrients, the bioavailability of phytochemicals is relatively low (12). This is partly due to the fact that phytochemicals are recognized and handled by the body as xenobiotics (47). Although the liver was assumed to be the main site of xenobiotic metabolism originally, the small intestine has been found to have a significant capacity of xenobiotic metabolism and efflux (*e.g.* ABCC2 and *p*-glycoprotein) (12). Metabolism and efflux in the small intestine may therefore limit the absorption of dietary phytochemicals significantly (48). Absorbed phytochemicals may be further modified by xenobiotic metabolism after absorption, thus the molecular characteristics of phytochemicals found in the circulation is often quite different from the molecular forms found in food (49). Phenolics may be modified into conjugates (*e.g.* sulfates and glucuronates) of the original parent aglycone (47). The biological activity of these metabolized compounds may be increased, but appears more often to be decreased (12).

The phytochemicals that are not absorbed in the small intestine reach the large intestine where they may be hydrolyzed by the microflora (12). These metabolites

can be absorbed, and the bioavailability of certain phytochemicals actually seems to depend on the individual composition of the colon microflora (*e.g.* daidzein) (50).

A significant amount of consumed phytochemicals will not be absorbed. This fraction may however still exert beneficial health effects through its presence in the intestine, as local effects in the large intestine have been suggested to influence the "systemic health" (12;51). This may occur by elimination of reactive species present in the large intestine (52), and several studies have shown that dietary phytochemicals may reduce peroxidation of polyunsaturated lipids in the gastrointestinal tract (reviewed in (51)).

As for the absorption and metabolism, the elimination of phytochemicals follows the same route as xenobiotics. The elimination mainly occurs through two pathways; via bile or urine (12). For extensively conjugated metabolites, the elimination is more likely to be through the bile, while small conjugates are excreted through the urine (49).

1.3 Genetic regulation

Nutrients and gene regulation

Nutrients may affect cellular signalling systems that regulate gene expression through different transcription factors (53). In addition to the relatively well established influence of vitamin A and D, dietary compounds such as fatty acids (54), glucose (55), and several phytochemicals (56), have been shown to influence gene expression. The most studied nutrients and their target transcription factors are presented in **Table 1.5**.

Table 1.5 Transcription factors influenced by nutrients (adapted from Muller *et al.* (53))

Transcription-factor pathways mediating nutrient-gene interactions		
Nutrient	**Compound**	**Transcription factor**
Macronutrients		
Fats	Fatty acids	PPARs, SREBPs, LXR, HNF4, ChREBP
	Cholesterol	SREBPs, LXRs, FXR
Carbohydrates	Glucose	USFs, SREBPs, ChREBP
Proteins	Amino acids	C/EBPs
Micronutrients		
Vitamins	Vitamin A	RAR, RXR
	Vitamin D	VDR
	Vitamin E	PXR
Minerals	Calcium	Calcineurin/NF-ATs
	Iron	IRP1, IRP2
	Zinc	MTF1
Other food components		
	Flavonoids	ER, NFkB, AP1
	Sulphoraphane	Nrf2
	Xenobiotics	CAR, PXR

AP1, activating protein 1; CAR, constitutively active receptor; C/EBP, CAAT/enhancer binding protein; ChREBP, carbohydrate responsive element binding protein; ER, oestrogen receptor; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; IRP, iron regulatory protein; LXR, liver X receptor; MTF1, metalresponsive transcription factors; NFkB, nuclear factor κB; NF-AT, nuclear factor of activated T cells; Nrf2, Nuclear factor E2 related protein 2; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SREBP, sterol-responsive-element-binding protein; USF, upstream stimulatory factor; VDR, vitamin D receptor.

The influence of nutrients on gene expression has been suggested to have a significant role in the beneficial health effects of dietary plants (53). It is therefore a growing realization that we need to understand the effects of nutrition at the molecular level. The *nutrigenomics* has been developed as a new branch in nutritional research to study the genetic influence and cellular response of nutrients (53). Together with analysis of the proteome (proteomics) and the metabolome (metabolomics), nutrigenomics aims to increase the understanding of how nutrients influence the whole organism, and how such influences can affect the health. To understand the relationship between nutrients and genetic transcription however, basic gene regulation needs to be considered.

Basic gene regulation

The nucleotides along the DNA strand codes for different genes. A gene is generally defined as a DNA segment that contains the instructions for making a particular protein (57). Genes consist of two functional parts: the coding region, containing the information needed to produce the gene-product; and the promoter, regulating the expression of the gene.

The expression of genes can be regulated in many ways **(Figure 1.3)** (58), primarily at five different levels: (i) transcriptional control, (ii) during splicing and processing of the primary RNA transcript, (iii) during transport of the mRNA out of nucleus, (iv) during translation of the mRNA, or (v) by activation/inactivation of proteins.

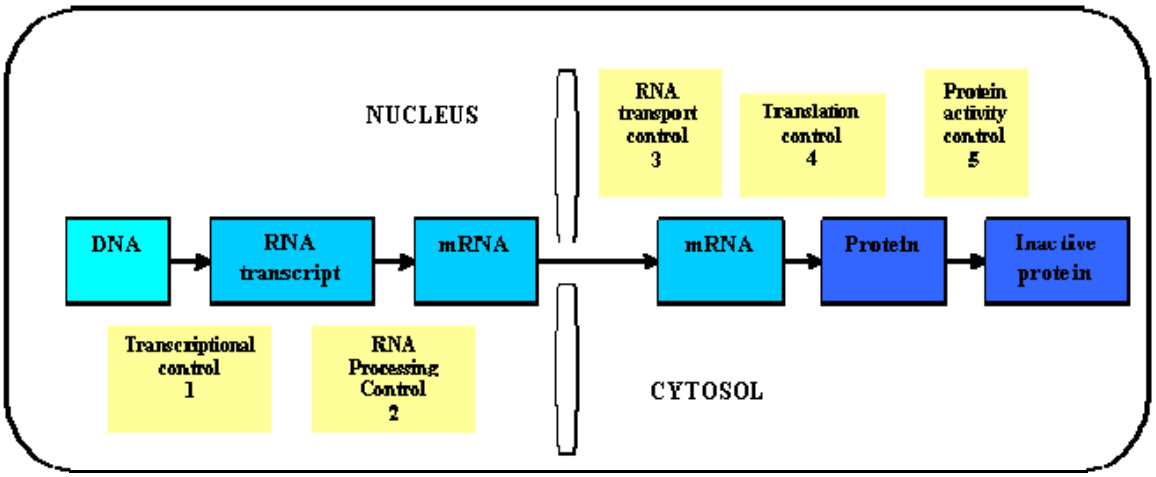


Figure 1.3 Overview of the regulation of gene expression

The promoter includes an initiation site, where the transcription actually starts, sites where general transcription factors bind, and a sequence approximately 50 bases upstream with sites where the RNA polymerase II initially attaches. In order to aid the assembly of general transcription factors and RNA polymerase onto chromosomal DNA, nearly all eukaryotic promoters require activator proteins. The DNA sites where these factors are bound are called “enhancers”, and these sites can reside thousands of nucleotide pairs away from the promoter. By looping and using other proteins as adaptors, the DNA allows the proteins to influence the events that take

place at the promoter. The repressor proteins however, prevent the assembly of the transcription complex and thus inhibit transcription.

Gene activator and repressor proteins also exploit the chromatin structure to turn genes on or off. A tightly packed DNA will hamper access of the "transcription machinery" to the promoters, and nucleosomes may inhibit transcription if they are positioned over a promoter. By chromatin remodelling complexes and modification of the histone proteins, cells can alter the chromatin structure and render the DNA more or less accessible. Gene regulating proteins may utilize such mechanisms to alter the chromatin structure and allow greater (activator proteins) or less (repressor proteins) accessibility to the underlying DNA, resulting in an increased or reduced assembly of the general transcription factors and RNA polymerase at the promoter (58).

In this thesis we study whether genetic regulation of the cytoprotective mechanisms of the body, such the antioxidant defence and detoxifying enzymes, can be affected by components from dietary plants.

1.4 Cytoprotective mechanisms

1.4.1 The antioxidant defence

Our oxidative energy production takes place within the mitochondria (59). In this process, reactive oxygen species (ROS) are generated as byproducts. ROS, along with reactive nitrogen species (RNS) **(Table 1.6)**, are also produced by other endogenous processes, such as inflammation, and may further be generated by exogenous stimuli, such as UV radiation, chemotherapeutic agents, hyperthermia and growth factors (60).

Table 1.6 Examples of reactive oxygen and nitrogen species (59).

Reactive Oxygen Species
Superoxide, $O_2^{\cdot-}$
Hydroxyl, $OH^{\cdot}$
H_2O_2
Hydroperoxyl, $HO_2^{\cdot}$ (protonated superoxide)
Reactive Nitrogen Species
Nitric oxide, $NO^{\cdot}$
Nitrogen dioxide, $NO_2^{\cdot}$
Nitrate radical, $NO_3^{\cdot}$
Nitrous acid, HNO_2

Reactive species play a dual role within the organism: they have beneficial effect, *e.g.* as defence against infectious agents, while they may also cause oxidative stress and be harmful (61). Their toxicity owes to their ability to react with molecular components within the cell, generating a radical chain reaction. This may cause loss of function of proteins, brakes and cross-linking of DNA, and peroxidation of lipids which affects the membrane fluidity (59). An overproduction of ROS and RNS may thus result in oxidative damage (61). Oxidative damage accumulates during life, and has been coupled to a number of age-dependent chronic diseases, such as cancer, arteriosclerosis, arthritis, and neurodegenerative disorders (59;61).

Due to the damaging effects of reactive oxidative and nitrogen species, an effective antioxidant defence system has evolved. This defence is constituted by enzymatic and non-enzymatic components. The enzymatic system consists of superoxide dismutase (SOD), glutathione peroxidase (GP), glutathione reductase (GR), and catalase (CAT); while the non-enzymatic system consists of thioredoxin (Trx), lipoic acid, α-tocopherol (vitamin E), ascorbic acid (vitamin C), selenium, glutathione (GSH), ferritin, albumin, transferrin, lactoferrin, ceruloplasmin, uric acid, and other antioxidants (60;61). The enzymatic antioxidant defence is mainly found intracellularly: SOD is found in the mitochondria and cytosol, GP in the cytosol and mitochondria, and CAT in the peroxisomes (60). For the non-enzymatic compounds however, some are found both intra- and extracellular (*e.g.* ferritin, α-tocopherol, ascorbic acid, GSH, lipoic acid, and other antioxidants); others are found largely

extracellular (*e.g.* transferrin, lactoferrin, ceruloplasmin, and uric acid); and some are mainly intracellular (*e.g.* Trx) (60;61).

Dietary antioxidants

Some dietary compounds exert antioxidant properties; for example ascorbic acid, tocopherols, and several phytochemicals (56). Some phytochemicals have further been shown to induce the endogenous antioxidant defence (*e.g.* sulphoraphane and curcumin), thus exerting antioxidant function in a more indirect way (62). The latter way may be especially efficient since it involves up-regulation of proteins and enzymes that are not consumed in the redox reaction, have long half-lives, and catalyze chemical reactions that lead to detoxification (62). Dietary compounds that both scavenge reactive species directly and also up-regulates the endogenous antioxidant defence, may provide extensive protection against oxidative damage (62). Due to the correlation between oxidative damage and disease, the wide variety of compounds with antioxidant properties in dietary plants has been proposed as one of the mechanisms behind the beneficial health effects of dietary plants (63).

1.4.2 Xenobiotic metabolism

To minimize potential injury caused by xenobiotics that are introduced into the human body, compounds perceived as xenobiotics undergo a sequential three-step metabolism (64):

The first step is directed by the phase I enzymes. These enzymes consist primarily of the cytochrome P450 super-family, and catalyze reactions that introduce functional groups into hydrophobic organic molecules (65). In most cases the reaction products are inert metabolites, but sometimes the phase I enzymes produce highly reactive products harmful to biological molecules (*e.g.* strong electrophilic compounds) (62). The second step is directed by the phase II enzymes. These enzymes consist of several conjugating enzymes, such as glutathione S-transferase (GST), NADPH:quinone oxidoreductase (NQO1) (66) and UDP- glucuronosyltransferase (UGT)) (64). Conjugation of xenobiotic compounds by phase II enzymes generally

increases their hydrophilicity and enhances their excretion in the bile and/or the urine. The phase II enzymes thus exert a detoxifying effect. The xenobiotic metabolism has also been added a third step, called phase III, which consists of the efflux transporters (*e.g. p*-glycoprotein) that export the final metabolites.

Damaging molecules produced by phase I metabolism can potentially be detoxified by phase II metabolism (62). The consequences of such damaging compounds may therefore depend on the balance between the phase I and phase II enzymes (67). Some carcinogens have been found to enter the body as unreactive pro-carcinogens dependent on phase I enzymes to be converted into highly reactive carcinogens (67). The consequences of these carcinogens may thus rely on the efficacy of the phase II enzymes, and carcinogen treated GST-knock out mice have correspondingly been shown to develop significantly more tumours than wild type littermates (68).

Phase II enzymes have been found to be up-regulated by several different compounds (67). Induction of phase II enzymes has received great attention with respect to chemoprevention, and several chemopreventive compounds have been suggested to exert their chemoprevention by inducing phase II enzymes (for a review (69)). Oltipraz for example, a synthetic compound shown to have chemoprotective activity against several classes of carcinogens, has been shown to exert chemoprevention in mice by induction of phase II enzymes (70).

1.4.3 EpRE-dependent transcription of cytoprotective proteins

Together with the antioxidant defence, the phase II enzymes provide eukaryotic organisms with protection against damaging compounds such as oxidants and electrophiles (62). Interestingly, with respect to the preventive role of both of these systems, the inducers of phase II enzymes have been found to induce several components of the endogenous antioxidant defence as well (62). It has therefore been suggested that the components of these two cytoprotective systems should collectively be referred to as cytoprotective proteins (62).

As up-regulation of cytoprotective proteins may counteract the damaging effects of oxidants and electrophilic compounds, induction of these proteins may to be an effective means in the prevention of chronic diseases (62). Potent inducers have been found, and a special characteristic of these inducers was revealed by Talalay *et al*. (71), showing that the majority contain, or acquire by metabolism, an electrophilic centre.

Inducers of cytoprotective proteins may be divided into two main classes: The first class is called bifunctional inducers, and consists of planar aromatic compounds, *e.g.* polyaromatic hydrocarbons (PAH) and flavonoids, that up-regulate certain phase I enzymes in addition to cytoprotective proteins. The second class is called monofunctional inducers, and consists of compounds (*e.g.* sulphoraphane, cinnamates and coumarins), which exclusively up-regulate cytoprotective proteins. Due to the potential activation of pro-carcinogens by phase I enzymes, the monofunctional inducers have been assumed to provide more beneficial health effects than the bifunctional inducers (67).

In studies trying to elucidate the molecular mechanisms by which the monofunctional inducers up-regulate cytoprotective proteins, the 5' flanking region of the rat (72) and mouse (73) GST Ya subunit gene was found to contain a similar 41-bp DNA fragment. The DNA fragment had the consensus sequence 5'-$T^{A}/_{C}ANN^{A}/_{G}TGA^{C}/_{T}NNNGC^{A}/_{G}$-3' (74), and was located between base pair -754 and -713 in the mouse (73), and -682 and -722 in the rat GST Ya gene (72). The DNA fragment was further found to activate transcription of the GST gene in response to an electrophilic signal (75). Subsequent studies have identified the sequence within several promoters of cytoprotective proteins, such as mouse HO-1 (76), rat and human NAD(P)H:quinine oxidoreductase (NQO1) (77;78), human γ-glutamylcysteine synthetase (GCS) (79), and Trx (80), in addition to the promoter of nuclear factor E2-related protein 2 (Nrf2) (81). These cytoprotective proteins may be transcriptionally induced through this enhancer element (62). The enhancer element is referred to as both the "antioxidant-responsive element" (ARE), and the "electrophile-responsive element" (EpRE). This thesis will use the term EpRE.

Regulation of EpRE-dependent transcription

Nrf2

In order for EpRE-dependent transcription to be induced, transcription factors need to associate with EpRE. The proteins in the Cap'n'Collar (CNC) family of basic region leucine-zipper (bZIP) transcription factors have been found able to activate EpRE-dependent transcription (82). The NF-E2-related factor 2 (Nrf2) of this family appears to be the main transcription factor involved in induction of cytoprotective proteins through EpRE (83). A study showed that Nrf2-deficient mice develop more spontaneous gastric tumours, and have no preventive effect of the chemo-protective compound oltipraz against carcinogen induced tumorigenesis (70). Nrf2 is expressed in an ubiquitously manner, but is expressed in rather high amounts in the intestine, lung and kidney where detoxification reactions occur routinely (83).

Nrf2 normally resides in the cytosol, but translocates to the nucleus upon activation by inducers of EpRE-dependent transcription (84). In nucleus Nrf2 associates with other transcription factors, and bind to EpRE as heterodimers with small Maf (Musculoaponeurotic fibrosarcoma) bZIP proteins, *e.g* MafG and MafK (83). Nrf2 is also described to interact with the co-activator CREB binding protein (CBP) before DNA-binding (85). The DNA bound Nrf2 complex recruits the basal transcriptional machinery and activates transcription of cytoprotective proteins (86).

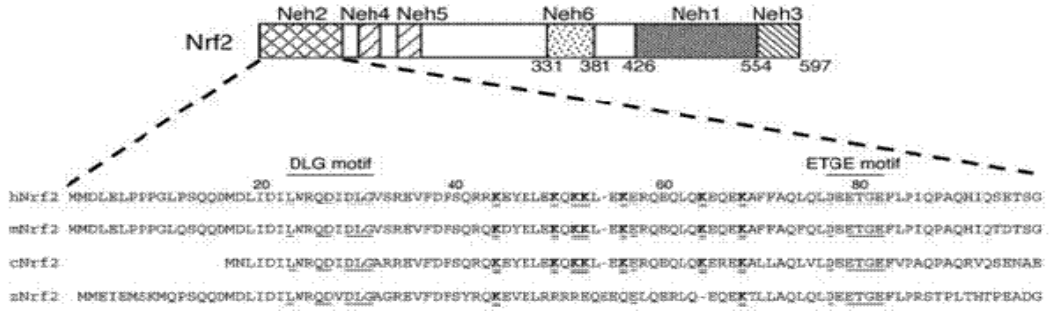


Figure 1.4 A model of the Nrf2 structure (87).

Nrf2 contains six highly conserved regions, named Nrf2-ECH homology (Neh) domain 1 to 6 **(Figure 1.4)**: Neh1 contains a CNC homology region and a bZIP domain; Neh2 and Neh3 contains the amino and carboxyl termini respectively; Neh4

and Neh5 are two acidic domains, shown to be important for CBP binding and transcriptional activation, while; Neh6 contains a conserved serine-rich region (83;88). The Neh2 domain has been shown to be necessary to retain Nrf2 in the cytoplasm and repress its activity under basal conditions (88). This repression is mediated by a protein called Kelch-like ECH-associated protein 1 (Keap1), and the Nrf2 Neh2 domain has been found to contain two motifs, ETGE and DLG, by which Keap1 recognizes and binds to Nrf2 (87).

