Additive and Synergistic Modulations of Nuclear factor-κB by Dietary Plant Extracts

Master Thesis

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Summary

Epidemiological studies have shown that a high intake of fruits and vegetables is associated with a reduced risk of chronic diseases. The mechanisms behind these beneficial effects are not fully understood, but it is widely believed that the numerous phytochemicals found in plants and interactions between them plays an important role. In the present work 8 different dietary plant extracts are selected on basis of their high phytochemical content and their ability to inhibit the transcription factor NF-κB. NF-κB is essential in normal immune responses and cell survival, but is also up-regulated in conditions like chronic inflammation and certain cancers. The purpose of this work was to elucidate whether combinations of the extracts have an additive or synergistic effect on NF-κB activation under an acute inflammatory condition in a monocytic cell line. In addition we studied the effect of a combination extract on NF-κB activation *in vivo* in transgenic mice.

Out of 22 combinations tested *in vitro*, 10 showed a synergistic effect on LPS-induced NF-κB activity. The combination of oregano and coffee showed the strongest synergistic effect with 46 % lower NF-κB activity than the sum of effects of the two individual extracts. Coffee, oregano and thyme were each present in 4 of the 10 combinations that showed synergistic effects. In addition, 10 of the 22 combinations showed an additive effect or a trend towards synergy.

The *in vivo* experiment showed that a combination of thyme, clove, oregano, coffee and walnut gave a trend towards inhibition of LPS-induced NF-κB activity in whole mice. The combination-extract significantly inhibited LPS-induced NF-κB activity in the intestine and showed a trend towards lower NF-κB activation in the liver.

This is the first study to show synergistic effect of dietary components on NF-κB activation. Based on these results further work with food synergy is warranted.

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List of Abbrevations

AMP	Adenosine Monophosphate
AP-1	Activator Protein 1
ATP	Adenosine Triphosphate
CAT	Catalase
CD14	Cluster of Differentiation 14
COX-2	Cyclooxigenase-2
CVD	Cardiovascular Disease
DHA	Dcosahexaenoic Acid
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ECM	Extra Cellular Matrix
EMSA	Electrophoretic Mobility Shift Assay
EPA	Ecosapentaenoic Acid
FBS	Fetal Bovine Serum
FRAP	Ferric Reducing Ability of Plasma
GPx	Glutathione Peroxidase
GSH	Glutathione
HBV	Hepatitis B
HCV	Hepatitis C
H_2O_2	Hydrogen peroxide
HPH	Hygromycin-B-Phosphotransferase
HPV	Human Papilloma Virus
IKK	Inhibitor of Nuclear Factor kappa B Kinase
IL	Interleukin
IκB	Inhibitor of Nuclear Factor kappa B
iNOS	inducible Nitric Oxide Synthase
LBP	LPS Binding Protein
LDH	Lactate Dehydrogenase
LDL	Low-density Lipoprotein
LPS	Lipoplysaccaride
LUC	Luciferase
MD-2	Myeloid Differentiation-2

MeOH	Methanol
mRNA	messenger Ribonucleic Acid
NEMO	NF-κB Essential Modulator
NF-κB	Nuclear Factor kappa B
NLS	Nuclear Localization Sequence
NO	Nitric Oxide
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PPAR	Peroxisome Proliferator-Activated Receptor
Redox	Reduction/Oxidation
RHD	Rel Homology Domain
RLU	Relative Luminicence Units
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SOD	Superoxide Dismutase
TEAC	Trolox Equivalent Antioxidant Capacity
TLR4	Toll-like Receptor 4
TNF	Tumor Necrosis Factor
TPTZ-Fe^{3+}	Ferric tripyrdyltriazine

1. Introduction

1.1 Dietary Plants and Health

The burden of chronic non-communicable diseases like obesity, diabetes, cardiovascular diseases, osteoporosis and cancers is a growing problem both in developed and developing countries. In 2001 chronic diseases contributed approximately 60% of total reported deaths in the world, and this proportion is expected to increase[1]. WHO concluded that unhealthy diet, physical inactivity and tobacco represent the major risk factors for chronic diseases. They estimate that 80% of heart disease, stroke and type 2 diabetes and 40 % of cancers would be prevented if these risk factors were eliminated[2].

Epidemiological studies suggest a protective relationship between consumption of fruit and vegetables and a large number of chronic diseases [3, 4]. The evidence is particularly strong for a protective effect against the development of cardiovascular disease (CVD) and cancers [5]. World Cancer Research Fund and American Institute for Cancer Research reported in 2007 that fruits and vegetables probably protects against a number of cancers[6]. Thus to improve quality of life and reduce public health expenditures, many countries have developed recommendations for an increased intake of fruit and vegetables[1].

The mechanisms and compounds involved in these protective effects of fruits and vegetables have however not been fully established. Fruits and vegetables contain well known biologically active compounds, including macro-and micro nutrients such as carbohydrates, vitamins and minerals, as well as non-nutritive constituents that could potentially reduce the incidence of diseases. Some of the potentially protective factors are carotenoids, polyphenols, dietary fibre, folic acid, vitamin C and vitamin E [4, 7, 8]. The protective effects are only seen when whole fruits and vegetables are consumed. Many trials have investigated if there is an beneficial effect

of multivitamin/mineral supplementation on chronic disease prevention, but they have failed to find this preventive effect [9]. For example, a diet rich in β-carotene protects against cancer development, however supplements of β-carotene do not have cancer preventive effects, rather increases the incidence of lung cancer in smokers [10, 11]. This may indicate that several of the compounds of fruits and vegetables work together in a integrated network to produce health benefits[12], and thus single components from supplements may not have the same effects as whole foods.

The research group of Professor Rune Blomhoff is involved in investigating the chemical and biological properties of dietary plants and the mechanisms behind the protective effect of fruits and vegetables. Since fruits and vegetables are rich in phytochemicals, much of the ongoing research focuses on screening for the total antioxidant content of a wide variety of dietary plants and how these dietary plants may modulate the regulation of genes involved in disease development.

1.2 Dietary Phytochemicals

The term "phytochemicals" refers to a wide variety of compounds produced in plant foods. So far many thousands of biologically active compounds in plants have been found which determine particular properties in the plant[13]. Phytochemicals often perform important functions in the plant, such as providing colour, flavour[6], protection against UV radiation and predators[14]. Many of these individual phytochemicals have been isolated and identified in fruits, vegetables and grains, but many still remain unknown and need to be identified and tested before we can fully understand their health benefits. Phytochemicls can be classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds and organoulfur compounds [15] (**Figure 1.1**) ,of which phenolics are the largest and most studied group [14].

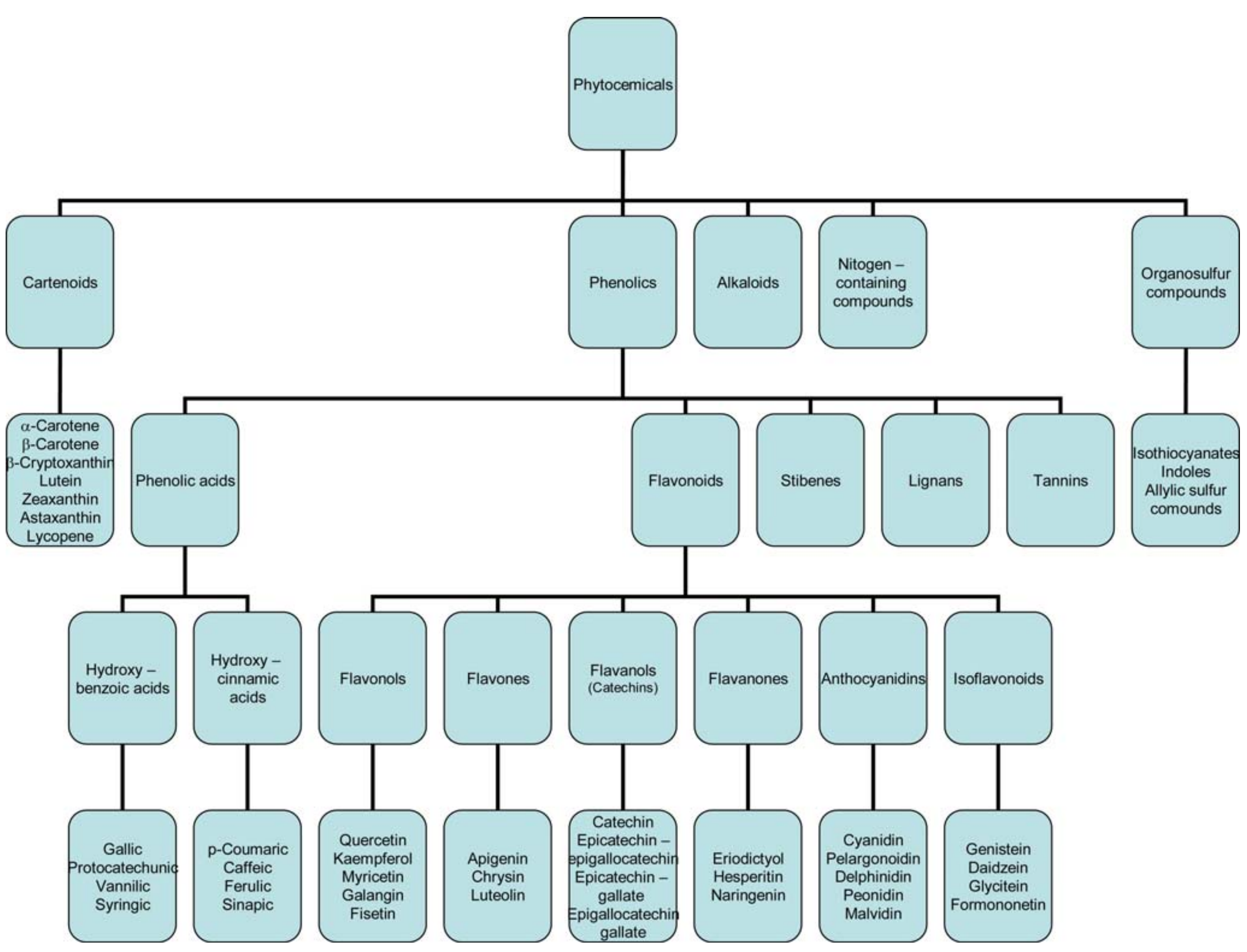


Figure 1.1 Classification of dietary phytochemicals (Modified from Liu *et al.*[16])

1.2.1 Phenolics

Phenolics are of great importance in plant physiology with their role in pigmentation, flavour, growth, reproduction and resistance to pathogens and predators [14]. Many thousand phenolics with at least one aromatic ring (phenolic ring) bearing hydroxyl groups have been characterized. The phenolics can be further divided into subgroups based on the structural components. Distinctions are thus made between the phenolic acids, flavonoids, stilbenes, and lignans [17] (**Table 1.1**).

Table 1.1. Phenols[17]

Phenolic class	**Chemical structure**	**Examples**	**Dietary sources**
Flavonoids			
Flavonols		Quercetin Kaempherol	Cherry tomato, onion, broccoli, tea, red wine, berries
Flavones		Apigenin Luteolin	Cereals, parsley, celery
Flavanones		Hesperetin Naringenin	Citrus fruits
Flavanols		Catechins	Chocolate, beans, apricot, tea, red wine, cherry, apple
Anthocyanidins		Cyanidin	Aubergine, berries, red wine, red cabbage
Isoflavones		Daidzein Genistein	Soy products
Phenolic acid			
Hydroxybenzoic acid		Gallic acid	Berries Onion
Hydroxycinnamic acid		Caffeic acid Ferulic acid	Blueberry, kiwi, cherry, plum, apple, grains
Lignans		Matairesinol	Linseed, lentils, cereals, garlic
Stilbenes		Resveratrol	Grapes, Red wine

Most phenolics have antioxidant properties and thus phenolics are the most abundant antioxidant in our diet. The estimated intake is suggested to be as high as 1 g/day where phenolic acid account for about on third of the total intake and flavonoids account for most of the remaining two thirds [18]. The main dietary sources of phenolics are fruits and beverages (fruit juice, wine, tea, coffee and chocolate) and, to a lesser extent vegetables, dry legumes and cereals. Certain phenolics are found in a wide variety of plants, while others are specific to particular plants. In most cases, foods contain complex mixtures of phenolics [17, 18]. Environmental factors such as soil type, sun exposure, temperature and rain fall, have major effects on the phenolic content of plants. The degree of ripeness also affects the concentration and proportion of phenolics, where phenolic acid concentration decrease during ripening, whereas anthocyanin concentrations increase[17].

Flavonoids:

Flavonoids are the most common phenolic compound in our diet and human intake is estimated to be between a few hundred mg/d to 650 mg/d [16]. Flavonoids can be further divided into 6 major subclasses: flavones, flavonols, flavanones, flavanols (cathechins and proanthocyanidins), anthocyanins, and isoflavones. Flavonoids provide flavour and colour to fruits and vegetables[14].

Flavonols are the most widely represented flavonoids in the food. Fruits often contain 5-10 different major flavonol glycosides (glycosylated form of flavonols). The flavonols accumulate in the skin and leaves of the fruit because their biosynthesis is stimulated by light. Thus the flavonol concentration of fruits and vegetables depend on the exposure to light [17].

Phenolic acid:

Phenolic acids can be divided into two subclasses: hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids are found only in a few plants eaten by humans such as certain berries and onions. Hydroxycinnamic acids are more common and are found in flour, coffee, aubergine, blueberry, kiwi and other fruits and vegetables[17].

1.2.2 Metabolism of Phytochemicals

The chemical structure and functional characteristics of the phytochemicals determines their rate and extend of intestinal absorption, metabolites circulating in plasma and biological properties [18], however the knowledge about the absorption of phytochemicals is limited and mainly focus on certain phenolic compounds. In foods most phenolics are usually found conjugated to sugars (glycosides), but aglycones may also appear. Aglycones can be directly absorbed from the intestine but glycosides, esters, or polymers must be hydrolyzed by intestinal enzymes (β-glucosidase and lacthase phlorizin hydrolase) or by the colonic microflora before they can be absorbed. Phytochemicals that are not absorbed in small intestine enters the colon, where they may undergo hydrolysis and be absorbed [17]. During transport through the enterocytes, phenolics may be conjugated. After absorption in the intestine, the phenolics enter the hepatic portal vein and further the hepatocytes in the liver where the conjugation of phenolics continues. These conjugations include methylation, sulfation and glucuronidation and are the same process as when detoxifying drugs[17, 19]. This processing of phytochemicals may greatly influence their bioactivity[20]. Conjugation of phytochemicals increases their hydrophilic properties which restricts their potential toxic effect and increase their excretion in urine and bile. When excreted in the bile, phytochemicals may be reabsorbed in the colon. This enterohepatic recycling may lead to a prolonged presence of phytochemicals within the body[17, 19].

In the circulation phytochemicals generally appear as conjugates and bound to plasma proteins, with albumin being the primary protein responsible for binding. Plasma concentration of phytochemicals after consumption of food varies highly according to the nature of the phytochemical and the amount in food source[17]. According to Scalbert *et al.*[18] the plasma concentration of single phytochemicals rarely exceeds 1 μM after consumption up to10 -100 mg of the compounds. They also conclude that more phenolic compounds may be present as metabolites of the original compound [18]. This is however after consumption of single phytochemicals and not after

consumption of whole dietary plants, which contains a wide variety of phytochemicals with different chemical structures and functional characteristics.

Phytochemicals are able to penetrate tissue and reach different organs, but their ability to accumulate in specific target tissues needs to be further investigated[17].

1.2.3 Health Benefits and Biological Effects of Phytochemicals

Biological effects of phytochemicals are dependent on the amount consumed and on numerous processes, including absorption, transport, metabolism and effects on cellular targets[17].

Epidemiological studies are useful for studying associations between long-term exposure of phytochemicals and possible effect on health. Epidemiological data suggest beneficial effects of flavonoids and lignans on risk of cardiovascular diseases, but are not convincing with regards to cancer[20]. A reduction of mortality risk caused by coronary artery disease up to 65 % with a high intake of flavonoids has been reported [20].

The mechanisms of action are proposed to be many and include trapping and scavenging of free radicals, regulation of nitric oxide (NO), decreased leukocyte immobilization, induced apoptosis, inhibition of cell proliferation and angiogenesis, and exhibition of phytoestrogen activity[14, 20, 21]. These mechanisms may play a part in the prevention of various diseases such as cardiovascular diseases, chronic inflammatory diseases, and cancers [21].

1.3 Oxidative Stress and Antioxidant Defence

1.3.1 Oxidative Stress

Humans and animals depend on oxygen for life, but the oxygen can also lead to oxidative stress and damage to our cells [22]. Oxygen is an essential molecule during normal cellular metabolic reactions. A result of these reactions is the production of energy, but also formation of reactive species such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive iron species [23]. Reactive species can also be formed by ultraviolet light, radiation, chemotherapeutics and environmental toxins [24]. If not eliminated these reactive species can react with and alter the structure and function of several cellular components, such as the cell membrane, lipids, cellular proteins, carbohydrates and DNA [12, 24]. The consequences of these modulations in the cells can be development of disease. The body contains an endogenous antioxidant system that protects us against such reactive species. The balance between the production of reactive species and the antioxidant defence determines the degree of oxidative stress and damage. Too high levels of reactive species and a limited antioxidant defence mechanism can damage cells [25].

Cellular DNA is susceptible to damage from a variety of free radicals and reactive species. Damage are normally corrected by the endogenous DNA repair system, but sometimes the repair system fails leading to loss of nucleotides, mutagenic or abortive repair events [26]. DNA damage can also occur indirect through attack of reactive species on proteins and lipids. All these alterations of DNA can lead to stimulation of cell growth, suppressed apoptosis or necrosis, which may result in cancer development [25].

It has also been suggested that accumulation of damaged products from the action of reactive species, may be critical to development of atherosclerosis [27], diabetes and chronic inflammation [28].

1.3.2 Antioxidant defence

The endogenous antioxidant defence system is a complex system including both enzymatic and non-enzymatic compounds. The most important enzymatic constituents of the endogenous antioxidant defence include the enzymatic scavengers superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase[29]. These enzymes neutralize and eliminate ROS and RNS thus preventing oxidative damage. Different compartments of the cell and organs contain different antioxidant defence molecules (**Figure 1.2**). SOD is found in mitochondria and cytosol, CAT is found in peroxisomes in most tissue, glutathione peroxidise in cytosol and mitochondria[28]. Body fluids are poorly protected by antioxidant enzymes, but plasma contains albumin, uric acid, ferritin and transferrin that protect against oxidative stress by binding reactive compounds and protecting proteins from reacting with radicals [3, 28].

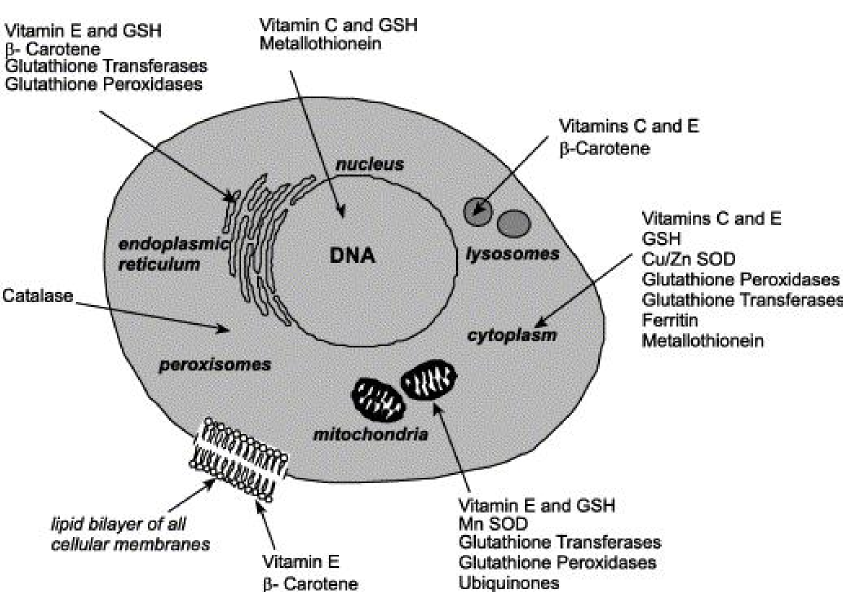


Figure 1.2 Antioxidant defence systems in the cells[7]

Non-enzymatic compounds in the antioxidant defence system include vitamin E (α-tocopherol), carotenoids, vitamin C (ascorbic acid) and probably the most important of them all glutathione[24]. Vitamin E, vitamin C and carotenoids can be obtained from the food, while glutathione is an endogenous compound. α-tocopherol scavenges reactive species of lipid membranes and lipoproteins such as LDL. It has been suggested that α-tocopherol and ascorbic acid act synergistically in antioxidative processes and inhibit oxidative stress in a greater extend than what they do individually[3]. Ascorbic acid scavenges aqueous radicals and acts as a synergist to regenerate α-tocopherol by reducing the α-tocopheroxyl radical formed when α-tocopherol reacts with a radical. An additive effect of α-tocopherol and β-carotene is also seen by their action on different phases of the cell membrane and other membranes inside the cell. α-tocopherol is located near the membrane surface while β-carotene is located mainly in the interior of the lipophilic domain in the membrane, which makes β-carotene able to scavenge lipophilic radicals more efficiently than α-tocopherol [3]. Carotenoids also prevent oxidative stress by scavenging reactive species generated by light exposure. Because of carotenoids ability to give pigmentation of the plant it also prevent the light to cause damage to the plant [30].

