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Internal Medicine / Dermatology and Venereology

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**Influence of *Myrtus communis* extracts
on keratinocyte barrier**

Dissertation

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Chapter 1

Introduction, Objectives and Hypothesis

1. Introduction

1.1 Skin and appendages

The skin is the largest organ in the body. In a 70-kg individual, the skin weighs over 5 kg and covers a surface area of approximately 2m². It also contains millions of nerve endings, and it has the ability to re-build and repair any damage itself. Its structure and functions are vital in maintaining body homeostasis, temperature control, and as a protective barrier for the body. Skin is composed of three layers; the epidermis which is the outer layer, the dermis, and the subcutis. The epidermis is typically 0.05-1 mm thick and contains cells that differentiate from the basal layer to the surface. The cellular progression from the basal layer to the skin surface takes about 30 days, and during this process, cells lose their nucleus and change shape. This layer is rather thick on the palms and soles. The epidermis contains no blood vessels, and it is nourished by blood vessels in the dermis. Dermis is thicker than epidermis and contains: connective fibers, muscle tissue (adhered to hair follicles), blood vessels, lymphatic ducts, glands and nerves. Connective tissue protects the epithelium and enables movement of the skin over the organs. The subcutis is a layer of tissue which lies immediately below the dermis of vertebrate skin. It is often referred to as subcutaneous tissue, though this is a less precise and anatomically inaccurate term. The subcutis consists primarily of loose connective tissue and lobules of fat. It contains larger blood vessels and nerves than those found in the dermis (Burns et al. 2012).

The epidermis consists of four layers. In the first layer (the basal layer), cells are undifferentiated, and it includes stem cells that are capable of division and proliferation (Strachan and Ghadially 2008). There are many layers of spinous cells (stratum spinosum) above the basal layer, which are identified by desmosome adhesions and spindle shapes caused by these adhesions. In addition, these cells express the early differentiation marker cytokeratin 10; cells in this layer are not homogeneous and differentiation occurs from the bottom upwards. For instance, this can be seen by the presence of the intermediate differentiation marker involucrin which is absent in the lower cells

of stratum spinosum, but can be found in the upper cells. Next, there is the granular layer (stratum granulosum) which contains 3-5 layers. In this layer, cells contain lamellar bodies and keratohyalin granules (indicator of granular layer in light microscope). Granular layer cells are responsible for the expression and processing of final differentiation markers; filaggrin and loricrin. Finally, the outermost epidermal layer (or the cornified layer) consists of dead cells and intracellular lipids. The cornified layer is an important part of the skin's defense barrier (Hashimoto 1971, Rothman and Flesch 1944).

Other cells in the epidermis are the melanocytes, Langerhans cells and Merkel cells. Melanocytes are dendritic cells that distribute packages of melanin pigment in melanosomes to surrounding keratinocytes to give skin its colour. The number of melanocytes does not differ much between white and black skin. Rather it is the nature of the melanin and the size of the melanosomes that account for the different appearances. The Langerhans cells are also dendritic in nature, although these are of mesenchymal origin and originate from bone marrow. The Langerhans cells are antigen-presenting cells and process antigens encountered by the skin to local lymph nodes and thus have a key role in adaptive immune responses in the skin. Merkel cells are probably derived from keratinocytes. They have, among others, a role as mechanosensory receptors in response to touch (Burns et al. 2012).

1.2 Tight junctions

Tight junctions (TJ) are intracellular connections, which are important for the trans-cellular distribution of water and minerals, and barrier-building against pathogens. They create cell polarity by creating a barrier between the upper and lower parts of the lateral plasma membrane (Hashimoto 1971, Rothman and Flesch 1944). Tight junction proteins are involved in different functions, including barrier function, cell proliferation, and differentiation (Figure 1.1). In transmission electron microscopy, TJs can be seen as so called, "kissing points," as the membranes of neighbouring cells are very close without any intermembraneous material between them (Figure 1.2)

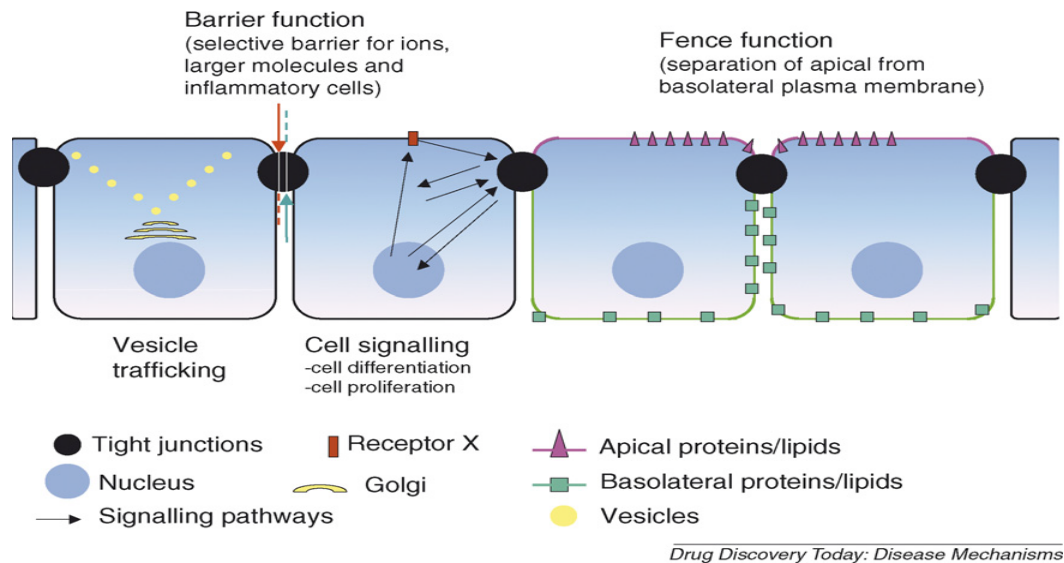


Figure 1.1 Schematic drawing of TJs in simple epithelial cells denoting the various functions of TJ (Brandner et al. 2008).

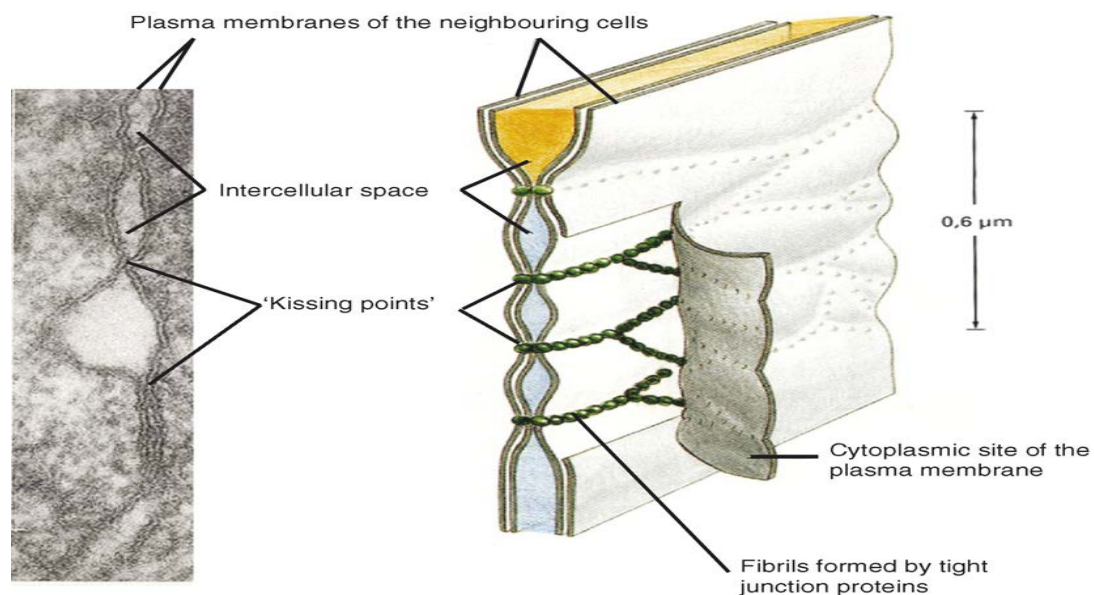


Figure 1.2 Structure of tight junctions. (a) Transmission electron microscopic picture showing the typical TJ kissing points. (b) Schematic drawing of TJs with TJ strands formed by TJ proteins (Alberts et al. 1995).

1.2.1 Molecular composition of tight junctions (TJs)

In recent years, many studies have been conducted to identify mechanisms affecting selective paracellular dispersion, which has led to further understanding of the molecular composition of TJs (Furuse 2010). A tight junction is formed by; transmembrane proteins of the claudin family, TAMPs

(TJ associated MARVEL proteins, e.g. occludin), and junctional adhesion molecules (JAMs), as well as cytoplasmic connection proteins, including: ZO-1-3, MUPP-1, and cingulin (junction plaques) (Figure 1.3) (Aijaz et al. 2006, Schneeberger and Lynch 2004). Transmembrane proteins are connected to the actin cytoskeleton via cytoplasmic proteins (Furuse 2010). Transmembrane proteins (claudin and occludin) have an essential role in creating a semipermeable barrier, while cytosolic proteins not only connect components of the membrane and membrane processes to the cytoskeleton, but they are also involved in signaling between the TJs and the cell nucleus (Figures 1.1) (Umeda et al. 2006).

