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# **CONIFEROUS RESIN SALVE, ANCIENT AND EFFECTIVE TREATMENT FOR CHRONIC WOUNDS – LABORATORY AND CLINICAL STUDIES**

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ACADEMIC DISSERTATION

To be presented with the permission of  
the Medical Faculty of the University of Helsinki,  
for public examination in Lecture Hall 4 of Meilahti Hospital,  
Haartmaninkatu 4, Helsinki, on May 24<sup>th</sup>, 2013, at 12 noon.

Helsinki 2013

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ISBN 978-952-10-8710-3 (paperback)  
ISBN 978-952-10-8711-0 (pdf)  
<http://ethesis.helsinki.fi>  
Unigrafia, Helsinki 2013

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## **ABSTRACT**

Sipponen, Arno. **Coniferous resin salve, ancient and effective treatment for chronic wounds - laboratory and clinical studies**

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Natural coniferous resins and other terpenic wood extracts have been raw materials for various products in industry, and have been used as traditional medicines in Finland for hundreds of years, particularly as a home-made salve for skin wounds and infections. Due to the author's own positive empirical experiences of natural coniferous resin salve in wound care, the present "resin-project" was set up in order to investigate (1) the antimicrobial properties of the resin and resin salve by microbiological laboratory techniques, and to study (2) the efficacy, feasibility and safety of the resin salve for wound care in objective clinical trials. The thesis comprises four microbiological investigations and two clinical trials.

Microbiological studies with an agar diffusion test and with the European Pharmacopoeia challenge test gave somewhat controversial results indicating that the water solubility of resin and resin salve may affect the assay results. The studies showed that coniferous resin and resin salve were strongly antimicrobial against a wide spectrum of both Gram-positive and Gram-negative bacteria, including the methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant enterococcus (VRE). The resin was, in addition, antifungal against the most common dermatophytes, and against the *Candida albicans* yeast. The antibacterial action of resin was observed to result from destruction of the bacterial cell wall and cell membrane structures. Microbes, both bacteria and fungi, exposed to resin or resin salve, showed thickening of the cell walls. The cells formed aggregations, showed arrested mitoses, and were finally destroyed according to the observations in scanning and transmission electron microscopy. In electrophysiological experiments with *Staphylococcus aureus*, the exposure of the micro-organisms to resin and resin acid (abietic acid) caused dissipation of the cell membrane potential and changed the branching of fatty acids in the cell walls. The observed changes were concluded to indicate that the coniferous resin influences the bacterial cell survival via damage to cell membrane functions.

In tests on safety aspects, the coniferous spruce resin or resin salve were not mutagenic in Ames tests. In skin irritation tests in rabbits (ISO 10993-10:2010 standard),

the resin salve containing purified coniferous resin 10% (w/w) was “negligibly” irritating. In *in vitro* cytotoxicity tests (EN ISO 10993-5:2009 and USP<87>) with mouse fibroblasts in HAM F12 culture medium the salve was markedly cytotoxic but slightly cytotoxic if diluted (1+3) with fresh culture medium.

In analyses with gas-liquid chromatography (GLC) and mass spectrometry, the acetone extracts of both pure spruce resin and resin salve contained coumaric acid, a group of diverse resin acids and lignans. All these compounds are potentially biologically active and were calculated to occur in total in purified spruce “callus” resin at a concentration of 300 mg/g of resin (w/w) and 30 mg/g (w/w) of the 10% resin salve, on average.

One of the clinical trials is a randomized, prospective investigation where the efficacy of resin salve treatment for 6 months was compared to the efficacy of hydrofiber dressings (with or without silver) in 37 patients with severe, grade II-IV, pressure ulcers. In this trial the resin salve treatment was, in both per protocol and ITT (intention-to-treat) analysis, significantly more effective ( $P=0.003$ ) in improving ulcer healing (regarding both the rate of healing per patient ( $N=23$ ) or per ulcers ( $N=29$ )) than the control treatment. All pressure ulcers in all compliant patients (per protocol analysis) in the resin group healed except for one ulcer in one patient (94% (95%CI:84–100%)) whereas less than half (36% (8–65%)) of the ulcers healed in the control group. Resin salve improved the ulcer healing independent of whether the ulcer was infected or not. The other clinical trial was an observational and prospective study on efficacy and feasibility of the resin salve treatment in chronic (complicated) surgical wounds in a cohort of 23 patients. In all 23 patients, the wound healed in  $43\pm24$  days on average without any draw-backs. In multivariate analysis, the length of the wound was an independent and significant predictor of the healing time. In resin salve therapy, the wounds progressively healed (re-epithelialized) at 2 mm per day on average. In the clinical trials, including a total of 45 compliant patients, one patient (2%; 95%CI:0–5%) had an allergic reaction (allergic contact dermatitis). No other complications or side effects were observed. In calculations of direct costs, the expenses of the pharmaceutical materials were  $1.2 \text{ €} \pm 0.5 \text{ €}$  per day on average among the patients treated with resin salve.

Descriptions of terms commonly used in the present paper are presented in Appendix I.

**Keywords:** Resin, rosin, Norway spruce, microbiology, pressure ulcer, wound healing, hydrofiber dressing, chronic surgical wound, surgical site infection, wound infection, cost analyses.

## **ACKNOWLEDGEMENTES**

The thought of writing a dissertation rose from my empirical observations as a GP in Kolari 12 years ago. Severe, chronic wounds did not react so well to generally used wound-care products but healed fast when using traditional resin salve. The positive experiences on resin salve's efficiency raised the question of whether the successes were just coincidental or whether this treatment is efficient "for real".

Research work is a requirement for developing new treatments. In my dissertation and the studies it is based on, I have aimed at finding answers to questions on whether the centuries-old resin salve treatment is an acceptable, safe and efficient treatment also when examined from the viewpoint of modern medical criteria and objective research.

Writing the dissertation has been a large challenge along with the daily work as a surgeon. The attitude of my superiors PhD Jussi Haapala and Docent Markku Luostarinen at Päijät-Häme Central Hospital, and Professor Eero Belt at the late Heinola Rheumatism Foundation Hospital, as well as the granting time off for research has been of crucial support, and I want to thank them all for this help.

The topic of the dissertation and the business born from it has, from time to time, caused conflicting thoughts and statements in my environment and colleagues, which has surely not made the research easier. Despite this, I have tried to keep in mind the general governmental guideline of "the goal of scientific study must be innovations that can also be utilised commercially and industrially".

For those who had a positive attitude towards the grant applications, I want to thank the Lapland Regional Fund of the Finnish Cultural Foundation for the grants in 2005 and 2008 as well as The Finnish Medical Society Duodecim for the grant in 2008.

The dissertation is interdisciplinary in nature and I have often needed the help of several specialists. I want to thank the researchers at The Finnish Forest Research Institute (METLA); PhD Pekka Saranpää, MSc Tapio Laakso, PhD Rainer Peltola and ScD Minna Männistö, and for electron microscope research, Docent Kari Lounatmaa. I thank all my colleagues who were co-workers in the clinical studies, Docent Anthony Papp, MD Harri Kauppinen, PhD Raine Tiihonen and Docent Petteri Carlson.

I want to direct special thanks to the microbiologists Docent Merja Rautio, Docent Kirsi Laitinen and MSc Pirkko Koukila-Kähkölä for their skilled and professional co-operation. I also want to direct my thanks to Wound Nurse Opri Kuokkanen for help in carrying out the patient studies.

Revising of the language of the publications and the thesis were carried out by Docent Robert Paul and by Docent Jan Dabek. I thank them both.

I thank Professor Seppo Sarna for the statistical advice and help in the fifth publication. I also want to thank Professor Eero Saksela for the advice, opinions and support during writing of the dissertation.

The official referees, Docents Veijo Saano, Hannu Syrjälä and Jyrki Vuola have done noticeably precise and detailed work. I thank them for their great help, professional directions and support.

I want to direct special thanks to my friend and colleague PhD Janne J Jokinen for his crucially important co-operation and contribution in dissertation affairs and many activities outside of it. The thousands of kilometres' travel related to the randomised patient study, travelling to 11 health centres and countless fast-food restaurants around Finland, have been one of the highlights of the whole research work.

I thank my friend, colleague and supervisor Docent Jouni Lohi for his help and support in the different phases of the dissertation and also for the “prepping course” he organised in Posio. It has been important to talk with him also in those moments when everything has not gone quite as planned.

I thank my friend and father, Professor Pentti Sipponen for all the support I have received from him, both in research activities and issues outside of the science.

My wife Pia, children Immo, Okko and Hilla; if the cobbler's children have no shoes, I believe that in our family we manage to get the wounds closed.

## LIST OF ORIGINAL PAPERS

This thesis is based on the following original articles, which are referred to in the text by the Roman numerals:

- I Rautio M, Sipponen A, Peltola R, Lohi J, Jokinen JJ, Papp A, Carlson P, Sipponen P. *Antibacterial effects of home-made resin salve from Norway spruce (Picea abies)*. APMIS 2007;115:335-340
- II Sipponen A, Laitinen K. *Antimicrobial properties of natural coniferous rosin in the European Pharmacopoeia challenge test*. APMIS 2011;119:720-724
- III Rautio M, Sipponen A, Lohi J, Lounatmaa K, Koukila-Kähkölä P, Laitinen K. *In vitro fungistatic effects of natural coniferous resin from Norway spruce (Picea abies)*. Eur J Clin Micr Inf Dis 2012;31:1783-1789
- IV Sipponen A, Peltola R, Jokinen JJ, Laitinen K, Lohi J, Rautio M, Männistö M, Sipponen P, Lounatmaa K. *Effects of Norway spruce (Picea abies) resin on cell wall and cell membrane of Staphylococcus aureus*. Ultrastructural Pathology 2009;33:128-135
- V Sipponen A, Jokinen JJ, Sipponen P, Papp A, Sarna S, Lohi J. *Beneficial effect of resin salve in treatment of severe pressure ulcers: a prospective, randomized and controlled multicentre trial*. Br J Dermatology 2008;158:1055-1062
- VI Sipponen A, Kuokkanen O, Tiihonen R, Kauppinen H, Jokinen JJ. *Natural coniferous resin salve to treat complicated surgical wounds: pilot clinical trial on healing and costs*. Int J Dermatology: 2012;51:726-732

## ABBREVIATIONS

BCE	Before the common era
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CFU	Colony forming unit
DiOC <sub>2</sub>	3,3'-diethyloxacarbocyanine iodide
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
FAB	Fastidious anaerobe broth
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GLC	Gas-liquid-chromatography
GMP	Good manufacturing practice
HA	Hyaluronic acid
HB-EGF	Heparin-binding epidermal growth factor
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
IL	Interleukin
ISO	The International Organization for Standardization
ITT	Intention to treat analysis
Ltd	Limited
METLA	Finnish Forest Research Institute
MFI	Mean fluorescence intensity
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MS	Mass spectrometry
NO	Nitric oxide
PDGF	Platelet-derived growth factor
PMNL	Polymorphonuclear leucocyte
ppm	Parts per million
RCT	Randomized controlled trial
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TGF	Transforming growth factor
TGF-β	Transforming growth factor-β
TMSI	Trimethylsilylimidazole
TNF-α	Tumor necrosis factor -α
VEGF	Vascular endothelial growth factor
VRE	Vancomycin-resistant enterococcus

# 1. INTRODUCTION

Treatment of chronic wounds is difficult and long-lasting. There are hundreds of topical treatment options available on the market with largely unproven clinical efficacy. Aging of the population results in higher and higher prevalence of chronic wounds and leads to increasing use of topical wound care products. Modern dressings do not always lead to satisfactory wound healing, which makes further research on wound treatment options including old forgotten treatment options used in folk-medicine relevant. Resin salve has been used for centuries in traditional medicine. The resin study project was set up owing to encouraging personal clinical experience with resin salve treatment in patients with severe pressure ulcer (Sipponen et al. 2003, Sipponen et al. 2007). The aim of the project was to investigate the efficacy and the mechanisms of action of coniferous resin and resin salve in the treatment and healing of skin wounds and ulcers.

## 1.1 MEDICAL USE OF RESIN IN THE PAST

The first reports of using resins or rosins in medicine are from the antiquity. Resins have been used for nearly every kind of human disorder and disease (Langenheim 2003). In mummification, the Egyptians first used simple linen bandages and plaster but began to use resin-impregnated linen bandages during the 11<sup>th</sup> dynasty, 4000 years ago (Proefke et al. 2008). As the technologies improved, the mummies were dried with natron and even filled with heavily resin-soaked linen, obviously to improve the preservation of the body and to inhibit the insects and microbes destroying the tissues (Dawson 1927, Ancient Egypt 1999, Dunn 2011).

First medical publication of the use of coniferous resin in medical practice in Finland is from 1578. Swedish physician Benedictus Olai wrote about natural resin in treatment of old leg wounds in the first medical textbook of the Swedish kingdom as follows: "...take wax and white spruce resin, both 2 pellets, rams tallow 2 pellets, ship pitch, and olive oil, both 6 pellets...keep in glass container. The wound has to be washed three times a day with sweet wine, and then spread with the above mentioned salve" (Olai 1578).

Elias Lönnrot (1802-1884) was a Finnish physician, botanist and philologist. In his publication on Finnish botany, in the Flora Fennica from 1866, Elias Lönnrot wrote about coniferous resin as follows: "when the tree rind is wounded in the summer, a resin-like liquid streams out, which will solidify to thick terpene that can be used externally as a detergent and wound curative" (Lönnrot et al. 1866).

Beside the use for medical purposes, resin and rosin have been used in various non-medical applications (Langenheim 2003). Such application range from religious purposes to production of incenses, and includes even the ship industry. Rosin has been a raw material for various products in the chemical industry, the end products being, for example, paints, lacquers, solvents, printing inks, chewing gums, spices and perfumes, soaps and other items of personal hygiene (Metsälä 2001). Pine resin has been an important commodity at least since biblical times, as attested by the story of Noah receiving instructions from God to “cover the ark inside and out with pitch” (Bible approx. 1000 BCE).

Salves manufactured from the Norway spruce (*Picea abies*) have been utilized for centuries in folk-medicine, at least in the northern part of Finland and Sweden. Traditionally the resin salve was prepared by boiling the resin with butter or animal fat. The resulting salve was used, and is still in use, as a home-made remedy for wounds, skin infections and abscesses. A typical indication for the resin salve treatment was, for example, the “resin suction” in cases where a foreign body had to be removed from the skin (e.g.; wooden splinter) (Timo Kyrö personal communication 2006, Pakkanen et al. 2011). In practice, the resin salve was used occasionally in primary care even by the authorized physicians in treatment of different kinds of skin wounds, in northern Finland in particular.

In the beginning of millennium, the author and his colleagues had positive experiences from some sporadic patient cases treated with the traditional resin salve in Kolari health care center, Finnish Lapland, suggesting that the resin salve is an effective and a well tolerated treatment option in skin wounds and ulcers (Sipponen et al. 2003, Sipponen et al. 2007). Therefore, the present “Resin Project” was set up to study objectively the effectiveness and the mechanisms of resin salve and natural coniferous resins in wound care.

The recipes and recommendations published by Benedictus Olai in 1578 or by Elias Lönnrot in the 19<sup>th</sup> century are obviously the only few publications of resin salve treatment for wound care before 2003. Although resin salve has been used in folk medicine for centuries, scientific and objective studies on its clinical efficiency in human medicine do not seem to exist. No scientific papers in English can be found in the PUBMED database before 2003 by the words “resin and skin”, “resin and skin wound”, or “coniferous resin and skin ulcer”.

## 1.2 CONIFEROUS RESIN

### 1.2.1 BIOLOGY

The conifers (e.g., pine, spruce, balsam, fir, etc.) synthesize resin (oleoresin) which is composed of pure resin acids mixed with various volatile and nonvolatile terpenes (Langenheim 2003, Keeling et al. 2006, Zulak et al. 2010). This resin is yellowish and liquid and can be trickled by artificially wounding of the tree trunk (Nagy et al. 2000). Some distilled fractions of the wood extracts (e.g., pine oil, tall oil) of coniferous trees are called rosins and contain only resin acids in varying ratios, being, thereby, largely similar in composition to the natural heartwood resin (oleoresin) (Holmbom et al. 2008, Zulak et al. 2010). By heating and pyrolysis (e.g., in tar kilns) the resins form tars, a process during which carcinogenic compounds are also formed.

In biology, the coniferous resin has been mainly studied by the wood physiologists and chemists. Coniferous resins are divided into physiological and non-physiological resins (pitch). The physiological one (oleoresin) appears mainly in resin canals of the heartwood and knots (Langenheim 2003). The heartwood resin (oleoresin) is a mixture of resin acids, other terpenes and fatty acids. In case of wounding of the tree rind, the heartwood resin, oleoresin, is exuded into the sapwood and further onto the tree surface (Holmbom et al. 2008, Zulak et al. 2010). Exudation of the oleoresin starts 3–4 weeks after the wounding. With time and evaporation of the volatile terpenic compounds, the oleoresin on the wound surface will be mixed with secretions and ingredients from the cells in wood sap and surface, resulting in knot-like masses composed of the sc. non-physiological resin. This non-physiological “callus” resin occurs as nodular swellings on the rim of the wound (Holmbom et al. 2008).

On the tree trunk, the initially liquid physiological resin (oleoresin) will be hardened with time resulting in multicolored, dark and semisolid material (Fig. 1). This material, “callus” resin, is mechanically removable, for example, by knife or axe. The callus resin of Norway spruce (*Picea abies*) has been the typical raw material for the home-made salves and is also the raw material of the resin used in the present study, or of salves industrially prepared and manufactured from the natural coniferous resin.

## INTRODUCTION



**Figure 1** Norway spruce (*Picea abies*) is a common conifer in the Taiga zone, also known as the boreal forest, which is the world's largest terrestrial biome. In North America it occupies most of inland Canada and Alaska as well as parts of the extreme northern continental United States. It also covers most of Sweden, Finland, Norway, Russia (Siberia in particular), northern Kazakhstan, northern Mongolia, and northern Japan (Hokkaido). The “callus resin” (insert) is formed as masses on the surface of the conifer trunk if the tree surface is wounded. Callus resin is multicolored, solid or semisolid and is composed of various ingredients synthesized and secreted by the heartwood and sapwood cells.

All coniferous trees synthesize and secrete resin that vary somewhat in content and type of resin acids (Holmbom et al. 2008). Resin is easily drawn off in liquid form from the trunk of pines, for example, whereas the solid or semisolid masses of the multicolored resin swellings on the tree trunk (“callus resin”) are formed in the spruce, and are the natural source of the resin.

Fungal and bacterial infections cause disease in the conifers, against which the trees form and secrete resins, obviously as a defense (Byun-McKay et al. 2006, Zukal et al. 2010). The conifers have likely evolved the terpene-based resin secretions to deter insect pests and to prevent microbial contact and invasion, a mechanism that resembles the sticking-plaster in prevention of the infections in human skin wounds (Trapp et al. 2001, Franceschi et al. 2005). As hydrophobic compounds, resin may also inhibit the influx of water and protect the tree from dehydration (Holmbom et al. 2008).

### **1.2.2 CHEMICAL COMPOSITION**

The chemical composition of plant resins depends on the definitions used and the source of the resin. The resin, in general terms, is a liquid hydrocarbon secretion of many plants, and is mainly composed of volatile and nonvolatile terpenes and essential oils (Langenheim 2003). Some resins, particularly in conifers, contain a mixture of diterpenic, organic carboxylic acids, called resin acids, in high quantity (Kimland 1972, Holmbom et al. 2008). The components of resin can be separated by fractional distillation. Distillation of the physiological resin (oleoresin) from wood extracts results in formation of rosins, which are mixtures of various resin acids (Zulak et al. 2010). The rosins and resins are solid at room temperature. The oleoresins that contain benzoic acid or cinnamic acids are called balsams.

