“Anti-inflammatory effects of herbal preparations in cytokine-challenged normal human colon cells”

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Acknowledgement

Abstract

Inflammatory bowel diseases (IBD) are chronic relapsing intestinal disorders characterized by up-regulation of pro-inflammatory cytokines followed by invasion of immune cells to the intestinal lamina propria. Standard therapies consist of anti-inflammatory or immunosuppressive drugs. Since clinical efficiency is not satisfactory and the established drugs have massive side effects, new strategies to treat IBD are required. Herein, we investigate the protective effect of the fixed combination herbal preparations STW5 and the investigational combination STW5-II and the contribution of the corresponding single components in an *in vitro* inflammation model. The normal human colon epithelial cell line, NCM460, was treated with STW5, STW5-II or their single components for 4 h followed by experimental conditions comparative to induction of colitis. A pro-inflammatory cytokine cocktail consisting of TNFα, IL-β and IFNγ was used to simulate inflammatory stimuli normally caused by immune cells. The effects on NCM460 cells were investigated by enzyme-linked immunoassay (ELISA) and Proteome Profiler®. Levels of IP-10, MCP-1, I-Tac, Groα and IL-8 were elevated in cytokine-treated cells compared to untreated cells, but significantly reduced upon pretreatment with STW5 extracts. However, the single extracts revealed only little effects on protein expression. Furthermore, we investigated the effect of STW5 extracts on pro-inflammatory transcription factor of the Stat family using Western blot. In addition, we tested effects on upstream MAPK p38 Both, STW5 and STW5-II did not show any effect on MAPK p38, but were effective in reducing phosphorylated levels of Stat1.

In conclusion, both extracts act in an anti-inflammatory manner by influencing cytokine secretion via reduced activity of the JAK/Stat1 pathway. Relevant differences between STW5 and STW5-II were not found indicating similar efficacies.

Zusammenfassung

Chronisch entzündliche Darmerkrankungen (CED) sind rezidivierende Krankheiten des Magen-Darm-Traktes, welche durch eine erhöhte Produktion von entzündungsfördernden Zytokinen und der massiven Einwanderung von Immunzellen in die Lamia Propria gekennzeichnet sind. Zur Standarttherapie gehören Mittel gegen die Entzündung und Medikamente zur Immunsuppression. Da die klinische Wirksamkeit dieser Mittel häufig nicht ausreichend ist und sie darüber hinaus auch zu teils schwerwiegenden Nebenwirkungen führen, ist die Suche nach neuen Strategien zur Behandlung von CED obligat. In dieser Studie untersuchen wir die schützende Wirkung von STW5 (Iberogast®), einer fixen Konbination aus neun Pflanzenextrakten, von STW5-II, einer Testkombination aus sechs Extrakten, sowie von den Einzelextrakten in einem *in vitro* Model der Entzündung. NCM460, eine humane Zelllinie aus dem Kolonepithel, wurde mit STW5, STW5-II oder den Einzelextrakten vorbehandelt und daraufhin Konditionen ausgesetzt, welche vergleichbar mit einer Entzündung sind. Ein entzündungsfördernder Mix aus TNFα, IL-1β und IFNγ wurde verwendet, um den normalerweise von Immunzellen verursachten Stimulus zu simulieren. Die Auswirkungen auf die NCM460 Zellen wurden mittels ELISA und Proteom Profiler® untersucht. In Zellen, welche mit Zytokinen behandelt wurden, zeigten sich erhöhte werde für die Chemokine IP-10, MCP-1, I-Tac, Groα und IL-8 im Vergleich zu unbehandelten Zellen. Eine Behandlung mit STW5 oder STW5-II konnte diesen Effekt reduzieren, jedoch nicht die Einzelsubstanzen. Weiterhin wurde mittels Western Blot der Effekt der pflanzlichen Extrakte auf die Stat Proteine, welche sich fördernd auf entzündliche Prozesse auswirken, untersucht. Zusätzlich wurden weitere für den Entzündungsprozess wichtige Parameter untersucht. STW5 und STW5-II zeigten keine Wirkung auf die Aktivität von p38 und Nf-κB. AP-1 wurde unter den untersuchten Bedingungen überhaupt nicht beeinflusst. Von den Stat Proteinen wurde die Phosphorylierung und damit Aktivierung von Stat1 durch STW5 und STW5-II reduziert.

Beide Extrakte wirken antientzündlich, indem sie die Ausschüttung von Zytokinen durch eine verringerte Aktivität des JAK/Stat1 Signalwegs beeinflussen. Relevante Unterschiede zwischen den Extrakten wurden nicht gefunden.

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1 List of Abbreviations

Abbreviation	Connotation
AKBA	Acetyl-11-keto-b-boswellic acid
AP-1	Activator protein-1
APC	Antigen presenting cells
ATF-2	Activating transcription factor 2
ATP	Adenosine triphosphate
CD	Crohn's disease
CD11b	Cluster of differentiation 11b
$CD4^+$	Cluster of differentiation 4 positive
$CD8^+$	Cluster of differentiation 8 positive
COX	Cyclooxygenase
DNA	Deoxyribonucleic acid
DC	Dendric cells
DMSO	Dimethyl sulfoxide
DSS	Dextran sodium sulfate
EAggEC	Enteroaggregative E. coli
EGCG	Epigallocatechin-3-gallate
ELISA	Enzyme-linked Immunosorbent Assay
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal regulated kinases
GALT	Gut associated lymphoid tissue
GAP	Good Agricultural Practice
Groα	Growth-regulated protein alpha
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HDAC	Histone deacetylase
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IFNγ	Interferon-γ
IgE	Immunoglobulin E
IKK	IκB kinase
IL-10	Interleukin 10
IL-1α	Interleukin 1α
IL-1β	Interleukin 1β
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	inducible nitric oxide synthase
IP-10	IFN-γ–inducible protein 10
I-TAC	IFN-inducible T-cell α chemoattractant
IκB	Inhibitor of kappa B

JNK	c-Jun N-terminal kinase
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
McD88	Myeloid differentiation factor 88
MCP	Monocyte chemoattractant protein 1
MDP	Muramyl dipeptide
Mif	Macrophage Migration Inhibitory Factor
MMP-12	Matrix metallopeptidase 12
MMP-9	Matrix metallopeptidase 9
MPO	Myeloperoxidase
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NLR	NOD like receptor
NO	Nitric oxide
NOD	Nucleotide-binding domain
PAI-1	Plasminogenactivator-inhibitor-1
PAMP	Pathogen-associated molecular pattern
PGE_2	Prostaglandin E_2
PKC-β2	Protein kinase C-β2
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SIRT1	Sirtuin 1
SOP	Standard operation procedure
STAT	Signal transducer and activator of transcription
T_h1 cells	T helper cells 1
T_h17 cells	T helper cells 17
T_h2 cells	T helper cells 2
TLR	Toll-like receptor
TNBS	2,4,6-Trinitrobenzenesulphonic acid
TNFα	Tumor necrosis factor alpha
TPA	12-O-tetradecanoylphorbol acetate
UC	Ulcerative colitis

2 Introduction

2.1 General information about inflammatory Bowel disease

Inflammatory bowel diseases (IBD) are chronic disorders in modern societies and comprise two major clinical forms: Crohn's disease (CD) and ulcerative colitis (UC). The prevalence of IBD is 10/100000 [1]. Although both forms of IBD occur worldwide, there is a north-south slope in incidence of IBD in Europe as well as in North America. General, incidence of IBD is higher in "westernized" civilizations for both CD and UC. In other countries the incidence is rising but seems to be stable in high-incidence areas [2]. The primary peak of incidence for both diseases, for both sexes in each disease is between 15-25 years, the second peak of incidence occurs in the sixth to seventh live decade [3]. The Progression of the diseases is characterized by chronic inflammation of the bowel with periods of exacerbation and remission. Clinical symptoms comprise are abdominal pain, (bloody) diarrhea and weight loss. Risk factors include environmental, immunological, microbial and genetic aspects. Beneficial aspects in case of ulcerative colitis are smoking and appendectomy [4]. Both inverse associations were confirmed in meta studies. The rising Incidence in western civilizations is also hypothesized to be linked to higher hygiene standards. Extremely high hygiene standards might affect the development of the immune system negatively and predispose children raised under these conditions to IBD [5]. Dietary antigens are one of the most common types of luminal antigens and might play a pivotal role in IBD development. In Addition, this might explain the varinace in Incidence of IBD in different geographical regions. However, consensus in clinical studies is still outstanding. High sugar and fat intake are suspected to increase the risk of IBD development [4].

2.2 Clinical Diagnosis

Colonic inflammation with known cause occur due to infection, ischaemia, physical damage such as irradiation, or a specific immunologic sensitivity such as to cow's milk protein. Since the cause of CD and UC remains unclear both disease conditions are distinguished based on anatomic and histological features. In CD, the inflammation affects the whole gastrointestinal tract. Sides of inflammation can be found from mouth to anus. These sides are discontinuous and can occur in separated areas. The inflammation is transmural and found through the whole epithelial wall. The criteria for a diagnosis of UC include a continuous mucosal inflammation without granulomata found in biopsies. UC affects the rectum and some parts of the colon. [6].

2.3 Therapy of inflammatory bowel disease

Since both Forms of IBD cannot be cured at present, the goal of the therapy is to induce and maintain remission, to reduce symptoms and to increase the quality of life for patients. Non-medical treatments include change of life style, diet and avoiding environmental factors. Medical intervention focuses on anti-inflammatory and immune modulary substances. First line medications to induce remission in mild to moderate active UC are oral and rectal formulations of 5-aminosalicylic acid (mesalazine), as well as oral prodrug formulations sulfasalazine (5-aminosalicylic acid linked to sulfapyridine), olsalazine (5-aminosalicylic acid dimer) and balsalazide (5-aminosalicylic acid linked to 4-aminobenzoyl-β-alanine) [7, 8]. The exact mechanism of action remains poorly understood. Mesalazine regulates the formation of pro-inflammatory prostaglandins and leukotrienes. Cyclooxygenase (COX) and lipoxygenase (LOX) pathways as well as the peroxisome proliferator activated receptor-g are believed to be targeds of 5-aminosalicylic acids. Also they may have anti-oxidative properties and play a role in inhibition of T cell activation[9]. Different formulations of mesalazine and prodrugs showed no difference. The optimal induction dose of 5-aminosalicylic acid compounds is controversially discussed. While early trails showed dose response between 800 and 4800 mg per day[10], later trials could not consistently show a dose response between 1500 and 4800 mg per day[11, 12]. Patients with special forms of UC like proctitis and left sided ulcerative colitis might respond better to rectal mesalazine or rectal cortisone, which includes hydrocortisone, budesonide and beclomethasone formulations, rather than to oral 5-aminosalicilic acid formulations. To reduce systemic complications of cortisones, foam formulations were developed to decrease bioavailability[13]. Rectal cortisone formulations were less effective compared to rectal mesalazine [12, 14]. Patients that did not respond to oral or rectal mesalazine therapy or both should be treated with systemic cortisones with oral prednisone 40 mg per day up to 1 mg/kg per day or equivalent. To prevent osteoporosis, which is a typical side effect of steroid therapy, patients taking at least 5 mg for 2 month should be considered for bone density measurement [15]. Cortisones regulate the transcription of inflammatory genes like inducible cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) by activating the cytoplasmic corticoid receptor [16]. Patients who often need steroid therapy should be considered for azathioprine (2,5 mg/kg bodyweight per day) or mercaptopurine (1,5 mg/kg bodyweight per day)[12]. An open label trial revealed no beneficial effects of azathioprine in CD when given early after diagnosis compared to conventional management [17]. Azathioprine and mercaptopurine are antimetabolites, but

immunomodulation is induced by T cell apoptosis by modulating cell signaling [7]. In serve cases of UC, patients who may not require hospitalization but continue to show symptoms with oral or topic steroid or mesalazine therapy should be given anti-Tumor necrosis factor alpha (TNFα) antibody infliximab at 5mg/kg [18]. Infliximab was superior to placebo in clinical trials enrolling patients who failed corticoid or aminosalicilate therapy. Patients who did not respond to the initial two doses were unlikely to respond to a third one. Side effects of infliximab include infusion reactions, autoimmunity and an increased risk for infections [19]. Patients who require hospitalization should be given intravenous corticosteroids. Cyclosporine and tacrolimus are effective in patients who did not respond to intravenous corticoid therapy [12]. First line medications to maintain remission in patients with UC are aminosalicylates. Thiopurines are also effective but should be reserved for patients frequently relapsing despite aminosalicylate therapy or steroid dependent patients. Steroids are ineffective maintaining remission [7]. Surgery is indicated when life threatening complications occur or in patients not responding to long-term immunosuppression [12].

For Crohn's disease, sulfasalazine at doses 3000–4500mg per day is beneficial in active disease in patients with mild to moderate CD [20]. The Effect of mesalazine is controversial [21-23]. Budesonide is recommended in patients with ileal or right colonic involvement or both [24]. The antimetabolites azathioprine and mercaptopurine were equal to placebo in inducing remission, but both lowered steroid consumption of the patients. They were inferior compared to infliximab, but combination of both was superior to infliximab alone therapy in inducing steroid-free remission [25]. Infliximab is given at a dose of 5 mg/kg. An alternative to infliximab is a fully human anti-tumour necrosis factor (anti-TNF) antibody, adalimumab, given subcutaneously with a loading dose of 160 mg at week zero and 80 mg at week two [12]. 5-Aminosalicylates did not show any benefit in maintaining remission. Budesonide at 6mg can be used to maintain remission for 6 month. Azathioprine and mercaptopurine and d methotrexate are effective in maintaining remission after induction with corticosteroids. Furthermore, azathioprine is effective after induction of remission with Infliximab [26].