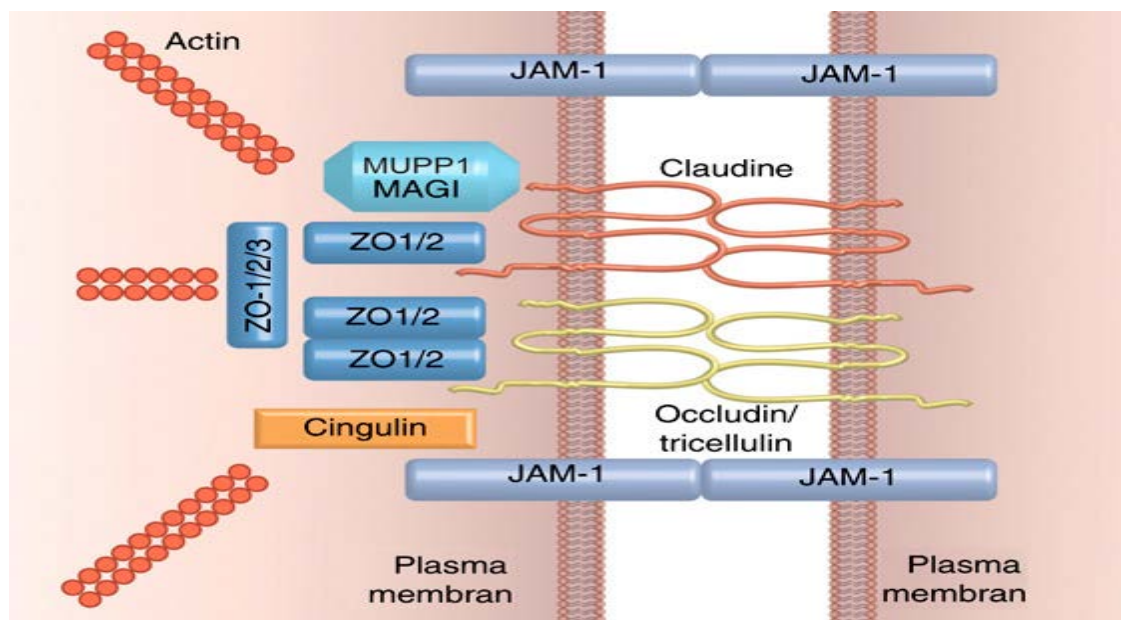


Figure 1.3 Principal components of tight junctions. ZO-1 and ZO-2 are important for the assembly of claudin and occludin, which lead to the formation of TJ strands. The plaque proteins ZO-1-3 and cingulin connect to the actin cytoskeleton (Niessen 2007).

1.2.2 Main components of TJs

A) TAMPs

Occludin

The most well-known member of the TAMP family is occludin. This protein is found in both epithelial and endothelial TJs (Niessen 2007). It was the first integral membrane protein recognized in TJs (Furuse et al. 1993). Even

though it has been shown that a high expression of occludin induces increased transepithelial electrical resistance (Karnaky 1992), it has also been demonstrated that the absence of occludin in occludin knock-out mice does not have an effect on barrier function (Saitou et al. 2000). Hence, the physiological function of occludin in TJs is still unclear.

B) Claudins

Claudins comprise a large family of proteins, which include a minimum of 24 homologous members in humans. They are important for the formation and selectivity of the paracellular barrier, especially for ions. Lack of claudin 16 causes structural disorders, which leads to impaired renal reabsorption of calcium and magnesium and as a result to hypomagnesia and hypercalciuria. Polymorphism in cld-11 and cld-14 cause inherent deafness, and in cld-11 it results in loss of myelin in the central nervous system and impotence in men induced by loss of TJ resistance in the sertoli cells.

A lack of cld-1 genes in humans results in sclerosing cholangitis, and this is associated with ichthyosis, which is a severe and chronic disease. These patients also suffer from erythema and alopecia areata (Rabia et al. 2004).

1.2.3 Tight junctions and tight junction proteins in the skin

TJ proteins are also found in mammalian skin. However, while the distinct tight junction structure, known as the, “kissing point,” is limited in the granular layer, the pattern of tight junction proteins located in the epidermis is much more complicated. Some tight junction proteins, including JAM-A, Cldn-1, Cldn-7, and MUPP-1 exist in all living epidermal layers, while some, including occludin, are only present in the granular layer and ZO-1 and Cldn-4 are restricted to the upper epidermal layers (Figure 1.4 and 1.5) (Brandner et al. 2002, Brandner et al. 2006, Pummi et al. 2001). In light microscopy, the Straum corneum (SC) is negative for TJ proteins, but Cldn-1 and Ocldn were detected in the lower layers of SC by electron microscopy. Therefore, it is likely that other TJ proteins, which have not yet been investigated by EM, are also present in the SC.

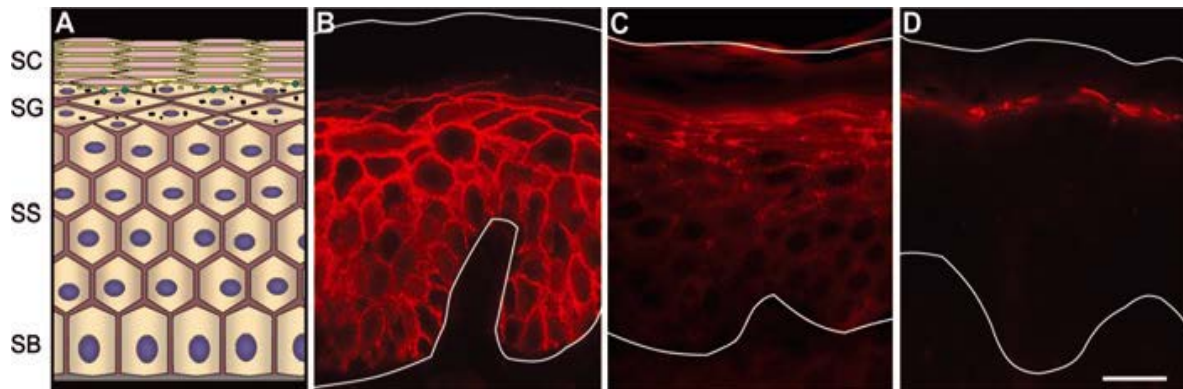


Figure 1.4 Representative localization patterns of TJ proteins in human epidermis. (A) Schematic overview of human epidermis. Typical tight junction (TJ) structures are shown as green dots. (B–C) Epifluorescence pictures of Cldn-1 (B), ZO-1 (C), and occludin (D) as examples of TJ proteins present at the cell–cell borders in all living layers (B), in the upper layers (C), and in the SG (D); bar 20 µm; white lines indicate the boundaries of the epidermis (Kirschner and Brandner 2012).

In 2002, Furuse et al. used the accepted Chen et al. (Chen et al. 1997) method to show that amine-reactive biotinylation reagent (557 D) injected into the dermis of newborn mice spread into the epidermis through intercellular passageways, and that it comes to a halt in granular layers where the TJ proteins, occludin and claudin-1, are located. In claudin-1 knock-out mice this interruption was no longer present. This finding was the first description of the TJ barrier in mammalian skin. In 2010, it was shown that this TJ barrier is not only present in newborn mouse skin, but it is also found in adult human skin (Figure 1.5) (Kirschner et al. 2010). These results confirmed the early studies of Hashimoto (Hashimoto 1971) on human epidermis that showed the presence of a barrier for the ion lanthanum in the granular layer. However, other researchers (Feldmeyer et al. 2006) attributed this barrier to deposits of extracellular lipids.

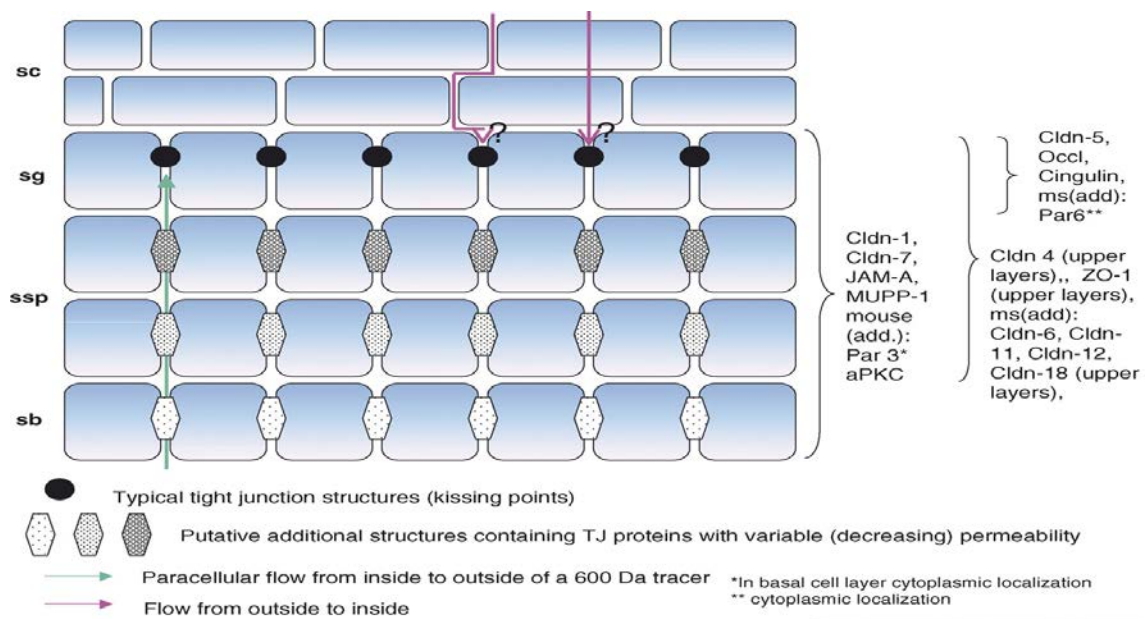


Figure 1.5 Schematic drawing of the epidermis showing localization of typical TJ structures as well as TJ proteins.sc: stratum corneum, sg: stratum granulosum ssp: stratum spinosum, sb: stratum basale. add: additional, ms: mouse (Brandner et al. 2008).