The resin acids in resins and rosins are diterpenic, tricyclic organic acids and are classified as abietic acid or pimamic acid types (Langenheim 2003, Zulak et al. 2010). These diterpenic resin acids are nonvolatile and are one of the major components of the solid spruce callus resin. Abietic acid, dehydroabietic acid, hydroxydehydroabietic acid and neoabietic acid are examples of common resin acids of the abietic type. Pimamic and isopimaric acids are the corresponding examples of acids of the pimamic type. The resin acids are soluble in alcohol, DMSO and various organic solvents but are poorly water soluble, excepting some solubility of the dehydroabietic acid and hydroxydehydroabietic acids (Peng et al. 2000, unpublished preliminary studys). At room temperature the resin acids are solid (melting point above 150°C).

### **1.2.3 ANTIMICROBIAL PROPERTIES**

The scientific literature on the microbiology of plant extracts is large. Many terpenes and terpenoids have been shown to be antimicrobial against bacteria, fungi, and even against viruses or protozoa (Cowan 1999, Peng et al. 2000). The microbiology of the resin acids of coniferous origin is relatively extensive but largely targeted on studies on bioaugmentation of bacteria and fungi for removal of the resin acids from pulp mill effluents (Liss et al. 1997, Mohn et al. 1999, Yu et al. 2001, Kallioinen et al. 2003, Kostamo et al. 2003). Resin acids and rosin in high concentrations are toxic to aquatic organisms, and may impair the proper function of paper machines (Yu et al. 2001). In addition, the isolation of microbes of genera *Sphingomonas*, *Zoogloea*, *Raistonia*, *Burkholderia*, *Pseudomonas* and *Mycobacterium* may grow on and utilize resin acids (Mohn et al. 1999, Yu et al. 2001).

The antibacterial and antifungal properties of the resin acids are examined by biologists and wood microbiologists with ordinary microbiological laboratory techniques and also by using pine wood blocks and spruce wood chips as culture media (Mohn et al. 1999, Savluchinske-Feio et al. 1999, Gigante et al. 2002, Kallioinen et al. 2003, Savluchinske-Feio et al. 2007).

## INTRODUCTION

The antimicrobial activity of the resin acids, even against multiresistant bacteria, is documented in several earlier papers in the literature in English (Smith et al. 2005, Savluchienske-Feio et al. 2006, González et al. 2009), suggesting that the resin acids are strongly antimicrobial. Also, the literature on applications of the technical rosins in dentistry is relatively large and indicates that the rosins intermixed with dental adhesives and cements are antimicrobial as well (Herrera et al. 2000).

Microbiological examinations specifically on the natural spruce callus resin, or on corresponding resin salves, do not exist, however. No publications can be found in the PUBMED database with key words “resin and antibacterial property” before 2008. On the other hand, there are, before and after 2008, studies in the medical literature on the antibacterial properties of purified resin acids and of Portuguese rosin (Söderberg et al. 1990, Söderberg et al. 1991, Johansson et al. 1995). These studies apply usual microbiological techniques and inevitably demonstrate a clear antimicrobial activity of the resin acids against bacteria, against the Gram-positive bacteria in particular. The investigations suggested that obviously dehydroabietic acid has the most potent antibacterial activity (Söderberg et al. 1990, Wang et al. 2012).

## 1.3 CHRONIC WOUNDS AND WOUND HEALING

Tissue repair is a necessity for life and well-being. The proper healing of skin wounds is an example of this well-being. Skin wounds are classified as acute and chronic. An acute wound is defined as injury of the skin, which heals in a predictable time through normal wound healing process (Diegelmann et al. 2004). A chronic wound is defined as a wound that heals within weeks or months, whereby the healing is delayed or cannot be predicted (Broderick 2009). Various mechanisms and factors may impair the normal, healing process, resulting in a chronic wound (Broughton et al. 2006).

Chronic skin wounds are extremely common throughout the world. It is estimated that 70% of all chronic wounds in Finland are pressure ulcers, venous leg ulcers or diabetic wounds (Eriksson et al. 1999). The crude prevalence of leg ulcers in adult populations varies in different studies in Finland and other countries between 0.1–0.9%, and the prevalence of pressure ulcers in geriatric wards is estimated to be around 22% (Eriksson et al. 1999, Woodbury et al. 2004). It is assumed that 1% of people in developed countries will suffer from a chronic leg ulcer during their life (O’Meara et al. 2010). The prevalence of active leg ulceration in Europe is reported to be 0.1–0.5% in the adult population on average, the prevalence rising even up to 8.3% in age groups over 85 years (Callam et al. 1985, Nelzen et al. 1991, Nelzen et al. 1996, Moffatt et al. 2004). Approximately 70% of all leg ulcers are considered to be related to venous hypertension and venous insufficiency (O’Meara et al. 2009). More rare types of skin ulcers are malignant wounds, or wounds due to vasculitis.

Costs of wound care are high in general. It is estimated in United States that the treatment of chronic wounds costs several billion dollars, annually (Fonder et al. 2008). In the UK, annual costs from pressure ulcers alone are estimated to be 2.5 billion €, corresponding to 2.6% of the National Health Service budget (Franks 2007). Unorganized treatment of chronic wounds in the district of Helsinki University Hospital (appr. 1 million inhabitants) cost 20 – 40 million € per year (Lepäntalo et al. 2009).

### 1.3.1 PRESSURE ULCERS (BED SORE)

Pressure ulcers (“bed sores”) result from ischemia of skin tissue due to pressure over the bony prominences. The most important predisposing factors are immobilization, malnutrition and ischemia, and a patient’s senility (Lyder 2003, Thomas 2006, Stechmiller et al. 2008, Ontario Health Technology Advisory Committee Meta-analysis 2009, White-Chu et al. 2011). The prevalence of pressure ulcers varies from 0.4% to 38% among patients in acute care units, from 2.2% to 29% in those in long-term care units, and from 0% to 17% among geriatric patients in homecare (Lyder 2003, Reddy et al. 2006, Thomas 2006, Franks 2007). The treatment of pressure ulcers is difficult, laborious, and expensive, and the ulcers are often colonized with bacteria (Kontiainen et al. 1988, Lohi et al. 2010). It is estimated that the care of chronic wounds in the USA, among which the pressure ulcer is a major disease, costs 6–15 billion US dollars annually (Markova et al. 2012). Correspondingly, the annual wound care costs in Denmark, a country with similar population to that of Finland, are estimated to be around 100 million € (Hjort et al. 2010).

Avoidance of the risk factors and preservation of the skin integrity are the main targets to reduce the ulcer risk (Stechmiller et al. 2008, Ontario Health Technology Advisory Committee Meta-analysis 2009, White-Chu et al. 2011). The main treatment principles of the ulcer are the reduction of pressure on the bone prominences, prevention of friction or shear forces, in addition to effective local ulcer care, surgical debridement of necrotic tissue, management of bacterial contamination or infection, and optimization of the patient’s nutritional status (Moore et al. 2005, Reddy et al. 2006, Ontario Health Technology Advisory Committee Meta-analysis 2009). Due to poor overall health, patients with severe pressure ulcers are often outside the surgical treatment options (plastic surgery), and topical ulcer care remains the prime approach in the management of these patients.

For topical management of pressure ulcers, there are hundreds of different products on the market with varying clinical effectiveness or applicability, and with high variance in price (Iivanainen et al. 2011). Generally the selection of the dressing should be based on the nature of the tissue in the ulcer bed. There is some evidence of clinical effectiveness (supported by direct or indirect evidence from properly designed clinical series on pressure ulcers in humans providing statistical results that

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support recommendations) for some commercially available products (European Pressure Ulcer Advisory Panel 2009).

### 1.3.2 THE CHRONIC SURGICAL WOUND

If the skin wound after surgical operation is not primarily healed in 2–3 weeks, the wound is considered chronic, and heals by secondary intention (Hirschmann 2011).

In clinical practice in Finland and elsewhere, approximately 4% of surgical wounds will be infected. *Staphylococci* are the most prevalent cause, and the infection results ultimately in chronic complicated surgical wound (Haley et al. 1985, Haukipuro 1996). Other well-known risk factors of the chronic complicated wounds are the use of corticosteroids or other immunosuppressive medicaments, systemic diseases like diabetes mellitus, and smoking (Hachenberg et al. 2010). Prevention of chronic surgical wounds requires the recognition and the correction of these risk factors. The treatment options include adequate topical wound care, surgical debridement of the necrotic tissue, and the removal of visible foreign materials (e.g., osteosynthesis material), management of the infection, and optimization of the nutritional status (Haukipuro 1996, Gottrup 2008). In topical treatment, however, no objective and high quality studies on effectiveness exist regarding the great majority of available products (Vermeulen et al. 2004).

### 1.3.3 NORMAL WOUND HEALING

Normal wound healing is a complex phenomenon that includes the up-regulation and down-regulation of various cellular and molecular mechanisms, such as phagocytosis, chemotaxis and mitogenesis of many cell types, collagen synthesis, and the synthesis of various matrix components in carefully controlled cascades that correlate with the appearance of distinct clinical stages, e.g., hemostasis, inflammation, proliferation and remodeling (Clark 1996, Broughton et al. 2006, Velnar et al. 2009). The healing stages involve interactions of many regulatory pathways, and include participation of various tissue and cell growth factors, cytokines and hormones, and may be summarized as follows based on several references (Winter 1962, Levenson et al. 1965, Leibovich et al. 1975, Kurkinen et al. 1980, Grinnell et al. 1981, Clark et al. 1982, Clark 1991, Clark 1993, Grinnell 1994, Slavin 1996, Herrick et al. 1997, Witte et al. 1997, Agren et al. 1998, Nwomeh et al. 1999, Tonnesen et al. 2000, Armstrong et al. 2002, Cross et al. 2003, Gabbiani 2003, Desmouliere 2005, Broughton et al. 2006, Hinz 2007, Barrientos et al. 2008, Kota et al. 2012):

*Stage of hemostasis:* Skin injury results in damage to blood vessels and in extravasation of blood into the tissues. After injury, the exposed skin collagen comes into contact with blood, promoting platelet aggregation and activation of chemotactic

factors. Vasoconstriction and coagulation will limit the blood loss and lead to clot formation and platelet aggregation. The clot, composed of fibrin, fibronectin, vitronectin, von Willebrand factor and thrombospondin, forms the primary matrix for the migration of cells in the injured tissue area. The platelets are trapped and promote the inflammatory response. The granules of the platelets contain growth factors, such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and transforming growth factor-beta (TGF- $\beta$ ), which start the wound healing cascades by attracting and by activating fibroblasts, endothelial cells and macrophages. The vasoactive serotonin in platelets increases the microvascular permeability which results in edema and in exudation of fluid into the extravascular space.

*Stage of early inflammation (24–48h).* The early inflammatory phase in wound healing begins with the activation of the complement cascade. Complement components, like C5a, promote infiltration of the polymorphonuclear leucocytes (PMNLs). The PMNLs adhere to endothelial cells and move through the vessel wall (diapedesis). The PMNLs are capable of phagocytosis of bacteria and foreign materials and hence prevent infection but may otherwise contribute little to the normal wound healing processes and debridement. The PMNLs kill micro-organisms by releasing degrading enzymes, oxygen-derived free radicals and reactive nitrogen species. This phase of wound healing ceases within a few days, once the infection is cleared. Matrix metalloproteinases (MMP) appear in the wound fluid in the early inflammatory stage but decrease in quantity within 24–48 hours, when particularly the decrease of MMP 9 activity, correlates positively with the deposition of collagen in the wound area.

*Stage of late inflammation.* Complement, blood clot, immunoglobulin G (IgG) fragments, collagen and elastin breakdown fragments, and cytokines such as leukotriene B4, platelet factor IV, PDGF and TGF- $\beta$  attract blood monocytes into the wound area. The monocytes are transformed to tissue macrophages and act as regulatory cells for wound repair in the late stage of the inflammatory phase. These cells release cytokines and growth factors which recruit and stimulate fibroblasts, keratinocytes and endothelial cells. For wound debridement, the macrophages release proteolytic enzymes (e.g., collagenase). To stimulate the inflammation, the tissue macrophages secrete transforming growth factor-alpha (TGF- $\alpha$ ), heparin-binding epidermal growth factor (HB-EGF), and fibroblast growth factor (FGF).

Lymphocytes enter the injured tissue in the inflammatory phase (>72 hours after the wounding), due to the influences of IgG and complement, and particularly due to the influence of interleukin-1 (IL-1) of macrophage and endothelial cell origin. The IL-1 further regulates the collagenase activity, and, thereby plays a role in the modeling of the extracellular matrix (ECM).

*Stages of proliferation, fibroblast migration and formation of granulation tissue (2–4 day).* In this phase, granulation tissue is formed from the fibrin/fibronectin matrix. PDGF and TGF- $\beta$  attract fibroblasts and myofibroblasts into the wound

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area at days 2 to 4. These cells migrate, proliferate and produce the new ECM composed of fibronectin, hyaluronic acid (HA), collagen, and finally of proteoglycans.

The granulation tissue, a pink granular tissue made up of numerous loops of capillaries easily bleeds if traumatized. It is composed of macrophages intermixed with collagen, glycosaminoglycans (GAGs), fibronectin and tenascin. It appears as early as 48 hours after wounding, and the fibroblasts become its predominant cell type at 96 hours.

*Stage of epithelialization.* Within a few hours from wounding of the skin, a single layer of epidermal cells loosen the attachment to the dermis and begin to migrate from the wound edges over the provisional matrix and granulation tissue. When the moving cells collide, a new basement membrane is generated. The re-epithelialization progresses if the wound is kept moist. The growth factors modulate this epithelialization. EGF, bFGF and keratinocyte growth factor stimulate epithelial mitogenesis and proliferation.

*Stage of remodeling.* The remodeling of ECM is characterized with an ongoing synthesis and breakdown of native and newly synthesized collagen. The MMPs that are produced by fibroblasts, granulocytes and macrophages degradate the collagen. As the remodeling is completed, the MMP activity decreases and the inhibitors of metalloproteinases appear and increase in activity. TGF- $\beta$  plays a role in accomplishment of the events.

The final remodeling of a wound takes place when the contractile ECM shrinks and the wound margins converge with interactions between fibroblasts and ECM, with the influences of TGF- $\beta$ , PDGF and FGF. During this remodeling, the capillaries decrease in number and the blood flow is reduced. An acellular, avascular scar is the final result.

It is considered that the collagen fibers in a scar never regain the same strength as they have in unwounded skin, and that 80 percent of the strength of the collagen in unwounded skin can be achieved at maximum.

*Angiogenesis.* Appearance of new blood vessels occurs during all stages of wound healing. It is a complex process including interactions between proangiogenic mediators (e.g. vascular endothelial growth factor (VEGF), TGF, PDGF, IL1 and IL6) and angiogenic inhibitors (e.g. inadequate ECK degradation, growth factor imbalance, problems in signal transduction). Angiogenesis is characterized by ECM degeneration and endothelial cell proliferation and migration. By means of chemo-tactic and mitogenic factors endothelial cells begin to elongate and form capillary sprouts. As the collagen forms a scar, and tends to accumulate in the granulation tissue, angiogenesis will diminish. Up- or downregulation of any factor in the normal process of angiogenesis can result in disruption of the wound healing process.

*Wound contraction, Myofibroblast.* Contraction of wound borders and secretion of new extracellular matrix are requirements for the final healing. Myofibroblasts arise from fibroblasts induced by mechanical stress, TGF- $\beta$  and cellular fibronectin. They synthesize collagens (type I and III), which are main components of the final

wound ECM. At the same time high contractile forces are generated by the myofibroblasts which are beneficial in the wound healing process. An overexpression of this phenomenon is the manifestation of a hypertrophic scar.

### 1.3.4 FAILURES IN WOUND HEALING

Several molecular processes may fail, when the wound will not heal properly. The failures in one or more molecular or regulatory factors can lead to a chronic wound, and may be summarized as follow (Witte et al. 1997, Cross et al. 2003):

*Inflammatory response and wound infection.* The normal inflammatory response can be altered and prolonged in chronic wounds. A microbial infection attracts PMNLs that may hamper the progress of the sequential phenomena in acute wound healing, and will, thereby, result in a chronic wound. The macrophage activation, essential for the release of cytokines and growth factors to recruit fibroblasts, keratinocytes and endothelial cells, may be suppressed, resulting in a distortion and prolongation of the inflammatory stage (Moore et al. 1997, Boyce et al. 2000). In non-healing leg ulcers, the pro-inflammatory cytokines, IL-1, IL-6, tumor necrosis factor - $\alpha$  (TNF- $\alpha$ ), prostaglandin E2 and thromboxane are present in high concentrations indicating an augmented inflammatory phase of wound healing (Trengove et al. 2000).

The continued presence of bacteria in a wound, and the subsequent wound infection, may lead to endotoxin production. Although inflammation is a part of normal wound healing, the repair process may be delayed if the inflammation is excessive and destructive. Chronic colonization by bacteria forms biofilms (permanent bacterial colonies in extracellular polysaccharide matrix) that are resistant to the actions of host defense, and resistant to antimicrobial agents, thereby contributing to delayed healing in the wounded area (Davey et al. 2000).

*Protease activity.* In chronic wounds the synthesis and degradation of granulation tissue and ECM may be disturbed. The levels of collagenase, gelatinase and neutrophil elastase have been shown to be elevated in tissue samples from pressure ulcers and chronic venous leg ulcers (Grinnell et al. 1996, Yager et al. 1996, Schultz et al. 1998).

*Cellular activity and cell morphology.* The function and metabolic activity of the tissue and inflammatory cells may be impaired. Fibroblasts tend to be larger and more polygonal in shape in chronic wounds compared with the normal skin fibroblasts (Stanley et al. 1997). Proliferation and mobility of the fibroblasts isolated from chronic venous leg ulcers, and from pressure ulcers, are reported to be abnormal in comparison with the fibroblast from normal skin (Van de Berg et al. 1998, Agren et al. 1999, Raffetto et al. 2001). A failure in re-epithelialization is perhaps the most prominent clinical feature in chronic wounds, this failure being associ-

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ated with deficiency in migration rather than in proliferation of the keratinocytes (Adair 1977, Andriessen et al. 1995).

*Extracellular matrix.* Failures in composition of the ECM are one class of deficiencies when the wound does not heal. The ECM proteins such as e.g. fibronectin are shown to be decreased in quantity in chronic wounds, and the quantity of collagen synthesized by fibroblasts tends to be decreased (Herrick et al. 1992, Herrick et al. 1996). Failures in expression of the MMPs may contribute when there are defects in collagen deposition (Agren et al. 1998). Failure in the accumulation of collagen in the granulation tissue may further be the cause of delayed wound healing (Lauer et al. 2000). Fibroblasts in chronic wounds have been demonstrated to have a reduced ability to synthesize type I collagen, suggesting failure in the appearance of proper ECM during the scar remodeling stage (Cook et al. 2000).

*Free radicals.* Appearance of reactive nitrogen (nitric oxide; NO) and oxygen-derived free radicals originating from the PMNL are implicated in development and persistence of chronic wounds. This appearance and action of free radicals can be opposed and reversed with internal antioxidants (e.g., synthesis of hyaluronic acid by fibroblasts and myofibroblasts), or with external addition of antioxidants (Salim 1991, Abd-El-Aleem et al. 2000, Hemmati et al. 2011). In diabetic patients with recurrent neuropathic and neuroischaemic leg ulcers, the plasma levels of NO have been shown to be significantly increased compared to the levels in patients with non-recurrent ulcers (Jude et al. 2001).

