UNIVERSITY OF FORWARD THINKING WESTMINSTER

Exploring In Vitro and In Vivo Pharmacology of Echinacea Purpurea (L.) Moench

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Contextual Statement

Doctoral Thesis

Based on Published Work

Exploring In Vitro and *In Vivo* Pharmacology of *Echinacea Purpurea* (L.) Moench

Final Version

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I, Roland Schoop, confirm that the work presented in this thesis is my own.
Where contributions or information has been derived from other sources, I confirm that this has been indicated in the commentary.

It is never too late to become what you might have been

George Eliot

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I finally dedicate this work to my parents, who gave me the opportunity to become a researcher and to study life sciences, the most fascinating topic ever.

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Contained Tables and Figures

List of Abbreviations

AdV	Adenovirus
AP	Associated Press
CoV	Coronavirus
DMSO	Dimethyl Sulfoxide
EDQM	European Directorate for the Quality of Medicines & Healthcare
EF	Echinaforce®
ELISA	Enzyme Linked Immunosorbent Assay
EMA	European Medicine Agency
ESCOP	European Scientific Cooperative on Phytotherapy
HA	Haemagglutinin
HMP	Herbal Medicinal Product
HMPC	Herbal Medicinal Product Committee
HPLC	High Performance Liquid Chromatography
HSV	Herpes Simplex Virus
ICAM	Intracellular Adhesion Molecule
ICD	International Classification of Diseases
IL	Interleukin
IFN	Interferon
IUCN	International Union for Conservation of Nature
LPS	Lipopolysaccharide
MIC	Minimal Inhibitory Concentration
MPV	Metapneumovirus
NCCAM	National Center for Complementary and Integrative Health
PBMC	Peripheral Blood Mononuclear Cells
Pfu	Plaque Forming Unit
Ph Eur	Pharmacopoea Europaea
PI	Parainfluenza
RV	Rhinovirus
RSV	Respiratory Syncytial Virus
RTI	Respiratory Tract Infections
THMP	Traditional Herbal Medicinal Products
TNF	Tumor Necrosis Factor
WHO	World Health Organization

WFO	World Flora Online

Abstract

Background

Echinacea purpurea has long been used for the treatment and prevention of respiratory tract infections. The medicinal plant has originally been discovered by the Native American population, who squeezed the sap from fresh plant or chewed dried roots. Today, commercially available preparations vary greatly in terms of Echinacea concentration, manufacturing methods, plant parts used, et cetera. This heterogeneity results in tremendously fluctuating bio-activities between products. Some of these products finally failed to show efficacy in large clinical studies raising principal questions about the value of the medicinal plant.

Objectives

The aim of this doctoral work was to explore *in vitro* and *in vivo* pharmacological effects, as well as clinical efficacy in prevention and acute treatment of respiratory tract infections for a single, phytochemically characterized and standardized *Echinacea purpurea* extract (Echinaforce®, EF).

Methodology

In vitro antiviral and anti-inflammatory (immune-modulatory) activities of EF were researched studying the most relevant respiratory pathogens including influenza, respiratory syncytial virus (RSV) or coronavirus (CoV) and using cytokine assays for interleukins IL-6 or IL-8 in airway epithelial cells. Test tube experiments were sought to be confirmed in organotypic tissues and upon peroral administration of EF *ex vivo*, whereas bio-availability of alkylamides in the extract was investigated to estimate systemic relevance. Two clinical studies aimed to investigate efficacy of EF for the treatment and the prevention of respiratory tract infections and subanalyses served to confirm above proposed pharmacological actions *in vivo*. Finally, EF's therapeutic potential beyond its traditional use for colds and flu was researched by looking at the prevention of bacterial superinfections including bronchitis or pneumonia.

Results

EF was demonstrated to inhibit a wide range of respiratory agents, showing a primary specificity to enveloped viruses (e.g. influenza, RSV and CoV) *in vitro* and in a clinical prevention study. The extract modulated overexpression of inflammatory cytokines in epithelial cells and in the presence of respiratory pathogens. It is thereby expected not only to impact occurrence but also the symptomatic development of viral infections. Alkylamides were found to play a role in the systemic immune-modulation as bio-availability was demonstrated after peroral ingestion of EF.

Next, EF was administered for the treatment of clinically diagnosed influenza (flu) to find non-inferiority to the gold-standard therapy Oseltamivir in this indication. Comparable recovery rates were observed for

virologically confirmed influenza infections, demonstrating clinical efficacy in the treatment of viral respiratory infections.

Another large clinical study investigated EF for the prevention of respiratory tract infections. In the placebo group a total of 188 cold episodes were identified, which lasted for 850 days in comparison to 149 episodes and 672 sick days with EF extract ($p < 0.05$). Enveloped viruses (influenza, RSV and CoV) were found in 24 patients with EF and in 47 with placebo ($p < 0.05$).

In vitro EF reduced the expression of bacteria-binding receptors (e.g. of intracellular adhesion molecule, ICAM-1) on airway epithelial cells after infection with respiratory viruses. Thereby, EF prevented the attraction of pathogenic bacteria potentially inducing bacterial superinfections of initial viral infections.

Conclusion

The evidence generated by this PhD work substantiates the medicinal value of *Echinacea purpurea* for the treatment and prevention of respiratory tract infections. By focussing on a single, chemically standardized extract (Echinaforce®) problems with heterogeneity between Echinacea products could be overcome to reach more consistent conclusions.

EF exhibits antiviral and anti-inflammatory effects to not only prevent occurrence but also the symptomatic development of infections. Alkylamides are bio-available and capable to systemically modulate the immune response. Bacteria-binding receptors on the epithelium are controlled with EF to finally prevent respiratory complications including bronchitis or pneumonia.

Aims and Objectives

The aim of this doctoral work was to explore *in vitro* and *in vivo* pharmacological effects, as well as clinical efficacy in prevention and acute treatment of respiratory tract infections for a single, phytochemically characterized and standardized *Echinacea purpurea* extract (Echinaforce®, EF).

The following aims and objectives were to be achieved and answered for Echinaforce® (EF) extract within this PhD work:

- Exploring the antiviral activity *in vitro*
- Exploring the anti-inflammatory (immune-modulatory) activity *in vitro*
- Confirming antiviral and immune-modulatory actions *in vivo* and *ex vivo*
- Assessing bioavailability of alkylamides after peroral administration of EF extract
- Assessing clinical efficacy of EF extract in preventing RTIs
- Assessing clinical efficacy of EF extract in treating influenza illness
- Assessing the potential of EF extract in preventing RTI complications (bronchitis or pneumonia) *in vivo* while
- Exploring the underlying mode-of-action for prevention of RTI complications *in vitro*

Original Contribution to Knowledge

The evidence accrued in this PhD work materially contributes to the modern scientific understanding of *Echinacea purpurea* and substantiates its medicinal value for the treatment and prevention of respiratory tract infections. By focussing on a single, chemically standardized extract (Echinaforce®) problems with Echinacea products' heterogeneity could be overcome to achieve more consistent conclusions.

The provided *in vitro* and *in/ex vivo* results suggest antiviral, anti-inflammatory and immune-modulatory activities. Two clinical studies were carried out demonstrating efficacy in the prevention and treatment of respiratory tract infections under randomized, double-blind, placebo-/active controlled conditions. Finally, EF showed potential in preventing bacterial complications of initial viral infections through regulation of bacteria-binding receptors on epithelial cells.

Maybe the most relevant outcome of this PhD work presents the unspecific activity of EF against a broad range of enveloped viruses. The current Covid-19 pandemic shows that nature harbours a plethora of pathogens for which the medical armamentarium has no immediate solution. Infections can globally spread within weeks and effective vaccines or therapies are not timely available. Readily accessible herbal treatments like Echinacea may in reality provide an immediate, affordable and effective therapeutic solution.

1 Introduction

Herbal medicine has always been essential in health care and today still plays an important role in the treatment and prevention of illnesses (Piletti et al., 2001). In antiquity no distinction between food, food-supplements, spices and therapeutic agents was found - nutrition and medicine were not seen as strictly separate entities (Fürst and Zündorf, 2015; Engebretson et al., 2002). Today, this distinction is very well developed and established as a scientific and political paradigm. Unprocessed plants may still be used as part of nutrition but preparations thereof fall into a tightly regulated sector of "plant-products". Even more so if they are declared as being used for medicinal purposes with specific indications and therapeutic claims, i.e. herbal medicinal products (HMPs, Gupta, 2015).

Such herbal medicinal products may gain market access (registration or licence) as "medicines" if produced and vended in compliance with internationally applicable standards (*Pharmacopoea Europaea, Ph.Eur.*) and monographs issued by the European Medicine Agency (Herbal Medicinal Product Committee, HMPC). A majority of HMPs achieve the level of a traditional HMP (THMP) due to a "plausibility" of efficacy as evidenced by 30 years in-use rather than any sound clinical evidence (Cox and Roche, 2004). As a consequence, many questions and uncertainties pertaining to pharmacology and effectiveness remain, leading authorities and medicine agencies to implement increasingly cautious restrictions regarding the use of plant-products due to lack of evidence.

For example, the HMPC (a subsection of the European Medicine Agency, EMA) granted registrations for the purple coneflower [*Echinacea purpurea* (L.) Moench] under the following precondition only (HMPC, 2015):

- Preventive continuous use of maximal 10 days
- No use in paediatric population
- Autoimmune illnesses stated as a contraindication

Limitations were introduced because of open questions concerning safety / pharmacology (e.g. Echinacea as a potential pure immune-stimulant), lack of data on use in children on the one side, and on the other side only limited evidence regarding its efficacy (Kreft and Razinger, 2014). For instance, the assessment report argues that beyond a duration of 10 days treatment efficacy was not proven convincingly, which would be an essential requirement for granting extension. In contrast, earlier monographs by the European Scientific Cooperative on Phytotherapy (ESCOP) or by World Health Organization (WHO) more closely stayed with the historical medicinal use of this plant with a maximal duration of use of eight weeks and no limitations in the paediatric population (WHO, 1999; ESCOP, 2009). In essence, the example demonstrates the importance of scientific research in preserving the historical use of herbal medicinal products and to introduce them into the modern evidenced based medicine.

1.1 History and Therapeutic Use of Echinacea

The medicinal properties of Echinacea have originally been discovered by the indigenous population Americas (Bauer and Wagner, 1990). They mainly used *Echinacea purpurea* (L.) Moench*, E. pallida* (Nutt.) Nutt. and *E. angustifola* DC. var. angustifolia, all of which belong to the Compositae/Asteraceae plant family (Riddel, 1835; Mitchel, 1909 and WFO, 2020). Geographically completely distinct ethnic groups independently discovered very similar medicinal uses. Anaesthetic and anti-inflammatory actions were found beneficial not only for the treatment of cold-related symptoms like sore-throat but also for toothaches and snake bites (Wishart, 2007).

In the beginning of the 20th century eclectic physicians primarily used *E. angustifolia* roots for wounds, poisonous bites, stings and acute infections, even for sepsis as a "corrector of body fluids" (Felter, 1906 and Felter, 1989). Despite condemnation of Echinacea in 1909 by the American Medical Association of being unworthy of further consideration until more reliable evidence is present in its favour, Echinacea continued to be very popular and ranked first in sales statistics of Lloyd Brothers Inc. (Puckner, 1909; Lloyd, 1923 and Lloyd, 1917).