2.4 Natural Products and IBD

2.4.1 Isoprenoids

Isoprenoids constitute an abundant class of chemical compounds consisting of two or more isoprene-units. They are structurally varying and play many roles in the physiological processes of plants and animals (pigments, fragrances, vitamins, precursors of sex hormones,

etc.)[27]. Some of them have been shown to affect human health. Recent studies have proclaimed an anti-inflammatory mechanism for several isoprenoids[28]. Andalusol ((1R,3S,4S,4aR,8S,8aS)-8-(hydroxymethyl)-3,4a,8-trimethyl-4-(3-methylene-4-penten-1-yl)decahydro-1,3-naphthalenediol;), a labdane diterpenoid, is a compound found and identified in the extract of *Sideritis foetens* Clemen [29]. It was able to reduce inflammatory processes in carrageenan or 12-O-tetradecanoylphorbol acetate (TPA) treated rats in vivo. In peritoneal rat mast cells, andalusol reduced histamine release after stimulation with calcium-ionophore [30]. In lipopolysaccharide (LPS) - or interferon-γ (IFNγ)- stimulated J774 macrophages, it inhibited NF-κB (nuclear factor kappa B) activity, as confirmed by electrophoretic mobility shift assay (EMSA) and western blot. Since the inducible nitric oxide synthase (iNOS) gene is under the control of NF-κB, iNOS expression and nitric oxide (NO) synthesis were also inhibited. Chemically related substances were also tested, but were not as effective as andalusol. Polyol labdadiene was more effective than its hydrophobic counterpart. Manoyl oxides failed to be effective. The inhibitory activity of andalusol may be due to its hydroxyl groups [31]. Kaurenes are intermediates in the synthesis of gibberellins. Three members of the kaurene family (foliol, linearol, ent-Kaur-16-ene-19-oic acid), all consisting of a tetrahydrophenantren basic structure, were also found to inhibit inflammatory pathways, particularly the NF-κB-pathway. They demonstrated the ability to decrease iNOS-levels in LPS-stimulated macrophages. An EMSA assay showed the inhibition of NF-κB at the iNOS promoter. Further, inhibitory activity towards IκB kinase (IKK, a kinase that marks the Inhibitor of kappa B (IκB) and thus causes its degradation and subsequent activation of NF-κB) was hypothesized by the authors, but they failed to show this effect in vitro. Since immunoprecipitation experiments did not confirm a direct inhibitory effect, an interaction with upstream targets of IKK has been suggested [31]. Further studies aimed to find anti-inflammatory semi-synthetic compounds using ent-kaurenes as a lead structure. There seems to be a correlation between the presence of a carboxylic acid group and iNOS inhibition. In addition, the presence of a D-ring seems to enhance the inhibitory activity [32]. Amethanolic extract from Isodon japonicus exerted an inhibitory effect on LPS-induced NO and prostaglandin E_2 (PGE_2) release from macrophages. Four members of the kaurane family (kambanin, kamebactel A, kamebaukarin, excisarin A;) were found to be responsible for the decreased levels of NO and PGE_2. They inhibited the NO and PGE2 production in a concentration dependent manner. An EMSA and a reporter gene assay demonstrated that the kauranes are capable of blocking the Deoxyribonucleic acid (DNA) binding of NF-κB (Hwang et al. 2001). Another promising anti-inflammatory diterpenoid is hypoestoxide,

which is extracted from *Hypoestes rosea*. The basic structure of hypoestoxide is a tertracyclic saturated carbon ring with two epoxide residues and an acteoxygroup. In concentration up to 100 µM, it reduced the release of TNFα, interleukin 1β (IL-1β) and interleukin 6 (IL-6) in LPS stimulated cells without demonstrating cytotoxic effects. Furthermore, hypoestoxide was tested for its ability to inhibit NF-κB and IKK. EMSAs performed with HeLa-cells pre-treated with hypoestoxide showed a dose dependent decrease in the activities of both proteins. The inhibition of phorbol ester-induced topical inflammation in mice confirmed the obtained data [33]. Several studies have reported the anti-inflammatory activity of sesquiterpene lactones. The efficiency of the single compounds varies due to their structural diversity and the different assay techniques used for their evaluation. There is a relation between the presences of certain groups including the isoprenoid ring, a lactone ring containing a conjugated exomethylene group, and an α,β-unsaturated cyclopentenone or a conjugated ester moiety and the capacity to inhibit iNOS-dependent NO production [34]. One compound belonging to the sesquiterpene lactones is parthenolide. This substance, which is extracted from *Tanacetum parthenicum*, is a potent inhibitor of NF-κB. Parthenolide interferes with NF-κB activation by preventing IκB-a phosphorylation, a step leading to its degradation by the proteasome. Experiments revealed that parthenolide impaired TNFα induced activation of the IKK complex, but did not act against the IKK-mediated NF-κB activation. In addition, EMSA experiments demonstrated the dose dependent reduction of NF-κB/DNA complex, but no reduction of the constitutive binding protein [35]. Helenalin, another sesquiterpene lactone, was isolated from *Arnica montana*. It showed anti-inflammatory properties by modulating the NF-κB/IκB complex in a way that prevented the degradation of the inhibitory subunit. Other transcription factors were not affected [36]. However, helenalin was also found to be cytotoxic due to its alkylating activity [37]. Triterpenoid saponins are a class of natural compounds present in plants and marine animals. Investigations into extracts of *Acacia victoria* lead to the dicovery of the avicin family, in particular avicin G, as promising substances. Avicin G reduced NF-κB activation without affecting IκB degradation. A possible target is the p65 subunit of NF-κB, which is responsible for nuclear translocation of the transcription factor. The results of the experiments suggest that avicin G also inhibits the DNA-binding ability of NFκB by affecting essential sulfhydryl groups [38]. Another triterpenoid, pristimerin (**Fig. 9**), inhibited NF-κB and reduced iNOS levels in LPS stimulated macrophages. However, there are some concerns with the use of pristimerin, since it shows some toxicity in MTT and sulforhodamine assays [34].

2.4.2 Stilbenes

The most abundant stilbene is resveratrol (5-[(E)-2-(4-hydroxyphenyl) ethenyl] benzene-1,3-diol;). It is found in red grapes, in certain berries and in peanuts. The bioavailability of resveratrol is very low because of rapid metabolism. Nevertheless, beneficial effects, including anti-inflammatory, cell growth-modulatory and anti-carcinogenic effects, have been described. Resveratrol demonstrated the capacity to decrease clinical scores in dextran sodium sulfate (DSS)-induced colitis in mice. The amount of neutrophils and $CD4^+$ (cluster of differentiation 4 positive) T-cells decreased after treatment with resveratrol in both the mesenteric lymph nodes and the lamina propria of the colon [39]. These properties of resveratrol are partially due to its inhibition of NF-κB and activator protein-1 (AP-1). The mechanism of action may be reduced phosphorylation and translocation of the p65 subunit. The inhibition of NF-κB and the suppression of associated reporter genes could be observed even after NF-κB activation by treatment with TNFα, phorbol 12-myristate 13-acetate (PMA), LPS, Hydrogen peroxide (H_2O_2), okadaic acid or ceramide. The effect was demonstrated in myeloid (U-937), lymphoid (Jurkat) and epithelial (HeLa and H4) cells [40]. Furthermore, resveratrol was shown to alleviate oxidative events in 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced colitis as assessed by myeloperoxidase (MPO) activity. It also reduced the colonic injury score in the same experiment [41]. Levels of cytokines and enzymes associated with inflammatory processes were also diminished (IL-1β, IL-6, iNOS, cyclooxygenase-2 (COX-2)). In addition, levels of prostaglandin E_2 were restored to basal values [41, 42]. Another effect of resveratrol is to enhance sirtuin 1 (SIRT1) activity. SIRT1 is a deacetylase that downregulates p53, therefore negative regulation of NF-κB by SIRT1 is presumed [42]. In order to optimise the therapeutic attributes and to increase the potency of resveratrol, lead optimization studies were performed to investigate structural features such as substitution on each of the two aryl rings and substitution on the alkene. A library consisting of 78 resveratrol analogues was generated and tested for biological activity. The assay revealed that COX-1 and COX-2 inhibition requires a conformation where both aryl rings are rotated out-of-plane. NF-κB inhibition, in contrast, appears to require a coplanar conformation. The resorcinol ring is present in all potent inhibitors. Only COX-2 inhibition permits a greater variation. NF-κB inhibition is enhanced in analogues with large hydrophobic rings instead of the phenolic residue [43]. Another stilbene, pterostilbene, was able to reduce aberrant crypt foci, lymphoid nodules and tumours in experimental mice models. It diminished NF-κB activation by blocking phosphorylation of protein kinase C-β2 (PKC-β2). In addition, it enhanced the

production of antioxidant enzymes. Its structure is similar to resveratrol. However, in pterostilbene the hydroxyl residues are esterified with methyl groups [44].

2.4.3 Flavonoids

Flavonoids are a chemically related and widespread class of plant secondary metabolites. They are divided according to their chemical backbone into five groups: flavones (2-phenyl-4H-chromen-4-one), flavonols (2-phenyl-4H-chromen-4-one), flavanols (2-phenyl-3,4-dihydro-2H-chromen-3-ol), flavanones (2-phenyl-2,3-dihydro-4H-chromen-4-one) and anthocyanins (2-phenylchromenium). All flavonoids contain an aromatic ring A, which is fused to a heterocyclic carbon ring C. A second aromatic ring B is attached by a carbon–carbon bond to ring C at position 2. Because flavonoids show several beneficial health effects, such as modulation of cytokine secretion and inhibition of inflammatory enzymes, SAR-studies were performed on quiescent and concanavalin A-stimulated rat splenocytes to reveal the effect of different flavonoid structures. The 3-hydroxy residue seems to diminish the anti-inflammatory activity. This effect is compensated by additional B-ring hydroxylation, as in case of quercetin. Further experiments revealed that the presence of a 2–3 double bond, 4'-hydroxylation, 3'-hydroxylation and the presence of 5-OH favour inhibition. The fact that the structural requirements were similar for inhibition of iNOS, COX-2, interleukin 2 (IL-2) and interferon-γ (INFγ) reflects a common mechanism of action [45]. The plant derived flavonol, quercetin, was tested for its antioxidant properties in HepG2 cells. It reduced the DNA-binding ability of NF-κB in H_2O_2-stimulated cells, but did not affect the DNA-binding in untreated cells [46]. A CaCo-2 cell model was used to investigate the anti-inflammatory features of quercetin. It was found that it suppressed allergic inflammation induced by IL-4 or immunoglobulin E (IgE) allergens. Modulation of the p38 MAPK (mitogen-activated protein kinases) pathway was suggested as a target. In macrophages challenged with gliadin, quercetin lowered the activation of STAT-1 (signal transducer and activator of transcription-1) and NF-κB pathways [47]. Another promising property of quercetin is that it increased levels of claudin-4. This protein is part of the tight junctions responsible for barrier function of epithelial cells. Hence, quercetin may have a positive effect on preventing intestinal barrier disruption, which seems to play an important role in the pathogenesis of IBD [48]. Quercetin was also shown to inhibit several inflammatory cytokines [49]. Despite these promising effects, quercetin itself failed to ameliorate DSS-induced colitis in mice. However, its glycoside rutin (3-O-rhamnosyl-glucosyl-quercetin) was effective in protecting animals from intestinal inflammation in the same model. Furthermore, protein levels of IL-1β were

quantified by Enzyme-linked Immunosorbent Assay (ELISA) in both mucosal epithelial cells and peritoneal macrophages. Interestingly, rutin, but not quercetin, significantly attenuated IL-1β levels. In DSS-colitis (dextran sodium sulfate induced colitis), gene expression of pro-inflammatory cytokines such as IL-1β was diminished by dietary intake of rutin, but not by quercetin intake [50]. Nobiletin, a citrus polymethoxyflavonoid, was found to inhibit the production of PGE_2 and pro- matrix metallopeptidase 9 (MMP-9) in rabbit synovial cells and to decrease the levels of IL-1α, IL-1β and TNFα messenger RNA (mRNA) in macrophages. These findings support the postulation that nobiletin might also be a promising candidate in treating inflammatory bowel diseases [28]. Green tea is one of the most common beverages throughout the world and provides many health benefits. The green tea polyphenols have been identified to be responsible for these effects. The mode of action of one derivative, theaflavin-3,30-digallate, a flavanol, has been investigated in RAW267.4 macrophages where it was able to decrease iNOS activity and subsequent NO production. An EMSA showed that it inhibited NF-κB [34]. Epigallocatechin-3-gallate (EGCG) is a further flavanol demonstrating NF-κB inhibitory activity as observed in TNFα stimulated human epidermal keratinocytes and human epidermal carcinoma cells. The treatment of non-stimulated cells resulted in cell cycle arrest in the carcinoma cell line, but not in normal human epidermal keratinocytes [51]. The Met tyrosine kinase, as well as its downstream proteins AKT and extracellular signal regulated kinases (ERK), were hypothesized to be targets for EGCG. These proteins are involved in both tumour development and inflammatory processes [52]. EGCG also enhanced adenosine and its receptors, which play a role in anti-inflammatory pathways. The suppression of TNFα induced NF-κB activation could be blocked with stable folate, supporting the theory that the folate cycle is involved in EGCG activity [53]. In a study designed to elucidate the mechanism of EGCG on the behaviour of peripheral cluster of differentiation 8 positive ($CD8^+$) cells, the cells were incubated either with or without catechin. The researchers observed that expression of Cluster of differentiation 11b (CD11b) molecules on the surface of $CD8^+$ cells was impaired by incubation with catechin, resulting in a decreased ability to adhere to intercellular adhesion molecule-1, and subsequently in a reduced ability to migrate in response to chemokines [54]. Within the class of flavones, luteolin, wogonin and oroxylin A are considered active. Luteolin targets various molecules in cellular pathways, resulting in inhibition of NF-κB and induction of apoptotic cell death in TNFα activated intestinal epithelial cells [55]. Interestingly, luteolin showed beneficial effects in vivo in spontaneous colitis in IL-10 deficient transgenic mice, but aggravated DSS-induced colitis in mice using a reporter gene under the control of NF-κB, probably by blocking NF-κB dependent protective

molecules in enterocytes [56]. Wogonin, an ingredient of *Scutellaria baicalensis*, showed potent inhibition of NF-κB activity in RAW264.7 macrophages and C6 rat glia cells, both of which had been stimulated with LPS. It was also able to reduce NO levels via inhibition of iNOS expression. Oroxylin is another constituent of *S. baicalensis* with anti-inflammatory activity in LPS-stimulated macrophages

[34]. Isoflavones have mainly estrogenic properties and thus have been investigated for their use in hormone sensitive cancers. However, experiments have also revealed that the isoflavone genistein modulates cytokine concentrations in enterocyte-like cells. In CaCo-2 cells challenged with a cytokine cocktail, it was shown to down regulate the inflammatory response [57]. Among the flavanones, hesperidin was found to improve the health of mice treated with DSS to induce colitis. Hesperidin lowered oxidative stress as it reduced inflammation via an immunomodulatory effect. Oral administration of hesperidin significantly decreased serum levels of IL-6 [58]. Microarray studies to investigate changes in human colonic fibroblasts after treatment with a citrus extract revealed reduced mRNA levels of plasminogenactivator-inhibitor-1 (PAI-1) and increased mRNA levels of Matrix metallopeptidase 12 (MMP-12). Both proteins are involved in modulating the extracellular matrix, tissue repair and fibroblast migration. Flavanones contained in the extract may have an effect on fibroblast activity and thus may modulate wound healing. A prominent finding is that flavanones may remain for several hours in the colon. This enables them to modify gene activities in the mucosa [50]. Delphinidin is a member of the class of anthocyanidins. Dietary ingestion of anthocyanidins results in amelioration of antioxidant activity. Antimicrobial actions have also been observed. Experiments on HCT116 cells revealed that delphinidin was able to induce cell cycle arrest in the G2/M phase by activating caspase-3. In addition, it was found to inhibit the phosphorylation and degradation of IκB-a, thus preventing NF-κB activation [59]. Sylimarin is found in the seeds of *Silybum marianum* and is a mixture of the flavonoids silybin, silychristin, and silydianin. It's commonly used as a liver protective drug. There is no adequate characterisation of the distinct components, because experiments were performed with the mixture [34]. Sylimarin exhibited pathway dependent inhibition of both NF-κB DNA-binding ability and its gene expression. The effect was observed in the hepatoma cell line HepG2 challenged with okadaic acid, but not with TNFα [60]. However, studies performed with human histiocytic lymphoma U-937 cells revealed an inhibitory effect after treatment with TNFα. Inhibition was specific for NF-κB, since AP-1, another transcription factor that controls growth and differentiation in response to a variety of stimuli

such as TNFα [61], was not affected. The hydrophobic fraction of liquorice extract contains glabridin, a flavonoid known to have beneficial effects such as anti-inflammatory, antimicrobial, anti-atherosclerotic and anti-nephritic activities. In a DSS-induced colitis model in BALB/c mice, the therapeutic effects of glabridin were examined. Administration of glabridin resulted in improved clinical scores including survival rate, body weight, and disease activity index. Glabridin treatment also resulted in lowered levels of pro-inflammatory cytokines and enzymes. Colon length reduction through DSS treatment was moderated after administration. Inflammatory status was also assessed by histological examination. DSS-induced colitis mice showed typical crypt dilation and goblet cell depletion. In contrast, tissue sections from glabridin treated mice revealed decreased mucosal injury [62]. The precursors of flavonoids, the chalcones (1,3-diphenyl-2-propenones), also display anti-inflammatory abilities and are able to inhibit NF-κB signalling. Since NF-κB activation is regulated by histone deacetylase enzymes (HDACs), 21 chalcones were screened in a fluorescence assay for HDAC inhibitory activity. Among these isoliquiritigenin, butein, homobutein and marein were active [63].