1.2.4 Tight junctions in cultured keratinocytes

Much of the data about the formation and maintenance of TJ barrier function in keratinocytes has been gathered from cultured cells. Calcium is an important inducer of TJ formation in keratinocytes. An increase in extracellular Ca^{2+} concentration causes the formation of cell to cell contacts and cell differentiation (Schneeberger et al. 1992), and this results in a continuous localization of TJ proteins at the cell–cell borders (Kirschner and Brandner 2012). In addition it leads to the formation of a barrier for ions resulting in transepithelial resistance (TER) (Kirschner et al. 2010). This TER is mainly formed by TJ-based paracellular resistance and only a minor part is attributed to membrane-based transcellular resistance (Kirschner et al 2013). Furthermore, tight junctions in keratinocytes form a barrier to water and intermediate-size and larger molecules (Kirschner et al 2013). Cell polarity complex Par3/Par6/aPKC is responsible for the formation of a TJ barrier

(Kirschner and Brandner 2012). During cell to cell contact formation in cultured keratinocytes, first primordial junctions form, these include tight and adherens junction proteins and they are essentially found in the basolateral part of the plasma membrane. Calcium plays an important role in this stage, since adherence of the cells initially depends on mediation of the calcium-dependent adherens junction protein E-cadherin (Kirschner et al. 2009). TJs or TJ proteins influence differentiation in keratinocytes (Kirschner and Brandner 2012, Rachow et al. 2013). Downregulation of Cldn-1 in human cells can cause an increase in keratinocyte proliferation (Benedetto et al. 2011).

1.2.5 TJs and skin infection

The skin is one of the main targets in microbial attacks. For an infection to occur, bacteria must enter the body through the stratum corneum, and then through the layers of living epidermal cells, by paracellular or transcellular routes. An investigation was conducted to clarify the role of TJ proteins during an infection with skin pathogens (*S. aureus*). A cell line of keratinocytes and infected pig skin were examined in vitro, and it was found that claudin and ZO-1 levels are downregulated due to *S. aureus* infection. In addition, tests have indicated that a total loss of PKC typically plays a key role in the influence of *S. aureus* on TJs. Interestingly, early infection with *S. aureus* as well as colonization of the skin by *S. epidermidis* resulted in the increased expression of TJ proteins and, for *S. epidermidis*, as well as in an increase of TER. These results indicate possible TJ assistance with the skin barrier function during a skin infection (Ohnemus et al. 2007).

Toll-like receptor 2 (TLR2) is important for immune responses to a number of microbes, such as *Staphylococcus aureus* and herpes simplex virus and it can also enhance TJ function in cultured keratinocytes (Yuki et al. 2011, Kuo et al. 2012). TLR2 synthetic agonists (Pam3CSK4 and Malp-2) also enhance the TJ protein expression. Kuo et al. found that mRNA expression of claudin-1, claudin-23, occludin, and ZO-1 was significantly induced after 24 hours of stimulation with confluent primary human keratinocytes in the presence of *S. aureus*-derived peptidoglycans (Kuo et al. 2012).

1.3 Myrtus (*M. communis*)



Figure 1.6 *M. communis*

1.3.1 Botanical background, history and general utilization

M. communis (botanical name), with the common name Myrtle, is an ancient sacred plant which was used in ceremonies and as a symbol of youth and beauty. It has been known to the Persians, Greeks, and other civilized nations since ancient times and is native to South America, Australia, north-western Himalayas, Middle East, West Asia, North Africa, and Zagros mountain range regions but was first introduced to the world by the British in 1597. *M. communis* grows in mediterranean countries like Italy, along the coastline and inland hills, and in Turkey, in spruce and pine forests and near riversides, and especially around the Taurus Mountains, and it is known by such names as: Hambeles, Mersin, and Murt. In Iran, Myrtus grows in semi-humid and steppe regions with a mild climate. It is found in abundance in Lorestan, Khorasan, Fars (especially around Lake-Maharlou), and Kerman, Bandar Abbas and Yazd heights (Asif et al. 2011, Akin et al. 2010, Zanneti et al. 2010, Gerbeth et al. 2011, Aydin and Özcan 2007, Annis remedy 2012)

M. communis is a small shrub of the Myrtaceae family, which can grow as tall as 5m (15 ft). It has ever-green, opposing, sharp, leathery, dark green, and fragrant 3 to 5 cm leaves (sweet camphor-like smelling herb), with erect, branched, and numerous gray stems. The relatively large and beautiful

flowers of *M. communis* are white with a unique fragrance, and they blossom between April and June in Iran. *M. communis* fruit are bluish-purple edible berries, with several seeds (Asif et al. 2011, Begum et al. 2012).

The leaves contain 1.5 to 2% (by volume) of a pale yellow oil; if dried in the shade, they become dark with a pungent odor, and if dried in the sun, they turn yellow and develop a mild scent (Asif et al. 2011, Akin et al. 2010, Begum et al. 2012).

Myrtle essential oil is obtained through steam distillation of the myrtle plant's; flowers, leaves and stem. Its main constituents are 1, 8-cineole, myrtenol, pinene, myrtenyl acetate, geraniol, Linalool, camphene and borneol. α -pinene and 1,8-cineol (eucalyptol) together represent around 86% of the oil content (Curini et al. 2003).

M. communis has evidence-based medicinal properties such as: antimicrobial, antiinflammatory, antioxidant, antidiabetic, anticarcinogenic, antimutagenic, antihyperglycemic, antihemorrhagic, antiparasitic and antifungal properties, which have been associated with its important chemical compounds such as; α -pinene, limonen, linalool, α -terpineol, carophyllin and 1,8-cineole (Teseng et al. 2005, Zeng et al. 2011, Messaoud and Boussaid 2011, Sepici et al. 2004, Elfellah et al. 1984, Al-Saimary et al. 2002, Bonjar 2004).

Moreover, myrtle contains oligomeric, nonprenylated acylphloroglucinols such as myrtucommulone and semi-myrtucommulone, which are considered to be responsible for the antioxidative and antibacterial activities of myrtle preparations (Appendino et al. 2002, Rosa et al. 2003). This plant was also used in bronchitis infections and urogenital problems in the 19th century (Annis remedy 2012, Begum et al. 2012).

Mansouri in 1999, conducted research to find new antibacterial agents that were effective against *Staphylococcus aureus* isolated from healthy carrier (nose or throat) or clinical samples. From 10 plants screened for their antibacterial activity, *M. communis* (leaves) had the greatest activity, inhibiting the growth of 99% of isolates (Mansouri 1999). This was confirmed in another study in Iran in which a hydroalcoholic extract of myrtle herb leaves was evaluated at four different concentrations including 10-80 mg/ml on four

strains of pathogenic bacteria. The results showed that the extract of *M. communis* leaves has the greatest antibacterial effect against *S. aureus* and *Vibrio cholerae* serotype Ogawa (Taheri et al. 2013).

In addition, a study in Iran also showed that myrtle essential oil (*M. communis*) is very active against *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenza*, in vitro in disk diffusion tests (Yazdi et al. 2008). *M. communis* has antifungal activities too. The essential oil of *M. communis* exerted a 60% growth inhibition against *R. solani* at a dose of 1600 ppm (Curini et al 2003).

1.3.2 *Myrtus communis* modern medicinal forms

Today, *M. communis* is used in various forms.

Myrtoplex cream contains 10% extract of the leaves of *M. communis* and it is used to treat herpes simplex type 1, 2. The main ingredients of this cream are tannins, polyphenols and volatile oils, such as cineole and flavonoids.

Myrtex solution contains 10% extract of the leaves of *M. communis* with a final concentration of 9 mg/ml, 8-cineole and it is used for minor aphthosis and herpes simplex. The main ingredients of this solution are essential oils such as; 1, 8-cineole, α -pinene, myrtenol, limonene, and myrtol. Myrtlx® is another medical form of *M. communis* which contains 10% oil (see Materials) and it is used for the same indications as Myrtex.

Dineh inhaler powder (a 50 gram pack) containing 20% *Foeniculum vulgare*, 30% *M. communis*, 15% *Thymus* sp, and 5% *Matricaria chamomilla*, is used to relieve the symptoms of; colds, nasal congestion, cough, and improve acne. The main ingredients of this powder are cineole, citronellol, thymol and flavonoid quercetin.

Rectol, made by Khorraman Laboratories, contains *M. communis* 5%. It is used for pain relief and in the treatment of hemorrhoids. Rectol acts by affecting myelinated nerves, with anti-itch and pain relief (analgesic) effects, which significantly reduces hemorrhage and inflammation after 72 hours.