Keap1

Keap1 is a cytoskeleton-bound cysteine-rich zinc-metalloprotein (84) which is widely expressed in various cell lines and tissues (88). Keap1 has been suggested to function as an adaptor protein for a ubiquitin ligase that targets lysine residues in the Neh2 domain, marking Nrf2 for degradation by the 26S proteasome (89). Thus, Keap1 regulates the steady state level of Nrf2, and maintains a low activation of EpRE-dependent transcription under basal conditions.

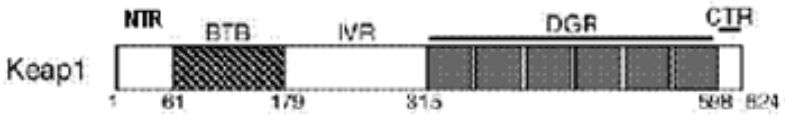


Figure 1.5 Model of the Keap1 structure (87).

Keap1 contains five distinct domains (90) **(Figure 1.5)**. The first domain consists of the amino-terminal region (NTR). The second domain contains a BTB/POZ (bric-a-brac, tramtrack, broad complex/Poxvirus zink finger) domain, by which Keap1 forms homodimers and probably associates with the ubiquitin ligase (89). The third domain consists of a cysteine-rich intervening region (IVR), also found to possibly interact with the ubiquitin ligase (90). The fourth domain consists of the double glycine repeat (DGR), also known as the Kelch domain, shown to be responsible for the binding of Keap1 to the cytoskeleton and to the Neh2 domain of Nrf2 (90). The fifth

domain consists of a short carboxy terminal domain (CTR), probably also involved in the binding to Nrf2 (87).

Homodimers of Keap1 bind with an overlapping binding surface of its DGR and CTR domains (Keap1-DC) to the ETGE and DLG motifs of Neh2; one monomer to each motif, but with a higher binding affinity for the ETGE motif (87).

Regulation of the Nrf2 release and translocation

In order to induce EpRE-dependent transcription, Nrf2 needs to be stabilized, dissociated from Keap1, and translocated to the nucleus (91). It is widely accepted that Nrf2 can accumulate in nucleus after exposure to oxidative stress or EpRE inducers (86;91), but the mechanisms by which this occur are not fully understood. Different mechanisms have been suggested, and both modification of Keap1 cysteine residues and phosphorylation and regulation of the transactivation activity of Nrf2 has been proposed as possible mechanisms **(Figure 1.6)** (86).

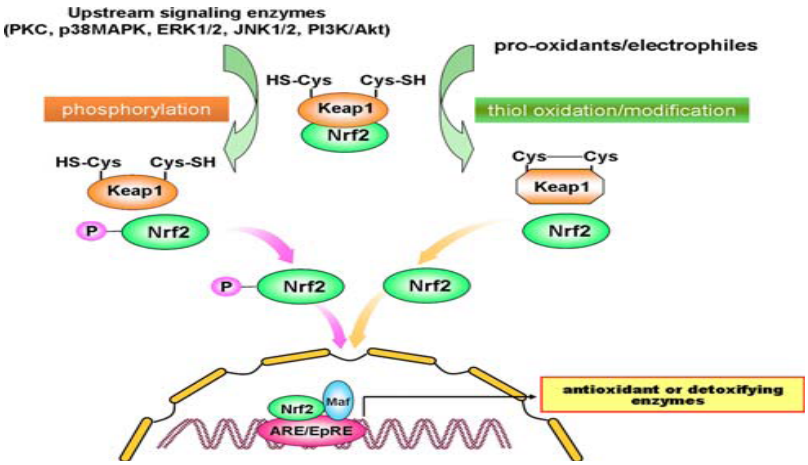


Figure 1.6 Model of Nrf2 regulation (92)

Modification of cysteine residues on Keap1

Most inducers of EpRE-dependent transcription contain electrophilic moieties that are capable of reacting with sulfhydryl groups; such as reactive cysteine residues (91). While the Neh2 domain bound to Keap1 lacks modifiable cysteine residues, Keap1 contains several of such, and its characteristic as a Nrf2-repressor has been

shown to be impeded by oxidative and electrophilic modification of some of these (93). Modification of cysteines on Keap1 by electrophilic inducers however appears to be insufficient to disrupt the complex between Keap1 and Nrf2 (94). Induction of EpRE-dependent transcription via modification of cysteine residues on Keap1 is rather suggested to be mediated by stabilization of Nrf2 through disruption of its ubiquitination and proteasomal degradation (95).

Between the two Keap1 binding motifs in the Neh2 domain of Nrf2, there is a α-helix containing seven lysine residues which have been shown to be necessary for the Nrf2 ubiquitination mediated by Keap1 (89). Correct spatial positioning between the target lysine residues and the ubiquitin ligase is important, and a distortion of this positioning may inhibit ubiquitination and degradation of Nrf2. The binding of the two Neh2 recognition motifs DLG and ETGE to the Keap1 homodimer appears to lock Nrf2 in a position that promotes ubiquitin transfer (87). As the DLG motif is only weakly bound to Keap1, an alteration in the conformation of Keap1 may disrupt the Keap1-DLG association and thus impede the ubiquitination of Nrf2 (96). This has led to proposal of the "hinge and latch" model to illustrate how Keap1 represses Nrf2, where the ETGE motif is characterized as the hinge, and the DLG as the latch (96). This model suggests that when the DLG and ETGE motifs are bound to Keap1, the lysine residues of the Neh2 α-helix are maximally exposed for ubiquitination and Nrf2 is efficiently degraded. If one of the motifs dissociates from Keap1-DC however, the ubiquitination may not be optimized and the degradation of Nrf2 may be abrogated (96).

Regulation of Nrf2 transactivation by cellular kinases

Phosphorylation is important in regulation of gene expression in eukaryotic cells (58). Several transcription factors have been shown to undergo phosphorylation, including Nrf2 (97). Specific kinase signals may regulate the nuclear translocation of Nrf2, and its ability to induce EpRE-dependent transcription (97;98).

MAPKs

MAPKs are serine/threonine kinases that convert various extra cellular signals into different phosphorylation cascades (99). Three MAPK signalling pathways have been identified; extracellular signal-regulated protein kinases (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK. They all consist of three subsequent kinases and work in the same way: a MAPK kinase kinase phosphorylates and activates a MAPK kinase, which phosphorylates and activates a MAPK that generally performs the ultimate task of the MAPK pathway.

All three identified MAPKs have been found able to regulate EpRE-dependent transcription (100). JNK1 has been suggested to be an important upstream activator of Nrf2, since induction of EpRE-dependent transcription by a known inducer appear to depend on this MAPK (101). Another study showed that ERK2 may be involved in up-regulation of cytoprotective proteins mediated by tert-butylhydroquinone (tBHQ), a known inducer of EpRE-dependent transcription (102). For p38 MAPK, Keum *et al.* (100) showed that an over-expression of this kinase suppresses the constitutive as well as the induced EpRE-dependent gene transcription, while certain inducers of EpRE-dependent transcription have been suggested to act through activation of this kinase (103). Thus, while ERK2 and JNK1 MAPKs appear to be activators of EpRE-dependent transcription, the effect of p38 MAPK on EpRE-dependent transcription remains debated (86).

PKC

Protein kinase C (PKC) is a serine/threonine kinase that in response to inter- and intracellular signals phosphorylates proteins controlling cell growth and differentiation (99). A study showed that Nrf2 was activated and translocated to the nucleus by a known PKC activator, phorbol 12-myristate 13-acetate (PMA), while the translocation was abrogated by an inhibitor of PKC (97). Nrf2 was further shown to be phosphorylated by inducers of EpRE-dependent transcription as well as PKC activators, but not after pre-treatment with a PKC inhibitor. A following study showed that Nrf2 contains a serin-40 that is necessary for the observed Nrf2-phosphorylation by PKC (104). As this is situated in the Neh2 domain of Nrf2, it is possible that phosphorylation of serin-40 abrogates the association between Nrf2 and Keap1 and allows Nrf2 to translocate to the nucleus.

PI3K

Phosphatidyl inositol-3 kinase (PI3K) is an important lipid kinase controlling cell growth, differentiation and apoptosis (99). The downstream targets of PI3K are phospholipase C (PLC) and serine threonine kinase Akt/protein kinase B (Akt/PKB) (105). An involvement of this kinase in EpRE activation has been suggested, and PI3K inhibitors have been shown to repress hemin-induced Nrf2 nuclear translocation and expression of cytoprotective proteins in human neuroblastoma cells (105).

Inducers of EpRE-dependent transcription

EpRE-dependent transcription has been shown to be induced by cellular stress (oxidants, electrophiles and shear stress), signalling molecules (nitric oxide, growth factors), oxidized lipids (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, oxidized low-density lipoproteins), several synthetic compounds (*e.g.* BHQ), and also a wide range of components found in dietary plants (90;106-108). The inducers found in edible plants are of special interest since they can be supplied through the diet. A selection of dietary plant compounds found to induce EpRE-dependent transcription is presented in **Table 1.7**.

Table 1.7 A selection of EpRE inducers found in dietary plants (adapted from Surh (84))

EpRE inducers found in edible plants			
Compound	**Example of source**	**Experimental system**	**Reference**
Cafestol, kahweol	Coffee	Nrf2-wild type C57BL/6 mice	(109)
Carnosol	Rosemary	Rat kidney cell line	(110)
Curcumin	Turmeric	Human hepatoma cells	(103)
		Swiss albino mice	(111)
Diallyl sulfide	Allium family of vegetables (onion, garlic, leek, shive, shallot)	Nrf2-wild type C57BL/6 mice	(112)
Lycopene	Tomato	HepG2 cell line	(113)
Resveratrol	Grape skin (and thus red wine), peanuts, berries (*e.g.* mulberry, blueberry, cranberry)	Rat kidney cell line	(114)
		HepG2 cell line	(115)
Sulphoraphane	Cruciferous vegetables (*e.g.* broccoli)	HepG2 cell line	(100)
		Nrf2-wild type mice	(116)
Quercetin	Apples, onions	HepG2 cell line	(117)
Zerumbone	Tropical ginger	Rat liver epithelial cell line	(118)

1.5 Health effects of combinations of dietary plants

With identification of several bioactive compounds in food, a massive commercial industry has developed making supplements based on one or a few of these isolated compounds. However, although epidemiological studies indicate beneficial health effects of their plants of origin, individual phytochemical supplements studied do not appear to have consistent beneficial health effects (4;10;14). In fact, some studies have shown that high dose supplementation of single antioxidants may be harmful, and may even lead to increased cancer incidence among specific groups (4;119;120). It thus appears as the beneficial health effect of dietary plants not solely depends on one or a few bioactive components within these foods.

Dietary plants contain thousands of phytochemicals with different size, structure, and mechanism of action (14). A meal composed of different plant sources will thus contain a large variety of phytochemicals. Supplements however, commonly only contain one or a few compounds, and thus in that way can not be compared directly with a phytochemical rich diet. Supplements furthermore lack components from the food matrix, such as macronutrients, vitamins, and minerals, which may be important contributors to the beneficial health effect of dietary plants (121). Administration of single dietary compounds may therefore be a major simplification of the actual biological impact of foods, and this could explain the apparent lack of beneficial health effects of phytochemical supplements. Research where whole foods, rather than individual food components is the variable is thus essential in nutrition research (121).

Food synergy

Additive, synergistic and antagonistic effects are terms used in several scientific areas such as biology, chemistry and pharmacology (122). An additive effect defines a situation where the total effect of a combination of two or more agents is the same as the sum of the individual agents **(Figure 1.7 C)**. A synergistic effect however, is when the total effect of the combination is greater than the sum of the individual agents **(Figure 1.7 D)**, while an antagonistic effect defines a situation where the total effect of a combination of two or more agents is less than the sum of the individual agents **(Figure 1.7 E)**.

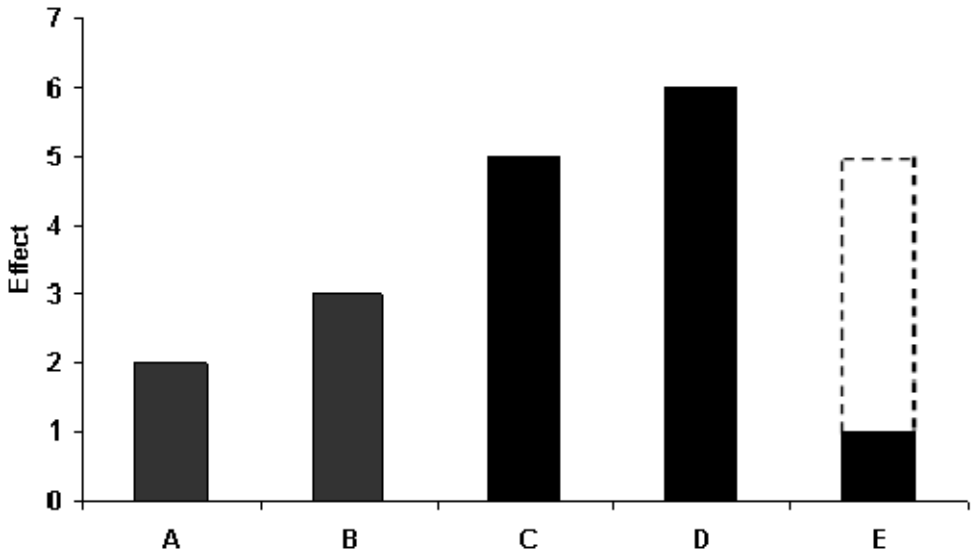


Figure 1.7 Presentation of additive, synergistic and antagonistic effects. A) Effect of compound A. **B)** Effect of compound B. **C)** Additive effect of combining A + B. **D)** Synergistic effect of combining A + B. **E)** Antagonistic effect of combining A + B.

These terms may also be applied in nutrition research to describe the health effects of combined foods or food constituents (123). From epidemiological studies it is apparent that the dietary pattern has a great influence on health. It has therefore recently been hypothesized that the beneficial health effects observed for some dietary patterns, *e.g.* the Mediterranean diet, results from the combined effect of several components within the respective diet (124). Potential additive or synergistic effects of different food components may explain the apparent lack of health effect of

isolated compounds such as dietary antioxidants, and could contribute to a better understanding of the mechanisms behind the beneficial health effects of dietary plants (121).

In a recent study investigating the effect of combinations of dietary plant extracts on NF-κB activity, several extract combinations were found to exert additive and synergistic effects on NF-κB inhibition (125). By showing that combinations of dietary plants may have additive and synergistic effects on molecular mechanisms such as NF-κB regulation, the results of this study supports the theories of food synergy. Few studies have however investigated the potential effects of dietary plants in combination on other signalling pathways, and it is therefore of interest to establish systems in which such combinations can be studied.

2. Aims of the thesis

Up-regulation of cytoprotective proteins is assumed to be protective against several chronic pathological conditions. Cytoprotective proteins can be transcriptionally induced through a common element in their promoter called electrophile responsive element (EpRE). Several pure phytochemical compounds, as well as dietary plant extracts, have previously been shown to be effective inducers of EpRE-dependent transcription. Up-regulation of cytoprotective proteins may therefore partly explain the beneficial health effect of dietary plants.

With the emerging interest on synergistic and additive effects among food components, studies investigating such effects on EpRE-dependent transcription are needed. This thesis focuses on the combinatorial effects of dietary plants on EpRE-dependent transcription. Also, to expand the knowledge beyond *in vitro* induction of EpRE-dependent transcription, transgenic EpRE mice were utilized.

The specific aims of this thesis are:

- To study the dose-response effect on EpRE-dependent transcription by dietary plant extracts not previously tested in EpRE-LUC HepG2 cells.
- To study the effect of combinations of dietary plant extracts on EpRE-dependent transcription *in vitro*.
- To study the effect of one dietary plant extract on EpRE-dependent transcription *in vivo*, using transgenic reporter mice.

3. Materials

3.1 Cell line

Cell line	Distributor	Location
HepG2 (HB-8065)	The American Type Culture Collection	Manassas, VA

3.2 Cell culture materials

Chemical/compound/ equipment	Manufacturer	Location
Cell culture flask 75 cm^2	BD Falcon, 353110	San Jose, CA
Cell culture plates 12, 24 wells	Corning Inc.	Corning, NY
EpRE luciferase reporter plasmid		
Fetal Bovine Serum	Sigma, F-7524	St. Louis, MO
L-Glutamine	Sigma, G-7513	St. Louis, MO
Lipofectamine	Invitrogen Corp.	Carlsbad, CA
MEM Non-essential Amino Acid solution (100x)	Sigma, M-7145	St. Louis, MO
Minimum Essential Medium Eagle	Sigma, M-4526	St. Louis, MO
Opti-mem	Invitrogen Corp., 11058-021	Carlsbad, CA
Penicillin/Streptomycin Solution	Sigma, P-4458	St. Louis, MO
Sodium pyruvate solution	Sigma, S-8636	St. Louis, MO

3.3 Plasmid isolation

Chemical/compound/ equipment	Manufacturer	Location
Agar	MERCK	Dermstadt, Germany
Ampicillin	Sigma, A0166	St. Louis, MO
Isopropanol	Arcus Kjemi AS	Vestby, Norway
Pepton	MERCK	Dermstadt, Germany
Yeast extract	MERCK	Dermstadt, Germany
Jetstar 2.0 Plasmid kits	Genomed GmbH	Löhne, Belgium
NaCl	MERCK	Dermstadt, Germany

3.4 Transgenic mice

Transgenic mice	Distributor	Location
EpRE-luciferase mice	Cgen	Oslo, Norway

3.5 Materials for *in vivo* experiments

Equipment	Manufacturer	Location
Cameo 25 Gas Syringe Filter, 0.22 micron?	Osmonics Inc, DGA02025SO	Minnetonka, MN
Centrifuge tubes, 15 and 50 ml	BD Biosciences	Franklin Lakes, NJ
Feeding tube for oral gavage feeding	Agn Tho's AB	Lidingö, Sweden
Micro tubes 1.5 ml	Sarstedt	Nümbrecht, Germany
Omnifix (syringes) 1 and 10 ml	Braun Melsungen AG	Melsungen, Germany
Sterican (needles) 0.15x16 mm	Braun Melsungen AG	Melsungen, Germany