2.4.4 Naphthodianthrone

Hypericin, a naphthodianthrone found in *Hypericum perforatum*, can act as an inhibitor of NFκB in a non-antioxidant way. Hypericin decreased NFκB levels in HeLa cells and HTC10 cells. The effect was observed after induction with TNFα and H_2O_2, but not H_2O_2, indicating that hypericin has no radical scavenging activity. HeLa cells were also transfected with a stable IL-6 promoter fused to luciferase gene. The assay showed a decreased enzyme activity after incubation with hypericin. In contrast, hyperforin, another constituent of *H. perforatum* did not reveal any effect [64].

2.4.5 Curcumin

Curcumin ((1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione;) is a polyphenolic substance found in *Curcuma longa*. It is the main constituent of turmeric and has been used as a culinary spice for centuries. In addition, recent investigations have revealed that it possesses promising anti-oxidative and anti-inflammatory features. In a study aimed to investigate the effects in a rat colitis model, curcumin was able to reduce inflammatory markers. The results were obtained by measuring MPO activity, production of cytokines, expression of iNOS, COX and MAPK signalling. In the case of the mitogen-activated-protein-kinases, curcumin inhibited p38 signalling but not c-Jun N-terminal kinase

(JNK) activity [65]. Treatment of RAW264.7 macrophages with curcumin resulted in decreased protein and mRNA levels of iNOS, as evaluated by western and northern blotting. Mobility shift experiments showed a reduced DNA-binding activity of NF-κB induced by LPS. The blocked NF-κB activity was due to the prevention of IκB-a degradation [66]. Neutrophils play a major role in epithelial dysfunction and are involved in colonic injury in inflammatory bowel disease. Curcumin prevented neutrophilic migration directly by impairing their motility. Furthermore, treatment of colonic epithelial cells and macrophages resulted in decreased LPS-induced expression of chemokines [67]. Clinical studies showed significant improvement in disease scores when patients were treated with curcumin versus placebo in ulcerative colitis and Crohn's disease [68, 69]. A Cochrane review of clinical trials examining the efficacy and safety of curcumin in ulcerative colitis concluded that curcumin may be a safe and effective therapy for maintenance of remission in quiescent ulcerative colitis when given as adjunctive therapy along with mesalamine or sulfasalazine [70]. Structure–activity-relationship studies with curcumin analogues revealed that there is no correlation between the anti-oxidative and anti-inflammatory properties of curcumin, although compounds that retain the enone function were most effective in reducing TNFα induced NF-κB activation. Substitutions on the aryl rings are not essential but may be effective [71]. Further lead optimizing studies revealed that curcuminoid pyrazoles may have similar anti-inflammatory properties and may offer new treatment possibilities [72].

2.4.6 Boswellic acids

Oleogum resins from *Boswellia species*, particularly *Boswellia serrata* are used in indigenous cultures for treatment of a variety of diseases. Animal models revealed the resin's anti-inflammatory properties, making it an interesting subject for further investigations into its efficiency in treating inflammatory diseases including Crohn's disease and ulcerative colitis. The resin's effect is mediated by boswellic acids. Chemically, boswellic acids belong to the class of triterpenes and can be distinguished between pentacyclic and tetracyclic triterpenes. Carrageenan-induced rat paw oedema was reduced by oral administration of an alcoholic extract of the oleogum resin, but not by ingestion of water extracts. This indicates both an anti-inflammatory ability and a poor water solubility of the active compounds. Further investigations indicated that boswellic acids do not inhibit prostaglandin synthesis but rather leukotriene formation. A mixture of boswellic acids was able to inhibit immune haemolysis of antibody-coated sheep erythrocytes, indicating an anti-complement activity, in particular inhibition of C3-convertase in the classical complement pathway. In addition, the proliferation

of lymphocytes was reduced after stimulation with LPS. Acetyl-11-keto-b-boswellic acid (AKBA) was found to be most effective [73, 74]. Clinical studies confirmed that preparations from gum resin of *B. serrata* could be effective in the treatment of colitis [74].

2.4.7 Nicotine

One of the unexplained differences between Crohn's disease and ulcerative colitis is the enhanced incidence and prevalence of ulcerative colitis in ex-smokers and non-smokers. In order to examine the responsible substances, experiments have been performed with ethanolic extracts from filtered and non-filtered smoke and nicotine (3-[(2S)-1-methylpyrrolidin-2-yl] pyridine). Nicotine as well as filtered smoke was able to reduce colonic injury, MPO activity, and monocyte chemoattractant protein 1(MCP-1) expression, which is associated with diminished neutrophil infiltration. In contrast, non-filtered smoke aggravated the colonic injury implicating that the smoke contains damaging factors [75].

2.4.8 Further substances

Among the plant derived steroids, guggulsterone, isolated from the resin of *Commiphora mukul*, was found to attenuate signs and symptoms of inflammatory bowel diseases in TNBS- and oxazolone-induced colitis models in mice. However, only E-guggulsterone was effective in both models. Z-guggulsterone failed to show an effect in TNBS colitis and had only a partial effect in oxazolone colitis. In vitro studies, performed in $CD4^+$ cells obtained from the lamina propria of the model mice revealed that guggulsterone modulates the CD3/CD28 activation pathway and attenuates cytokine release [76]. Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic) is an organic molecule produced by bacteria. In a TNBS colitis model, ectoine was tested against sulfasalazine as reference. Treatment with ectoine resulted in dimished loss of body weight, reduced ulcerative area and colonic mass index (ratio of colon weight to total bodyweight). Glutathione (GSH) activity was increased and MPO activity was decreased. All results were comparable to sulfasalazine treated animals. Ectoine stabilizes the intestinal membrane and thus improves the function of the disrupted barrier [77]. *Picorrhiza kurroa* is a plant growing in Himalaya, where it is used in traditional medicine. Among its constituents, apocynin (4-hydroxy-3-methoxyacetophenone) was found to attenuate reactive oxygen species (ROS) during oxidativeburst through antagonizing Nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase. In a TNBS-colitis model in Wistar rats, apocynin lowered the macroscopical damage score, decreased MPO activity and

reduced the number of granulocytes and macrophages. It also inhibited NF-κB and STAT3 activation in DSS-colitis [78].

2.5 The intestinal immune system

IBD are imitated due to a dysregulated immune system. The origin of inflammation remains unclear. However, the continuous interaction between innate and adaptive immune system and the commensal gut microbiota is needed to maintain the non-pathological state. It is suggested that an aberration in this process led to an over activated immune response to nonpathogenic antigens. The intestinal epithelial cells are also involved in the immune response, since they build the mucosal barrier between gut bacteria and the lymphoid system. This barrier strongly depends on tight junctions between the epithelial cells. The cytoskeletal structure of tight junctions is important for the epithelial barrier function. A dysregulation of the epithelial barrier increases the cellular space between the cells and eases the contact between antigens and immune system [79]. Specialized epithelial cells like Paneth cells or goblet cells regulate the production of the mucus, which builds a protective layer over the barrier, and factors which help regulate the immune response. In IBD, the immune response results in continued epithelial injury, which causes erosions, ulcerations, and a decrease in the production of defensins [80]. The lamia propria mucosea is a thin layer of connective tissue beneath the epithelial wall. It hosts a complex set of immune cells which are needed to balance the tolerance of luminal microbiota and the requirement of defense against pathogens. As mentioned before, this balance is disturbed in IBD. A hallmark of active disease is an increased infiltration of the lamina propria of innate immune cells and adaptive immune cells[81]. Cells of the innate immune system involve for example neutrophils, macrophages, dendritic cells, and natural killer T cells. Furthermore, there are specialized M cells within the epithelial barrier sampling luminal antigens and subsequent present them to the gut associated lymphoid tissue (GALT) [82]. Cells of the innate immune system as well as epithelial cells recognize conserved strucutures on microbes known as pathogen-associated molecular patterns (PAMPs)[83] with pattern recognition receptors (PRR) and induce an immune response. PRRs include the family of Toll-like receptors (TLRs), C-type lectins, nucleotide-binding domain (NOD) and leucine-rich repeat-containing receptors (NLRs), and retinoic acid-inducible gene I-like receptors [84]. TLRs are the best studies class of receptors within the PRR group. Phylogenetic analysis revealed at least six families of TLRs in vertebrates with each family recognizing a general class of PAMPs[85]. The best-characterized microbial ligands form TLRs are lipopolysacchride (endotoxin) of gram-negative bacteria (TLR4),

bacterial lipoproteins and lipotechoic acid, fungal zymosan (TLRs 1, 2, and 6), bacterial flagellin (TLR5), a profilin-like molecule from the protozoan Toxoplasma gondi (TLR11), unmethylated CpG motifs present in DNA (TLR9), double-stranded RNA (TLR3), and single-stranded RNA (TLR7)[86]. TLR activation on antigen presenting cells such as dendric cells and macrophages led to a migration into secondary lymphoid organs and subsequent to the production of T helper cells. Since commensal bacteria produce PAMPs, there are two hypotheses, how constant inflammation as a response is avoided. On the one hand PAMPs and PPRs might be spatial separated. Pathogens from gut microbiota are located to the luminal side whereas PPRs are found intracellular or on the basolateral membrane. This inhibits constitutive ligation with microfloral PAMPs. On the other Hand, recognition might be actively down regulated by regulatory T cells and secreted factors like Il-10 and Il-2. Mice models proofed the critical role of IL-10 in the regulation of TLR driven inflammation. Mice lacking IL-10 developed t helper cell driven colonic inflammation, while mice lacking IL-10 and myeloid differentiation factor 88 (MyD88), an adaptor protein in the TLR signaling cascade, showed no evidence of disease[87]. MyD88 deficient mice were also more susceptible to colonic injury after radiation and showed abnormal cell proliferation in the epithelium. Furthermore, production of cytoprotective factors was reduced in MyD88 deficient mice in an animal model for IBD. This indicates that activation of PRR by commensal bacteria is also needed for epithelial balance and protection from injury [88]. Patients with IBD showed an altered TLR expression pattern on the epithelial cells, leading to an disturbed innate immune mechanism [89] . Another class of PRRs is the intracellular NLR, which is expressed in immune cells and in nonimmune cells like epithelial cells. NRL signaling is similar to TLR signaling with shared downstream targets like adaptor protein MyD88 and Nuclear factor kappa B (Nf-κB). The earliest-identified and best-characterized NLRs are NOD1 and NOD2. NOD mediated Nf-κB activation leads to production of inflammatory cytokines, chemo attractants, adhesive molecules and inducible enzymes. Both NOD1 and NOD2 might play a role in orchestrating adaptive immune response by regulating antigen presentation together with TLRs. The NOD2 agonist, muramyl dipeptide (MDP), can act as an effective adjuvant for antigen-specific T cell responses and antibody production [90]. Mutations in NOD2-gene were found to be linked with Crohn's Disease [91]. However, the role of NOD2 in some cell lines remain uncertain

Activated antigen presenting cells (APC) migrate to local or secondary lymphoid tissue, where they initiate the maturation of adaptive immune cells. Dendric cells (DC) play a key

role in keeping the homeostasis in balance. They produce a variety of TLRs and NLRs and are able to distinguish between pathogen and commensal bacteria and so to activate or to silence T cell maturation [92]. Studies suggest that dendric cells incorrectly recognize commensal antigens in IBD and induce a pro inflammatory response normally directed to pathogens. Also DCs seem to have a prolonged life [93, 94]. The adaptive immune response causes the tissue damage and is responsible for the manifestation of IBD. Involved cells comprise B cells are producing Immunoglobulin A (IgA) and IgG and a mixture of T helper cells 1 (T_h1); T_h2 and T_h17 cells. T helper cells are classified according to the secreted cytokine pattern. In simplistic terms, UC is T_h2 driven while CD is more associated with T_h1 response. Recent findings suggest a more complex overlap between the two major forms and also involve T_h17 cell lineage[95]. The interleukin 23 pathway is essential for T_h17 cell development and genes involved in this pathway or in T_h17 cell function have been associated with both UC and CD [96].

2.6 Role of epithelial cells in immune response

The intestinal epithelial cells function is not limited to nutrient absorption, electrolyte transport and physical protection against pathogens and injurie. They are able to secrete soluble mediators known to be important for communication with immune system cells. Interleukin 8 (IL-8) is an important chemokine and mediator of immune response shown to be produced by intestinal cell lines. In vitro, T_{84}, Caco-2, SW620, and HT29 cells constitutively produce IL-8 mRNA. $T_{84,}$ SW620, and HT29 constitutively express and secrete low levels of IL8 Secretion can be differentially stimulated with TNFα, interferone gamma (IFN-γ); interleukin 1 beta (IL-1β) or lipopolysaccharide (LPS)[97, 98]. Pathologic bacteria were also found to be involved in IL-8 response of intestinal cells. Enteroaggregative E. coli (EAggEC) is suspected to cause persistent diarrhea. Children with EAggEC showed elevated IL-8 levels. Furthermore, two strains of EAggEC were found to increase IL-8 release in vitro [99]. Clostridium difficile toxin A plays an essential role in the development for pseudomembranous colitis. Cell culture studies indicate that colitis is induced by epithelial IL-8 secretion followed by detachment and apoptosis of the epithelial cells[100]. In diseased mucosa of IBD patients the level of IL-8 correlates with macroscopic damage and neutrophil accumulation.[101]. Monocyte-chemoattractant protein 1 (MCP-1) is produced by a variety of cells including epithelial cell. Biopsies of inflamed mucosa revealed multiple MCP-1 positive cells after immunohistological staining, whereas in non-inflamed tissue MCP-1 was restricted to the surface epithelium[102]. In vitro, MCP-1 secretion was increased rapidly after

stimulation with TNFα in HT29 cells, with IL-1β in Caco-2 cells and after [103]. Colonic epithelial cells also produced increased levels of IL-8 and MCP-1 after infection with invasive strains of bacteria, whereas gene expression was not changed after infection with non-invasive bacteria [104]. IFN-γ–inducible protein 10 (IP-10) and IFN-inducible T-cell α chemoattractant (I-TAC) are chemokines that bind to receptor CXCR3 mainly found on activated T cells. Activation of receptor CXCR3 leads to chemotaxis and subsequent infiltration of the inflammation side with T cells [105, 106]. In vitro cell lines constitutively express IP-10 and I-Tac mRNA in varying degrees depending on the cell line. Both chemokines are inducible by IFN-γ in mRNA expression and Protein secretion. The effect was intensified by simultaneous stimulation with TNFα or IL-1α. Caco-2 cells grown on Transwell inserts showed that IP-10 and I-TAC are secreted basolateral rather than apical. Enteroinvasive bacterial infection is not able to increase chemokine production but also augments IFN-γ stimulation [107].