*Patient specific reasons.* Many diseases (e.g. diabetes mellitus, malignancies), medicines prescribed (e.g. immunosuppressive medication), living habits (e.g., smoking) and mechanical or even psychological factors may impair wound repair, resulting in chronic and complicated wounds or ulcers. None of the treatment efforts in topical wound care will result in wound healing, if these patient-related background factors and diseases are not taken into account and controlled (Park et al. 2010).

### 1.3.5 WOUND CARE IN CLINICAL PRACTICE

The target of chronic wound treatment in clinical practice is its full healing. Treatment is multidisciplinary, and involves, if necessary, surgical wound revision and tissue reconstruction in addition to the control and management of mechanical, social and medical background factors (Gottrup et al. 2001, Stechmiller et al. 2008, Park et al. 2010). In addition to the topical tools, several surgical (reconstructive surgery) and device-based options (e.g., negative pressure wound therapy, laser therapies, maggots, etc.) for treatment of skin wounds and ulcers are available (Vikatmaa et al. 2008, Jokinen et al. 2009, White-Chu et al. 2011). These surgical and device-based options are out of the scope of the present study project, and the author focuses only on the topical, “conservative” tools in wound care. A recently

published systematic review with recommendation is available on the efficacy and safety of device-based negative pressure wound treatment in Finland (Juutilainen et al. 2007).

The target of non-surgical and non-device-based topical wound care is the creation of preconditions for proper healing of the wound including the prevention and treatment of wound infections (Clark 1996, Broughton et al. 2006, Velnar et al. 2009). Wound care is focused on maintaining and supporting the processes of normal wound healing mechanisms, and, in cases with deficiencies in proper healing, by applying methods and techniques that may reverse the failures.

In clinical practice, chronic skin wounds and ulcers are classified according to their clinical appearance as follow: (a) pink wounds indicating that the wound is epithelializing, (b) red wounds indicating that the wound is granulating, (c) yellow wounds indicating that the wound is covered with fibrin and (d) black wounds indicating that the wound is necrotic, and is not healing (Cuzzell 1988).

The topical wound care can be divided into debridement, control of the infection, and control of the moisture balance.

Debridement of the wound from all necrotic and foreign material is one of the most important and first steps in wound care (Steed et al. 1996, Attinger et al. 2001). Selection of the right methods for the debridement (surgical vs. chemical) is dependend on the wound characteristics, the patient's overall status, and on the facilities of the clinic. The combinations of different methods may be useful and beneficial (Dissemond et al. 2004). The goal of the debridement, regardless of the method, is to achieve a clean wound bed.

Surgical debridement is the most effective method to remove necrotic tissue and impurities. In inborn autolytic debridement, the patient's own proteolytic enzymes and macrophages dissipate necrotic material in the wound. For activation of these internal phenomena, the preservation of a moist wound environment and proper topical wound care are the requirements. These can be achieved and supported with wound care products. Externally applied proteolytic enzymes may be used to dissipate and debride necrotic material from the wound base. A combination of surgical debridement with external enzymatic debridement has been shown to be effective (Ramundo et al. 2008).

Biological debridement may also be carried out with maggots (*Lucia sericata*). The effectiveness of this method in wound healing, as well as its cost-effectiveness, has not yet been reliably proven (Hall 2010).

### 1.3.6 CONTROL OF INFECTION

The differential diagnosis between wounds with an infection or with simple contamination with microbes is essential but problematic in clinical practice (Rondas et al. 2009). Oral antibiotics are recommended if systemic complaints and clinical

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signs of a wound infection appear. Contamination or colonization of wounds by bacteria is not an indication for treatment with antibiotics. Antibiotics do not accelerate wound healing in these cases (Alinovi et al. 1986, Wikström et al. 1999). Topical antibiotics are not useful and are not accepted in the treatment of chronic wound and ulcers, mainly due to problems with bacterial resistance (O'Meara et al. 2010). Sugar, honey and silver are antimicrobial and considered as acceptable topical agents for infection control even in chronic wounds (Meaume et al. 2005, Mphande et al. 2007).

### 1.3.7 CONTROL OF MOISTURE

The wound should not dry up but should not be too wet either. Careful control of the moisture balance helps the proper evolution of all molecular and cellular stages of wound repair. Necrotic and infected wounds with abundant exudation are recommended to be covered with hydrofiber, alginates or polyurethane dressings, which have enough absorption capacity. In dry wounds or in wounds with slight exudation, moisture maintaining dressings, such as hydrogels and hydrocolloids, are recommended (Iivanainen et al. 2011).

### 1.3.8 DRESSINGS IN PRACTICAL WOUND CARE

Topical dressings in wound care are divided in passive, active and interactive dressings. Passive dressings protect the wound, active dressings affect the wound healing process and interactive dressings react with exudation and can thereby have an effect on wound moisture. Wound care products in general are classified to the category "medical device" with one exception. Collagenase (Clostridiopeptidase A), used in enzymatic cleaning of the wound is categorized in Finland as a medicinal product.

In available literature, there is no "gold standard" or strong consensus recommendations for the selection of topical treatment tools, dressings, or equipments for wound care (Chaby et al. 2007, Peltonen 2007, Canadian Agency for Drug and Technologies in Health 2011). In practice, the treatment methods chosen for wound care are based on subjective observations, on personal experience in the wound care, and on the availability and price of the available tools in the treatment units. Selection of the consumables is not easy. On the market in Finland, there are over 300 different local wound care products with varying properties and with a large variance in consumer price (Iivanainen et al. 2011). A summary of different types of wound dressings and other consumables available in Finland at present is presented in Appendix II (Iivanainen et al. 2011).

## **2. OBJECTIVES OF THE PRESENT STUDY PROJECT**

Answers were sought to the following specific questions:

1. Is the natural coniferous resin and resin salve antimicrobial?
2. Is the antimicrobial activity specific and what are the mechanisms?
3. Is the resin salve treatment safe?
4. Is the coniferous resin salve effective in the treatment of pressure ulcers and chronic surgical wounds in clinical trials?
5. What are the direct costs of the pharmaceutical materials in treatment of chronic surgical wounds with the coniferous resin salve?

### **3. MATERIALS AND METHODS**

#### **3.1 SPRUCE RESIN AND RESIN SALVE (paper I-VI)**

The resin for all studies in the present project was collected in Kolari, Finnish Lapland, from the trunks of full-grown Norway spruce (*Picea abies*) with knives, with the permission of the landowners. Bark and other impurities were removed from the resin by mechanical cleaning and the resulting solid resin (crude raw material) was stored at room temperature until further processed.

The resin salve used in the randomized controlled trial (paper V) was a mixture of Norway spruce resin and salt-free butter (Valio Ltd., Helsinki, Finland). The salve was prepared according to the common practice in traditional medicine (Timo Kyrö, personal information, 2006). The raw coniferous resin was mixed and boiled with salt-free ordinary butter (Valio Ltd., Helsinki, Finland) by stirring in a weight proportion of 1:3 (w/w), filtered and allowed to cool to room temperature, and finally packed into tubes in the University Pharmacy, Helsinki, Finland.

The resin salve applied in the observational clinical trial, mycological trials and morphological and electrophysiological investigations was a mixture of purified resin with a standardized salve base produced by the GMP standards (Abilar 10% resin salve, Repolar Ltd., Espoo, Finland) (papers II–IV, VI). The resin (raw material) was, after mechanical purification, dissolved for 3–4 weeks in ethanol at room temperature, and filtered resulting in an ethanol solution with a resin content around 65% (w/w) (Patent FI 122095 B). This alcohol-coniferous resin solution was used as the active ingredient in the industrially manufactured, “modern” resin salve. This salve and its dilutions (dilutions with salve base) were used in the challenge tests and in the mycological investigations (papers II and III).

#### **3.2 MICROBIOLOGY (paper I-III)**

To study the antimicrobial properties of resin and resin salve various techniques were used. This was done because of the poor water solubility of the compounds in resin and resin salve. Agar diffusion test is largely based on the solubility of the test material in solid agar. The zone of inhibition may be small due to poor solubility even though the test material is highly antimicrobial. The liquid media test is a sensitive method and not dependent on diffusion in solid agar. Instead of

assessing zonal inhibition, visual monitoring of turbidity is used to evaluate the antimicrobial property.

In the agar diffusion and agar liquid tests, the test material is placed on bacteria grown in culture. In the challenge test, by contrast, the bacteria are placed on the test material after which the antimicrobial effect of the test material is assessed by the number of micro-organisms recultured after a specific incubation period. Antimicrobial action in this test is not dependent of water solubility.

Minimum inhibitory concentration (MIC) is the lowest concentration of tested material showing antimicrobial activity. A low MIC is an indication of a potent antimicrobial effect.

The microbial strains used in the microbiological studies are presented in appendix III.

### **3.2.1 AGAR DIFFUSION TESTS (paper I)**

In the Agar diffusion tests, Mueller-Hinton media were used. Wells with a diameter of 8 mm were punched into the agar medium and filled with the resin salve. The plates were incubated for 18 h after which zones of inhibition were measured and noted. Wells filled with sterile saline without resin were used as controls.

### **3.2.2 MIC ANALYSES (paper I)**

The MIC values of the coniferous resin and resin salve were analyzed by mixing the purified resin in different concentrations with the agar. Lowest antimicrobial concentration was considered as the MIC value. The pulverized resin was added into the Mueller-Hinton liquid agar to produce the following final concentrations of resin in the medium: 1.0, 0.8, 0.6, 0.4 and 0.2% (w/v). Detailed information is given in paper I. The media/resin solutions were autoclaved and poured onto the Petri dishes. Bacteria (*S. aureus*, *S. epidermidis*, *E. faecalis*, *St. pyogenes* and *St. agalactiae*) tested in the MIC experiments were delivered as 1 ml aliquots onto the surface of three parallel Mueller-Hinton/resin plates. Inoculated plates were incubated and bacterial growth was inspected after 16–20 h.

### **3.2.3 LIQUID MEDIA EXPERIMENTS (paper I)**

The liquid growth medium used in the microbiological tests was the Fastidious Anaerobe Broth (FAB) (Lab M, Bury, England). Due to bacterial growth, the broth becomes turbid or - depending on the extent of the bacteria growth - visible colonies

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are formed. The FAB medium was used because of its semisolid property which made it possible to directly observe or even count the number of colonies of bacteria growing in the medium. The experiments with the FAB medium were performed in two different ways.

First, the bacteria tested were inoculated into the FAB media with or without resin salve. The colonization of the bacteria was visually examined and recorded as absent, mild, moderate, or heavy.

Second, the FAB media with or without a pretreatment with the resin salve were prepared. In preparing the resin-pretreated FAB medium, a layer of resin salve was spread on the bottom of a Petri dish, after which the FAB solution was layered on this resin bed. After incubation of 3 hours, the FAB medium was collected. This FAB medium pretreated with the resin salve did not differ macroscopically from the FAB medium that was not pretreated with the resin salve. The culture experiments were carried out in the FAB media with and without the pretreatment with the resin salve.

The growth of bacteria in the test tubes was visually monitored. The antibacterial activity was detected as a decreased turbidity in cultures containing resin as compared to those without resin. After the liquid culture tests, subcultures on agar plates were performed in order to determine whether the resin killed the bacteria or only inhibited their growth (bactericidal *vs.* bacteriostatic action).

### 3.2.4 EUROPEAN PHARMACOPOEIA (Ph. Eur.) CHALLENGE TESTS (paper II)

In challenge tests, the antimicrobial action of the resin was tested against bacteria and fungi. The tests were performed by adding bacteria ( $10^5$ - $10^7$  cfu/ml) or fungi/yeasts ( $10^4$  or more cfu/ml) to 10 g of test medium (resin, resin salve or control) as challenged micro-organisms. If the microbes remain viable and re-cultivable in the challenge test, the test substance is considered to exert no bacteriocidic or fungicidic influences.

The re-growth of microbes from the challenged test medium was assayed by sampling at different time intervals (up to 7 days). Samples were re-cultured in specific media and a reduction in growth intensity of the challenged micro-organism was assessed as colony forming units (cfu). According to the European Pharmacopoeia (European Pharmacopoeia 5.0) challenge test criteria, a reduction of the bacterial count by at least by  $10^2$  cfu within 48 h and by at least by  $10^3$  within 7 days is considered to indicate a significant bactericidal activity of the substance under test. Correspondingly, a reduction in yeasts of at least  $10^2$  cfu within 14 days is considered fungicidal.

### **3.2.5 AGAR PLATE DIFFUSION TESTS IN MYCOLOGY (paper III)**

Coniferous resin or resin salve in different dilutions with the salve base was applied and poured into 9 mm diameter holes in specific agar gel. Samples of sterile saline without resin were used as controls. The plates were incubated and the size of the inhibition zone was measured for yeasts at 24-48 hours and for dermatophytes at 7 days.

## **3.3 MORPHOLOGICAL AND ELECTROPHYSIOLOGICAL STUDIES (paper III-IV)**

### **3.3.1 ELECTRON MICROSCOPY, BACTERIOLOGY (paper IV)**

Diluted cultures of *S. aureus* were transferred onto a layer of purified resin or 10% (w/w) resin salve on the Petri dishes and incubated for 18 hours. Thereafter, the specimens for the electron microscopy were prepared from the samples of cultivated bacteria from the Petri dish. Pure *S. aureus* bacteria cultures without exposure to the resin or resin salve were used as controls.

Specimens for the electron microscopy were prepared at the Institute of Biotechnology, Electron Microscopy Unit, University of Helsinki, Helsinki, Finland, (Kari Lounatmaa Sc.D. (Biol.)). Transmission electron microscopy (TEM) examinations were done in the same institute. The scanning electron microscopy (SEM) examinations were done in the Department of Electronics, School of Electrical Engineering, Aalto University, Espoo, Finland, (Kari Lounatmaa Sc.D. (Biol.)).

Morphometric analyses of 55 randomly assigned TEM micrographs (1–5 *S. aureus* bacteria in each) were carried out by counting the cell diameter, cell wall thickness, number of mitoses, and the cross-sectional area of the bacteria.

The cell diameter and the cell wall thicknesses at opposite sites were measured in all cells that were clearly and fully visible in the micrograph. The number of mitoses, defined as two “sister” cells with a complete or incomplete common cell wall and cell membrane, were assessed individually for each image, and the cumulative numbers of such cells were calculated.

### **3.3.2 ELECTRON MICROSCOPY, MYCOLOGY (paper III)**

Samples for TEM and SEM were taken from the *Trichophyton mentagrophytes* agar diffusion culture plates (see 3.2.5 Agar plate diffusion test in mycology) from 2 sites: first, from a site where no inhibition to growth of the fungi was observed and, second, from a transitional zone between the areas of the inhibition zone

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and zone of normal growth. The electron microscopy specimens were prepared as described above and in detail in paper IV.

### 3.3.3 ELECTROPHYSIOLOGICAL STUDIES (paper IV)

The effect of the spruce resin and abietic acid on the bacterial membrane potential was measured by flow cytometry and the BacLight Bacterial Membrane Potential Kit. The resin and abietic acid were transferred to glass beakers, and the diluted *S. aureus* suspension (4ml) was poured to the beaker in duplicates. *S. aureus* without the exposure to the resin or abietic acid was used as control. After incubation, 1ml of bacterial suspension from each beaker was transferred into plastic cuvettes with specific commercial reagents to measure the fluorescence shift from the red emission toward the green emission. This shift takes place if the membrane potential of the bacterial cells diminishes.

Fluorescence in flow cytometry was measured at 615nm (red) and 518nm (green). The intensities of the red and green fluorescence were recorded for each sample. The mean fluorescence intensity (MFI) ratiometric parameter ( $MFI_{red} : MFI_{green}$ ) describes the strength of the membrane potential.

The electrophysiological studies were done in METLA (Finnish Forest Research Institute, Rovaniemi) (Rainer Peltola, Ph.D. and Minna Männistö, Ph.D.).

### 3.3.4 ANALYSES ON FATTY ACIDS (paper IV)

Resin diluted in DMSO/Tryptic Soy Broth (TSB-medium) with a resin concentration of 0.1% (w/v) was prepared. DMSO without resin was used as a control medium. 1ml of *S. aureus* was transferred onto both media, and incubated. After measuring the bacterial density, the solution was centrifuged and washed to get pellets. Bacterial fatty acids were extracted and the amount of branched chain and straight chain fatty acids was measured with gas chromatography in METLA (Finnish Forest Research Institute, Rovaniemi) (Rainer Peltola, Ph.D. and Minna Männistö, Ph.D.).

## 3.4 OTHER ANALYSES

### 3.4.1. GLC/MS ANALYSES

The gas-liquid-chromatographic (GLC) analyses with mass spectrometry (MS) of the coniferous resin and resin salve were performed in METLA (Finnish Forest Research Institute, Helsinki) (Tapio Laakso, M.Sc. and Pekka Saranpää, Ph.D.). Water or acetone extracts of resin or resin salve were obtained by sonication for 1 hour in

an ultrasonic bath then evaporated dry under nitrogen. Before the GLC/MS analysis, dried samples were silylated with 0.5 ml 20% TMSI (trimethylsilylimidazole)-pyridine mixture. The GLC/MS analyses were performed with the HP 6890 GLC-system equipped with mass selective detector 5973 and an HP-5 capillary column (30 m x 0.25 mm i.d., 0.25 micrometer film thickness). Helium was used as carrier gas, flow 1.5 ml/min. The chromatographic conditions were as follows: initial temperature 180°C, temperature rate 5°C/min, final temperature 300°C for 5 min; injection temperature 280°C and split ratio 1:20. The MS-interface temperature was 300°C and the ion source temperature 230°C. Mass spectra were obtained by electron impact (EI mode) at ionization energy 70 eV (Sipponen et al. 2007).

#### **3.4.2 AMES TESTS**

The Ames genotoxicity tests of resin and resin salve were performed with *Salmonella typhimurium* (TA98 and TA100 strains) with or without S9 rat liver metabolic activation factor by Jorma Mäki-Paakanen, Ph.D., at the THL (National Institute for Health and Welfare, Kuopio, Finland) with the standard and usual techniques (Mortelmans et al. 2000).

#### **3.4.3 SKIN IRRITATION TEST**

Skin irritation tests and *in vitro* cytotoxicity tests on the coniferous 10% resin salve were done by LAB Research (Scantox), Copenhagen, Denmark (unpublished data). The skin irritant effect was investigated according to the ISO (The International Organization for Standardization) 10993-10:2010 standard. Three female rabbits were exposed to 0.5 g of the resin salve at each of two sites on the back. After a 4-hour exposure period, the test item was removed and the skin was examined 1, 24, 48, 72 hours after termination of the exposure, and on day 8. The skin reactions to salve were read according to the appearance of erythema and edema. Finally the primary irritation index was calculated (scale: 0-8).

#### **3.4.4 CYTOTOXICITY TEST**

The *in vitro* cytotoxicity test for 10% coniferous resin salve was done according to the ISO 10993-5:2009 and USP<87> (U.S. Pharmacopeia biological reactivity tests, *in vitro*) guidelines. Salve was extracted to the HAM F12 medium (containing 10% foetal bovine serum and 50 microg/ml gentamycin). The extract was obtained by mixing of 4.26 g salve with 21.3 ml of cell culture medium for 24 hours at 30°C. L929 mouse fibroblasts (ECACC No. 85011425) were added to this medium, or to

a diluted medium (1+3), and were cultured at ca. 37°C in a humidified atmosphere of 5% carbon dioxide in air for 48 hours. Thereafter, the number and percentage of viable cells were calculated. The scale of cytotoxic reactivity in the test is 1–4. Grade 4 indicates severe cytotoxicity (nearly all cells are destroyed). Grades 1, 2 and 3 indicate slight ( $\leq 20\%$  of cells are destroyed), mild ( $\leq 50\%$  of cells are destroyed) or moderate ( $\leq 70\%$  are destroyed) cytotoxicity, respectively.