3.6 Chemicals

Chemical/compound	Manufacturer	Location
2,4,6-tripyridyl-s-triazine (TPTZ)	Fluka Chemie AG	Deisenhofen, Switzerland
2-Propanol (HPLC – grade)	Merck	Darmstadt, Germany
Acetic acid	Merck	Damstadt, Germany
Albumin	Bio-Rad Laboratories Inc., 500-0007	Hercules, CA
Argon	AGA, 100325	Oslo, Norway
ATP (adenine triphosphate)	Roche Diagnostics	Ottweiler, Germany
Bio-Rad Protein Assay	Bio-Rad laboratories Inc.,500-0006	Hercules, CA
Coenzyme A	Roche Diagnostics	Ottweiler, Germany
Corn oil Biochemica	Sigma, C-8267	St. Louis, MO
D-Luciferin	Biosynth AG	Staad, Switzerland
DMSO (dimethylsulphoxide)	Sigma, D-5879	St. Louis, MO
DTT (dithiothreitol)	Sigma	St. Louis, MO
EDTA (diethylenediamine tetra-acetic acid)	Merck	Darmstadt, Germany
Ethanol	Arcus AS	Oslo, Norway
$FeCl_3$ • 6H20	BDH Laboratory Supplies	Dorset, England
$FeSO_4$ • 7H2O	Riedel-deHaën AG	Seelze, Germany
Hydrochloric acid	Merck	Darmstadt, Germany
Isoflourane	Baxter AS	Oslo, Norway
Methanol (HPLC-grade)	Merck	Darmstadt, Germany
$MgSO_4$ • 7H2O	Merck	Darmstadt, Germany
MilliQ water	Millipore	Bedford, MA
PBS (Phosphate buffered saline)	Bio Whittaker, BE17-512F	Verviers, Belgium
Reporter Lysis Buffer	Promega, E3971	Madison, WI
Sodiumacetate trihydrate	Riedel-deHaën AG	Seelze, Germany
Tricine	Sigma, T5816	St. Louis, MO

3.7 Dietary plant extracts

Name	Latin Name/Commercial name	Producer	Location
Broccoli (provided by Garmannvik Johansen)	*Brassica oleraceae var. italia*	Thor Graff	ICA, Grünerløkka, Norway
Coffee	*Coffea arabica*	Friele	Bergen, Norway
Cocoa (provided by Garmannvik Johansen)	Freia Regia originalkakao	Freia Kraft Foods	Oslo, Norway
Dog rose (provided by Kolberg)	*Rosa canina*		Oslo, Norway
Oregano	*Oreganum vulgare*	Hindu	Bergen, Norway
Red Onion	*Allium cepa*	Unknown producer	Deli de Luca, Bislett, Norway
Red Wine (provided by Garmannvik Johansen)	Da Luca, Primitive Merlot Tarantino	MGM Mondo del Vino	Italy
Rosemary (cells)	*Rosmarinus officinalis*	Santa Maria	Mölndal, Sweden
Rosemary (mice)	*Rosmarinus officinalis*	Hindu	Bergen, Norway
Thyme	*Thymus vulgaris*	Hindu	Bergen, Norway
Cherry tomato	*Solanum, lycopersicum*	Henning Ruud	ICA, Bislett, Norway
Turmeric (provided by Kolberg)	*Curcuma longa*	Rajah	Enfield, England

3.8 Instruments

Instrument	Manufacturer	Location
Biofuge Fresco	Heraeus Instruments	Osterode, Germany
2510 Branson	Branson Ultrasonics Corp.	Dansbury, CT
IVIS Imaging System 100 Series	Xenogen Corporation	Alameda, CA
Labofuge 400e	Heraeus Instruments	Osterode, Germany
Luminometer TD 20/20	Turner Designs	Sunnydale, CA
Megafuge 1.0 R	Heraeus Sepatech GmbH	Harz, Germany
Nano Drop Spectrophotometer ND-1000	Saveen Werner	Limhamn, Sweden
Synergy 2	Bio Tek® Instruments, Inc	Winooski, VT
Technicon RA 1000 system	Technicon Instruments Corporation	New York, NY
Titertek Multiskan Plus	ELFAB	Finland
Orbital Incubator	Weiss Gallenkamp	Leicestershire, England
Avanti J-26 XP	Beckmann Coulter	Fullertone, CA

3.9 Software

Software	Manufacturer	Location
Adobe Illustrator 10	Adobe Systems Incorporated	San Jose, CA
Gen 5™ PC	Bio Tec® Instruments, Inc	Winooski, VT
Living Image Software	Xenogen Corporation	Alameda, CA
Microsoft Office XP	Microsoft Corporation	Redmond, WA
Reference manager 11	ISI Research Soft	Carlsbad,CA
SPSS 16 for Windows	SPSS Inc.	Chicago,IL

4. Methods

4.1 Dietary plant extracts

Spices, cherry tomatoes, coffee, and red onion were bought in local grocery stores in Oslo. The extracts of broccoli, cocoa, dog rose, red wine, and turmeric, were generously provided by previous masterstudents in Rune Blomhoffs group, Marit Kolberg and Siril Garmannvik Johansen.

The foods were homogenized by a food processor, and 10 g of the products were measured out into tubes. For dry foods, 10 ml of methanol (MeOH) and 10 ml of MilliQ H_2O (MQ-water) were added. For some products, like thyme, 20 ml of these solvents were needed to make a homogenous dilution of the material and the liquid. For vegetables, 10 ml of MeOH, and MQ-water depending on their water content was added; tomatoes contains 94 % water, and therefore 10 ml of MeOH and 10 ml – (10 g x 0.94) = 0.6 ml MQ water was added. The tubes were vortexed for 30 seconds, and thereafter sonicated in a 0ºC water bath for 30 minutes.

After sonication, the extracts were transferred into falcon tubes and then centrifuged at 4ºC and 4000 rpm for ten minutes. The supernatant was transferred into Erlenmeyer flasks, and to collect as much as possible of the liquid phase the centrifugation was repeated. The Erlenmeyer flasks were then placed under nitrogen gas, to evaporate all the alcohol and as much as possible of the water, concentrating the liquid to a viscid fluid at a total amount of < 4 ml.

4.1.1 Extracts for cells

For cell experiments, the concentrated extracts were diluted to a total amount of 5 ml with Phosphate Buffered Saline (PBS), or Dimethyl Sulfoxide (DMSO) + PBS if the content was difficult to dissolve in PBS alone. The final concentration of DMSO in cell culture never exceeded 0.2 ‰. The extracts were then sterile filtered with a 0.22

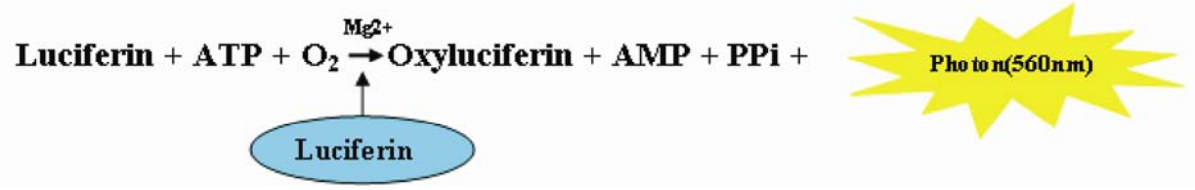
µm filter, transferred into airtight tubes and stored under argon at -70ºC. Final concentrations of the extracts were 2 g/ml.

4.1.2 Extracts for animals

For animal experiments the concentrated extract was diluted to a total amount of 5 ml with corn oil. The extract was transferred into airtight tubes, and stored under argon at -70ºC. Final concentration of the extract was 2 g/ml.

4.2 Luciferase as a reporter system

The regulation of a DNA sequence can be studied by linking the sequence to an easily detectable reporter gene, such as firefly luciferase. The firefly luciferase protein catalyzes bioluminescent oxidation of luciferin in the presence of ATP, magnesium and oxygen. This generates oxyluciferin and measurable luminescent light with a wavelength of 520-570 nm and peak density at 560 nm (126). The gene coding for firefly luciferase can therefore be used as a reporter gene, by inserting a reporter construct containing the luciferase gene into a genome and add luciferin to the system. If expressed, the luciferase will produce luminescent light by oxidation of the luciferin, and when luciferin and ATP are available in surplus the luminescence will be linear to the luciferase expression (126). The luminescence will signify the activity of the element that is attached to the luciferase reporter gene.



To incorporate a gene of interest, *e.g.* reporter gene constructs, into a genome, plasmids can be used. A plasmid is an extra-chromosomal DNA molecule separate from the chromosomal DNA, capable of replicating independently from the chromosomal DNA. Plasmids usually occur naturally in bacteria, and are often circular and double-stranded. A previously described luciferase reporter plasmid

construct containing two EpRE sequences (127) was used in the experiments in this thesis to study the effect of different extracts on EpRE activity **(Figure 4.1)**.

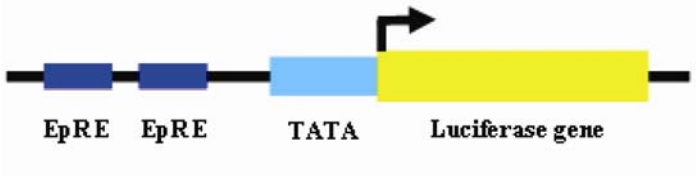


Figure 4.1 Illustration of the luciferase gene coupled to a promoter containing two EpRE motifs.

4.3 *In vitro* experiments

4.3.1 Cell culturing

Cell line

The *in vitro* experiments in this project were carried out in HepG2 cells. The HepG2 cell line is a human hepatocellular carcinoma cell line, isolated from a Caucasian male (128).

Cell medium

The cells were grown in Eagle's Minimum Essential medium (MEM), supplemented with 10 % heat-inactivated fetal bovine serum, streptomycin (50 mg/ml), non-essential amino acids (0.01 %), sodium-pyruvate (1 mM) and L-glutamin (2 mM). The medium was changed every 2 – 3 days. The cell cultures were contained in a humidified atmosphere with 5 % CO_2 at 37ºC. Transient transfection was performed using cell culture medium without penicillin according to manufacturer's instruction.

Cell culturing

The cells were split 1:3 – 1:5 when confluent, approximately twice a week and always the day before transfection. Cell viability and morphology was determined using a phase contrast microscope.

When confluent, the old medium was removed, and the cell layer in the flasks was rinsed with 4 ml 0.25 % trypsin containing 0.02 % (w/v) EDTA to remove the bovine serum and any protease inhibitors from the cells. Then 3 ml of 0.25 % trypsin was added, and the cell flasks were placed in 37ºC for 5 – 10 minutes. When the cells were dispersed, 6 ml of complete growth medium was added to the solution and the cells were gently aspirated by pipetting. The cell suspension was transferred in appropriate aliquots to new 75cm^2 cell flasks, depending on the confluence of the cell culture.

Freezing and thawing

Stocks of HepG2 cells were stored in liquid nitrogen in the presence of DMSO to prevent formation of ice crystals and lysis of cells during storage. The cells were thawed rapidly by gentle agitation in a 37ºC water bath and transferred into 75cm^2 cell flasks with 20 ml cell culture medium. Medium was changed after 24 h to remove the DMSO, and after about two weeks the cells had resume normal growth rate and were used for experiments.

Muliplication and isolation of the EpRE-LUC plasmid

The 2 x EpRE-LUC construct used in the experiments had been made previously and inserted into plasmids containing an ampicillin resistant gene (127). The plasmids, containing an ampicillin resistant gene, had been inserted into competent DH5α E.Coli bacteria by transformation. Bacteria reproduce rapidly, and each of the new bacteria contains one or more copies of the plasmid.

To multiply the plasmid, glycerol stocks of plasmid containing bacteria were thawed and transferred to flasks containing 100 ml LB- medium supplemented with 50 µg/l ampicillin. The flasks were incubated over night at 37ºC in an orbital incubator, and then the E.Coli bacteria were then pelleted by centrifugation. To isolate the plasmid, a Jet Star Maxi preparation was performed, which employs a modified alkaline/SDS method to prepare a cleared lysate. After neutralization, the lysate was applied onto a Jet Star Column, where the plasmid is bound to an anion exchange resin. The resin

was washed to remove RNA and other impurities, and finally the plasmid was eluted from the column and concentrated by alcohol precipitation. The precipitated plasmids were dissolved in MQ-water and DNA concentration was quantified by a photometer prior to transfection of plasmids into HepG2 cells.

4.3.2 Experimental outline

Transfection

The cells were cultured at approximately 60 % confluence the day prior to transfection, in 12-well cell culture plates (22 mm). For the dose-response relationship studies of extract combinations however, 24-well cell culture plates (11 mm) were used. The plates were contained in a 37ºC incubator over the night, in order to let the cells attach to the wells and grow.

The HepG2 cells were transiently transfected using Lipofectamin 2000, a cationic lipid-based transfection agent. According to the manufacturer's protocol, each well of the 12-well cell culture plates was added 1.6 µg DNA diluted in 100 µl Opti-mem, and 2 µl Lipofectamin 2000 diluted in 100 µl Opti-mem. Each well of the 24-well cell culture plates was added 0.8 µg DNA diluted in 50 µl Opti-mem, and 1 µl Lipofectamin 2000 diluted in 50 µl Opti-mem. The dilution of Lipofectamin 2000/Opti-mem was incubated in room temperature for 5 minutes before it was combined with the DNA/Opti-mem dilution, and the final mixture was incubated at room temperature for 20 minutes to allow the DNA-Lipofectamin 2000 complexes to form. Each well of the 12-well culture plates were added 800 µl of a culture medium without penicillin, and 200 µl of the DNA/Lipofectamin 2000 mix. Each well of the 24-well culture plates were added 400 µl of the same culture medium, and 100 µl of the DNA/Lipofectamin 2000 mix. The cell-plates were placed in an incubation cabinet for 6 h to optimize the transfection, before the DNA/Lipofectamin 2000 mix was removed and replaced with 1000 µl complete culture medium.

Incubation with food extracts

The following day, the cells were incubated with food extracts. The cell culture medium was replaced with fresh medium, and the cell wells were incubated with extracts in different concentrations or vehicle control for 17 h. A total amount of 15 µl extract was added each well, diluted with PBS or PBS/DMSO (4:1) depending on the solvent used when making the extracts. As a control, PBS or PBS/DMSO (4:1) was added in the same amount as the extracts.

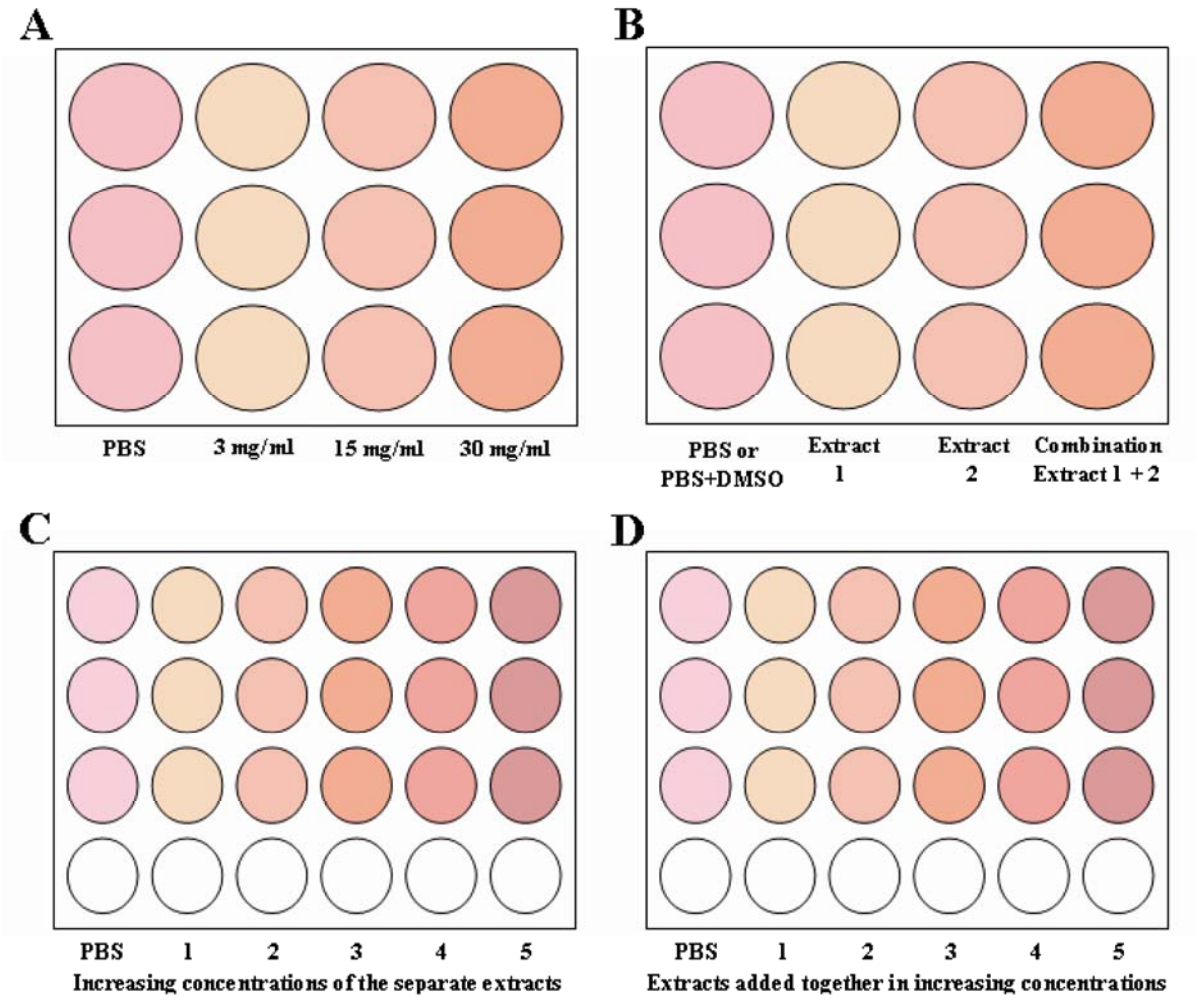


Figure 4.2 Extract stimulation of transiently transfected EpRE-LUC HepG2 cells. Extracts were administrated both separately and in combination. **A)** To study single extracts, three different concentrations of the extract were administrated in 12-well cell culture plates. **B)** To study the effect of extract combinations in 12-well cell culture plates, a single concentration of each extract was added individually and in combination. To study the effect of extract combinations in 24-well cell culture plates, several different concentrations of each extract were administrated individually **(4.2 C)** and in combination **(4.2 D)** on different cell plates.

When testing single plant extracts in 12-well cell plates, three concentrations of the extract were used (**Figure 4.2 A**). To study the effect of a combination of two extracts, a single concentration of each extract was administrated individually and in combination (**Figure 4.2 B**). In the dose-response relationship studies of extract combinations however, 8 concentrations of each extract were administrated individually **(Figure 4.2 C)** and in combination **(Figure 4.2 D)**. All cell plates were incubated at 37ºC for 17 h after extract stimulation.

Luciferase measurements

After extract incubation, the luciferase activity was measured. Two different methods for luciferase measurement were applied.

Effect of single plant extracts and extract combinations in 12-well cell plates

The medium containing the extracts was removed, and each well was added 300 µl of lysis buffer. The cell plates were placed in 4ºC for 20 minutes to optimize cell lysis. After lysis the cells were collected by a cell scraper and transferred into eppendorf tubes, which were vigorously vortexed and then centrifuged at 4ºC for 5 minutes. An amount of the supernatant (20 µl) was transferred into luminometer tubes, and 100 µl Luciferase assay was added (see below). The firefly luciferase assay system contains ATP, luciferin, Mg^{2+}, Coenzyme A (CoA) and a buffer that maintains a pH of 7.8 to optimize the luciferase bioluminescent reaction in lysed cells. The luciferase activity was measured in a TD 20/20 luminometer after 3 seconds of vortexing.