2.7 STW5 and STW5-II

STW5 (Iberogast®) is a mixture of nine plant extracts by Steigerwald Arzneimittelwerke (Darmstadt, Germany). The preparation consists of the 50% (v/v) hydroethanolic fresh plant extract of *Iberis amara L.*(*Brassicaceae*) whole plants and the 30% hydroethanolic extracts of *Melissa officinalis L.* (*Lamiaceae*) leaves, *Matricaria chamomilla* (*Compositae*) flowers, *Carum carvi L.* (*Apiaceae*) fruits, *Mentha piperita L.* (*Lamiaceae*), *Angelica archangelica L.* (*Apiaceae*) roots, *Silybum marianum (L.) Gaertn.* (*Compositae*) fruits, *Chelidonium majus L.* (*Papaveraceae*) herbs and *Glycyrrhiza glabra L.* (*Leguminosae*) roots. STW5-II is an investigational mixture consisting only of six plant extracts mentioned before. All plants were cultivated according to Guidelines for Good Agricultural Practice (GAP). To ensure quality all drugs and *I. amara* fresh plant were analyzed according to to the Pharmacopoeia Europaea German Pharmacopoeia (Deutsches Arzneibuch or Steigerwald monographs. All parameters for extraction are defined by standard operation procedures (SOP) [108, 109]. STW5 is established for treatment of functional dyspepsia. A systemic review consisting of three placebo-controlled trials and a reference-controlled trial proofed STW5 to be superior in reducing gastrointestinal symptom-scores [110]. STW5 also showed anti-inflammatory properties in gastroesophageal reflux disease models [111, 112]. Animal models of gastrointestinal inflammation revealed that interaction of STW5 and Adenosin receptor $A_{2A}R$ mediate this effect [113].

Table 1: Composition of STW5 and STW5-II.

Code (batch number)	**Plant origin**	**Extraction solvent (V/V)**	**Drug-Extract ratio**	**Content in STW5**	**Content in STW5-II**
STW5 (430392)	mixture	-	-	proprietary	-
STW5-II (141008)	Investigational mixture	-	-	proprietary	-
STW6 (13187)	*Iberis amara* L.	50% Ethanol	1 : 1.5–2.5	15%	15%
STW5 KII (13182)	*Mentha piperita L*	30% Ethanol	1 : 2.5–3.5	5%	10%
STW5 KIII (13176)	*Matricaria chamomilla* L.	30% Ethanol	1 : 2–4	20%	30%
STW5 KIV (13192)	*Glycyrrhiza glabra* L.	30% Ethanol	1 : 2.5–3.5	10%	10%
STW5 KV (13175)	*Angelica archangelica* L.	30% Ethanol	1 : 2.5–3.5	10%	-
STW5 KVI (13188)	*Carum carvi* L.	30% Ethanol	1 : 2.5–3.5	10%	20%
STW5 KVII (23033)	*Silybum marianum* (L.) Gaertn.	30% Ethanol	1 : 2.5–3.5	10%	-
STW5 KVIII (13195)	*Melissa officinalis* L.	30% Ethanol	1 : 2.5–3.5	10%	15%
STW5 KIX (23044)	*Chelidonium majus* L.	30% Ethanol	1 : 2.5–3.5	10%	-

2.8 Aim of the pharmacological study

STW5 and STW5-II showed anti-inflammatory effects in animal models of intestinal inflammation . Since standard therapy of inflammatory bowel disease often fails and is associated with severe side effects, the need of alternative therapies is urgent. Natural products revealed promising results in cell and animal models and to some extend even in

clinical trials. STW5 is established in the therapy of functional dyspepsia and thus known to be safe for use. Animal models suggest that patients suffering from IBD might benefit of the multi-component therapy, since serological and histological markers of inflammation were reduced under STW5 therapy. Herein, we used a normal human colon cell line to examine the effects of the extracts on their potential to regulate the response of the epithelial cells to inflammatory stimuli. Further on, we investigate the mechanisms of action which is responsible for changes in the chemokine profile.

3 Results

3.1 Cytotoxicity of the extracts

As a starting point, toxic concentrations of the STW5, STW5-II as well as of the single extracts towards human colonic cell line NCM460 were determined. Cells were treated with various concentrations of lyophlilisated extracts re suspended in cell medium. Effects are displayed in **Figure 1.**

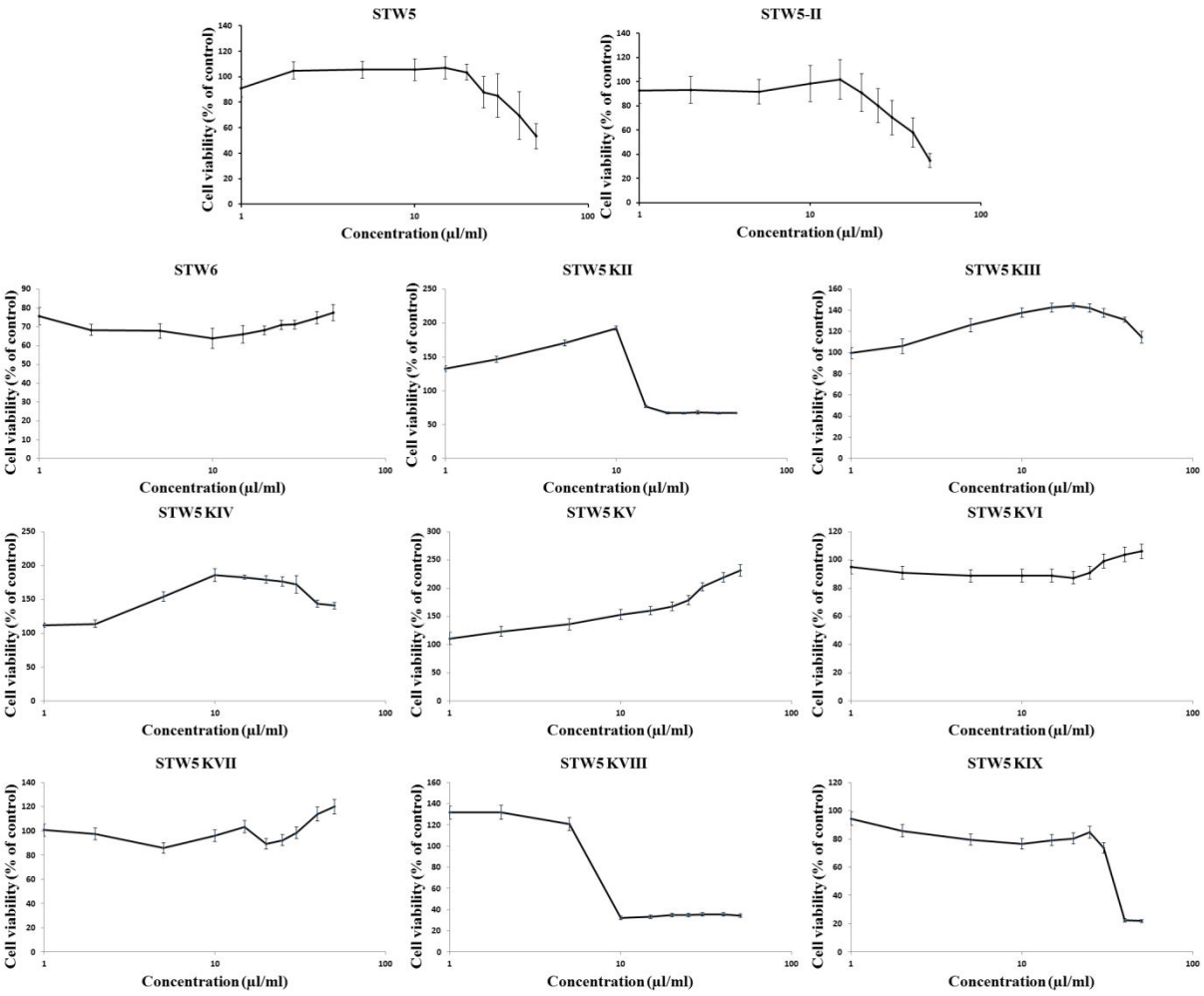

Figure 1: Cytotoxic effects of plant extracts: NCM460 cells were treated with extract combinations or single extracts for 24h. Cytotoxicity was measured by resazurin assays by normalizing to untreated control.

To avoid false results due to toxic effects, we determined safe concentrations of each extract. The multi-extract combinations STW5 and STW5-II were nontoxic for concentrations up to 30µl/ml. *Matricaria chamomilla* L. (STW5 KIII), *Glycyrrhiza glabra* L. (STW5 KIV), *Angelica archangelica* L. (STW5 KV), *Carum carvi* L. (STW5 KVI) and *Silybum marianum* (L.) Gaertn. (STW5 KVII) were not toxic for tested concentrations up to 50µl/ml. *Iberis*

amara L. (STW6) revealed a stable cell viability of about 80% (compared to untreated control). *Glycyrrhiza glabra* L. (STW5 KIV) and *Angelica archangelica* L. (STW5 KV) improved the cell viability to 150% (STW5 KIV) and 200% (STW5 KV). *Melissa officinalis* L. (STW5 KVIII) treatment led to a decrase of cell viability to 20% at a concentration of 10µl/ml. Decline of cell viability was shown for *Mentha piperita L.* (STW5 KII) at 15µl/ml and *Chelidonium majus* L. (STW5 KIX) at 40µl/ml.

3.2 Chemokine profile of NCM460 cells

First step to get insights in the response mechanisms of NCM460 cells is to treat them with inflammatory stimuli and to determine the response regarding the secreted chemokine Profile. IL-8 as prominent chemokine known to be secreted by epithelial cells was used as reference to validate the method. As a starting point, we checked for appropriate stimuli to increase the IL-8 secretion of NCM460 cells. We decided to check TNFα; IL-1β and IFNγ as immunological factors and LPS and MDP as bacterial factors. Strongest induction of IL-8 secretion was obtained with a combination of TNFα; IL-1β and IFNγ. To determine the influence of the extracts on the signal of an immune based assay, standard IL-8 dilution series were solved in different concentrations of STW5-II in cell medium and analysed by ELISA (**Figure 2**).

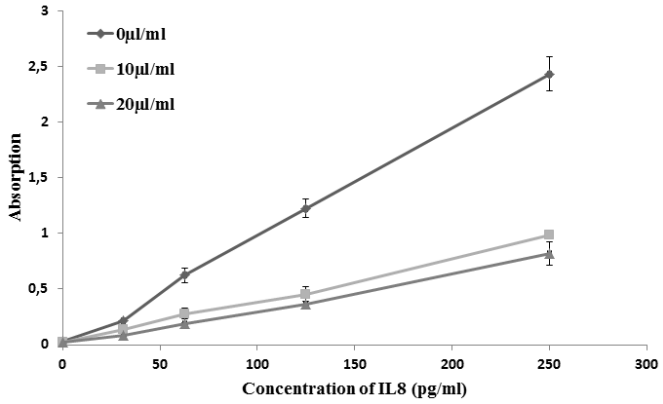

Figure 2: Effect of STW5-II on absorbance values. IL-8 standard was diluted in 0, 10 and 20µl/ml STW5-II and analysed by ELISA.

In the linear range between 0,2 and 0,8 absorbance an increased concentration of STW5-II led to reduced levels absorbance levels for high concentrations of IL-8. The effect was strong between 10µl/ml STW5-II and pure medium. A small difference existed between 10µl/ml and 20µl/ml. This indicates that STW5-II disturbs the assay and might lead to false positive results. To avoid the effect, we tested systems with extract free test supernatant at the end.

First we checked and spatial separated model. Cells were grown on Transwell inserts. Treatment was performed in the basal chamber whereas the supernatant of the apical chamber was collected for further analyses.

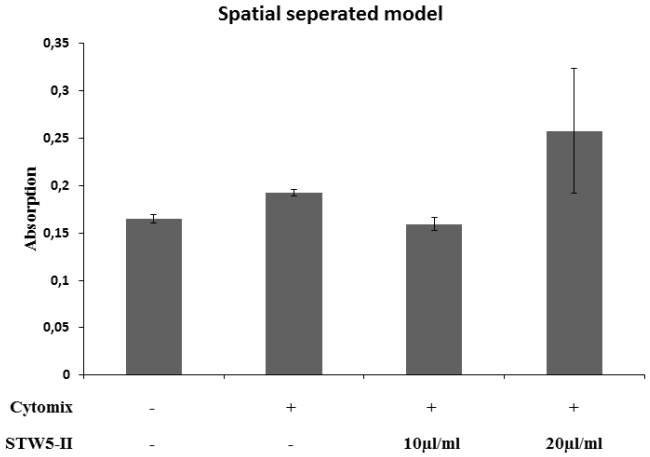

Figure 3: NCM460 cells grown on Transwell inserts were treated or not basal with STW5 and simultaneously induced with a mix of cytokines (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ). Effect on IL-8 secretion was measured via ELISA in the apical chamber.

The absorption levels which correspond with the IL-8 secretion were stable after both cytokine and extract treatment. The higher absorption resulting from the treatment with 20µl/ml STW5-II is relativized by the standard deviation.

In a second approach, we tested a model were induction and treatment were added sequential to the cells. Cells were grown in a multi-well plate. Growth medium was removed and replaced with medium containing diluted extract. After treatment time, medium was removed again and replaced with medium containing the mix of cytokines. After induction time supernatant was collected, purified and kept for further analysis.

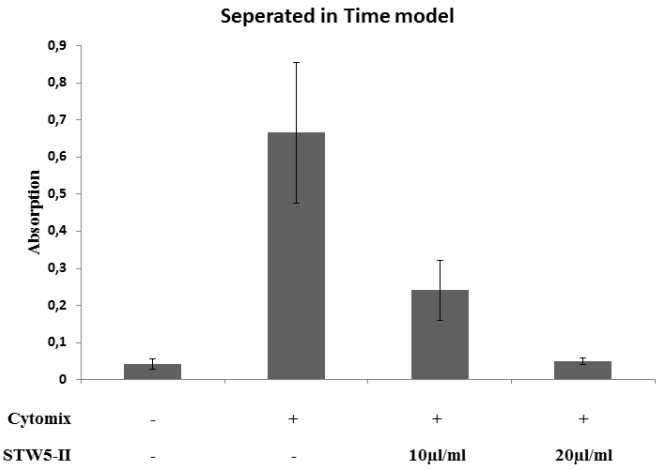

Figure 4: NCM460 cells grown in a multi-well chamber were pre-treated for 4h with STW5-II and subsequent induced for 24h with a mix of cytokines (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ). Effect on IL-8 secretion was measured in the supernatant

The absorption and thus the IL-8 secretion were markedly increased after treatment with cytomix as indicated before. Addition of plant extract reduced the IL-8 secretion even if the extaract was not present during the induction with cytokines. 10µl/ml STW5-II were able to decrease the absorption value more than 50%. 20µl/ml led to levels comparable with untreated control.

The "Seperated in time model" was decided to be used in further experiments. To gain insights in the whole spectrum of secreted cytokines, we used the membrane-based sandwich immunoassay Proteom Profiler® (R&D Systems). Supernatants from cells treated sequential with extracts and cytokines were mixed with a cocktail of biotinylated antibodies and then added to a membrane spotted with fixed antibodies. The signal was produced by treating the membrane with streptavidin-horseradish peroxidase (HRP) solution and a chemiluminescent substrate of HRP. The detected signal density was corresponding to the chemokine secretion.