E. purpurea had not seen similar appreciation in Europe until the Swiss naturopath Alfred Vogel and Gerhard Madaus imported seeds from this species (Bauer and Wagner, 1990). With its introduction in Europe in the early fifties and the subsequent large-scale production, the today still dominant players were introduced in the pharmacy market: Echinaforce®, a hydroalcoholic extract (65% V/V) of *E. purpurea* herb and roots (95:5) by A. Vogel AG, Switzerland and Echinacin®, a hydrophilic pressed juice from *E. purpurea* herb (Madaus GmbH, Germany). Today, both *E. purpurea* and *E. angustifolia* are not listed in the International Union for Conservation of Nature's Red List of Threatened Species (IUCN, 2020).

Regardless of the broad historical use of Echinacea for a wide range of ailments, scientific research commenced exploring potential benefits in respiratory tract infections more specifically. In consequence, authoritative textbooks published by the HMPC, WHO and ESCOP mention beside the topical use for wounds, mainly the treatment and prevention of colds and flu.

1.2 Echinacea Product Heterogeneity

As shown by Gilroy and colleagues (2003), commercially available products containing Echinacea vary considerably in terms of

- plant species used (*E. purpurea, E. angustifolia* or *E. pallida*)
- source material quality (fresh, dried or lyophilized),
- plant parts used (above-ground herb and/or roots, whole plant)
- manufacturing techniques (dried powder, tea, alcoholic extractions, CO_2 extracts or pressed-juice)

An incredible heterogeneity results within available Echinacea products and their content of bio-active substances, which has analytically been investigated using High Performance Liquid Chromatography (HPLC) technique (Osowski et al., 2000; Tobler et al., 1994). The fact that some Echinacea preparations were given intra-muscular or intra-venously further complicates the comparability of products and undermines any general conclusion on efficacy for the medicinal plant (Barnes, 2005).

When considering the quality of Echinacea products a few classes of chemical markers are discussed to be pharmaceutically important (Bauer and Wagner 1990). Those comprise the caffeic acid derivatives echinacoside, verbascoside, cichoric and chlorogenic acid, or cynarin, flavonoids (e.g. quercetin, luteolin), aetheric oil, asteracea-specific polyacetylenes, polysaccharides, whereas alkylamides or echinacoside are relatively specific for the genus Echinacea and cichoric acid for the species *E. purpurea*. The latter were suggested as reliable markers to approach the quality of Echinacea within its particular indication and use (Bauer, 1995; Bauer and Wagner 1990; Barnes, 2005).

A systematic analysis of cichoric acid and alkylamides (e.g. dodeca-2E,4E,8Z,10E/Z tetraenoic acid isobutylamide, abbreviated as “tetraene”) found significant differences between plant species and plant parts used, lipophilicity of extractant and finally the underlying manufacturing process (Osowski et al., 2000). Tetraene concentration amongst spagyric / homeopathic dilutions often ranged below the detection limit (<0.1 μg/ml) whereas mother tinctures contained reasonable amounts between 2.2 and 60.5 μg/ml. Significant differences were even found within preparations, which are both proposed by the HMP Committee for the treatment of respiratory tract infections: hydrophilic pressed juices and lipophilic hydroalcoholic extractions (both derived from *E. purpurea*) yielded concentrations as low as 1.1 μg and up to 60.5 μg of tetraene per ml, respectively. Very similar quantitative variations resulted from the analysis of cichoric acid.

Bacterial impurity is another factor to be considered when assessing quality and pharmacology of natural products, in particular of Echinacea. Pugh and colleagues (2013) showed that macrophage activation observed *in vitro* was largely attributed to bacterial Braun-type lipoprotein and lipopolysaccharides (LPS) contained in certain Echinacea products. Similar biological effects were identified also for endotoxins, which seem to be quite abundant in preparations (Gertsch et al., 2004).

Impurities should be regarded critically as they tend to produce immune-stimulatory effects *in vitro,* including induction of inflammatory mediators TNF-alpha or interleukins (Ulevitch et al., 1995). These effects most reasonably should be classified as artefacts, because plant-derived products may contain these substances even if they are not taken for treatment of respiratory tract infections. Essentially, impurities are not Echinacea-specific but rather depend on or are introduced by manufacturing processes. Important articles even have pointed out that any unspecific induction of inflammatory meditators (e.g. TNF-alpha or IL-6) may produce adverse effects in acute respiratory infections, which themselves are the symptomatic manifestation of inflammatory processes (Johnston, 1997).

Overall, it can be concluded that Echinacea preparations vary considerably, quantitatively as well as qualitatively. This is inevitably resulting in diverging *in vitro* bioactivities and might therefore present a reasonable justification for heterogeneities observed in clinical trials results as well (Karsch-Völk et al., 2014).

1.3 Research on Echinacea and Plant Extracts in General

As exemplified by author instructions of scientific journals (e.g. Phytomedicine or Planta Medica), research on plant products increasingly abstains from investigating unspecific bio-activities or substances that are widely abundant and probably ingested at higher quantities from other sources (i.e. polyphenols). Anti-oxidative, radical-scavenging or antiproliferative effects are not of primary interest for investigating Echinacea within its indication. Concentration is another critical parameter for the judgement of (physiological) relevance of observed *in vitro* effects. Bio-active substances in herbal preparations often demonstrate limited bioavailability and it is recognized that effects at concentrations above 100 μg/ml are not likely to be of physiological relevance (Epriliati and Ginjom, 2012).

Below biological activities are currently discussed as determinants for the therapeutic potency in preventing and treating respiratory tract infections, for which Echinacea is taken. Even when focusing on these more relevant assay systems, the available literature continues to show great heterogeneity between products mentioned above.

1.3.1 Anti-viral Effects

A review by Hudson and Vimalanathan (2011) identified for most Echinacea species and products antiviral effects *in vitro*, but for some only at elevated concentration, i.e. above 100 μg/ml (Hudson and Vimalanathan, 2011). Wacker and Hilbig found a 50 – 80% protection against infections by herpes simplex virus (HSV-1), influenza A or vesicular stomatitis viruses (VSV) when pre-treating cell lines for 4 - 6 hours with either aqueous or methanolic *E. purpurea* extracts. Concentrations of 20 μg/ml showed significant

effects, the “protection” persisted for 24 hours and could be reversed by the addition of hyaluronidase enzyme (Wacker and Hilbig, 1978). Echinacea was proposed to transform cells into a virus-resistant state through the induction of interferons (e.g. IFN-γ) as shown by mice *in vivo* experiments by Bodinet and colleagues / Zhai and colleagues (Bodinet et al., 2002; Zhai et al., 2007).

Results complemented earlier findings from May und Willuhn (1978) that demonstrated for *E. pallida* aqueous extracts, direct virus inhibition at a concentration of 27 μg/ml. Results also resemble data obtained from *E. purpurea* pressed juice and Cheminat and colleagues suggested caffeoyl-derivatives (e.g. caffeic and cichoric acid) as active players in hydrophilic preparations (Cheminat et al., 1988). However, cichoric acid was found to inhibit 50% infectivity only at concentrations of 125 μg/ml, raising the question of physiological relevance of those findings. Others have, in contrast, postulated the importance of lipophilic extraction (i.e. hexane or ethanol) of *E. purpurea* for optimal inactivation of herpes simplex virus (Binns et al., 2002). A minimal 100% inhibitory concentration (MIC_{100} value) against HSV and influenza was found in the low microgram range for aqueous and ethanolic extracts. Cichoric acid was attributed an at least moderate activity. Vimalanathan and colleagues finally investigated several solvent fractions from *E. purpurea* to find most impressive anti-influenza and anti-HSV activity in an ethyl acetate fraction upon light activation. Polysaccharide-enriched fractions (hydrophilic) showed a relatively weak activity in the same experimental setting (Vimalanathan et al., 2005).

In conclusion, the medicinal plant Echinacea principally provides a rich source for antiviral substances, which are however not completely retrieved by all manufacturing procedures. On the other side, a clear correlation of antiviral activity with any particular substance classes cannot be made based on the available evidence. Comparison of the available literature is hampered by methodological differences and the high variability in Echinacea sources and specimen preparations.

1.3.2 Anti-inflammatory Effects

Inflammation represents the by-product of a pathogen eliminating process called immune response. It is the result of an excessive production of pro-inflammatory mediators (acute phase proteins) including tumor necrosis factor alpha (TNF-α) or interleukins (IL-1 and IL-6). It has been shown that the severity of respiratory symptoms tightly correlates with the production of mentioned mediators in the airways and their regulation may represent a mechanism for effective treatment (Johnston SL, 1995). Also, in this respect different Echinacea preparations produced different effects as briefly outlined in the following and more extensively elsewhere (Barnes, 2005).

In vitro an endotoxin-free *E. purpurea* pressed-juice (herba) and purified polysaccharides thereof possessed immunostimulatory activity to murine and human macrophages and mononuclear cells as evidenced by induced TNF- α and interleukin levels (Burger et al., 1997, Stimpel et al., 1984). A dried *E. purpurea* extract activated production of TNF-α, IL-1 and IL-6 by natural killer cells obtained from human peripheral blood

mononuclear cells (hPBMCs) and enhanced the phagocytic activity (See et al., 1997). Rininger (2000) found clear stimulation of TNF-α and IL-6 in RAW264.7 macrophages and hPBMC only upon simulated (*in vitro*) digestion of *E. purpurea* herb and root priorly dissolved in dimethyl sulfoxide (DMSO). However, different Echinacea preparations (laboratory and commercial) showed great effect variations in this experiment. Interestingly, extracts standardized to discussed marker substances like phenol contents (4%), polysaccharides or to echinacoside/alkylamide proved inactive in induction of said cytokines (Rininger et al., 2000). Roesler et al. (1991) postulated immunostimulatory effects *in vivo* but only upon bypassing digestion via intraperitoneal administration. These results exemplify how *in vitro* observed effects may not fully develop upon peroral administration due to digestive degradation or low bioavailability of potentially active substances.

In sharp contrast, more recent studies found that an endotoxin-free, alcoholic *E. purpurea* extract alone did not induce TNF-α neogenesis but modulated its expression upon lipopolysaccharide or viral stimulation (Gertsch et al., 2004; Zhai et al., 2007). Although TNF-α mRNA transcription was induced by Echinacea, its translation into TNF-α protein was not affected and protein secretion balanced. Here, alkylamides seem to play an important role in regulating inflammation through modulation of TNF-α, beside their known inhibition of cyclooxygenase and 5-lipoxygenase (Wagner and Jurcic, 1991). Likewise, Randolph et al (2003) found a general cytokine mRNA induction *in vitro* but a reduced production of IL-1β, ICAM-1, IL-8 and TNF-α and concomitant increase of interferon IFN-γ upon 2 days oral administration of an endotoxin-free Echinacea preparation. Finally, Sharma and colleagues (2006) could in preliminary experiments identify intracellular activating effects of *E. purpurea* on STATS or NF-KB, some proinflammatory transcription factors.

Overall, Echinacea effects on the immune system are qualitatively and quantitatively highly heterogeneous even somewhat contradictory. It appears that hydrophilic extracts (e.g. Echinacea pressed juices) may contain compounds which are able to broadly stimulate cytokine production at least *in vitro*. It remains to be ascertained, whether these substances are Echinacea-specific or rather ubiquitously abundant (i.e. polysaccharides or endotoxins). On the other side were lipophilic extracts (if free of endotoxins) able to more selectively modulate particular components of the immune system: typical inflammatory mediators (TNF-α or IL-1) were down-regulated by Echinacea in the presence of viral stimuli, whereas antivirally acting IFN-γ was purposefully upregulated. Finally, the phagocytic capacity was enhanced by lipophilic Echinacea preparations and contained alkylamides (Goel et al., 2002; Bauer et al., 1988).