### 3.5 CLINICAL TRIALS (paper V-VI)

#### 3.5.1 A RANDOMIZED, CONTROLLED TRIAL (RCT) IN PATIENTS WITH PRESSURE ULCERS (paper V)

The study was a prospective, randomized, open-label but controlled multicentre investigation designed to compare the effectiveness of the resin salve treatment with a generally accepted control treatment (carboxymethylcellulose hydrofiber polymer dressing with or without silver (Aquacel® or Aquacel Ag®)) in patients with severe pressure ulcers (grade II–IV).

In each study center, the patients were randomized into the resin salve and control group by using mixed block randomization (closed envelopes in blocks of four cases).

The primary outcome measure was complete healing of the ulcer within 6 months. The healing rate was recorded in both study arms (resin salve group *vs.* control group) as a function of time (percentage of patients healed in time units and percentage of ulcers healed in time units). The secondary outcome measures were the improvement of the ulcer grade (ulcer size), and the prevalence rate of successful eradication of the pathogenic bacteria cultured from the ulcers at study entry. Safety was followed with adverse event reports.

The study population was recruited in 11 primary care hospitals (health centers). After the randomization, there were 21 and 16 patients in the resin salve group and in the control group, respectively. Altogether 22 patients (9 males and 13 females; mean age  $80 \pm 10$  years and  $74 \pm 8$  years) with 45 pressure ulcers went through the 6 month study course. There were 8 and 7 patients, respectively in the resin group and control group who dropped out for various reasons. All these dropouts were included in the intention-to-treat (ITT) analysis of the study results. The treatment was considered to be failed in the ITT analyses in all of these dropouts.

The patients recruited and selected into the present study population were considered ineligible for surgical treatment options (plastic surgery), or had undergone one or more surgical treatment efforts for ulcer without success in the past. The inclusion criterion was one or several severe pressure ulcers (grade II–IV) with or without infection. The exclusion criteria were a life expectancy of less than 6 months, or the patient's unwillingness to participate.

All ulcers were photographed, and planimetric analyses of the ulcer size were recorded at study entry and monthly thereafter. If the ulcer was not healed in 6 months, the treatment was considered unsuccessful, and follow-up was discontinued.

### **3.5.2 AN OBSERVATIONAL CLINICAL COHORT STUDY IN PATIENTS WITH CHRONIC SURGICAL WOUND (paper VI)**

The trial was a prospective observational clinical investigation of a cohort including 23 patients (mean age  $49 \pm 17$  years; 8 males and 15 females) whose surgical wound (length of the open wound  $29 \pm 19$  mm; range 5-60 mm, at study entry) did not heal by primary intent after elective surgery, and the wound was considered, therefore, chronic and complicated. The primary outcome measure was the time in days to complete wound healing. The secondary outcome measures were to assess the contributors (clinical parameters) that may delay the wound healing, to estimate the direct costs of the pharmaceutical materials in resin salve therapy and the rate of allergic contact dermatitis, or the appearance of other possible side effects. The study population was collected from patients referred to routine postoperative controls in the outpatient departments of two hospitals, the Rheumatism Foundation Hospital (Heinola, Finland) and the Jorvi Hospital (Helsinki University Central Hospital, HUS, Espoo, Finland). In all cases without exception, the resin salve treatment was carried out by the patients themselves (self treatment) as home care.

### **3.5.3 CALCULATION OF THE DIRECT COSTS OF THE PHARMACEUTICAL MATERIALS (paper VI)**

Calculations of direct costs of the pharmaceutical materials from the study entry until wound healing were assessed in all individuals separately and extrapolated, thereafter, to the whole study population. The consumer prices of the Abilar® 10 % resin salve and the Tyke HealthCare cotton gauze in the University Pharmacy in Finland in April 2011 were used as prices of the salve and accessories (dressings). The following variables were taken into account in assessments of the consumption and need of the salve and the accessories in every patient: 1) wound size, 2) daily consumption of salve extrapolated in relation to the wound size, 3) wound healing time, 4) number of 20 g salve tubes purchased during the treatment period per patient, 5) price of the 20 g salve tube, and 6) price and package sizes of the accessory products (dressings).

## MATERIALS AND METHODS

### 3.5.4 STATISTICAL ANALYSES (paper V-VI)

In the randomized, controlled trial (paper V), data analyses and reporting were based on the CONSORT statement and in the observational cohort study (paper VI) on the STROBE guidelines (Moher et al. 2001, von Elm et al. 2008). Mean and standard deviation were computed for quantitative data (continuous variables), while frequencies and percentages were computed for qualitative data (dichotomous variables). Continuous variables and proportions were compared using the Mann-Whitney non-parametric U-test or Student's t-test.

In the randomized study differences between the groups were compared with the  $\chi^2$  test or Fischer's exact test. Healing of ulcers over time was defined with Kaplan-Meier analyses. The log-rank test was used to compare healing times in the two study groups. A p-value of  $< 0.05$  was considered statistically significant.

## 4. RESULTS

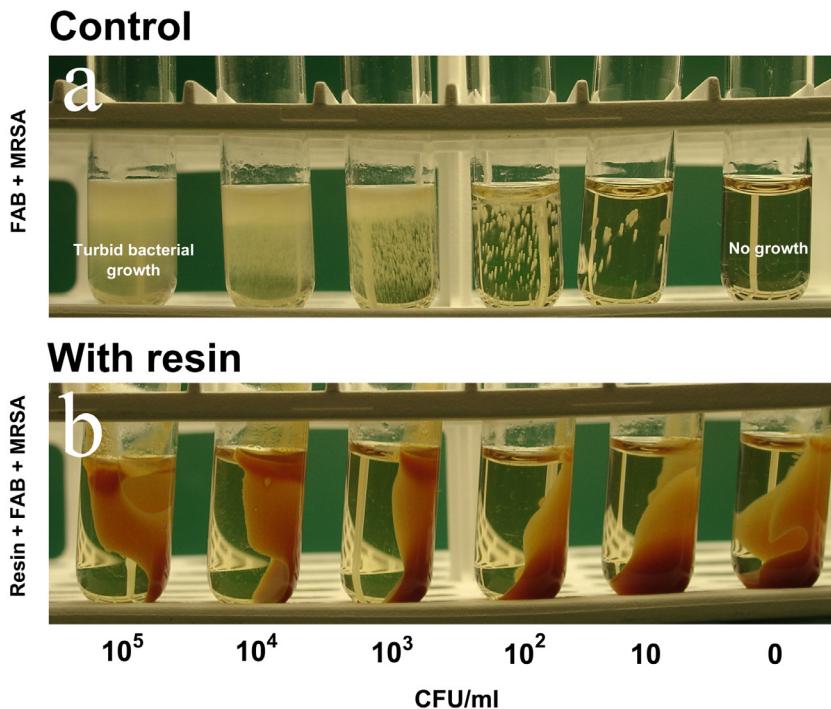
### 4.1 MICROBIOLOGY (paper I-III)

#### 4.1.1 CULTURE MEDIA TESTS ON BACTERIA (paper I)

Coniferous resin and the resin salve exhibited a clear antimicrobial effect against all Gram-positive bacteria tested including the methicillin-resistant *Staphylococcus aureus* (MRSA) and the vancomycin-resistant enterococcus (VRE), but only against *Proteus vulgaris* of the Gram-negative bacteria tested in the agar diffusion tests.

In the agar diffusion test, a drop of resin salve laid on the agar showed a narrow but clearly visible inhibition zone against the susceptible bacteria (e.g., *S. aureus*). The addition of sterile saline on the salve drop resulted in inhibition of the growth of bacteria in the whole area where the saline was spread out on the plate. In the FAB liquid growth medium, a drop of resin salve in the culture medium showed clear antibacterial activity and inhibited the growth of the bacteria in the medium (Fig. 2). Equally, the resin-pretreated FAB medium, up to a dilution of 1:2 with fresh FAB, was antibacterial in agar tests against *S. aureus*. Inhibition disappeared at dilutions of 1+5, or more.

The minimal inhibitory concentration values (MIC) of the spruce resin varied among bacterial strains tested. The MIC values of the purified spruce resin were 0.2% (w/v) against the bacteria of genus *Streptococcus*, followed by *Staphylococcus* 0.4% (w/v) and *Enterococcus* 0.4–0.6% (w/v). The MIC values against the Gram-negative bacteria and *P.vulgaris* bacterium were over 1% (w/v).



**Figure 2** The culture of the MRSA bacterium in the FAB medium in six test tubes without (a) or with an addition of a drop (0.05 ml) of 10% resin salve (b). The inoculated suspension of MRSA was adjusted to match the 0.5 McFarland turbidity standards and was serially diluted (10-fold) from  $10^1$  to  $10^{-6}$ . An inoculum of 100 microL of undiluted suspension and appropriate dilutions were added to each tube containing 900 microL of medium. The tubes were incubated at  $35 \pm 2^\circ\text{C}$  for 18 hours.

#### 4.1.2 EUROPEAN PHARMACOPOEIA (Ph. Eur.) CHALLENGE TESTS (paper II)

The resin dissolved in ethanol (A12t) immediately killed all bacteria tested in the challenge tests. All microbes inoculated were, however, re-cultivable from the resin or resin salve medium within 1 hour when the resin was alcohol-free. However, the number of re-cultivable bacteria diminished significantly in the inoculums when exposed to the purified resin or resin salve in the challenge test for longer than 1 hour. The challenge of microbes with 10% resin salve medium reduced the number (reduction  $>10^4$  in colony forming units) of *Staphylococci* within 24 h and, correspondingly, also significantly reduced the colonization of all other microbes tested within 4 days. The pure salvebase medium (control) or the 0.5% resin salve did not show any reduction in the re-growth of the microbes within 7 days.

The 2% resin salve medium showed a significant antimicrobial effect against staphylococci and *C. albicans* within 7 days, but it did not influence *B. subtilis* and showed only mild activity against *E. coli* and *P. aeruginosa*.

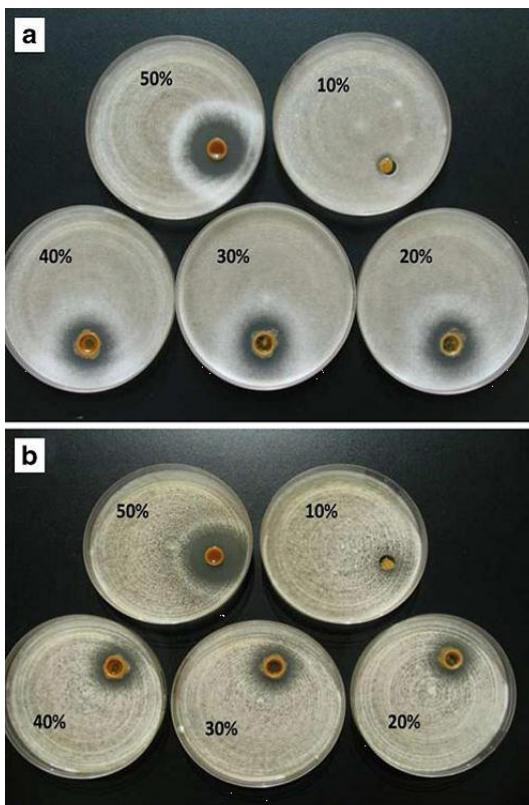
The challenge tests gave somewhat controversial results in microbial sensitivity to resin or resin salve compared to the agar diffusion test results (Table 1). The

growth of some Gram-negative rods (e.g., *E. coli* and *P. aeruginosa*) were not inhibited by the resin in the agar diffusion test but were clearly sensitive to resin in the challenge test.

#### 4.1.3 AGAR PLATE DIFFUSION TESTS IN MYCOLOGY (paper III)

In the agar diffusion tests, an antifungal effect of the resin salves was observed against all dermatophytes tested, particularly against the fungi of the *Trichophyton* species. The strength of the antifungal activity (size of the inhibition zone) was dependent on the concentration of resin in the salve. Resin in a concentration of 10% showed a clear but slight antifungal action, whereas the salve with a resin concentration of 20% or more caused a strong and significant fungistatic effect (Fig. 3).

In the agar plate diffusion test, *Candida* yeasts were insensitive to the resin, with the exceptions of *C. glabrata* and *C. krusei* where a narrow inhibition zone, however, appeared.



**Figure 3** Examples of the agar plate diffusion tests of the resin salve against *Trichophyton rubrum* (a) and *Trichophyton mentagrophytes* (b). The antimycotic activity tends to increase (size of the inhibition zone increases) with an increase in the concentration of the resin in the salve.

## RESULTS

**Table 1** A summary table on the results of the antimicrobial effects of the Norway spruce callus resin and resin salve against selected microbes in the FAB culture medium and in the European Pharmacopoeia challenge test. Note that the tests provide somewhat controversial results regarding the Gram-negative bacteria. These micro-organisms tend to be insensitive to resin in tests with the FAB medium (and in agar plate test) but sensitive in the challenge test.

Microbe species studied	Growth of bacteria in FAB medium		European Pharmacopoeia challenge test. Growth of micro-organisms after the challenge. Re-growth in cfu/ml units			
	FAB	FAB-resin	Salve base only 24 h	Resin 10% in salve base 24 h	Salve base only 4 days	Resin 10% in salve base 4 days
<b>Gram-positive cocci</b>						
<i>Staphylococcus aureus</i>	+++	-	>log 3	log 1/-	>log 3	-
<i>Staphylococcus epidermidis</i>	+++	-				
<i>Enterococcus faecalis</i>	+++	-				
<i>Streptococcus pyogenes</i>	+++	-				
<i>Streptococcus agalactiae</i>	+++	-				
<b>Gram-positive rods</b>						
<i>Arganobacterium haemolyticum</i>	+	-				
<i>Bacillus subtilis</i>			>log 3	log 3	>log 3	log 1
<b>Gram-negative rods</b>						
<i>Escherichia coli</i>	+++	+++	>log 3	log 3	>log 3	log 1
<i>Klebsiella pneumoniae</i>	+++	+++				
<i>Proteus vulgaris</i>	+++	-				
<i>Proteus mirabilis</i>	+++	+++				
<i>Pseudomonas aeruginosa</i>	+++	+++	>log 3	log 3	>log 3	log 1
<b>Yeasts</b>						
<i>Candida albicans</i>			>log 3	log 1	>log 3	-

Abbreviations: +++, ++, +, - = strong, moderate, mild or no growth growth of bacteria in the culture medium.

Log<sup>3</sup>, log<sup>2</sup> and log<sup>1</sup> indicate the growth of microbes in intensities of 10<sup>3</sup>, 10<sup>2</sup> or 1-10 cfu/ml. The abbreviation (-) indicates that no no-micro-organisms could be re-cultivated from the sample.

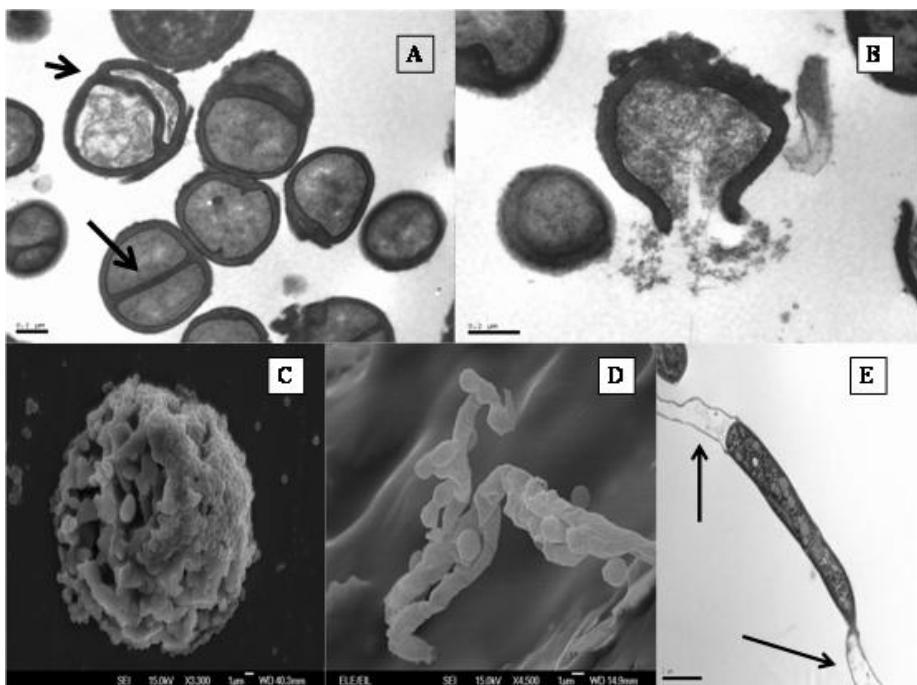
## 4.2 MORPHOLOGICAL AND ELECTROPHYSIOLOGICAL STUDIES (paper III-IV)

### 4.2.1 ELECTRON MICROSCOPY, BACTERIOLOGY (paper IV)

The exposure of the *S. aureus* cultures to the coniferous resin or resin salve resulted in alterations in structure of the microbial cell walls (Fig. 4). In the TEM micrographs, no changes in cell volumes were seen but the cell walls in bacteria exposed to resin were significantly thicker than those in the normal cells, or in the cells exposed to the salve base alone. The adhesion properties of the bacteria were also affected. In the SEM and TEM micrographs, the bacteria exposed to the resin tended to adhere and formed multicellular aggregations. In the TEM images the number of cells with septa-like structures was significantly more numerous in the exposed cultures than in the control cultures.

#### 4.2.2 ELECTRON MICROSCOPY, MYCOLOGY (paper III)

The exposure of the *Trichophyton mentagrophytes* cultures to the resin salve caused obvious destructions in the cell walls of the fungal hyphae. In the samples taken from the outside area of the inhibition zone in agar cultures plates, the fungal hyphae looked normal with plentiful spores, whereas in the samples from the inside area of the inhibition zone, the fungal cells were visualized with distorted remnants of short hyphae. In the samples from the margin area, the cell hyphae looked folded and irregular, and the cell walls were granular and flaky. Some hyphae were short and looked amputated. Resin seemed to destroy the fungal hyphae segment by segment (Fig. 4).



**Figure 4** A panel of photomicrographs of *S. aureus* bacteria (A-C) and *T. mentagrophytes* fungi (D,E) exposed to the Norway spruce callus resin salve. The exposure resulted in thickenings of the microbial cell walls, septa-like cell wall structures (arrows; A), disruptions of the cell wall (B) and cell aggregations (C). The fungal hyphae are damaged (D) and destroyed segmentally (arrows; E).

## RESULTS

### 4.2.3 ELECTROPHYSIOLOGICAL STUDIES (paper IV)

CCCP (carbonyl cyanide m-chlorophenylhydrazone) is an H<sup>+</sup> ionophore that disrupts the transmembrane proton gradient in prokaryotic cells. The DiOC<sub>2</sub> (3,3'-diethyloxacarbocyanine iodide) -stained *S. aureus* cells that were exposed to CCCP, spruce resin, or abietic acid, emitted more green than red fluorescence (radiometric parameter MFI<sub>red</sub>:MFI<sub>green</sub> decreased) compared to intact *S. aureus* cells. The observations imply that the proton gradient across the cell membrane in these bacteria had been disrupted when exposed to the resin or abietic acid.