It is also believed that phytochemicals may have an inhibiting effect on damage caused by reactive species. Other phytochemicals act like carotenoids in the interface of the phospholipid layer and scavenge lipophilic radicals. Phytochemicals are also believed to reduce incidence of DNA damage and exert indirect antioxidant abilities through protection or enhancement of endogenous antioxidants like glutathione [14]. Gluthathione scavenge free radicals and peroxides and protects the cells against oxidative stress and damage. Glutathione is synthesised enzymatically by γ-glutamyl synthetase (γGCS) and glutathione synthetase. *In vitro* experiments has showed that flavonoids increase expression of γ-glutamyl syntethase and *in vivo* experiments has showed an increased level of glutathione after feeding of berries rich in flavonoid [31].

1.4 Nutrients and Gene Regulation

In the past decade there has been more focus on how nutrition influences metabolic pathways and homeostatic control. This is a result of a growing realization that the effect of nutrition on health and disease cannot be understood without a profound understanding of how nutrients act at the molecular level and affect the regulation of our genes. This field within nutritional research is called nutrigenomics. From a nutrigenomic perspective, nutrients are dietary signals that influence gene expression (transcriptomics), protein expression (proteomics) and metabolite production (metabolomics) in response to particular nutrients [32]. The main goal for the nutrigenomics in nutrition science is to find the underlying mechanism of diet-related diseases and strategies for prevention of these, and to contribute to public health in a long-term perspective.

Micro- and macronutrients are known to function as fuels and co-factors for the body. It is now also evident that they have important effects on gene- and protein expression and further metabolism. The nuclear receptor super family of transcription factors, with 48 members, are the most important nutrition sensors[32]. Dietary compounds like fatty acids, glucose, amino acids, vitamin A, vitamin D, vitamin E and flavonoids, mediate their effect on gene expression through influence on transcription factors. An overview of these transcription factors and their nutritive substrates is shown in **Table 1.2**. Flavonoids, for example, can modulate the activity of Nuclear Factor-κB (NF-κB) [32], which is pivotal in regulation of immunity and inflammation, apoptosis, cell proliferation and development [33].

Table 1.2. Transcription factors influenced by nutrients[32]

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

AP1, activating protein1; 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, metal-responsive transcription factors; NFκB, nuclear factor κB; NF-AT, nuclear factor of activated T cells; 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.

1.5 NF-κB

Nuclear Factor kappa B (NF-κB) is a family of transcription factors expressed in all mammalian cells. The first article about NF-κB family of transcription factors was published in 1986 by Sen and Baltimore[34]. Since then more than 25 000 articles about NF-κB have been published. This high number of scientific studies is due to NF-κBs essential role in immune- and inflammation responses and that NF-κB has showed to be up-regulated in conditions like chronic inflammation and certain cancers.

1.5.1 The NF-κB proteins, structure and signaling

Five members of the NF-κB transcription factor family have been identified. These are p50/p105, p52/p100, RelA (p65), RelB and c-Rel (**Figure 1.3**). These proteins are able to form hetero- and homodimers, of which p50/RelA is the most common dimer. The NF-κB proteins are characterized by the presence of the Rel homology domain (RHD), which is an N-terminal region of approximately 300 amino acids. This domain plays an important role for DNA binding, dimerization and interactions with inhibitory IκB proteins [35, 36]. IκB use a core domain composed of six to seven ankyrin repeats to bind to the RHD. p105 is the precursor to p50 and p100 is the precursor to p52. These two precursors contain ankyrin repeats at their C-terminal and thus may act like an inhibitory IκB like domain. The precursors can dimerize with the different Rel proteins, but these dimers are trapped in cytoplasm. However p105 undergoes continuous processing to active p50. Activated forms of NF-κB translocate to nucleus and binds to DNA. Activation of most forms of NF-κB depends on phosphorylation of IκB proteins[37].

The IκB proteins (**Figure 1.3**) are cytoplasmic inhibitors, which are bound to the NF-κB dimers and prevent translocation from the cytoplasm to the nucleus. Normally, IκBα inhibits the transportation of the NF-κB dimer to the nucleus by masking the nuclear localization sequences (NLS)[35]. IκBα can undergo phosphorylation, ubiquination and proteasome-mediated degradation, which result in the liberation of NF-κB followed by translocation into nucleus. IκBα is rapidly degraded, but is resynthezised as a result of negative feedback since IκBα is a target gene of NF-κB[38]. The IκB-kinase (IKK) complex is responsible for the phosphorylation and degradation of IκB [39]. The IKK complex contain two kinase subunits (IKKα and IKKβ) and a regulatory subunit NEMO (NF-κB essential modifier, also known as IKKγ)[40].

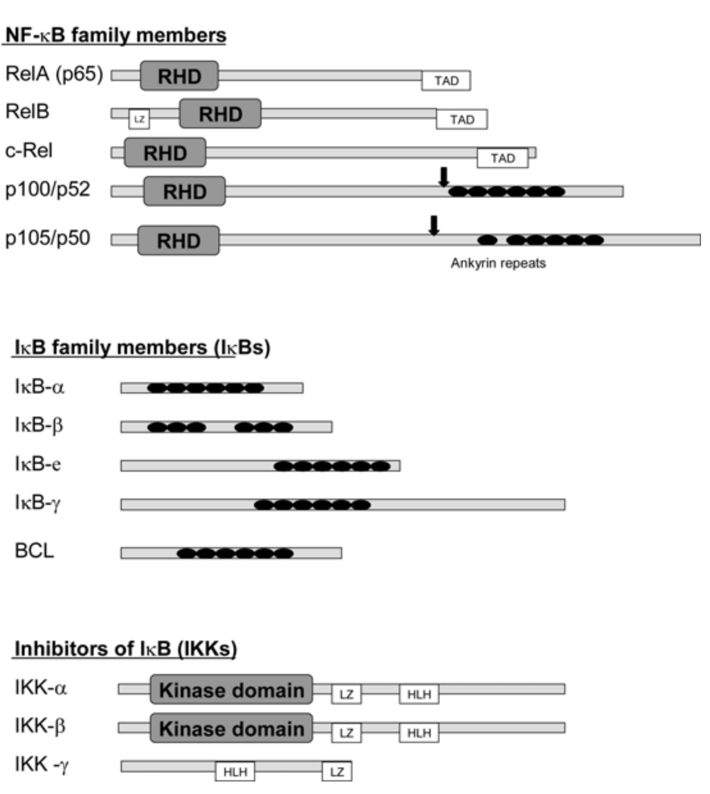


Figure 1.3 The NF-κB, IκB proteins and IKK proteins (Adapted from Hayden *et al.* [40])

NF-κB is activated through two main pathways (**Figure 1.4**). The classic pathway involves the dimer of p50/RelA. It is normally triggered in response to microbial and viral infections or exposure of pro-inflammatory cytokines. These substances activate the IKK complex via binding to membrane receptors, leading to a degradation IκBα and translocation of the p50/RelA dimer to nucleus. IκBα phosphorylation depends mostly upon the IKKβ catalytic subunit. The alternative pathway (**Figure 1.4**) includes the dimer of p52/p100 and RelB. This pathway is triggered by members of tumor-necrosis factor (TNF) cytokine family that selective activates the catalytic subunit IKKα [33, 41].

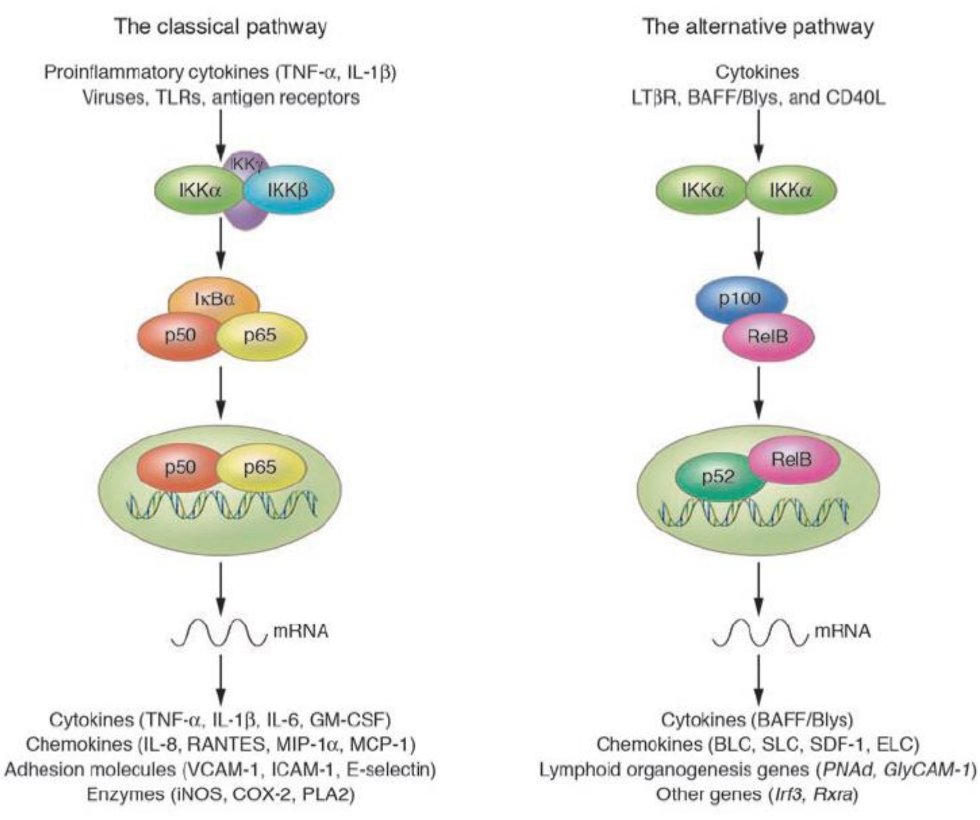


Figure 1.4. The two NF-κB signalling pathways[41]

When translocated into the nucleus, dimers of the NF-κB family binds to specific DNA sequences, called κB-sites, in the promoter of target genes and activate transcription [40]. Different dimers recognise different sequences and target genes [35]. To increase the strength and duration of NF-κB transcription, posttranslational modifications like phosphorylation and acetylation occur [39, 40]. More than 150 genes have been described as being NF-κB regulated (**Table 1.3**) [42]. These genes, based on their function and regulation, can be organized into four main categories: immunity and inflammation; anti-apoptosis; cell proliferation and negative feedback of the NF-κB signal [33]. Both the classical and alternative pathway regulates cell survival and death. The classical pathway is responsible for inhibition of programmed cell death (PCD), which again protects the cell from apoptosis and necrosis[41]. This pathway also has target genes coding for cytokines, chemokines, immunoreseptors, and cell adhesion molecules [43] (**Figure 1.4**). The alternative pathway is important for survival of premature B cells and development and maintenance of secondary lymphoid organs and it thus important in adaptive immunity.

Table 1.3 NF-κB target genes [33, 42]

Category	Target gene
Immunity and inflammation	IL-1, IL-2, IL-6
	Interferon-γ
	TNF-α
	Acute phase proteins
	Adhesion molecules (VCAM, ICAM)
	iNOS (inducible nitric oxide synthase)
	COX-2 (cyclooxigenase 2)
	LPS binding protein (LBP)
	SOD (superoxide dismutase)
Anti-apoptosis	cIAP (cellular inhibitors of apoptosis)
	Bfl 1/A1 (pro-survival Bcl-2 homologue)
Cell-proliferation	Granulocyte colony stimulating factor (G-CSF)
	c-MYC (proto-oncogene)
	PDGF (platelet activated growth factor)
Negative Feedback on NF-κB signal	IκBα, IκBβ, transcription factors that downregulate NF-κB-activity (p53, IRF-1)

1.5.2 Activators of NF-κB

NF-κB is a central mediator of the human immune and stress response. It is induced by a wide variety stressors (**Table 1.4**), including bacteria, bacterial products and viruses, cytokine and chemokines. Many of these inflammatory molecules are also target genes of NF-κB [42]. NF-κB is also activated by other stress factors including: physiological conditions such as haemorrhage and ischemia, ultraviolet irradiation, environmental hazards such as cigarette smoke and heavy metals.

Table 1.4 Activators of NF-κB (Modified from Pahl[42])

Inducers	**Examples**
Bacteria	E-coli
	Helicobacter pylori
	Gardenella vaginalis
	Staphyllococcus aureus
Bacterial products	Lipopolysaccaride (LPS)
	Enterotoxins
Viruses	Hepatitt B
	HIV-1
	Herpes simplex 1
	Influenza virus
Growth factors and hormones	Human Growth Hormone
	Insulin
	Platelet-Derived Growth Factor
Cytokines	IL-1
	IL-2
	TNF-α
Physiological stress	Hemorrhage
	Hyperglycemia
	Ischemia
Physical stress	Ultraviolet irradiation
Environmental hazards	Cigarette smoke
Therapeutic drugs	Anthralin (psoriasis treatment)
	Ciprofibrate (dyslipidaemia treatment)
	Methamphetamine (psychostimulant)
Modified proteins	Advanced glycated end products (AGEs)
	Oxidized LDL

1.5.3 Inhibitors of NF-κB

Over 750 inhibitors of NF-κB have been described and include a wide range of both natural and synthetic molecules[44]. The inhibitors can target any step in the NF-κB signalling pathway and the inhibition can occur by three mechanisms: 1) Blockage of incoming stimulating signals. 2) Interference with one of the steps in the cytoplasmic signalling cascade. 3) Blockage of NF-κB nuclear activity[44].

Inhibition of the early steps blocks the signal before it activates IKK, hindering phosphorylation of IκB and NF-κB will stay inactivated in cytoplasma[44]. Inhibitors acting in cytosol can inhibit IKK and IκB phosphorylation. An inhibition of proteases and proteasome activity can also occur, decreasing the degradation of IκB. The third step includes inhibition of translocation of NF-κB to nucleus, binding to DNA and modifications of the NF-κB dimer within the nucleus [44].

Both drugs for therapeutic use and different components in foods have been shown to inhibit NF-κB. Some of these compounds are listed in **Table 1.5**. Different phytochemicals like flavonoids exhibit an inhibiting effect on NF-κB[45, 46]. Curcumin (from turmeric) has been shown to inhibit IKK α and β, and also downregulate upstream kinases .Curcumin has also been shown to suppress the TNF-α-induced nuclear translocation and DNA binding of NF-κB[13]. Reservatrol (a stilbene found in grapes) block the activation of IKK and inhibit expression of iNOS and decreases production of NO (nitric oxide) in macrophages, which is associated with inhibition of IκB phosphorylation and NF-κB DNA binding[37].

Dysregulation of NF-κB is believed to play a key role in chronic inflammatory diseases and cancer development. An inhibition of NF-κB through drugs and food can thus be of importance in both therapy and prevention of disease.

Table 1.5 Inhibitors of NF-κB [37, 47]

Category	Examples
Dietary plant components:	Quecetin
	Vitamin C
	α-tochopherol
	Caffeic acid
	Curcumin
	Epigallocatechin
	Glutathione
Drugs:	Cyclosporin A (proteasome inhibitor)
	NSAIDs (inhibit phosphorylation of IκB)
	Ibuprophen (inhibit phosphorylation of IκB)
	Glucocorticoids (inhibit TNF-α)
	Thalidomide (inhibiting IKKβ)

1.5.4 NF-κB in health and diseases

NF-κB is crucial for normal life. It is important for immune function both for innate and adaptive immunity and it plays an important role for cell survival and normal cell proliferation.

Even though NF-κB is pivotal for normal physiology it is also associated with different diseases. NF-κB is dysregulated in various diseases such as chronic inflammation and several cancers[48, 49]. Pathways of NF-κB signalling are constitutively active during situations like rheumatoid arthritis, asthma and inflammatory bowel diseases[49]. Furthermore abnormally high levels of active NF-κB is found in leukaemia, breast cancer and some virus-induced cancers[48].

A causal connection between inflammation, infection and cancer has been suspected for many years. Viruses like human papilloma virus (HPV), hepatitis B and C (HBV, HCV) and bacteria helicobacter pylori are pathogens that can lead to cancer[50].

Because NF-κB plays an important role on immune system, anti-apoptosis and cell proliferation, it has also been associated with cancer[33]. NF-κB has various tumour promoting functions depending on the cell type in which is activated. In tumour cells activation of NF-κB leads to up-regulation of anti-apoptotic genes and ensuring survival and proliferation of tumour cells. NF-κB activation in inflammatory cells promotes tumour development through the induction of genes that encode cytokines and growth factors that again stimulate cell growth and angiogenesis (**Figure 1.5**). In addition NF-κB activation leads to production of adhesion molecules that may increase the invasion of cancer cells and thus metastasis[51].

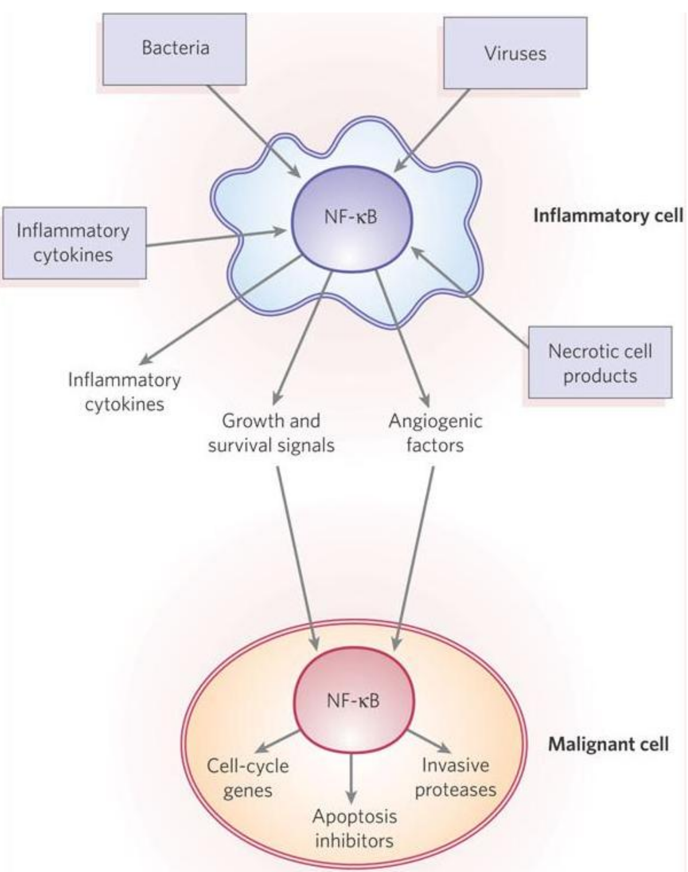


Figure 1.5. Activation of NF-κB in various cell types and influence on cancer. NF-κB has various tumour promoting functions depending on the cell type in which is activated. In tumour cells activation of NF-κB leads to up-regulation of expression of anti-apoptotic genes and ensuring survival and proliferation of tumour cells. In inflammatory cells NF-κB activation promotes tumour development through inducing the expression of genes that encode secreted cytokines and growth factors that stimulate tumour growth and angiogenesis[51, 52]

In cancers caused by viral infections, the NF-κB signalling pathway can be chronically stimulated by viral transforming proteins. The mechanisms of NF-κB activation in most other cancers, however, remain to be elucidated[48]. IKK activation is frequently seen in cancer, but how this critical step of NF-κB is dysregulated remains unknown. Inhibition of NF-κB in cancer cells may induce apoptosis. The currently available therapeutic NF-κB inhibitors target IκBα phosphorylation, IκBα degradation, and nuclear translocation. A major problem of these therapeutic inhibitors is that they lack specificity. They will thus affect the physiological function of NF-κB in normal cells as well as in cancer cells[48], and this can lead to profound side effects. It is therefore now of great interest and challenge to find inhibitors of NF-κB activation with minimal side effects [33, 48].

1.6 Food Synergy

For many years researchers have been focusing on identifying the bioactive compounds behind the health beneficial effect of eating fruits and vegetables. Antioxidants have for a long period been suggested to play a central role in this preventive effect. This focus on single antioxidants has formed a massive commercial industry that produces vitamins and antioxidants for supplementation. Concentrations of single nutrients provided by such supplements often exceed doses achieved by the diet and we still do not know the health effects of such a high intakes. It is now however widely believed that single antioxidants taken as supplements do not appear to have preventive effects[53, 54], and thus we need new strategies to study the positive health effects of a diet rich in fruits and vegetables.

Dietary plants contain thousands of phytochemicals. These compounds differ in molecule size, polarity, and solubility, which may affect their bioavailability and distribution of each phytochemicals in different cells, organs and tissue[16]. A natural combination of phytochemicals present in fruit and vegetables may thus be better than single antioxidants. With this large variety of phytochemicals, it is likely that the mechanisms of action vary accordingly, and that different phytochemicals may act in different compartments of the cell, or even on different targets in one signalling pathway. These multiple targets of action could be expected to lead to additive and possibly synergistic effects of combinations of phytochemicals.