In conclusion, substances in Echinacea principally have demonstrable effects on cells of the immune system. However, results from *in vitro* tests do not fully match with *in vivo* experiments and are prone to artefacts as digestion, metabolism or bioavailability are not taken into account. Bacterial contamination and endotoxins may further blur interpretation of any Echinacea-specific effects. Despite those experimental short comings, Echinacea was until the beginning of the 21st century considered a pure immune stimulant, albeit a consistent direction of activation was not reliably documented.

1.3.3 Echinacea Clinical Results

In view of Echinacea products' heterogeneity, it seems little surprising that clinical results vary as well. As mentioned earlier, *in vivo* research mainly focussed on the indication "treatment and prevention of colds and flu" and the long-proposed immune-stimulatory effect of the plant. A recent Cochrane-analysis by Karsch-Völk and colleagues (2014) looked at 10 prevention and 14 treatment studies of low (14), high (9) and unclear (1) risk of bias studying overall 4631 participants. The analysis concluded modestly positive effects for prevention overall (10 – 20%) and a tentative, exploratory pooling yielded a risk ratio of contracting a cold of RR = 0.83, 95% CI 0.75 to 0.92 ($p< 0.001$). Results on acute treatment effects were more consistent at least for Echinacea above-ground plant parts but not overall (Karsch-Völk et al., 2014). The parameter "sum scores after 5 to 10 days of treatment" provided more convincing treatment benefits than "duration of colds", which was reported in only two clinical studies (Karsch-Völk et al., 2014).

A comparison with earlier Cochrane reviews shows that the past decade of research has not created enough evidence to overcome problems associated with heterogeneity (Linde et al., 2006). In 2005, the publication of a very large, methodologically sound clinical trial attained much attention in the public domain (Turner et al., 2005). The study found no significant benefits for three different *E. angustifolia* preparations for the prevention and treatment of rhinovirally induced clinical colds. This project was funded by the National Center for Complementary and Alternative Medicine (NCCAM) of the National Institute of Health and the associated press (AP) and Bloomberg Business News considered the result as the "nail in the coffin' for Echinacea (Reuters, 2010).

Turner and colleagues included a total of 437 subjects in the study but allocated them to eight different treatment arms. Thus, the relatively large sample was divided into small groups of less than 50 subjects. This sample size was finally too small to reach statistical significance.

Consequently, Schoop (the author of this PhD work [2006]) and colleagues proceeded to analyse and identify further methodological weaknesses of above and of other available, non-significant studies and in their meta-analysis

- pooled patient data in order to achieve greater sample sizes and more statistical power
- defined the researched indication by introducing the cold definition by Jackson et al. (1958) and
- focussed on methodologically high-level studies reaching Jadad-scores > 3 (Jadad et al., 1996)

to finally find statistically significant effects in preventing cold episodes by Echinacea, despite the considerable variability in treatment effects between individual studies. The general perception of Echinacea being unworthy for further investigation was thereby refuted, but the fundamental question regarding the observed heterogeneity of treatment effects remained to be answered.

1.4 Research Strategy of Doctoral Work

The 10 years of research retrospectively documented in this contextual statement aimed to explore *in vitro* and *in vivo* pharmacological effects, as well as clinical efficacy in prevention and acute treatment of respiratory tract infections for a single, phytochemically characterized and standardized *Echinacea purpurea* extract (Echinaforce® extract, abbreviated as "EF"). It was not the intention to find overarching conclusions regarding the plant but at least to overcome the repeatedly mentioned issue of heterogeneity for this particular Echinacea preparation.

Echinaforce® extract is a hydroalcoholic tincture (65% V/V) from freshly harvested *E. purpurea* consisting of 95% herba and 5% radix, produced by A. Vogel AG, Switzerland. The plants are cultivated on proprietary fields at A.Vogel AG company (Switzerland) under organic growing conditions (according to the valid EC Directive). Cultivation, harvesting, storage, primary processing and documentation are in accordance with the Guideline on Good Agricultural and Collection Practise (EMEA/HMPC/246816/2005). The grower's certification organization is BIO-SUISSE. The seeds are propagated at A.Vogel AG, using a wild type that has been self-propagated since at least the early 1950s. Identity of produced plant material is tested according the European Pharmacopoeia (Ph. Eur.) release by the European directorate for the quality of medicines & healthcare (EDQM) to ensure the identity of plant species used (EDQM, 2004). The extract shows a composition of known pharmacologically active marker substances, which is given in below table 1. As per product analysis sheet the extract is demonstrated to be free of impurities and endotoxins (i.e. lipopolysaccharides), which potentially could influence outcomes from bio-assays as mentioned in Section *Echinacea Product Heterogeneity*.

Table 1: Pharmacologically active substances in Echinaforce® (EF extract), means of four determinations as obtained from [Sharma et al., 2009a]. PID 8/9 refers to dodeca2E,4E,8Z, 10E/Z tetraenoic acid-isobutylamide ("tetraene").

Compound	Concentration (µg/mL)
Caffeic acid	0 ± 0
Caftaric acid	264.4 ± 13.0
Chlorogenic acid	40.2 ± 2.0
Cichoric acid	313.8 ± 0
Cynarin	0 ± 0
Echinacoside	6.9 ± 0.4
PID 8/9	36.3 ± 1.8

The aim of this PhD work was to explore pharmacodynamics of EF extract together with clinical health benefits using straight-forward techniques, while steering off the now known pitfalls in plant product research, like testing of bacterially contaminated specimen at too high concentrations using irrelevant assays (also see Section *Echinacea Product Heterogeneity*). Suitable bio-assays were selected to optimally

reflect the pathophysiology of viral respiratory tract infections in humans and with reference to expected points of actions upon peroral administration of EF extract as illustrated in figure 1 (Johnston, 1995).

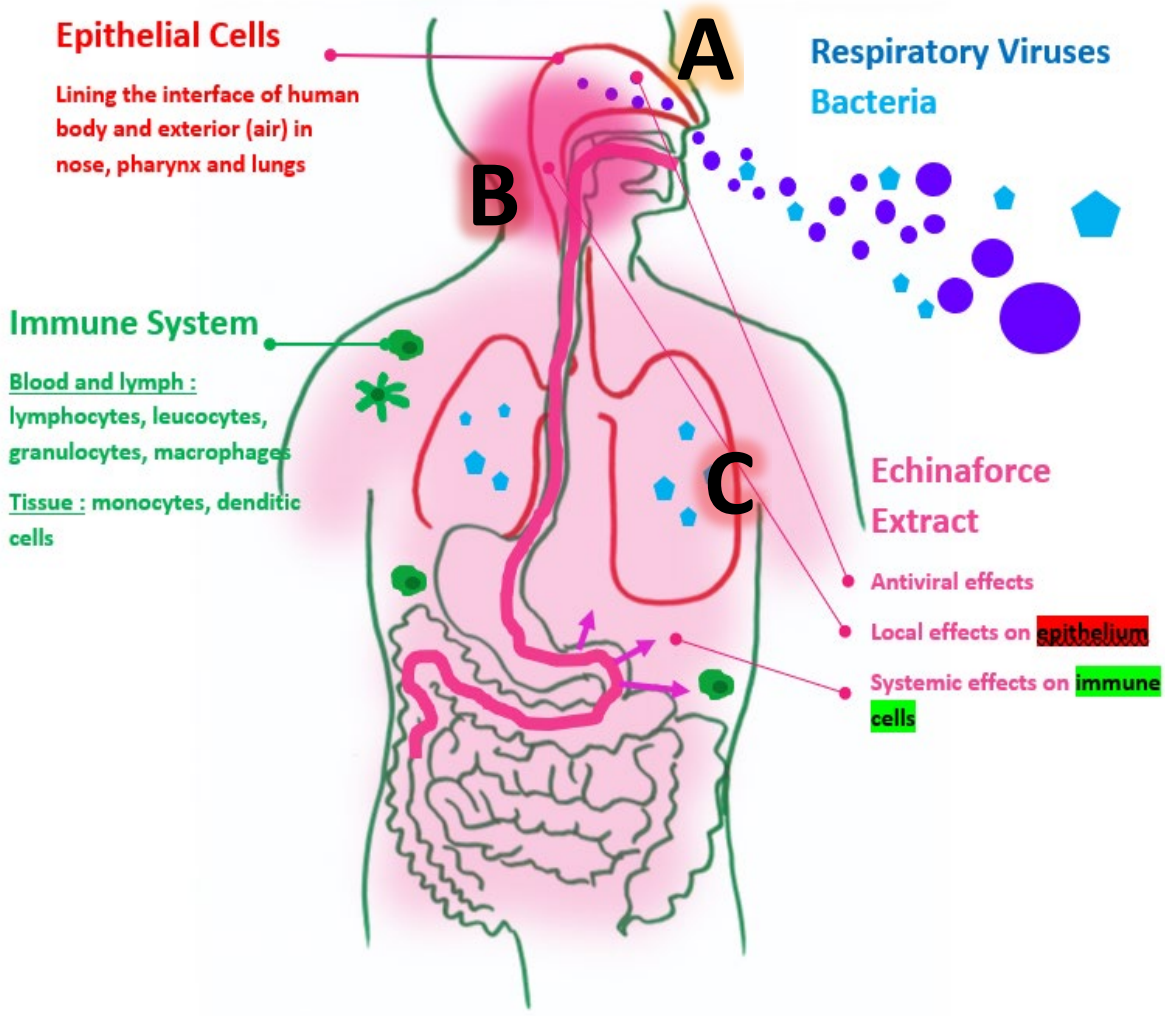


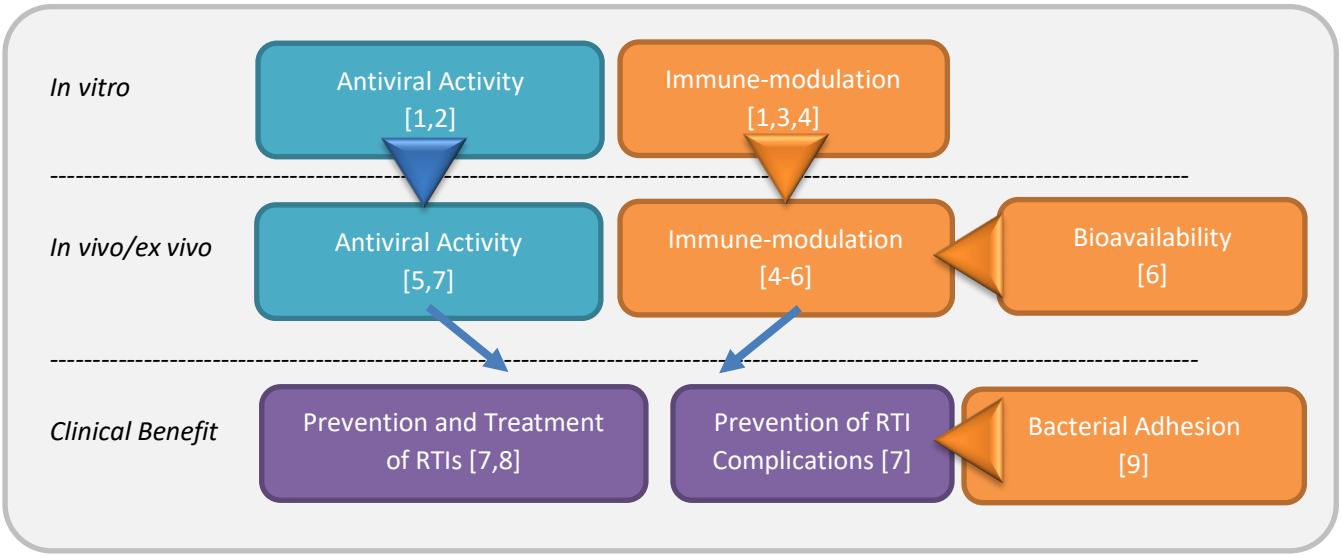
Figure 1 Pharmacological points of action controlling respiratory tract infections. Respiratory viruses (blue) attack epithelial target cells (red) in the nasopharyngeal region/lungs, whereas pathogenic bacteria can subsequently be attracted to the site of infection to produce complicated infections (light blue). The immune system (green) comprises different cell types found in the tissue, blood and lymph. Oral ingestion (especially sucking tablets) delivers EF extract (purple) first to pharyngeal epithelial cells, where antiviral (**A**) and anti-inflammatory effects (**B**) unfold. Thereafter, EF is swallowed for systemic resorption into the blood stream and the tissue to reach cells of the immune system (**C**).