Luciferase assay solution

Reagents	Amount
ATP (Adenosine triphosphate)	52,1 mg
Coenzyme A (CoA)	20,7 mg
Tricine	358,4 mg
$MgSO_4$ x 7 H_2O	92,2 mg
DTT (dithiothreitol)	513,5 mg
EDTA (ethylenediaminetetraacetic acid)	15 µl
ddH_2O	80 ml + Adjustment volume
d-Luciferin (20 mg/ml)	650 µl
Ingredients were mixed, pH adjusted to 7,4 by a pH meter, and ddH_2O was added to a total volume of 100 ml.	

Effect of dose-response relationship of extract combinations in 24-well cell plates

All wells were added 10 µl Luciferin, and incubated 5 minutes at 37 ºC before luminescence was measured in a Synergy 2 instrument. This is a computer controlled single-channel microplate reader, using BioTec's Gen 5™ PC software. Gen 5 reports the results of the measurements as Relative Luminescence Units (RLU), which is the average of data points in each well during the integration time (10 sec). Luciferin was given in excess so that the luciferase is the limiting factor of the reaction.

4.4 *In vivo* experiments

4.4.1 Work in advance of the animal experiments

The animals used in this thesis are bred and attended to at the Department of Comparative Medicine, Faculty of Medicine, University of Oslo.

Animal research has had a vital role in many scientific and medical advances of the past century, such as in production of insulin, penicillin, antidepressive medicines, and several vaccines. However, the use of living animals in research raises essential ethical questions. It is therefore important that all experiments involving living animals are well considered and strictly planned, so that unnecessary use of animals in research is avoided. The mice used in this thesis were attended to as required by the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). All experiments were further performed according to the national guidelines for animal welfare.

To provide for the best possible environment for the animals, the access to the animal department is severely restricted. This ensures that the people who handle the animals are well trained for this purpose, and that the appropriate ethic standards are being met. Thus, in order to get access permission, we made an agreement with the person in charge at the animal department to read curriculum on “laboratory animal science” before the project was started. We then received internal instructions in the routines and methods at the animal department by our co-supervisors Ingvild Paur and Trude Rakel Balstad.

4.4.2 Transgenic reporter mice

To study the effects of phytochemical rich extracts on EpRE activity *in vivo*, an experiment with transgenic reporter mice was performed. These mice have been developed by the research group, and contain the same EpRE luciferase construct as

used in the HepG2 cells were transiently transfected with (129). Transgenic reporter mice are generated by pro-nuclear micro-injection, injecting the reporter construct into one of the nuclei of a fertilized mouse zygote. This technique is further explained by Carlsen *et al.* (130).

4.4.3 Experimental outline

An *in vivo* experiment administrating rosemary extract was carried out on 4 different days, involving a total number of 14 male mice. At the first and second day of the experiment, the mice were followed for 24h after administration of the rosemary extract to measure the EpRE luciferase activity in the mice during this time **(Figure 4.3)**. These data was used to establish the time point with the largest difference in luciferase activity between the extract and control group, at which the mice were sacrificed at the third and fourth day of the experiment. The same 6 mice were used in the first three days of the experiment, although only four of these were included at the third day, and eight new mice were included at the fourth day. The mice were randomized into an extract or a control group, each group including the same number of mice.

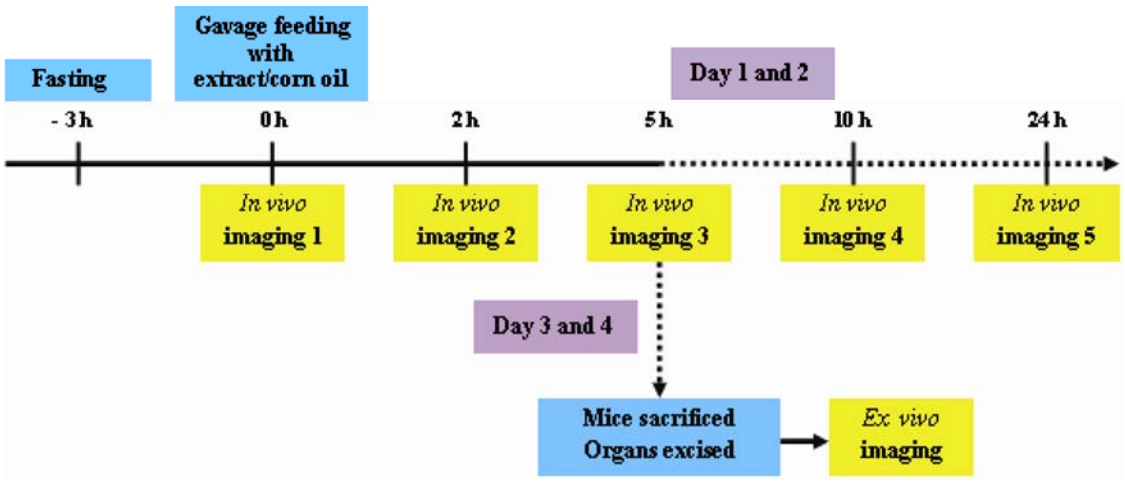


Figure 4.3 Experimental outline of the *in vivo* experiment

Prior to the start of the experiment, the mice were fasted for three hours. After the first *in vivo* imaging, the mice were tube fed with either 300 µl of a rosemary extract (extract group) or 300 µl corn oil (control group). *In vivo* imaging was performed at 0, 2, 5, 10 and 24h in the first study, and at 0, 2 and 5h in the second study.

After *in vivo* imaging at 5h in the first study, the mice were sacrificed. The organs were excised, and *ex vivo* imaging were performed on the liver, spleen, kidney, thymus, heart, lung, skin, muscle, intestine, testis, epididymis, adipose tissue and the brain. The organs, except for the intestine, were then transferred to separate tubes for storage at – 70 ºC for later analysis.

4.4.4 *In vivo* and *ex vivo* imaging

In vivo imaging was performed at the given time points in an IVIS 100 System. The mice were anesthetized using 2.5 % isofluoran, and shaved on the abdominal region before imaging. D-luciferin (160 mg/kg) was injected intraperitoneal, and mice were placed in a lightproof chamber where the isofluoran anaesthesia was maintained. Seven minutes after the injection of D-luciferin, the luminescence emitted from the mice was recorded for one minute or thirty seconds depending on the intensity of the luminescence. *Ex vivo* imaging was performed in the same way, utilizing the D-luciferin that was injected before the last *in vivo* imaging.

The luminescence in the images was quantified using Living Image Software. The luminescence captured by the *in vivo* imaging was quantified by placing circles around the abdomen of the mice, using the same sized circle in each picture of the same mouse. The *ex vivo* imaging was quantified by placing a circle or a square around the separate organs. The intestine was quantified by placing multiple circles and squares along the organ, measuring the luminescence of the large intestine and the small intestine separately to study whether there were any differences between the two sections.

4.4.5 Luciferase activity in tissues

To compare the imaging with the luciferase activity of the organs, the organs were homogenized and the luciferase activity of the homogenates was measured by the luciferase assay.

The organs were transferred into 5 ml tubes and added 1 ml of 1x Reporter Lysis Buffer (diluted with MQ-water from 5x Reporter Lysis Buffer). The brain and the liver however, were transferred into 10 ml tubes, and added 2 ml of Reporter Lysis Buffer. The tissue samples were kept on ice. The organs were homogenized by Ultra Thorax. The homogenates were transferred into Eppendorf tubes, and centrifuged for 15 minutes at 13000 rpm and 4 ºC in a Biofuge.

The supernatant was transferred into luminometertubes in duplicates of 20 µl. After addition of 100 µl of room-tempered Luciferase Assay Solution, the samples were vortexed for three seconds and the luminescence of the homogenates was measured by a TD 20/20 luminometer.

4.4.6 Protein concentration of the tissues

Since the tissue samples differ in size, the luciferase activity was adjusted for the protein content of the sample in order to compare the luciferase activity of the respective organs between the two groups. The protein concentration of the homogenates was measured by the Bradford protein assay. This colorimetric protein assay is a simple and accurate procedure of determining concentration of proteins, and relies on the binding of the dye Coomassie Brilliant Blue G-250 to aromatic amino acid residues of proteins. When binding to protein occurs, the absorbance maxima for an acidic solution of the dye shifts from 465 to 595 nm, and the colour of the dye changes from brown to blue.

The supernatant of the tissue homogenates was diluted with MQ-water from 10 to 200 times depending on the organs size and protein content. Solutions of known albumin concentrations were used as standards. Triplicates of each organ and

standards were added to a 96-well plate, 10 µl in each well. One well contained a blank of 10 µl MQ-water. Bio-Rad protein assay was diluted 1:4 in milliQ-water and filtrated through a paper before 200 µl was added to each well. The absorbance of the dye in each well was measured at 595 nm in a Titertek microplate reader, and the protein concentration was determined by relating the measurements to different albumin concentrations as a standard curve.

4.5 The Ferric Reducing Ability of Plasma (FRAP) assay

The FRAP assay was performed to measure the reducing capacity of the extracts applied in this study. This method was originally developed by Benzie and Strain to detect the reducing capacity of biological fluids (131), but can also be performed on extracts of dietary plants (132).

The FRAP method is based on the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). When occurring at low pH, reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) develops an intense blue colour with an absorption maximum at 593 nm. This is a non-specific reaction, and all reductants with higher half reaction reduction potentials than Fe^{3+}/Fe^{2+}-TPTZ half reaction will drive the Fe^{3+}-TPTZ reduction. Excess Fe^{3+}-TPTZ is used, so that the limiting factor of the reaction, and colour formation, is the reducing ability of the sample. By measuring the light, one can detect the concentration of electron donating antioxidants in the sample (132).

A Technicon RA 1000 system was applied to measure FRAP in the extracts. FRAP reagent (300 µl) was heated to 37ºC, and then added 10 µl of the samples. Each sample was measured in triplicates. The mixture of the sample and FRAP reagent was incubated for 4 minutes at 37ºC, and then the absorbance was measured at 600 nm. An aqueous solution with a known concentration of Fe^{2+} (1000 µmol/$FeSO_4$ x $7H_2O$) was used to calibrate the FRAP assay.

FRAP reagents

Reagents	Component	Volume in FRAP
Acetat buffer	300 mM acetate buffer, pH 3.6 + $C_2H_4O_2$ 16 ml/l buffer solution	25 ml
HCl	40 mM	2.4 ml
FeCl3 x 6 H_2O	20 mM	2.5 ml
TPTZ solution	250 mg in 5 ml methanol	155 µl

4.6 Statistical analysis

All the statistical analysis was performed using SPSS 16.0, and the statistical significant difference was set to $p< 0.05$ for all the analysis.

4.6.1 *In vitro* experiments

A one way ANOVA was used to examine the effects of dietary plant extracts and their combination on luciferase activity in EpRE-LUC HepG2 cells. To identify differences, a Dunnett's post hoc test was performed. In experiments studying separate extracts at different concentrations, the Dunnett's post hoc test was used to compare the effect of the different extract concentrations to the control. While in experiments studying extract combinations, the Dunnett's post hoc test was used to compare the individual extracts and the control to the extract combination.

To examine the effect of the extract combinations, the observed effect of the combinations was compared to the expected effect (the sum of the effects of the two individual extracts) by a Student's T-test. In the dose-response relationship studies of extract combinations, a Student's T-test was also used to study the effect of the extract combinations to the average effect of the individual extracts at the same total amount of extract.

4.6.2 *In vivo* experiments

In order to examine the effect of the rosemary extract on EpRE-dependent luciferase activity *in vivo*, a non-parametric Mann-Whitney U test was employed to compare the extract and control groups.

5. Results

5.1 Effect of dietary plant extracts on EpRE-activity in HepG2 cells

5.1.1 Effect of dietary plant extracts on EpRE-dependent luciferase activity

Several pure phytochemicals have been tested and shown to be effective inducers of EpRE mediated transcription in HepG2 cells transfected with EpRE-LUC reporter plasmid (129). A selection of extracts made of dietary plants high in at least one of these phytochemicals have been shown to induce EpRE mediated transcription as well.

Three separate extracts which had not previously been tested for their ability to induce EpRE activity in HepG2 cells were tested in this thesis; namely, cherry tomato, cocoa, and red wine. These extracts were selected based on their high phytochemical contents: cherry tomato was selected due to its lycopene content; cocoa was selected due to its high flavonoid content; and red wine was selected due to its content of resveratrol. Each extract was tested in three concentrations; 3mg/ml, 15 mg/ml and 30 mg/ml, and compared to a control of PBS. All of the experiments were repeated 2 - 3 times, and in each experiment, each treatment was performed in triplicates.

Results are presented in **Figure 5.1** as fold change as compared to the control**.** Figures display the mean ± the standard error of the mean (SEM).

Of these three extracts only cocoa **(Figure 5.1 C)** gave a statistically significant increase in EpRE mediated luciferase activity compared to the control. For cocoa, there was a 1.92 ± 0.12 fold increase at 15 mg/ml ($p= 0.02$), with a further increase to 2.60 ± 0.06 at 30 mg/ml ($p < 0.01$), as compared to the control.

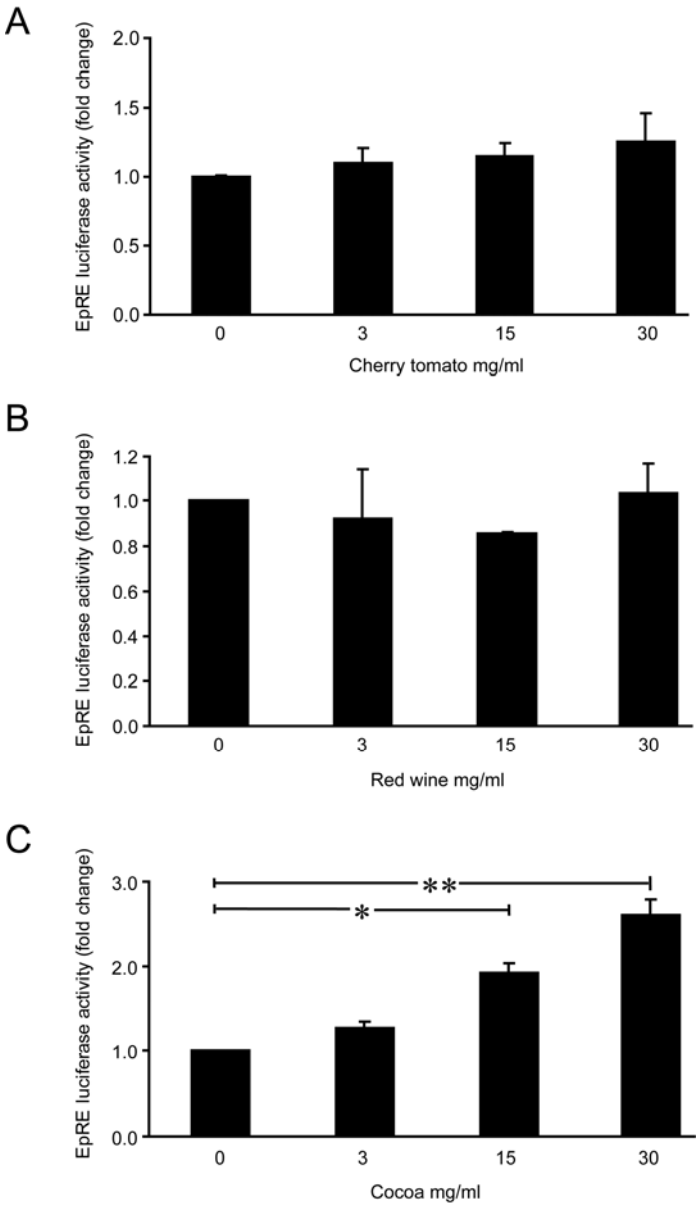


Figure 5.1 Effect of food extracts on EpRE luciferase activity in HepG2 cells. EpRE LUC cells were incubated with the indicated concentrations of extracts for 17h, and the EpRE luciferase activity was measured in a luminometer. The effect of the different concentrations on EpRE-dependent luciferase activity was compared to a control of PBS (0 in the figure). The bars represent mean ± SEM (n = 3 for tomato, and n = 2 for red wine and cocoa). * = $p < 0.05$, ** = $p < 0.01$. **A)** Cherry tomato. **B)** Red wine. **C)** Cocoa.

5.1.2 Effect of combinations of dietary plant extracts on EpRE-dependent luciferase activity

To study the effect of combinations of dietary plant extracts on EpRE-dependent luciferase activity, two extracts were administrated individually and in combination to EpRE-LUC HepG2 cells. To evaluate the effect of the extract combinations, the EpRE-dependent luciferase activity of the extract combinations was compared to the sum of the effect of the individually added extracts. The measured luciferase activity of the extract combination is described as the "observed" effect, while the sum of the effect of the extracts added individually is described as the "expected" effect. An observed effect significantly higher or lower than the expected could indicate a synergistic or an antagonistic effect respectively.

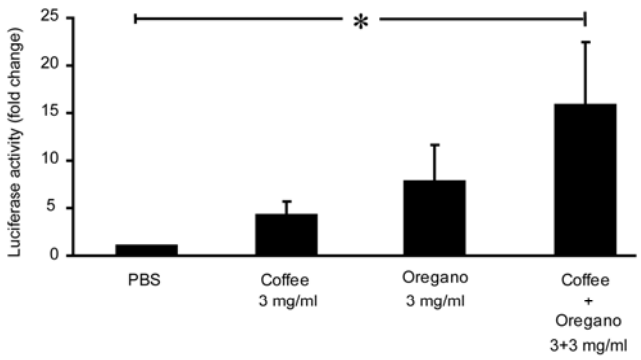
Based on previous experiments with dietary plant extracts in EpRE-LUC HepG2 cells (129), and the extracts tested separately in this thesis **(Figure 5.1)**, eleven extract combinations were tested in this thesis **(Table 5.1)**. The lowest extract concentrations observed to give a notable induction of EpRE-dependent luciferase activity in previous experiments were selected for the combinations. One exception from this was made, namely for red wine, as we wished to study the effect of a combination where one of the extracts did not have any inducing capacity on EpRE-dependent transcription when administrated individually. Rosemary, broccoli, turmeric, coffee, thyme, oregano, and red wine were tested at 3 mg/ml, while dog rose, red onion and cocoa were tested at 15 mg/ml.

Table 5.1 Extract combinations tested in the *in vitro* experiments

Extract combinations tested
Broccoli and rosemary
Broccoli and turmeric
Coffee and oregano
Coffee and thyme
Coffee and turmeric
Dog rose and rosemary
Dog rose and turmeric
Red onion and rosemary
Red onion and thyme
Red wine and rosemary
Rosemary and oregano

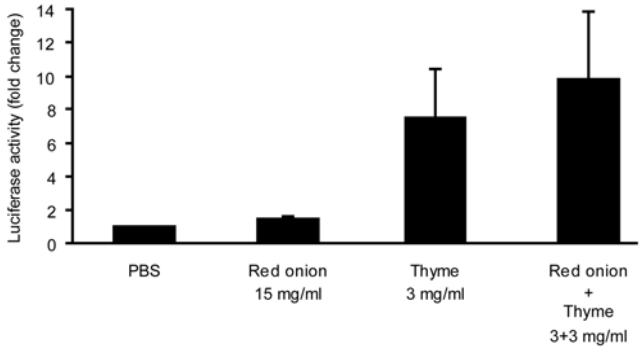
All experiments were repeated at least three times, and in each experiment each treatment was performed in triplicates. The results are presented as mean fold change ± SEM of EpRE-dependent luciferase activity compared to a PBS or PBS/DMSO control (**Figure 5.2)**. Also listed are the expected and observed effects, the percent difference between these values, and the p-value of a comparison of these two values.