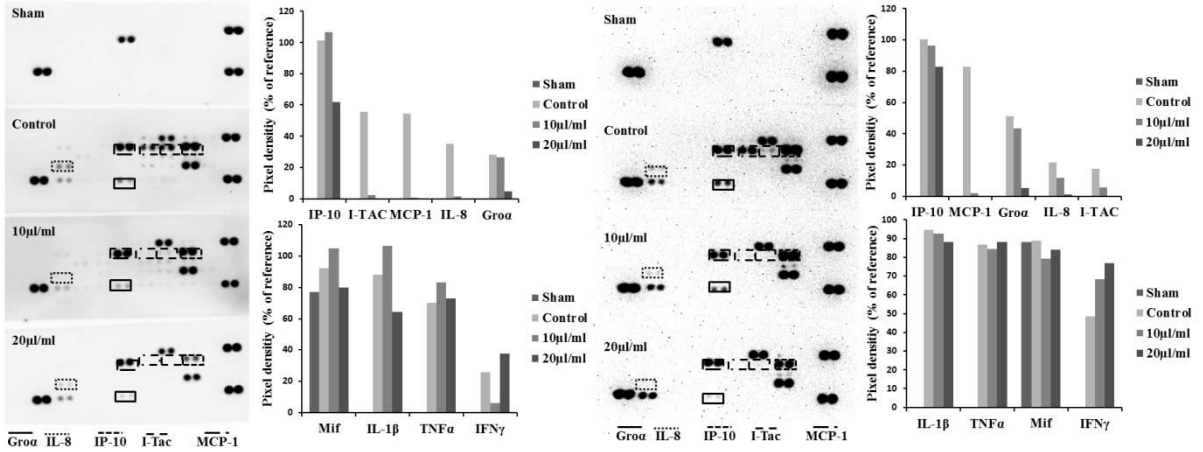

Figure 5: Effect of STW5 and STW5 on the cytokine profile of NCM460 cells. Cells were treated with STW5 (left) or STW5-II (right) (10 µl/ml or 20 µl/ml)) for 4 h prior to induction with the cytokine mixture (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ) for 24 h. Supernatants were collected and analyzed by Proteome Profiler® and evaluated for Pixel density using ImageJ software.

Signals were detected for IP-10, I-TAC, MCP-1, IL-8, Groα, Mif, IL-1β, TNFα and IFNγ. Due to the semi quantitative nature of the assay tendencies were reproducible but not absolute effects. IL-1β, TNFα and IFNγ were used to stimulate the cells, so the signal was found in every membrane except for the untreated control. Levels of IL-1β, TNFα were stable; IFNγ seemed to be slightly increased at high conentrations for both extracts. Macrophage Migration Inhibitory Factor (Mif) is present in all samples even in the untreated control and is not affected by any treatment. IP-10, I-TAC, MCP-1, IL-8 and Growth-regulated protein alpha (Groα) were significant increased after exposition to the cytokine cocktail. I-TAC, MCP-1and IL-8 were all significant decreased after treatment with both STW5 and STW5-II. As mentioned before, the Profiler is not quantitative. So we used the enzyme linked immunoabsorbant assay (ELISA) to gain insight in the quantitative nature of chemokine secretion inhibition. Cells were treated as described before and supernatant was analysed for single chemokines IP-10, I-TAC, MCP-1 and IL-8.

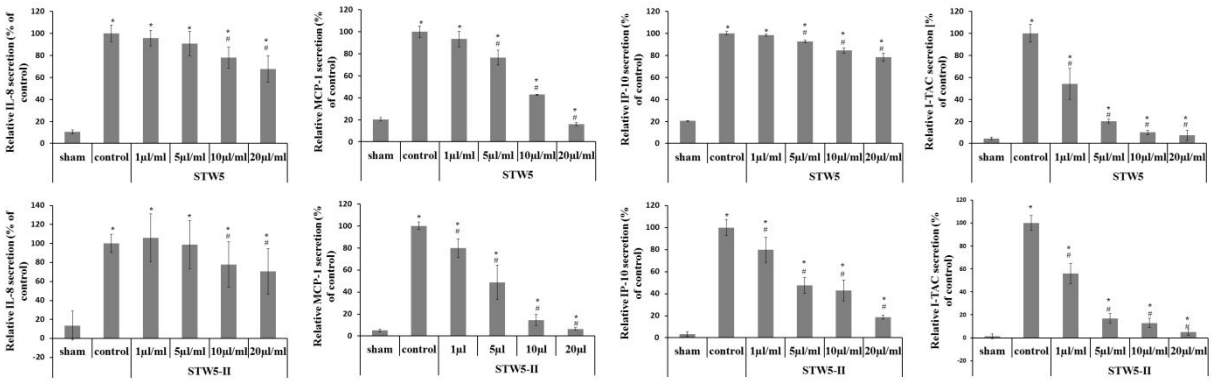

Figure 6: Dose dependent effects of STW5 and STW5-II on cytokine release. NCM460 cells were treated with STW5 or STW5-II (1 µl/ml; 5 µl/ml; 10 µl/ml; 20 µl/ml)) for 4 h prior to induction with the cytokine mixture (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ) for 24 h. Supernatants were collected and analyzed by ELISA.

As indicated by the Proteom Profiler®, all tested chemokines were induced after cytokine treatment. The strongest effect was observed for I-Tac. Both STW5 as well as the investigational mixture STW5-II were able to reduce the secretion of I-Tac and MCP-1 significantly in a concentration dependent manner with reaching basic levels at 20µl/ml. In case of IP-10 secretion, only STW5-II was able to reverse the effect of the cytokines significantly. IL-8 secretion declined after treatment with both extracts but to a lower extend compared to other cytokines.

The influence of the chemokine secretion was also tested for the single extracts. Since 10µl/ml was effective in both STW5 and STW5-II we chose it as standard and tested the single extracts in a concentration according to its content in 10µl/ml STW5-II (in case of STW5 KV, STW5 KVII and STW5 KIX we used the concentration in STW5.

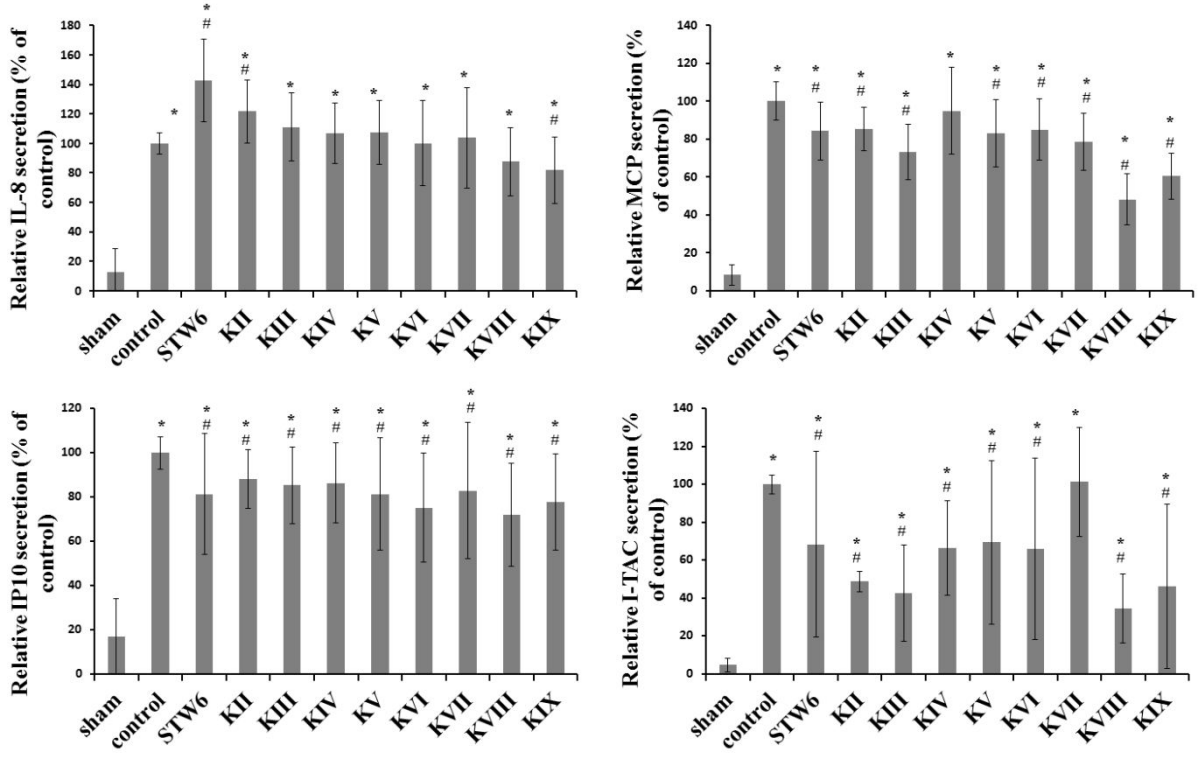

Figure 7: Effects of single extracts on cytokine release. NCM460 cells were treated with extracts (percentage of STW5-II calculated for 10 µl/ml according to content in herbal preparation) for 4 h prior to induction with the cytokine mixture (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ) for 24 h. Supernatants were collected and analyzed by ELISA.

Cytokine induced IL-8 secretion was not or only slightly effected by the single extracts. Most of the extracts (STW6, STW5 KII, STW5 KIII, STW5 KIV, STW5 KV, STW5 KVII) even increased the secretion. MCP-1 secretion was slightly reduced after treatment with single extracts compared to non-treated, cytokine induced control. Highest effects were observed for STW5 KVIII with a reduction about 50%. IP-10 secretion was also only slightly downregulated with every extract. I-Tac was affected by every extract but with confusing results and high standard deviation. STW5 KVIII reduced the secretion of I-Tac by 40%.

3.3 Influence of cytokines on intracellular proteins

To gain insights into the cellular mechanisms behind the chemokine secretion and to figure out the signal transduction affected by the extracts, we examined the effects on different proteins within the class of transcription factors and Mitogen-activated protein kinases (MAPK). As a first step we evaluated the effect on Nf-κB. Extracts of NCM460 cells were added to plates to which consensus binding site oligonucleotides were bound. Content of captured transcription factors were measured in an ELISA manner.

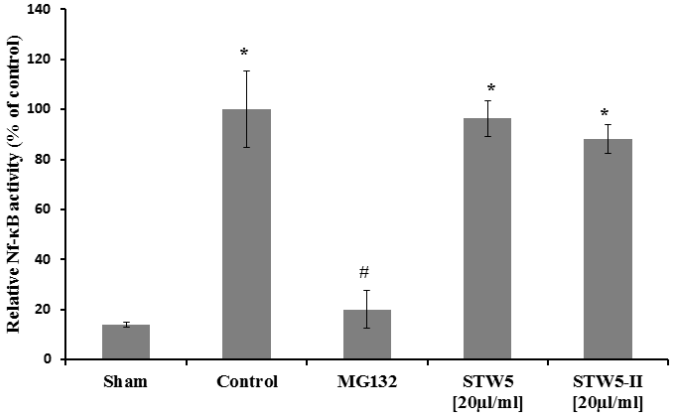

Figure 8: Effects of STW5 and STW5-II on NF-κB activity measured by TransAM ELISA. NCM cells were treated with extracts for 4h before they were induced with a mix of cytokines (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ) for 30min. Protein extracts were analyzed

Although there were observable effects regarding the activity after extract treatment for both STW5 and STW5-II, the results were not reliable since the induction of activity after cytokine treatment was very low and the activity after treatment with 20µl/ml of STW5-II was much lower than the activity of an untreated sample. For this reason we used a commercial available HEK-Blue™ Null1 cells which express SEAP reporter gene under the control of the IFN-b minimal promoter fused to five NF-κB binding sites. The cells were treated or not with STW5 or STW5-II or MG-132 as reference inhibitor and subsequent stimulated with cytokines.

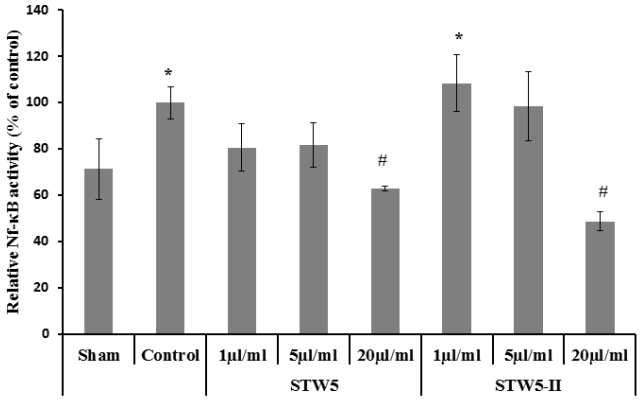

Figure 9: Effects of STW5 and STW5-II on NF-κB activity analyzed in HEK-Blue™ Null1 cells. Cells were pretreated with Extracts for 4h before they were induced with a TNF-α (10 ng/mL) for 24h. MG-132 was used as reference.

Nf-κB activity was strong induced after cytokine treatment. Reference inhibitor MG-132 reversed the effect of the cytokines nearly complete. STW5 and STW5-II only revealed a weak effect regarding the Nf-κB activity.

To analyse the involvement of AP1, NCM460 cells were transfected with a luciferase reporter gene. Transfected cells were treated with the known inductor phorbol-12-myristate 13-acetate for 1h and for 24h to determine the better time period. The inducible AP1-responsive firefly luciferase construct signal was compared to the constitutively expressing Renilla luciferase construct signal.

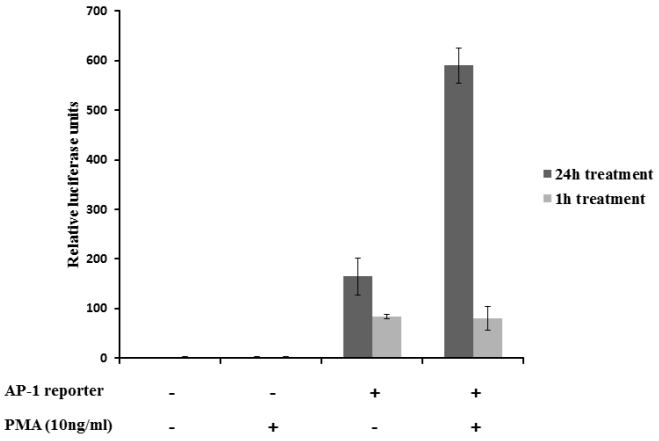

Figure 10: NCM460 cells transfected or not with AP-1 reporter were treated with known inductor PMA for 1h or 24h.. Cells lysates were analyzed for luciferase activity

Cells non transfected with AP-1 reporter did not show any signal with or without PMA treatment. Cells transfected with AP-1 reporter treated with solvent showed a signal at about 200 units for 24h treatment and a weaker signal at about 100 units for 1h treatment. Cells transfected with AP-1 reporter treated with PMA (10ng/ml) showed an increased signal after 24h treatment compared to transfected but untreated controls. 1h treatment with PMA (10ng/ml) of cells transfected with AP-1 reporter did not change the signal compared to solvent treated control. NCM460 cells were then treated with either Cytokine mixture and compared to PMA as a reference. As Time period 24h was chosen.

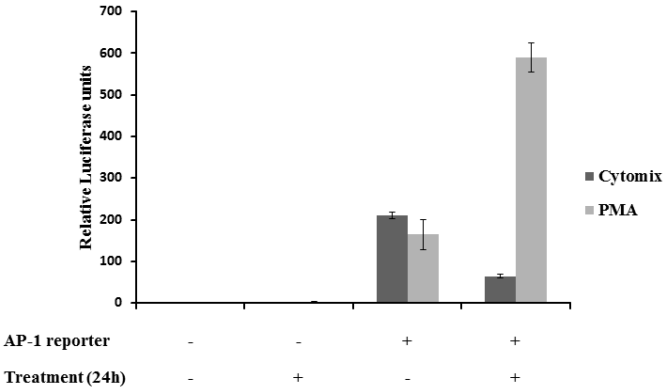

Figure 11: NCM460 cells transfected or not with AP-1 reporter were treated with known inductor PMA or with a mix of cytokines (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ) for 24h. Cells lysates were analyzed for luciferase activity

NCM460 cells not transfected with AP-1 reporter did not show any signal with or without Treatment (Cell declared as not treated were solvent treated). Cells transfected with AP-1 reporter showed weak signals when treated with solvents. The signal was slightly lower for PMA solvent (Dimethyl sulfoxide (DMSO)). Cells transfected with AP-1 reporter and treated for 24h with the cytokine mixture revealed a strongly decreased signal.