To this end, the antiviral research investigated a wide range of relevant and actual pathogens (Elliot and Fleming, 2009; **A** in figure 1). Immunological tests distinguished **local** (**B** in figure 1) from **systemic actions** (**C** in figure 1) by using epithelial cells and peripheral blood immune cells, respectively (Roitt et al., 1998).

Figure 2 illustrates the overall research strategy on which test system was employed and how results were intertwined in the overall context. Correlation between *in vitro (*1st level*), ex vivo* and *in vivo* (2nd level) and

finally clinical results (3rd level) was sought to scrutinize biological relevance and consistency of findings. For this purpose, bioavailability and pharmacokinetic properties of pharmaceutically active substances were investigated. Finally, the work explored EF's therapeutic potential beyond the traditional use for colds and flu and looked at the prevention of bacterial superinfections like bronchitis or pneumonia (Kenealy and Arroll, 2009).

Figure 2: Research strategy for exploring the pharmacology of EF extract. Correlation of *in vitro* antiviral, immune-modulatory effects (**1st level**) with in *vivo/ex vivo* results (**2nd level**) was to be explored and finally linked to clinical efficacy (**3rd level**). Relevant citations are indicated in brackets.



The following aims and objectives were to be achieved and answered for EF extract, while referring to the respective publications given in the following (Section 1.5 List of Referenced Publications):

- Exploring the antiviral activity *in vitro* [1,2]
- Exploring the anti-inflammatory (immune-modulatory) activity *in vitro* [1,3,4]
- Confirming anti-antiviral and immune-modulatory actions *in vivo* and *ex vivo* [4-7]
- Assessing bioavailability of alkylamides after peroral administration of EF extract [6]
- Assessing clinical efficacy of EF extract in preventing RTIs [7]
- Assessing clinical efficacy of EF extract in treating influenza illness [8]
- Assessing the potential of EF extract in preventing RTI complications *in vivo* [7] while
- Exploring the underlying mode-of-action for prevention of RTI complications *in vitro* [9]

1.5 List of Submitted Publications

The following **pivotal publications** form the core of this PhD work, full texts are attached in the **Annex**. Citations to below publications in the text are highlighted in bold and underlined letters.

Sharma, M., Anderson, S.A., Schoop, R., Hudson, J.B. (2009a). Induction of multiple pro-inflammatory cytokines by respiratory viruses and reversal by standardized Echinacea, a potent antiviral herbal extract. *Antiviral Research*, 83(2), 165-70. Cited in tables and figures as [1].

Pleschka, S., Stein, M., Schoop, R., Hudson, J.B. (2009). Anti-viral properties and mode of action of standardized Echinacea purpurea extract against highly pathogenic avian influenza virus (H5N1, H7N7) and swine-origin H1N1 (S-OIV). *Virology Journal*, 6, 197. Cited in tables and figures as [2].

Sharma, M., Schoop, R. and Hudson, J.B. (2009b). Echinacea as an antiinflammatory agent: the influence of physiologically relevant parameters. *Phytotherapy Research*, 23(6), 863-7. Cited in tables and figures as [3].

Sharma, M., Schoop, R., Hudson, J.B. (2010). The efficacy of Echinacea in a 3-D tissue model of human airway epithelium. *Phytotherapy Research*, 24(6), 900-4. Cited in tables and figures as [4].

Ritchie, M.R., Gertsch, J., Klein, P., Schoop, R. (2011). Effects of Echinaforce® treatment on ex vivo-stimulated blood cells. *Phytomedicine*. 18(10), 826-31. Cited in tables and figures as [5].

Woelkart, K., Marth, E., Suter, A., Schoop, R., Raggam, R.B., Koidl, C., Kleinhappl, B., Bauer, R. (2006). Bioavailability and pharmacokinetics of Echinacea purpurea preparations and their interaction with the immune system. *International Journal of Clinical and Pharmacological Therapies*, 44(9),401-8. Cited in tables and figures as [6].

Jawad, M., Schoop, R., Suter, A., Klein, P., Eccles, R. (2012). Safety and Efficacy Profile of Echinacea purpurea to prevent common cold episodes: A randomized, double-blind, placebo-controlled trial. *Evidence Based Complementary and Alternative Medicine*, 841315, Epub 2012, Sep 16. Cited in tables and figures as [7].

Raus, K., Pleschka, S., Klein, P., Schoop, R., Fisher, P. (2015). Effect of an Echinacea-Based Hot Drink Versus Oseltamivir in Influenza Treatment: A Randomized, Double-Blind, Double-Dummy, Multicenter, Noninferiority Clinical Trial. *Current Therapeutic Research*, 77, 66-72. Cited in tables and figures as [8].

Vimalanathan, S., Schoop, R. and Hudson, J. (2017). Prevention of influenza virus induced bacterial superinfection by standardized Echinacea purpurea, via regulation of surface receptor expression in human bronchial epithelial cells. *Virus Research*, 233, 51-59. Cited in tables and figures as [9].

Essentially, experiments were designed and carried out to explore *in vitro* and *in vivo* pharmacology of *Echinacea purpurea*. The work aimed to understand and estimate Echinacea's medicinal value for the treatment and prevention of respiratory tract infections. By focussing on a single, chemically standardized extract (Echinaforce®) problems with Echinacea products' heterogeneity were to be overcome, in order to achieve more consistent results.

A basic knowledge on the pathology of respiratory tract infections and challenges for therapy is indispensable to understand the context of test systems/results and shall be given in the following chapter.

1.6 Respiratory Tract Infections (RTIs)

Respiratory tract infections (RTIs) like colds and flu represent the most frequent illnesses in Western civilization (Rotbart and Hayden, 2000). Infections present with a variety of symptoms, including nasal complaints (sneezing, congestion or runny nose), sore throat, coughing as well as systemic symptoms like shivering, malaise, headache and sometimes fever. Most colds and flus are self-limiting illnesses and recovery is expected within 7 – 10 days but sometimes residual coughing can persist for up to 2 -3 weeks (Fendrick, 2003; Aherne et al., 1970). In children below 5 years of age, RTIs represent the leading cause of mortality (Denny, 1995). An estimate of cost of illness in the United States shows that non-influenza illness ranges amongst the 10 most expensive diseases with 40 billion dollars annually spent for medication, working absences and doctor's visits (Fendrick, 2003). Influenza certainly represents a unique viral illness amongst RTIs seasonally producing excess morbidity and mortality mainly in the elderly (Treanor and Falsey, 1999). The World Health Organization estimates 25 to 50 million influenza infections, 150'000 hospitalizations and up to 40 000 fatal outcomes every year in the United States alone (Fleming, Elliot and Nguyen-van Tam, 2009)

1.6.2 RTI Complications

RTIs have debilitating effects on our immune defence and tend to recur or exacerbate. Approximately 20 - 30% of infections develop into complications, and an increased incidence is observed in COPD patients, children and in the elderly – basically in those with weak immune defences (Kaiser et al.,2003; Kenealy and Arroll, 2009). RTI complications include *Otitis media*, Tonsillitis, *Sinusitis*, *Bronchitis*, *Bronchiolitis* and even life-threatening *Pneumonia*. The fear of complications is the main motive for prescription of antibiotics by physicians because these conditions are frequently associated with bacterial super-infections of the originally viral illness (Kenealy and Arroll, 2009; Elliot and Fleming, 2009).

1.6.3 Pathogenesis and Treatment of RTIs

RTIs are caused by a wide variety of viruses, while Rhinovirus, Coronavirus, Respiratory Syncytial Virus (RSV), Metapneumovirus (MPV), Boccavirus, Parainfluenza (PI) and Influenza constitute 90% of colds and flu (Elliot and Fleming, 2009). It is impossible to timely identify the causative agent during acute illness without quick bed-side virus testing to adapt therapy. Only Influenza and to a certain degree RSV show a distinct window of appearance and a characteristic symptomatic picture to allow identification solely on basis of clinical diagnosis. In practice, treatment of RTIs thus aims at reducing inflammatory responses by the host to the viral infection and to reduce specific symptoms like cough (mucolytic and/or cough suppressants) or nasal complaints (decongestants).

Vaccination provides an effective means of combating infections by influenza but there are currently no agents to protect against other respiratory viruses. The development of a specific prophylactic against colds and flu is ultimately hampered by the multiplicity of viruses and their propensity to mutate.

An alternative approach would be to support the body's own immune mechanism, falling back on the principle that the human organism is able to defend itself naturally against viruses and bacteria. It is here, that *Echinacea* has ever played a unique therapeutic role (Barrett et al., 2003).

2 Pharmacology of *Echinacea*: Summary of PhD Pivotal Research Work

The following section reviews the body of research carried out by the candidate to explore the (pre-) clinical pharmacological profile of Echinacea (on the example of EF extract). As shown in figure 1 the provided evidence is grouped into three levels

1. Level: Basic *in vitro* research work,
2. Level: Secondary confirmation by *ex vivo*, *in vivo* and bioavailability assays and finally
3. Level: Clinical research data to correlate pharmacology with patient-reported outcomes

in order to achieve the aims and objectives as set out in section *Research Strategy of Doctoral Work*.

The applicant has mainly been involved in the conceptualization of study designs and project lead. He developed the overall research idea and strategy. He has made essential contributions to study protocols, evaluation, interpretation and publication of study results. His contribution to the respective research work is estimated giving percentage indicating the approximate proportion of resource (time). A detailed listing of inputs to the cited articles is included as **Appendix 1** to show the range of contributed work. A brief summary is included per individual paragraph in the following as well.

2.1 Antiviral Activity of *Echinacea purpurea*

The predominance of viruses in cold and flu infections has been outlined earlier (Rotbart and Hayden, 2000; Fendrick, 2003). A main scope of the applicant's research thus focussed on the antiviral activity of EF extract as explored in more depth in the two pivotal scientific articles and a supplementary poster outlined below [Sharma et al., 2009; Pleschka et al., 2009; Signer et al., 2020]. The research question to be answered was whether EF extract would exhibit antiviral activity and at what concentrations, which marker substances are important and whether it would exhibit any specificity towards particular pathogens, including newly occurring virus strains. Employed techniques adhered to well-established methods for testing antiviral activities of pharmaceuticals and are detailed in the referenced publications.

2.1.1 Antiviral Research

[1] Sharma, M. et al. (2009a). *Antiviral Research*, 83(2), 165-70.