Synergy and additive effects are terms used in different scientific area such as biology, chemistry, anthropometry and pharmacology[55]. These terms are often associated with other terminology such as complementarities, interaction and cooperation. Additive effect can be defined as when the total effect of a combination of two or more agents is the same as the sum of the effects of each individual agent (**Figure 1.6** (A+B)). The definition of synergy is when two or more agents acting together and creates an effect greater than can be expected by combining the effects of each individual agent (**Figure 1.6** (A+B+C))

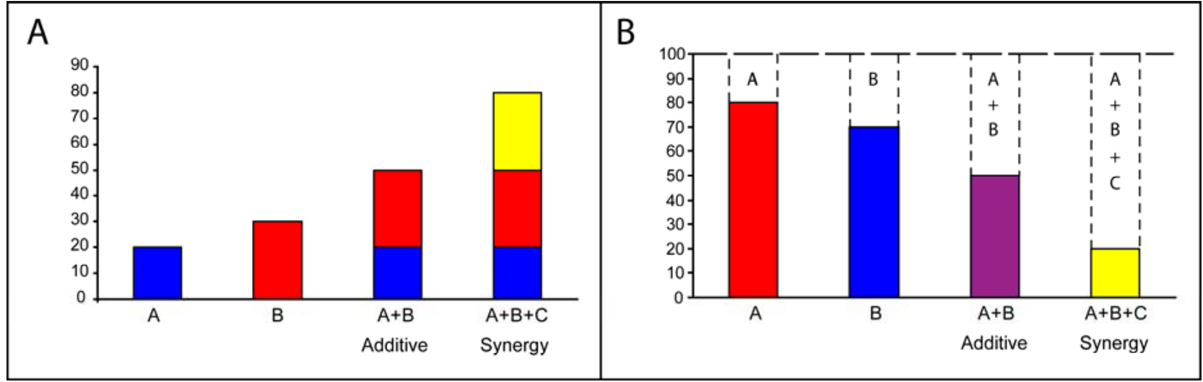


Figure 1.6. Additive and synergistic effects A) Additive and synergistic induction B) Additive and synergistic inhibition

The term synergy is also used in nutrition research and refers to food synergy. The definition on food synergy is more than additive influences of foods or food constituents on health [56].

All foods, both plant and animal sources, contain constituents that interact in complex biological systems [57]. Constituents of foods and especially plant based foods go beyond macronutrients and micronutrients and include plant enzymes, enzyme inhibitors, hormones, signal transducers, sterols, and phytochemicals[57]. Phytochemicals alone appear to have diverse functions in the plants such as colour the leaves, flowers and fruit, anti-microbial function, anti-fungal function, protects the plant from insects and damage from solar UV-radiation, chelation of toxic heavy metals and antioxidant protection from free radicals[58]. It is widely believed that the health benefits of diets rich in fruit and vegetables are likely to be due to their high content of phytochemicals[6]. A large portion of phytochemicals still remain to be widely studied and their bioactive effect on human physiology is not yet fully understood[6]. But because different plants have different content of phytochemicals with different structures and protective mechanisms[59], it is likely that constituents in whole foods act simultaneously on one or several targets giving a synergistic effect also in humans[58].

Some of the mechanisms that may lead to these effects include scavenge reactive oxygen species, up-regulation of endogenous antioxidant enzymes through induction

of e.g EpRE (electrophile-responsive element) regulated transcription and regulating numerous aspects of intracellular signalling cascades involved in the regulation of cell growth and inflammation[58]. Studies have showed that modulation of one signalling pathway by phytochemicals also can lead to activation or inhibition of other signalling pathways, leading to a dual positive effects[60]. Also, in signalling pathways, such as the NF-κB pathway, phytochemicals have been shown to act at multiple steps of the pathway[13]. It is possible that multiple targeting of one pathway and crosstalk between pathways produce additive or synergistic effects, and these processes may help elucidate the beneficial health effects of fruits and vegetables.

2. Aims of the Thesis

This thesis is a part of a project where the overall aims is to investigate mechanisms behind the disease-preventive effects from a high intake of dietary plants. Previous work by the research group of Professor Rune Blomhoffs has reported that the plant extracts chosen in this thesis has high total antioxidant content and also the ability to inhibit activation of the transcription factor NF-κB. The main focus of this work is to study combinations of dietary plant extracts rich in phytochemicals and elucidate if they have an additive or synergistic effect on NF-κB activity under acute inflammatory conditions.

The specific aims of this thesis are:

- To study combinations of a selection of dietary plant extracts to elucidate potential additive or synergistic effects on lipopolysaccaride-induced NF-κB activity in a human monocytic cell line.
- To select a combination of dietary plants extracts and study the effect of this combination on NF-κB activation in transgenic reporter mice.

3. Materials

3.1 Cell line

Cell line	Distributor	Location
U937 3xκB-LUC	The American Type Culture Collection	Manassas, VA

3.2 Cell culture equipment

Chemical/compound/ equipment	Manufacturer	Location
Cell culture flask, 25, 75, 225 cm^3	Corning Inc.	Corning, NY
Culture Plate 24	PerkinElmer, 6005168	Shelton, CT
Cell culture plates, 24 wells	Corning Inc.	Corning, NY
RPMI-1640	Sigma, R-0883	St. Louis, MO
Fetal Bovine Serum	Sigma, F-7524	St. Louis, MO
Hygromycin B	Invitrogen, 10687-010	Carlsbad, CA
L-Glutamine	Sigma, G-7513	St. Louis, MO
Penicillin/Streptomycin Solution	Sigma, P-4458	St. Louis, MO

3.3 Transgenic mice

Transgenic mice	Distributor	Location
NF-κB-luciferase mice	Cgen	Oslo, Norway

3.4 Transgenic mice equipment

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		
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
96 well plates	Sigma, M2936	St.Louis, MO

3.5 Chemicals

Chemical/compound	Manufacturer	Location
2-Propanol (HPLC – grade)	Merck	Darmstadt, Germany
2,4,6-tripyridyl-s-triazine (TPTZ)	Fluka Chemie AG	Deisenhofen, Switzerland
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
Coenzyme A	Roche Diagnostics	Ottweiler, Germany
Corn oil Biochemika	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
FeCl3 • 6H20	BDH Laboratory Supplies	Dorset, England
FeSO4 • 7H2O	Riedel-deHaën AG	Seelze, Germany
Hydrochloric acid	Merck	Darmstadt, Germany
Isoflourane	Baxter AS	Oslo, Norway
Methanol (HPLC-grade)	Merck	Darmstadt, Germany
MgSO4 • 7H2O	Merck	Darmstadt, Germany
MilliQ water	Millipore	Bedford, MA
PBS (Phosphate buffered saline)	Bio Whittaker, BE17-512F	Verviers, Belgium

Recombinant luciferase (1 mg/ml)	Promega	Madison, WI
Reporter Lysis Buffer	Promega, E3971	Madison, WI
Sodiumacetate trihydrate	Riedel-deHaën AG	Seelze, Germany
Tricine	Sigma, T5816	St. Louis, MO
Trypan blue	Sigma, T-6146	St. Louis, MO

3.6 Dietary plant extracts

Name	**Botanical name**	**Producer**	**Location**
Clove	Syzygium aromaticum	Krydd Huset	Ljung, Sweden
Arabica coffee	Coffea arabica	CIRAD	Montepellier, France
Dog rose	Rosa canina		Oslo, Norway
Oregano	Oreganum vulgare	Santa Maria	Mölndal, Sweden
Sunflower seed	Helianthus annuus	Gogreen	Linköping, Sweden
Thyme	Thymus vulgaris	Hindu	Bergen, Norway
Turmeric	Curcuma longa	Rajah	Enfield, England
Walnut	Juglands regia	Diamond	California, USA

3.7 Kits

Kit name	**Manufacturer**	**Location**
Bio-Rad Protein Assay	Bio-Rad laboratories Inc.,500-0006	Hercules, CA

3.8 Instruments

Instrument	**Manufacturer**	**Location**
Biofuge Fresco	Heraeus Instruments	Osterode, Germay
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
Synergy 2	Bio Tek® Instruments, Inc	Winooski, VT

Technicon RA 1000 system	Technicon Instruments Corporation	New York, NY
Titertek Multiskan Plus	ELFAB	Finland

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 15 for Windows	SPSS Inc.	Chicago,IL

4. Methods

4.1 Dietary Plant Extracts

Previous data have shown that dietary plant extracts rich on phytochemicals may inhibit NF-κB activation[61]. Dietary plants in this thesis are selected on basis of their ability to inhibit NF-κB and their high content of phytochemicals. Spices, walnuts and sunflower seeds were bought from local grocery stores in Oslo and dog roses were picked in the forest surrounding Rikshospitalet University Hospital in Oslo. The coffee extract was provided by Ingvild Paur, my co-supervisor. Spices, nuts and seeds were extracted directly after storage at room temperature and dog roses were frozen at -20 ºC few days prior to extraction.

Equal amounts milliQ water and methanol (MeOH) were used to extract the samples. Dry samples like spices were pulverized into small particles with a mortar. Walnuts, sunflower seeds and dog roses (seeds were excluded) were homogenized with a food processor. Dry samples (10 g) were added 10-20 ml milliQ water and 10-20 ml MeOH depending on the products properties. Wet products (10 g) were added 10-20 ml MeOH, but the amount of milliQ water added depended on the water content in each food sample. If the sample contained 80% water, and 10 ml MeOH was added, only 2 ml milliQ water (corresponding to the remaining 100%-80% = 20%) was added.

All samples were sonicated in a water bath for 30 minutes. After sonication the samples were transferred to 50 ml centrifuge tubes and centrifuged at 4000 rpm at 4 ºC for 10 minutes (Megafuge 1.0 R). The liquid phase was transferred to a small Erlenmeyer flask. The liquid phase was then concentrated under nitrogen gas to viscid fluid where the entire MeOH and most of the water was evaporated. The viscid fluid was diluted to a total liquid volume of 5 ml in phosphate buffered saline (PBS) and/or dimetyl sulfoxide (DMSO) depending on solubility. Some food items like

walnuts and sunflower seeds needed more DMSO than other food items like dog rose. Final concentration of DMSO in cell culture never exceeded 0.2 ‰.The extract was thereafter sterile filtrated with a 0.2 µm polyethersulfone membrane filter in sterile surroundings. The extract was stored in airtight tubes under argon gas at -70 ºC. The final concentration of the extract was 2 g original sample per ml.

We also made a combination extract to be used both in cell culture and in experiments with mice. The procedure for making this combination extract was the same as for the individual samples, except 10 g walnuts and coffee, 1 g clove, thyme and oregano was used. All samples were pulverized / homogenized and mixed together and further extracted as described above. The extract to be used in animal experiment was diluted in corn oil instead of PBS/DMSO. The final combination extract contained 2 g/ml coffee and walnuts, and 0.2 g/ml thyme, oregano and clove

4.2 In vitro Experiments

4.2.1 Cell line

In this project a U937 3xκB-LUC cell line was used. The original U937 cell line was established by Sundström and Nilsson in 1974[62].The U937 cell line is a human monocytic cell line isolated from malignant cells of a patient with histiocytic lymphoma. The U937 cells exhibits characteristics common to cells in the monoblastic stage [62], and thus the cells are similar to monocytes. Monocytes are released into the circulation after differentiation in bone marrow, and circulate in the blood approximately 24 hours. Cells from this circulating pool migrate through the blood vessel walls into various organs to become macrophages. Monocytes and macrophages are attracted to sites of inflammation or infection, and help fighting foreign substances in the body, micro organisms in particular.

The U937 3xκB-LUC cell line is a subclone of the U937 cell line. This subclone is stably transfected with a construct that contains the luciferase gene, coupled to a

promoter with three NF-κB-DNA binding sites (3xκB-LUC) (**Figure 4.2**).The luciferase activity in these cells is thus a measure of NF-κB activity. In addition to this a plasmid (pMEP 4, Invitrogen, Carlsbad, CA) containing a hygromycin resistant gene is stably transfected into the U937 3xκB-LUC cell line. This gene is called Hygromycin-B-phosphotransferase (HPH) gene and is isolated from E.*coli*. Hygromycin B is an antibiotic that kills bacteria, fungi and higher eukaryotic cells. It is used for selection of the U937 3xκB-LUC cells in culture since these cells are stably transfected with the HPH gene. The protein from the HPH gene detoxifies hygromycin-B by phosphorylation.

4.2.2 Cell Culturing

Cell culture medium

The U937 3xκB-LUC cells were cultured in RPMI-1640 medium. This medium is formulated to support lymphoblastoid cells in suspension cultures such as the U937 3xκB-LUC cell line. RPMI-1640 was supplemented with 10 % fetal bovine serum (FBS), L-glutamine (2 mM), streptomycin (50 mg/ml) and penicillin (50 U/ml) to make a complete medium. Hygromycin B (75μg/ml) was added to the cell suspension each time the cells were split. The cell suspension was incubated in 37°C and 5 % CO_2. The cells were counted and medium changed 3 times a week.

Counting and splitting

The cells were counted and split to 0.4 mill cells/ml 3 times a week and always the day prior to the experiment. Cells were counted visually, using a haemocytometer with two chambers. Each chamber has 16 squares. One square is 1x1 mm and when the chamber is filled, the height of liquid is 0.1 mm. The total volume of liquid in one square is therefore $0.1mm^3$. The average number of cells in one square, multiplied by 10^4 is thus the number of cells in 1 ml. At least 2 squares each chamber was counted.

Cells were visually examined prior to every splitting and experiment by looking at shape, size and color. The cells were also examined visually to exclude the presence

of bacteria. A mycoplasma test was done to exclude the presence of mycoplasma in the cell culture. The test was negative.

Freezing and thawing

Stocks of U937 3xκB-LUC cells were stored in liquid nitrogen. To prevent crystal formation and lysis of cells during storage, the stocks were frozen in the presence of DMSO.

A stock of U937 3xκB-LUC was thawed in 37°C water bath. Immediately after thawing the cells were transferred to a centrifuge tube with 10 ml of complete cell medium and centrifuged at 1200 rpm for 3 minutes. The supernatant was discarded and the cell pellet was resuspended with medium. The cell suspension was transferred into a 75 cm^2 cell flask with 18 ml complete cell medium and 75μg/ml Hygromycin B. The cell flask was placed in an incubator. Medium was changed the day after thawing and cells were split to 0.4 mill/ml two days after thawing. U937 3xκB-LUC cells were kept in culture at least one week prior to experimental use.

4.2.3 Experimental Outline

The day prior to the experiment the U937 3xκB-LUC cells were counted and split into 0.4 mill cells/ml, in order to be in a growth phase for the experiment. During the experiment the medium was changed from RPMI 1640 with 10 % FBS to RPMI 1640 with 2% FBS. This change of medium to 2 % FBS is necessary to minimize binding of phytochemicals to proteins. Protein binds phytochemicals greatly and can affect their activity[20]. The cells were seed in 24 well plates, 1ml in each well.

The extracts were diluted with medium to reach the correct concentration for the experiment. Dry samples (spices and coffee) were used in final concentration of 1.0 and 1.5 mg/ml. Wet samples (walnuts, sunflower seeds and dog roses) were used in final concentration 10 and 15 mg/ml. DMSO was added in the same amount to the controls and treatments. Dilutions of the extracts were added to each well as indicated in the **Figure 4.1**:

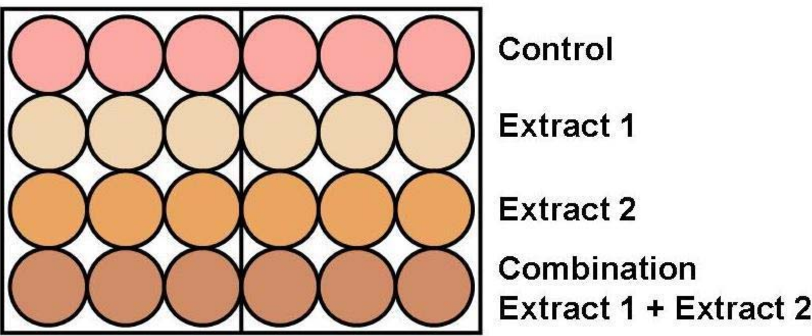


Figure 4.1. Well plate for experimental use

To simulate a bacterial infection lipopolysaccaride (LPS) was added to the U937 3xκB-LUC cells in the wells. This stimulation was done 30 minutes after addition of extracts. LPS is a component of the gram negative bacterial outer membrane and is a potent stimulator of the immune system. In the circulation LPS binds to LPS binding protein (LBP). This LPS/LBP complex is recognized by Cluster of Differentiation 14 (CD 14) which transfers LPS to a Toll-like Receptor 4(TLR 4)/myeloid differentiation 2 (MD-2) heterodimer. This will activate a downstream signalling pathway that in turn will activate NF-κB[63].

The cells with extracts and LPS were incubated at 37ºC for an additional 6 hours before luminescence was detected in a Synergy 2 instrument (see section 4.2.5).

All experiments were repeated at least three times and were performed in triplicates each time.

4.2.4 Luciferase Reporter

Bioluminescence is light produced by a chemical reaction within a living organism where chemical energy is converted to light energy. Fireflies are one species that produce bioluminescence and the firefly luciferase can be used for biological research. Luciferase is the enzyme that catalyses the conversion of luciferin to oxyluciferin, and bioluminescence/photons are produced in this reaction. See below:

Luciferin + ATP + O_2 $\rightarrow$ Oxyluciferin + AMP + PPi + photon (560 nm)

Luciferase

In both the U937 3xκB-LUC cells (see section 4.2.1) and transgenic mice (**Figure 4.2**) used in this thesis a luciferase gene coupled to a promoter of three NF-κB-DNA binding sites is utilized (**Figure 4.2**). Luciferase is thus utilized as a reporter gene for NF-κB

activation.

κB κB κB TATA Luciferase gene

Figure 4.2 3x κB-LUC construct. The construct used in both the subclone of U937 cells and reporter mice.

4.2.5 Measuring luminescence

After 6 hours of incubation with extract and LPS, the luciferase activity was measured. All wells were added 10 µl luciferin and incubated 5 minutes at 37C° before luminescence was read. Luciferin was given in excess so that the luciferase is the limiting factor of the reaction.

To measure the luminescence from U937 3xκB-LUC cells a Synergy 2 instrument was used. The Synergy 2 is a single-channel microplate reader which is computer-controlled using BioTec`s Gen 5™ PC software. Gen 5 reports the measurements results as Relative Luminicence Units (RLU). RLU is collection of data in each well for the duration of integration time (10 sec), and then average the data points. The luminescence is detected through an empty filter.

4.2.6 Cell Viability

Cell viability was examined by using trypan blue to distinguish between viable or non-viable cells. Non-viable cells absorb trypan blue in their membrane and become

dark blue while viable cells remains in normal colour and shape. Cells were counted visually, using a haemocytometer as previously described. Cells from control group and cells incubated with extract were added trypan blue in 0.4 % solution (50 µl trypan blue in 1 ml cells). One chamber of the haemocytometer was filled with control cells, while the other chamber was filled with the highest concentration of extract combination. The number of viable and non-viable cells was counted immediately, in at least four squares each chamber. A cut off value of 10 % non-viable cells was used. Concentrations or combinations with cell toxicity higher than 10 % were excluded from the results. The majority of eksperiments had cell toxicity under 10 %.

4.3 In Vivo Experiment

4.3.1 Transgenic reporter mice

To find out whether the extracts rich in phytochemicals are able to exert the same effects on NF-κB *in vivo*, experiments were performed with transgenic mice. We used transgenic mice that express the luciferase gene under the control of NF-κB. This mice model is developed by Carlsen et al[64] using the same NF-κB-luciferase construct as in the U937 3xκB-LUC cells . The production of transgenic NF-κB reporter mice was performed by the use of pro-nuclear microinjection where reporter construct was injected into one of the nuclei of a fertilized mouse zygote. This is further explained by Carlsen et al.[64].

4.3.2 Experimental Outline

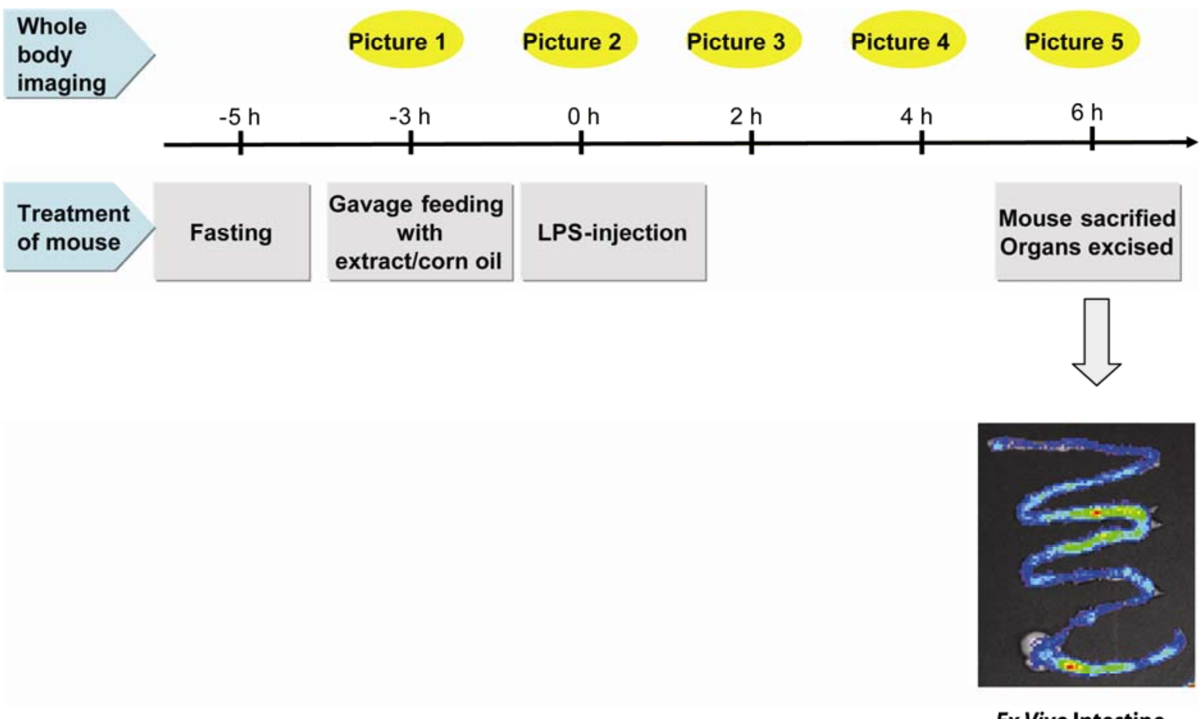


Figure 4.3 ***In Vivo*** **Experimental outline**

Transgenic NF-κB reporter mice were randomized into 2 groups. One group (Extract group) was given a combination-extract (clove, thyme, oregano, walnuts and coffee) and the second group (Control group) was given vehicle control (corn oil) (n = 12 for both groups). The combination extract used in this experiment was made as described in **4.1**.The combination extract contains 2 g/ml coffee and walnuts, and 0.2 g/ml clove, thyme and oregano.