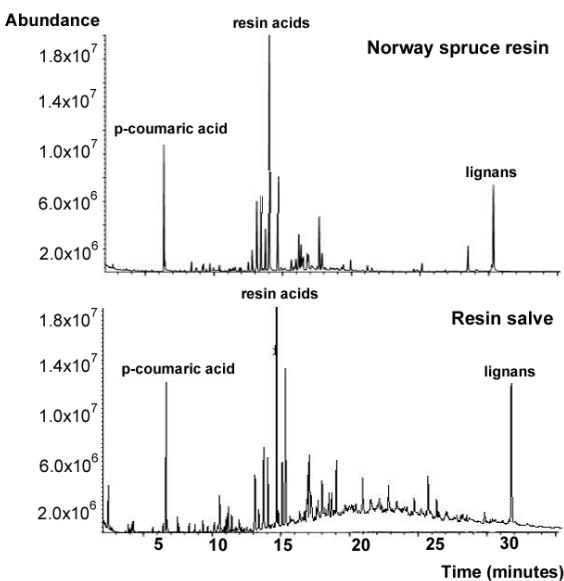
### 4.2.4 ANALYSES ON FATTY ACIDS (paper IV)

When the *S. aureus* cells were cultured in the presence of resin the share of the branched-chain fatty acids increased and the proportional share of straight fatty acids, correspondingly, decreased. This implies that resin exposure induces structural changes in the microbial cell walls.

## 4.3 OTHER ANALYSIS

### 4.3.1 CHEMICAL COMPOSITION OF THE CONIFEROUS “CALLUS” RESIN AND RESIN SALVE (UNPUBLISHED DATA)

In the gas-liquid-chromatography with mass spectra (GLC/MS), the acetone extracts of both pure coniferous callus resin of the Norway spruce, and the salve manufactured from this resin, gave very similar and stable chromatograms in repeated analyses (Fig. 5). The samples tested showed constantly the presence of coumaric acid (hydroxycinnamic acid), a group of resins acids and a group of lignans, similarly in both resin and resin salve extracts. The mean concentrations of coumaric acid, different resin acids and lignans in acetone extracts from 8 different salve samples are presented in Table 2. Among the resin acids, hydroxyabietic acid and abietic acid were the most abundant compounds. Pinoresinol was the most abundant lignan. The GLC chromatograms or the concentrations of coumaric acid, resin acids or lignans in the resin or resin salve remained stable in samples stored over 3 years at room temperature. Neither were there changes in the GLC/MS of the resin or salve samples kept in a heat chamber (+40°C) for 6 months.



**Figure 5** Example of the gas-liquid-chromatography (GLC) of purified coniferous callus resin from the Norway spruce (*Picea abies*) and from the salve manufactured from this resin. The GLC/MS analyses were done in METLA laboratories (Helsinki, Finland) from the acetone extracts of the purified coniferous resin or resin salve.

**Table 2** Concentrations of various resin acids, lignans and coumaric acid in 10% resin salve. Concentrations were calculated from the GLC/MS data of the acetone extracts of the resin salve (Metla, Helsinki, Finland). The analyses are the means of eight salve samples. Each analysis was done in duplicate.

Compound	Concentration mg/g
<b>Resin acids</b>	
dehydroabietic acid	8.8 ±1.0
abietic acid	6.0 ±1.3
isopimaric acid	3.1 ±0.3
pallusteric acid	2.8 ±0.4
neoabietic acid	2.0 ±0.4
pimaric acid	0.9 ±1.4
levopimaric acid	0.8 ±0.2
<b>Lignans</b>	
pinoresinol	7.6 ±1.0
isolariciresinol	1.7 ±0.9
larinol	1.6 ±0.3
secoisolariciresinol	0.8 ±0.2
<b>Total</b>	
resin acids, total	24.0 ±3.2
lignans, total	10.0 ±1.1
resin acids + lignans, total	32.6 ±5.5
Coumaric acid (hydroxycinnamic acid)	4.6 ±0.8

## RESULTS

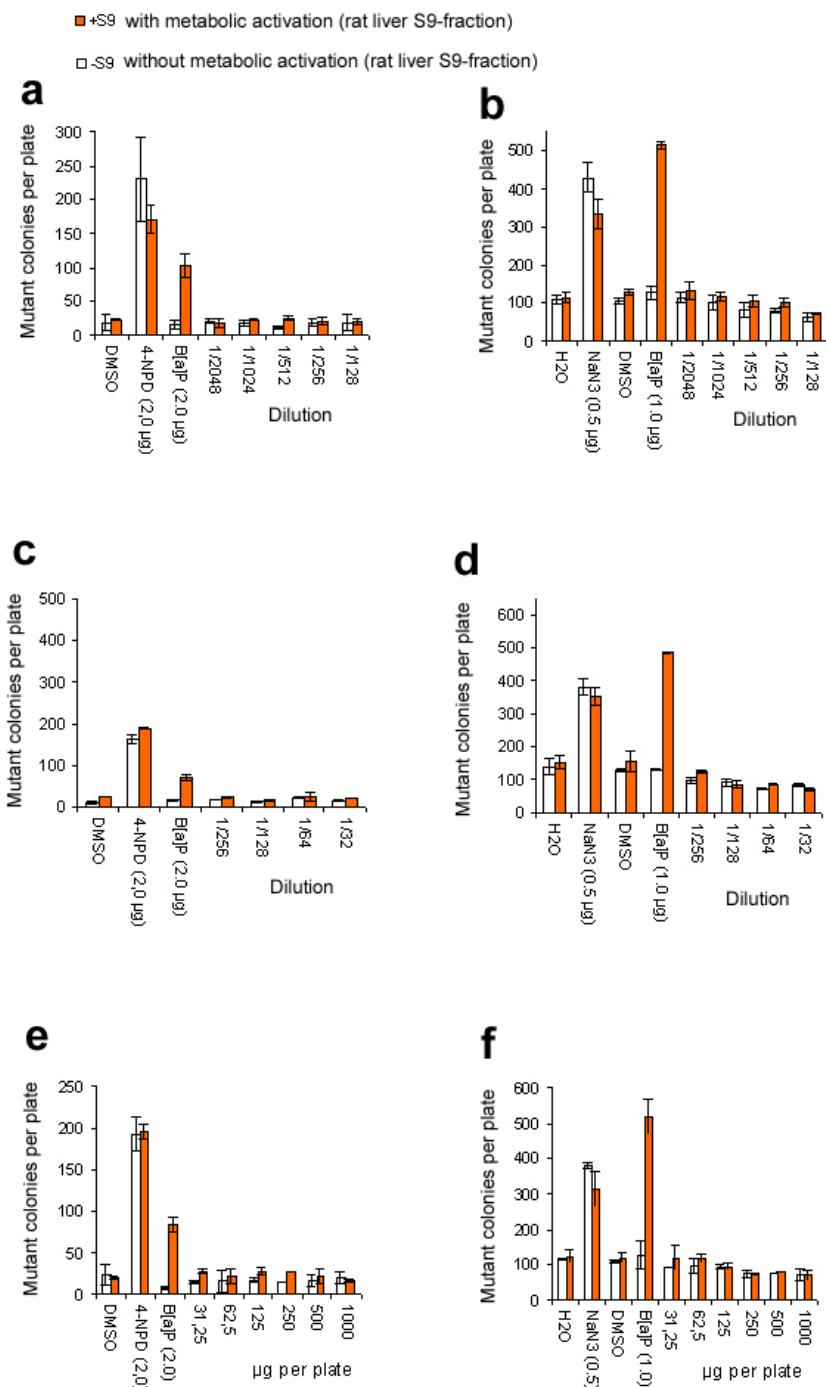
### 4.3.2 ALLERGIC REACTIONS, TOXICITY AND COMPLIANCE (paper V-VI)

In the trial of patients with pressure ulcers (paper V), one patient showed symptoms indicating allergic contact dermatitis, and was, therefore, dropped out from the study. In the cohort trial in patients with chronic complicated surgical wounds (paper VI) there were no observations of any adverse events or allergic reactions. In both studies (papers V and VI), altogether 45 patients underwent the scheduled treatment up to the end of the study and received resin salve daily for weeks or several months. Among these patients, the prevalence rate of allergic contact dermatitis was 2% (95%CI:0–5%; i.e., one patient out of 45).

The possible mutagenicity of the coniferous resin and resin salve was tested with the Ames tests (unpublished data). The test results are presented in Fig.6. In repeated tests with two *Salmonella typhimurium* strains, and with or without the metabolic activator, the resin or resin salve did not show any mutagenic properties, not even in maximal resin concentrations.

The skin irritation tests and *in vitro* cytotoxicity tests were performed with the coniferous 10% resin salve by an authorized laboratory (unpublished data). In the skin irritation test performed according to the ISO 10993-10:2010 standard, the 10% resin salve caused, in skin of three rabbits, slight (negligible) erythema 24 and 48 hours after the termination of exposure. The “irritation Index” was 0.1 (scale 0-8.0), which corresponds to the response category: “negligible” in the ISO 10993-10:2010 standard.

In the *in vitro* cytotoxicity test, the extracts of 10% coniferous resin salve in HAM F12 culture medium were cytotoxic against mouse fibroblasts in a 24 hour assay (unpublished data). Based on the EN ISO 10993-5:2009 and USP<87> criteria, the cytotoxicity was severe (cytotoxicity grade 4). The diluted extract (dilution 1+3) of the resin salve showed slight to mild cytotoxicity (cytotoxicity grade 1–2; scale of the index: 0–4).



**Figure 6** Results of the Ames test with the Norway spruce callus resin (a, b, e, f) and with the resin salve manufactured from the callus resin (c, d). The tests were performed with *Salmonella typhimurium* strain TA98 (a, b, d, e) or strain TA100 (c, f). The test was carried out with or without metabolic activation (rat liver S9-fraction). The samples of resin or salve were serially diluted in dimethyl sulfoxide (DMSO). Note that all sample results on mutagenicity were at the same level or less than in the results with the DMSO diluent or water control. All positive controls (4-NPD, B[a]P and NaN<sub>3</sub>) showed a clear mutagenicity.

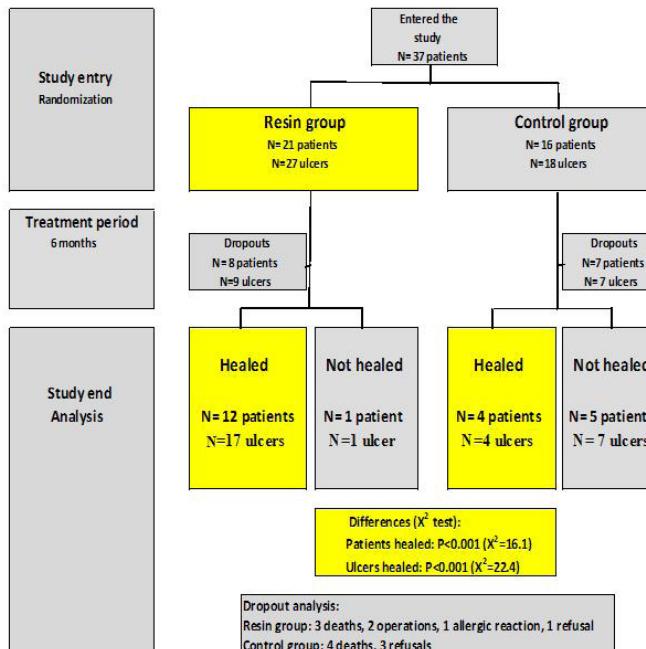
## 4.4 CLINICAL TRIALS (paper V-VI)

### 4.4.1 A RANDOMIZED, CONTROLLED TRIAL (RCT) IN PATIENTS WITH PRESSURE ULCERS (paper V)

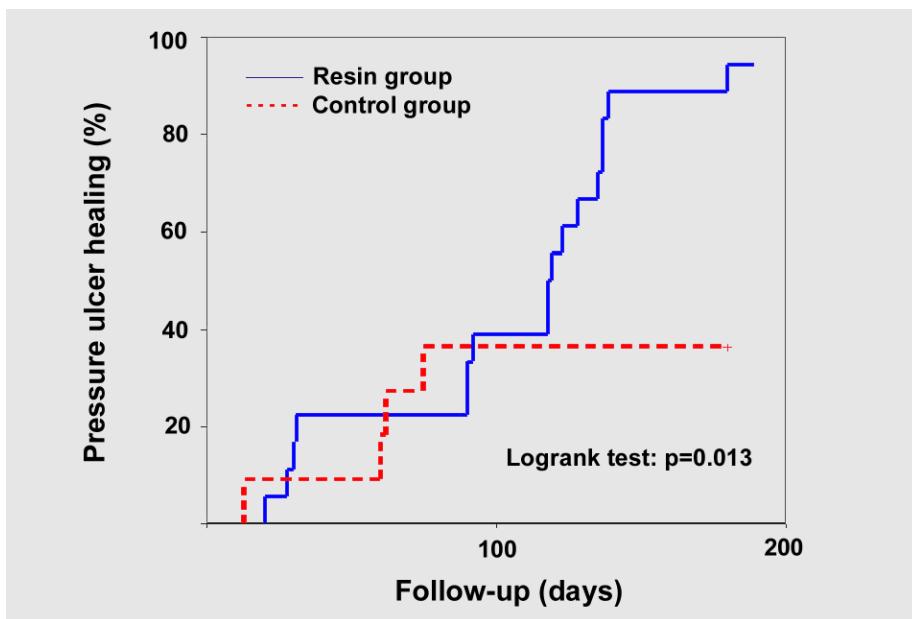
After recruitment of 37 patients, 13 eligible patients in the resin group and 9 patients in the control group underwent the 6 month treatment trial (Fig. 7). The corresponding numbers of ulcers were 18 and 11, respectively. Between the treatment groups, there were no significant differences in terms of the patient demography, clinical appearances or ulcer characteristics.

**Healing rate:** In the per protocol analysis, all ulcers healed in 12 of 13 compliant patients in the resin group and in four of nine compliant patients in the control group (92% (95%CI: 78–100%) vs. 44% (95%CI: 12–77%); P = 0.003; power 73%) during the 6-month treatment period (Fig. 7). The ulcer healing rate (as a function of time) was significantly better in the resin group than in the control group (log-rank test, P = 0.013; Fig. 8). Complete healing of ulcers occurred significantly more often in the resin group (94% (95%CI: 84–100%) of the ulcers healed within 6 months) than in the control group (36% (95%CI: 8–65%); P = 0.003).

In the intention-to-treat (ITT) analysis, and by assuming that all ulcers in patients dropped out in both study arms did not heal, the healing rate for ulcers were 63% (95%CI: 45–81%) and 22% (95%CI: 0–41%).



**Figure 7** Flow chart of the study design in the randomized controlled trial (RCT) and of the prevalence rate of patients and ulcers healed in 6 months in the resin and control groups (hydrofiber dressing with or without silver ( $\text{Ag}^+$ )).



**Figure 8** Graph of the healing rate (relative frequency of ulcers healed in time units) of severe pressure ulcers in the resin salve group and in the control group (hydrofiber dressing with or without silver ( $\text{Ag}^+$ )) in the randomized patient trial.

**Secondary outcomes measures:** During the 6-month treatment period, only one ulcer (6% of all ulcers) was not healed in the resin group. Correspondingly, in the control treatment group, six ulcers (55% of all ulcers) were not healed. One ulcer (9%) in the control group became worse.

At baseline, 10 different bacterial strains were cultured in both study groups. One isolate in one patient in the resin group and one in the control group was the MRSA bacterium. In both cases the culture was negative for MRSA one month later. *Staphylococcus aureus* bacteria seemed to be more susceptible to eradication with the resin salve than with the control treatment.

**Dropout analysis:** There were eight (8 of 21; 38%) dropout patients in the resin group, and seven (7 of 16; 44%) in the control group (Fig. 7). Reasons for the dropouts in the resin group were three deaths, two admissions for operative treatment, one allergic skin reaction, one misdiagnosis and one patient-related refusal without any specific reason. The reasons for dropouts in the control group were four deaths, two patient-based refusals without any specific reasons and one patient-related refusal because of the randomization to the control group that the patient did not accept, and therefore, finally refused.

## RESULTS

### 4.4.2 AN OBSERVATIONAL CLINICAL COHORT STUDY IN PATIENTS WITH CHRONIC SURGICAL WOUND (paper VI)

The healing rate of chronic, complicated surgical wounds was 100% (23/23) among patients who received the resin salve treatment. There were no dropouts. The average healing time was  $43 \pm 24$  days. Immobilization, use of immunosuppressive regimen, length and depth of the wound were independent factors of the healing time in multivariate regression analyses (Table 3). The wound length was an independent factor of the healing time in the multivariate regression analysis. Mathematically and theoretically, the wound healed a mean of 2 mm per day, indicating, for example, that the healing time was 10 days on average in wounds that were 2 cm long.

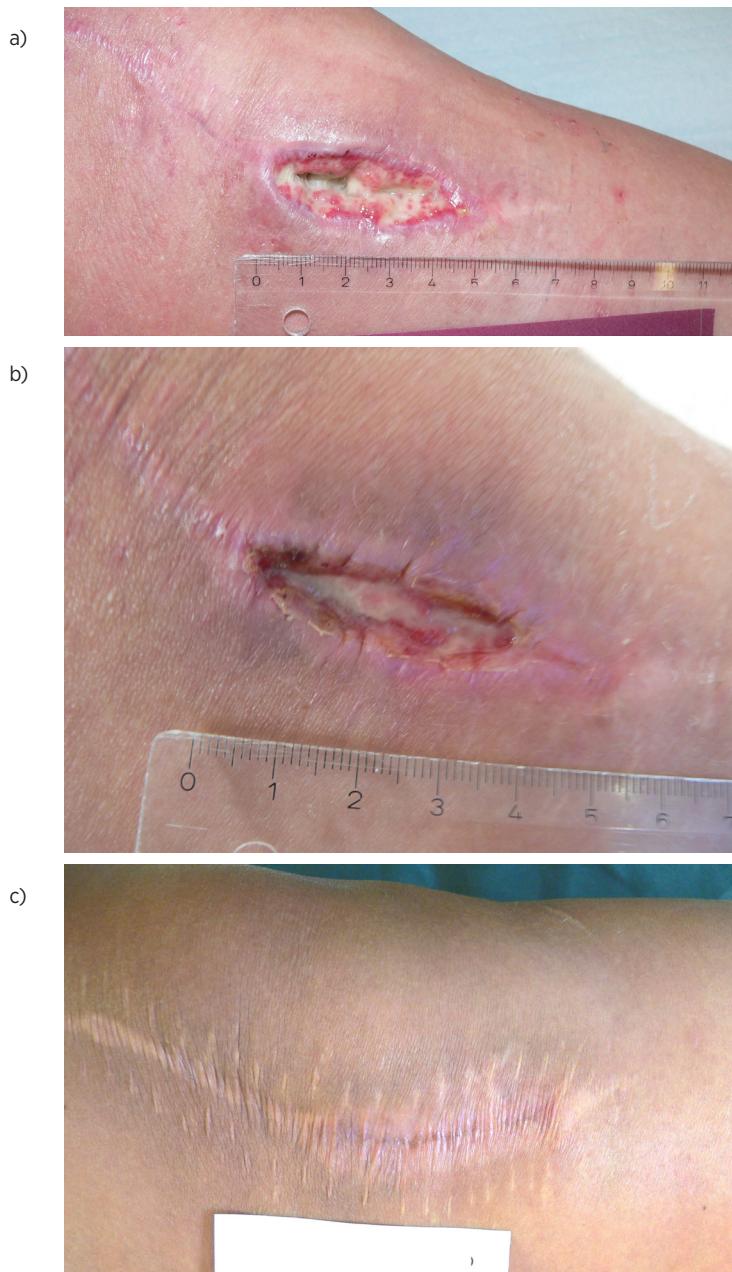
**Table 3** Table of results of the univariate and multivariate analyses, and of the influence of selected variables on the healing time of chronic surgical wounds in 23 patients. Linear regression analysis. All significant variables associated positively with the healing time.