A



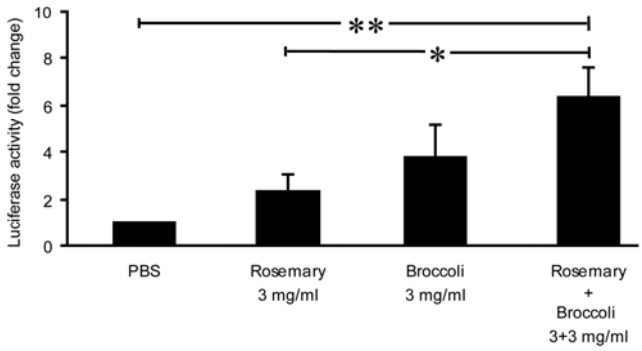
Expected	12. 12
Observed	15. 84
Difference (obs. – exp.)	3. 72
P (diff.)	0. 67
% difference (of exp.)	30.7

B



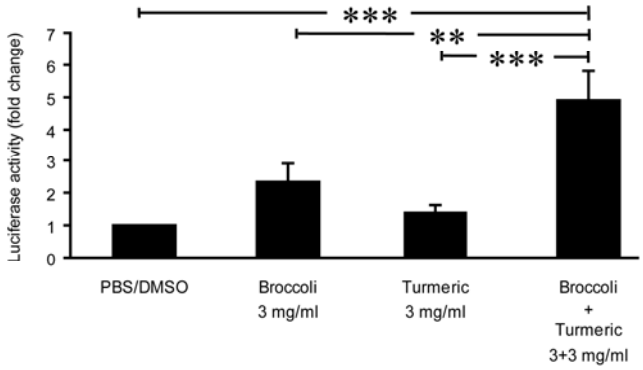
Expected	8. 93
Observed	9. 86
Difference (obs. – exp.)	0. 93
P (diff.)	0. 88
% difference (of exp.)	10. 4

C



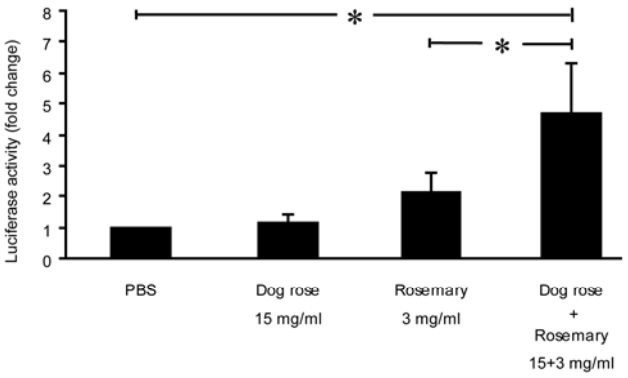
Expected	6. 14
Observed	6. 37
Difference (obs. – exp.)	0. 23
P (diff.)	0. 93
% difference (of exp.)	3. 7

D



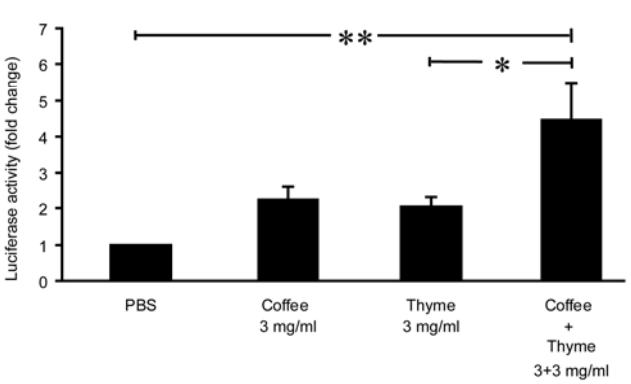
Expected	3. 73
Observed	4. 88
Difference (obs. – exp.)	1. 15
P (diff.)	0. 31
% difference (of exp.)	30. 8

E



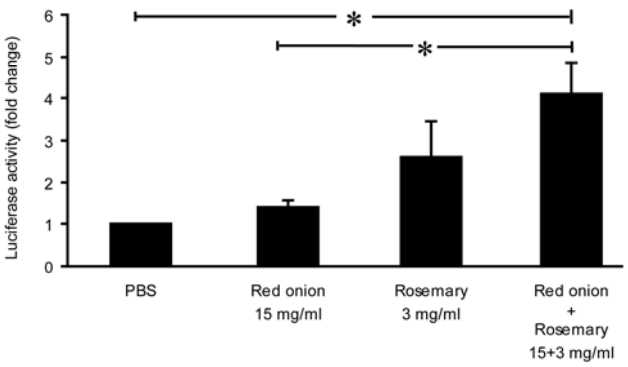
Expected	3. 28
Observed	4. 68
Difference (obs. – exp.)	1. 40
P (diff.)	0. 42
% difference (of exp.)	42. 7

F



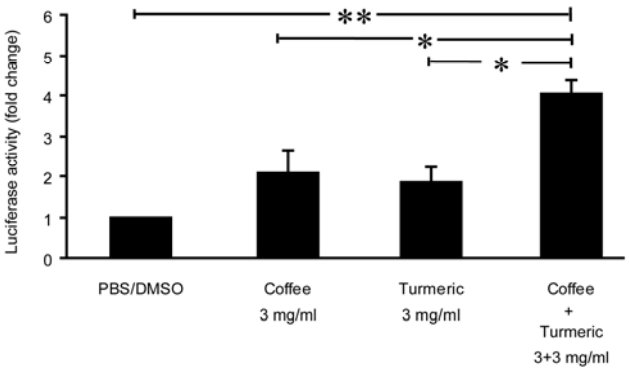
Expected	4. 23
Observed	4. 46
Difference (obs. – exp.)	0. 17
P (diff.)	0. 89
% difference (of exp.)	4. 0

G



Expected	4. 03
Observed	4. 13
Difference (obs. – exp.)	0. 09
P (diff.)	0. 94
% difference (of exp.)	2. 2

H



Expected	3. 93
Observed	4. 05
Difference (obs. – exp.)	0. 12
P (diff.)	0. 91
% difference (of exp.)	3. 1

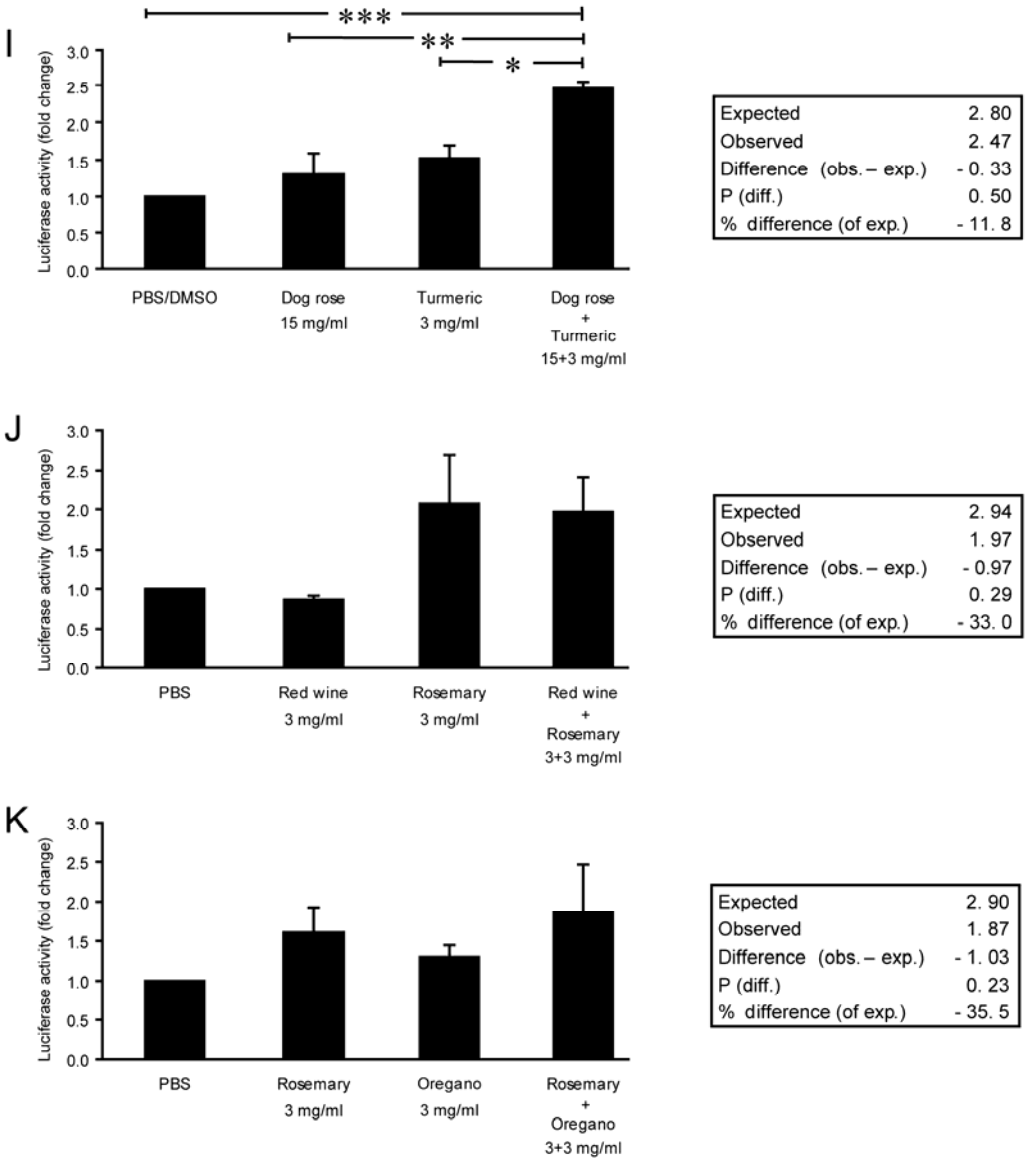


Figure 5.2 Effect of combination of plant extracts on EpRE-dependent luciferase activity. Transiently transfected EpRE-LUC HepG2 cells were stimulated with different extracts, individually and in combination. Their effect on EpRE-dependent luciferase activity was compared to a control of PBS or PBS/DMSO. The expected effect, *i.e.* the sum of the effect of the two extracts added individually, was compared to the observed effect of the extract combination. The bars represent mean EpRE luciferase activity ± SEM (n= 3 – 5). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. The text box presents the expected and observed values of the combinations, their difference and the p-values of their comparison.

A combination of coffee (3 mg/ml) and oregano (3 mg/ml) gave the highest induction of EpRE-dependent luciferase activity. The observed fold change was 15.84 ± 6.68, and significantly higher than the control ($p < 0.05$) **(Figure 5.2 A)**. The observed effect was further 3.72 higher than the expected (30.7 % difference), however not statistically significantly different ($p = 0.67$).

For the combination of red onion (15 mg/ml) and thyme (3 mg/ml) a fold change of 9.86 ± 4.57 in EpRE-dependent luciferase activity was observed, however not statistically higher than the control ($p = 0.14$) **(Figure 5.2 B)**. The observed luciferase activity of the combination was 0.93 higher than the expected (10.4 % difference), but not statistically significantly different ($p = 0.88$).

With a fold change of 6.37 ± 1.20, the combination of rosemary (3 mg/ml) and broccoli (3 mg/ml) gave a statistically significant increase in luciferase activity compared to the control ($p = 0.01$), and rosemary administrated individually ($p = 0.03$) **(Figure 5.2 C)**. The observed value was 0.23 higher than the expected value (3.7 % difference), but the two values were statistically significantly different ($p = 0.93$).

The combination of broccoli (3 mg/ml) and turmeric (3 mg/ml) gave a fold induction of 4.88 ± 0.91 in the EpRE-dependent luciferase activity, which was statistically significantly higher than the control ($p < 0.01$), and the broccoli ($p < 0.01$) and turmeric ($p < 0.01$) extracts added individually **(Figure 5.2 D)**. The observed value was 1.15 higher than the expected value (30.8 % difference), however not statistically significantly different ($p = 0.31$).

With a fold change of 4.68 ± 1.65 in EpRE luciferase activity, the combination of dog rose (15 mg/ml) and rosemary (3 mg/ml) increased the EpRE-dependent luciferase activity significantly compared to the control ($p = 0.02$) and dog rose added individually ($p = 0.02$) **(Figure 5.2 E)**. Despite an observed value 1.40 higher than the expected (42.7 % difference), the two values were not statistically significantly different ($p = 0.42$).

A combination of coffee (3 mg/ml) and thyme (3 mg/ml) (**Figure 5.2 F)** gave a fold change of 4.46 ± 1.00 in the EpRE-dependent luciferase activity, and the effect of the combination was significantly higher than the control (p= 0.01) and thyme added individually (p= 0.04). The expected value was further 0.17 higher than the observed value (4.0 % difference), but the two values were not statistically significantly different (p= 0.89).

The combination of red onion (15 mg/ml) and rosemary (3 mg/ml) gave a fold induction in the luciferase activity of 4.12 ± 0.73, which was significantly higher than the control (p= 0.02) and the red onion extract administrated individually (p= 0.03) **(Figure 5.2 G)**. With an observed value 0.09 higher than the expected value (2.2 % difference), the two values were not statistically significantly different (p= 0.94).

A fold change of 4.05 ± 0.35 was observed for the EpRE-dependent luciferase activity for a combination of coffee (3 mg/ml) and turmeric (3 mg/ml). This was statistically higher than the control (p< 0.01), and the coffee (p= 0.02) and turmeric (p= 0.01) extracts added individually **(Figure 5.2 H)**. The observed value was further 0.12 higher than the expected value (3.1 % difference), but not statistically significantly different (p= 0.91).

For a combination of dog rose (15 mg/ml) and turmeric (3 mg/ml), with a fold change of 2.46 ± 0.08 in the EpRE-dependent luciferase activity, the effect of the combination was statistically higher than the control (p< 0.01), and the dog rose (p< 0.01) and turmeric (p= 0.01) extracts added individually **(Figure 5.2 I)**. The observed effect was however 0.33 lower than the expected (11.8 % difference), but the expected and observed values were not statistically significantly different (p= 0.50).

The observed fold change of 1.97 ± 0.45 for the combination of red wine (3 mg/ml) and rosemary (3 mg/ml) was not significantly higher than the control (p= 0.69) **(Figure 5.2 J)**. The observed effect of the combination was further 0.97 lower observed than expected (33.0 % difference), however not statistically significantly different (p= 0.29).

The combination of rosemary (3 mg/ml) and oregano (3 mg/ml) had the lowest induction of EpRE-dependent luciferase activity, with a fold change of 1.57 ± 0.60, not statistically significantly different from the control (p= 0.71) **(Figure 5.2 K)**. The observed value was 1.03 lower than the expected value (35.5 % difference), although not statistically significantly different (p= 0.80).

Dose-response relationships of extract combinations

In the first experiments studying the effect of dietary plant extracts in combination, a single concentration of each extract was administrated. To further understand the relationships between the single extracts and their combinations, experiments with a wider range of concentrations were performed. Based on the previous results showing a strong induction of EpRE luciferase activity by the combination of coffee and oregano, and a difference in expected and observed value of 3.72 in fold change (30.7 % difference), this extract combination was selected for the extended study. The experiment was repeated three times, and in each experiment each treatment was performed in triplicates. The EpRE-dependent luciferase activity of the different treatments was compared to a control of PBS.

The results are presented as mean fold change of the EpRE-dependent luciferase activity ± SEM (n= 3 for all concentrations except n= 2 for 6 mg/ml) (**Figure 5.3**). The figure shows a comparison of the extracts added separately and together in different concentrations.

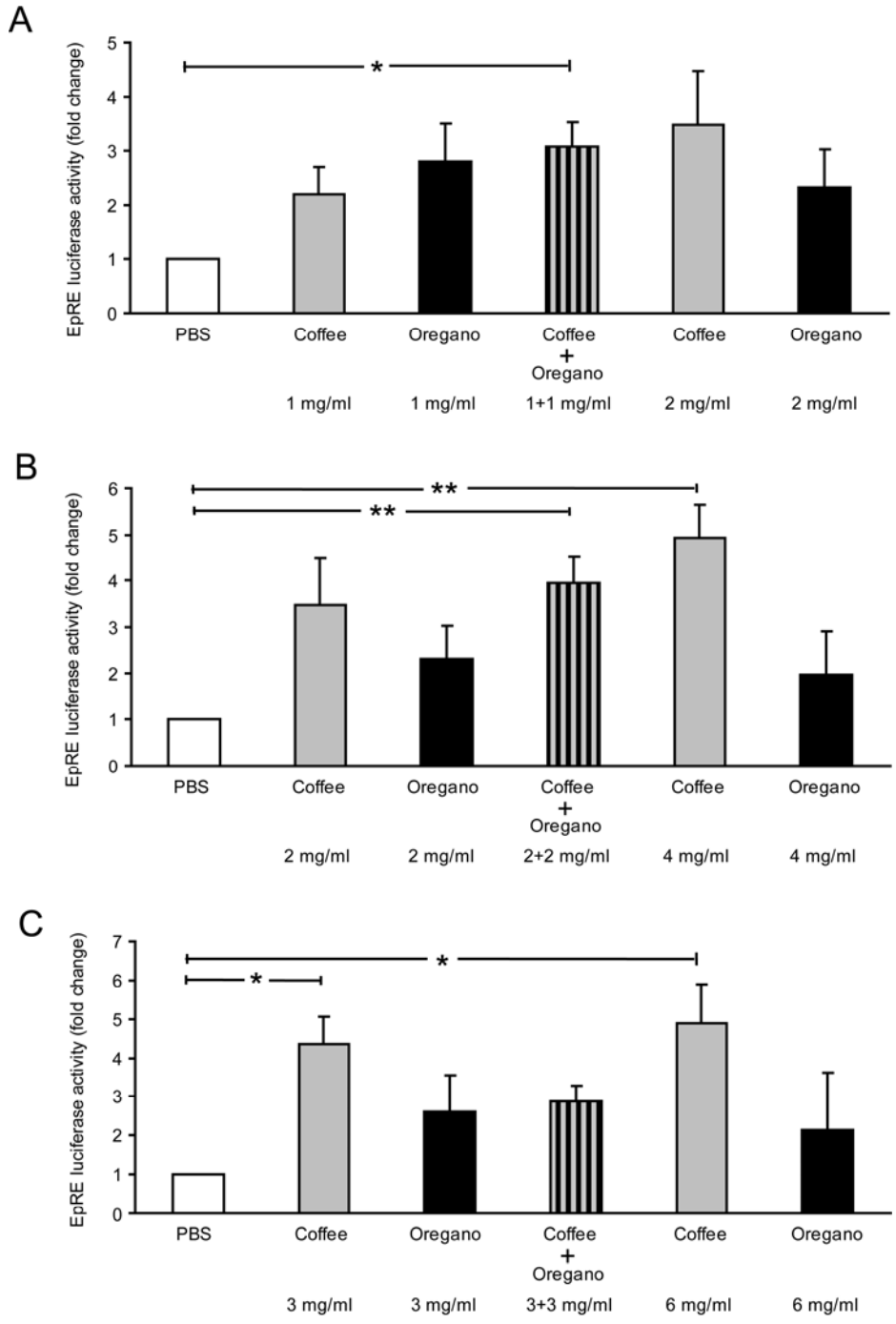


Figure 5.3 Effect on EpRE luciferase activity of coffee and oregano extracts. Transiently transfected EpRE-LUC HepG2 cells were stimulated with the indicated concentrations of coffee and oregano extracts, individually and in combination. EpRE luciferase activity was measured to compare the effect of the extract combinations with the separate extracts. The bars represent mean ± SEM (n= 3 for all concentrations except for n= 2 at 6 mg/ml). * = $p< 0.05$, ** = $p< 0.01$.