To evaluate the effect on the MAPK p38, proteins were extracted from treated cells. Phosphorylated p38 was precipitated with antibody bound to microbeads. The precipitates were incubated together with adenosine triphosphate (ATP) and the p38 substrate activating transcription factor 2 (ATF-2). The level of phosphorylated ATF-2 (pATF-2) reflects the activity of p38. At first, different induction times were checked.

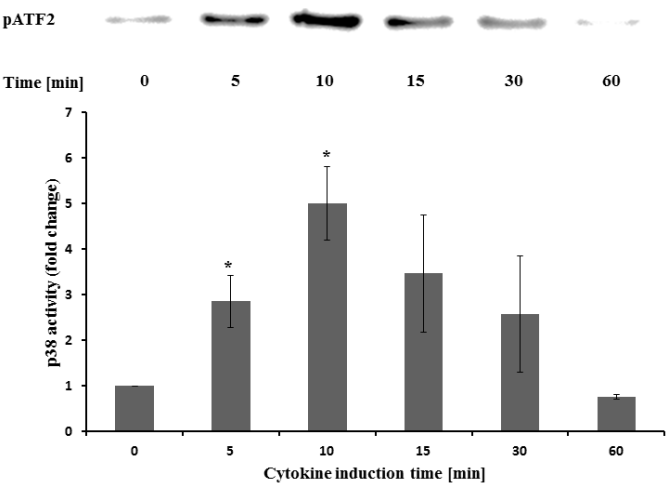

Figure 12: NCM460 cells were treated with the cytokine mixture (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ) for the indicated time points prior to harvesting and p38 activity measurement by ATF-2 phosphorylation. The upper panel shows a representative western blot

for pATF-2. The lower panel displays the quantification of three independent western blot experiments (pixel density fold change compared to 0 min; three independent experiments)

Compared to non-treated cells, the amount of pATF-2 was nearly triplet after 5min of treatment with the cytokine mixture. After 10min induction, the pATF-2 level reaches the highest value with a fold change of about 5 compared to non-treated cells. After 15min induction the fold change decreases to about 3,5; after 30 min the fold change is about 3. The pATF-2 level after 60min of induction is nearly the same as for non-treated cells. To determine plant extract effect on p38 activity, cells were pre-treated for 4h with 20µl/ml extract. As induction time, 5min and 10 min were used.

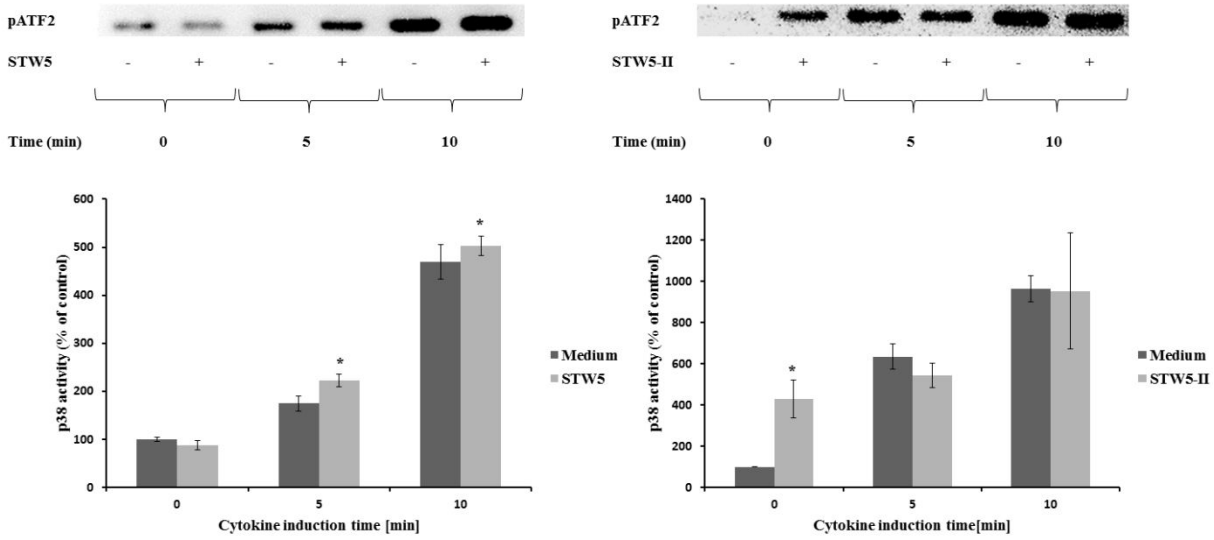

Figure 13: NCM460 cells were treated with STW5-II or STW5 at 20 µl/ml for 1 h prior to induction with the cytokine mixture (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ) for the indicated time points. Cell lysates were used to measure p38 activity by ATF-2 phosphorylation.

The Activity of p38 was significantly increased after 5 and 10 min treatment with or without STW5 or STW5-II. Compared to the medium control, STW5 treated cells revealed almost the same p38 activity or even a slightly increased activity. STW5-II treatment without cytokine induction showed increased p38 activity. After 5min or 10min cytokine treatment, STW5-II treated cells contained less active p38 compared to medium pre-treated cells.

To evaluate the effect on Stat protein we pre-treated cytokine induced cells with STW5 or STW5-II. The protein extracts were analysed by western blot to see if the level of phosphorylation was changed for Stat proteins.

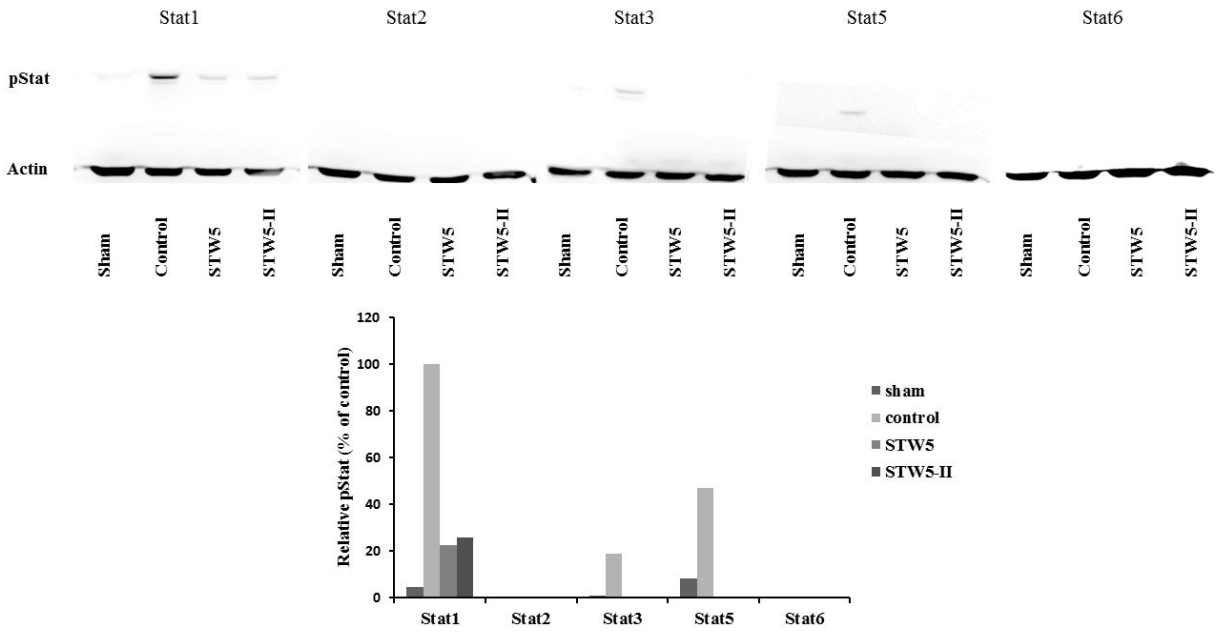

Figure 14: NCM460 cells were treated with each 10 µg/ml STW5 or STW5-II for 4 h prior to induction with the cytokine mixture (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ) for 30 min. Cell extracts were analyzed for phosphorylated Stat proteins by western blotting. Blots from left to right: Stat1, Stat2, Stat3, Stat5, Stat6.

Phosphorylated Stat1 was found in unstimulated cells. Stimulation with the cytokine mixture led to an increased number of pStat1. Pre-treatment with STW5 or STW5-II reduced the phosphorylation of Stat1. There was no detectable level of phosphorylated Stat2 in neither of the samples. Stat3 was also phosphorylated after cytokine treatment and seemed to be prevented by STW extracts. Level of phosphorylated Stat5 increased after cytokine treatment and seemed to be reduced in pre-treated cells. Levels of pStat6 could not be detected. Since signals of pStat3 and pStat5 were low and could not be reproduced, Stat1 was used for further experiments.

First, the effect of STW5 and STW5-II towards Stat1 phosphorylation was examined by adding different concentrations of the extracts prior to cytokine induction.

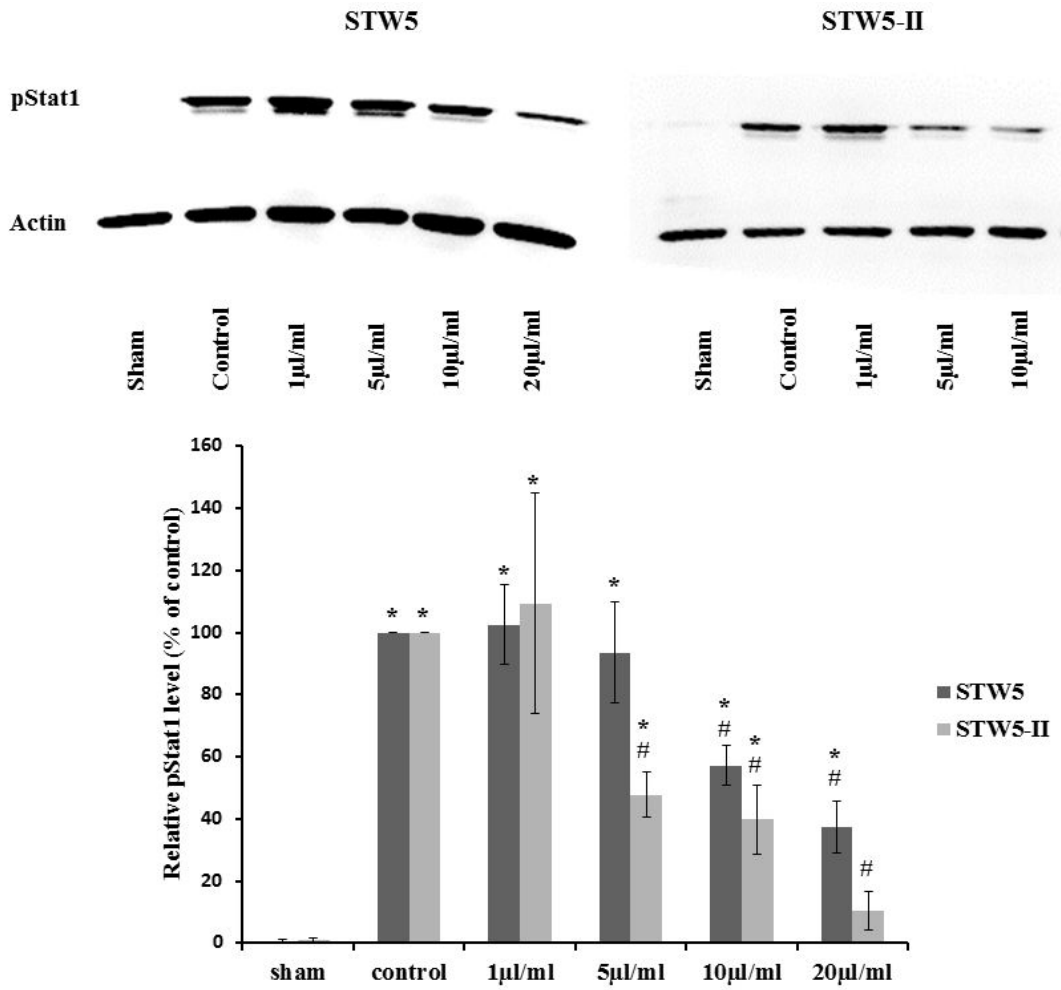

Figure 15: Effect of STW5 and STW5-II on Stat1 phosphorylation: NCM460 cells were treated with STW5 or STW5-II at indicated concentrations for 4 h prior to induction with the cytokine mixture (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ) for 30 min. Cell extracts were analyzed for phosphorylated Stat1 protein by western blotting. The lower panel displays the ratio of pStat1 to actin in relation to the control sample (n= Three independent experiments).

Stat1 was again strong phosphorylated after cytokine treatment. Pre-treatment with STW5 showed first effects at a concentration of 5µl/ml. pStat1 levels were cut in half at 10µl/ml and were only 40% after adding 20µl/ml STW5. Adding STW5-II prior to cytokine induction led to stronger effects compared to STW5. At 1µl/ml was little higher compared to untreated induced control cells. Values of pStat1 were below 50% of Cytokine induced cells for the concentrations 5µl/ml and 10µl/ml. For 20µl/ml of STW5-II the phosphorylation of Stat1 was reduced to 10%. The next step was to evaluate the effect of the single extracts within the mixture. Concentrations were chosen like before.