Covariate	R <sup>2</sup>	P value
<b>Univariate analysis</b>		
<b>Immobilization</b>	<b>0.375</b>	<b>0.001</b>
<b>Corticosteroids or immunosuppressive agents</b>	<b>0.295</b>	<b>0.004</b>
<b>Wound size</b>		
<b>depth</b>	<b>0.351</b>	<b>0.002</b>
<b>length</b>	<b>0.271</b>	<b>0.016</b>
<b>width</b>	<b>0.0</b>	<b>NS</b>
<b>area</b>	<b>0.109</b>	<b>NS</b>
<b>Age</b>	<b>0.093</b>	<b>NS</b>
<b>Sex</b>	<b>0.0</b>	<b>NS</b>
<b>Infection (positive culture)</b>	<b>0.0</b>	<b>NS</b>
<b>Diabetes or smoking</b>	<b>0.0</b>	<b>NS</b>
<b>Multivariate analysis</b>		
<b>Model including all above variates</b>	<b>0.679</b>	<b>0.001</b>
<b>Wound length</b>		<b>0.001</b>
<b>Corticosteroids or immunosuppressive agents</b>		<b>0.014</b>
<b>Immobilization</b>		<b>0.041</b>
<b>Others</b>		<b>NS</b>

No conspicuous or unusual clinical observations were seen in the patients who received the resin salve treatment suggesting that the compliance and feasibility were good. Clinical worsening (e.g., increase in size or change in appearances of the wound) was not observed in any of the patients.

Examples of the cases with chronic surgical wounds before and after treatment with the coniferous resin salve are presented in Fig. 9 and 10. An additional example of a case with a chronic, certainly “sterile” surgical wound before and after treatment with coniferous resin salve is presented in Fig. 11.

**Figure 9** A 50 year old woman with rheumatoid arthritis for 30 years. She received methotrexate, hydroxylchloroquine, folic acid and paracetamol medication. After arthrotomy of the left knee, she got a complicated surgical chronic wound, which was treated with the coniferous resin salve. Photograph a) at the beginning of the treatment b) in 20 days c) in 49 days when the wound was healed.



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**Figure 10** A 61 year old male with psoriatic arthritis, treated with methotrexate. After arthrodesis of the right ankle with an intramedullary nail, he got a complicated surgical chronic wound on the medial side of the lower leg extending to the bone. The wound was treated with coniferous resin salve. Photograph a) at the beginning of the treatment b) at 13 days representing granulation tissue on the wound base c) at 84 days when the wound was healed with only a small ulcer remnant in the center.





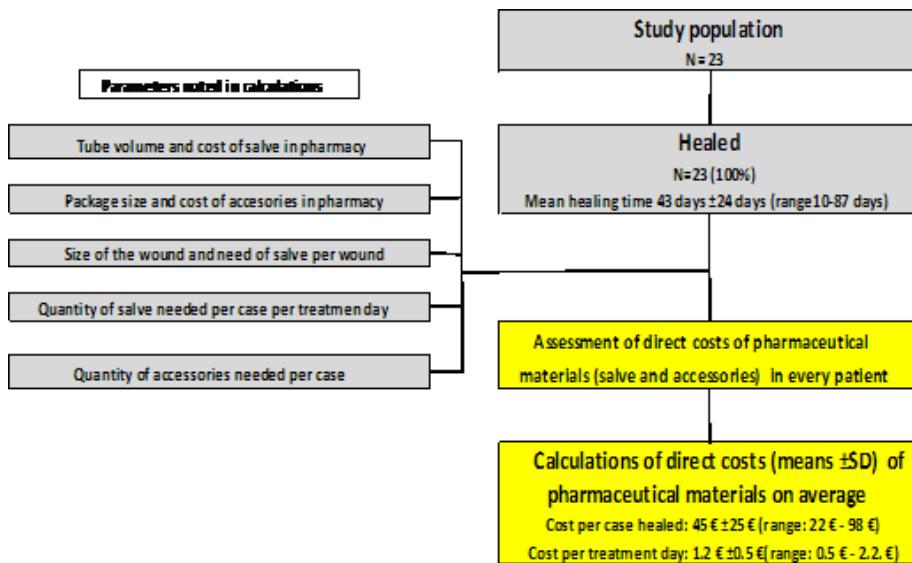
**Figure 11** An 81 years old male with prostate cancer. Fasciectomy was performed on the right hand due to severe Dupuytren's contracture (palmar fibromatoses) affecting the fourth and fifth finger. The wound didn't heal because of tension and wound edge necrosis. No clinical or microbiological evidence of infection was found. The chronic wound was treated with coniferous resin salve. Photographs A) at the beginning of the treatment B) at 22 days when the wound was healed.

#### 4.4.3 CALCULATION OF THE DIRECT COSTS OF THE PHARMACEUTICAL MATERIALS (paper VI)

Analysis of the direct costs of the pharmaceutical materials in the resin salve treatment was performed for the 23 patients in the observational cohort study on chronic surgical wounds (paper VI). The costs were first assessed in every patient separately, after which the mean costs  $\pm$ SD were calculated. A flowchart of the parameters taken into account in this assessment is presented in Fig. 12.

In the beginning of the study, the average wound area was  $424 \text{ mm}^2$  and the amount of resin salve needed for the treatment of a wound of such a size was empirically assessed (measured) to be 0.4 g per day. A salve tube of 20 g was correspondingly assessed to be sufficient for 50 days in patients with a wound of the average size ( $424 \text{ mm}^2$ ). Based on these estimations and by taking the consumer prices into account, regarding need and package sizes of the salve and accessories, the total mean costs of the resin salve treatment per patient during a treatment period of 43 days were  $45 \pm 26 \text{ €}$ , ranging from 22 € up to 102 € per patient. The average daily costs were  $1.2 \pm 0.5 \text{ €}$ , ranging from 0.5 € to 2.2 € per day.

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**Figure 12** The flow chart on analysis and parameters taken into account in assessment of the direct costs of the pharmaceutical materials in the observational trial, for 23 patients with a chronic surgical wound (paper VI).

## 5. DISCUSSION

### 5.1 GENERAL DISCUSSION

Although the microbiological studies gave somewhat controversial results, the predominant antimicrobial property of resin and resin salve was clear. However, owing to the small number micro-organisms tested generalizations cannot be fully justified. Our studies indicate that resin and resin salve seem to have a broad antimicrobial property, against both Gram-positive and Gram-negative bacteria. The morphological and electrophysiological studies were considered to support the microbial findings and gave evidence that the antimicrobial actions were unspecific and based on destruction of the cell walls and membranes of the micro-organisms.

The clinical studies in this thesis are also small and include a relatively few patients so that again generalizations are not defensible. The results suggest, however, that resin salve treatment tends to have a positive influence on wound healing, supporting the positive effect of resin salve on skin wounds as experienced in traditional medicine for hundreds of years.

In traditional medicine, resin salves have been used for long periods without major problems in tolerability. A predicted side effect of resin salve was resin allergy (colophony allergy / contact dermatitis) that was observed in 2% of the treated patients. This finding is in line with the prevalence described in the literature. For a more comprehensive picture of the safety of resin salve treatment, larger clinical trials are needed.

### 5.2 MICROBIOLOGY

The microbiological observations in this study project show that the coniferous resin and the salve manufactured from this resin are strongly antimicrobial against a wide range of microbes including both Gram-positive (e.g., *S. aureus*, MRSA, *B. subtilis*) and Gram-negative bacteria (e.g., *E. coli*, *P. aeruginosa*). They are also antifungal against yeasts, like *C. albicans*, and against the most common dermatophytic fungi (genus *Trichophyton*). These observations are in line with those reported in the previous and in some recent investigations (Söderberg et al. 1991, Cowan 1999, Savluchinske-Feio et al. 1999, Savluchinske-Feio et al. 2007, Wang et al. 2012).

The agar diffusion test and the European Pharmacopoeia challenge test provided markedly controversial results regarding the sensitivity of the different species

## DISCUSSION

of microbes to coniferous resin. In the agar diffusion tests, coniferous resin was antibacterial against all Gram-positive bacteria tested, like *S. aureus* and MRSA, *S. epidermidis*, *E. faecalis*, *S. agalactiae*, but not, or in lesser extent only, against the Gram-negative bacteria. In the challenge study, on the other hand, the resin was bactericidal also against the Gram-negative bacteria. In the agar diffusion test, resin was not antifungal against the *C. albicans* yeast whereas the resin was anti-fungal against *C. albicans* in the challenge tests. Several explanations are possible for these controversies regarding the sensitivity of bacteria and fungi in different tests, to the resin.

Firstly, the antimicrobial susceptibility tests on agar plates may not always be fully sensitive and will occasionally give false negative results due to methodological problems (Woolfrey et al. 1978; Catry et al. 2007). Secondly, the agar diffusion test is likely dependent on the water solubility and on the diffusion and dilution of the test substances in the aqueous agar medium. Most of the resin acids are poorly water soluble, and therefore may diffuse poorly in sufficient antimicrobial concentrations in the agar. Subsequently, the susceptibility of the microbe to the resin may be exhibited in agar diffusion tests only for the micro-organisms with the highest sensitivity. Since the resin sensitivity of Gram-positive and Gram-negative bacteria was different, the observations also indicate that the microbes genuinely vary markedly in their antimicrobial sensitivity to the coniferous resin.

In the challenge tests, the microbes are directly exposed to the resin in highest possible resin concentration. Therefore in this test, the antimicrobial actions may not be solely dependent of diffusion and dilution of resin or resin acids in the test medium. This may mean that antimicrobial effectiveness of the poorly water soluble coniferous resin against even the most resistant microbes is seen when the resin concentration is high enough. The threshold concentration of the resin in a salve for it to act microbically against most of the bacteria in a liquid physiological culture media was observed to be approximately 1% (w/w).

The bacteriostatic minimal inhibitory concentrations (MIC) of the resin were tested for Gram-positive bacteria (*S. aureus*, *S. epidermidis*, *E. faecalis*, *St. pyogenes*, and *St. agalactiae*) in the Mueller-Hinton medium. The MIC values obtained were 0.2–0.4% (w/v) that roughly correspond to resin acid concentrations of 2–4 mg resin in 1 g of the medium. In GLC analyses, the resin acid concentration in acetone extracts of the 10% resin salve was around 25 mg in 1 g of salve, suggesting that the concentration of the resin acids in 10% resin salve exceed manyfold the MIC values required for the antimicrobial influences in general. In challenge tests, the microbicidal effect (no regrowth of microbes) was obtained against all bacteria tested, both against Gram-positives and Gram-negatives, or *C. albicans*, with 10% resin salve in 4–7 days.

The MIC estimates obtained in our study represent the minimal concentrations of the purified crude spruce resin in the agar but these MIC values are not equivalent to the concentrations of the antimicrobial resin acids dissolving from

resin into the water phase of the agar. Natural resins and rosin are slightly water soluble. According to the published data, the water solubility of resin acids is variable and the maximal water solubility of resin acids has been reported to be around 1–5 ppm (parts-per-million); dehydroabietic acid has the highest solubility (Peng et al. 2000). Considering that the MIC value of the purified spruce resin is between 100–1 000 ppm (concentration of purified crude resin in salve or agar 0.1–1% (w/w)), and that the concentration of the resin acids in the aquatic agar would be 1–5 ppm, only a very small proportion of the resin (resin acids) is dissolved from the resin into the aquatic phase of the medium or the tissues. Nevertheless this small proportion (approximately one millionth of the concentration in MIC values), is capable of exerting an antimicrobial effect.

The exact mechanisms of the antimicrobial actions of the coniferous resin or resin acids against the microbes are not known. These mechanisms cannot be, however, specific since practically all micro-organisms seem to be more or less sensitive to resin, either in the agar diffusion, liquid media, or in the challenge tests. It is considered that the terpenes in general, a subgroup of the chemicals the resin acids also belong to, have direct toxic influences on the microbial cell walls and membranes (Sikkema et al. 1995, Wang et al. 2012). This explanation is supported by the present morphological and electrophysiological observations. In our study, the destruction as seen in the cell walls of both bacteria and fungi, suggesting also that this damage is diffuse injury, and is not species dependent as is often the case with the antibiotics.

The resin acids are considered to be the major antimicrobial factor of the coniferous resin as seen in the literature (Söderberg et al. 1991, Savluchinske-Feio et al. 1999, Wang et al. 2012). In our study and in concordance with earlier observations, abietic acid showed identical antimicrobial influences to those of the resin or resin salve (Söderberg et al. 1990, Söderberg et al. 1991, Smith et al. 2005). Lignans and coumaric acid are, on the other hand, compounds with anti-inflammatory and antioxidative properties (Kangas et al. 2002, Willför et al. 2003, Miyake et al. 2005).

There are some resemblances in biological actions between resin and ionic silver. Silver ions damage cell walls and cytoplasmic membranes, this damage appearing, however, more slowly than with the common chemical disinfectants like sodium hypochlorite or phenols (Chamakura et al. 2011, Li et al. 2011). The antimicrobial actions of coniferous resin and ionic silver differ from those of medical honey, the actions of which are obviously due to the acidity, hydrogen peroxide, an osmotic effect, or to the presence of unidentified compounds in the medical honey (Al-Waili et al. 2011).

### 5.3 MORPHOLOGY AND ELECTROPHYSIOLOGY

Our electron microscopical investigations indicated that the exposure of the cultures of *S. aureus* to spruce resin, resin salve, or purified abietic acid resulted in changes in structure and function of the microbial cell wall, indicating that the injuries of cell wall and membranes are the cause of the microbicidal actions of the resins.

It was shown by our electron microscopical observations that the exposure to resin or to 10% (w/w) resin salve for 18 h thickened the bacterial cell walls and increased the cell adherence. The exposure of the *S. aureus* cultures to resin in a concentration of 0.1% (w/v) increased the proportion of branched fatty acids in cell wall lipids, and the exposure of the microbes to resin or abietic acid for 90 min dissipated the membrane potential in bacterial cells. Since *S. aureus* cultures recovered even after long-term (3 days) contact with resin in the ordinary microbiological cultures, this electrophysiological dissipation of the membrane potentials may not result from simple cell death, but may, instead, be caused by failures in the proton-motive force across the cell membranes. The transmembrane potential may partially disappear even though the bacteria do not immediately die. Based on these observations, one may assume that the antimicrobial action of the coniferous resin against the microbes is more detergent-like and bacteriostatic than simple and fast necrotic killing of the cells.

Some resin acids (e.g., dehydroabietic acid and its hydroxylated derivates in particular) are compounds that can dissolve in small amounts in water. In GC/MS analysis of methylene chloride extracts of resin – water dispersions, dehydroabietic acid and its hydroxylated derivates ( $7\alpha$ - or  $7\beta$ -hydroxydehydroabietic acid 15-hydroxydehydroabietic acid,  $7\alpha,15$ - or  $7\beta,15$ -dihydroxydehydroabietic acid) dissolve in water in concentrations of 0.5 – 16 mg/L (unpublished observations). At physiological pH in an aquatic milieu, these compounds may be in both protonated and non-protonated form. The non-protonated forms may act as proton acceptors. The protonation of the resin acids may increase their fat solubility, and it can also enhance the protonophoric transport of H- ions across the cell membrane. The enhanced ionophoric transport of protons may interfere with the proton transport in the membrane-bound ATPase, resulting in uncoupling of the oxidative phosphorylation. Subsequently, the cell metabolism may cease and the supply of energy may be lost (Wikström 2009).

Our observations of an increase in the proportion of the branched-chain fatty acids in *S. aureus* lipids after exposure to the resin or abietic acid indicate that the resin or resin acids do not act only as simple protonophores. The structural alterations of the cell wall macromolecules may change the permeability and fluidity of the cell membrane and cell wall, and may, thereby, also disturb the normal physiological functions of the cell walls and cell membranes.

## 5.4 CLINICAL TRIALS

Our randomized controlled trial in patients with pressure ulcers (RCT, paper V) and the observational study in a cohort of patients with chronic surgical wounds (paper VI) are, to the author's knowledge, the first studies on the clinical efficacy of coniferous resin salve in skin wounds and ulcers. There are no earlier (before 2008) scientific publications in the MEDLINE database available with key words "resin salve and clinical trial" or "resin and skin ulcer".

The RCT documented a significantly higher healing rate of severe pressure ulcers with the coniferous resin salve with ordinary gauze dressings compared to the healing rate of pressure ulcers with the nonadhesive hydrofiber dressings. The ulcer healing rate was significantly ( $P<0.05$ ) higher in the resin salve treatment group than in the control group, in both the per protocol analysis, and in the ITT analysis.

In a systematic review of the Royal College of Nursing and National Institute for Health and Clinical Excellence (NICE) in 2005, modern dressings like hydrofibers or hydrocolloids, instead of saline gauzes, paraffin gauze or simple dressing pads, are recommended as treatment tools in the pressure ulcers. The hydrofiber and hydrocolloid dressings represent advanced technology and have been demonstrated to improve objectively the healing of pressure ulcers as compared with ordinary saline gauze treatment (Ontario Health Technology Advisory Committee Meta-analysis 2009). The hydrocolloid dressings are considered to create the optimum healing environment (NICE 2005). Since the resin salve with ordinary gauzes in the present trial gave better treatment results than the modern hydrofiber dressings, one may conclude that the superiority of the resin salve in pressure ulcers is due to the salve itself, not due to any additional facilities or dressings.

In previous studies, the treatment of pressure ulcers with the hydrofiber dressings for 6 months have been reported to result in total healing in 55 % of cases in general and in 89% of cases with stage II ulcers (Alm et al. 1989, Xakellis et al. 1992). In the present RCT, the treatment of stage II-IV pressure ulcers with hydrofiber dressings resulted (in the per protocol analysis) in healing of the ulcer in 44 % of the patients (95%CI:12–77%; 4 of 9 patients). This is a healing rate that is in line with the earlier published data.

In our RCT on pressure ulcers, the patients with an infected ulcer in the control arm received hydrofiber dressings impregnated with silver ( $\text{Ag}^+$ ). Silver has antimicrobial properties but the superiority of the silver impregnated dressings over those without silver is controversial and based on very few investigations of moderate quality only (Rosén 2010). In a multicenter RCT on leg ulcers, 60% and 57% of ulcers healed in 12 weeks using the hydrocolloid dressings with silver and without silver, respectively (Michaels et al. 2009).

Silver has clear antibacterial influences and reduces the number of the microorganisms in microbial cultures in *in vitro* experiments, the bactericidal effect re-

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sulting from the damage to cell wall and cytoplasmic membranes (Ivanova et al. 2011, Monteiro et al. 2011, Percival et al. 2011). Studies on the antibacterial influences of ionic silver at tissue level, *in vivo*, are, however, scanty (Rosén 2010). In one available study on leg ulcers, the silver impregnated dressings did reduce the count of bacteria in the wound (Sibbald et al. 2007). In our RCT, both the resin salve treatment and the hydrofiber dressings with or without silver resulted in microbiological clearance of pressure ulcers, this clearance taking place, however, somewhat faster with the resin salve than with the hydrofiber dressings.

In designing the study protocol of our RCT, the treatment was expected to take months because of the advanced age of the patients, and because of the large size and long clinical history of the pressure ulcers in the patients. The duration of the study was designed to last up to 6 months, and the *a priori* calculations of power (above 70%) indicated that approximately 20 cases had to be included in both study arms by assuming that the resin salve treatment would result in ulcer healing 25% more often than in the control treatment. It was finally observed that all ulcers in all patients in the resin group, except one ulcer in one patient, healed during this 6 month treatment period, and that the resin salve treatment was significantly better than the control treatment, the power of the study being 73%.