The mice were fasted for 2 hours (time:-5 hours) before starting the experiments (**Figure 4.3**). A single dose of combination-extract (300 µl) or control vehicle was administrated by oral gavage 3 hours prior (time: -3 hours) to s.c. injection of LPS (2.5 µg / kg) (time: 0 hours). Prior to imaging the mice were anesthetized with isoflurane, injected i.p. with 160 mg/ml D-luciferin and placed on their back in a light-proof chamber. The luminescence was detected for 1 min, 5 minutes after D-luciferin was injected. Whole body imaging was performed before oral gavage

feeding and at 0, 2, 4, 6 hours after LPS injection. After 6 h imaging the mice were sacrificed by cervical dislocation and organs were rapidly excised. *Ex vivo* imaging of the intestine was performed.

Imaging of the transgenic mice was performed with The IVIS Imaging System 100 Series. This imaging system detects photons generated from luciferase activity in the mouse. Results were calculated by using Living Image Software.

4.3.3 Luciferase activity in organs

A Luciferase assay was used to measure luminescence in each individual organ. Reporter lysis buffer (1ml) was added to the organ before homogenization to destroy the cell walls in the tissue. The homogenates were transferred to eppendorf-tubes and centrifuged at max speed for 15 minutes at 4°C. The supernatants were used for further measurement of luminescence and protein concentration. Luciferase activity was measured by adding 100 µl room tempered luciferase assay substrate to 20 µl of the supernatant. Luminescence was detected in a TD-20/20 luminometer. Luciferase substrate assay contains luciferin that is necessary to produce photons.

Luciferase assay solution

Reagent	Amount	Final concentration
ATP	52.1 mg	
Coenzyme A	20.7 mg	
Tricine	358.4 mg	
$MgSO_4$ x 7 H_2O	92.2 mg	
DTT	513.5 mg	
EDTA 0.5 M	15 µl	Final [0.075 mM]
dd H_2O	80 ml	
		Adjusted to pH to 7.4
Luciferin (20 mg/ml)	650 µl	Final [0.13 mg/ml]
		Volume adjusted to 100 ml

4.3.4 Total protein content in organs

Protein concentration in tissue homogenates was measured for correction of the luciferase activity to total protein content in each organ. Bio-Rad Protein Assay is based on the method of Bradford. It is a simple and accurate procedure of determining concentration of proteins. It is a dye-binding assay in which a differential colour change of a dye occurs in response to various concentrations of protein. It involves the addition of acidic dye called Coomassie® Brilliant Blue G-250. The Coomassie blue binds to basic and aromatic amino acid residues. When binding to proteins a colour change from brown to blue occur and the absorbance maximum for this blue colour is 595 nm.

The supernatant of the tissue homogenates were diluted with milliQ-water from 10 to 200 times depending on the organs size and protein content. Different known

concentrations of protein (albumin) were used as standards. Protein standards (10µl) and the different organ samples (10 µl) were added to a 96-well plate in triplicates. Coomassie blue was added to each well (200µl) before measuring the absorbance at 590 nm. (The Bio-Rad Protein Assay Solution was diluted 1:4 in milliQ-water, and filtrated through a filter paper prior to use). For measuring the absorbance a Titertek microplate reader was used. The sample was compared with a standard curve from protein standards of 0.041, 0.061, 0.091, 0.273 and 0.410 mg albumin/ml. Every result within this range provides a quantitative measurement of protein concentration

4.4 Effect of Extracts on Recombinant Luciferase

In the U937 3xκB-LUC cells luciferase is utilized as a reporter for NF-κB activity. It is possible that various treatments can affect the luciferase enzyme directly and further affect the results on NF-κB activity. To test for possible effects of the extracts on luciferase, a recombinant luciferase was used.

Recombinant luciferase was diluted in RPMI medium with 2 % FBS to 1 ng/ml. Extracts were diluted in this recombinant luciferase solution into the same concentrations as used in the cell experiments.

Luciferase activity was measured as in section 4.3.3, by adding 100 µl room tempered luciferase assay solution to 20 µl of the recombinant luciferase-extract solution. Luminescence was detected in a TD 20/20 luminometer.

4.5 The Ferric Reducing Ability of Plasma (FRAP) Assay

The FRAP assay was used to measure the total antioxidant content of the extracts in the experiments. This method is based on the method of Benzie and Strain. The FRAP assay measures the ferric reducing ability of plasma, but can also be used for fluids other than plasma, extracts of plant, herbs and other dietary samples. This method is based on measurements of absorption changes that appear when a ferric

tripyrdyltriazine (TPTZ- Fe $^{3+}$) complex is reduced to ferrous tripyrdyltriazine (TPTZ-Fe^{2+}). When this reduction occur an intense blue colour with an absorption maximum at 593 nm will appear. This reaction takes place at a low pH. The colour appearance is thus a measure on the reducing ability of the sample and the presence of a reductant (antioxidant). In the FRAP assay, excess Fe^{3+} is used, so that the rate limiting factor of the reaction is the reducing ability of the sample. The reaction is non-specific, and any half- reaction which has a less positive redox potential than the Fe^{3+} / Fe^{2+} -TPTZ half reaction will drive the reduction from TPTZ-Fe^{3+} to TPTZ-Fe^{2+}.[65]

Dietary plant extracts were diluted in a dilution containing 10% milliQ water and 90 % methanol. Sunflower seed, walnut, coffee, and turmeric were diluted 100 times. Combination mix for mice and cells, thyme, oregano and dog rose were diluted 1000 times. Clove was diluted 10 000 times.

FRAP was determined in extracts by the method of Benzie and Strain [65]. A Technicon RA 1000 system was used for the measurements of absorption change that appear when TPTZ-Fe^{3+} complex reduce to TPTZ-Fe^{2+} in the presents of antioxidants. The measurements were performed at 600 nm. An aqueous solution of 1000 µmol/ $FeSO_4$ x $7H_2O$ reagent was used to calibration of the instrument.

Reagents

Reagents	**Component**	**Volume in FRAP reagent**
Acetate buffer	300 mmol/l acetate buffer, ph 3.6 + $C_2H_4O_2$ 16 ml/l buffer solution	25 ml
HCl	40mmol/l	2.4 ml
$FeCl_3$ x 6 H_2O solution	20 mmol/l	2.5 ml
TPTZ solution	250 mg in 5 ml methanol	155 µl

4.6 Statistical Analysis

When normal distribution one way ANOVA was used to examine possible effects of dietary plant extracts and combination of those extracts on NF-κB activity in U937 cells. Differences were identified using Dunnett (combination compared with individual extracts) Comparison. ANOVA was performed using SPSS 15.

When examine possible synergistic effect of the combination group compared to expected group (the sum of effects of the two individual extracts) Two-tailed Student`s T-test was performed in Microsoft Excel.

One way ANOVA was also used to examine the possible effect of LPS pre-conditioning. Differences between groups were identified using Bonferroni Correction.

The Pearson correlation coefficient was performed to examine correlation between FRAP-values and NF-κB activity for the respective extracts.

When not normally distributed Mann-Whitney U test was performed using SPSS 15 to examine possible effects of combination extract on NF-κB reporter mice.

Statistical significant difference was set to $P<0.05$ for all analysis.

5. Results

5.1 Basal and LPS-induction of NF-κB activity

U937 cells stably transfected with NF-κB-luciferase reporter were used to study the possible effect of foods rich in phytochemicals on NF-κB activation. To simulate an infection bacterial lipopolysaccharide (LPS) was added to the cell culture medium during experiments. We compared basal and LPS-induced NF-κB activity to look at LPS influence on the U937 3xκB-LUC cells.

LPS induced NF-κB activity increases 29.9-fold ($P < 0.001$) compared to basal NF-κB activity (n = 12) (**Figur 5.1 A**). Concentrations of 0, 0.01 and 1 μg/ml of LPS give a dose dependent increase in NF-κB activation where 1 μg/ml exhibits the highest NF-κB activity (**Figure 5.1 B**).

Previous experiments measured NF-κB activation over time in U937 3xκB-LUC cells stimulated with 1 μg/ml LPS. At this concentration the luciferase activity was reaching a plateau after 6 hours incubation with LPS[61].

Based on these experiments incubation with 1 μg/ml LPS for 6 h was used as standard set-up for later experiments in this thesis.

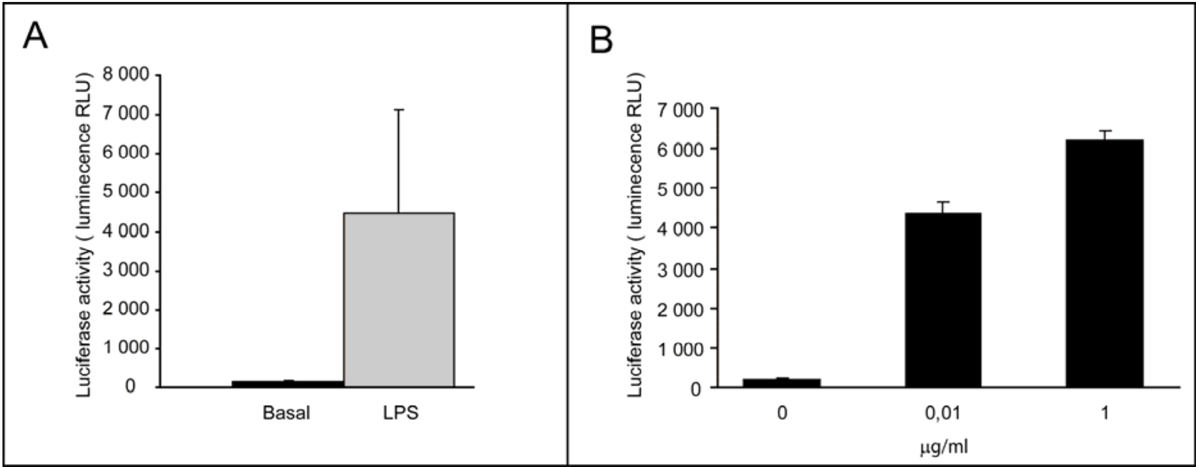


Figure 5.1 Elevated NF-κB activity in U937-3xκB-LUC cells incubated with LPS. Cells were incubated with and without LPS in 24-well plates for 6 hours. Data shown is absolute values of luminicence and are given in Relative Luminescence Units (RLU).The bars show the mean ± SD. **A**) Luciferase activity in U937 3xκB-LUC cells incubated without LPS and with 1 μg/ml LPS (n = 12). **B**) Dose-response relationship for U937 3xκB-LUC cells incubated with LPS (n = 3).

5.2 Effect of Dietary Plants on U937 3xκB-LUC cells

The effect of various individual dietary plants extracts on luciferase reported LPS-induced NF-κB was investigated. These dietary plant extracts include oregano, thyme, clove, turmeric, coffee, walnut, sunflower seed and dog rose. Each extract was tested in two different concentrations in at least 7 experiments. The table represents mean of all experiments as percent of control, where control is set to 100 %. Control cells are treated with LPS only (LPS-induced NF-κB activity). Spices and coffee were tested in 1 mg/ml and 1.5 mg/ml and walnut, sunflower seed and dog rose were tested in 10 mg/ml and 15 mg/ ml. Walnut, sunflower seed and dog rose are food samples that contain more water than spices and coffee. They are also eaten in greater amounts than spices. This is why a 10 fold higher concentration is used for walnut, sunflower seed and dog rose than for spices and coffee.

Oregano, thyme, clove, coffee, walnut, sunflower seed and dog rose show an ability to inhibit LPS-induced NF-κB activity in U937 3xκB-LUC cells (**Figure 5.2**). The difference between the NF-κB activation in control cells and cells incubated with

dietary plant extract is statistically significant at both concentrations tested. Of all extracts, walnut (15 mg/ml) shows the greatest ability to inhibit NF-κB activity to 20.7 % of controls. Looking at extracts tested with 1 and 1.5 mg/ml, clove (1.5 mg/ml) is the most potent extract to inhibit LPS-induced NF-κB activity with 44.8 % of control. All these extracts inhibit NF-κB activation in a dose dependent manner.

Turmeric exhibits a different effect than the other extracts (**Figure 5.2**). When incubating U937 3xκB-LUC cells with 1 or 1.5 mg/ml turmeric extract, LPS-induced NF-κB activity increased to 122.3 % ($P < 0.01$) of control and 121.8 % ($P < 0.01$) of controls.

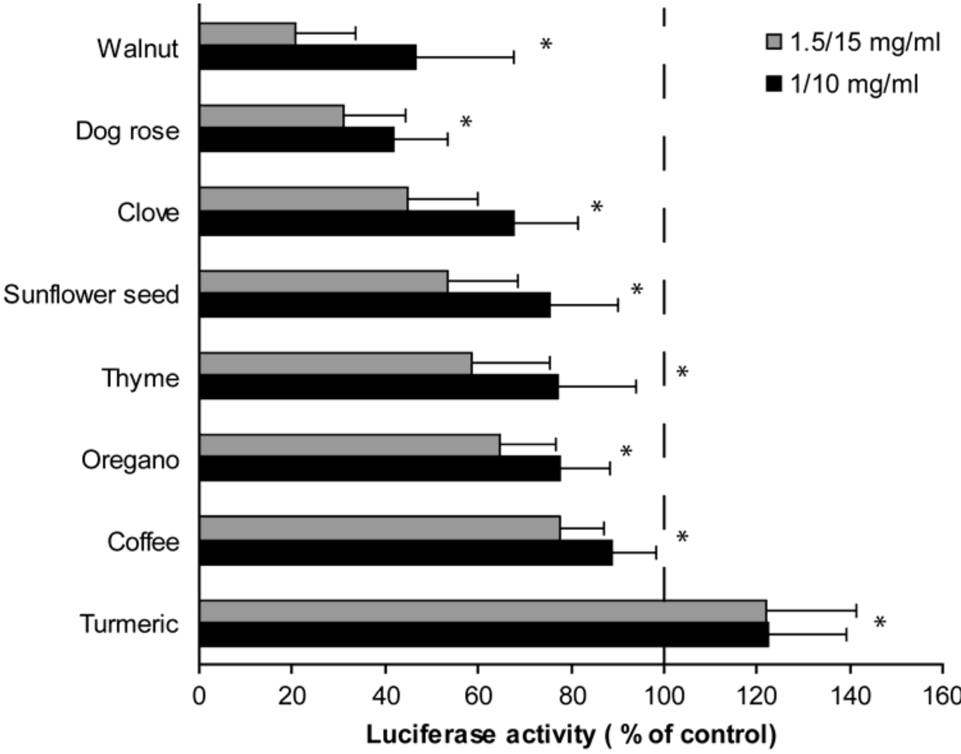


Figure 5.2 LPS-induced NF-κB activity of individual extracts. Bars show the mean ± SD % of control (control = 100 %) in two different concentrations. * $P < 0.05$. n > 7. Each experiment was performed in triplicates.

5.3 Synergistic Effect of Dietary Plants on U937 3xκB-LUC cells

Synergistic effect is present when two or more agents acting together and create an effect greater than that predicted by knowing only the separate effects of the individual agents. In this case it would mean that the combination of two extracts shows a greater inhibition of NF-κB than the sum of the separate effects of two individual food extracts (**Figure 1.6 B** Introduction).

In this project the effect of combinations of pairs of extracts on luciferase reported LPS- induced NF-κB was investigated to look for possible synergistic effects. Both single extracts and combinations of these extracts were included in each experiment for comparison. All together 23 combinations where tested of which one combination (turmeric and clove) was excluded due to cytotoxicity. The remaining 22 combinations were all tested in two different concentrations, but only one concentration of each combination is presented in this thesis because the results were similar. In the experiments showing a synergistic effect the lowest concentration is chosen to avoid the "floor effect" (see section 6.2.2). All experiments were repeated at least three times and in each experiment, each treatment was performed in triplicates.

The figures represent the mean of at least three different experiments and are presented as percentage of control (cells treated with LPS only). The table next to all figures present calculations for possible synergistic effects[66]. Expected values are calculated from the observed values from the individual extracts whereas the observed values are the effect of combination of two extracts. Also percent difference between expected and observed value and significance level are listed.

5.3.1 Synergistic effect on NF-κB activation

Of the 22 combinations tested, 10 showed a statistical significant synergistic effect compared to each single extract. Looking at the expected values compared to observed values, there is a synergistic effect when the NF-κB activity in observed value is lower than the expected value. Coffee, oregano and thyme were each present in 4 of the 10 combinations that shows a synergistic effect, whereas walnut and sunflower seed were each present in 3 of the 10 combinations. Clove and turmeric were each present in one combination that shows synergistic effect.

Combination of oregano & coffee (**Figure 5.3 A**) exhibit a strong synergistic effect where observed value is 46 % (P = 0.01) lower in LPS-induced NF-κB activity than the expected value.

Combination of oregano & turmeric (**Figure 5.3 B**) also exhibit a synergistic effect where the observed LPS-induced NF-κB activity is 26 % (P = 0.03) lower than the expected value. Despite of turmeric alone increasing the NF-κB activity to 114 % of controls, the combination of oregano & turmeric show a significant inhibiting synergistic effect on NF-κB activation.

Combinations of clove & coffee (**Figure 5.3 C**) and walnut & coffee (**Figure 5.3 D**) exhibit a synergistic effect with respectively 24 % (P = 0.03) and 22 % (P = 0.04) lower LPS-induced NF-κB activity in observed value than expected value.

The combinations of oregano & sunflower seed (**Figure 5.3 E**) and thyme & coffee (**Figure 5.3 F**) had similar synergistic effects. Observed values show a significant lower NF-κB activity than the expected values with a 20 % (P = 0.02 and P = 0.04 respectively) difference.

For the thyme & sunflower seeds (**Figure 5.3 G**) in combination the observed LPS-induced NF-κB activation was 18 % (P = 0.04) lower than the expected value, whereas for the walnut & sunflower seed combination (**Figure 5.3 H**) the observed

LPS-induced NF-κB activation was 12 % (*P* = 0.01) lower than the calculated expected value.

Thyme & oregano (**Figure 5.3 I**) and thyme & walnut (**Figure 5.3 J**) exhibit the lowest synergy effects of all combinations with respectively 8 % ($P < 0.001$) and 6 % ($P < 0.001$) lower LPS-induced NF-κB activity for observed value than for the expected value.