Myrtex topical lotion which is standardized based on 30 mg 1, 8-cineol in each ml and contains essential oil of *M. communis* with α -pinene, 1, 8-cineol (Eucalyptol), limonene, α -terpineol, and linalyl acetate, is used to treat

hemorrhoid symptoms like; bleeding, pain, itching, cramping and retention in the rectum, tenesmus, and anal fissures.

Belfarol, *M. communis* oil lotion (myrtol) in 5% almond oil, with anti-fungal and antibacterial properties (especially against gram-positive bacteria), is used to strengthen eyebrows and eyelashes, and as anti-dandruff and anti-inflammation of the eyelids, and it is found in Iranian pharmacies. Its composition is unknown.

The oral type of *M. communis*, with the trade name "Gelomyrtol", is made in Germany. 1 enteric-coated capsule contains 120 mg of distillate from a mixture of eucalyptus oil, sweet orange, myrtle oil and lemon oil. Other ingredients are medium chain triglycerides, gelatin, vegetable glycerin, Gelomyrtol is used to treat bronchitis and acute and chronic Rhinosinusitis, and after paranasal sinus surgeries (Shariati et al. 2010).

While many studies have investigated the effect of *M. communis* on bacteria, there have been no studies conducted on its effects on the skin defense barrier. Thus, this study was conducted with the aim to determine the effect of *M. communis* extract on the keratinocyte (TJ) barrier function.

1.4 Research goals

- The main goal

-Determining the effect of *M. communis* extract on the barrier function of epidermal cells.

- Specific goals

1. Determining cell toxicity of two different *M. communis* extracts on keratinocytes.
2. Determining the effect of *M. communis* extracts on transepithelial resistance of keratinocytes during barrier (TJ) formation
3. Determining the effect of *M. communis* extracts on transepithelial resistance in keratinocytes with a preformed barrier (TJs).

1.5 Hypothesis

Dissemination of skin diseases that are resistant to certain chemical drugs justify the search for new types of drugs and compounds with fewer side-

effects that can affect pathogenic factors and improve the skin's defense systems (Teseng et al. 2005). This has led to research into new drugs, especially medicinal plants and compounds extracted from them, in order to discover their healing properties. Among natural resources, *M. communis* enjoys significant importance because it has been described as having; antiseptic, antibacterial, antiviral, antiparasitic, and antifungal properties (Zeng et al. 2011).

This study was conducted with the aim to determine the effect of *M. communis* extract on barrier function (tight junctions) in human skin cells (keratinocytes).

We wondered whether the positive effects seen for *M. communis* on skin and mucosa infections are not only mediated by their antibacterial/antifungal effects, but also by a keratinocyte barrier strengthening activity which leads to a decrease in the uptake of pathogens.

Chapter 2

Material and Methods

2.1 Materials

2.1.1 Myrtus Extracts:

A) Ethanol-based Myrtus extract 10% (Myrtus/EtOH)

This extract was prepared by the Pharmacognosy Department of Shahid Beheshti University of Medical Sciences, Iran. To prepare the Myrtus extract, a maceration method was used. To that end, 70 grams of dried Myrtus leaf from Iran were ground and poured into an Erlenmeyer flask, and 500 ml of 96% alcohol was added and left at room temperature for 48 hours. During this period, the container was stirred and shaken several times. After this time, the contents of the flask were filtered, and the solvent was distilled in a vacuum at 40°C.

To determine the extract's dry weight, 5 ml of the filtered solution was poured into an hour-glass (weight measure precise to 3 decimal points). Next, to ascertain complete dryness, it was maintained at 40°C for 48 hours. After drying, the hour-glass was weighed again, and its weight, minus the weight of the empty hour-glass, was determined to be the weight of the dry extract. The extract was passed through a 0.2 nm syringe filter and then placed at -20°C. Finally, 10 grams of the extract was diluted in 90 ml of alcohol 96%. The extract contained α -pinen, 1,8-cineol, linalool, α -terpineol, geraniol, camphen, and limonen (Figure 2.1).



Figure 2.1 Ethanol-based Myrtus extract 10% (Myrtus/EtOH)

B) Water-based Myrtus extract 10% (Myrtus/Oil)

This extract with the brand name Myrtlx® was bought from Giah Essence Phytopharm Company, Gorgan-Iran. For the production of the extract, 100 grams of chopped fresh leaves from Iran were distilled in a 1000 ml distillation device with 500 ml of water for 4 to 6 hours. Then, the oily Myrtus extract floating on the surface of the water was collected and diluted with 72% ethanol to produce a Myrtus 10% solution. The 10% solution contained as its main ingredients; 21mg/ml 1,8-cineol and 2.5% α -pinen. Furthermore, it also contained limonene and myrtenol (Figure 2.2) .



Figure 2.2 Water-based Myrtus extract 10% (Myrtus/Oil)

2.1.2 Generally required devices, tools, and materials

Material	Manufacturer
PBS	Biochrom AG (Berlin, DE)
Calcium Chloride	Promocell (Heidelberg, DE)
EDTA	Merck (Darmstadt, DE)
Ethanol	Merck (Darmstadt, DE)
Isopropanol	Merck (Darmstadt, DE)
Thiazolyl Blue Tetrazolium Bromide (MTT)	Sigma-Aldrich, DE
Trypsin	Biochrom AG (Berlin, DE)

Device	Type	Manufacture
Centrifuge	Laborfuge 400e	Heraeus Instruments (Hanau, DE)
Clean bench	Hera Safe 12	Heraeus Instruments (Hanau, DE)
Incubator	BBD 6220	Heraeus Instruments (Hanau, DE)
Rotary shaker	Tiny Turner	CTI (Idstein, DE)
Light microscope	Leica DMIL	Leica, Wetzlar, DE
Neubauer Counting Chamber	Standard version	LO - Laboroptik GmbH- DE
Ohmmeter	Millicell-ESR	Merck Millipore DE
Petri Dishes	100x20 mm	Becton Dickinson Falcon USA
Pipettus	N 1/008/886	Hirschmann DE
Pipets different sizes		Becton Dickinson Falcon USA
Tissue Culture Plate	Wellplate (96 well)	Becton Dickinson Falcon USA
Vortex	REAXtop	Heidolph (Schwabach, DE)
Trans well Plates	Transwell Permeable Supports, 0,4µm Polyester Membrane, 6,5 mm Insert, 24 well Plate	Corning Incorporated, USA

2.1.3 Media

Dermalife Medium low calcium (Lifeline Technology, Frederick, MD, USA; Cat-No: LM004 + LS-1030)

Contains:

L-Glutamin
Rh-insulin
Hydrocortisone
Epinephrine
Rh-TGF-alpha
Extract P
Apo-Transferrin

Dermalife Medium high calcium

CaCl₂ was added to Dermalife Medium low calcium to a final concentration of 1.8 mM calcium

Epilife Medium low calcium (Life Technologies (Gibco Cascade Biologics), Carlsbad, CA, USA Cat. no. M-EPI-500-CA + S-001-5; 0.06 mM Ca²⁺)

Contains:

Bovine pituitary extract (BPE),
Rh- insulin-like growth factor-I
Hydrocortisone
Bovine transferrin
Human epidermal growth factor

Epilife Medium high calcium

CaCl₂ was added to Epilife Medium low calcium to a final concentration of 1.8 mM calcium

2.1.4 Tissues

Primary human keratinocytes were cultured from the foreskin of boys < 5 years. The usage of the tissue was approved by the Ethics Committee of the Ärztekammer Hamburg (WF-061/12).

2.2 Methods

2.2.1 Preparation and culture of keratinocytes

All of the following steps were done under sterile conditions!

A small tube containing biopsy samples of human skin tissue (from the foreskin), was transferred to a sterile workbench. The skin tissue was transferred to a petri dish and divided into smaller pieces with a scalpel, washed in PBS and then incubated in trypsin 0.25% in PBS solution at 4°C for 24 hours. After this time the epidermis was separated from the dermis with fine forceps and transferred to 10% FCS in PBS and separated in a single cell suspension by gently pipetting up and down.

The single cell suspension was centrifuged at 800 x g for 5 minutes. Next, the supernatant was removed and the pellet was resuspended in Derma Life Medium and transferred into petri dishes and incubated at 37 °C, 10% CO₂, and 90% humidity. The medium was changed 3 times a week. When the cells reached the density of 70% confluence, they were subcultured.

To subculture the cells, the medium was removed and the adhered cells were treated with 0.1%/0.02% Trypsin/EDTA in PBS for 5 min at 37°C. The trypsin reaction was stopped with 10%FCS in PBS and the cells were counted in a Neubauer counting chamber.

2.2.2 Cell count and transfer of cells to Epilife/Dermalife

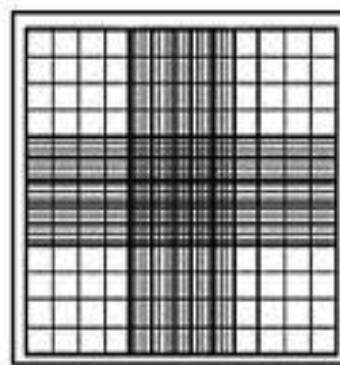
To determine the number of cells, 10 µl of the cell suspension (see 2.2.1) were transferred to the Neubauer counting chamber (Figure 2.3). Counting

the cells was performed in 16 small squares under a microscope with magnification $\times 10$, in the contrast phase.

Counting the cells was performed according to the formula below:



C-Chip Neubauer improved



Neubauer-improved chamber counting detail.