The observational clinical cohort study (paper VI) was carried out to investigate the feasibility and safety aspects of the resin salve treatment in complicated and chronic surgical wounds. Even though the trial did not include control cases, it is noteworthy that all consecutive wounds in patients admitted to the trial from two centers, with the length of the open wound varying between 5 mm and 60 mm (mean  $29 \pm 19$  mm), healed in a reasonable time, 43 days on average (range: 10–87 days), without any clinical drawbacks. This wound healing time in the present investigation was somewhat shorter than the healing time reported in two earlier similar studies (Shealy et al. 2006, Ubbink et al. 2008). For comparison, it was reported in a recently published RCT on chronic venous leg ulcers using modern hydrofiber dressings with silver (Ag+), progression of wound healing occurred in 52–67% of the cases in 8 weeks (Harding et al. 2011).

There are only few high quality studies in the literature on the efficacy of various conservative (non-surgical and non-device based) topical treatment options of chronic surgical wounds. In a recent Cochrane Library systematic review and meta-analysis, 13 RCTs on the efficacy of various dressings or topical agents in postoperative chronic wounds were analyzed (Vermeulen et al. 2004). The results published in these studies were concluded to be non-comparable and unreliable because of the many inconsistencies and flaws. Exceptions were a small study on Aloe vera and one study on plaster cast applied to an amputation stump. Otherwise, there were no statistically significant differences in the healing rates of the chronic surgical wounds between the various treatment tools applied (e.g., gauze, modern hydrofiber and hydrocolloid dressings, foams, alginate, etc.; 11 trials) (Vermeulen et al. 2004). In a RCT in patients with chronic surgical and traumatic wounds,

treatment with hydrofiber dressings with silver for 2 weeks was not significantly better than treatment with iodine gauzes, the hydrofiber treatment resulting in the wound healing in 23% of the patients (Jurczak et al. 2007). In another systematic review, the modern hydrogel dressings were recommended for wound debridement only (Vaneau et al. 2007). In a British review on four RCTs including altogether 221 patients, by comparing the hydrocolloid dressings with the saline gauzes, the odds of wound healing in 6 weeks to 6 months varied from 1 to 11 in favor to the hydrocolloids (NICE 2005). In the present RCT, the OR (odds ratio) for ulcer healing in 6 months was 30 (95%CI: 4–218) in favor of the resin salve as compared with the hydrofiber dressings.

In the multivariate linear regression analysis of the data from the observational cohort study on chronic surgical wounds, the wound length was found to be negatively correlated with the healing time and to be an independent parameter for predicting wound healing. Under resin salve treatment, the re-epithelialization (healing) of wounds occurred at 2 millimeters per treatment-day, on average, according to the regression analysis. This observation may mean that, if the wound healing takes place *per primam* with the resin salve, the surgical complicated wound heals (re-epithelializes) along the longitudinal axis by one millimeter per day at both wound ends, the late remnants of wound remaining in the wound center before the final closure of the wound (Fig. 10). This is certainly a simplification and a theoretical model only. Wound healing in clinical practice is more complicated and also dependent on factors other than the wound length, e.g. on contraction of the wound edges.

#### 5.4.1 LIMITATIONS OF THE PRESENT CLINICAL TRIALS

There are several limitations in the present clinical investigations. Firstly, the exact knowledge of the past history of patients in the RCT was not available and, therefore, the disease history could not be used as an objective parameter. In an earlier investigation in Finland on chronic skin wounds and ulcers among the geriatric ward patients, the disease history of ulcers varied from 3 months to 4 years indicating that the pressure ulcer is an extremely long-lasting disease (Eriksson et al. 1999).

On the other hand, the baseline characteristics (length, width and depth) of the wounds were known and noted in all patients in the observational cohort study. In these patients, the resin salve treatment was, however, instituted as a primary treatment method when the wound was observed and classified chronic and complicated (wound open 2 weeks after the elective operation). Therefore, the past history was considered irrelevant and need not be taken into account.

Secondly, in our RCT, the requirement of blind outcome measures, or concealment in the allocation, could not be fulfilled because of the characteristic physical features (color, smell, etc.) of the resin salves. The present RCT was a multicenter

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study (11 health centers) which may reduce the bias caused by possible preconceptions in clinical assessments or in patient inclusions.

Thirdly, the number of patients in both trials was relatively small, particularly in the trial on pressure ulcers (RCT; paper V), but corresponds to the number of patients in two previous RCTs on skin wounds in the available literature (Burke et al. 1998, Griffiths et al. 2001). Long-continued therapy (6 months) is laborious, expensive and difficult to carry out which forces limitation of the patient number. Patients are, in addition, old and, due to natural deaths, the frequency of drop outs is high which handicaps the final conclusions.

Fourthly, there are no accepted standards (“gold standard”) in wound care in the cases outside the scope of the surgical procedures (Gottrup et al. 2010). This makes the selection of reference treatment difficult. The skin wounds and ulcers are treated with manifold of tools and with varying methods, of which there are not much evidence of effectiveness or data of the costs (Iivanainen et al. 2011). In the present RCT, the resin salve treatment was to be compared with the hydrofiber dressing (with or without silver; Ag<sup>+</sup>) treatment. These dressings are widely used in Finland and are considered to be capable, corresponding with the antimicrobial activity of the resin salve, of absorbing bacteria even though the evidence of this antimicrobial ability is sparse (Barnea et al. 2010). In Sweden, more than 60% of the health care centers practicing wound care use the silver (Ag<sup>+</sup>) impregnated hydrofiber dressings (Rosen 2010). At the time of institution of the present RCT (in 2008), the hydrofiber bandage was a generally accepted and widely used treatment option, and was, therefore, chosen as a reference (control) treatment option.

### 5.4.2 DIRECT PHARMACEUTICAL COSTS

The consumer prices, the product sizes and the daily consumption of the wound care products vary greatly which makes it difficult to compare the costs between the different treatment options. To achieve as objective estimation of the expenses of the pharmaceutical material as possible, the costs of these consumables were calculated and estimated individually in every 23 patient in the observational cohort study (paper VI).

The costs of the occlusive and gauze-based dressings commonly used in the wound care have been investigated earlier in one study (Ubbink et al. 2008). In this investigation, the overall costs of the occlusive and gauze dressings per treatment day and per patient were 7.48 €, and 3.98 €, respectively. In our study, the direct costs of the pharmaceutical materials in the resin salve treatment in the present study was  $1.2 \pm 0.5$  € per patient and treatment day. It seems that these daily costs of the resin salve treatment are considerably lower than those with, e.g., medical honey or sodium carboxymethylcellulose hydrocolloid polymer or hydrofiber dressings.

The skin substitute products from cell culture or other special techniques markedly exceed the consumable costs of the ordinary topical wound care items. The costs of the skin substituent was estimated to be over 5,000 € per treated patient in Switzerland (Ortega-Zilic et al. 2010). In a patient case from USA, the total costs of the treatment of stage III-IV ischial pressure ulcer for twelve months with wet-to-dry dressings and for 15 months with biological dressings and negative pressure wound therapy was 183,000 € when extrapolated from the insurance records (Schessel et al. 2012). In these perspectives, the modern dressing therapies, and particularly the resin salve, seem to be inexpensive treatment options which even allow the ulcer care at home.

No analyses on expenses were done in the present RCT. The daily cost of the pharmaceutical consumables in the resin salve treatment is probably quite similar in the pressure ulcer patients and those with chronic surgical wounds. Therefore, it is possible that the direct cost of the pharmaceutical materials in the treatment of pressure ulcers for up to 6 months is cheaper with the resin salve than with the hydrofiber dressings. One may extrapolate that the cost of the consumables, without labour expenses, in the treatment of one pressure ulcer for 6 months was around 250 €. In Germany, the cost of treatment of pressure ulcers for 2 months in a hospital setting was estimated to be more than 6,000 € per patient (Assadian et al. 2011). In a multi-centre study of economics over a period of 8 months in three community hospitals in Germany, the mean cost of treatment of one leg ulcer was 1,342 € (48 € per day), of which the share of the cost attributable to consumables was 458 € (34% of the total cost) (Assadian et al. 2011).

## 5.5 ALLERGY, SIDE EFFECTS AND SAFETY ASPECTS

Resins and rosins occur as ingredients in thousands of products of our everyday life without major health problems (Färm et al. 1994, Krob et al. 2004). In the past, coniferous resins have been used as chewing gums and were even ingested by people and animals (Metsälä 2001).

Allergy to natural resins and rosins (colophony allergy) is, however, a well known side effect of resin salve therapy, and is a result of the exposure of the sensitized patients to the resin acids (Färm 1998). The allergy risk may vary between different types of rosin products and between different resin acids. Colophony allergy is relative rare, but seems to occur among people in patch tests in frequencies that resemble the prevalence rates of allergy to neomycin or lanolin (Uter et al. 2002, Bajaj et al. 2007, Dotterud et al. 2007, Lindberg et al. 2007).

In objective studies, colophony allergy appears in 1–3% of people in general (Färm 1998, Downs et al. 1999). In Sweden, approximately 5% of patients patch tested for dermatitis symptoms in a special dermatology clinic showed allergy to colophony (Färm 1998). In our clinical studys (papers V and VI) the total prevalence

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rate of the allergic contact dermatitis to resin was 2% (95%CI: 0-5%). However, the small number of patients in the trials have to be taken into account when making conclusions.

The tars that are manufactured from the coniferous resins and rosins by heat (pyrolysis), and by distillation, contain carcinogenic compounds (Roelofzen et al. 2007). For this reason the use of tars is prohibited in the EU and tar is considered toxic or carcinogenic. Coal tar was commonly used in the past as a treatment option for psoriasis in the dermatology clinics (Roelofzen et al. 2007). Manufacturing of salve including coniferous resins without heating, they do not contain carcinogens as demonstrated by the present negative Ames tests. Neither are the purified resin acids mutagenic (Nestmann et al. 1979). Both the resin acids and rosins in high concentrations are, on the other hand, toxic to some aquatic organisms, as is the case with pulp and paper mill effluents (Oikari et al. 1982, Peng et al. 2000). Accordingly, the 10% resin salve was cytotoxic against mouse fibroblasts in the present *in vitro* cytotoxicity tests. However, the *in vitro* toxicity was strongly concentration dependent. The cytotoxicity diminished to mild when the salve was diluted to 2.5% (1+3) in its resin content. When used *in vivo* for skin wounds and ulcers, the salve with 10% resin content did not show any adverse effects, indicating that the resin components dissolved in water and tissue fluids are quickly diluted to levels that cause no significant toxic effects.

### 5.6 WHY DOES THE CONIFEROUS RESIN PROMOTE WOUND HEALING?

Although our “resin salve project” gives evidence that coniferous resin salve improves the healing of skin wounds and ulcers, it does not provide ultimate answers as to why this happens.

The optimal wound care product should include a wide-range antimicrobial property, be able to enhance and stimulate epithelialization and the healing processes in a chronic wound, and be inexpensive and safe. Resin salve may fulfill many of these requirements. The salve (and resin) is antimicrobial against a very large range of microbes, and this antimicrobial action certainly is one of the critical factors in healing wounds (Clark 1996, Broughton et al. 2006, Velnar et al. 2009). Furthermore, it is not expected that the salve induces resistant microbial strains as is the case with antibiotics. In addition, the salve also seems to promote the healing of ulcers and wounds that are not clinically infected, suggesting that in the wound care the salve also acts by mechanisms other than by being antimicrobial.

The beneficial effects of coniferous resin in wound care must partly be linked with the physiological processes of wound healing. This conclusion is supported particularly by our observation in the RCT that pressure ulcers without infection also healed with the resin salve. Coniferous resins and the resin salve contain cou-

maric acid (hydroxycinnamic acid) and lignans. Both of these compounds are potentially biologically active and may promote the repair processes (Ferguson et al. 2005, During et al. 2012).

The lignans are “phytoestrogens” which are, after ingestion, by intestinal bacteria metabolized to compounds (enterolactone and enterodiol) with hormone-like properties (Adlercreutz 2007). Such hormonally active metabolites may also therefore be formed from the lignans in skin wounds by actions of the skin bacteria. This may further result in enhancements of the molecular and cellular mechanisms in wound repair: lignans or their active derivates may enhance the synthesis of collagen and hyaluronic acid, or stimulate the re-epithelialization. Such effects would resemble the actions of the estrogen hormones on the aging skin (Jackson et al. 2011, Shu et al. 2011).

Lignans and coumaric acid (hydroxycinnamic acid) are polyphenols and have antioxidative and anti-inflammatory properties (Ferguson 2001, Kangas et al. 2002, Dragsted 2003, Ferguson et al. 2005, Saleem et al. 2005, Adlercreutz 2007, Li et al. 2007, Cosentino et al. 2010, Maurya et al. 2010). Both of these properties are likely to affect the cellular and molecular processes in wound repair. Lignans and coumaric acid may enhance wound healing, e.g., by inhibiting the release of free radicals, reactive nitrogen species (NO), and by inhibiting proteinase expression in the PMNs, by improving the wound debridement and by reducing, thereby, growth factor inhibitions (Salim 1991, Cosentino et al. 2010). Anti-inflammatory agents are reported to enhance wound re-epithelialization, to improve macrophage infiltration and to reduce PMN activity in experimental wound repair models, and they may also diminish wound pain (Jorgensen et al. 2008, McLennan et al. 2008). By possessing anti-inflammatory properties due to the presence of coumaric acids and lignans in high quantity, the coniferous resin and resin salve may enhance the proliferation of fibroblasts and promote the synthesis of fibronectin, collagen I and III, all which are impaired in chronic wounds (Mariggio et al. 2009).

In contrast to the resin acids, coumaric acid and the lignans are water soluble and can reach the tissues in concentrations high enough to arouse significant biological influences. All these possible mechanisms and actions of the lignans and coumaric acid remain, however, speculative.

## 5.7 CHEMICAL COMPOSITION OF SPRUCE RESIN

Coniferous callus resin is a complex mixture of various compounds, of which many may be biologically active. In the chemical analysis of natural resins from varying sources, the resin acids (dehydroabietic, abietic, neoabietic, levopimaric, pimaric, palustric, isopimaric acids, etc.) were observed to be the major components in acetone extracts of the coniferous callus resins, in addition to lignans (lariciresinol, pinoresinol and matairesinol, etc.) and coumaric acid (Holmbom et al. 2008). In

## DISCUSSION

line with these observations, our GLC/MS analyses indicated that the diterpenoid resin acids and lignans together are the major components and occur in 10% resin salve (acetone extracts) in a concentration of more than 30 mg/g (w/w). Accordingly, the concentration of resin acids and lignans together is at least 300 mg/g (w/w) in the crude purified spruce resin. The high quantity of the coumaric acid was a surprise. GLC/MS analyses of the acetone extracts of the resin salves showed that the concentration of coumaric acid in 10% resin salve was around 4 mg/g (w/w), and in crude coniferous spruce resin 40 mg/g (w/w), respectively.

The total mass of resin acids, lignans and coumaric acid was less than half of the total mass of the crude resin. This is in some controversy with earlier analyses which indicate that only the resin acids would form 70% of the total resin mass (Langenheim 2003, Holmbom et al. 2008). The discrepancies may be due to methodological differences, and depend on how the extractions are carried out and also depend on the kind of resin analyzed (pathological vs. physiological resin). Other components than resin acids, such as fatty acids and uncharacterized terpenes or terpenoids, may vary in concentration in resins of different origin or type. In our analysis, however, the concentrations of resins acids, coumaric acid and lignans remain similar and stable in different samples of coniferous “callus” resin from Norway spruce collected in Western Lapland in Finland. In addition, the concentrations of these compounds remained stable and unchanged over many years in the resin salves preserved at room temperature, or even when preserved in stringent environmental conditions (6 months in 40°C).

## **6. CONCLUSIONS**

The author's answers to the study objectives are:

1. The pure coniferous resin from Norway spruce and 10% resin salve manufactured from this resin are antimicrobial against a wide range of bacteria, and fungi.
2. The antimicrobial action is unspecific and results in damage to the cell membranes and walls in the micro-organisms. This damage results in structural and electrophysiological changes in the cell walls and membrane.
3. Allergic reactions are rare and occur in 2% of patients with long-term use (weeks or months). Natural coniferous resin is not mutagenic in Ames tests. Salve is negligibly irritating in the rabbit skin irritation test and is cytotoxic against mouse fibroblasts in the *in vitro* cytotoxicity tests
4. Natural coniferous resin salve is a promising treatment option in the local treatment of pressure ulcers and chronic surgical wounds. It promotes the healing of advanced, severe pressure ulcers more effectively than standard treatment with hydrofiber dressings. In chronic surgical wounds, resin salve treatment positively associates with progressive healing of the wound. The improvement of skin ulcers and wounds is not limited to the healing of infected wounds only, suggesting that the resin has positive influences on mechanisms that play a role in wound repair.
5. The direct costs of the pharmaceutical materials (resin salve and accessories) in resin salve treatment are low: the mean cost per patient per treatment day is  $1.2 \pm 0.5$  €.

### **6.1 ASPECTS FOR THE FUTURE**

Chronic skin wounds and ulcers are a common and increasing problem in our society. The prevalence accumulates with age, their treatment is laborious and costly and the current practices are largely based on subjective impressions of efficacy. In wound infections multiresistance of micro-organisms is an increasing problem. Therefore, new therapeutic options and practices are needed. Resin salve may provide some opportunities in this respect but more controlled studies, knowledge and experience are required to confirm the safety and efficacy of resin salve.

## 7. TIIVISTELMÄ

Sipponen, Arno. **Pihkasalva, vanha mutta edelleen tehokas hoito kroonisiin haavoihin – laboratorio-ja kliininen tutkimus**

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Havupuun, varsinkin kuusen pihkasta valmistettu kotitekoinen salva on ollut käytössä haavojen ja ihoinfektioiden kansanlääkinnässä vuosisatojen ajan. Kirjoittajan omiin positiiviisiin empiiriisiin havaintoihin perustuen käynnistettiin ”pihkasalvaprojekti”, jonka tarkoituksena oli selvittää (1) kuusen runkopihkan (”calluspihka”; resin) ja pihkasalvan antimikroisia ominaisuuksia käyttäen objektiivisia laboratoriottkimusmenetelmiä, sekä (2) tutkia pihkasalvan tehoa, soveltuvuutta ja turvallisuutta haavanhoidossa objektiivisten kliinisten potilastutkimusten perusteella. Väitöskirja koostuu neljästä projektiin kuuluvasta mikrobiologisesta tutkimuksesta sekä kahdesta klinisestä potilastutkimuksesta.

Mikrobiologiset tutkimukset Agar diffuusiotesteissä, European Pharmacopoeia'n challenge testeissä ja mikrobiien kasvatusnesteissä osoittivat, että kuusen pihka, 10%:nen pihkasalva ja puhdistettu hartsihappo (abietiinihappo) olivat voimakkaasti ja laajakirjoisesti antimikroisia varsinkin Gram-positiivisia, mutta myös Gram-negatiivisia baktereereja kohtaan, mukaan lukien metisilliini-resistantti *Staphylococcus aureus* (MRSA) sekä vankomysiini-resistantti enterokokki (VRE). Pihka ja pihkasalva olivat lisäksi vahvasti antifungaalisia yleisimpiä kynsisientä aiheuttavia dermatofyttejä sekä *Candida albicans* hiivasientä vastaan. Pihkan ja pihkasalvan todettiin vaurioittavan mikrobiien soluseinämää ja solukalvoa. Pyyhkäisy- sekä läpäisyelektromikroskooppisten tutkimusten perusteella pihkalle tai pihkasalvalle altistettujen bakteerisolujen seinämät paksuuntuivat, solut muodostivat ryppäitä ja mitoosit keskeytyivät. *Staphylococcus aureus* baktereilla tehdyt elektrofysiologiset tutkimukset osoittivat, että mikrobien altistaminen kuusen pihkalle tai hartsihappolle (abietiinihappo) aiheuttivat solukalvopotentiaalin häviämisen sekä muutoksia soluseinämän rasvahappojen haarautumisessa. Todettujen muutosten tulkittiin viittaavan siihen, että pihkan antimikroinen ominaisuus on seurausta mikrobiien soluseinämään kohdistuvasta vauriosta, jonka vaikutuksesta mikrobiolujen energiatuotanto estyy.