For the combination of 1 + 1 mg/ml of the two extracts, the observed fold change of EpRE-dependent luciferase activity was 3.07 ± 0.45. This was significantly higher than the control (p= 0.049), but not significantly higher than any of the two separately added extracts at 1 mg/ml **(Figure 5.3 A)**. When comparing the observed and expected value of the combination, the observed value was significantly lower than the expected value at 5.00 ± 0.09 (p= 0.01). Secondly we compared the effect of the combination to that of the individual extracts at the same total concentration of food (1 + 1 mg/ml in the combination towards 2 mg/ml of the individual extracts). The average value of 2 mg/ml coffee and oregano was 2.90 ± 0.56, and thus somewhat lower than the combination of 1 + 1 mg/ml of the extracts, although not significantly different (p= 0.83).

With a fold change of 3.96 ± 0.55, the combination of 2 + 2 mg/ml of the two extracts gave a statistically significant induction of the EpRE-dependent luciferase activity compared to the control (p< 0.01) (**Figure 5.3 B**). A statistically significant induction of EpRE was also observed for coffee at 4 mg/ml (p< 0.01), with a fold change of 4.94 ± 0.40 compared to the control. When comparing the extract combination with the separate extracts at 2 mg/ml, their average value at 4 mg/ml (3.46 ± 0.46), or the expected value (6.30 ± 0.89), no statistically significant differences were found.

For the results displayed in **Figure 5.3 C**, both 3 mg/ml and 6 mg/ml of coffee gave a statistically significant induction of EpRE compared to the control, with a mean fold change of 4.40 ± 0.70 (p= 0.01) for 3 mg/ml and (3.52 ± 1.24) (p= 0.01) for 6 mg/ml. For the extract combination of 3 + 3 mg/ml a fold change of 2.88 ± 0.39 was observed, while the expected value at this concentration was 7.24 ± 1.20 and nearly significantly different from the observed (p= 0.06). The average EpRE induction of 6 mg/ml coffee and oregano extract was further higher than the observed value for the combination, however not statistically significantly different (p= 0.59).

5.2 Effect of dietary plant extracts on transgenic EpRE LUC reporter mice

To investigate the effect of a dietary plant extract on the EpRE activity *in vivo*, experiments were performed using transgenic EpRE-LUC reporter mice. Previous studies have found a strong induction of EpRE-dependent activity *in vitro* by carnosol from rosemary, as well as rosemary extract (110;129). An extract of rosemary was also found to induce EpRE-dependent luciferase activity in the *in vitro* studies of this thesis, although not statistically different probably due to a low extract concentration and thus a relatively low luciferase induction. Carnosol has further been found to be an exceptionally strong inducer of EpRE-dependent activity *in vivo* (129), and an extract of rosemary was therefore selected to study the effect of a dietary plant extract on EpRE-dependent activity *in vivo*.

5.2.1 *In vivo* imaging

In vivo imaging was performed at 0, 2, 5, 10 and 24h after gavage feeding. For 0 - 5h 12 mice are included in each group, while for 5 - 24h 3 mice are included in each group (**Figure 5.4).**

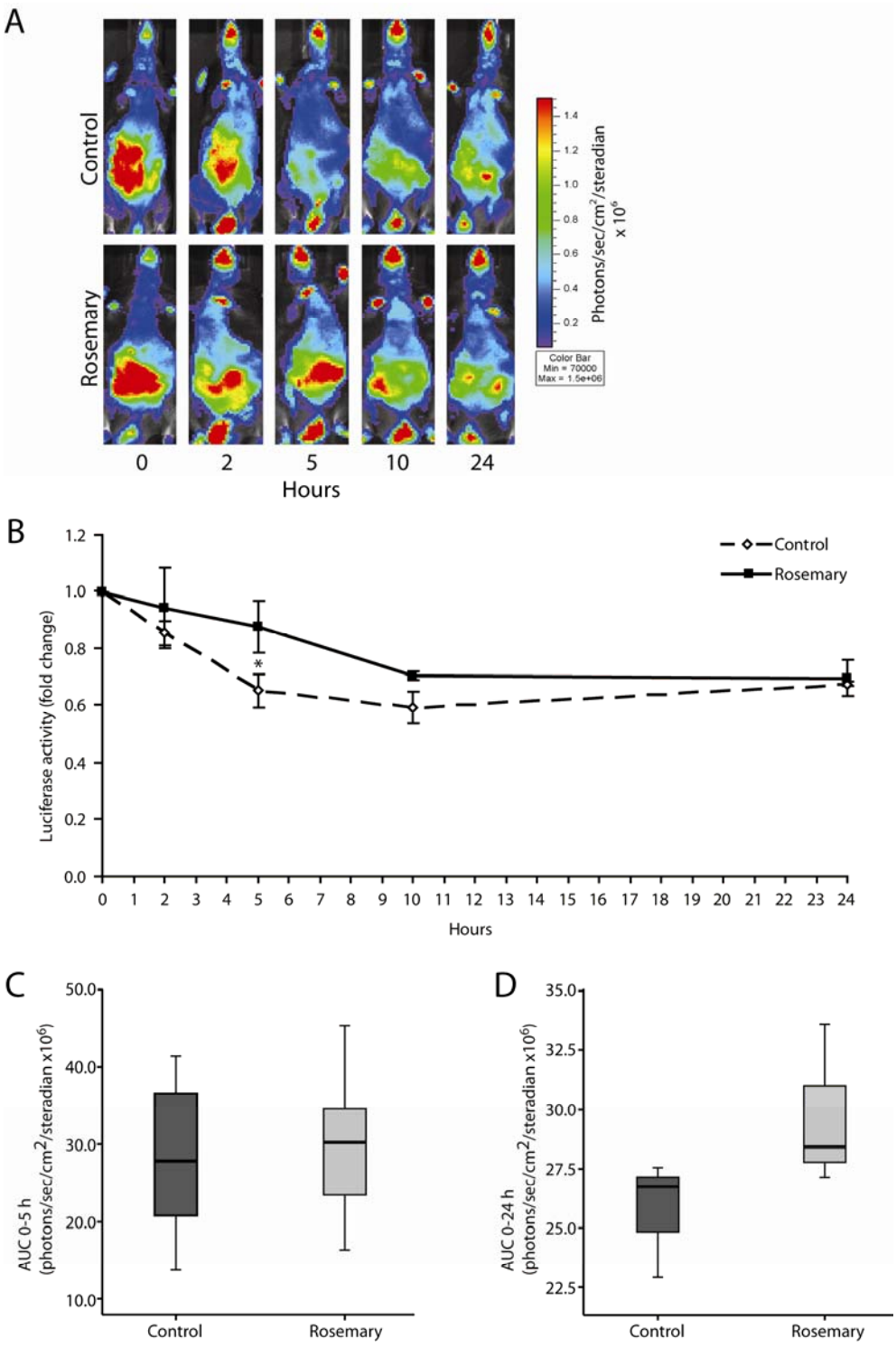


Figure 5.4 *In vivo* imaging of EpRE luciferase activity after administration of rosemary extract. EpRE LUC mice were gavage fed with rosemary extract or corn oil (control), and *in vivo* imaging of the EpRE luciferase activity was performed after 0, 2, 5, 10 and 24 h (n= 12 for 0 – 5h, n= 3 for 5 – 24h). Luciferase imaging is measured in photons/sec/cm^2/steradian. * = $P < 0.05$. **A)** Comparison of the *in vivo* imaging of a mouse from each of the two groups. **B)** Fold change of EpRE-dependent luciferase activity 0 – 24h after gavage feeding. **C)** Box plot of the area under curve (AUC) for the average luciferase radiance 0 – 5h. **D)** Box plot of the AUC for the average luciferase radiance 0 – 24h.

Figure 5.4 A displays a comparison of the *in vivo* imaging 0 – 5h after gavage feeding with either corn oil (control) or rosemary extract. With a mean luciferase activity measured at the start of the experiment at 700591 ± 63666 p/s/cm2/steradian for the control group, and 695616 ± 76814 p/s/cm2/steradian for the extract group, the two groups were not significantly different (p= 0.89).

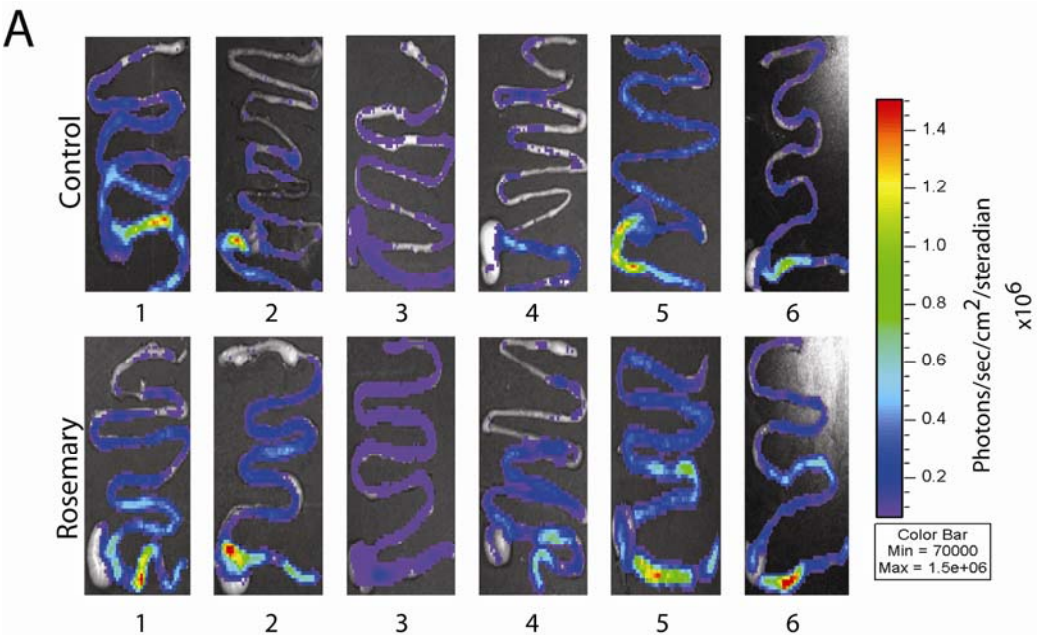
To measure the response to the rosemary extract, fold change of the luciferase activity from the base line value was calculated for the two groups at each time point **(Figure 5.4 B)**. Although the luciferase activity was decreased in both groups during the experimental period, the extract group seemed to retain a higher luciferase activity than the control group. The largest difference between the luciferase activities of the two groups was seen at 5h, with a fold change compared to the base line value of 0.65 ± 0.06 for the control group and 0.88 ± 0.09 for the extract group, and at this time point the fold change values of the two groups were significantly different ($p < 0.05$). The fold change values were however not statistically significantly different between the two groups at any of the other time points.

To display the total response in EpRE-dependent luciferase activity of the two groups, the area under curve (AUC) for the average radiance ($p/s/cm^2/sr$) is presented (**Figure 5.4 C** + **D**)**.** The mean AUC of the imaging performed 0 – 5h was $2.81 \times 10^6 \pm 2.54 \times 10^5$ for the control group and $2.98 \times 10^6 \pm 2.31 \times 10^5$ for the extract group (**Figure 5.4 C**), not statistically different (p= 0.20). The mean AUC for the imaging performed 0 – 24h was further $2.57 \times 10^7 \pm 1.43 \times 10^6$ for the control group, and $2.97 \times 10^7 \pm 1.97 \times 10^6$ for the extract group, however not statistically different (p= 0.71).

5.2.2 *Ex vivo* imaging

The time point with the largest difference in EpRE-dependent luciferase activity of the two groups after gavage feeding was decided from the *in vivo* imaging (**Figure 5.4 B**). The last two experimental days, the mice were sacrificed at this time point to do *ex vivo* imaging and further analysis of the organs **(Figure 5.5)**. The luminescence

was measured in photons/sec/cm^2/steradian, and the mean fold change of the control group is set to one.



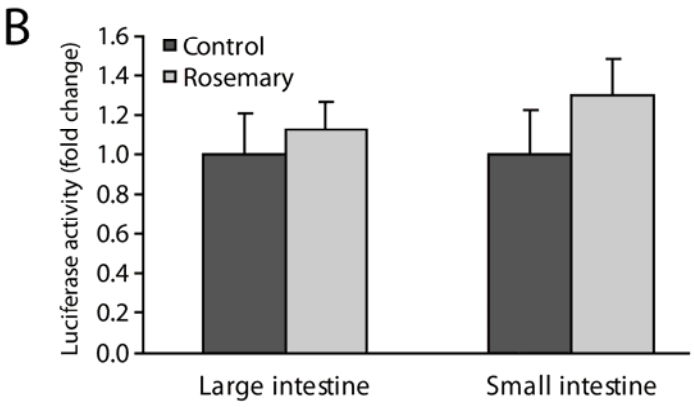


Figure 6.5 ***Ex vivo*** **imaging of the intestine after administration of rosemary extract.** The mice were gavage fed with either corn oil (control) or rosemary extract, and after 5h the mice were sacrificed and the organs excised. The luciferase activity was measured by *ex vivo* imaging in photons/sec/cm^2/steradian. The bars represent mean ± SEM (n= 6 in each group). **A)** *Ex vivo* imaging of the intestine in the control and extract group. **B)** Luciferase activity of the large and small intestines of the two groups.

Figure 5.5 A presents the *ex vivo* imaging of the intestines in the control and extract group at 5 h after gavage feeding. For the extract group, a fold change of 1.12 ± 0.14 was observed in the luciferase activity of the large intestine, while a fold change of 1.30 ± 0.18 was observed in the luciferase activity of the small intestine **(Figure 5.5 B)**. The luciferase activities were however not statistically different between the two groups in the large intestine (p= 0.59) or the small intestine (p= 0.31).

5.2.3 Luciferase activity in tissues

In addition to the luminescence measured *ex vivo* in the IVIS system, the luciferase activity was also measured by a luminometer in homogenates of the organs. To adjust for the size of the tissue samples, the measured luciferase activity was corrected for the protein content of the sample.

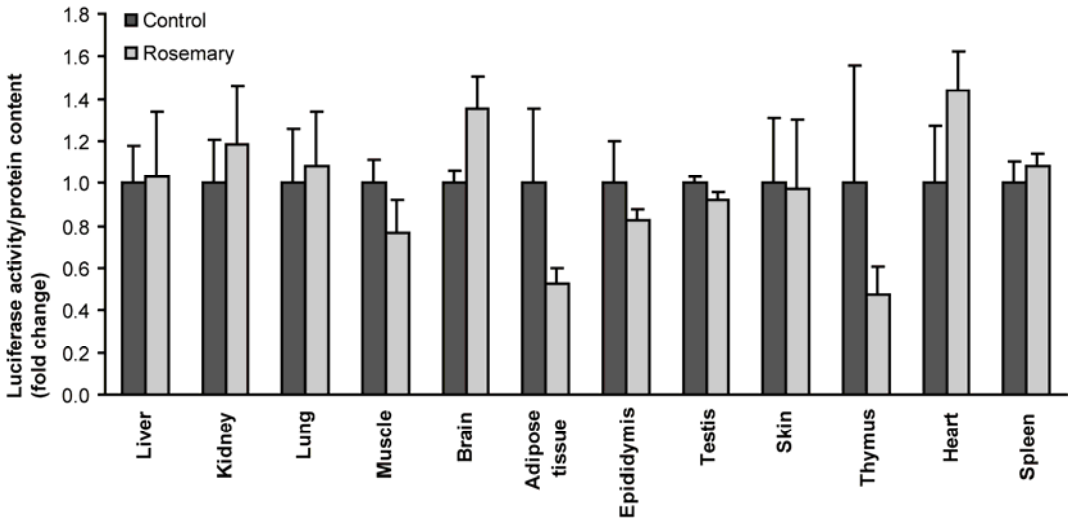


Figure 6.6. Luciferase activity in various organs after gavage feeding with rosemary extract. The organs were excised 5h after gavage feeding with either extract or corn oil (control). The EpRE activity in homogenates of the organs was measured and corrected for total protein content. The bars represent mean ± SEM. n = 6 in each group.

The organs with the largest difference between the two groups were the brain, adipose tissue, thymus and heart **(Figure 5.6)**. For the extract group, the fold change of luciferase activity was 1.36 ± 0.15 in the brain, 0.53 ± 0.07 in the adipose tissue, 0.48 ± 0.13 in the thymus, and 1.44 ± 0.18 in the heart, as compared to the control group. The luciferase activities were however not statistically different between the two groups in any of the organs.

5.3 FRAP analysis

As an evaluation of the amount of potentially bioactive compounds present in the food extracts, FRAP assay was used to assess their total reducing capacity. The results are presented as mmol/100g of the original product (mean ± SD).

5.3.1 FRAP analysis of food extracts used in cell experiments

Eleven extracts were used in the *in vitro* experiments of this thesis, and their FRAP-values are listed in **Table 5.2**. The oregano extract had the highest FRAP value, with 78.25 ± 0.52 mmol/100g. The lowest reducing ability was measured in the broccoli extract, with a FRAP value of 0.77 ± 0.01 mmol/100 g.

Table 5.2 FRAP values of food extracts used in the *in vitro* experiments. *= extract and FRAP value was provided by Marit Kolberg and Siril Garmannvik Johansen.

Extract	FRAP (mmol/100 g) Mean (n=3)	SD (mmol/100 g)
Oregano	78.25	0.52
Dog rose *	74.8	7.40
Thyme	46.83	0.24
Rosemary	30.82	0.93
Coffee	19.35	0.81
Cocoa *	8.48	0.61
Cherry tomato	4.02	0.01
Red wine *	3.37	0.06
Red Onion	2.57	0.09
Turmeric *	1.30	0.40
Broccoli *	0.77	0.01

5.3.2 FRAP analysis of extracts used in animal experiments

One extract of rosemary was used in the *in vivo* experiments, and the FRAP value is presented in **Table 5.3**.