Figure 2.3 C-Chip Neubauer-improved

Neubauer-improved counting chamber details:

Total number of cells = $n \times f \times v \times 10,000$

Where:

N = counted cells

F = diluting factor

V = total volume

Chamber factor 10,000 = 1 mm^3

Vitality [%] = $\text{number of viable cells} / \text{total number of cells} \times 100$

Cells in 10% FCS (see 2.2.1) were centrifuged (5 min $800 \times g$), the supernatant discarded and the pellet was resuspended in Dermalife Medium (for MTT assays) or in Epilife Medium (for the TER and MTT assays).

2.2.3 Preparation of the cells for MTT assay

For MTT assays, 7500 cells/well were seeded with Dermalife low calcium into 96 well plates. After 3 days, cells were fed with Dermalife +/- myrtus extracts for low calcium cells or with Dermalife + 1.8 mM Ca +/- myrtus extracts for high calcium cells. Cells were cultured for 20h (short-term experiments) or 5 d (long term experiments) and Myrtus extracts were applied every 48 h.

2.2.4 Determination of cell viability (MTT assay)

Using the MTT assay, cell survival in a 96-well plate (in quantitative terms) was determined as follows:

After 20 hours, respectively 5 days, the keratinocytes that had been stimulated with different concentrations of Myrtus/EtOH and Myrtus/Oil (undiluted, 1/100, 1/500, 1/1,000, and 1/5000) were taken out of the incubator, and the supernatants were removed using a multi-channel pipette. Then, MTT assay medium was prepared with a 1:5 mixture of Dermalife medium and MTT stock solution (5 mg/ml in PBS). 100 μ l of this MTT assay medium was added to each well of the 96-well plate, and incubated at 37°C, 10% CO₂, and 90% humidity for 4 hours.

The MTT assay medium was removed using a multi-channel pipette, and then 100 μ l 2-propanol was added to each well, and incubated at RT for one hour in the dark.

Next, the quantitative cell survival rate was determined using an ELISA digital colorimetric reader device. The color intensity, as a result of formazan production, was measured at 570 nm against a 2-propanol blank control. Color intensity was proportional to the number of viable cells.

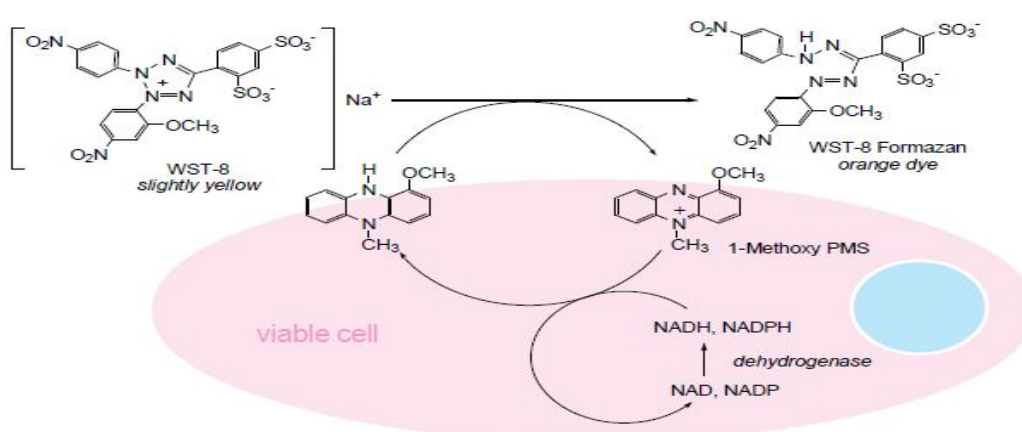


Figure 2.4 Mechanism of the MTT test and cell viability evaluation

The MTT mixture is added to the medium during incubation and revitalized by the succinate dehydrogenase system (enzymes of the mitochondrial respiratory cycle). Breaking this substance produces blue formazone crystals. The depth of color produced is directly associated with metabolically active cells. After performing this test, light absorbance of the solution is read at 570 nm wavelengths, and the cell count is calculated using a standard curve. Increased light absorbance is indicative of increased cell survival (Figure 2.4). To control the keratinocytes, the above groups were stimulated with ethanol and Dermalife.

2.2.5 Preparation of cells for transepithelial resistance (TER) measurements

In total, 40,000 cells in Epilife low calcium medium were seeded into transwell chambers (Corning, 24 well). After 3 days of incubation at 37°C, 10% CO₂ and 90% humidity medium were replaced by Epilife high calcium medium (time point 0). From this point on, TER was measured every 24h and the medium was changed directly after the measurement.

Myrtus/Oil was applied at 1:1,000 and 1:5,000 dilution in Epilife high calcium medium. In the experiments determining the influence of Myrtus extracts during barrier formation, Myrtus/Oil was applied directly from the beginning (0 days) and TER measurement was performed every 24 hours for 7 days. In the experiments determining the influence of Myrtus extracts on a preformed barrier, Myrtus/Oil was applied from day 5 onwards. TER was measured from day 0 to day 10.

For the controls, Epilife high calcium medium was used. Previous experiments have shown that there is no difference between the TER of Epilife high calcium medium and Epilife high calcium medium containing ethanol at a dilution of 1:1,000 and 1:5,000.

2.2.6 Determination of trans-epithelial electrical resistance (TER)

Transepithelial electrical resistance is an epithelial barrier integrity measurement method in which passive ion flux is determined through an epithelial model. To this end, the following sequence was performed:



Figure 2.5 Transwell Permeable Supports

2.2.6.1 Preparation of measuring electrodes

Before measurement, the electrode was placed in ethanol 70% to disinfect for 15 minutes. It was then left in the open air to dry. Next, it was placed in PBS (phosphate-buffered saline) solution to balance for 15 minutes. Prior to measurement of resistance, the Ohm-meter was set at zero as suggested by the guidebook.

2.2.6.2 Measurement of transepithelial electrical resistance

Note should be taken that measurement of transepithelial resistance is only possible when cell density is suitable. Thus, cell density should be monitored regularly under a microscope, and measurement can begin when the correct cell density is observed.

Transwell plates, containing cell suspensions were removed from the incubator, and measurement began immediately. The measuring probe was placed in a Transwell plate in such a way that the short leg was in contact with the suspension in the well and the long leg with the suspension was outside the well. Measurement began by pressing the measuring button. The button was held down until the display screen showed a constant digit (about 5 seconds) (Figure 2.6).

The blank was determined by measuring a cell culture insert without cells, but with culture medium. The blank had to be subtracted from the values measured for the cell cultures.

2.2.6.3 Calculation of Unit Area Resistance ($\Omega \times \text{cm}^2$)

The resistance is inversely proportional to the area of the tissue. The larger the membrane, the lower the resistance.

NOTE: Resistance readings for 6-well or larger devices should not be converted to unit area resistance.

Typically the product of the resistance and the area is calculated and reported. This value is independent of the area of the membrane used. The unit area resistance is obtained by multiplying the meter readings by the effective surface area of the filter membrane. The dimension is $\Omega \times \text{cm}^2$.

Resistance of a unit area = Resistance (Ω) \times Effective Membrane Area (cm^2)

The transwells used in our experiments had a unit Area = 0.33 cm^2

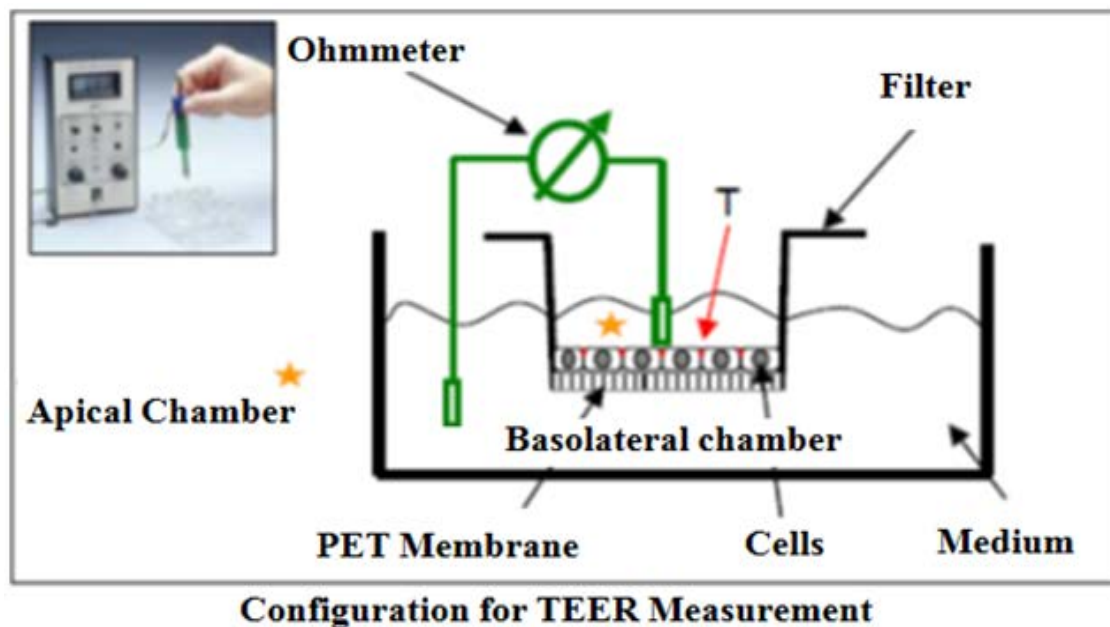


Figure 2.6 TER Measurement (Schlüter, Holger (2006))

Chapter 3

Results

3. Results

For a general characterization of the influence of the two *Myrtus* extracts (Ethanol-extract = *Myrtus*/EtOH, oily-extract collected from the surface of water = *Myrtus*/Oil) on human primary keratinocytes, the viability of the cells after the application of different concentrations of the extracts was tested by MTT assay in short-term (20h) and long-term (5d) experiments. Furthermore, the effect was tested in low-calcium (undifferentiated) and high-calcium (differentiated) cells.