Pihkan turvallisuutta koskevat tutkimukset osoittivat, että pihkalla ja pihkasalvalla ei ole mutageenisiä ominaisuuksia Ames-testin perusteella eikä 10%:nen pihkasalva aiheuta merkittävää ärsytystä (irritaatiota) kanin terveellä iholla tehdys-

sä kontrolloidussa ja sertifoidussa ärsytystestissä. Sertifoidussa *in vitro*- testissä 10%:nen pihkasalva oli fibroblasti- soluviljelmässä sytotoksinen.

Kaasu-neste kromatografia (GLC) sekä massaspektrometritutkimukset osoittivat pihkan ja pihkasalvan (asetoniuutos) sisältävän ainakin kolme biologisesti potentiaalista ainerryhmää: p-kumarihappo, hartsihapot (abietiinihappo, dehydroabietiinihappo, isopimarihappo, jne.) sekä lignaanit (pinoresinoli, larisiresinoli, jne.). Kuusen puhdistetun runkopihkan asetoniuutteessa hartsihappojen ja lignaanien kokonaispitoisuus oli keskimäärin 300 mg/g sekä 10 %:ssa pihkasalvassa 30 mg/g. Pihka sisältää myös rasvahappoja sekä monoterpeenejä, flavonoideja ja tanniineja, joiden pitoisuutta tutkimuksessa ei määritetty.

Satunnaistetussa, prospektiivisessa kliinisessä potilastutkimuksessa verrattiin perinteisen pihkasalvan tehoa gradus II-IV painehaavaa sairastavilla potilailla 11 terveyskeskuksen vuodeosastolla. Pihkasalvaa verrattiin hydrofiberihoitoon (ilman hopeaa tai hopean kanssa) 37 potilaalla. Pihkasalvahoito tai vertailuhoito voitiin toteuttaa 22 potilaalla 6 kk hoitoperiodin kuluessa. Sekä *per protocol* että ITT (intention-to-treat) analyysien mukaan pihkasalva oli tilastollisesti ( $P = 0.003$ ; *per protocol* analyysi; parantuneet haavat) vertailuhoitoa merkittävästi tehokkaampi. Pihkasalvaryhmässä kaikki haavat paraniivat 6 kuukauden hoitojakson aikana yhtä haavaa lukuun ottamatta (94% (95%CI 84-100%)). Vertailuryhmässä alle puolet (36% (8-65%)) haavoista parani kuuden kuukauden hoitojakson aikana. Pihkasalvan tehokkuus haavan paranemisessa oli riippumaton siitä oliko haava infektoitunut vai ei.

Toisessa potilastutkimuksessa (observatiotutkimus) selvitettiin 10%:sen pihkasalvan tehokkuutta sekä käytettävyyttä 23 kroonista, komplisoitunutta leikkaushaavaa (avoimen haavan pituus  $29 \pm 19$  mm; vaihteluväli 5-60 mm) sairastavalla potilaalla kotihoitona/itsehoitona. Kaikki haavat paraniivat keskimäärin  $43 \pm 24$  päivää. Haittavaikutuksia ei todettu ja hoitoa ei jouduttu lopettamaan yhdelläkään potilaalla. Laskennallinen haavaan paranemisnopeus (re-epitelisaatio) oli keskimäärin 2 mm päivässä.

Potilastutkimukset (satunnaistettu tutkimus ja observatiotutkimus; *per protocol*) kohdistuivat yhteensä 45 potilaaseen, joista yksi (2%; 95%CI: 0-5%) potilas satunnaistetussa painehaavatutkimuksessa sai allergisen reaktion (allerginen kontaktidermatiitti). Muita allergisia reaktioita tai haittavaikutuksia ei tutkimuksissa todettu. Pihkasalvahoidon hoitolarvikkeiden (pihkasalva, sidetaitokset, ym.) hintaksi observatiotutkimuksessa komplisoituneissa leikkaushaavoissa laskettiin  $1.2 \text{ €} \pm 0.5 \text{ €}$  per hoitopäivä.

Tutkimukset osoittavat, että kuusen pihkasta valmistettu salva on objektiivisesti arvioituna tehokas, halpa ja turvallinen hoito ihohaavoissa. Pihka ja 10%:nen pihkasalva, ja niiden sisältämät hartsihapot, ovat laajakirjoisesti antimikrobisia, mutta salvan haavojen paranemista nopeuttava vaikutus täytyy johtua potilastutkimusten perusteella myös pihkan positiivisesta vaikutuksesta haavan paranemista ohjaaviin mekanismiin, ei pelkästää pihkan antimikrobisesta ominaisuudesta.

## 8. ZUSAMMENFASSUNG

Sipponen, Arno. **Harzsalbe, altbewährte und effektive Behandlung chronischer Wunden - Labor- und Klinikstudien**

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Natürliche Nadelbaumharze und andere terpenische Holzextrakte werden als Rohstoffe in verschiedenen Industrieprodukten verarbeitet und in Finnland schon seit hunderten von Jahren in der traditionellen Medizin verwendet, vor allem als hausgemachte Salben zur Behandlung von Hautverletzungen, chronische Wunden und Infektionen. Aufgrund der positiven empirischen Erfahrungen des Autors mit Harzsalbe in der Wundpflege wurde das "Harzprojekt" entwickelt, um (1) die antimikrobiischen Eigenschaften des Harzes und der Harzsalbe mittels labortechnischer Verfahren zu erforschen und (2) die Effizienz, Anwendbarkeit und Sicherheit der Harzsalbe zur Wundpflege in objektiven klinischen Studien zu testen. Die Dissertation besteht aus vier mikrobiologischen Untersuchungen und zwei klinischen Studien.

Mikrobiologische Studien mit einem Agardiffusionstest und mit dem Provokationstest des Europäischen Arzneibuchs (European Pharmacopoeia challenge test) zeigten, daß Harz und Harzsalbe starke antimikrobiische Eigenschaften gegenüber einer Vielzahl von sowohl Gram-positiver, als auch Gram-negativer Bakterien aufzeigten, darunter auch das methicillinresistente *Staphylococcus aureus* (MRSA) und die vancomycinresistente *Enterococcus* (VRE). Das Harz und die Harzsalbe waren darüber hinaus antifungal gegenüber den gängigsten Dermatophyten, sowie gegen die *Candida albicans*-Hefepilz. Die antibakterielle Wirkung des Harzes wurde als Ergebnis der Zerstörung der bakteriellen Zellwand- und Zellmembranstrukturen beobachtet. Laut Beobachtungen durch Scanning- und Transmissionselektronenmikroskopie zeigten Mikroben, sowohl Bakterien, wie auch Pilze, die dem Harz oder der Harzsalbe ausgesetzt wurden, eine Verdickung der Zellwände, Bildung von Zellaggregate und Hemmung von Mitosen. In elektrophysiologischen Experimenten mit *Staphylococcus aureus* verursachte die Aussetzung von Mikroorganismen gegenüber Harz und Harzsäure (Abietinsäure) die Auflösung des Zellmembranpotentials und veränderte die Ramifikation von Fettsäuren in den Zellwänden. Aus den beobachteten Veränderungen wurde der Schluss gezogen, daß das Harz und die Harzsalbe durch den Schaden an den Zellmembranfunktionen Einfluß auf das Überleben von Bakterienzellen nimmt.

In Versuchen im Hinblick auf Sicherheitsaspekte sind Fichtennadelharz oder Harzsalbe in Ames-Tests nicht mutagen und die 10%ige Harzsalbe hat keine be-

deutende reizende (irritative) Eigenschaft laut kontrollierten Hautreizungstests und Irritationstests. Bei zertifizierten *in vitro* Tests mit Fibroblastenzellkultur erwies sich die 10%:ige Harzsalbe als zytotoxisch.

Gas-Flüssigkeits-Chromatographie (GLC) und Massenspektrometrie- Studien zeigten, daß Harz und Harzsalbe (Acetonextrakte) mindestens drei biologisch potente Stoffgruppen enthalten: p-Cumarsäure, Harzsäuren und Lignane. Diese Stoffe treten insgesamt mit einer durchschnittlichen Konzentration von 300 mg/g in Harz (w/w) und 30 mg/g (w/w) in Harzsalbe auf. Zusätzlich enthält Harz Fettsäuren, Monoterpane, Flavonoide und Tannine, von denen keine Konzentrationsberechnungen durchgeführt wurden.

Einer der klinischen Studien ist eine randomisierte, vorausblickende Untersuchung, bei der die Wirksamkeit von Harzsalbenbehandlung über einen Zeitraum von 6 Monaten mit der Wirksamkeit von Hydrofaserband (mit oder ohne Silber) an 37 Patienten mit schweren, Grad II-IV, Druckgeschwüren verglichen wurde. Bei diesem Versuch erwies sich die Harzsalbe sowohl laut Protokoll-, als auch laut ITT (intention-to-treat)- Analyse als deutlich wirksamer ( $P=0.003$ ) bei der Verbesserung der Geschwürsheilung (sowohl im Hinblick auf die Heilungsrate pro Patient ( $N=23$ ), als auch pro Geschwür ( $N=29$ )) als die Kontrollbehandlung. Alle Druckgeschwüre der Patienten (laut Protokollanalyse) in der Harzgruppe heilten, mit Ausnahme eines Geschwürs bei einem Patienten (94% (95%CI 84-100%)), wohingegen weniger als die Hälfte (36% (8-65%)) der Geschwüre in der Kontrollgruppe heilten. Harzsalbe verbesserte den Heilungsprozess von Geschwüren unabhängig davon, ob das Geschwür infiziert war oder nicht.

Die andere klinische Studie war eine beobachtende und vorausblickende Untersuchung zur Wirksamkeit und Anwendbarkeit der Harzsalbenbehandlung bei chronischen, komplizierten Operationswunden in einer Kohorte bestehend aus 23 Patienten. Bei allen 23 Patienten heilte die Wunde durchschnittlich innerhalb von  $43 \pm 24$  Tagen ohne jegliche Nachteile. Bei der Harzsalbentherapie heilten die Wunden schrittweise (re-epithelisiert), im Durchschnitt 2 mm pro Tag.

In den klinischen Studien (randomierte Studie und Observationsstudie; *per protocol*), die insgesamt 45 Patienten umschlossen, wies ein Patient (2%; 95%CI: 0-5%) eine allergische Reaktion auf (allergische Kontaktdermatitis). Es wurden keine anderen Komplikationen oder Nebenwirkungen beobachtet. Die direkten Kosten für pharmazeutisches Material waren durchschnittlich  $1,2 \text{ €} \pm 0,5 \text{ €}$  pro Tag pro Patient, welche mit der Harzsalbe behandelt wurden.

Die Studien zeigen, daß die Harzsalbe objektiv betrachtet effektiv, günstig und gefahrlos in der Behandlung von Hautwunden ist. Harz und die 10%:ige Harzsalbe sind gegen eine Vielzahl von Mikroorganismen antimikrobiisch, aber der positive Einfluß der Salbe auf die Heilungsgeschwindigkeit der Wunde, muß auf andere Mechanismen zurückzuführen sein als auf die Antimikrobität alleine.

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## 10. CONFLICTS OF INTERESTS

Arno Sipponen is a shareholder of Repolar Oy. The company was founded in 2006 to develop and market coniferous resin-based products for medical practice.

This thesis also includes unpublished data.

## 11. APPENDICES

### 11.1 Appendix I.

Descriptions of the terms commonly used in the present papers

Terms resin, rosin, colophony, pitch, etc. are widely used confusingly in the literature and in the labels of various natural or industrial products. The terminology is not fully established, and the terms are often used intermixed without definitions and, therefore, may cause misunderstandings. In the present context, the definitions are:

**Resin:** Resin is a natural hydrocarbon secretion of many plants, especially of coniferous trees like the spruce and the pine. The resin is a natural product and is a mixture of resin acids (e.g., abietic acid, hydroxyabietic acid) with other natural terpenic compounds and fatty acids. In the present papers, the term “resin” refers to the coniferous non-physiologic “callus” resin from the Norway spruce (*Picea abies*).

**Rosin and colophony:** Rosins are manufactured from the resin by fractional distillation to vaporize the volatile terpenes and terpenoids, resulting in products that are composed practically of only the resin acids. Thus, the rosins can be considered technical and chemical products originating from the natural resins or wood extracts (e.g., tall oil). Rosins may also be products that are mixtures or derivates of various chemically purified natural resin acids. Rosins are used in manufacturing a wide range of different products, e.g. varnishes, adhesives, cosmetics, paints. Occasionally, the term colophony is used as a synonym for rosin.

**Resin acid:** Resin acids (e.g.; dehydroabietic, levopimaric, pimaric, palustric, isopimaric, sandracopimaric, abietic acid and neoabietic acids) are specific tricyclic and diterpenic organic acids in both resins and rosins.

**Lignans:** Lignans (e.g.; lariciresinol, pinoresinol and matairesinol) are, in addition to the resin acids, a group of ingredients of the natural non-physiologic callus resin. They do not exist in the technical rosins and oleoresins. Lignans are water soluble and are metabolized by enteric bacteria to enterolactone and enterodiol. Lignans have been used as food additives.

**Coumaric acid** (hydroxycinnamic acid; hydroxy derivate of cinnamic acid): A component in natural wood extracts. Coumaric acid is considered to have antioxidative properties. Its exact biological role is unclear. Coumaric acid is found, besides in conifers, in different plants like carrots, tomatoes and garlic, or in wine and vinegar.

**Terpenes and terpenoids:** Terpene hydrocarbons are derived biosynthetically from the isoprene ( $C_5H_8$ ) units. They form of a large group of dissimilar

compounds in the plant extracts, some of which are volatile, and some of which are not. When the terpenes are modified chemically (e.g., methyl groups are modified, or oxygen atoms are added) the resulting compounds are generally referred to as terpenoids. Some authors will also use the term terpene to include all terpenoids. Terpenes are classified by number of terpene units in the molecule. Diterpenes are composed of four isoprene units (molecular formula  $C_{20}H_{32}$ ). The resin acids are nonvolatile diterpenes. Steroids and vitamin A are other examples of terpenes.

## 11.2 Appendix II.

### Wound care products and dressings

In Finland there are six proprietary medicinal products available and authorized for the treatment of wounds and ulcers:

- Products containing dexamethasone as the effective agent. Dexamethasone turns into panthenol in the skin and mucosal tissue. It is considered to have positive influence on re-epithelialization and collagen durability.
  - » Bepanthen (marketing authorization holder: Bayer)
  - » Bepanthen (Orion)
  - » Dexpanthenol (Ratiopharm)
- A product containing platelet-derived growth factor (PDGF) beclapertin. It is intended for the treatment of neuropathic and ischemic diabetic wounds extending into subcutaneous tissue. An increased risk of manifestation of malignancy has been observed in patients treated with beclapertin.
  - » Regranex (Janssen-Cilag)
- Product containing clostridiopeptidase A. Clostridiopeptidase A is only active in a moist milieu, and as an enzymatic agent it is indicated for enzymatic debridement of necrotic wounds.
  - » Irixol and Irixol mono (Smith & Nephew)

Many types of dressings are available in Finland in 2012 for the wound care. The recommendations and properties are listed below, as presented by the manufacturers and as collected by Iivanainen and Seppänen (2011). It is noteworthy that most of the properties mentioned in the list have not been scientifically examined and are only the opinions of the commercial manufacturers.

#### **Passive dressings**

These dressings (gauzes) protect the wound and do not contain any active components that might influence the wound healing. The occlusive gauzes can be air- or watertight and they provide a seal from the environment.

## **Interactive dressings**

The interactive dressings contain properties considered to actively influence the wound healing processes. These dressings can be separated into different categories:

**Dressings containing active carbon:** The dressings remove bad odor from the wound and can be used, for example, in infected and malignant wounds.

**Alginate dressings:** The dressings contain brown alga that will change to a gel in contact with wound secretions. The dressings are considered to have autolytic and haemostatic properties. They can be used in wounds with moderate or heavy exudation.

**Silver dressings:** The silver impregnated dressings have antimicrobial properties and recommended to be used in infected wounds. Silver has antimicrobial properties but is reported to induce antimicrobial resistance.

**Hydrofiber dressings:** The dressings compose of sodiumcarboxymethylcelulose with a high absorption capacity. They can be used in wounds with moderate or heavy exudation. Hydrofiber dressings will change to a gel in contact with wound exudates. The dressings are considered to have autolytic properties.

**Hydrophobic dressings:** These are dressings with hydrophobic property. Microbes are considered to adhere to the dressing and are removed when the dressing is changed.

**Hydrogels:** The hydrogels contain abundant water and the dressings moisturize the wound permitting autolytic debridement.

**Hydrocolloids:** Hydrocolloid dressings moisturize the wound permitting autolytic debridement. These dressings are not recommended to be used in infected wounds.

**Polyurethan dressings:** Polyurethan dressings are recommended in superficial wounds like abrasions, burn wounds and surgical wounds. The dressings have no absorption capacity.

**Zink dressings:** Zink dressings are designed for venous leg ulcers below the compression bandages. Zink protects from excessive moisture.

**Resin dressings:** A salve with antibacterial, antifungal, and with debridement properties and recommended to be used in skin wounds when impregnated into an appropriate dressing, or by spreading the salve onto the wound directly and then covering the wound with an appropriate dressing or band. The salve enhances re-epithelialization.

### 11.3 Appendix III.

#### Microbial strains used in microbiological studies

	Microbial strain
<b>Gram-positive cocci</b>	
<i>Staphylococcus aureus</i>	ATCC 25923
<i>Staphylococcus aureus</i>	ATCC 6538
<i>Staphylococcus aureus (MRSA)</i>	NEQAS 4937/98
<i>Staphylococcus aureus (MRSA)</i>	ATCC 43300
<i>Staphylococcus epidermidis</i>	ATCC 49461
<i>Enterococcus faecalis</i>	ATCC 29212
<i>Enterococcus faecalis (VRE)</i>	EARSS UA605/01
<i>Enterococcus faecalis</i>	EARSS UA1527/01
<i>Streptococcus pyogenes (A)</i>	ATCC 19615
<i>Streptococcus agalactiae (B)</i>	NEQAS 6098/01
<b>Gram-positive rods</b>	
<i>Arcanobacterium haemolyticum</i>	LABQ 237/95
<b>Gram-negative rods</b>	
<i>Escherichia coli</i>	ATCC 25922
<i>Escherichia coli</i>	ATCC 10538
<i>Enterobacter cloacae</i>	ATCC 23355
<i>Klebsiella pneumoniae</i>	ATCC 13883
<i>Proteus vulgaris</i>	ATCC 8427
<i>Proteus mirabilis</i>	ATCC 12453
<i>Pseudomonas aeruginosa</i>	ATCC 27853
<i>Pseudomonas aeruginosa</i>	ATCC 15442
<i>Bacillus subtilis</i>	ATCC 6633
<b>Dermatophytes</b>	
<i>Trichophyton rubrum</i>	ATCC 28188
<i>Trichophyton rubrum</i>	Clinical isolate 05PI5613
<i>Trichophyton mentagrophytes</i>	ATCC 9533
<i>Trichophyton tonsurans</i>	Lab quality strain 426/2000
<b>Yeasts</b>	
<i>Candida albicans</i>	ATCC 90028
<i>Candida albicans</i>	ATCC 10231
<i>Candida glabrata</i>	NL 2238
<i>Candida krusei</i>	ATCC 6258
<b>Opportunistic fungal species</b>	
<i>Fusarium solani</i>	NEQAS 5204/1999
<i>Chrysosporium keratinophilum</i>	Clinical isolate 05PI5899