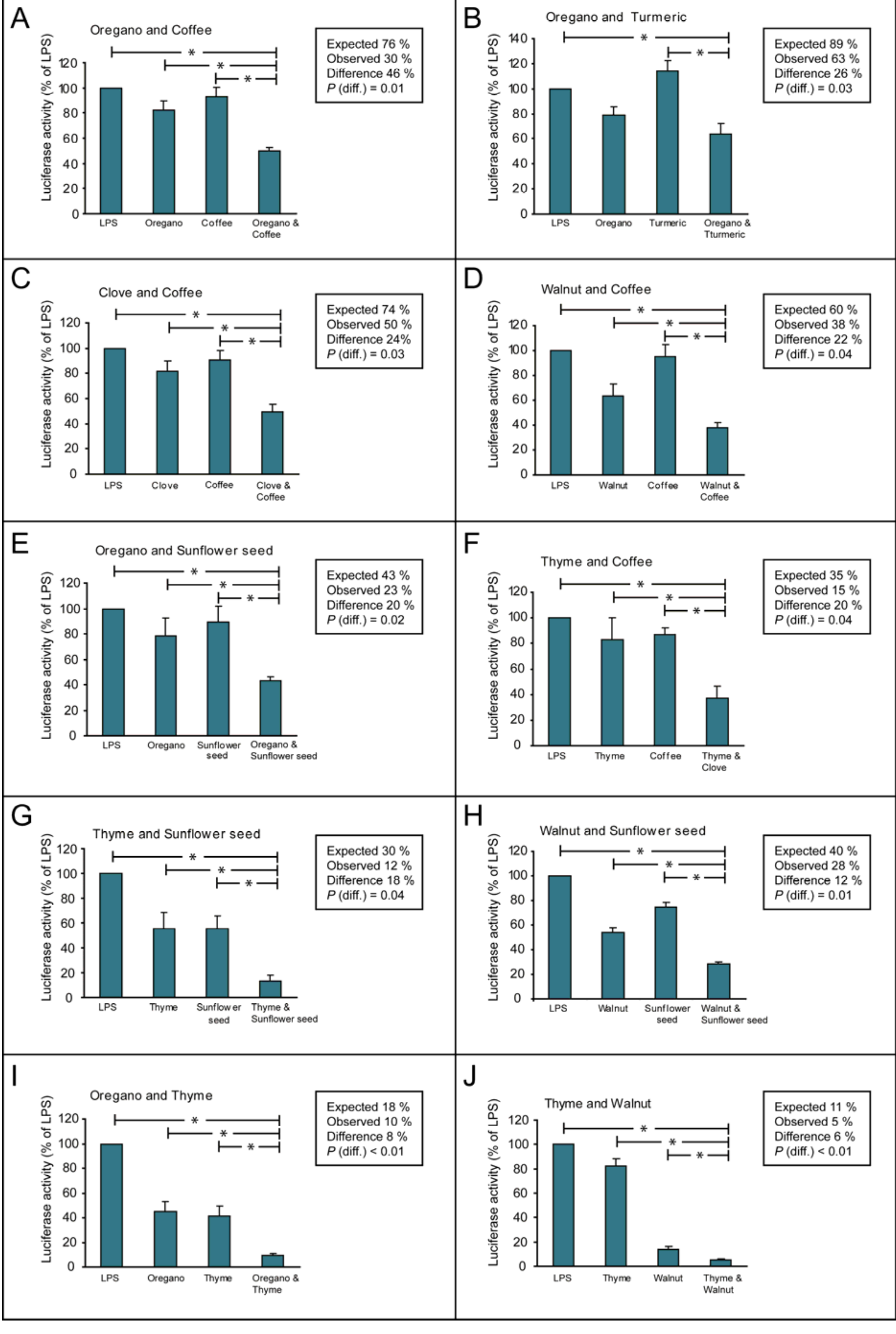

A
Oregano and Coffee
Expected 76 %
Observed 30 %
Difference 46 %
P (diff.) = 0.01
Luciferase activity (% of LPS)
LPS
Oregano
Coffee
Oregano & Coffee
B
Oregano and Turmeric
Expected 89 %
Observed 63 %
Difference 26 %
P (diff.) = 0.03
LPS
Oregano
Turmeric
Oregano & Tturmeric
C
Clove and Coffee
Expected 74 %
Observed 50 %
Difference 24%
P (diff.) = 0.03
LPS
Clove
Coffee
Clove & Coffee
D
Walnut and Coffee
Expected 60 %
Observed 38 %
Difference 22 %
P (diff.) = 0.04
LPS
Walnut
Coffee
Walnut & Coffee
E
Oregano and Sunflower seed
Expected 43 %
Observed 23 %
Difference 20 %
P (diff.) = 0.02
LPS
Oregano
Sunflower seed
Oregano & Sunflower seed
F
Thyme and Coffee
Expected 35 %
Observed 15 %
Difference 20 %
P (diff.) = 0.04
LPS
Thyme
Coffee
Thyme & Clove
G
Thyme and Sunflower seed
Expected 30 %
Observed 12 %
Difference 18 %
P (diff.) = 0.04
LPS
Thyme
Sunflower seed
Thyme & Sunflower seed
H
Walnut and Sunflower seed
Expected 40 %
Observed 28 %
Difference 12 %
P (diff.) = 0.01
LPS
Walnut
Sunflower seed
Walnut & Sunflower seed
I
Oregano and Thyme
Expected 18 %
Observed 10 %
Difference 8 %
P (diff.) < 0.01
LPS
Oregano
Thyme
Oregano & Thyme
J
Thyme and Walnut
Expected 11 %
Observed 5 %
Difference 6 %
P (diff.) < 0.01
LPS
Thyme
Walnut
Thyme & Walnut

Figure 5.3 Synergistic effects of dietary plant extracts on LPS-induced NF-κB activity. U937 3xκB-LUC cells were treated with dietary plant extracts plus LPS and incubated for 6 hours. Bars shows mean ± SD. * $P < 0.05$. n = 3.Each experiments performed in triplicates. Textbox shows expected and observed value % of control, percent difference between expected and observed value and *P*-value for the difference.

5.3.2 Additive effect on NF-κB activation

Additive effect is when the effect of the sum of two individual food extracts is equal to the effect of the combination of the two food extracts. In other words expected effect of two extracts is equal to the observed value. In this thesis we have categorized additive effect as: observed value ≤ expected value, with no statistically significant difference between the two groups.

Of the combinations tested,10 of 22 combinations exhibit additive effects on NF-κB activation.

Four combinations exhibit an additive effect with < 5% difference between observed and expected value. These combinations are oregano & walnut (P= 0.98) (**Figure 5.4 A**), oregano & clove (P = 0.81) (**Figure 5.4 B**), oregano & dog rose (P = 0.57) (**Figure 5.4 C**), and sunflower seed & dog rose (P = 0.83) (**Figure 5.4 D**).

Thyme & clove (P = 0.39) (**Figure 5.4 E**), clove & dog rose (P = 0.39) (**Figure 5.4 F**), sunflower seed & coffee (P = 0.52) (**Figure 5.4 G**) and clove & walnut (P = 0.08) (**Figure 5.4 H**) show an additive effect where observed value exhibit a lower LPS-induced NF-κB activity than expected value. This difference is small and range from 5 % to 9 %, but is not statistical significant.

Clove & sunflower seed (**Figure 5.4 I**) and turmeric & thyme (**Figure 5.4 J**) are two combinations where the difference between observed and expected group seems to be distinct and point to a synergistic direction. The observed values are 15 % (P = 0.27) and 25 % (P = 0.26) lower than the expected value, however the differences are not statistically significant and thus these combinations are categorized as additive.

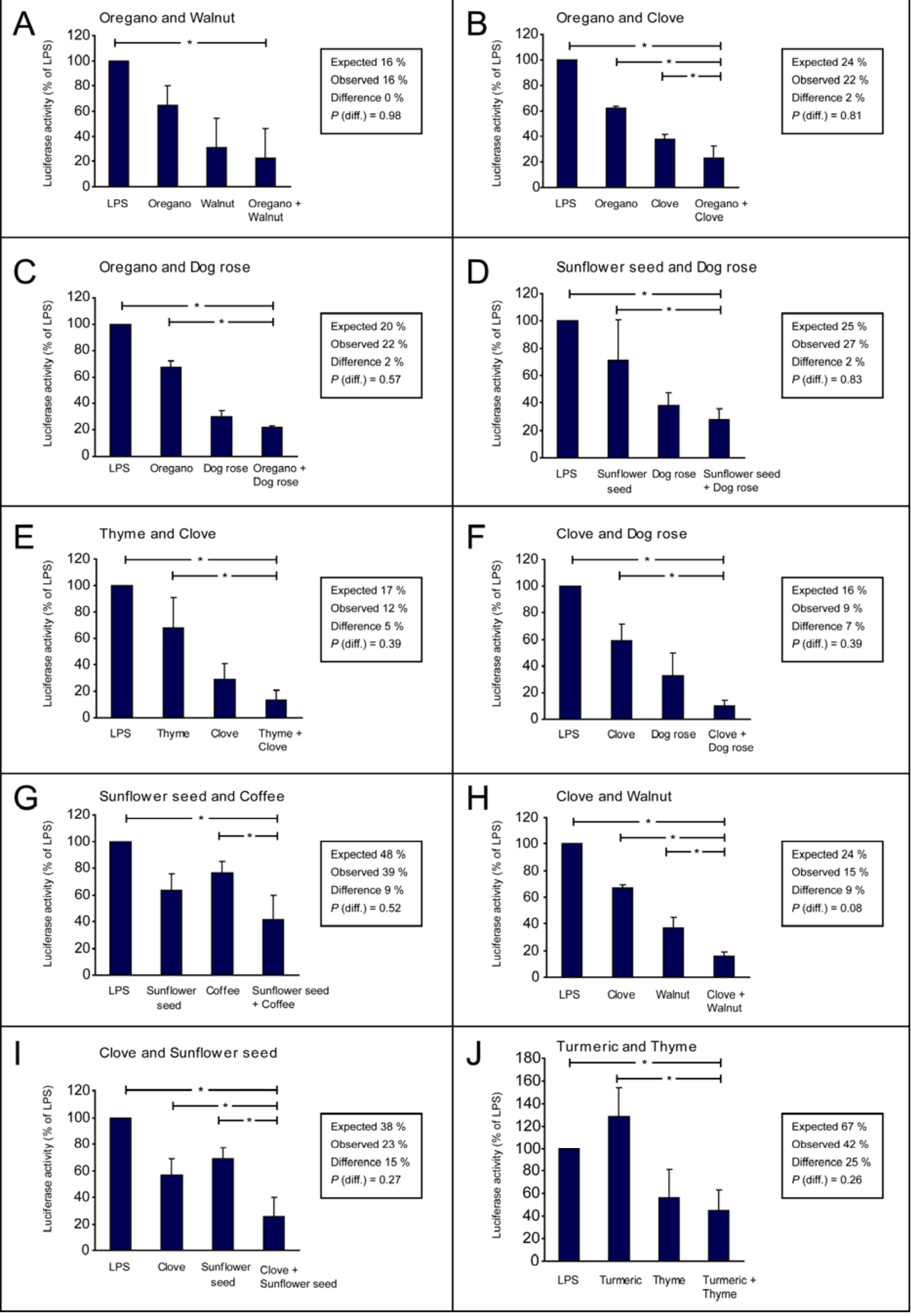

A
Oregano and Walnut
Luciferase activity (% of LPS)
LPS
Oregano
Walnut
Oregano + Walnut
Expected 16 %
Observed 16 %
Difference 0 %
P (diff.) = 0.98
B
Oregano and Clove
LPS
Oregano
Clove
Oregano + Clove
Expected 24 %
Observed 22 %
Difference 2 %
P (diff.) = 0.81
C
Oregano and Dog rose
LPS
Oregano
Dog rose
Oregano + Dog rose
Expected 20 %
Observed 22 %
Difference 2 %
P (diff.) = 0.57
D
Sunflower seed and Dog rose
LPS
Sunflower seed
Dog rose
Sunflower seed + Dog rose
Expected 25 %
Observed 27 %
Difference 2 %
P (diff.) = 0.83
E
Thyme and Clove
LPS
Thyme
Clove
Thyme + Clove
Expected 17 %
Observed 12 %
Difference 5 %
P (diff.) = 0.39
F
Clove and Dog rose
LPS
Clove
Dog rose
Clove + Dog rose
Expected 16 %
Observed 9 %
Difference 7 %
P (diff.) = 0.39
G
Sunflower seed and Coffee
LPS
Sunflower seed
Coffee
Sunflower seed + Coffee
Expected 48 %
Observed 39 %
Difference 9 %
P (diff.) = 0.52
H
Clove and Walnut
LPS
Clove
Walnut
Clove + Walnut
Expected 24 %
Observed 15 %
Difference 9 %
P (diff.) = 0.08
I
Clove and Sunflower seed
LPS
Clove
Sunflower seed
Clove + Sunflower seed
Expected 38 %
Observed 23 %
Difference 15 %
P (diff.) = 0.27
J
Turmeric and Thyme
LPS
Turmeric
Thyme
Turmeric + Thyme
Expected 67 %
Observed 42 %
Difference 25 %
P (diff.) = 0.26

Figure 5.4 Additive effects of dietary plant extracts on LPS-induced NF-κB activity. U937 3xκB-LUC cells were treated with dietary plant extracts plus LPS and incubated for 6 hours. Bars shows mean ± SD. * *P* < 0.05. n = 3.each experiment performed in triplicates. Textbox shows expected and observed value % of control, percent difference between expected and observed value and *P*-value

5.3.3 No synergistic or additive effect on NF-κB activation

There are two combinations that exhibit a different effect on LPS-induced NF-κB activation than the combinations previously explained. For these two combinations the sum of effects of the two individual extracts together exhibit a lower LPS-induced NF-κB activity than the effect of the combination of the same extracts.

The combination walnut & dog rose (**Figure 5.5 A**) show a significant difference between expected and observed value (*P* = 0.04) where expected LPS-induced NF-κB activity is 12 % lower in than the observed value.

The combination of thyme & dog rose (**Figure 5.5 B)** gave no statistically significant difference (*P* = 0.45) between the observed and the expected NF-κB activation

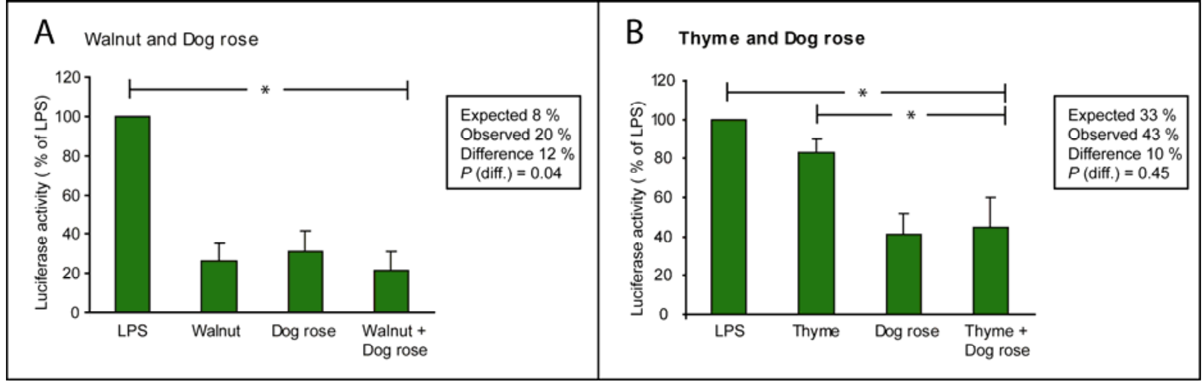


Figure 5.5 Effects of dietary plant extracts on LPS-induced NF-κB activity. U937 3xκB-LUC cells were treated with dietary plant extracts plus LPS and incubated for 6 hours. Bars shows mean ± SD. * *P* < 0.05. n = 3.Each experiment was performed in triplicates. Textbox shows expected and observed value % of control, percent difference between expected and observed value and *P*-value

5.3.4 Modulation of NF-κB by Combination extract

After having tested combinations of two extracts for their effects on NF-κB activation in cell culture, we wanted to test the effect of a combination extract containing 5

extracts that have showed inhibiting effect on NF-κB activation. This experiment was mainly to test the combination extract *in vitro* before using it *in vivo*, however also to identify the minimum concentration of this extract with the ability to inhibit NF-κB activation. The combination extract was made of thyme, oregano, clove, walnuts and coffee. This extract was tested in U937-3xκB-LUC cells with concentrations ranging from 0.01 to 1mg/ml for thyme, clove and oregano and 0.1 to 10 mg/ml for walnut and coffee.

The combination extract inhibited LPS-induced NF-κB activation in a dose-dependent manner (**Figure 5.6**), inhibiting NF-κB activity to 1.7 % (P <0.001) of controls at the highest concentration 10 mg/ml for walnut and coffee and 1 mg/ml for thyme, oregano and clove. The lowest concentration that showed a significant difference in NF-κB activity between control cells and the treated cells, were 1 mg/ml for walnut and coffee, and 0.1 mg/ml for thyme, clove and oregano. The NF-κB activity dropped the most between the concentrations of 0.1 and 0.3 for thyme, clove and oregano and 1 and 3 mg/ml for walnut and coffee, from 73 % of control cells to 8 % of control cells.

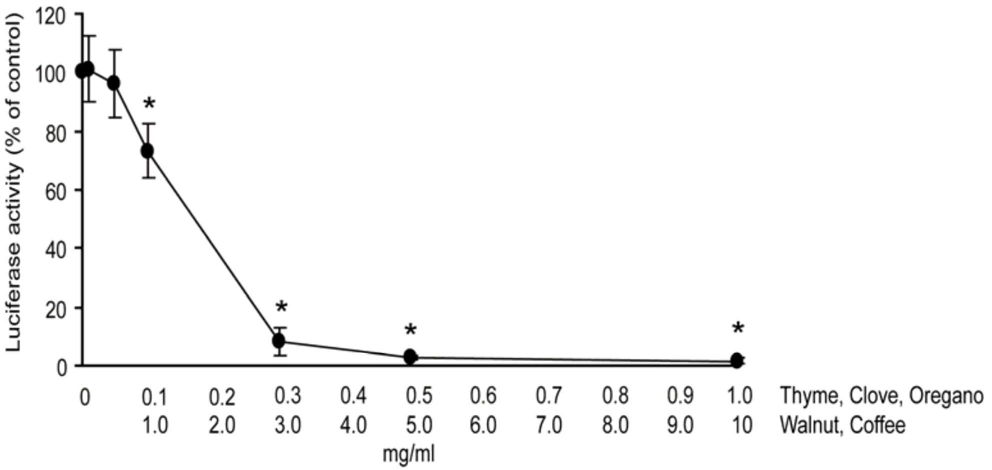


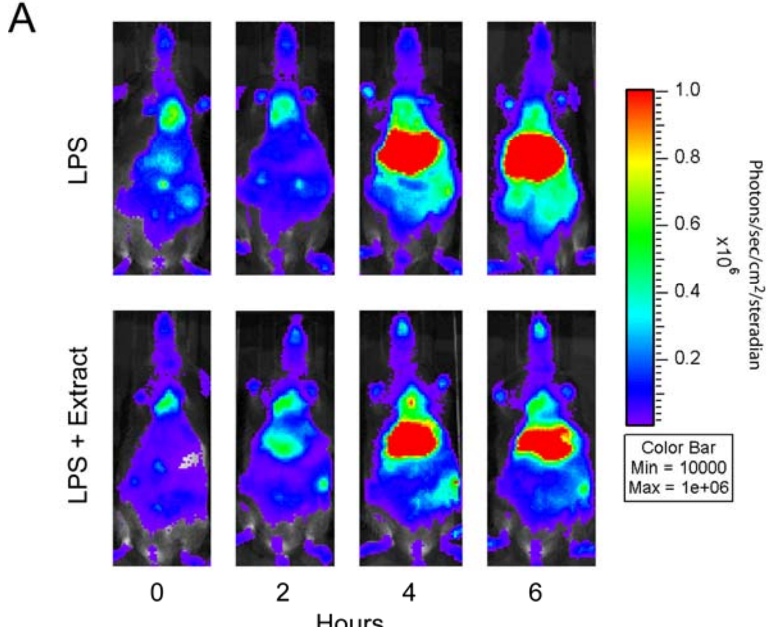
Figure 5.6 Dose-response relationship between combination extract and NF-κB activity. U937 3xκB-LUC cells were incubated with a combination extracts containing thyme, oregano, clove, walnuts and coffee plus LPS for 6 hours. Mean % of control ± SD.* $P< 0.05$. n =3. Each experiment performed in triplicates.

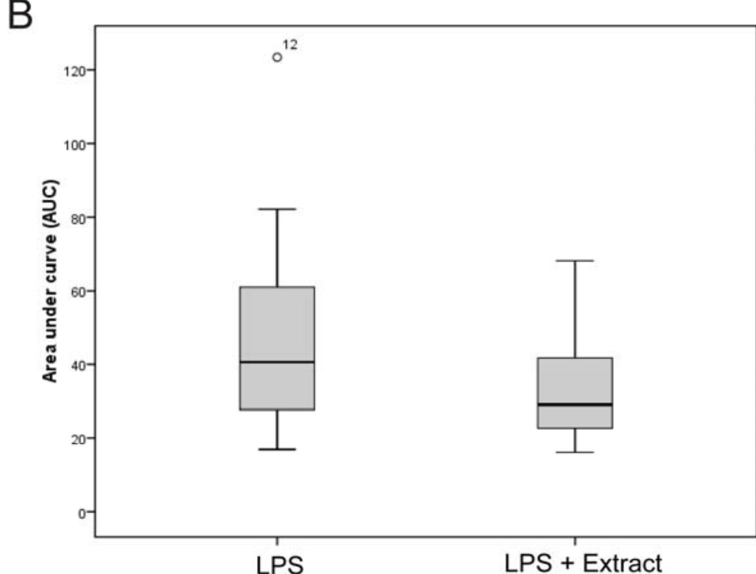
5.4 *In Vivo* Modulation of NF-κB by Dietary Plants

After having tested the combination-mix on U937 3xκB-LUC cells and seen a dose dependent inhibition of LPS-induced NF-κB activity, the next step was to test the same combination extract, containing clove, thyme, oregano, walnut and coffee, in the NF-κB-luciferase reporter mice.

5.4.1 *In Vivo* Imaging: Modulation of NF-κB by Combination-mix

Transgenic NF-κB-luciferase reporter mice were given either one dose of the combination extract or vehicle control prior to an s.c. LPS injection, and NF-κB activation was monitored by *in vivo* imaging. When light from whole mice were quantified we found that mice given the combination extract exhibit a trend towards inhibition of NF-κB activation compared to control mice. The area under curve (AUC) (**Figure 5.7 B**) for the NF-κB activation from 0-6h show that median for the mice receiving the extract is 28.9 (95% CI for median: 18.7-43.5), whereas for control mice the AUC is 40.6 (95 % CI for median: 26.8-66.1). Extract group exhibit a 1.4 fold lower LPS-induced NF-κB activation compared to the control mice. This difference is however not statistically significant ($P = 0.18$).