3.1 Short term effect of *Myrtus* extracts on keratinocyte cell viability (incubation 20 hours)

3.1.1 Influence of *Myrtus*/EtOH on cell viability (20h)

Under short term experimental conditions, incubation with ***Myrtus*/EtOH** resulted in a significant decrease of cell viability of primary human keratinocytes at 1:100 (reduction to 10-13%) and 1:500 dilution (reduction to 23-34%) under low calcium conditions (Figure 3.1). The results obtained with *Myrtus*/EtOH at 1:100 dilution, and to some extent at 1:500 dilution, might be artificially high, because the extract itself showed a dark brown color and was difficult to remove completely at 1:100 and, only partly at 1:500 dilution. With a non-diluted extract all of the cells were killed (data not shown). At a 1:1,000 dilution a very slight, but significant increase of viability (to 108% +/- 1.1%), was seen in cells from one donor, at 1:5,000 a decrease of 77% +/- 1.4% was seen in the same cells. For a different donor, a significant decrease of cell viability to 86% +/- 3.7% was seen with *Myrtus*/EtOH 1:1,000. There was no significant influence of *Myrtus*/EtOH at 1:1,000 on keratinocytes from the third donor and in the 1:5,000 dilution on keratinocytes from the two other donors, however, a trend of reduced viability was observed (Figure 3.1).

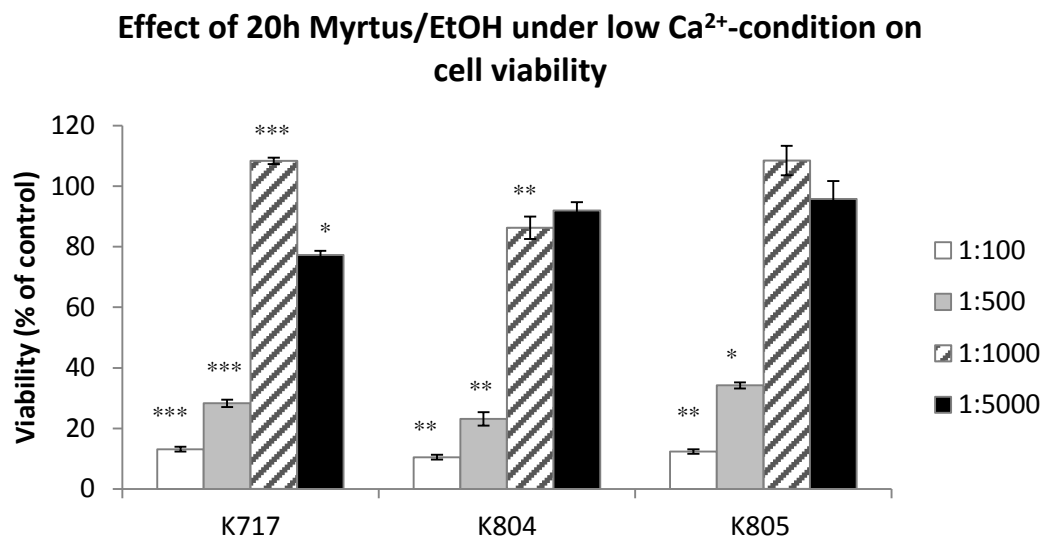


Figure 3.1 Viability of keratinocytes incubated with different doses of Myrtus/EtOH after 20 hours of incubation under low Ca²⁺-conditions. Means +/- STD. Experiments were performed in triplicates. K717, K804 and K805 denote different keratinocyte donors. Control: Medium containing corresponding concentrations of EtOH. Dilutions were performed in medium*: p<0.05, **: p<0.01, * p<0.001 compared to the respective controls.**

In addition, under high-calcium conditions there was a decreased viability of cells incubated with 1:100 and 1:500 dilutions of Myrtus/EtOH (reduction to less than 15% for both dilutions). Again, the results obtained with Myrtus/EtOH at 1:100 dilution might be artificially high, because of the color of the extract (see low calcium cells). There was no significant influence at higher dilutions except for a slightly reduced viability (92% +/- 3%) in cells from donor K804 treated with 1:1,000 dilution (Figure 3.2).

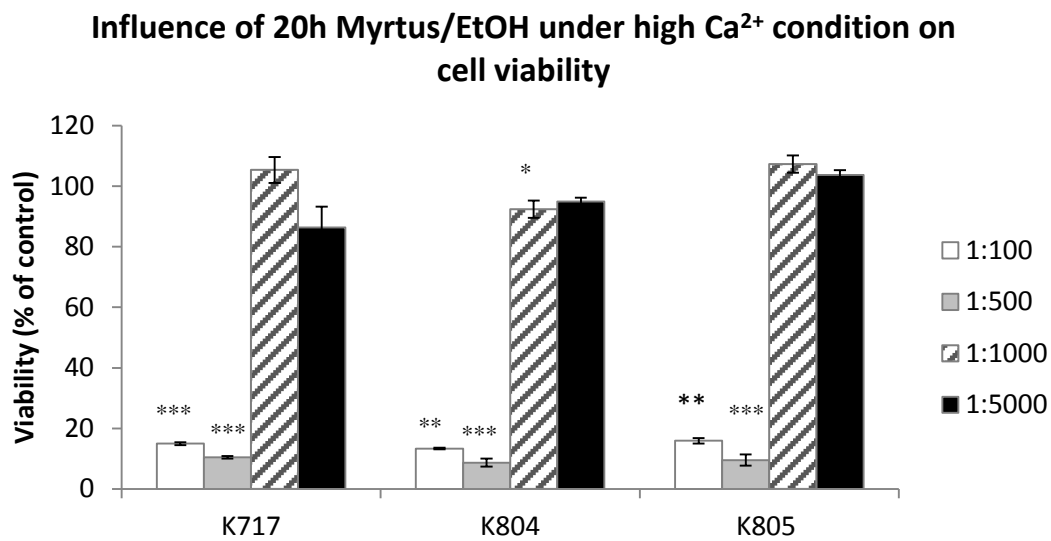


Figure 3.2 Viability of keratinocytes incubated with different doses of Myrtus/EtOH after 20 hours of incubation under high Ca^{2+} -conditions. Means \pm STD. Experiments were performed in triplicate. K717, K804 and K805 denote different keratinocyte donors. Control: Medium containing corresponding concentrations of EtOH. Dilutions were performed in medium *: $p < 0.05$, **: $p < 0.01$, * $p < 0.001$, compared to the respective controls.**

3.1.2 Influence of Myrtus/Oil on cell viability (20h)

Myrtus/Oil under low-calcium conditions undiluted (data not shown) and at 1:100 dilution (Figure 3.3) resulted in a significant reduction to less than 2% of cell viability of primary keratinocytes from three different donors. There was also a significant reduction at 1:500 (to 79% \pm 6%) and 1:1,000 (to 87% \pm 6%) dilution for cells derived from one donor (K805), while there was no significant effect on the cells from the two other donors. Moreover, under high-calcium conditions, it reduced cell viability at 1:100 dilution in all three keratinocyte lines, in cells from donor K717, and also at 1:500 dilution (Figure 3.4).

In the Myrtus/Oil experiments, the medium was used as a control because previous experiments had shown that ethanol in dilutions used for our experiments had no influence on cell viability (Figure 3.5).

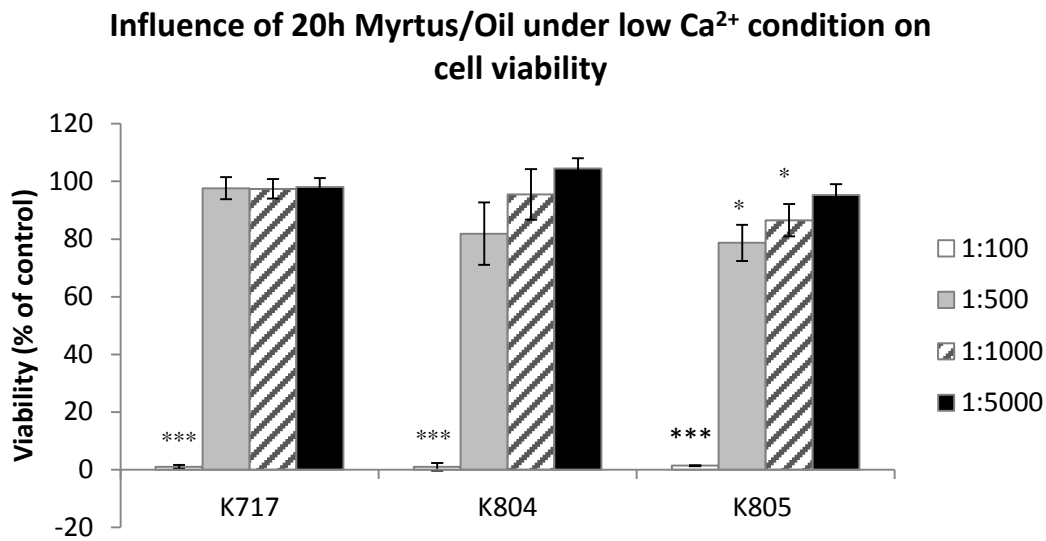


Figure 3.3 Viability of keratinocytes incubated with different doses of Myrtus/Oil after 20 hours of incubation under low Ca^{2+} -conditions. Means \pm STD. Experiments were performed in triplicate. K717, K804 and K805 denote different keratinocyte donors. Control: medium. Dilutions were performed in medium *: $p < 0.05$, **: $p < 0.01$, *** $p < 0.001$ compared to control.