Table 5.3 FRAP value of the rosemary extract used in the *in vivo* experiments

Extract	FRAP (mmol/100 g) Mean (n=3)	SD (mmol/100 g)
Rosemary	60.30	0.43

6. Discussion

6.1 Discussion of methods

6.1.1 Luciferase as a reporter system

Reporter genes have become a valuable tool in studies of gene expression. The luciferase reporter system can be used to study the activation of promoters or promoter regions both *in vitro* and *in vivo*, and was utilized to determine the activity of EpRE-dependent transcription in this thesis.

The advantage of the luciferase reporter is the high sensitivity due to low background luminescence, making it possible to detect low levels of gene expression. When luciferin is given in excess, there is a good correlation between the expressed luciferase and the intensity of luminescence. The translation of luciferase is very rapid, with maximum activity short time after initiation of transcription, and the enzyme has a relatively short half-life of about 2h due to high turn-over rate (133). The luciferase reporter system may therefore be a good detector of dynamic changes in EpRE-dependent transcription compared to alternative reporter genes, such as β-galactosidase and green fluorescent protein, which do not have these advantages (134).

As alternative methods to study activation of the Nrf2/EpRE pathway, electrophoretic mobility shift assay (EMSA) could be used to detect the interaction of Nrf2 and EpRE, while a TransAM™ Nrf2 assay could be used to detect activation of Nrf2. Such methods do however not report whether EpRE-dependent transcription is activated or not. The luciferase reporter system signifies the transcriptional activity of a specific promoter element, and therefore provides a well suited method to study the effect of dietary plants on EpRE-dependent transcription.

Use of the luciferase reporter gene in vivo

By molecular imaging of the luciferase activity in transgenic EpRE-LUC mice, it is possible to study the activity of EpRE-dependent transcription in a living animal over time. Molecular imaging thus represents an interesting way to study the effect of dietary plants on EpRE-dependent transcription *in vivo*.

Molecular imaging further allows non-invasive treatment of animals. It is possible to perform several measurements of the EpRE activity in the same animal, and the animal can thus serve as its own control. The number of animals needed in an experiment is reduced, and the effect of differences between the animals is lowered.

The luminescence detected by molecular imaging is emitted from several tissues and organs within the mice. Since mammalian tissues can scatter and absorb photons, the detected luminescence may be different from the actual light emitted (130). Haemoglobin for example, absorbs light at wavelength range 450 - 570 nm, which includes the wavelength of the luciferase luminescence (130). Organs rich in blood, such as the liver, heart, spleen and kidney, may thus emit a lower proportion of its produced luminescence as compared to organs such as thymus and lungs. Therefore, to study the organs individually, the mice were sacrificed and the organs excised, imaged *ex vivo*, and homogenized for further luciferase analysis.

6.1.2 *In vitro* studies

Since it is easy to control the factors of influence in cell cultures, they are well suited for mechanistic studies. *In vitro* studies are further a non-invasive way to perform experiments, and the ethical dilemmas around most *in vitro* studies are generally minimal.

Conclusions from *in vitro* studies can however not be directly transferred to intact organisms. While the cell culture conditions of HepG2 cells are very stable and controlled, the hepatocytes in the liver are under varying influence of different signalling molecules, nutrients, and hormones. In cell cultures, extracts are further added directly to the cells and not processed in the intestine. The compounds that

reach the cells in an *in vitro* experiment may therefore not be exactly the same compounds as those that would reach the corresponding cell type *in vivo*. *In vitro* studies are however of major importance to perform mechanistic studies, as well as screening studies of the effect of different treatments on molecular mechanisms; such as the effect of dietary plant extracts on EpRE-dependent transcription.

Use of HepG2 cells

The HepG2 cell line used in the *in vitro* experiments of this thesis is derived from a human hepatocarcinoma (128). Because of its characteristic as a cancer cell line it is capable of multiple replications in culture, and has a relatively long life compared to primary hepatocytes (135). The morphological characteristics and epithelial cell shape of HepG2 is compatible with those of liver parenchyma cells. The liver plays a major role in human metabolism, and is central for metabolism and function of the phytochemicals. The liver is further one of the main sites for phase I and II metabolism (12), and the HepG2 cell line has maintained many of the specialized functions that characterize normal human hepatocytes (136). The HepG2 cell line is therefore a good model for studies on the biological effects of phytochemicals (127), and may be particularly suited for studies on regulation of EpRE-dependent transcription.

HepG2 is an adherent cell line, and the cell viability and morphology can be determined by a phase microscope. Cells viability was closely followed with respect to confluence, growth, detachment, and infections. Cells viability and potential cytotoxicity of treatments can be further studied by measuring the metabolic activity of the cells (*e.g.* incubating the cells with a tetrazolium salt that is cleaved into a measurable product by metabolic active cells), or by measuring the cells membrane integrity (*e.g.* by adding trypane blue that selectively colour dead cells, or by evaluation of the amount lactate dehydrogenase in the cell medium) (137). Some of these methods may however be unsuited to determine the HepG2 cell viability in this thesis. As several of the plant extracts contain reducing compounds; the ability of some of the methods to report cell viability would have been influenced (*e.g.* lactate

dehydrogenase). Studies on antiproliferative and cytotoxic effect of resveratrol, epicatechin, and phytochemicals derived from apples, have shown that the concentrations of treatment and the time-course necessary for cytotoxicity of phytochemicals in HepG2 cells is higher and longer than the concentrations and incubation-time used in this experiment (138-140).

Experimental outline

Transfection enables introduction of an experimental DNA element such as a reporter gene construct, into a cell line. In this study HepG2 cells were transiently transfected with a 2 x EpRE LUC plasmid construct. The Lipofectamine 2000 transfection reagent contains liposomes of cationic and neutral lipids, which can interact electrostatically with nuclear acids and merge with cell membranes to deposit their cargo inside (141). The efficiency of the transfection depends on the cell density, the cell passage, the amount of DNA:Lipofectamine 2000, and the incubation time of the Lipofectamin 2000 and DNA:Lipofectamine 2000 (141). It is possible to co-transfect a second reporter vector or protein as an internal control of the transfection efficiency, *e.g.* renilla luciferase, green fluorescent protein, or β-galactosidase (142). As transient transfection of HepG2 cells by the Lipofectamine 2000 is well established in our laboratory (127), such co-transfections were however not performed in the experiments of this thesis. The luciferase activity in stimulated cells was further always compared to a control.

The cells were stimulated with plant extracts and incubated at 37ºC for 17h, due to previous experiments showing a maximal EpRE-dependent luciferase activity after 17h of extract incubation (129). To study the effect of combinations of dietary plant extracts on EpRE activity in HepG2 cells, extracts were incubated both individually and in combination to the cells. In the first experiments with extract combinations, the luciferase activity was measured in a TD 20/20 luminometer using the luciferase assay system after cell lysis. This is a well established method used to measure luciferase activity in HepG2 cells in our laboratory, and has been used in several previous experiments with dietary plant extracts (127;129).

When combining the two extracts the final concentration of extract (not taking into account the type of extract) is higher than in each of the two concentrations alone. We can therefore not rule out the possibility that the effect of the extract combination is due to a higher amount of extract (higher "food count"), or whether the effect is dose-dependent. We therefore wished to examine a wider range of concentrations in each experiment, and a new experimental setup was planned.

In this new setup, a new method to measure luciferase activity was used. In this method luciferin was added directly to the cell culture medium, and a Synergy 2 luminescence plate reader was used to measure luciferase activity. This method has been used by the research group to measure the luciferase activity in U-937 3xκB-LUC cells, but has not previously been used to measure the EpRE-luciferase activity in HepG2 cells. To verify the new method against the previously used, two identically experiments were performed with the two methods and they were found to correspond well (data not shown).

6.1.3 *In vivo* experiments

A cell line should consist of only one type of cells, and the physiological interactions occurring within an intact animal can not be imitated by these. EpRE activity may be influenced by several intracellular and extracellular factors, and the regulation of EpRE may be far more complex in an intact animal than in a single cell line. *In vivo* studies are therefore an important tool in nutritional and pharmacological research to study how EpRE activity can be affected *in vivo*.

Transgenic reporter mice have offered a valuable opportunity to study *in vivo* regulation of genetic transcription. Compared with *in vitro* cell culture experiments, transgenic EpRE mice provide a unique possibility to study the EpRE activity at the whole body level, reflecting the dynamics of the complex biological networks.

6.1.4 Food extracts

To extract as much as possible of these bioactive compounds from dietary plants, the extraction method should be able to extract polar as well as non-polar compounds. The extraction procedure used in this thesis was based on the method developed for FRAP analysis by Benzie and Strain (131), which has been modified by Halvorsen *et al*. (132) to include water and methanol as solvents. The method was further developed with evaporation of the liquid to concentrate the extracts for *in vitro* and *in vivo* experiments.

Since the extraction method employs both water and methanol as solvents, it will be able to extract both hydrophilic and hydrophobic substances. The composition of the compounds being extracted is however not known. To characterize the profile of the compounds, procedures like high performance liquid chromatography (HPLC) (143) and liquid chromatography mass spectroscopy (144) could be performed.

6.1.5 FRAP assay

The reducing capacity of the extracts used in this study was measured by the FRAP assay. The FRAP assay measures the capacity of present antioxidants in a sample to reduce Fe^{3+} to Fe^{2+}, and is therefore a direct measure of antioxidants or reductants. Other methods used to assess the antioxidant capacity are the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC) assay, and the oxygen radical absorbance capacity (ORAC) assay. These methods are however based on the ability of antioxidants to neutralize reactive species generated in the sample mixture, and may thus be more indirect than the FRAP method. The results from TEAC and ORAC may further depend on the reactive species used, and the FRAP assay therefore provides a good measurement of the concentration of reductants in a sample (132).

The FRAP assay gives fast and reproducible results, which are linear over a wide range of concentrations (131). The FRAP assay has further been found to correlate well with the total phenolic content of different samples (145;146). However, one

possible limitation of the FRAP assay is the low reactivity of thiols with ferric ions, due to lower reduction potential of thiols than the Fe^{3+}/Fe^{2+}-TPTZ half reaction. Few antioxidant thiols are nonetheless present in dietary plants, and the FRAP assay is therefore assumed to be a well suited method to measure the total antioxidant capacity of plants (132).

In accordance with earlier studies (125;147), the highest FRAP values measured in this thesis were for the spices and coffee, with oregano reaching the highest value. For the other extracts, the FRAP values were also found to be in relatively good accordance with previous studies (125;147;148). For cherry tomatoes and red onion however, previous studies have published a FRAP value of 0.62 mmol/100g for cherry tomatoes (149), and 0.69 mmol/100g for red onion, respectively (132). The FRAP values obtained for the cherry tomato and red onions extracts in this thesis are thus much higher than previously measured.

The differences seen in the FRAP values measured for the same plant species may be due to differences in species, manufacturer, land of origin, place of growing, and time of harvesting. This may lead to large variety in the composition of phytochemicals, and thus their reducing capacity. In tomatoes for example, the phytochemical distribution and amount of antioxidants is highly dependent on the conditions during cultivation (150). As the extracts are prepared by an extended extraction method compared to that used for the FRAP measurements on the foods, this is probably the largest source of variation in the FRAP values.

6.2 General discussion

Epidemiological studies have showed an inverse relationship between consumption of dietary plants and the incidence of several chronic diseases, such as cancer, cardiovascular disease and diabetes (2;40). Therefore, with the high global burden of chronic diseases, research on the molecular mechanisms underlying the beneficial health effect of dietary plants should be highly prioritized.

Dietary plants contain a wide range of phytochemicals. Several phytochemicals have been shown to induce cytoprotective proteins through the EpRE motif (84), and this induction has been suggested to contribute to the disease preventive effects afforded by dietary plants (62). Phytochemicals have further been suggested to work in an additive and synergistic manner, and the concept of synergy among phytochemicals has been postulated as important for the beneficial health effect of a diet high in dietary plants (14).

Because of the link between induction of EpRE-dependent transcription and up-regulation of cytoprotective proteins (75;76), cell lines and animal models with EpRE reporter constructs can be utilized to study the cytoprotective and chemopreventive potential of dietary plants. Several dietary phytochemicals, and a selection of dietary plant extracts high in at least one of these, have been studied and found to be potent inducers of EpRE-dependent transcription (84;129). Few studies have however investigated the effect of combinations of dietary plant extracts on EpRE-dependent transcription. Thus the main purpose of this study was to investigate and compare the individual versus the combined effect of dietary plant extracts on EpRE- dependent transcription. An *in vivo* experiment was also performed to study the ability of a dietary plant extract to induce EpRE-dependent transcription in living animals.

The *in vitro* experiments showed that combinations of dietary plant extracts are potent inducers of EpRE-dependent transcription. Of the 11 extract combinations studied, 8 gave a statistically increased EpRE-dependent luciferase activity compared to the control, and 7 of these were further statistically higher than one or both of the

individually administrated extracts. There appear to be additive effects of several of the combinations. Three combinations however, showed a trend towards synergistic effects (rosemary + dog rose, broccoli + turmeric and coffee + oregano), while two other combinations showed a trend towards antagonistic effects (rosemary + oregano and red wine + rosemary). When a combination of oregano and coffee was tested in a wider range of concentrations, antagonistic effects were indicated at some of the concentrations. Furthermore, transgenic EpRE mice given rosemary extract had a significantly higher EpRE-dependent luciferase activity compared to control mice 5h after administration of the extract. In particular, the EpRE-dependent luciferase activity was higher in the intestine, kidney, heart and brain of the mice receiving rosemary extract, although not statistically significantly different. Thus, dietary plant extracts known as *in vitro* inducers of EpRE-dependent transcription may also induce EpRE-dependent transcription *in vivo*.

6.2.1 Effect of dietary plant extracts on EpRE-dependent luciferase activity *in vitro*

Tomato, red wine and cocoa extracts

Tomato, red wine and cocoa extracts, not previously tested for their ability to induce EpRE in EpRE-LUC HepG2 cells, were studied in this thesis. Although lycopene and resveratrol, found in tomatoes and red wine, have been found to induce EpRE-dependent transcription and up-regulate cytoprotective proteins in HepG2 cells as well as other cell lines (113-115;129), only the cocoa extract gave a significant induction of EpRE-dependent luciferase activity in this thesis.

Effect on EpRE-dependent transcription by combinations of dietary plant extracts

The results of the extract combinations are summarized in **Figure 6.1**. The combinations of coffee + oregano, red onion + thyme and rosemary + broccoli gave the highest increase in EpRE activity. The induction of EpRE-dependent luciferase activity observed for the different extracts are in accordance with previous studies conducted by Balstad *et al*., who showed a relatively high fold change of luciferase activity in EpRE-LUC HepG2 cells at 3 mg/ml of coffee (fold change 3.8), oregano (fold change 4.1), thyme (fold change 8.8), and broccoli (fold change 4.0) (129). Coffee has also been shown to induce EpRE-dependent transcription in transgenic EpRE-LUC mice (151).

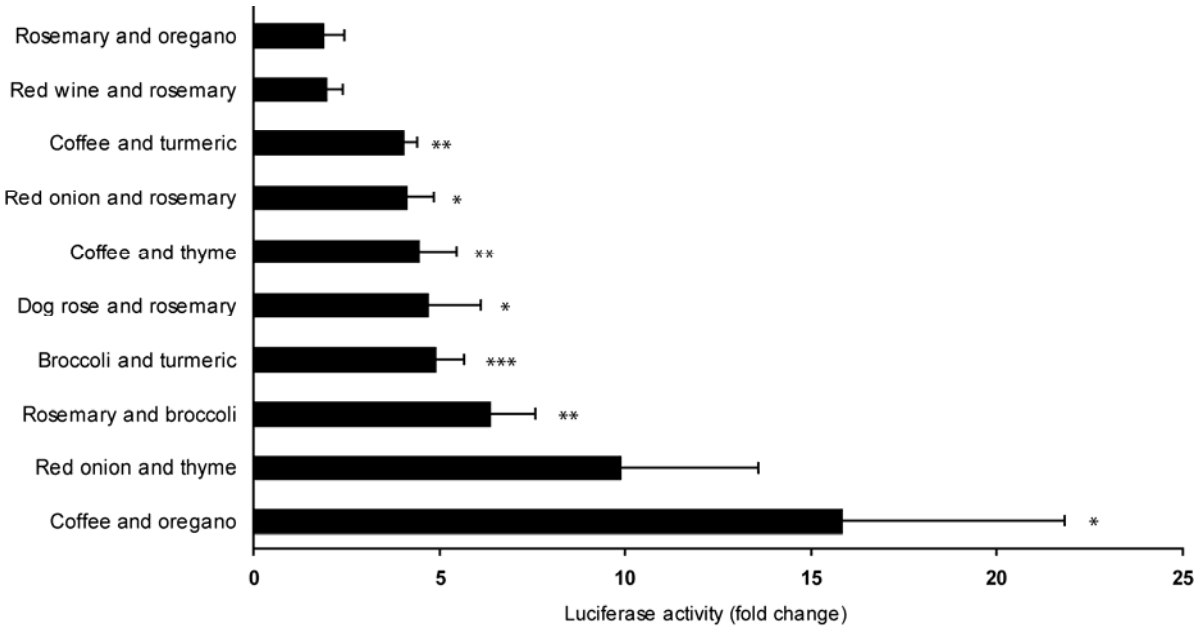


Figure 6.1 Summary of the effect of extract combinations on EpRE-dependent luciferase activity. Eleven extract combinations were administrated to EpRE-LUC HepG2 cells, and their respective induction of EpRE-dependent transcription was measured by their luciferase activity. The bars represent mean ± SEM (n= 3 – 5).

As food synergy may have an important role in the beneficial health effect of dietary plants, it is of great interest to establish systems to study the combinational effects of dietary plants. In this thesis we compared the observed effect of the extract combinations to the sum of the individually administrated extracts. This was done to study whether the actual effect of the combination was equal, larger or smaller than the sum of the effect of the individual extracts. As there were no statistically significant differences between the expected and observed values, no observed effects were significantly higher or lower than the expected value. Several of the combinations had a relatively low difference between the expected and observed value; while a total of 6 combinations had a difference < 12 %. This indicates that there may be additive effects of several of the extract combinations tested in this thesis. Additive effects of dietary plants on EpRE-dependent transcription may contribute to the understanding of the health benefits of a diet rich in dietary plants.

Some of the differences between the expected and observed values of the extract combinations were however noteworthy. For 3 combinations the observed effect was > 30 % higher than the expected, and these were rosemary + dog rose, broccoli + turmeric, and coffee + oregano. Although the combination of rosemary and dog rose did not statistically significantly increase EpRE-dependent luciferase activity, there may be some synergistic effect of these three extract combinations. On the contrary to these 3 combinations, 2 other combinations had an observed value > 30 % lower than the expected value, namely the combination of rosemary + oregano, and red wine + rosemary. For these combinations, there may be antagonistic effects between the extracts. The p-values of the comparison of the expected and observed value are correspondingly the lowest of all the combinations tested.