The applicant designed, initiated and guided the research project, helped with interpretation of results and writing of publication (parts on in vivo relevance and correlation with clinical evidence). His contribution to the overall work: ***15 – 25%*** *(exclusively intellectual input)*

In this first study, a wide variety of respiratory viruses was investigated for their sensitivity against EF extract.

Antiviral activity was found against influenza A (H3N2), respiratory syncytial virus (RSV) and herpes simplex virus (HSV) at minimal inhibitory concentrations of $MIC_{100} < 50$ μg/ml. Activity against the most common rhinovirus was observed at higher concentrations of 800 μg/ml, which was still considered relevant upon topical application (sucking, gargling) to the pharynx region (Sharma et al., 2009). Very recent research data completed investigations on anti-viral activity of EF extract with data on coronaviruses [CoV-229E, CoV-MERS and CoV-SARS] and parainfluenza viruses, to show high sensitivity to the extract at inhibitory concentrations of IC < 50 μg/mg as well (Signer et al., 2020).

The generated evidence nourishes the hypothesis that EF extract may have antiviral potential to broadly block membranous, rather than non-membranous viruses like adenoviruses or poliovirus (Sharma et al., 2009; Signer et al., 2020). The latter were less sensitive to the extract and only at concentrations above 800 μg/ml. Results show, that direct exposure to the extract is mandatory for maximal inhibition, whereas the addition of EF after viral inoculation ("*intracellular protocol*") produced a significantly weaker inhibition. The observed differences in activity called for the underlying mechanistic mode-of-action capturing membranous, rather than non-membranous pathogens. This was further explored in the following experiments:

2.1.2 Antiviral Research (Influenza)

[2] Pleschka, S. et al. (2009). *Virology Journal*, 6, 197. *The applicant designed and guided the research work, in particular the resistance experiments. He coordinated the work/exchange between laboratories and was involved in writing of publication (Echinacea and in vivo relevance). Contribution to the overall work:* ***20 – 30%*** *(exclusively intellectual input).*

Different influenza strains (A/Victoria/3/75; A/Thailand/KAN-1/2004, A/FPV/Bratislava/79, A/Puerto Rico/8/34 and A/Hamburg/1/09) were cultivated in MDCK cells or embryonated chicken eggs prior exposure to EF. Again, minimal inhibitory concentrations (MIC) were estimated at differing plaque forming units (pfu) to find that all influenza strains were susceptible to EF extract at concentrations of 0.1 to 50 μg/ml.

A hemagglutination assay investigated influenza's ability to aggregate erythrocytes via interaction of viral docking receptor haemagglutinin (HA) with cellular sialic acid to form virus – erythrocytes precipitates. Already at 50 μg/ml EF prevented this agglutination, indicating that the extract modified influenza surface receptors (HA), which are essential for viral attachment to host cells during the infection process. The viral replication process was thus found affected by EF at the earliest possible step (prior to cellular infection) (**Sharma et al., 2009**). Intracellular virus replication was however not further influenced by the extract, or only at higher, probably non-physiological concentrations, as demonstrated for influenza or coronavirus (**Sharma et al., 2009**; Signer et al., 2020).

We found that prolonged exposition of influenza to a neuraminidase inhibitor (Oseltamivir) produced the emergence of resistances, whereas continuous passaging in the presence of EF extract did not have the same effect. Obtained Oseltamivir-resistant influenza viruses finally kept their susceptibility towards EF extract (**Pleschka et al., 2009**).

Interestingly, two research laboratories at the University of British Columbia, Canada (**Sharma et al., 2009**) and the University Giessen, Germany (**Pleschka et al., 2009**) have found varying inhibitory concentrations (IC_{50} and MIC_{100}) for EF extract in inhibiting influenza. This might be due to altering viral infectiousness/concentration, i.e. plaque forming units (pfu). Whereas Pleschka and colleagues used stock solutions of 10^7 to 10^8 pfu, Hudson's group used pfu's ≤ 10^6 in their experiments. As already demonstrated by Pleschka et al. (2009) changing a virus pfu has implications on measured inhibitory concentrations, simply because more HA molecules need to be converted by more extract. Future research work should aim to standardize infectious units to make outcomes more comparable. Moreover, endpoints should be harmonized to express either IC_{50}, MIC_{100} or to any other threshold. So far, experiments tested the direct virus blocking potential of EF as well as a treatment-like situation of administering EF to virally-infected cells. Further settings should include a prolonged (up to 72 hours) pre-treatment of target cells prior to viral infection to mimic a prevention-like situation.

The above cited work confirms the presence of antivirally active compounds in the medicinal plant *E. purpurea*. Particular extracts thereof (e.g. EF extract) show a specificity towards membranous respiratory viruses like Influenza, Respiratory Syncytial-, Herpes simplex, Corona- or Parainfluenza viruses. Due to the multiplicity of bio-active substances in herbal extracts, it is reasonable to assume that resistances are less likely to develop and activity is maintained over time (Wagner and Merzenich, 2009). Direct contact of virus and extract however is important to entail maximal biological activity, which advocates the use of *Echinacea* for prevention – i.e. prior to viral infiltration of host cells. Once infection has established EF extract is only partially or not effective in blocking replication intracellularly and only budding off-springs might be neutralized thereafter.

2.2 Immune-modulatory Effects of *Echinacea purpurea*

E. purpurea is widely used by consumers for the support of immune defences (Barrett et al., 2003). Functions of the immune system are however highly complex and depend not only on typical immune cells like T-, B-, dendritic cells but also on macrophages in their respective differentiation modes. RTI symptoms for instance are primarily a result of a local inflammatory response of airway epithelial cells to viral infection (Johnston, 1997). Clinical examinations have shown that the nasal production of cytokines like interleukin-6 (IL-6, IL-8 or TNF-α) directly correlates with symptom severity and the course of infections (Grünberg et al., 1997). As a consequence, **local** anti-inflammatory actions at the site of infection (airway epithelium) effectively reduce the symptomatic evolution of cold and flu infections at an early stage. On the other side may **systemic** effects be different from locally applied, because here all kinds of immune cells are involved to build up primary and secondary immune responses, including immunological memory mainly during later stages of an infection (Roitt et al., 1998).

We therefore aimed to explore the effects of EF extract on aspects of an immune response and differentiated local actions on epithelial cells from systemic actions on peripheral blood monocytes (PBMC). First, we investigated how and at which concentration EF extract affected the virally induced production of inflammatory IL-6, IL-8 and TNF-α by alveolar and bronchial epithelial cells. Time- and dose-dependence as well as specificity of effects were determined by varying exposure times, EF concentrations and viral stimuli. A common, well-established and valid technique (enzyme linked immunosorbent assay, ELISA) was employed, using most relevant target cells and respiratory agents in order to estimate expression profiles of important cytokines (see Section 1.3.2. Anti-Inflammatory Effects). Secondly, it was investigated if effects still prevail in a more *in vivo*-like approach, i.e. in organotypic 3-D tissue model of human airway epithelium.

2.2.1 Immunological Research (*in vitro*)

[1] Sharma, M. et al. (2009a). *Antiviral Research*, 83(2), 165-70.

[3] Sharma, M., Schoop, R. and Hudson, J.B. (2009b). *Phytotherapy Research*, 23(6), 863-7.

The applicant designed, initiated and guided the research project, helped with interpretation of results and writing of publication (parts on in vivo relevance and correlation with clinical evidence). His contribution to the overall work: ***20 – 30%*** *(exclusively intellectual input)*

Human alveolar epithelial (A459) and bronchial cell-lines (BEAS-2B) were cultivated *in vitro* prior to infection with rhinovirus (RV14 or RV1A). As expected, viral infection augmented the production of IL-6, IL-8 and TNF-alpha, whereas EF dose-dependently reversed their expression levels after 24 and 48 hours. The experiment also showed that 20 to 40 μg/ml of EF extract was sufficient to down-regulate inflammatory processes secondary to virus infection. Since EF was administered after viral infection it is reasonable to assume that the extract would still work even when infection had already established (i.e. for acute treatment). The addition of EF could be delayed until 48 hours post infection and inhibitory effects on cytokines still prevailed.

Local anti-inflammatory effects finally were reproduced employing alternating infectious stimuli: Influenza, RSV, Adenovirus types 4 and 11 and Herpes simplex virus (HSV). A highly consistent picture of viral induction and reversal by EF extract became evident, indicating a generally applicable mode-of-action.

2.2.2 Immunological Research (*ex vivo*)

[4] Sharma, M., Schoop, R., Hudson, J.B. (2010). *Phytotherapy Research*, 24(6), 900-4.

The applicant designed and guided the research project, introduced EpiAirway tissue to the research group, and contributed writing of publication. His contribution to the overall work: ***30%***

Above *in vitro* results were then to be confirmed in an *ex vivo* model using organotypic 3-D tissue model of human airway epithelium. The EpiAirway™ tissue (MatTeck, Ashland, MA, USA) represents a histological reconstruction of human airway epithelium composed of epithelial cells from human donors, mucin-producing goblet cells and mechanically functional cilia. Rather than using immortalized cell lines the EpiAirway™ re-builds the natural human airway epithelium retaining tissue architecture and differentiation (Klausner et al., 2007). The model is essentially appropriate because *in vivo* expressed interleukins by airways resemble the expression pattern by this ex vivo assay (Message and Johnston, 2004). Astonishingly, RV infection did not affect the histology of epithelium, which corresponds well with the reported lack of lytical potential for this type of virus. Again, the viral induction of inflammatory cytokines and of mucin was potently reversed by EF as measured by ELISA technique 24 and 48 hours post inoculation.

So far, no single chemical substances contained in EF extract were investigated in above experiments and it would be interesting and point of future research to understand, which ingredients are mediating said effects. Combination with a permeability test system (e.g. Caco-2) would deliver information whether bio-active substances are systemically available after all.

In conclusion, effects of EF on the airway epithelium were seen under *in vitro* as well as *ex vivo* conditions supporting the hypothesis of *Echinacea* acting as a potential local anti-inflammatory agent. This action is considered of high importance in view of the early inflammatory genesis of typical cold symptoms. In fact, inhibition of inflammation by Echinacea was also observed *in vivo* in the context of clinically induced rhinovirus infections (inoculation studies). Thereby, the development of symptoms (inflammation) was significantly reduced by Echinacea, rather than the infection (rhinovirus titers) itself (Schoop et al., 2006).

Local anti-inflammatory effects were herewith ascertained and the next experiment addressed the question how EF extract influences aspects of the **systemic** immune system *in vivo*. To this end, the number and function of immune cells were measured by means of full blood counts and measuring cytokine/chemokine production during oral administration of EF extract.

To this end, the Instant Leucocyte Culture System (ILCS®) was employed for leukocyte activation immediately upon drawing the blood from Echinaforce-treated subjects under standardized conditions. This was accomplished by using a peculiar type of blood-sampling syringe, pre-filled with cell culture medium, including already the stimulants used to activate leukocytes. This eliminated the need for any cell preparation and avoided artificial, undesired manipulation-dependent changes in cell behaviour. Moreover, the immediate activation upon bleeding prevented storage- and shipping-induced impairment of cell functions guaranteeing a maximum of standardisation. The sequence of a) drug ingestion and b) extra-corporeal immune cell stimulation mimics also widely the events upon drug intake, where the drug first is absorbed in the intestine. Then the active ingredients occur in the blood and take effect on circulating leukocytes, which are later recruited to the diseased tissues, where finally their activation takes place. Stimulants were selected to activate essential receptors of the most important leukocyte subtypes differently involved in the regulation of immune responses and inflammation: lipopolysaccharide (LPS) and staphylococcal enterotoxin B (SEB, a so-called superantigen), excellently activating e.g. T-cells.