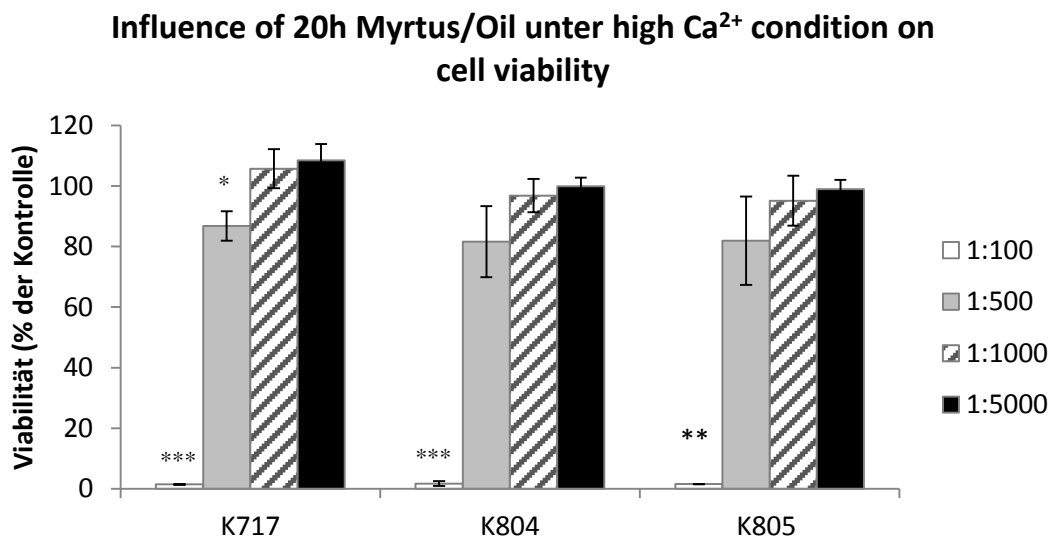


Figure 3.4 Viability of keratinocytes incubated with different doses of Myrtus/Oil after 20 hours of incubation under high Ca^{2+} -conditions. Means \pm STD. Experiments were performed in triplicate. K717, K804 and K805 denote different keratinocyte donors. Control: medium. Dilutions were performed in medium *: $p < 0.05$, **: $p < 0.01$, *** $p < 0.001$ compared to a control.

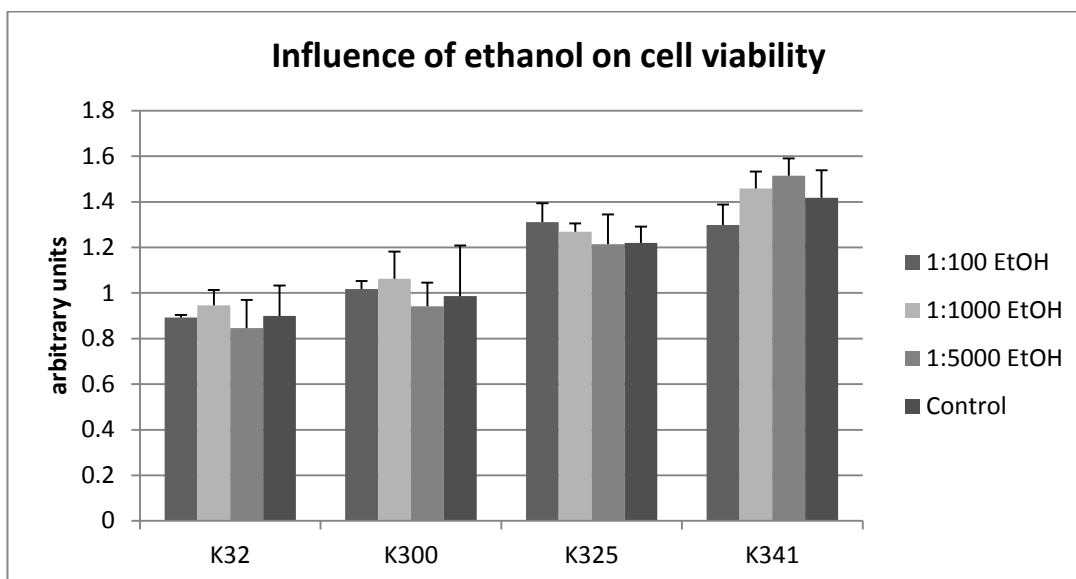


Figure 3.5 Viability of keratinocytes incubated with different doses of ethanol after 20 hours of incubation under low Ca^{2+} -conditions. Means \pm STD. Experiments were performed in triplicate. K32, K300 and K325 and K341 denote different keratinocyte donors. Control: medium. Dilutions were performed in a medium.

3.1.3 Comparison of Myrtus/EtOH and Myrtus/Oil under low and high calcium conditions in short-term cultures

The negative influence of Myrtus/EtOH at 1:500 dilution was significantly ($p < 0.001$ for K717 and K805, $p < 0.01$ for K804) more pronounced in high-calcium than in low-calcium cells, while there was no gross difference at the other dilutions (Figures 3.1 and 3.2). For Myrtus/Oil there was no clear difference at any dilution (Figures 3.3 and 3.4).

3.2 Long-term effect of Myrtus extracts on keratinocyte cell viability (incubation 5 days)

3.2.1 Influence of Myrtus/EtOH on cell viability (5d)

Myrtus/EtOH significantly reduced cell viability to values $< 12\%$ in keratinocytes from all three donors at dilutions of 1:100 to 1:500 in cells incubated for 5 days with the extracts under low (Figure 3.6) and high (Figure 3.7) calcium conditions. Again, the results obtained with Myrtus/EtOH at 1:100

dilution, and, to some extent also at 1:500 dilution, might be artificially high, because of the color of the extract and the resistance to complete removal.

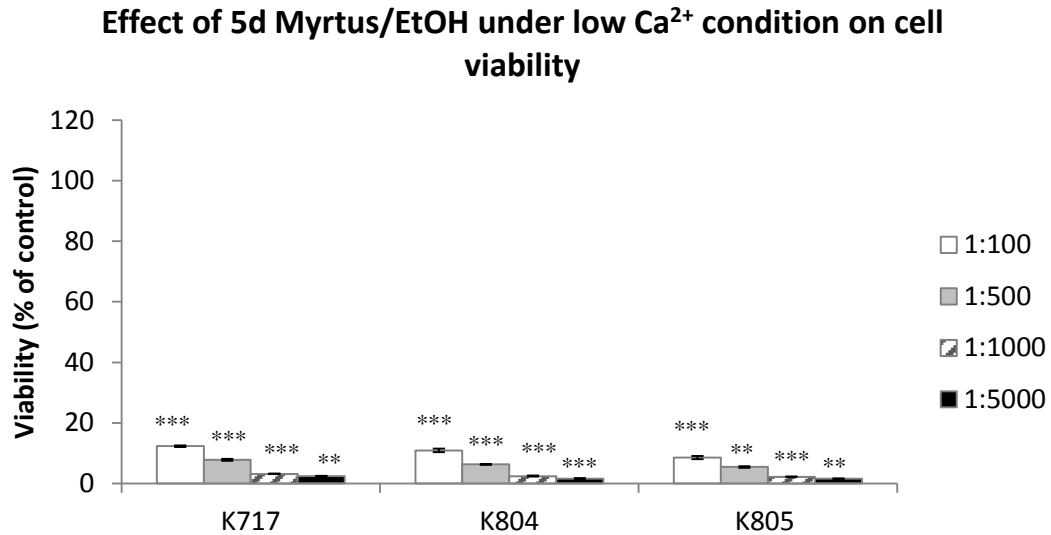


Figure 3.6 Viability of keratinocytes incubated with different doses of Myrtus/EtOH after 5 days of incubation under low Ca²⁺-conditions. Means +/- STD. Experiments were performed in triplicate. K717, K804 and K805 denote different keratinocyte donors. Control: Medium containing corresponding concentrations of EtOH. Dilutions were performed in medium *: p<0.05, **: p<0.01, * p<0.001 compared to the corresponding controls.**