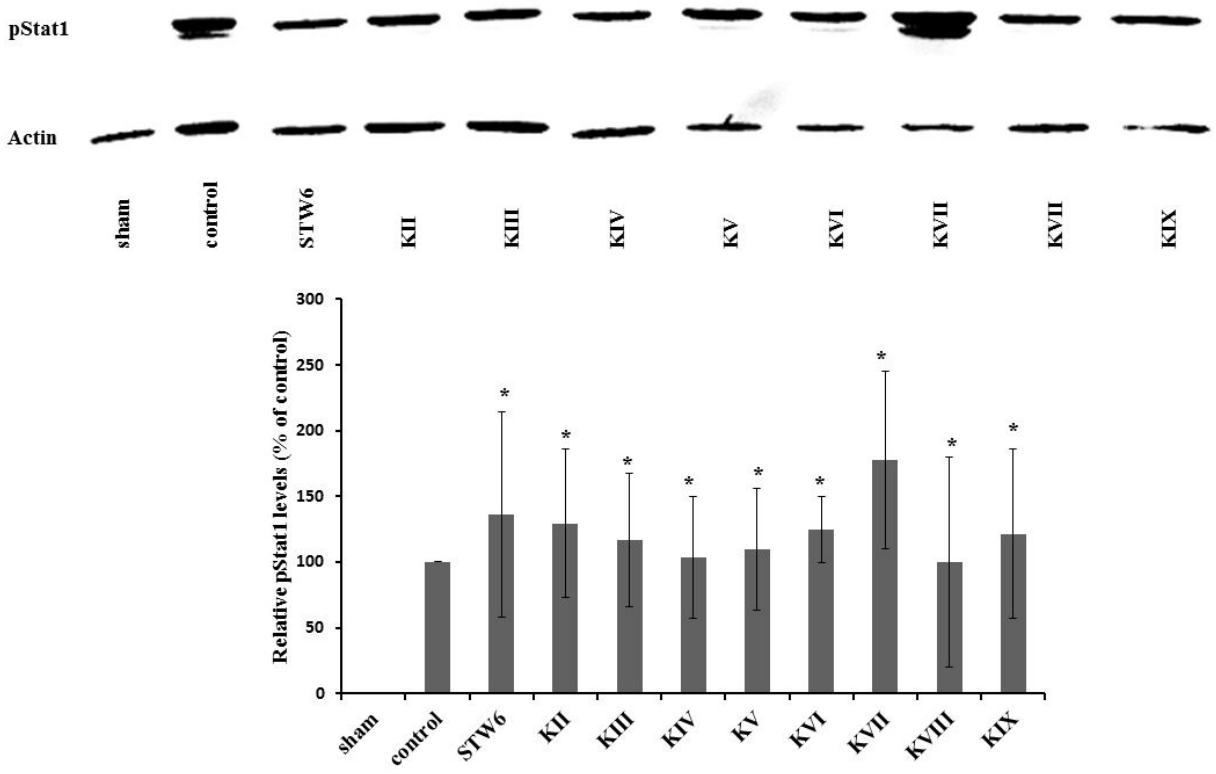

Figure 16: Effect of STW5 and STW5-II on Stat1 phosphorylation: NCM460 cells were treated with isolated extracts (percentage of STW5-II calculated for 10 µl/ml according to content in the herbal preparation) for 4 h prior to induction with the cytokine mixture (10 ng/ml TNF-α, 5 ng/ml IL-1β and 10 ng/ml IFN-γ) for 30 min. Cell extracts were analyzed for phosphorylated Stat1 protein by western blotting (n= Three independent experiments)

The single Extracts were not able to reverse the effects of the cytokine mixture. Most of the single Extracts even increased the Stat1 phosphorylation (STW6: 136,1% ± 78,4; STW5 KII: 129,3% ± 56,5; STW5 KIII; 116,9% ± 50,7; STW5 KIV: 103,5% ± 46,4; STW5 KV: 109,7% ± 46,4; STW5 KVI: 125,1% ± 25,1; STW5 KVII: 177,8% ± 67,6; STW5 KVIII: 99,8% ± 80,2; STW5 KIX: 121,4% ± 64,6). Single extracts did not reproduce the effect of the fixed combinations STW5 and STW5-II

4 Discussion

STW5 (Iberogast®) was shown to be effective in a TNBS model of intestinal inflammation via Adenosine A2A receptor activation, and hence inhibition of the TNF-alpha pathway. Furthermore, STW5 increased IL-10 levels in mucosal preparations of mice and reduced TNFα secretion in LPS-induced human monocytes [114, 115]. In a DSS model of inflammation STW5 was able to reduce biochemical markers and attenuated histological parameters. Nevertheless, its influence on the epithelial cells and their contribution to the inflammation process has yet to be investigated.

In the present study, we have evaluated the effects of the clinically established fixed combination preparation STW5 (Iberogast®) and the modified STW5-II extract combination, which contains only 6 of the 9 herbal extracts in STW5, for their immunomodulatory effects on human colonic epithelial cells and the underlying molecular mechanisms.

Cell models can be used to mimic an inflammatory state usually by treating the cells with an appropriate stimulus [116, 117]. To avoid artefacts, we used the normal human colon cell line NCM460 [118] rather than colon cancer cell lines. Therefore, we examined whether NCM460 is responsive to stimuli and useful as a model of inflammation. As inflammatory stimulus, a cytokine mixture consisting of TNFα, IL-1β and IFNγ was used. These cytokines are known to be released in inflammatory bowel disease [119, 120] and are effective in inducing inflammatory reactions in cell models [121]. First, we profiled the cytokine release of the cells by a membrane-based sandwich immunoassay. NCM460 cells were able to respond and secrete a variety of chemokines, some of which are known to be involved in chemotaxis and were linked to IBD. We decided to focus on IL-8; MCP-1; I-TAC and IP-10, as those were the ones most strongly modulated in our experimental setting. IL-8 is a powerful neutrophil chemoattractant and activator, whose levels are increased in the mucosa of IBD patients [122]. It is also an established inflammatory marker in other cell lines [123-125]. Levels of MCP-1 were shown to be increased in IBD [126] and were associated with the response towards infliximab therapy [127]. I-TAC facilitates the development of Th17 cells and stimulates Th1 cells to produce IL-6 [128]. Human intestinal cell lines were shown to produce I-TAC after cytokine stimulation [129]. IP-10 plays a role in the inflammatory process, as it was shown to be increased in mucosal biopsies from patients with UC or CD [130] and was induced *in vitro* after IFNγ stimulation in epithelial cell lines [107, 129]. It also presents a

promising target for treatment of IBD, as a phase II study demonstrated efficacy of anti-IP-10 antibodies in patients suffering from moderate to severe UC [131].

To investigate a dose-effect relationship, we tested the effect of the herbal preparations on chemokine release via ELISA. STW5 was able to reduce IL-8 in a dose dependent manner, while STW5-II pre-treatment led to an increased secretion at low concentrations and decreased release at higher ones. *Chelidonium majus L.* (STW5 KIX) was the only single extract that significantly reduced IL-8 levels. MCP-1 release from colon cells was significantly reduced after pretreatment with both STW5 and STW5-II. Furthermore, almost all single extracts except for *Glycyrrhiza glabra L.* (STW5 KIV) decreased the secretion of MCP-1 significantly, but not as effectively as STW5 or STW5-II. Both combination preparations reduced the cytokine induced IP-10 secretion. Here STW5-II was a lot more effective than STW5. Interestingly, STW5-II showed dose-dependent effects on the IP-10 release over 1-20µg/ml, but no great difference between 5 and 10µg/ml and an immense decrease between 10 and 20µg/ml. This inconsistency indicates further mechanisms of IP-10 inhibition which occurs only in high doses of STW5-II. All single extracts inhibited IP-10 release comparable to STW5 but not to STW5-II. The secretion of I-TAC was iinhibited dose-dependently over 1-20µg/ml for both extract combinations. Again, chemokine levels were stable over 5-10µg/ml for STW5-II, supporting the theory of various mechanisms involved in the inhibition.

To examine which signaling pathway might be involved in the anti-inflammatory mechanism of STW5 and STW5-II, we first identified molecules that are affected by the cytokine treatment. Since we used TNFα, IL-1β and IFNγ as stimuli, activated pathways should lead to the activation of transcription factors like AP-1, NF-κB and STAT proteins. Activation of the IL-1β receptor (IL-1R) or the TNFα receptor (TNFR) results in recruitment of adaptor proteins which in turn activate secondary proteins. The mitogen-activated protein kinase kinase 3 (MEKK3) is a central kinase in both receptor signaling pathways and is able to activate the MAPKs p38 and c-Jun NH(2)-terminal kinase (JNK) [132, 133]. JNK subsequently activates the transcription factor AP-1 [134]. MEKK3 also plays a pivotal role in activating the IκB kinase (IKK, a kinase that phosphorylates the Inhibitor of kappa B (IκB) and thus causes its degradation and subsequent activation of NF-κB). Other adaptor proteins in the TNFα signaling pathway also lead to activation of IKK. Activation of the IFNγ receptor results in the activation of STAT proteins [135]. We decided to focus on these key points in the signaling pathways to gain insights in the molecular mechanisms of STW5. Most of the

investigated proteins were shown to be involved in IBD. The MAPK p38 is a key regulator in the activation process of different transcription factors [133, 136, 137].

AP-1 regulates cell responses to a variety of stimuli including cytokines [138]. Because AP-1 was previously shown to be involved in IL-8 release *in vivo* and *in vitro [139, 140]*, we examined if AP-1 is also induced with the cytokine mixture in our experimental setting. The cytokine mixture was not able to increase the activity of AP-1, indicating that it is not responsible for the cytokine release.

NF-κB is a central transcription factor involved in many cell functions including the regulation of cytokine expression. Cytokines found to be involved in IBD like IL-8 are influenced by NF-κB. Inhibition of the latter was shown to attenuate signs of experimental colitis in mice [141]. To determine the activity of NF-κB in NCM460 cells, protein extracts were added to plates in which multiple copies of double stranded consensus DNA were immobilized. Induction of NF-κB was insufficient in this setting, but the results hinted to some activity of STW5 and previous literature data showed a potent effect on NF-κB in colon adenocarcinoma cells (Bonaterra et al. 2013). Therefore, we further examined the influence of cytokines and extract in commercial available HEK-Blue™ Null1 cells which express the SEAP (secreted alkaline phosphatase) reporter gene under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1 binding sites. In these cells, NF-κB activity was sufficiently increased after cytokine treatment. Nevertheless, STW5 and STW5-II had only week effects, not sufficient to explain the inhibition of cytokine release. We concluded that NF-κB activity is not affected by the plant extract combinations in our model.

p38 mitogen-activated protein kinase (MAPK) signaling pathways were shown to be involved in inflammatory bowel disease and can be induced by inflammatory cytokines like IL-1β and TNFα [142]. Furthermore, p38 is involved in the activation of transcription factors. To measure the activation of p38, protein extracts were precipitated with immobilized anti-pp38 antibody beads. Activity was measured by the ability of p38 to phosphorylate ATF-2. As expected, the treatment with cytokines increased the activity of p38 which peaked after 10 min induction time. Pretreatment with the extract combinations showed no effect at all during the activation of p38. This indicates that p38 is not affected by STW5 or STW5-II.

STAT proteins are cytoplasmic transcription factors, which dimerize and translocate to the nucleus upon phosphorylation, where they interact with DNA and induce transcription. STAT1 protein expression and phosphorylation were increased in mucosal samples from IBD

patients [143]. Also, increased phosphorylation of STAT1 was observed in cell models of IBD [144, 145]. STAT1 phosphorylation can be induced by IFNγ, making STAT1 a possible target of the plant extract combinations. We showed that pSTAT1 levels increased after cytokine treatment. Furthermore, pretreatment with STW5 or STW5-II revealed a dose-dependent inhibition over 1-20µg/ml. STAT3 and STAT5 were also inhibited by the combination preparation. However their induction was rather week in our experimental setting, therefore their contribution to the overall inflammatory state is probably minor.

Thus, inhibiting STAT1 might explain the reduced cytokine expression, which in turn could partly explain the anti-inflammatory effects of STW5 & STW5-II.

4.1 Conclusion

Human epithelial cells react to inflammatory stimuli (*e.g.* Th1 cytokines) by releasing a subset of chemokines, which in turn attract immune cells such as macrophages. Our study revealed that the herbal preparation STW5 (Iberogast®) and the modified investigational combination STW5-II reversed these effects without affecting NF-κB. Further studies showed that the extracts reduced tStat1 phosphorylation. We conclude that these herbal preparations may have a therapeutic potential for patients with IBD.

5 Materials and Methods

5.1 Chemicals and equipment

STW5 extracts were kindly provided by Steigerwald Arzneimittelwerk GmbH (Darmstadt, Germany) as Lyophyllisates. Stock solutions were prepared by solving the lyophylisates in DMEM supplemented with 1% NEAA according to the percentage of the dry residue. Resolved extracts were used for two weeks and stored at room temperature.

Table 2: STW extracts and percentage in the combination extracts

Code	Plant origin of extract	% in STW5	% in STW5-II
STW5	Combination of nine plants	-	-
STW5-II	Combination of six plants	-	-
STW6	Iberis amara	15%	15%
STW5 KII	Menthae X piperitae	5%	10%
STW5 KIII	Chamelia recutita	20%	30%
STW5 KIV	Glycyrrhiza glabra	10%	10%
STW5 KV*	Angelica archangelica	10%	-
STW5 KVI	Carum carvi	10%	20%
STW5 KVII	Silybum marianum	10%	-
STW5 KVIII	Melissa officinalis	10%	15%
STW5 KIX	Chelidonium majus	10%	-

Table 3: cell culture media, reagents and disposable material

Product	Supplier
6-well cell culture microplate, clear, Nunclon®	Thermo Scientific, Germany
12-well cell culture microplate, clear, Nunclon®	Thermo Scientific, Germany
96-well, flat bottom cell culture microplate, clear, Nunclon®	Thermo Scientific, Germany
96-well, flat bottom cell culture microplate, white, Nunclon®	Thermo Scientific, Germany
Cell culture flasks (25 cm2), Nunclon®	Thermo Scientific, Germany
Cell culture flasks (75 cm2), Nunclon®	Thermo Scientific, Germany
Cell scraper	Greiner Bio-One, Germany

Centrifuge tube (15 ml)	Sarstedt, Germany
Centrifuge tube (50 ml)	Sarstedt, Germany
Cover Glass 24 × 32 mm	VWR International, Austria
DMEM, High Glucose, GlutaMAXTM, Pyruvate	Life Technologies, Germany
DPBS, no calcium, no magnesium	Life Technologies, Germany
Fetal Bovine Serum (FBS)	Life Technologies, Germany
L-Glutamine	PAA Laboratories, Germany
Microplates 96-well for ELISA	R&D Systems, Germany
Micro tubes (1.5 mL, 2.0 mL)	Sarstedt, Germany
Penicillin (10000 U/mL)/Streptomycin (10000 µg/mL)	Life Technologies, Germany
Plate seal foils	R&D Systems, Germany
Pipette tip (10, 200 and 1250 µL)	Sarstedt, Germany
Pipette with tip (5 and 10 mL)	Greiner BIO-ONE, Germany
Roti® PVDF blot membrane (0.45 µm)	Roth, Germany
RPMI 1640	Life Technologies, Germany
Trypsin-EDTA 0.25% (1×), phenol red	Life Technologies, Germany

Table 4: Chemicals, dyes, antibodies, enzymes and kits

Product	**Supplier**
30% acrylamide/bis solution 29:1	Bio-Rad, Germany
5 × Hot Start Taq EvaGreen qPCR Mix (no ROX)	Axon Labortechnik, Germany
Ammonium persulfate (APS)	Sigma-Aldrich, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, MO, USA
CCL2/MCP-1 DuoSet ELISA	R&D Systems, Germany
CXCL8/IL-8 DuoSet ELISA	R&D Systems, Germany
CXCL10/IP-10 DuoSet ELISA	R&D Systems, Germany
CXCL11/I-TAC DuoSet ELISA	R&D Systems, Germany
Bromophenol Blue	Merck, Germany
Complete Mini Protease Inhibitor	Roche, Germany
Cignal AP1 Reporter (luc) Kit	Quiagen, Netherlands
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Germany
Dual-Luciferase® Reporter Assay System	Promega, Wisconsin, USA
Ethanol (EtOH)	Sigma-Aldrich, Germany
Glycerol	AppliChem, Germany
Glycine	AppliChem, Germany
HRP-linked anti-mouse IgG	Cell Signaling, Germany
HRP-linked anti-rabbit IgG	Cell Signaling, Germany
Human Cytokine Array Panel A	R&D Systems, Germany
Hydrochloric Acid 37% (HCl)	AppliChem, Germany
IL1B Recombinant Human Protein	Life Technologies, CA, USA
LuminataTM Classico Western HRP substrate	Merck Millipore, Germany
MagicMark™ XP Western Standard	Life Technologies, CA, USA
Methanol	J. T. Baker, NJ, USA
M-PER® Mammalian Protein Extraction Reagent	Thermo Scientific, IL, USA
Normocin™	Invivogen, France
p38 MAP Kinase Assay Kit (Nonradioactive)	Cell Signalling, Germany
Phorbol 12-myristate 13-acetate	Sigma-Aldrich, Germany
Phospho-Stat (Ser473) (D9E) XP® Rabbit mAb	Cell Signalling, Germany
PhosSTOP Phosphatase Inhibitor Cocktail Tablets	Roche, Germany
QUANTI-Blue™	Invivogen, France
Recombinant Human TNF-alpha Protein, CF	R&D Systems, Germany
Recombinant Human IFN-gamma Protein, CF	R&D Systems, Germany
Resazurin	Sigma-Aldrich, Germany
Sodium bicarbonate	Sigma-Aldrich, Germany
Sodium chloride (NaCl)	Grüssing, Germany
Sodium dodecyl sulfate (SDS)	J. T. Baker, NJ, USA
Sodium hydroxide (NaOH)	Sigma-Aldrich, Germany
Tetramethylethylenediamine (TEMED)	AppliChem, Germany