As the first experimental setup for studying combinations of dietary plant extracts included only single concentrations of each extract, a new experimental setup including a wider range of concentrations was planned. The advantage of this setup is that it allows comparison of the dose-response relationships for the extracts added individually and in combination. Due to a high induction of EpRE-dependent luciferase activity and a large difference between the expected and observed value

observed, the combination of coffee and oregano was selected. Interestingly, when comparing the observed effect of the 1 + 1 mg/ml extract combination with the expected value, the observed was significantly lower than the expected. It thus appears to be an antagonistic effect of the coffee and oregano extracts at this concentration. Furthermore, at the combination of 3 + 3 mg/ml, the observed effect was nearly significantly lower than the expected, indicating a trend towards antagonism at this concentration. This is intriguing with respect to the observed trend towards synergy when tested at this concentration in the first experiments. The overall fold induction from the oregano extract was lower in this extended experiment compared to the first experiments, and this discrepancy seems account for the difference between these two sets of experiments. This underlines the difficulties of working with food extracts.

Mechanisms behind induction of EpRE-dependent transcription by dietary plant extracts

Since most previous studies on dietary plants and EpRE-dependent transcription, only a few mechanistic studies have investigated the induction of EpRE-dependent transcription by dietary plant extracts. For the dietary plant extracts containing phytochemicals of which a mechanism for EpRE-induction has been described, it may however be assumed that the induction will be at least partly mediated by the same mechanisms as their respective phytochemicals. It is, however, important to note that the dietary plant extracts may contain other compounds that could also affect regulation of EpRE-dependent transcription.

A selection of the phytochemicals, including vitamins and minerals, that may be present in the extracts is presented in **Table 6.1**.

Table 6.1 A selection of phytochemicals in the dietary plant extracts (14;109;152;153)

Food	Phytochemicals (including vitamins and minerals)
Broccoli	Vitamin A Vitamin B_1, B_2, B_3, B_6 Vitamin C Vitamin E Folic acid β-carotene Isothiocyanates (phenetyl isothiocyanates, sulphoraphane) Cyanides Thiocyanates Nitriles
Cocoa	Vitamin A Vitamin B_1, B_2, B_6 Vitamin B_3 Folic acid Flavanols Flavonols Anthocyanins Procyanidins
Coffee	Diterpenes (cafestol, kahweol) Caffeine Phenolic compounds (caffeic acid)
Dog rose	Vitamin C Flavonoids Phenolic acids Lycopene
Oregano	Phenolic acids (caffeic acid, coumaric acid, rosmarinic acid) Flavonoids
Red onion	Vitamin B_1, B_2, B_3, B_6 Vitamin E Folic acid Quercetin Anthocyanidins Gallic acid Diallyl sulfide
Red wine	Flavonols Flavanols Resveratrol
Rosemary	Phenolic acids (caffeic acid, rosmarinic acid, caffeoyl derivatives) Phenolic diterpenes (carnosol, carnosic acid, epirosmanol)
Thyme	Phenolic acids (gallic acid, caffeic acid, rosmarinic acid) Phenolic diterpenes Flavonoids
Tomato	Vitamin A Vitamin B_1, B_2, B_3, B_6 Vitamin C Vitamin E Folic acid Lycopene β-carotene Phytoene Phytofluene Flavanones
Turmeric	Curcumin

Induction of EpRE-dependent transcription has been suggested to be directed by modification of cysteine residues on Keap1, or by activation of cellular signalling systems regulating the Nrf2 translocation or transactivation, such as MAPK, PKC and PI3K (98). These mechanisms of action have been demonstrated for a number of phytochemical inducers of EpRE-dependent transcription, and some inducers appear to work through multiple mechanisms (*e.g.* sulphoraphane) (84;102).

Induction of EpRE-dependent transcription by individual extracts

A previous study has found a potent induction of EpRE-dependent transcription by a broccoli extract in EpRE-LUC HepG2 cells (129), and a broccoli extract was also shown to induce EpRE-dependent luciferase activity in this thesis. Broccoli contains high amounts of sulphoraphane, in addition to several vitamins, minerals, and other phytochemicals **(Table 6.1)**. Sulphoraphane has been suggested to be an effective cancer preventive compound from several epidemiological studies (154;155). A mice study has shown that sulphoraphane is able to induce cytoprotective proteins such as GP, GSR, NQO1, GST, GCL, and ferritin in the small intestine, through an Nrf2 activated EpRE-dependent transcription (116). By studies in cell lines, the Nrf2 activation has been suggested to be mediated by an activation of JNK1 and ERK2 (84;102), and inhibition of p38 MAPK (100). Since sulphoraphane is an electrophile component, it may also activate Nrf2 translocation by modification of Keap1 cysteine residues directly (62;84).

A cocoa extract was recently found to induce cytoprotective proteins in HepG2 cells, and this was suggested to be mediated by an activation of the ERK1/2 pathway (156). In line with these studies, a cocoa extract was found to give a significant induction of EpRE-dependent transcription in the EpRE-LUC HepG2 cells in this thesis. Cocoa contains both vitamins, minerals, and several phytochemicals **(Table 6.1)**, and several of its components may thus contribute to its induction of EpRE-dependent transcription.

A previous study has showed an induction of EpRE-dependent transcription in EpRE-LUC HepG2 cells by a coffee extract (129), and this was confirmed here. A

study administrating coffee to mice found a significant induction of cytoprotective proteins in the liver and gastrointestinal tract of Nrf2-wild type mice compared to Nrf2-knock out mice, and this induction was further suggested to be mediated by the phytochemical compounds cafestol and kahweol (109) **(Table 6.1)**. The mechanisms behind induction of EpRE-dependent transcription by coffee is not fully elucidated, but a study has shown that the degree of roasting may be an important determinant for the extent of the induction (151).

Dog rose, containing vitamin C in addition to several phytochemicals **(Table 6.1)**, has been shown to induce EpRE-dependent luciferase activity in HepG2 cells (fold change 1.9 at 15 mg/ml) (129). An induction of EpRE-dependent luciferase activity by dog-rose was also observed in this study. Lycopene has been shown to induce EpRE-dependent transcription (129) and up-regulation of cytoprotective proteins in a HepG2 cell line (113). Lycopene may therefore have contributed to the induction of EpRE-dependent transcription observed for the dog rose extract in this thesis. The mechanisms by which lycopene induce EpRE-dependent transcription are however not fully elucidated.

An extract of oregano has been found to induce EpRE-dependent luciferase activity in HepG2 cells (129), and this result was confirmed in this study. Oregano contains several different phytochemicals, such as phenolic acids and flavonoids, which may be contributing to its induction of EpRE-dependent luciferase activity **(Table 6.1)**.

A red onion extract was recently tested and found to induce EpRE-dependent luciferase activity in HepG2 cells (fold change 1.2 at 15 mg/ml) (129), and an induction of EpRE-dependent luciferase activity by a red onion extract was confirmed in this thesis. Red onions contain a wide range of potentially bioactive compounds **(Table 6.1)**, of which diallyl sulphide and quercetin have been shown to induce EpRE-dependent transcription. The organosulphur compound diallyl sulphide has been shown to induce the cytoprotective protein NQO1 in Nrf2-wild type but not in Nrf2-knock out mice, indicating an induction of EpRE-dependent transcription by

diallyl sulphide (112). The phytochemical quercetin has been shown to induce EpRE-dependent transcription in HepG2 cells, potentially by modification of Keap1 (117).

An extract of rosemary has been shown to induce of EpRE-dependent transcription in EpRE-LUC HepG2 cells (fold induction 2.4 at 3 mg/ml) (129), and an induction of EpRE-dependent luciferase activity of a rosemary extract was also observed in this thesis. Carnosol is found in high amounts in rosemary **(Table 6.1)**, and studies conducted in a rat kidney cell line has shown that carnosol induce expression of HO-1 by activating Nrf2 and inducing its association with EpRE (110). This was further indicated to be mediated by an up-regulation of the PI3K. The carnosol may therefore contribute to the induction of EpRE-dependent transcription observed for rosemary in this study, although rosemary may contain other EpRE inducing compounds as well.

Thyme is a phytochemical-rich herb **(Table 6.1)**, which has been shown to induce EpRE-dependent luciferase activity in HepG2 cells when administrated as an extract (129). This is in accordance with the thyme extract used in this thesis. As thyme contains several phytochemicals, there may be several contributors to its induction of EpRE-dependent transcription.

In addition to its wide use as a spice, turmeric has been used as an anti-inflammatory and anti-bacterial agent in traditional Indian medicine (157). These properties have been ascribed to its phenolic phytochemical curcumin, which has been found to possess potent anti-inflammatory and antioxidant properties, which has also been investigated as a chemopreventive agent (158;159). Curcumin has been shown to up-regulate cytoprotective proteins by stimulating Nrf2 translocation and DNA binding both in human hepatoma cells (103) and in mice (111). This up-regulation has further been suggested to be mediated by activation of PKC and p38 MAPK (103). Both curcumin and turmeric has been found to induce EpRE-dependent luciferase activity in HepG2 cells (fold change 2.2 at 3 mg/ml) (129), and an induction of EpRE-dependent luciferase activity was confirmed in this thesis. Due to its content of curcumin, turmeric may induce EpRE-dependent transcription at least partly via the same pathways as curcumin.

Induction of EpRE-dependent transcription by extract combinations

From the previously discussed mechanisms by which dietary plant extracts may induce EpRE-dependent transcription, it is apparent that incubation of cells with a combination of extracts may introduce a wide range of compounds with potential effects on the Nrf2/EpRE-pathway. These food extracts may contain a variety of both known and unknown inducer compounds. Their mechanisms of inducing EpRE-dependent transcription can be through studied mechanisms of single compounds present in these extracts, but possibly by other compounds and other pathways as well. The effects of these compounds may either be enhanced or inhibited compared to the corresponding effects of each compound alone, leading to additive, synergistic or antagonistic effects. It is possible that a particular mechanism of acting may be saturated at high concentrations of a compound or if several compounds target the same point of the pathway, so that the induction of EpRE-dependent transcription will stagnate.

By activating different targets in the pathways, combinations of compounds may potentiate and multiply the EpRE-activation. Compounds, such as sulphoraphane, can modulate the cysteine residues on Keap1, and thus abrogate the ubiquitination of Nrf2. If the combination additionally contains components that induce EpRE-dependent transcription through cellular kinases, the induction of EpRE-dependent transcription mediated by the two extracts may be particularly strong.

An additive or synergistic effect of foods on signalling pathways such as the Nrf2/EpRE pathway may provide beneficial health effects of dietary plants when consumed in the diet. So far, only a few studies have explored additive and synergistic effects of foods, and thus further research in this complex field of nutrition is warranted.

Comparison of FRAP value and EpRE induction of extracts

It has become more and more accepted that phytochemicals exert functions *in vivo* extending beyond direct antioxidant properties. The induction of EpRE-dependent transcription of cytoprotective proteins appears to be mediated by compounds both with and without *in vivo* redox activity according to Dinkova-Kostova and co-workers (62).

Although the highest FRAP-value was measured for the oregano extract, and the lowest FRAP-value was measured for the broccoli extract, both induce EpRE relatively strong and to a quite similar extent. This is in line with the theory presented by Dinkova-Kostova and co-workers (62) that several compounds induce cytoprotective proteins independently of their redox activity, of which one example has been sulphoraphane. This observation may emphasize the importance of phytochemicals beyond their function as antioxidants.

6.2.2 Effect of rosemary extract on EpRE-dependent luciferase activity *in vivo*

The experiments in this thesis, as well as other studies (129), have indicated an induction of EpRE-dependent transcription by rosemary extract *in vitro*. To our best knowledge however, no previous studies have tested the effect of a rosemary extract on EpRE-dependent transcription *in vivo*.

A significantly higher EpRE activity in the extract group as compared to the control was found 5h after gavage feeding. This timing is in accordance with previous similar studies administrating dietary plant extracts to transgenic EpRE luciferase mice (129;151). The statistically higher EpRE activity in the extract group as compared to the control group at 5h shows that rosemary extract has an effect on EpRE activity *in vivo*.

We observed that the overall EpRE activity was slightly decreased relatively to the base line level in both groups during the 24h experimental period. Living organisms

have developed a fluctuating system called circadian rhythms, generating 24-h periodicities in various physiological and behavioural processes (160). It may therefore be assumed that the EpRE-dependent luciferase activity also may be affected by circadian system. Interestingly, the mice in the extract group seemed to maintain a higher EpRE activity, at least during the first 5h.

From the *ex vivo* imaging, the extract group appeared to have a higher EpRE-dependent luciferase activity in both intestinal segments compared to controls, although not statistically significant. This observation is particularly interesting with respect to the increasing awareness of the local cytoprotective effects of phytochemicals in the intestine (12;51). Nrf2 is highly expressed in the intestine (83), and an induction of cytoprotective proteins in this segment may reduce damage elicited by potentially reactive compounds in food. This may contribute to the observed beneficial effects of phytochemicals in the intestine.

6.2.3 *Potential health effects of inducers of cytoprotective proteins*

The cytoprotective proteins are important to reduce damage by oxidants and electrophilic compounds within the body. The family of GSTs for example, are important contributors to the cellular biotransformation of electrophilic compounds (161). Some EpRE-induced cytoprotective proteins may further catalyze reactions that generate small direct antioxidant molecules. Examples of such proteins are HO-1, generating carbon monoxide and biliverdin/bilirubin (162), and γGCS, catalyzing the rate-limiting step in the synthesis of GSH (163). EpRE-dependent transcription also up-regulates small molecule antioxidants directly, such as Trx; in addition to enzymes responsible for catalyzing the reduction of such small molecule antioxidants, such as ThR (62). The intrinsic network of cytoprotective proteins is thus very complex **(Figure 6.2)**, and provides protection against damaging oxidative and electrophilic compounds at several levels. Induction of this system is therefore suggested to decrease the risk of cancer and other chronic diseases whose pathogenesis depends on oxidative stress and electrophilic toxicity.

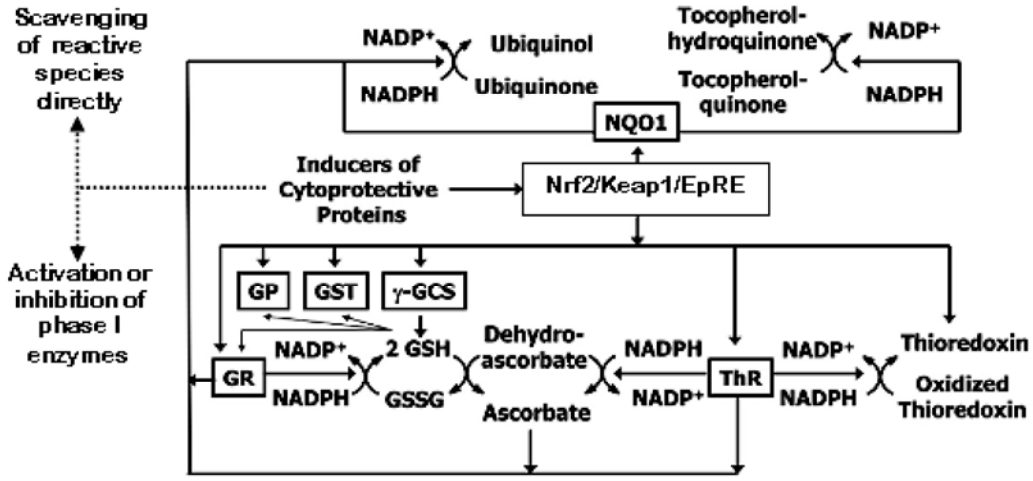


Figure 6.2 Potential effects of EpRE-inducers (adapted from Dinkova-Kostova *et al.* (62)). EpRE-dependent transcription up-regulates several cytoprotective proteins, both small redox active proteins, *e.g.* Trx, and enzymes that catalyze reduction of the oxidized form of small redox active proteins, *e.g.* ThR. EpRE-dependent transcription may thus increase the reducing capacity of an organism, and reduce damage potentially caused by oxidative stress and electrophiles.

Originally the inducers of EpRE-dependent transcription of cytoprotective proteins were divided into two different categories: the bifunctional inducers, and the monofunctional inducers (section 1.4.3). Due to the potential activation of pro-carcinogens by the cytochrome P450 enzymes up-regulated by the bifunctional inducers, the monofunctional inducers have been hypothesized to be more beneficial (71). Studies have further shown that a number of monofunctional inducers, *e.g.* sulphoraphane (164), may inhibit certain cytochrome P450 enzymes (165). This may thus additionally increase the beneficial health effect of monofunctional inducers.

In addition to induction of cytoprotective proteins, some inducers have been found to be strong antioxidants, *e.g.* curcumin (62). Inducers with antioxidant activity may therefore work as direct scavengers of reactive species in addition to up-regulating cytoprotective proteins, and thus increase the total cytoprotective effect provided by these inducers (62).

Recently, there has also been increasing interest in a potential cross-talk between the transcription factor nuclear factor kappa B (NF-κB) and the Nrf2/EpRE signalling pathway (92). NF-κB regulates transcription of many genes involved in immune and inflammatory responses, such as proinflammatory cytokines, chemokines, and

enzymes that generate mediators of inflammation (92). Several compounds have been shown to inhibit NF-κB activity while inducing EpRE activity, *e.g.* sulphoraphane (166;167) and resveratrol (114;168). Lipopolysaccharide induced NF-κB activation has further been shown to be attenuated by the Nrf2 activators sulphoraphane and curcumin (95). It has therefore been hypothesized that these two signalling pathways may cross-talk, and such cross-talk has been suggested to be mediated through competition of the transcriptional co-factor CBP (92). Phytochemicals that are both inhibitors of NF-κB and activators of Nrf2 may have extensive beneficial health effects, by inhibiting detrimental inflammation processes in addition to up-regulating cytoprotective mechanisms. The strong indications from epidemiological studies of beneficial health effects of sulphoraphane and resveratrol may thus be at least partly explained by their dual function as inhibitors of NF-κB and activators of Nrf2.

7. Conclusion

The aims of this thesis were to study the effect of dietary plant extracts on EpRE activity in EpRE-LUC HepG2 cells, both separately and in combinations, and to study the effect of a dietary plant extract on EpRE-dependent transcription in transgenic mice.

Of the 3 separate extracts tested in HepG2 cells, only cocoa gave a statistically significant increase in EpRE activity.

Of 11 extract combinations tested in HepG2 cells, 8 gave a statistically significant induction of EpRE-dependent transcription, and 7 of these were also statistically higher than one or both of the separately added extracts. The combinations appeared to exert additive effects on EpRE-dependent transcription, although some showed a trend toward a more synergistic or antagonistic effect. When coffee and oregano was tested in a wider range of concentrations, a trend towards antagonistic effect was observed. Thus, we found that there are mostly additive effects of combinations of dietary plant extracts on EpRE activity *in vitro*.

In the *in vivo* experiment, we found a statistically higher EpRE-dependent transcription for the group of mice receiving rosemary extract 5h after extract administration, as compared to the mice receiving vehicle control only. Interestingly, in the *in vivo* experiments the intestine of the extract group appeared to have higher EpRE activity than the control group, although this difference was not statistically different.

Additive effects of dietary plants may provide extensive beneficial health effects. By showing that there may be additive effects between dietary plants on induction of EpRE-dependent transcription, this thesis may contribute to a better understanding of the beneficial health effects of dietary plants. Further studies are however needed to elucidate the relationship between dietary plants and EpRE activity more thoroughly.

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