A
LPS
LPS + Extract
0
2
4
6
Hours
1.0
0.8
0.6
0.4
0.2
x10^6
Photons/sec/cm²/steradian
Color Bar
Min = 10000
Max = 1e+06


B
Area under curve (AUC)
120
100
80
60
40
20
0
12
LPS
LPS + Extract

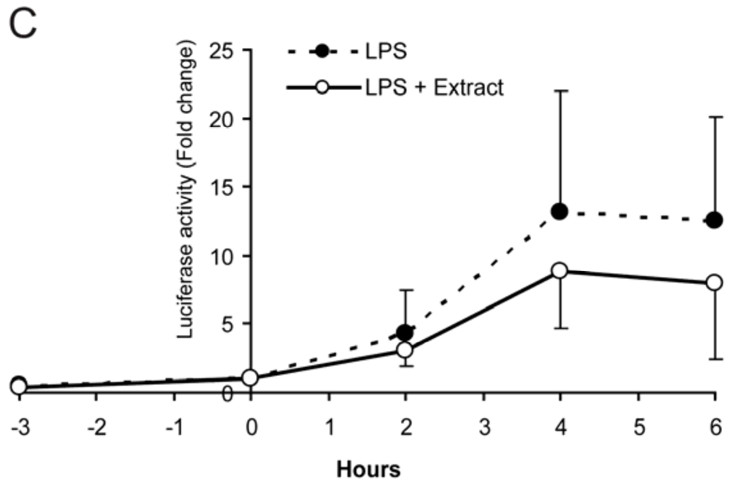

C
LPS
LPS + Extract
Luciferase activity (Fold change)
25
20
15
10
5
0
-3
-2
-1
0
1
2
3
4
5
6
Hours

Figure 5.7 Effect of combination extract in transgenic NF-κB reporter mice. Transgenic mice were gavage-fed with a combination extract or control vehicle (corn oil) 3 hours before s.c. injection of LPS. *In vivo* imaging was performed at 0h, 2h, 4h and 6h. n = 12 in each group. **A**) *In vivo* images of one representative mouse from all time points are shown. **B**) Box plots of the area under the curve for both groups **C**) The mean ± SD of photons/sec/cm^2 /steradian (normalized to 0h) of the whole mouse minus the head and hind limbs.

5.4.2 *Ex Vivo* Imaging: Modulation of NF-κB by Combination-mix

NF-κB activity in specific organs can not be determined by whole mouse *in vivo* imaging. For that reason the organs are excised from the mouse and NF-κB activation is tested for each organ separately. The NF-κB activation in the intestine is tested by *ex vivo* imaging.

Ex vivo imaging after administration of combination-extract or vehicle control show that the combination-extract inhibits NF-κB activation in the intestine compared to control mice. The mean fold change compared to control is 0.51± 0.28 (P = 0.02) (**Figure 5.8 A**). *Ex vivo* images of one representative intestine from each treatment is shown in **Figure 5.8.B**.

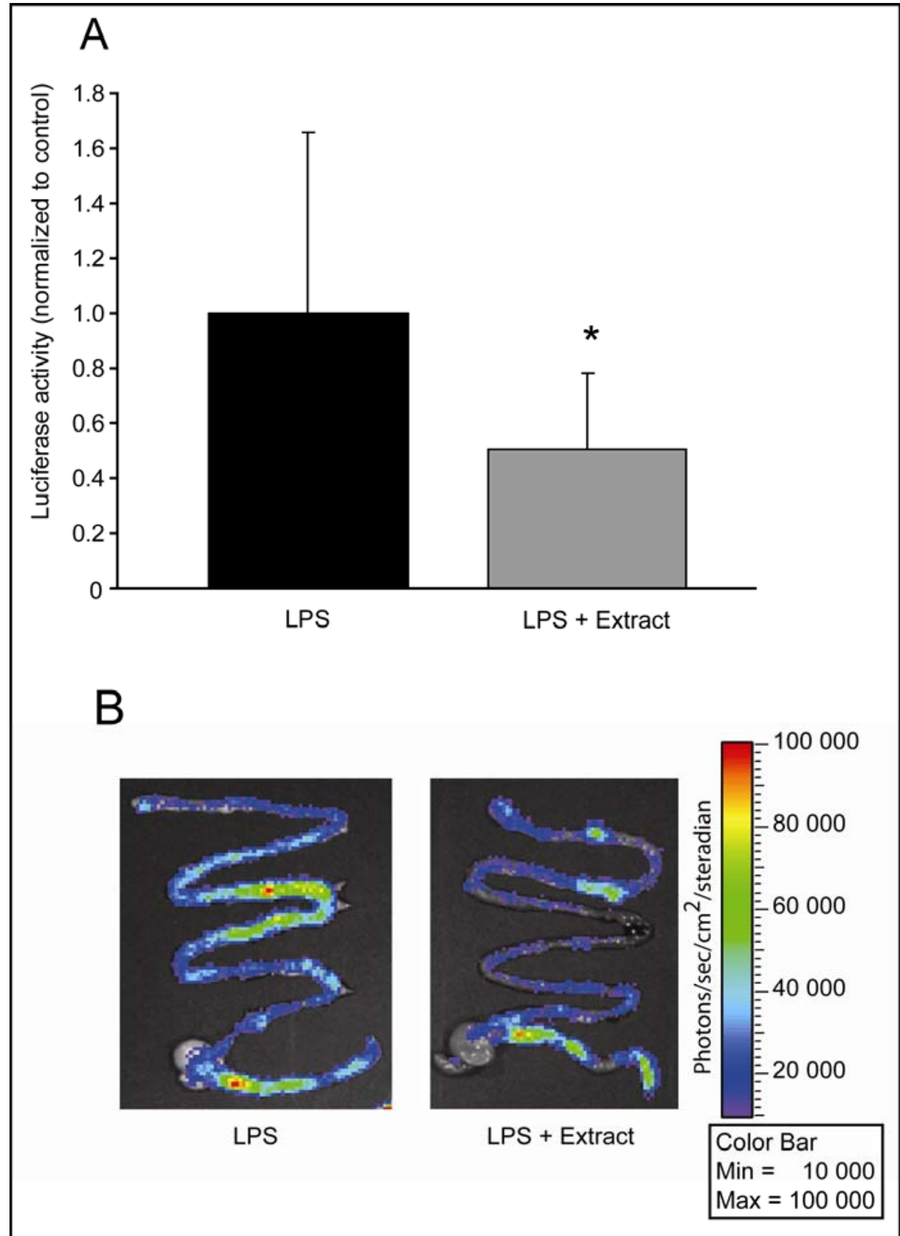


Figure 5.8 *Ex vivo* imaging of LPS-induced NF-κB activity in intestine of transgenic mice after administration of combination extract or vehicle control. After *in vivo* imaging of the transgenic NF-κB reporter mice, the animals were sacrificed. Organs were excised from the mice and the intestine was immediately imaged. **A**) Bars shows mean luciferase activity normalized to control ± SD. * $P = 0.02$. **B**) *Ex vivo* image of one representative intestine from each group (photons/sec/cm^2/steradian).

5.4.3 NF-κB activity in specific organs

NF-κB activity is measured in homogenates of the specific organs excised from the mice.

NF-κB activity near significantly ($P = 0.056$) decreased in liver of mice receiving the combination extract compared to the control mice (**Figure 5.9**). The mean for extract group is 0.7 ± 0.4 fold change compared to controls. No other organ tested exhibit statistically significant difference in NF-κB activity between extract group and control group.

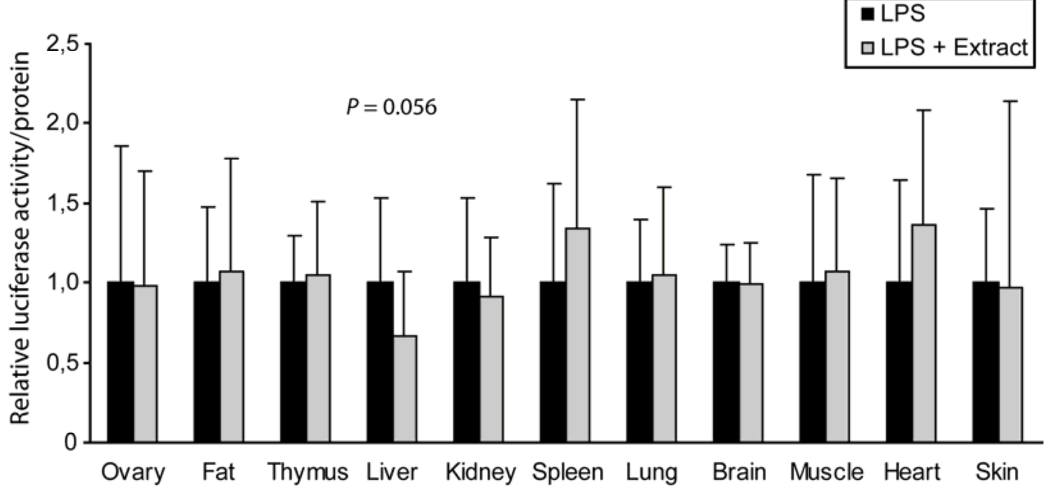


Figure 5.9 LPS-induced luciferase activity in various organs after administration of combination extract. After organs were excised from the transgenic NF-κB reporter mice 6 h after LPS injection, NF-κB activity in homogenates from organs were measured and corrected for total protein content. n = 12 for each organ in each group. The bars represent mean ± SD.

5.5 Effect of Extracts on Recombinant Luciferase

Measuring luciferase reported NF-κB activity is dependent on the oxidation of luciferin to oxyluciferin. This reaction is catalysed by the enzyme luciferase. Various treatments may affect the luciferase enzyme directly and further influence the results on NF-κB activity. Dietary plant extracts used in the experiments were all tested for their ability to affect the production of light from a recombinant luciferase enzyme. In

these experiments the same concentrations of the extracts as in the cell culture experiments were used. All the extracts exhibit the ability to decrease the activity of the recombinant luciferase enzyme compared with control (**Figure 5.10 A**). This inhibition was most prominent in walnut, clove, thyme, oregano, sunflower seed and coffee where the recombinant luciferase activity ranged from 0.03 to 3.4 % of control.

The combination mix used in cell culture and *in vivo* experiments was also tested for its ability to affect the recombinant luciferase enzyme. This extract also decreased the recombinant luciferase activity (**Figur 5.10 B**). Different concentrations of the extract were tested inhibited the recombinant luciferase activity in a dose dependent manner.

This inhibition of recombinant luciferase could indicate that the extracts may affect the results on NF-κB activity in experiments. In cell culture experiments the extracts will not be directly in contact with the luciferase enzyme as the components will have to be absorbed through the cell wall, however based on these results we can not rule out an effect of the extracts on luciferase activity in our cell culture experiments. This will be further discussed in section 6.1.4.

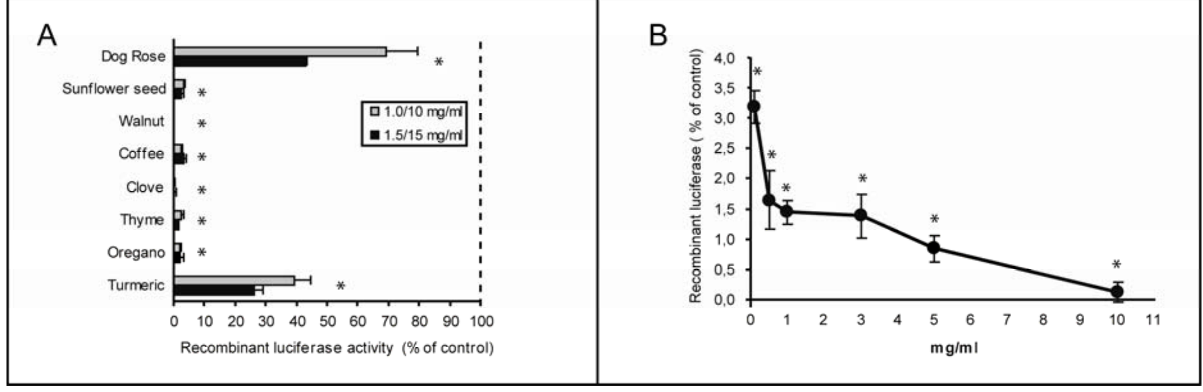


Figure 5.10 Effect of plant extracts on recombinant luciferase activity. Dietary plant extracts and recombinant luciferase was combined and luciferase activity measured with TD 20/20 luminometer. Bars and points shows mean % of control ± SD **A**) Effect of individual extract on recombinant luciferase. *$P < 0.01$. **B**) Dose-response relationship between combination extract (thyme, oregano, clove, walnut and coffee) and activity of recombinant luciferase. * $P < 0.01$.

5.6 FRAP Measurements

The total reducing capacity in each extract used in the experiments was assessed by the FRAP assay. Results are given as mmol/100 g original product.

The FRAP values of spice-, walnut-, sunflower seed- and coffee- extracts used in the experiments are listed in **Table 5.1.** Clove extract is exceptionally high in total reducing capacity with a FRAP-value of 137.3 mmol/100g. Next is extract of oregano with a FRAP value of 111.0 mmol/ 100g, followed by extracts of dog rose, thyme, coffee and sunflower seed. The lowest FRAP values of extracts used in these experiments is walnut and turmeric with 1.8 and 1.3 mmol/100g respectively.

Table 5.1 FRAP-values for individual dietary plant extract.

Extract	**FRAP mmol/100 g n =3**	**SD FRAP**
Clove	137.3	1.3
Oregano	111.0	2.7
Dog rose	74.8	7.4
Thyme	51.4	1.4
Coffee	13.3	0.0
Sunflower seed	8.4	0.1
Walnut	1.8	0.1
Turmeric	1.3	0.4

The FRAP assay revealed a high total reducing capacity in both combination extracts for mice experiments and combination extract for cell culture experiments with FRAP-values of 82.3 and 77.1 mmol/100g respectively (**Table 5.2**).

Table 5.2 FRAP values for combination extract.

Extract	**FRAP mmol/100 g n = 3**	**SD FRAP**
*Combination extract to mice	82.3	0.9
*Combination extract to cells	77.1	0.0

* Contains 0.2 g/ml clove, oregano, thyme, 2 g/ml coffee and walnut

5.7 Correlation between FRAP and NF-κB activity

Phytochemicals are reported to inhibit NF-κB activity *in vitro*[46]. Many phytochemicals also exhibit antioxidant properties[46, 58]. These findings suggest that phytochemicals may inhibit NF-κB activity through their antioxidant properties. FRAP assay detect most of the components in the extract that exhibit antioxidant properties including phytochemicals.

A possible association between FRAP-values from the combination of two extracts and their combined ability to modulate LPS-induced NF-κB activity was studied. No correlation between these two variables was detected (r = - 0.28, P = 0.20) (**Figure 5.11**).

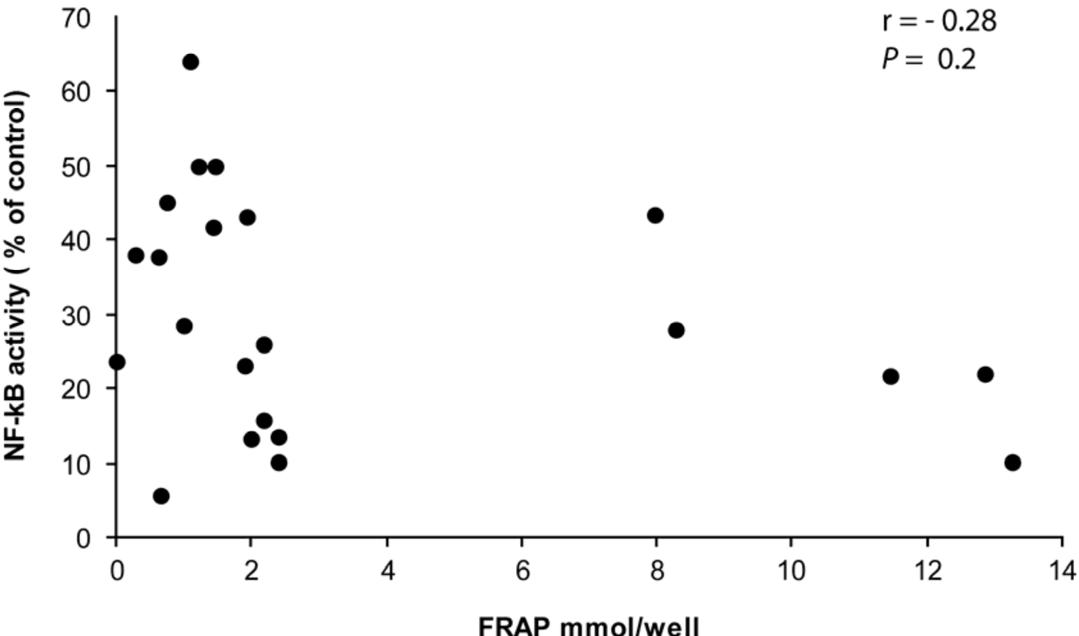


Figure 5.11 Correlation between FRAP-values of two combining extracts and their ability to modulate LPS-induced NF-κB activity in U937 3x κB cells. The correlation is measured using Pearson correlation of FRAP-values of extracts and mean luciferase activity from the cell-plate-well containing combination of two extracts. n = 3. each experiment performed in triplicates.

6. Discussion

Epidemiological studies have shown that regular consumption of fruit and vegetables reduces the risk of developing chronic diseases like cancer and cardiovascular diseases[3, 4]. It is widely believed that the health benefits from consumption of fruits and vegetables are due to the many bioactive phytochemicals in the plant[6]. Their role and mechanisms in the human body are not fully understood, but they may be bioactive components that act together to influence physiological responses beneficial to health. Whole dietary plant contains thousands of phytochemicals with different functions[6]. A natural combination of phytochemicals present in fruit and vegetables may thus be better than single antioxidants.

There are a wide variety of reports describing substances with the ability to modulate NF-κB activity[42, 44]. Reported substances are both for therapeutic use and of food origin. NF-κB is often dysregulated and constitutively active in various chronic inflammatory diseases and some cancers[48, 49]. Because of these observations NF-κB modulators and especially inhibitors are of great interest due to their potential use in prevention and treatment of several chronic diseases.

Investigation has mostly been focusing on single constituents in plant based food and their role in modulating NF-κB activity. Reports about whole dietary plants, combinations of different plant based food items and their potential synergistic effect on NF-κB are not found. There is limited investigation of food synergy in general and especially about biological mechanisms behind synergy. Methods and model system that are used in food synergy research varies. Due to this lack of research on food synergy and variation of methods and model systems, further research on food synergies is greatly needed.

This thesis focuses on extracts of whole dietary plants rich in phytochemicals and their ability to affect NF-κB activity under an inflammatory condition in cell culture and mice. The plant extracts have previously been reported as inhibitors of NF-κB

when stimulated with LPS[61]. Combining the inhibitory plant extracts into culturing cells and mice will give an exposure of numerous of different bioactive components. It is believed that all these components act together and that these interactions may lead to an alteration of the individual effects causing additive, synergistic or antagonistic effect on LPS-induced NF-κB activity. The present work addresses potential additive and synergistic effects of combining different dietary plant extracts *in vitro*. Of the 22 combinations tested, 10 showed synergistic effects on NF-κB activation, and oregano, thyme and coffee were most frequently present in the synergistic combinations.

In addition an *in vivo* experiment with a combination of 5 dietary plant extracts was performed to elucidate the effect on LPS-induced NF-κB in transgenic mice. This combination extract significantly inhibited NF-κB activation in intestine, and gave a trend towards inhibiting NF-κB activity in liver and the whole body.

6.1 Discussion of Methods

6.1.1 The luciferase reporter

The transcription factor NF-κB was chosen because of its important role in regulation of immune and inflammatory response. Even though NF-κB plays an essential role in normal physiology, inappropriate regulation of NF-κB activity has been implicated in the pathogenesis of several diseases. Thus the luciferase reporter system offers a valuable tool for studying NF-κB regulation in conditions such as chronic inflammation and infections.

In this thesis the enzyme luciferase is used as a reporter system to determine the activity of NF-κB. This is achieved by using a DNA construct that contains the luciferase gene, coupled to a promoter of three NF-κB-DNA-binding sites which make the transcription of luciferase dependent on NF-κB activation. The NF-κB-luciferase reporter is used in both U937 3xκB-LUC cells and transgenic mice.

The advantage of using the luciferase reporter is that it has a high sensitivity, which makes low levels of gene expression easy to detect. There is also a correlation between amount luciferase and the light produced, as long as luciferin is used in excess[64]. Luciferin is thus given in excess in the experiments to prevent it from being the limiting factor in the reaction. In addition, the production of the active enzyme luciferase is very rapid and it also has a short half life (2-3 h)[67]. These advantages make *in vivo* imaging realistic and close to real time.

Limitations of luciferase as a reporter *in vivo* includes haemoglobin as an absorber of light emitted from luciferase[64]. Due to this luciferase activity will vary depending on the content of blood in different organs. The greatest limitation using luciferase is the low penetration of tissue. This makes the luminescence occurring in deeper layers of tissue difficult to detect with *in vivo* imaging.

The luciferase reporter model gives an overall picture of the NF-κB activity, but it does not say anything about the target genes of NF-κB. Those target genes can be studied by measuring mRNA levels using Northern blots, reverse transcriptase polymerase chain reaction (RT-PCR) or in situ hybridisation. To elucidate the expression of multiple genes cDNA microarray techniques can be used. The latter method makes it possible to assess the effect of a specific diet or nutrient for example phytochemicals on the expression of a large proportion of the genome[32]. The luciferase reporter does not say anything about where the treatment/dietary plant extracts acts in the NF-κB signalling cascade.