TransAM® Nf-κB Transcription Factor ELISA Kits	Active Motif, Belgium
Tris (hydroxymethyl) aminomethane (Tris)	AppliChem, Germany
Trypsin (modified, sequencing grade)	Promega, WI, USA
Tween20	Sigma-Aldrich, Germany
Ultravision Quanto Detection System HRP	Thermo Scientific, Germany
Water, nuclease-free	Thermo Scientific, Germany
β-Actin (13E5) rabbit mAb	Cell Signalling, Germany
β-Mercaptoethanol	AppliChem, Germany
Zeocin™	Invivogen, France

Table 5: Technical equipment and software

Device	**Supplier**
Adobe Photoshop CS5 v 12.0.0.2	Adobe Systems, USA
Agilent 2100 Bioanalyzer	Agilent Technologies GmbH, Germany
Alpha Innotech FluorChem Q system	Biozym, Germany
Centrifuge 5424	Eppendorf, Germany
ChemSketch	ACD, Canada
Coulter Counter Z1	Beckman Coulter, Germany
ENVAIR eco air V 0.8m vertical laminar flow workbench	ENVAIR, Germany
Eppendorf 8-channel electric pipette	Eppendrof, Germany
FluorChem® Q imaging system	Alpha Innotech, CA, USA
Forma Steri-Cult 3310 CO_2-Incubator	Thermo Scientific, Germany
Heraeus Cytospin	Thermo Scientific, Germany
Heraeus Fresco 21 microcentrifuge	Thermo Scientific, Germany
Heraeus Labofuge 400 R centrifuge	Thermo Scientific, Germany
ImageJ2 v 2.0 Snap 1.49d	NIH, MD, USA
Infinite M2000 Pro™ plate reader	Tecan, Germany
Maxisafe 2020 laminar flow hood	Thermo Scientific, Germany
Microsoft Office	Microsoft Corporation, WA, USA
Milli-Q ultrapure water purification system	Millipore, Germany
Mini-PROTEAN® Tetra Cell	Bio-Rad, Germany
NanoDrop 1000 Spectrophotometer	PEQLAB, Germany
Neubauer counting chamber	Marienfeld, Germany
Optika XDS-2 trinocular inverted microscope	Optika, Italy
Precisa BJ2200C balance	Precisa Gravimetrics AG, Switzerland
REAX 2000 vortexer	Heidolph, Germany
Safe 2020 Biological Safety Cabinets	Thermo Scientific, Germany
Sartorius R 160 P balance	Sartorius, Germany
Sonorex RK 102 H Ultrasonic Cleaning Unit	Babdelin, Germany
Spectrafuge™ Mini Centrifuge	Labnet, Germany
SUB Aqua 26 waterbath	Grant Scientific, Germany
Thermomixer comfort	Eppendorf, Germany
TopMix vortexer	Fisher Scientific, Germany
VMD 1.9 software	University of Illinois at Urbana Champaign, IL, USA

5.2 Cell culture

All cell lines were maintained in a humidified environment at 37 °C with 5% CO2. Passaging was performed twice per week. Adherent cells were detached by treatment with 0.25%

trypsin/EDTA solution (Life Technologies). All experiments were performed on cells in the logarithmic growth phase. Cell counting was carried out by the use of Coulter Counter Z1 (Beckman Coulter) or a Neubauer counting chamber (Marienfeld).

CaCo2 cell line was purchased from American Type Culture Collection (Manassas, USA). Cells were cultivated in complete DMEM culture medium with GlutaMAX (Invitrogen) supplemented with 10% fetal bovine serum, L-glutamine (2 mM), and 1% of a 10,000 U/mL penicillin G and 10 mg/mL streptomycin. For experiments cells were maintained in DMEM culture medium without phenol supplemented with 1% NEAA.

NCM460 cell line was purchased from Incell Cooperation (San Antonio, USA). Cells were cultivated in complete M3BaseF culture medium (Incell) supplemented with 10% fetal bovine serum, L-glutamine (2 mM), and 1% of a 10,000 U/mL penicillin G and 10 mg/mL streptomycin. Prior to experiments, cells were cultivated in RPMI 1640 medium with 2 mM L-glutamine (Invitrogen) supplemented with 10% fetal bovine serum and 1% of a stock solution of 10,000 U/mL penicillin G and 10 mg/mL streptomycin. During experiments cells were maintained in DMEM culture medium without phenol supplemented with 1% NEAA.

HEK-Blue™ Null1 cells were purchased from Invivogen (Toulouse, France). Cells were cultivated in complete DMEM culture medium with GlutaMAX (Invitrogen) supplemented with 10% fetal bovine serum, L-glutamine (2 mM), and 1% of a 10,000 U/mL penicillin G and 10 mg/mL streptomycin, 100 mg/ml Normocin™ and 100mg/ml selective antibiotic Zeocin™.

5.3 Cytotoxicity assay

Cell viability was evaluated by the resazurin assay. This test is based on the reduction of the indicator dye, resazurin, to the highly fluorescent resorufin by viable cells. Nonviable cells rapidly lose the metabolic capacity to reduce resazurin and, thus, do not produce a fluorescent signal [169].

In brief, adherent cells were harvested with 0.25% trypsin/EDTA (Invitrogen) and diluted to a final concentration 5×10^4 cells/mL. One hundred micro-liters of the cell suspension were sowed into the wells of a 96-well culture plate one day before treatment. For suspension cells, 2×10^4 cells were directly sowed prior to the assay in a 96-well culture plate in a total volume of 100 µL for each well. Marginal wells were filled with 200 µL of pure medium in order to minimize effects of evaporation. Besides, wells filled with medium served as the negative

control to determine background fluorescence that may be present. Then cells were treated with different concentrations of the extracts of interest alone or combined. After 24 or 72 h, 20 µL resazurin (Sigma-Aldrich) 0.01% w/v in ddH2O was added to each well and the plates were incubated at 37 °C for 4 h. Fluorescence was measured on an Infinite M2000 Proplate reader (Tecan) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each assay was done at least two times, with six replicates each. The cytotoxic effect of the treatment was determined as percentage of viability and compared to untreated cells. The toxicity of extracts was determined by means of the formula:

$$cell\ viability\ (\%\ of\ control) = \frac{absorption\ from\ treated\ cells - absorption\ from\ Medium}{absorption\ from\ untreated\ cells - absorption\ from\ Medium} * 100\%$$

The calculated cell viability (y-axis) was plotted against the log drug concentration (x-axis) using Microsoft Excel. The obtained curve was used to determine nontoxic concentrations of the extracts, which could be used in further experiments

5.4 Induction of Inflammation

Adherent cells were harvested with 0.25% trypsin/EDTA (Invitrogen) and seeded at an concentration of 20000cells/cm² in an appropriate plate. After three days of growth cells were pretreated or not with diluted stock solutions of STW extracts for 4h. Subsequently the extract containing medium was removed. Inflammation was induced by adding DMEM supplemented with 1% Non-essential amino acids (NEAA) containing a human cytokine mixture (CM) consisting of 10ng/ml TNF-α, 5ng/ml IL-1β and 10ng/ml IFN-γ to the cells. Incubation was performed for various time periods according to the following test

5.5 Proteom Profiler

Cells were treated like described before and induced with CM for 24h. Supernatants were collected and purified by centrifugation. The Proteome Profiler Human Cytokine Array Kit, Panel A (R&D Systems) is a membrane-based sandwich immunoassay. Supernatants were mixed with a cocktail of biotinylated detection antibodies for 1h and then incubated overnight at 4°C with the array membrane which is spotted in duplicate with capture antibodies to specific target proteins. Captured proteins were visualized using LuminataTM Classico Western HRP substrate (Merck Millipore). The produced signal was detected with Alpha Innotech FluorChem Q system (Biozym) and was proportional to the amount of analyte

bound. Density analysis of the signals was performed with ImageJ Software (NIH, MD, USA). All values were normalized with the internal reference signal.

5.6 Enzyme linked immunoassay (ELISA)

ELISA was performed with the DuoSet Kits from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany) according to manufactures instructions. In brief, cells were treated like described before and induced with CM for 24h. Supernatants were collected and purified by centrifugation. Microplates(R&D Systems) were incubated with capture antibody overnight to certify the antibodies attach to the well bottom. Plates were washed three times and incubated with undiluted sample or reference concentrations of the examined protein. Plates were washed again three times and incubated with biotinylated detection antibody. Subsequent to the next washing step, streptavidin-HRP was added to each well. After 20min incubation, the plates were washed and developed with a mixed color solution for another 20min before stop solution was added to each well. During all incubation steps the microplates were covered with sealing foil (R&D Systems). Optical density was determined using an Infinite M2000 Proplate reader (Tecan) set to 450nm with a wavelength correction at 540nm. The inhibitory effect was described as the percentage of the optical density compared to non-treated inflammatory cells. Non-inflammatory cells were also normalized in this way. Each assay was done at least two times, with three replicates each.

5.7 HEK-Blue Null 1 reporter cell assay

HEK-Blue™ Null1 cells express the SEAP (secreted alkaline phosphatase) reporter gene under the control of the IFN-β minimal promoter fused to five NF-kB and AP-1 binding sites. Stimulation of HEK-Blue™ Null1 cells with an NF-kB activator will induce the production of SEAP. Levels of SEAP were detected by using the alkaline phosphatase reagent Quanti-Blue™. The cells were cultured, according to the manufacturer's recommendations. The cells were treated with varying concentrations of STW5 or STW5-II for 4 h and then induced with TNF-α (10 ng/mL) for 24h at 37°C. Quanti-Blue™ reagent was added to the isolated supernatant of the cells and incubated 4h at 37 °C. Absorbance was measured on an Infinite M2000 Proplate reader (Tecan) with a wavelength ai 630nm. Nf-kappaB activity was determined as percentage of signal intensity and compared to cytokine treated cells. Each assay was performed with three replicates.

5.8 Protein extraction

NCM460 cells were seeded at 20,000 cells/cm² in a 6-well plate and grown for 3 days in RPMI media supplemented with 10% FBS , 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 95% humidified atmosphere with 5% CO_2. Cells were treated with STW5 or STW5-II and inflammation was induced for 30min as described before. Cells were washed three times with DPBS buffer, incubated with cell lysis buffer (New England biolabs) for 5 min and collected. Lysated cells were sonicated 4 times for 5 min and centrifuged at 14,000×g for 10 min. The supernatant was collected. Protein content of the supernatant was determined using NanoDrop 1000 Spectrophotometer with standard protein setting.

5.9 Western Blot

Protein aliquots (60 µg/well) were loaded on a discontinuous SDS-PAGE (Table5) for separation. After blotting proteins to a PVDF-membrane, the membrane was incubated overnight with primary antibody. After washing with TBST three times for 10min, membranes were incubated for 1 h with secondary HRP-linked antibody (New England Biolabs). Before the proteins were detected with Luminata^{TM} Classico Western HRP substrate (Millipore), the membrane was washed again three times for 10min with TBST. Pictures of the membranes with chemifluorescent signal were taken using Alpha Innotech FluorChem Q system (Biozym, Germany). Documentation of the pictures was performed with ImageJ2 v 2.0 Snap 1.49d software (NIH, MD, USA).

Table 6: Recepine for discontnous SDS-PADE

Compound	**Loading Gel [ml]**	**Stacking Gel[ml]**
H_2Odd	7,1	6,05
Tris-HCL	3,75 (pH=8,8	2,5 (pH=6,8)
SDS 20% in H_2Odd	0,075	0,05
30% Acrylamid	4	1,35
Temed	0,01	0,01
APS 10% in H_2Odd	0,075	0,05

5.10 TransAM ELISA for NF-κB activity

NCM460 cells were treated and Inflammation was induced as described before.. Proteins were extracted as described before. Protein aliquots of whole cell extracts (60µg) were added to a 96 plate in which multiple copies of double stranded consensus DNA were immobilized. Active Nf-κB binds to specifically to these bound oligonucleotides. A primary antibody specific for an epitope on the bound and active form of Nf-κB is then added followed by subsequent incubation with secondary antibody and Developing Solution. Optical density was measured using the Infinite M2000 Pro™ plate reader (Tecan) at a wavelength of 450 nm. Nf-κB activity was determined as percentage of signal intensity and compared to cytokine treated cells. Each assay was performed with three replicates.

5.11 AP1 reporter assay

NCM460 cells were transfected with cignal AP1 reporter kit (Quiagen) according to manufacturer's instruction. In brief, cells were suspended and added to a mixture of reporter and transfection reagent. After 24 hours of transfection, medium was changed to assay medium (Opti-MEM + 0.5% FBS + 0.1mM NEAA + 1mM Sodium pyruvate + 100 U/ml penicillin + 100 µg/ml streptomycin) and cells were treated with 10 ng/ml of Phorbol 12-myristate 13-acetate (PMA) or cytokine mixture in mentioned concentrations. PMA is a known inducer of AP1 activity and was used as a positive control. The AP1 reporter contains a mixture of inducible AP1-responsive firefly luciferase construct and constitutively expressing renilla luciferase construct (40:1). The assay was developed by using Dual-Luciferase Reporter Assay System from Promega. Cells were lysed within the wells and luciferase assay reagent was added. Luminescence was measured on an Infinite M2000 Pro™ plate reader (Tecan). After measuring firefly luciferase activity, the stop solution was added and the measurement was repeated to measure renilla luciferase activity. Relative Luciferase activity was determined as luminescence signal of firefly luciferase activity normalized to the signal of renilla luciferase activity.

5.12 MAPK p38 activity assay

NCM460 cells were grown for three days before treatment. For kinetic measurements, cells were treated with the cytokine mixture for different periods. For evaluation of STW5 effects, cells were pretreated with extracts for 4h before treatment with cytokine mixture. Proteins were extracted as described before. Protein aliquots (200ng) were incubated overnight with

immobilized anti-pp38 antibody beads. The immunoprecipitates were washed and incubated with ATF-2 fusion protein and ATP for 30 min at 30°C. ATF-2 is a known substrate for p38. The level of pATF-2 was measured via western blot as described before.

5.13 Statistical Analysis

Results are represented as mean ± SEM. *P*-values were calculated by Student's *t*-test. Differences were considered as significant at $P < 0.05$.

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7 Publications

M. Schneider, T. Efferth , H. Abdel-Aziz, „Anti-inflammatory Effects of Herbal Preparations STW5 and STW5-II in Cytokine-Challenged Normal Human Colon Cells," *Front Pharmacol*. 2016 Oct 26;7:393. eCollection 2016.

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	2016 – 2016	Pharmacist at Stern Apotheke Mainz
	2012 – 2012	Intership at Fliederbergapotheke in Darmstadt
	2011– 2012	Intership at Merz Pharmaceuticals in Frankfurt.
Languages:	German	Mother tongue.
	English	Fluent in speaking, reading and writing.
Computer Skills:		MS Windows, Mac OS, MS Office and Internet.

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