2.3.3 Immunological Research (*in vivo/ex vivo*)

[5] Ritchie, M.R. et al. (2011). *Phytomedicine*. 18(10), 826-31.

The applicant designed and guided the research project and implemented the Instant Leukocyte Culture System, ILCS®. He was involved in study set up, patient inclusion and informed consenting at the investigational site. Writing of publication (introduction/conclusion, parts on in vivo relevance and correlation with clinical evidence). His contribution to the overall work: ***40-60%*** *(experimental and intellectual input)*

This study aimed to examine effects of peroral application of EF extract on *ex-vivo* stimulated PBMCs to localize any systemic effects. After a run-in phase of two days (baseline), EF was administered over eight days to 30 healthy individuals. Daily collected blood (isolated PBMCs) was stimulated with LPS (lipopolysaccharide, 100ng/ml) and SEB (staphylococcal enterotoxin B, 25ng/ml) for 24 hours using the Instant Leukocyte Culture System (ILCS®). EF increased production of chemotactic interleukin-8 (IL-8), MCP-1 and reduced the expression of inflammatory TNF-α and IL-1 upon *ex vivo* stimulation. In consistency with an anti-inflammatory response, IL-10 was increased to baseline levels. Anti-virally acting interferon gamma (IFN-γ) was purposefully induced in volunteers with elevated risk of infection and in stressed subjects. Individuals with low cortisol levels (n = 11) experienced significant down-regulation of acute-phase proteins IL-1-beta, IL-6, IL-12 and TNF- α, while those with higher cortisol levels showed no such down-regulation upon treatment with EF. The respective numbers of immune cells (erythrocytes, leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils and thrombocytes) remained unchanged during treatment. This *ex vivo* trial suggests a support of weak immune systems by *Echinacea*, i.e. during phases of increased stress or susceptibility to cold infections, while well-performing immune systems are not over-stimulated. Effects are best described as immune modulation.

Effects were observed upon peroral application of EF extract using an *ex vivo/in vivo* approach and are in agreement with and further confirmation of anti-inflammatory effects seen *in vitro* [**Sharma et al., 2009b and 2010**]. Through elevated IFN-γ antiviral immune defences are supported and an increased MCP-1 and IL-8 production contributes the attraction of immune cells to the site of infection (chemotaxis).

Results were generally afflicted by large standard deviations, as typically seen with this kind of *ex vivo* experiments. Often statistical significance was not reached for individual cytokines and particular treatment days necessitating a pooling of single measurement into a global analysis (whole treatment period). Also, no placebo control was included and reference was made to baseline. Future experiments may consider to include larger patient numbers to reduce variance of results and include a parallel control treatment. Also, it would be interesting to collect blood samples from infected rather than healthy subjects to gain results from an acute treatment scenario where figures might be different.

Earlier studies by Gertsch (2004) highlighted the importance of alkylamides (e.g. dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides or "*tetraene*") in this immune-modulatory process (Gertsch et al., 2004). Through interaction with the human endocannabinoid system, i.e. binding to cannabinoid receptor (CB-2), *tetraene* and its derivatives are able to modulate the production of TNF- α to provide a possible mode-of-action for the effects observed by Ritchie (2011). The next set of experiments therefore aimed to clarify whether alkylamides are resorbed after all at detectable concentrations in blood serum for their purposed interaction with PBMCs. Galenic variations (drops, tablets) containing EF extract were ingested by healthy volunteers to determine kinetic profiles and levels of bioavailability by applying routine methods for bioavailability investigations. Another goal of the study was to re-confirm above mentioned effects on immunological markers *ex vivo,* again using the established ELISA technique mentioned in Section 2.2 Immune-modulatory effects of *Echinacea purpurea*

2.4 Immunological Research (Bioavailability and Pharmacokinetics)

[6] Woelkart, K. et al. (2006). *International Journal of Clinical and Pharmacological Therapies*, 44(9),401-8.

The applicant designed the research project, wrote study protocol and was involved in the registration process of the clinical study. Writing of publication (clinical and pharmacodynamics parts). His contribution to the overall work: ***20-30%*** *(protocol development and study setup)*

Dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide („*tetraene*") was found to be bioavailable from *E. purpurea* in the form of EF tablets and tincture in a randomized, open, single-dose cross-over study involving 10 patients. Maximal concentration (C_{max}) of *tetraene* was found to be 0.12 ng/ml after a dose of 12 Echinaforce tablets and was reached after T_{max} of 45 minutes. C_{max} of the same marker substance after a dose of 4 mL Echinaforce tincture (EF extract) was found to be 0.40 $\pm$ 0.11 ng/mL after 30 minutes. *Tetraene* levels returned to baseline within three to four hours (**Woelkart et al., 2006**).

For reaffirmation of observed anti-inflammatory effects, PBMC were isolated from EF-treated subjects and stimulated with endotoxin (lipopolysaccharide, 100 pg/ml). After 23 hours of stimulation, supernatant was collected and a significant reduction of TNF-alpha was observed in contrast to IL-6, which remained unchanged. Results were therefore well in agreement with observations by **Ritchie et al., (2011).**

A second study tested the bioavailability of another tablet formulation (Echinaforce Junior tablets) in comparison with above conventional Echinaforce tablets. For both formulations a very similar C_{max} = 0.22 ± 0.15 ng/mL and 0.23 ± 0.16 ng/mL was reached and a comparable amount of marker substance „*tetraene*" resorbed. Echinaforce Junior tablets, which galenically complex alkylamides (e.g. *tetraene*) by β-cylcodextrine "molecular tubes", seemed to release the analytical marker slightly slower. The time to

maximum concentration was reached after one hour, while for the prior formulation the time was approximately half an hour (**Woelkart et al., 2008**).

In conclusion, local and systemic anti-inflammatory effects were identified for EF extract *in vitro*, which were confirmed by two independent *in vivo / ex vivo* assays. Bioavailability studies show that the immune-modulatory substance "*tetraene*" is absorbed after oral application of various EF galenic forms to exert systemic immune-modulation. The latter includes anti-viral and chemotaxis-inducing effects to support the hypothesis of Echinacea as an immunologically active plant.

In our experiments we focussed on the resorption of "tetraene", the main alkylamide in EF extract but did not test derivatives thereof or other typical chemical markers mentioned in table 1. Little evidence is available on the bioavailability of caffeic acid conjugates (cichoric acid or echinacoside), which according to Matthias et al. (2005) do not cross the intestinal barrier *in vivo*. Finally, no evidence of bioavailability is known for other Echinacea chemical markers, which for this reason were not included.

A third and major part of the body of evidence presented in this PhD work form two large clinical trials that were carried out to estimate preventive and acute treatment benefits of EF extract. The first clinical trial administered EF extract continuously over 4 months to investigate safety and efficacy of long-term prevention. The study also aimed to further confirm pharmacological actions of EF mentioned in Section 2.1 to Section 2.4: Pathogen analysis in nasal secretions from patients with colds allowed for reconfirmation of anti-viral effects and by assessing individual's immune status we aimed to re-demonstrate any tailored immune-modulation by EF extract as proposed earlier.

Methods principally adhered to already published study protocols (as reviewed by Karsch-Völk, et al. 2014) whereas cold episodes were defined according to the definition by Jackson and colleagues (1958). This was considered to be highly relevant and best practice to investigate cold therapeutic's effectiveness.

2.5 Clinical Study on Prevention of RTIs by Echinacea (Clinical Research)

[7] Jawad, M. et al. (2012). *Evidence Based Complementary and Alternative Medicine*, 841315, Epub 2012.

The applicant designed the clinical study, wrote study protocol and patient diaries/case report files (CRF), was involved in the registration process and study setup. He was involved in clinical monitoring and finally statistical evaluation, writing of publication and study report. His contribution to the overall work: ***30-40%*** *(protocol development, study setup, analysis and interpretation)*

This randomised, double blind, placebo-controlled clinical trial investigated the safety and efficacy of EF extract prevention on N=755 subjects over 4 months of continuous use. Nasal secretions from participants with acute colds were analysed for the presence of Rhinovirus, Influenza, RSV, Corona-, Parainfluenza-, Adeno-, Bocca- and Metapneumovirus using RT-PCR technique.

EF extract was found non-inferior to placebo with regard to adverse events, adverse drug reactions (ADRs), laboratory blood parameters and finally the assessment of tolerability. Allergic reactions, leukopenia or autoimmune diseases were not increased under Echinacea treatment.

In the placebo group a total of 188 cold episodes were identified, which lasted for 850 sick days in comparison to 149 episodes and 672 sick days with EF extract (Chi-Square test, $p<0.05$). Echinacea reduced virally confirmed infections from 74 to 54, while membranous viruses like Influenza, RSV, Parainfluenza and Coronavirus were found in 24 patients with Echinacea and in 47 with placebo (Chi-Square test, $p<0.05$). Thereby, earlier observations of antiviral activity against membranous viruses were confirmed *in vivo* (**Sharma et al., 2009; Sharma et al., 2010**; Signer et al., 2020).

Preventive effects were also seen on the level of recurring cold infections: 100 episodes observed in 43 subjects with placebo were reduced to 63 episodes in 28 subjects with Echinacea, corresponding to a ratio of recurring infections of 1.59 ($p=0.017$).

The preventive effect of EF was further studied in subjects who reported stress, while reaching >14 points on the perceived stress scale (PSS-10). Both, cold episodes and episode days were significantly increased by 66.6% and 40.7% with placebo treatment ($p<0.05$). Similar results were seen in more susceptible individuals (reporting >2 colds/year, N=343), those with poor sleep (<8 hours sleep, N=160) and finally those who smoked, although the last population was very small with N=43 subjects (Schapowal, 2013).

The primary variable in this study regarded safety rather than efficacy, which was evaluated descriptively. This could eventually be considered as a weakness, however, sample size analysis showed that the study was large enough and appropriately designed to estimate the preventive efficacy with sufficient power.

Data from this large clinical study provides sound evidence for the clinical relevance (3rd level) of *in vitro/ex vivo* observed pharmacodynamic actions (1st and 2nd level as per Figure 1). Firstly, efficacy of EF extract was demonstrated for the prevention of respiratory tract infections. Given the prevalence of viruses in RTIs, this result alone points towards an antiviral efficacy. This was effectively confirmed by a reduced number of viral infections in the EF group. A high specificity towards membranous viruses was again reported

Immune modulation seems to play a central role in Echinacea's mechanism of action. As shown by **Ritchie (2011)**, EF extract modulates immune functions primarily in case of increased susceptibility and during phases of stress. Very similar effects were seen in this clinical trial, where preventive benefits were predominant in individuals with stress, sleeping disturbance or in smokers. In the latter groups, the difference (EF to placebo) in experiencing cold episodes ranged between 40 and 67% and was therefore higher than the 26% relating to the overall population.

This clinical study suffered from the typical limitations of long-term prevention trials, where compliance of study subjects over 4 months cannot be fully guaranteed. Some participants did not report colds at all and it cannot be ascertained whether no infections occurred or whether those were simply not reported. A closer observation of participants would be warranted to monitor intake of study medication and reporting of infections to the study centre. In fact, the Jawad study provides no further substantiation of anti-inflammatory effects because no immunological parameters were assessed in patients. Instead, clinical evidence can be retrieved from artificial rhinovirus infection studies, where *Echinacea* was able to prevent the inflammatory, symptomatic development of clinical colds rather than the virus infection rate (Schoopet al., 2006). In conclusion, pre-clinical and clinical evidence suggests EF extract as active immune-modulator, that specifically reverses virally-induced secretion of pro-inflammatory mediators on the one side and supports immune functions (i.e. chemotaxis or IFN-γ) where required.