Another way of measuring NF-κB activity is by using electrophoretic mobility shift assay (EMSA). In this method a radiolabelled oligonucleotide with high-affinity DNA binding sequence for NF-κB is used. The NF-κB transcription factor will then bind the oligonucleotide and the amount of binding will be measured by electrophoresis assay. This protein-DNA complex will move more slowly than free DNA and is possible to detect in gel electrophoresis[68]. This is a method that gives quantitative information of NF-κB, but it does not tell whether the transcription factor is able to activate transcription.

It is not known where the NF-κB luciferase construct is incorporated in the mouse genome, and thus affects genes involved in the NF-κB signalling pathways. This may affect the results in some study designs where gene regulation and target genes or signalling pathways are of particular interest, however probably not in this model system.

The use of U937 3xκB-LUC cells

Human monocytes are attracted to sites of inflammation or infection, and help fight foreign substances in the body. U937 3xκB-LUC cells are human monocytic cells that are responsive to bacterial infection through their Toll-like receptors that recognise LPS. They are also responsive to other stimuli like phytochemicals[61]. The monocytes are stably transfected with a construct that contains the luciferase gene, coupled to a promoter with three NF-κB-DNA binding sites (3xκB-LUC).This *in vitro* system is an excellent model for initial testing of NF-κB modulation. Stably transfected cells with transcription factor-reporting construct like this cell line enable rapid and simple screening of how various factors regulate NF-κB. U937 3xκB-LUC cells are also easy to culture and can be grown in sufficient quantities for large and frequent experiments.

Even though this is a good *in vitro* model, it can not represent the effect of phytochemicals in an intact organ or even an animal or human. Cells in such organisms are exposed to and react on signals from a wide range of different cells. They also interact with the extra cellular matrix (ECM). Cell/cell and cell/ECM interactions establish a communication network that maintains the specificity and homeostasis of the tissue[69]. This communication is difficult to model in an *in vitro* system.

After a period of 2-3 months of culturing, the cells showed a lower LPS-induced luciferase activity than right after thawing. Due to this we replaced the culturing cells with a new stock of frozen cells twice during the period of experiments. Considering

these variations of the response to LPS, the control is set to 100% and all results are expressed as % of control cells treated with LPS and vehicle only.

In vivo usage of the luciferase reporter gene

After having screened the extracts for their effect in cell culture, the next step was to look at the extracts effect in an intact animal using luciferase reporter mice. This model enables investigation of how an intact animal and each tissue respond to dietary plant extracts.

There are many advantages of using transgenic reporter mice: Cell culture conditions are based on a single cell type and cannot replicate all processes that occur in an intact animal. Bioavailability of the compounds in dietary plant extracts differs in with the tissues and nutrients also undergo metabolic changes during digestion. It is therefore necessary to use animal models both to confirm *in vitro* data and to extend the knowledge to the *in vivo* situation.

To detect the luciferase activity in mice *in vivo* imaging of the whole mice are performed. *In vivo* imaging or molecular imaging can be defined as visual representation, characterization, and quantification of biological processes at the cellular and subcellular level within intact living organism[70]. Use of transgenic mice and *in vivo* imaging makes it possible to view complex biological processes within an intact, living mouse without doing invasive procedures to obtain results. This is much easier and more rapid than e.g. measuring mRNA levels of a target gene or DNA-binding of a transcription factor. The luciferase reporter gene is dependent on both the binding to DNA and the ability of NF-κB to activate transcription. Consequently, the luciferase production is the net result of the regulation of NF-κB and will not be influenced by other transcription factors present [71].

Furthermore, *in vivo* imaging provides the possibility to follow the same animal over time and do repetitive measures of the same mouse. This allows the animal to serve as its own control, thus reducing the number of animals needed and also minimizing the effects of differences between individual animals.

6.1.2 Cell viability

In the experiments of this thesis we have tested cell viability by trypan blue colouring of cells, with a 10 % cut off value. Another commonly used method to measure cell viability is the LDH assay. This method is based on measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells. This assay detects the reducing capacity of LDH by measuring a colour change. Most of the extracts used in this work are strong reductants that can interfere with the LDH assay, producing the same colour change as LDH. The LDH method is therefore not suitable for these experiments.

6.1.3 Extracts of dietary plants

The method of extraction in this work includes both water soluble and fat soluble substances, but the whole composition of the extracts are not known. Previous experiments have shown that spices and walnuts contain high levels of polyphenols[72-75].

Shan *et al.* [74] observed a strong total antioxidant capacity (TEAC) and total phenolic content in clove, oregano and thyme. They also showed that there is a highly positive linear releationship ($R^2 = 0.95$) between the total antioxidant content and total phenolic content in these spices, indicating that phenolic compounds in the tested spices contributes to their antioxidant capacity.

The whole composition of bioactive compounds in these extracts is not identified. A separation and analysis of the components and phytochemicals in each extract can be acquired by using chromatography method[76]. This involves the extract dissolved in a mobile phase passing through a stationary phase to separate and isolate molecules. Future work with our extracts should involve characterization and identification of the particular substances or combination of substances in each extract.

The metabolism of certain phytochemicals is reasonably well understood. During metabolism the size, polarity and form of phytochemical alters and the molecular

form reaching the peripheral circulation and tissues are different from those present in foods. A possible contribution to the knowledge on the mechanisms of action of phenolics is to design *in vitro* experiments with conjugated phenolics instead of aglycones that is present in the plants natural form[19]. Scalbert *et al.*[18] suggests less focus on the differences between the natural occurring form of phytochemicals and the conjugated form found in plasma. Instead the focus in research should be on biological activities of the metabolites present in our tissue and especially the conjugated forms[18]. In this thesis we do not know in what form phytochemicals are present after extraction.

6.1.4 Effect of extracts on Recombinant Luciferase

When testing the extracts ability to affect the production of light from a recombinant luciferase enzyme, all the extracts exhibit the ability to decrease the luciferase activity. The extracts are in this situation in direct contact with the recombinant luciferase enzyme and it should be kept in mind, that only a low percentage of the plant compounds are expected to be taken up by the intact cell. The extracts will therefore not be in direct contact with the luciferase enzyme during a cell experiment.

To further elucidate the effect of extracts on the luciferase enzyme, *in vitro* experiments could be set up as usual, but with the addition of extract directly before measurement of luminicence. Similar experiments have previously been performed for thyme, turmeric, oregano and clove [77] and showed that the effect of the extracts on the luciferase enzyme in intact cells was almost eliminated compared to the direct effects on recombinant luciferase. This indicates that the extracts do not affect the luciferase enzyme in intact cells.

Another way of studying NF-κB activity without including luciferase is to examine the extracts ability to modulate NF-κB DNA binding activity. Previously work has showed that oregano, thyme, clove, turmeric and walnut inhibit LPS-induced p65 DNA binding[61], which also indicate that the present extracts inhibit NF-κB activity.

Such experiments as mentioned above were beyond the capacity of this master thesis, however should be considered for further work with these extracts.

6.1.5 FRAP-measurements

There are several different ways of measuring total antioxidant content of samples like extracts of food and spices [78]. Three of the methods that are commonly used are Trolox (6-hydroxy-2,578-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay[79], the oxygen radical absorbance capacity (ORAC) assay[80] and Ferric-reducing ability of plasma (FRAP)assay[65]. The TEAC and ORAC assays are based on the antioxidants ability to react with or neutralize free radicals generated in the assay system. FRAP assay measure the reduction of Fe^{3+} (ferric iron) to Fe^{2+} (ferrous iron) in the presence of antioxidants [78]

The results in this work include combinations of walnut, coffee, sunflower seeds, turmeric, thyme, oregano, clove and dog rose. The total antioxidant capacity of the extracts was measured in this thesis using FRAP assay. The FRAP values of such extracts has been measured before[61], but since new extracts were made the measurements were repeated for the extracts used in this thesis.

Paur *et al*.[61] has reported the FRAP values of dietary plant extract in their work. Extracts of clove, dog rose, thyme, oregano, turmeric, coffee, walnut and sunflower seeds had high total antioxidant content. Extract of clove showed the highest FRAP value of 141.5 mmol/100 g, followed by all spice (29.8 mmol/100g), dog rose (27.8 mmol/100g) thyme (21.0 mmol/100g), oregano (19.9 mmol/100g), and coffee (18.5 mmol/100g). Walnut, sunflower seeds and turmeric comes further down on the list. These food samples were chosen for *in vitro* experiments because of their high total antioxidant content reported by Halvorsen *et al*. [78] and Dragland *et al* [81].

The FRAP-values found in the oregano-, dog rose-, thyme- and sunflower seed-extracts of this thesis were higher than those reported by Paur et.al[61]. The FRAP values in this thesis showed a 5.6 fold higher antioxidant content for oregano than

those reported by Paur *et al*. The other extracts ranged from 2.5 to 2.7 fold higher antioxidant content than the previous work. Values found in clove, coffee, walnut and turmeric were from 1.03 to 4.3 fold lower than those reported by Paur *et al*. The method of extraction is the same for both these projects.

Dragland *et al.*[81] have reported FRAP values on clove oregano and thyme. Clove and thyme showed a 3.4 and 1.2 fold higher FRAP value from their measurements that in this thesis, while oregano is 2.5 fold higher in this thesis than reported in their article. Halvorsen *et al.*[78] have investigated the FRAP values in dog rose, walnut and sunflower seeds. Here the main difference between my work and their work was the values of walnuts, which was 21 mmol/100g compared to 1.8 mmol/100g in the extract used in this thesis.

These differences between different works can probably be explained by variations in antioxidant content between food samples and differences in the extraction of food samples. Another explanation may be different amounts of DMSO when re-dissolving the final extract as some phytochemicals are more easily dissolved in DMSO than in aqueous solutions. It is unlikely that the total antioxidant content in the dietary plant extracts explains the total inhibitory effect on NF-κB. Other components and combination of all constituents may however contribute to the effect as well.

6.2 General Discussion

6.2.1 LPS-induced NF-κB activity by individual extracts

In this work 8 different dietary plant extracts were tested. Thyme, oregano, clove, coffee, walnut, sunflower seed and dog rose showed an ability to inhibit LPS-induced NF-κB activity (**Figure 5.2**). Of extracts tested at 1 and 1.5 mg/ml clove was the strongest inhibitor, and of extracts tested at 10 and 15 mg/ml walnuts was the

strongest inhibitor. Only one dietary plant extract, turmeric, showed an elevation in LPS-induced NF-κB activity (**Figure 5.2**).

In the report of Paur *et al.*[61] all these dietary plant extracts showed a strong ability to inhibit LPS-induced NF-κB activity. They have used higher concentrations which can explain that oregano, thyme, clove, coffee and sunflower seed showed stronger inhibitory effect in their results than in this thesis. The effect of walnuts on LPS-induced NF-κB activity in our work showed a stronger inhibition than in the report of Paur *et al*. even though we used a lower concentration. This may indicate that walnut is a potent inhibitor even at low concentrations. Another explanation of this difference of NF-κB activity can be due to variation in the quality and composition of food samples. Walnut contains a large amount fat which makes extraction and further work difficult. Variations between different extracts and experiments because of water soluble and fat soluble phases may occur.

Turmeric showed a strong ability to inhibit LPS-induced activity at a concentration of 3 mg/ml in previously work by Paur *et al*[61]. In this work turmeric at a concentration of 1.5 mg/ml gave an increased LPS-induced NF-κB activity which may indicate that turmeric need to reach a certain concentration to exhibit a inhibitory effect on NF-κB. Curcumin a polyphenol found in turmeric, is previously found to suppress NF-κB activation induced by various inflammatory stimuli[82], which is similar to what Paur *et al*. found both when testing curcumin and also turmeric[61].

In the *in vitro* experiment were the combination extract was tested on LPS-induced NF-κB, we observed that the lowest concentration that showed a significant difference between control cells and treated cells were at 0.1 mg/ml for thyme, clove and oregano, and 1 mg/ml for walnut and coffee (**Figure 5.6**). In order for these experiments to be nutritionally relevant in a human the concentrations must reflect concentrations in the circulation. A dose of 0.1 mg/ml of spice extract in cell experiments will correspond to an intake of 0.5 g each of thyme, clove and oregano for an adult with 5 L blood. For walnut and coffee a dose of 1 mg/ml extract will correspond to an intake of 5g each. This is presumed with a 100 % absorption rate.

With 10 % absorption the dietary intake will correspond to 5 g for thyme, clove and oregano, while 50 g for walnut and 0.7 L coffee (70g coffee/L). This is an amount that is possible to consume during a day, even though it might be not common for the spices.

6.2.2 Synergistic Effect of Dietary plant extracts on LPS-induced NF-κB activity.

In Vitro experiments

The main purpose of this work was to determine the effect of combining dietary plant extracts and how the combinations are able to alter LPS-induced NF-κB activity compared to the effects of individual extracts. Out of 22 experiments, 10 showed a synergistic effect where combining extracts showed a significant lower NF-κB activity than when summarizing the effect of the individual extracts. Oregano and coffee together exhibit the strongest synergistic effect (**Figure 5.3 A**). Another 10 combinations showed an additive effect or a trend towards synergy. Two combinations showed either synergistic or additive effect. Summary of the effects of combining two extracts are shown in **Table 6.1**.

Table 6.1 Summery of effect of combining extracts.

Synergistic effect	*Additive effect*	*No synergistic/additive effect*
Oregano + Coffee	Oregano + Walnut	Walnut + Dog rose
Oregano + Turmeric	Oregano + Clove	Thyme + Dog rose
Clove + Coffee	Oregano + Dog rose	
Walnut + Coffee	Sunflower seed + Dog rose	
Oregano + Sunflower seed	Thyme + Clove	
Thyme + Coffee	Clove + Dog rose	
Thyme + Sunflower seed	Sunflower seed + Coffee	
Walnut + Sunflower seed	Clove + Walnut	
Oregano + Thyme	Clove + Sunflower seed	
Thyme + Walnut	Turmeric + Thyme	

Remarkably 20 of the 22 combinations showed either an additive or synergistic effect on LPS-induced NF-κB activity. The extracts in the present work were selected based on their high content of antioxidants/phytochemicals and their strong ability to inhibit NF-κB[61]. The idea behind the selection was to maximize the possibility to find a potent synergistic or additive effect on LPS-induced NF-κB by combining the extracts. Due to these promising results it is likely that many other combinations and concentrations of extracts and phytochemicals may also give a synergistic effect even though they individually exhibit a different modulation on NF-κB.

Some individual extracts like walnut, clove and dog rose showed a strong inhibition of LPS-induced NF-κB activity (**Figure 5.4.**). When summarizing values from single extracts using % of control values, the level of NF-κB activity gets very low and

sometimes indicating more than a total block of NF-κB activity. A synergistic effect is then impossible to detect even if it is present. This phenomenon is called “floor effect” and is defined in statistics as: “an artificial lower limit on the value that a variable can attain, causing the distribution of scores to be skewed”[83]. To avoid the “floor effect” and make it possible to detect a potential additive or synergistic effect, we have log transformed the % of control values for all the extracts prior to calculations of synergistic effects. Another possibility of avoiding this effect is to lower the concentrations of the extracts with strongest inhibitory effect on NF-κB.

The effect of turmeric extract alone increases the LPS-induced NF-κB activity compared to control. Still when combining turmeric with oregano (**Figure 5.3 B**) a strong synergistic inhibitory effect is present. This illustrate an effect more than an additive effect and the two extracts somehow strengthen each others effect.

No previously work about food-synergy and NF-κB is found, but studies using other model systems have looked at extracts of food rich in phytochemicals and food-synergy. Liu.*et al.*[16] showed that a combination of orange, apple, grape and blueberry displayed a synergistic effect in antioxidant activity *in vitro*. From this work they proposed that the additive and synergistic effect of phytochemicals in whole fruit and vegetables are responsible for their potent antioxidant and anticancer activities. This group have used the median effective dose (EC50 = the dose exhibiting 50 % of total antioxidant activity) to detect the synergistic effect. To use this method an experiment design with a wide range of concentrations of each extract are needed.

Eberhart *et al.*[84] demonstrated that vitamin C in whole apples contributed to less than 0.4% of total antioxidant activity. They also observed that apples with skin had a higher ability to inhibit cell-proliferation in liver-tumor cells and colon-cancer cells than apples without skin. From these results they suggested that most of the antioxidant activity in apples may be due to phytochemicals and that a combination of phytochemicals from whole apples play a beneficial role in cancer cell proliferation.

Food synergy and the nature of food synergy are complicated to study. Effects of single nutrients are simple to test in cell cultures without complex interactions with other bioactive compounds. But the association between dietary plants and the effect on biological systems can be difficult to uncover when hundreds of different phytochemicals are present, acting at different target sites and also synergistically.

Correlation between FRAP values and NF-κB activity

In this experiment no correlation was found between FRAP-values from combinations of extracts and LPS-induced NF-κB activity in U937 3xκB-LUC cells. Most phytochemicals have antioxidant properties and due to this the FRAP value would probably reflect the phytochemical content in the extracts. Because of the absence of correlation between FRAP values and inhibition of NF-κB activity, we can not conclude that the only mechanism behind the inhibition of LPS-induced NF-κB activity are the antioxidant properties. It is likely that the compounds in the extracts targets different places in the NF-κB signalling pathways or crosstalk with other signalling pathways, causing the additive and synergistic effects seen in this work. Further investigation is needed to fully elucidate the mechanisms behind the inhibitory effect of dietary plant extracts on NF-κB.

6.2.3 Effect of combination extract on LPS-induced NF-κB activity: In Vivo

After having tested combinations of dietary plant extracts in cell cultures, we performed an *in vivo* experiment treating the mice with an extract combination of potent *in vitro* NF-κB inhibitors. This experiment demonstrates the ability of dietary plant extracts to dampen a strong inflammatory stimulus (LPS). Mice treated with the combination extract had a lower overall NF-κB activity than mice treated with control vehicle however this difference was not statistical significant. When looking at the NF-κB activity in the homogenates of the organs the extract treated livers had close to significant lower activity than the control group. These results may indicate

that some of the bioactive compounds in the dietary plant extracts are absorbed and distributed into various tissues. It is a trend towards showing a bioavailability, but we don't know how much of the phytochemicals and which components that is absorbed.

One interesting observation found by performing *ex vivo* imaging of organs was that mice treated with the combination extract, showed a significant lower LPS-induced NF-κB activity in intestine compared to controls. This may indicate that bioactive constituents in the combination extract have the ability to inhibit NF-κB activity in a direct and local manner in intestine. NF-κB activity is increased in patients with Crohn`s disease and ulcerative colitis[85]. Inhibition of NF-κB activity has been suggested as a major component of the anti-inflammatory activity of glucocorticoids. Long term treatment of glucocorticoids can give severe side effects[85]. Therefore, it must be of great interest to find combinations of natural food items to inhibit NF-κB in patients with inflammatory bowel disease to avoid these toxic side effects of drugs.

In *in vivo* experiments the amount of combination-extract (300µl) corresponds to an intake of 1.5 kg walnuts, 21.4 L coffee (70g coffee/L) and 150 g thyme, oregano and clove for a person weighing 75 kg. This is large amounts and exceeds the typical dietary intake of these food items. But it is a possibility that consumption over a period of time will have the same physiological effect as consuming everything in one meal. Further experiments are needed to elucidate the ability of lower doses or other combinations of dietary plant extracts to inhibit NF-κB.

6.2.4 Mechanisms behind NF-κB modulation of dietary plant extracts

Different phytochemicals exhibit an inhibiting effect on NF-κB[45, 46]. How and where phytochemicals targets NF-κB is not fully understood[51], but there is many suggested mechanisms of action: Phytochemicals have been proposed to play a beneficial role against oxidative stress[86]. Phytochemicals are belived to scavange reactive oxygene species (ROS) like superoxide anion ($O_2^{\bullet -}$), hydrogene peroxide (H_2O_2) and nitric oxide (NO). Phytochemicals may also maintain and increase the level of antioxidant

enzymes like superoxide dismutase, catalase and gluthatione peroxidise[86].NF-κB is proposed to be activated by oxidants and pro-oxidants[87] and this scavenging of ROS and increase of antioxidant enzymes by phytochemicals can thus indirectly reduce activation of NF-κB. At the same time there have been observations that show that compounds exhibit oxidative damage may repress activation of NF-κB and thus induce apoptosis[87, 88]. This may reflect that phytochemicals also exert their inhibitory effect on NF-κB through other mechanisms than as antioxidants.

The extracts used in this thesis contains a wide variety of bioactive compounds that can potentially interact with NF-κB signalling pathway (summarized in **Table 6.2**)

Table 6.2 Possible bioactive compounds in the dietary plant extracts [72-74, 89]

Food item	Bioactive compounds
Clove	Phenolic acids (gallic acid)
	Flavonol glucosides
	Phenolic volatile oils (eugenol, isoeugenol)
	Tannins
Oregano	Phenolic acids (caffeic acid, coumaric acid, rosmarinic acid)
	Flavonoids
Thyme	Phenolic acids (gallic acid, caffeic acid, rosmarinic acid)
	Phenolic diterpenes
	Flavonoids
Turmeric	Curcumin
Coffee	Cafestol and kahweol
	Caffeine
	Thocpherols
	Phenolic compounds (caffeic acid and quinic acid)
Sunflower seed	Linoleic acid
	Fiber
	Vitamin E
	Phytosterols
	Minerals
Dog rose	Vitamin C
	Flavonoids
	Phenolic acids
	Lycopoene
Walnuts	ω-3 and ω-6
	Tannins
	Folate
	Phenolic compouds (ellagitannins, ellagic acid)

Turmeric contains high levels of the phytochemical curcumin[82, 90]Curcumin has been shown to inhibit IKK α and β, and also downregulate upstream kinases .In addition curcumin has been shown to suppress phosphorylation of IκB and also TNF-α-induced nuclear translocation and DNA binding of NF-κB[13].