With its specificity to broadly inhibit influenza viruses *in vitro*, EF extract was consequently investigated for the treatment of flu by means of an active controlled, randomized, double-blind clinical trial (**Raus et al., 2015**). This study aimed to show non-inferior of EF extract to the gold standard therapy, Oseltamivir (Tamiflu®) in terms of resolution of clinically and virologically confirmed flu.

2.6 Clinical Study on Acute Treatment of Flu by *Echinacea* (Clinical Research)

[8] Raus, K. et al. (2015). *Current Therapeutic Research*, 77, 66-72.

The applicant designed the clinical study, wrote study protocol, case report files and patient diaries, contributed to the registration process and study setup. He was involved in monitoring, statistical evaluation and finally writing of publication and study report. His contribution to the overall work: ***30-40%*** *(protocol development, study setup, analysis and interpretation)*

Overall, 473 patients with clinically diagnosed influenza (ICD-19-GM-2014 classification J11.1) were enrolled in the study, taken a nasal sample for virus analysis and were randomly allocated to receive either EF extract (in form of Echinaforce Hotdrink, also containing *Sambucus nigra*) or Oseltamivir (Tamiflu®) for 10 days treatment under blind conditions. The European Commission granted a marketing authorisation for Tamiflu® for the acute treatment of influenza, for which it is considered the gold standard therapy. This study was closely adapted to the large phase III clinical studies with Oseltamivir, which showed significant superiority over placebo.

The primary efficacy parameter of the study was the confirmation of non-inferiority of *Echinacea* compared to Oseltamivir in the proportion of recovered patients after 1, 5 and 10 days of treatment in the per-protocol collective (PP). The rate of recovery from illness was equivalent in the two treatment groups. After one day of treatment 1.5% of patients with EF extract and 4.1% with Oseltamivir fully recovered. After 5 days of treatment the rates of recovered patients increased to 50.2% and 48.8%, respectively. After 10 days 90.1% and 84.4% of the patients with *Echinacea* and Oseltamivir recovered from illness, respectively.

Very similar recovery rates were obtained from patients with virologically-confirmed influenza as for the whole study population, as included after clinical diagnosis (0% vs. 0% after 1 day; 45% vs. 42.9% after 5 days and 95% vs. 76.2% after 10 days with *Echinacea* and Oseltamivir, respectively). This implies a high prevalence of influenza virus infections among the included study subjects (whole population) although the virus recovery rate with approximately 10% was fairly low, this maybe due to suboptimal sample handling or recovery limits.

Individual influenza symptoms alleviated quickly with both treatments despite marginally higher baseline values in the *Echinacea* group at inclusion. Interestingly, recovery of influenza-lead symptoms (cough, headache, myalgia and feverishness) occurred slightly faster (trend) with EF extract than with Oseltamivir. Fever resolved within 2 days and there was no difference between therapies. Respiratory and gastro-intestinal complications were lower with EF extract (2.46%) than with Oseltamivir (6.45%, p=0.076). Antibiotics were administered only in 2% of patients and no hospitalization occurred, while the total use of rescue-medication was similar in both groups. With respect to safety, EF extract proved superior to Oseltamivir, due to a reduced incidence of gastrointestinal complaints including vomiting.

No patients with underlying health conditions were included into the study and thus no implications on influenza risk populations can be made (i.e. immune suppression, hypertonia or pregnancy). Subjects between 12 and 70 years of age were included but any conclusion regarding the paediatric and senescent population might be poorly conceived due to low patient numbers.

The here investigated product has been developed to further optimize treatment effects in a novel galenic form, Echinaforce® Hotdrink. Beside EF extract as active substance the formulation contains several excipients including concentrated sap from *Sambucus nigra* berries (elderberry) and citric acid. The syrup was to be diluted in hot water. This pharmaceutical form has been shown to contribute to the subjective impression on symptom alleviation, including nasal complaints, sore throat and cough (Sanu and Eccles, 2008). Moreover have clinical studies shown efficacy in the treatment of colds and flu for *Sambucus nigra*, but the resulting evidence was still considered insufficient for establishing an HMPC monograph (Hawkins et al., 2019 and HMPC, 2014). Whereas from a patient's perspective the addition of beneficial components to EF extract is definitely sensible it still raises the question to which extent the observed treatment effects can be attributed to EF extract. With this respect the present study provides evidence for treatment effects for this particular galenic EF formulation rather than the EF extract. Also, this particular work does not further unravel the issues associate with heterogeneity in Echinacea preparations.

This clinical trial nevertheless further corroborates *in vivo* relevance (3rd level) of the *in vitro* observed antiviral activity of EF extract (1st level). Non-inferiority to the antiviral gold-standard Oseltamivir in confirmed influenza infections suggests a similar (antiviral) mode-of-action for Echinacea. This study does however not provide additional evidence of immune-modulatory action as special groups have not further been analysed.

Of particular interest was the low RTI complication rate observed in both treatments. Respiratory infections like colds and flu have a tendency to develop into RTI complications, associated with *sinusitis, bronchitis or pneumonia*. Some respiratory viruses like influenza or RSV are known to impair the physical barrier of airway epithelium and to even down-regulate immune functions giving access to bacteria - finally leading to viral / bacterial superinfections. *Staphylococcus aureus, Haemophilus influenza* or *Streptococcus pneumonia* are common agents involved in pathological illness exacerbation.

A recent meta-analysis showed that *Echinacea* may have a potential not only to prevent viral RTIs but bacterial complications thereof as well. Thereby, lipophilic extracts prepared from *E. purpurea* prevented approximately 50% of complicated developments of RTIs (Schapowal et al., 2015). The very latest publication aimed to mechanistically explain the reported benefits using an *in vitro* approach. It researched in more detail if and how EF extract would be able to prevent bacterial adherence to the airway epithelium secondary to viral infections. In this context, we tested the relevance of cell surface receptors in the binding behaviour of pathogenic bacterial strains.

2.7 Research on Prevention of Superinfections (*in vitro*)

[9] Vimalanathan, S., Schoop, R. and Hudson, J. (2017). *Virus Research*, 233, 51-59.

The applicant has given intellectual input in design and conduct of the study. He was involved in writing publication. His contribution to the overall work: ***20-30%***

Human bronchial epithelial cells (BEAS-2B) were cultivated to confluence prior to infection with H3N2 Influenza virus. After removal of inoculum, cells were treated for 48 hours with EF extract, which was thereafter removed. Then, cellular binding of *Staphylococcus aureus* or non-typeable *Haemophilus influenza* bacteria was measured. EF treatment of BEAS-2B at concentrations of 1:200 and 1:400 lead to a significant reduction of bacterial adherence to virus-infected cells. The proceeding experiment examined involvement of cellular surface receptors (ICAM-1, platelet activation factor (PAF)-receptor or fibronectin), which have previously been shown to play a role in bacterial binding. Indeed, EF down-regulated the expression of all three binding structures as shown by immunohistochemical staining. It was further demonstrated, that binding of *S. aureus* could specifically be inhibited through a monoclonal blocking antibody to ICAM-1, highlighting the importance of receptors in the pathological process of bacterial adherence.

The experimental approach used in this study is relatively new and involves many handling steps, i.e. cell cultivation followed by viral infection and subsequent exposition to EF extract whereas bacterial adherence is examined as a last procedure. One experimental cycle takes several days until completion and thereby is affected by significant fluctuation. Results should thus be considered qualitatively rather than to quantitatively assess the exact extent of reduction. Nevertheless, the experiment provides a reasonable explanation for clinically observed effects of Echinacea in reducing RTI complications.

3. Overall Discussion

Medicinal plants are a rich source for potent bio-active substances and even the pharmaceutical industry is reverting to chemical scaffolds from nature for drug discovery (Newman and Gragg, 2012). Isolation of individual compounds and activities however seems not trivial and different extraction techniques yield products, which are hardly comparable. Decades of Echinacea research investigated highly heterogeneous products from variable species and plant parts, using differing extraction methods. As a result, overall conclusions on Echinacea-derived preparations remain vague, leading to much confusion about sense or non-sense in using it. The goal of this PhD work was therefore to accurately address pharmacology and clinical efficacy for a single *E. purpurea* extract (Echinaforce®) by employing state-of-the art scientific methods. The research focussed on test systems deemed relevant for the estimation of its potential in respiratory tract infection prevention and treatment: Antiviral, anti-inflammatory and immune modulatory effects were studied *in vivo, ex vivo* and under *in vivo* conditions.

3.1 Antiviral Activity of EF Extract

Results clearly indicate a broad antiviral potential directed against membranous viruses. Experiments were carried out at three independent research facilities:

- University of British Columbia, Canada: Sharma et al., 2009
- University of Giessen, Germany: Pleschka et al., 2009 and
- Laboratory Spiez, Switzerland: Signer et al., 2020.

Results were across laboratories highly consistent and were finally confirmed by a large clinical prevention trial conducted at the University in Cardiff (Jawad et al., 2012). Whereas *in vitro* experiments yielded significant results on the level of individual viruses, in the clinical study (Jawad et al., 2009) only the pooled result of membranous viruses gave p-values below 0.05. This was mainly a consequence of low sample numbers when breaking down to the single pathogen rather than a lack of effect. A very recent clinical study investigated EF extract in children (4 – 12y) and for the first time provided a significant reduction also on the basis of particular virus species (i.e. influenza) (Ogal et al., 2019). Rhino- just like adenoviruses seem to be more resistant to EF extract that could not reduce infections neither in spontaneously occurring nor in artificially induced RV infection studies. A meta-analysis by Schoop, however, identified a potential of Echinacea in reducing the symptomatic development, rather than the infectivity of RV pointing towards anti-inflammatory activity of the extract being of importance, too (Schoop et al., 2006). In a patient's perspective it might be irrelevant whether prevention of RTIs is achieved through suppression of viral infection or simply of symptom development – since both would result in maintenance of health.

Rhinoviruses are not known to be particularly lytic nor to cause significant pathology to the respiratory tract (Mosser et al., 2005). This in sharp contrast to for instance influenza, which has been demonstrated to cause more serious epithelial disruption and pathology (Short et al., 2016) Therefore, a reduced antiviral activity against RV may be acceptable from a clinical perspective, despite its frequency in causing colds.

3.2 Local and Systemic Anti-inflammatory Effects of EF Extract

Cytokines and chemokines are important immunological players that are complexly regulated during acute respiratory tract infections. Their modulation presents an effective means for managing the disease through a therapeutic intervention (Johnston, 1997). Suppression of (locally expressed) TNF- α or IL-6 is expected to correlate with symptom reduction, whereas anti-virally acting IFN-γ or chemotactic cytokines (e.g. MCP-1) are still required to constitute a systemic immune response and their activation might be meaningful (Roitt et al., 1998).

Several *in vitro* experiments showed down-regulation of TNF-α and IL-6 by EF extract in the airway epithelium (local reaction) and this was also confirmed *ex vivo* in peripheral blood mononuclear cells (PBMCs) after peroral ingestion (systemic reaction) (**Sharma et al., 2009a/b; Ritchie et al., 2011**). IL-8 was down-regulated by EF extract only in epithelial cells *in vitro* (contributing to the local anti-inflammatory effect) but at the same time upregulated systemically by PBMCs *ex vivo* (**Ritchie et al., 2011 and Woelkart et al., 2006**). Further experiments are required to explain EF's versatile effects concerning this parameter on different cell types. A possible explanation could be variable CB-2 expression patterns (PBMC >> epithelial cells), any other yet undefined receptor accounting for differential regulation or systemic feedback loop mechanisms.

Anti-inflammatory effects thus are evident for EF extract, as shown by the local and systemic regulation of TNF-α and IL-6 (**Sharma et al., 2009a/b**; **Ritchie et al., 2011; Woelkart et al., 2006**). Importantly, this effect only applies for endotoxin-free Echinacea, whereas contamination with bacteria produces a contrary impact (please refer to Section 1.2 Echinacea Product Heterogeneity). Finally, anti-inflammation appeared exclusively upon cellular stimulation with lipopolysaccharide (LPS) or viruses whereas EF extract alone in an un-induced setting did not show any effect (**Sharma et al., 2009a/b; Ritchie et al., 2011,** Gertsch et al., 2004).

3.3 Systemic Immuno-modulation by EF-Extract

An intact immune response towards exogenous pathogens depends on further factors, which EF extract is able to modulate. Interferon-gamma (IFN-γ), a potent stimulator of antiviral immune defences experienced

ex vivo a marked up-regulation when EF extract was administered perorally (**Ritchie et al., 2011**). In the same study, chemotactic cytokines MCP-1 and IL-8 were upregulated. Again, cytokine protein modulation was exclusively observed in the presence of viral / bacterial stimuli (LPS), whereas EF alone did not alter MCP-1 or IL-8 secretion - neither on epithelial cells nor in PBMC (Gertsch et al., 2004; **Sharma et al., 2009**). Intracellular signalling molecules (STAT or NF-κB) and cytokine mRNA may be induced by EF but are not translated into biologically active protein. Whether this represents a form of immune cell "priming" remains to be determined.

Effects seen on cytokine production may best be described as immuno-modulation as they not only unfold in dependence of absence/presence of pathogens but also of immunological conditions (stress, lack of sleep or susceptibility). The concept of Echinacea as immune-modulator is further substantiated by the fact, that preventive benefits were primarily observed in subjects with expected weak immune functions as described by **Jawad (2012)**. In his meta-analysis, Schapowal et al. (2015) came to the very same conclusion when looking at recurrent RTIs and complications.

Interestingly, EF seems to solely act on cytokine expression whereas the differential blood count did not detect any changes in lymphocytes, monocytes, granulocytes or thrombocyte levels even after prolonged exposure to EF extract over 4 months (**Jawad et al., 2012**; Schoop et al., 2006).

3.4 Bioavailability of Alkylamides

It was found that many above observations might result from interaction of alkylamides with the endocannabinoid system, in particular CB-2 receptor expressed on immune cells (Gertsch et al., 2004). Alkylamide derivatives were shown to permeate cultivated intestinal epithelial cells (Caco-2) and the main compound dodeca2E,4E,8Z, 10E/Z tetraenoic acid-isobutylamide ("tetraene") was found in blood serum after peroral administration of different formulations containing EF extract (Matthias et al. 2005, **Woelkart et al., 2006** and Woelkart et al., 2008). The overall resorption was fast, with C_{max} (< 1ng/ml) reached within 30 min to 1 hour to indicate a first-pass effect - maybe including permeation through mucous membranes. The latter however remains to be shown in more detail. Alcohol appears to represent a good carrier and we found that Echinaforce (EF) tincture outperformed tablet formulations with respect of the overall resorbed amount and time to maximal concentration of *tetraene* in blood serum. Despite pharmacokinetic differences both, EF tincture and tablets attenuated the production of TNF-α to the same extent and demonstrated similar bio-activity (**Woelkart et al., 2006**).

Bioavailability of immunologically active alkylamides provides a strong linkage between *in vitro* findings and evidence obtained from *ex vivo* and clinical experiments which are discussed as follows.

3.5 Clinical Trials on Prevention and Acute Treatment of RTIs

It was an essential part of this PhD work to finally find pre-clinically observed antiviral, anti-inflammatory and immune-modulatory effects peaking in clinical efficacy of EF extract.

In this regard, a comparable antiviral spectrum was identified for EF *in vitro* and *in vivo*, showing a high specificity against membranous viral pathogens. EF extract proved equivalent to the gold standard treatment Oseltamivir for the treatment of influenza. Increased preventive benefits were seen in patients with weak immune defences and those in need for effective immune support. It is reasonable to assume that the proposed pharmacological actions result in both, preventive and acute treatment benefits. It would be interesting to estimate the individual contribution of antiviral, anti-inflammatory and immune-modulatory effects to the respective treatment situation. Currently, however, this remains a point of speculation.

EF prevented viral infections by 26% overall and by up to 50% in subjects with stress or susceptibility but bacterial complications (superinfections) like pneumonia were reduced by as much as 68%. This implicates another pharmacological principle in EF, namely a potential activity against bacteria. *In vitro* tests have shown direct inhibition of *Streptococcus pyogenes, Haemophilus influenza or Legionellae pneumophilia* (**Sharma et al., 2010**). An anti-bacterial mode-of-action is largely unknown and it is questionable whether sufficient concentrations of EF compounds are reached in the human organism for physiological relevance. Therefore, another – maybe more plausible – explanation for prevention of bacterial RTI complications was sought.

3.6 Prevention of Bacterial RTI Complications

Viral infections tend to affect barrier functions of the airway epithelium facilitating subsequent bacterial co-infection and development of RTI complications like *Pneumonia*, *Otitis Media* or *Sinusitis* (Matsukura et al., 1996). The prevention of viral infections thus has an impact on secondary complications as well because barrier functions remain intact (McCullers, 2011). Mechanistically it could be shown that EF even if administered after viral attack to epithelial cells reversed the expression of bacteria-binding receptors like ICAM-1, PAF-receptor or fibronectin (**Vimalanathan R et al., 2017**). Otherwise, those receptors would be virally induced on surfaces of the airways in high quantities to attract bacteria and facilitate super-infections. Once more, EF effects unfolded only upon viral activation, whereas under unstimulated conditions no significant receptor reduction was observed.

These very latest findings suggest potential for EF extract beyond the traditional treatment and prevention of colds and flu, as a potential alternative to antibiotics which are still routinely used to treat or prevent bacterial RTI complications (Cantrell et al, 2002).

4. Implications for Future Research

Phytochemical characterization of herbal preparations is essential prior to determination of their biological activities and clinical benefits. Manufacturing methods may greatly influence the analytical fingerprint and consequently therapeutic outcomes. However, any one-way correlation of bio-activities with particular chemical markers might be as tempting as misleading because other - yet undiscovered - substances could mediate the same effect and / or work synergistically (Wagner and Jurcic, 2011). Plant preparations should preferably be regarded in a "holistic" way rather than a mixture of particular chemicals. Importantly, influenza viruses are able to develop resistances towards the chemical Oseltamivir but not against the multicomponent EF extract (Cheng et al., 2009). Consequently, manufacturers of herbal medicinal products should pay prime attention on the production process rather than the concentration of particular chemicals in their product ("*quality by design*" or "*the process is the product*"). On the example of Echinacea, research is still not convincing enough (and will probably never be) to trace down its efficacy to single markers. Alkylamides appear to modulate production of TNF-α but show no antiviral potential. The latter activity could so far not be attributed to any known marker substance.

In this context, research on herbal medicinal products remains highly challenging and the comparability between differently manufactured products illusive. For this reason, this PhD work concentrated on a single Echinacea preparation. The above proposed antiviral, anti-inflammatory and immune-modulatory actions principally comply with the current understanding of managing RTIs. The measured bioactivities remained stable across different manufacturing lots and production years and thereby proved to be robust. Although the chosen research strategy produced convincing results overall, other pharmaceutical activities have not been researched yet. Future research should aim to explore Echinacea's effects on the human microbiome or on microbial metagenomics in the gut or airways, which are discussed to have a major impact on the human immune system and health. Furthermore, the pharmacology of alkylamides is still not well understood. Yet unpublished data showed a wide variety of isobutylamide derivatives in alcoholic extracts whereas their individual contribution to immune modulation remains to be determined. On the other hand, it would be interesting to elucidate mechanisms and *in vivo* relevance of antibacterial effects of the extract. Ethnopharmacology might provide valuable guidance on the direction of future Echinacea research, whereas it is of utmost importance to always refer to the nature of preparation used.

Maybe the most promising and most convincingly researched potential of EF extract is the unspecific activity against a broad range of membranous viruses. The current (2020) Coronavirus (SARS-CoV-2) outbreak shows that nature harbours a plethora of pathogens for which the medical armamentarium has no solution. Infections can globally spread within weeks and newly developed vaccines or therapies are not available fast enough. Readily accessible herbal treatments like EF extract may under real-life conditions provide an immediate, affordable and effective therapeutic solution. More in-depth pharmacological

research on how therapeutic benefits manifest might be required to convince health authorities and organizations prior to their official recommendation.

5. Overall Conclusion

This PhD work is based on research that has been carried out and published in the past 10 years (so-called PhD by publication). The aim of this doctoral work was to explore *in vitro* and *in vivo* pharmacological effects, as well as clinical efficacy in prevention and acute treatment of respiratory tract infections for a single, phytochemically characterized and standardized *Echinacea purpurea* extract (Echinaforce®, EF).

The provided evidence suggests antiviral, anti-inflammatory and immune-modulatory activities, for which Echinacea has long been used in tradition. Two clinical studies were conducted to estimate preventive and treatment benefits in respiratory tract infections under randomized, double-blind, placebo-/active controlled conditions. EF has potential in preventing bacterial complications of initial viral infections through regulation of bacteria-binding receptors on epithelial cells.

The presented work demonstrates, that the applicant has successfully designed and carried out a complete research program including pre-clinical, clinical as well as pharmacokinetic studies on *Echinacea purpurea*. Overall, this work substantially contributes to the modern scientific understanding of this medicinal plant and is herewith proposed for consideration as doctoral thesis based on publication work.

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Appendix 1

Table 2: Tabular listing of applicant's contribution (marked in green, X) to the individual research work forming the body of evidence referring to in this application for PhD. More specific contributions are explicitly indicated in the respective boxes.

Reference	Invention Novelty	Design	Project Lead	Conduct	Registration Authorization	Interpretation	Publication Writing
Sharma M (2009a) [1]	-	X	X	-	n.a.	Relevance of immune mediators	Correlation in vitro – clinical situation
Pleschka S (2009) [2]	Resistance experience	X	-	Project Coordination	n.a.	X	Echinacea, clinical relevance
Ritchie M (2011) [3]	ILCS instant leucocyte culture system	X	X	Informed consent Patient inclusion	MHRA	Biological action cytokines	Introduction and Conclusion
Sharma M (2009b) [4]	-	X	X	-	n.a.	X	In vivo relevance and clin. Evidence
Sharma M (2010) [5]	Epi-Airways, 3D tissues	X	-	-	n.a.	X	-
Woelkart K (2006) [6]	-	Study protocol	X	-	X	-	Pharmacodynamics
Jawad M (2012) [7]	Nasopharyngeal virus detection	Study protocol CRF diaries	X	Monitoring site setup	MHRA	Statistical Evaluation	Full publication
Raus K (2015) [8]	-	Study protocol CRF diaries	X	Monitoring	SUKL	Statistical Evaluation	Full publication
Vimalanathan S (2017) [9]	Superinfections	X	X	-	n.a.	-	Respiratory Tract Complications

Annex 1: Full Publications