Clove contains high levels of phenolic acids and phenolic oils (eugenol and isoeugenol) which displays antioxidant properties[91]. These essential oils from clove have earlier been reported to have a antibacterial activity and fungicidal activity in *in vitro* studies[91]. In addition major compounds of clove, isoeugenol and eugenol, decrease nitric oxide production (NO) due to decreased iNOS expression. The same report concluded that clove inhibit NF-κB activity by suppress translocation, DNA binding and upstream NF-κB activation[92].

Oregano and thyme has a high level of phenolic asids[74]. Paur *et al*.[61] found that extracts of clove, oregano, thyme, turmeric and walnuts inhibited both LPS-induces and TNF-α induced NF-κB (p65) DNA binding.

Walnuts have the reputation to be part of "heart healthy diet" with the effect of lowering the blood cholesterol both in humans and animals[93]. Walnuts contain multiple health-beneficial components, such as ω-6 and ω-3 fatty acids, vitamin E folate, fiber and phenolic antioxidants[72, 93]. It is a possibility that the strong inhibitory effect on NF-κB seen in this work and previously work[61] is related to other components than phytochemicals for example ω-3 fatty acids and α-tochopherols. α-tocopherols have a ability to resist oxidation which indirect inhibit the activation of NF-κB thorough reducing amount of ROS[42]. ω-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are proposed to inhibit at three levels on the NF-κB signalling cascade: 1)EPA and DHA interferes with TLR4 and block LPS induced activation with NF-κB. 2) EPA and DHA inhibit phosphorylation of IκB by inhibiting IKK. 3) EPA and DHA interferes with peroxisome proliferator- activated receptor (PPAR) that inhibit NF-κB binding to DNA[94]. It is also a possibility that the

high content of ω-3 fatty acids in walnuts may increases the diffusion of polyphenols over cell membrane and further affects the NF-κB signalling pathway.

Coffee contains bioactive compounds that both have showed a negative influence on health (cafestol, kawheol and caffeine) and proposed beneficial influence on health (phenolic compounds)[73].

Sunflower seeds contain polyunsaturated fatty acids, vitamin E, vitamin B. phytosterols and minerals and have been showed to have a cholesterol lowering effect and beneficial for heart health.

Dog rose has a high content of vitamin C and phytochemicals. It has been used for therapeutic use for ages and has previously showed to have a possible antioxidative and anti-inflammatory effect[89].

Studies have also showed that modulation of one signalling pathway by phytochemicals can simultaneously modulate other signalling pathways. Dinkova-Kostova *et al.*[60] found that triterpenoids (phytochemicals) elevated phase 2 enzymes, which is important compounds in the endogenous antioxidant defence system. They also found that iNOS were suppressed and that it was a close correlation between phase 2 gene induction and suppression of iNOS synthesis. They suggested that the mechanism behind this was that phase 2 enzymes decrease the amount of ROS and thus inhibit an activation of NF-κB generated by ROS. This will lead to an reduction of inflammatory response and a lower production of iNOS[60].

It is a possibility that different constituents in the different dietary plant extracts target different places in the NF-κB signalling pathway causing a stronger inhibitory effect than the single extract would exhibit alone. When one signalling cascade is modulated by phytochemicals other signalling pathways may be affected, giving a stronger and more complex response from the phytochemicals, which again can lead to a synergistic effect.

7. Conclusion

The purpose of this work was to study combinations of dietary plant extracts rich in phytochemicals, and elucidate potential additive or synergistic effects on NF-κB activity under acute inflammatory condition in U937 3xκB-LUC cells.

Of 22 combinations in the present work, 10 of them showed a synergistic effect on LPS-induced NF-κB activity. In addition 10 of the combinations showed an additive effect or a trend towards synergy.

The combination of oregano and coffee showed the strongest synergistic effect with 46 % lower NF-κB activity than summarizing the effects of the two individual extracts. Coffee, oregano and thyme were each present in 4 of the 10 combinations that showed a synergistic effect, whereas walnut and sunflower seed were each present in 3 of the 10 combinations.

In vivo experiments studying the effect from a combination-extract containing thyme, clove, oregano, coffee and walnut in transgenic reporter mice, showed a trend towards an inhibitory effect on LPS-induced NF-κB activity by whole body imaging. One interesting observation in this experiment was that we found a significant lower LPS-induced NF-κB activity in the intestine in mice treated with the combination-extract, indicating a local and direct effect of the combination extract. We also found a near significant inhibition of LPS-induced NF-κB activity in the liver, which indicates that the extract is bioavailable.

This thesis strengthens the connection between health benefits and consuming dietary plants, as this work shows that compounds in dietary plants can act in a synergistic manner. Further work is needed to elucidate the extent and possible health benefits of food synergies, as well as the mechanisms behind the synergistic effects.

8. Future perspectives

Reports of synergistic effects by combining whole dietary plants are limited, but of great interest due to the observed health benefits from consumption of fruits and vegetables. More studies on this area both on NF-κB activity but also other signalling pathways are necessary to elucidate potential combinations that can influence each others effect and contribute to possible prevention of different conditions like cancer and chronic inflammation.

In terms of understanding the mechanisms behind the synergistic effects when combining different plant extracts, it would be of great interest to measure the gene expression both in cells and mice to elucidate other potential genes that is involved during exposure of dietary plant extracts. This will give us a more holistic view and more information about other signalling pathways that is involved and their potential interactions with each other.

To understand the mechanisms behind the observed effects of the dietary plant extracts on NF-κB activity, a further characterization and isolation of different compounds in the extracts may be of interest. A characterization of compounds is also needed to understand where and how the phytochemicals inhibit the NF-κB signalling pathway, and how they interacts with each other and contributes to an additive, synergistic or antagonistic effect.

Extracts used in *in vitro* experiments were selected out of their strong inhibitory effect on LPS-induced NF-κB activity in previous work. The idea behind this selection was to maximize the possibility to find a potential synergistic or additive effect. It is still an open question whether different combinations of food extracts can exhibit synergistic or additive effects *in vivo*. It is also a possibility that experiments with lower and more biological concentrations enables synergistic effects to occur without reaching “floor effect”. Experiments with combining other extracts and concentrations could thus be implemented in further work.

The *in vivo* experiment shows only whether combination-extract is bioavailable and has the ability to modulate LPS-induced NF-κB activity or not. It is not possible to examine if there is a synergistic or additive effect of the extract. More *in vivo* experiments with single extracts and other combinations and concentrations are thus needed.

9. List of References

1. Report of a joint FAO/WHO Expert Consultation. Diet, Nutrition and the prevention of Chronic Diseases. No.916. 2003. 2003. Geneva, WHO Technical Report Series.
Ref Type: Report

2. World Health Organization. Prevention and control of noncommunicable diseases: implementation of the global strategy. 2007. World Health Organization.
Ref Type: Report

3. Hercberg,S., Galan,P., Preziosi,P., Alfarez,M.J., & Vazquez,C. The potential role of antioxidant vitamins in preventing cardiovascular diseases and cancers. *Nutrition* **14**, 513-520 (1998).

4. Steinmetz,K.A. & Potter,J.D. Vegetables, fruit, and cancer prevention: a review. *J. Am. Diet. Assoc.* **96**, 1027-1039 (1996).

5. van't,V.P., Jansen,M.C., Klerk,M., & Kok,F.J. Fruits and vegetables in the prevention of cancer and cardiovascular disease. *Public Health Nutr.* **3**, 103-107 (2000).

6. World cancer research fund,A.I.f.c.R. Food, Nutrition, Physical activity and the Prevention of Cancer : A global Perspective. 2007. American Institute for cancer research.
Ref Type: Generic

7. Lindsay,D.G. & Astley,S.B. European research on the functional effects of dietary antioxidants - EUROFEDA. *Mol. Aspects Med.* **23**, 1-38 (2002).

8. Willett,W.C. Goals for nutrition in the year 2000. *CA Cancer J. Clin.* **49**, 331-352 (1999).

9. Huang,H.Y. *et al.* Multivitamin/mineral supplements and prevention of chronic disease. *Evid. Rep. Technol. Assess. (Full. Rep.)*1-117 (2006).

10. Rowe,P.M. Beta-carotene takes a collective beating. *Lancet* **347**, 249 (1996).

11. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. *N. Engl. J. Med.* **330**, 1029-1035 (1994).

12. Blomhoff,R. [Antioxidants and oxidative stress]. *Tidsskr. Nor Laegeforen.* **124**, 1643-1645 (2004).

13. Surh,Y.J. Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer* **3**, 768-780 (2003).

14. Ross,J.A. & Kasum,C.M. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr.* **22**, 19-34 (2002).

15. Liu,R.H. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am. J. Clin. Nutr.* **78**, 517S-520S (2003).

16. Liu,R.H. Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J. Nutr.* **134**, 3479S-3485S (2004).

17. Manach,C., Scalbert,A., Morand,C., Remesy,C., & Jimenez,L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **79**, 727-747 (2004).

18. Scalbert,A. & Williamson,G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **130**, 2073S-2085S (2000).

19. Kroon,P.A. *et al.* How should we assess the effects of exposure to dietary polyphenols in vitro? *Am. J. Clin. Nutr.* **80**, 15-21 (2004).

20. Arts,I.C. & Hollman,P.C. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr.* **81**, 317S-325S (2005).

21. Nijveldt,R.J. *et al.* Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **74**, 418-425 (2001).

22. Halliwell,B. Biochemistry of oxidative stress. *Biochem. Soc. Trans.* **35**, 1147-1150 (2007).

23. McCord,J.M. The evolution of free radicals and oxidative stress. *Am. J. Med.* **108**, 652-659 (2000).

24. Finkel,T. & Holbrook,N.J. Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247 (2000).

25. Halliwell,B. Oxidative stress and cancer: have we moved forward? *Biochem. J.* **401**, 1-11 (2007).

26. Lindahl,T. & Wood,R.D. Quality control by DNA repair. *Science* **286**, 1897-1905 (1999).

27. Lusis,A.J. Atherosclerosis. *Nature* **407**, 233-241 (2000).

28. Halliwell,B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet* **344**, 721-724 (1994).

29. Young,I.S. & Woodside,J.V. Antioxidants in health and disease. *J. Clin. Pathol.* **54**, 176-186 (2001).

30. Stahl,W. & Sies,H. Antioxidant activity of carotenoids. *Mol. Aspects Med.* **24**, 345-351 (2003).

31. Moskaug,J.O., Carlsen,H., Myhrstad,M.C., & Blomhoff,R. Polyphenols and glutathione synthesis regulation. *Am. J. Clin. Nutr.* **81**, 277S-283S (2005).

32. Muller,M. & Kersten,S. Nutrigenomics: goals and strategies. *Nat. Rev. Genet.* **4**, 315-322 (2003).

33. Karin,M., Cao,Y., Greten,F.R., & Li,Z.W. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat. Rev. Cancer* **2**, 301-310 (2002).

34. Sen,R. & Baltimore,D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* **46**, 705-716 (1986).

35. Baldwin,A.S., Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu. Rev. Immunol.* **14**, 649-683 (1996).

36. Siebenlist,U., Franzoso,G., & Brown,K. Structure, regulation and function of NF-kappa B. *Annu. Rev. Cell Biol.* **10**, 405-455 (1994).

37. Karin,M., Yamamoto,Y., & Wang,Q.M. The IKK NF-kappa B system: a treasure trove for drug development. *Nat. Rev. Drug Discov.* **3**, 17-26 (2004).

38. Hoffmann,A., Levchenko,A., Scott,M.L., & Baltimore,D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* **298**, 1241-1245 (2002).

39. Chen,L.F. & Greene,W.C. Shaping the nuclear action of NF-kappaB. *Nat. Rev. Mol. Cell Biol.* **5**, 392-401 (2004).

40. Hayden,M.S. & Ghosh,S. Signaling to NF-kappaB. *Genes Dev.* **18**, 2195-2224 (2004).

41. Luo,J.L., Kamata,H., & Karin,M. IKK/NF-kappaB signaling: balancing life and death--a new approach to cancer therapy. *J. Clin. Invest* **115**, 2625-2632 (2005).

42. Pahl,H.L. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* **18**, 6853-6866 (1999).

43. Bonizzi,G. & Karin,M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol.* **25**, 280-288 (2004).

44. Gilmore,T.D. & Herscovitch,M. Inhibitors of NF-kappaB signaling: 785 and counting. *Oncogene* **25**, 6887-6899 (2006).

45. Bremner,P. & Heinrich,M. Natural products as targeted modulators of the nuclear factor-kappaB pathway. *J. Pharm. Pharmacol.* **54**, 453-472 (2002).

46. Muraoka,K. *et al.* Flavonoids exert diverse inhibitory effects on the activation of NF-kappaB. *Transplant. Proc.* **34**, 1335-1340 (2002).

47. Epinat,J.C. & Gilmore,T.D. Diverse agents act at multiple levels to inhibit the Rel/NF-kappaB signal transduction pathway. *Oncogene* **18**, 6896-6909 (1999).

48. Sun,S.C. & Xiao,G. Deregulation of NF-kappaB and its upstream kinases in cancer. *Cancer Metastasis Rev.* **22**, 405-422 (2003).

49. Tak,P.P. & Firestein,G.S. NF-kappaB: a key role in inflammatory diseases. *J. Clin. Invest* **107**, 7-11 (2001).

50. Karin,M., Lawrence,T., & Nizet,V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* **124**, 823-835 (2006).

51. Karin,M. & Greten,F.R. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat. Rev. Immunol.* **5**, 749-759 (2005).

52. Karin,M. Nuclear factor-kappaB in cancer development and progression. *Nature* **441**, 431-436 (2006).

53. Yusuf,S., Dagenais,G., Pogue,J., Bosch,J., & Sleight,P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N. Engl. J. Med.* **342**, 154-160 (2000).

54. Omenn,G.S. *et al.* Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N. Engl. J. Med.* **334**, 1150-1155 (1996).

55. Berenbaum,M.C. What is synergy? *Pharmacol. Rev.* **41**, 93-141 (1989).

56. Jacobs,D.R., Jr. & Steffen,L.M. Nutrients, foods, and dietary patterns as exposures in research: a framework for food synergy. *Am. J. Clin. Nutr.* **78**, 508S-513S (2003).

57. Jacobs,D.R., Jr. & Tapsell,L.C. Food, not nutrients, is the fundamental unit in nutrition. *Nutr. Rev.* **65**, 439-450 (2007).

58. Stevenson,D.E. & Hurst,R.D. Polyphenolic phytochemicals--just antioxidants or much more? *Cell Mol. Life Sci.* **64**, 2900-2916 (2007).

59. Chu,Y.F., Sun,J., Wu,X., & Liu,R.H. Antioxidant and antiproliferative activities of common vegetables. *J. Agric. Food Chem.* **50**, 6910-6916 (2002).

60. Dinkova-Kostova,A.T. *et al.* Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. *Proc. Natl. Acad. Sci. U. S. A* **102**, 4584-4589 (2005).

61. Paur,I., Austenaa,L.M., & Blomhoff,R. Extracts of dietary plants are efficient modulators of nuclear factor kappa B. *Food Chem. Toxicol.* **46**, 1288-1297 (2008).

62. Sundstrom,C. & Nilsson,K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer* **17**, 565-577 (1976).

63. Gangloff,M. & Gay,N.J. MD-2: the Toll 'gatekeeper' in endotoxin signalling. *Trends Biochem. Sci.* **29**, 294-300 (2004).

64. Carlsen,H., Moskaug,J.O., Fromm,S.H., & Blomhoff,R. In vivo imaging of NF-kappa B activity. *J. Immunol.* **168**, 1441-1446 (2002).

65. Benzie,I.F. & Strain,J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* **239**, 70-76 (1996).

66. David Jacobs Jr. University of Minnesota, USA. 2008.
Ref Type: Personal Communication

67. Thompson,J.F., Hayes,L.S., & Lloyd,D.B. Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene* **103**, 171-177 (1991).

68. Muller,J.M., Rupec,R.A., & Baeuerle,P.A. Study of gene regulation by NF-kappa B and AP-1 in response to reactive oxygen intermediates. *Methods* **11**, 301-312 (1997).

69. Kleinman,H.K., Philp,D., & Hoffman,M.P. Role of the extracellular matrix in morphogenesis. *Curr. Opin. Biotechnol.* **14**, 526-532 (2003).

70. Massoud,T.F. & Gambhir,S.S. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev.* **17**, 545-580 (2003).

71. Carlsen,H., Alexander,G., Austenaa,L.M., Ebihara,K., & Blomhoff,R. Molecular imaging of the transcription factor NF-kappaB, a primary regulator of stress response. *Mutat. Res.* **551**, 199-211 (2004).

72. Anderson,K.J. *et al.* Walnut polyphenolics inhibit in vitro human plasma and LDL oxidation. *J. Nutr.* **131**, 2837-2842 (2001).

73. Ranheim,T. & Halvorsen,B. Coffee consumption and human health--beneficial or detrimental?--Mechanisms for effects of coffee consumption on different risk factors for cardiovascular disease and type 2 diabetes mellitus. *Mol. Nutr. Food Res.* **49**, 274-284 (2005).

74. Shan,B., Cai,Y.Z., Sun,M., & Corke,H. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J. Agric. Food Chem.* **53**, 7749-7759 (2005).

75. Strimpakos,A.S. & Sharma,R.A. Curcumin: preventive and therapeutic properties in laboratory studies and clinical trials. *Antioxid. Redox. Signal.* **10**, 511-545 (2008).

76. Andlauer,W., Martena,M.J., & Furst,P. Determination of selected phytochemicals by reversed-phase high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection. *J. Chromatogr. A* **849**, 341-348 (1999).

77. Paur Knudsen Ingvild. Effect of Plant Extracts on Basal and LPS-induced NF-kB activity. 2004. University of Oslo, Department of Nutrition.
Ref Type: Thesis/Dissertation

78. Halvorsen,B.L. *et al.* A systematic screening of total antioxidants in dietary plants. *J. Nutr.* **132**, 461-471 (2002).

79. Miller,N.J., Rice-Evans,C., Davies,M.J., Gopinathan,V., & Milner,A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci. (Lond)* **84**, 407-412 (1993).

80. DeLange,R.J. & Glazer,A.N. Phycoerythrin fluorescence-based assay for peroxy radicals: a screen for biologically relevant protective agents. *Anal. Biochem.* **177**, 300-306 (1989).

81. Dragland,S., Senoo,H., Wake,K., Holte,K., & Blomhoff,R. Several culinary and medicinal herbs are important sources of dietary antioxidants. *J. Nutr.* **133**, 1286-1290 (2003).

82. Singh,S. & Aggarwal,B.B. Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. *J. Biol. Chem.* **270**, 24995-25000 (1995).

83. Andrew M.Coldman. A dictionary of Psychology, www.encyclopedia.com/doc/1087-flooreffect.html . 2001.
Ref Type: Electronic Citation

84. Eberhardt,M.V., Lee,C.Y., & Liu,R.H. Antioxidant activity of fresh apples. *Nature* **405**, 903-904 (2000).

85. Neurath,M.F., Becker,C., & Barbulescu,K. Role of NF-kappaB in immune and inflammatory responses in the gut. *Gut* **43**, 856-860 (1998).

86. Rahman,I., Biswas,S.K., & Kirkham,P.A. Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem. Pharmacol.* **72**, 1439-1452 (2006).

87. Karin,M. *et al.* Oxidative stress and gene expression: the AP-1 and NF-kappaB connections. *Biofactors* **15**, 87-89 (2001).

88. Bowie,A. & O'Neill,L.A. Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem. Pharmacol.* **59**, 13-23 (2000).

89. Chrubasik,C., Roufogalis,B.D., Muller-Ladner,U., & Chrubasik,S. A systematic review on the Rosa canina effect and efficacy profiles. *Phytother. Res.* **22**, 725-733 (2008).

90. Aggarwal,B.B. *et al.* Curcumin suppresses the paclitaxel-induced nuclear factor-kappaB pathway in breast cancer cells and inhibits lung metastasis of human breast cancer in nude mice. *Clin. Cancer Res.* **11**, 7490-7498 (2005).

91. Chaieb,K. *et al.* The chemical composition and biological activity of clove essential oil, Eugenia caryophyllata (Syzigium aromaticum L. Myrtaceae): a short review. *Phytother. Res.* **21**, 501-506 (2007).

92. Choi,C.Y. *et al.* Isoeugenol suppression of inducible nitric oxide synthase expression is mediated by down-regulation of NF-kappaB, ERK1/2, and p38 kinase. *Eur. J. Pharmacol.* **576**, 151-159 (2007).

93. Feldman,E.B. The scientific evidence for a beneficial health relationship between walnuts and coronary heart disease. *J. Nutr.* **132**, 1062S-1101S (2002).

94. Singer,P. *et al.* Anti-inflammatory properties of omega-3 fatty acids in critical illness: novel mechanisms and an integrative perspective. *Intensive Care Med.*(2008).