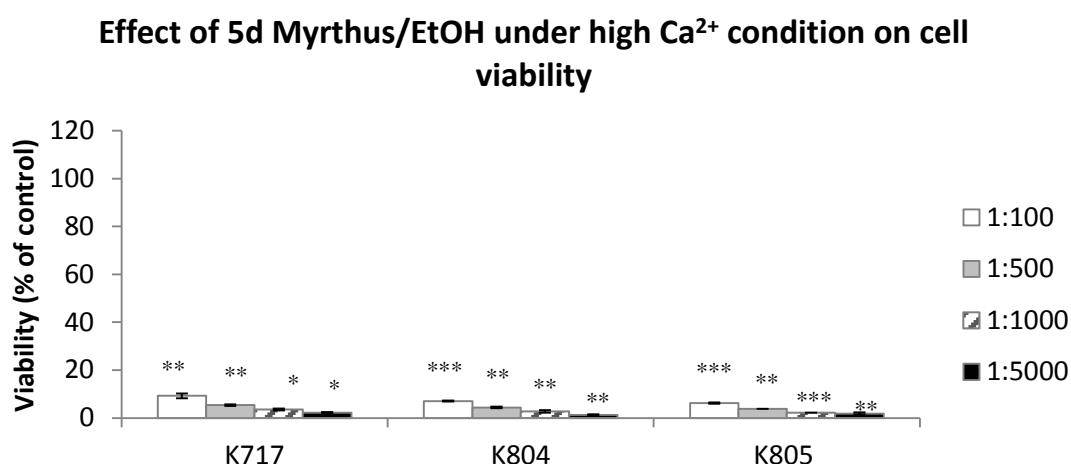


Figure 3.7 Viability of keratinocytes incubated with different doses of Myrtus/EtOH after 5 days of incubation under high Ca²⁺-conditions. Means +/- STD. Experiments were performed in triplicate. K717, K804 and K805 denote different keratinocyte donors. Control: Medium containing corresponding concentrations of EtOH. Dilutions were performed in medium *: p<0.05, **: p<0.01, * p<0.001 compared to controls.**

3.2.2 Influence of Myrtus/Oil on cell viability (5d)

Myrtus/Oil showed a dose-dependent influence on cell viability in keratinocytes after 5 days of incubation under low-calcium conditions. While only 1-2% of the cells survived at 1:100 dilution, there was a reduction to calcium 30-55% at 1:500 dilution and to 60-94% at 1:1,000 dilution. At 1:5,000 dilution the reduction was significant in keratinocyte cultures from two donors (82-93%), while there was no reduction for the third donor (Figure 3.8). Under high-calcium conditions, cells were also reduced to less than 2% at 1:100 dilution and there was a significant reduction to calcium 41-60% at 1:500 dilution. However, at 1:1,000 dilution, but only for the cells from donor K717, a significant reduction of cell viability to calcium 84% +/-1% was observed, while there was no significant reduction for the other donors. There was no significant influence of 1:5,000 dilution in any of the cell cultures (Figure 3.9).

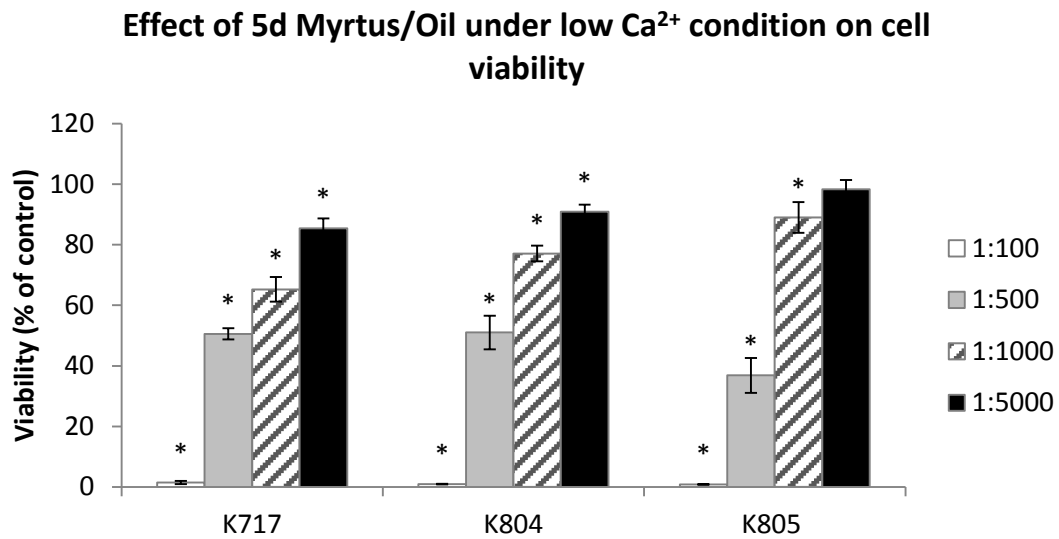


Figure 3.8 Viability of keratinocytes incubated with different doses of Myrtus/Oil after 5 days of incubation under low Ca^{2+} -conditions. Means \pm STD. Experiments were performed in triplicate. K717, K804 and K805 denote different keratinocyte donors. Control: medium. Dilutions were performed in medium *: $p < 0.05$, **: $p < 0.01$, *** $p < 0.001$ compared to controls.

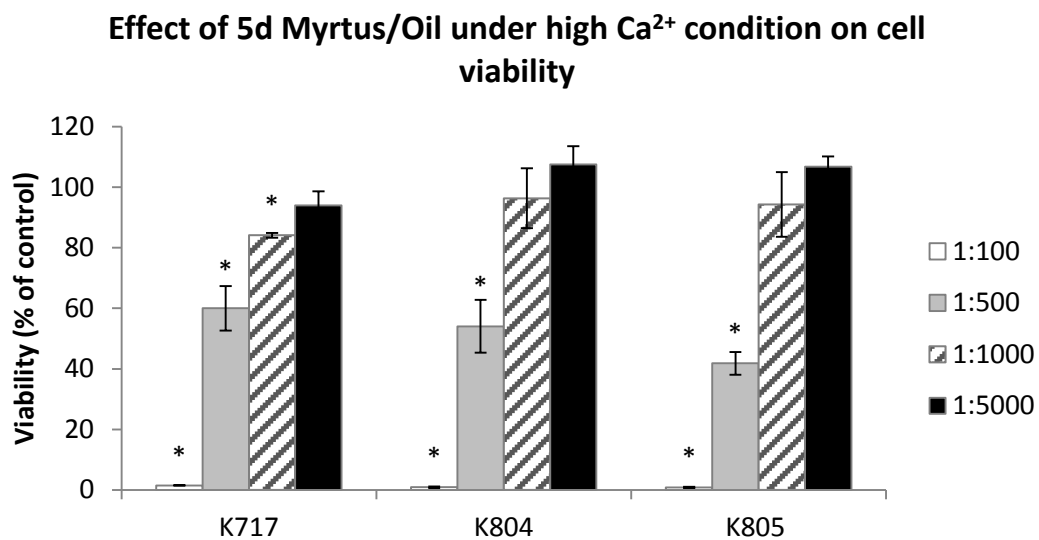


Figure 3.9 Viability of keratinocytes incubated with different doses of Myrtus/Oil after 5 days of incubation under high Ca^{2+} -conditions. Means \pm STD. Experiments were performed in triplicate. K717, K804 and K805 denote different keratinocyte donors. Control: medium. Dilutions were performed in medium *: $p < 0.05$, **: $p < 0.01$, *** $p < 0.001$.

3.2.3 Comparison of Myrtus/EtOH and Myrtus/Oil under low and high calcium conditions in long-term cultures

Myrtus/EtOH showed a strong cytotoxic effect on long term cultures of primary keratinocytes at all dilutions with less than 12% surviving cells. There was no difference between the low and high calcium conditions. Myrtus/Oil reduced cell viability under low and high calcium conditions to a very low amount at 1:100 dilution. At 1:500 dilution, 50% of the cells were still alive. At 1:1,000 and 1:5,000 dilution, there was a reduction to 60-90% at low calcium conditions, however, at high calcium conditions there was no significant effect except for a slight reduction at 1:1,000 dilution in cells from one donor.

3.3 Effect of Myrtus/Oil on the formation of barrier function in primary keratinocytes

To test the effect of Myrtus extracts on the formation of barrier function in primary keratinocytes, they were applied to confluent keratinocytes cultures directly after the induction of barrier (TJ) formation by the elevation of extracellular calcium concentrations (calcium switch). Barrier function was measured as transepithelial resistance (TER). As shown previously (Kirschner et al. 2013) this TER mainly reflects a block to paracellular permeability for ions due to tight junction function.

Because of the negative effect of Myrtus/EtOH on cell viability at all dilutions in long-term cultures, this extract was not used to test its effect on barrier formation. For Myrtus/Oil the dilutions 1:1,000 and 1:5,000 were used which showed no effect on cell viability in most cell cultures.

Using primary keratinocytes from three different donors, we observed that Myrtus/Oil at 1:1,000 dilution impairs barrier formation at days 2-7 after the calcium switch. The extent of impairment varied between the donors, but the overall tendency was similar. In cells from donor K301 1:1,000 dilution of Myrtus/Oil resulted in significantly impaired barrier formation from days 2-7 (Figure 3.10). In cells from donor K730 there was a significant impairment at days 4, 5 and 7 and a strong trend at days 2, 3 and 6 (Figure 3.11). In cells

from donor K832 there was a significant impairment at days 4-7 and a trend at days 2 and 3 (Figure 3.12).

At the 1:5,000 dilution, Myrtus/Oil significantly impaired barrier formation in cells from donor K301 at days 4, 5 and 7 (Figure 3.10), in cells from donor K730 at no time point (Figure 3.11), and in cells from donor K832 at day 7 (Figure 3.12). Interestingly, at days 3 and 6 there was a (mostly) significant improvement of barrier formation in the cells from all three donors (Figures 3.10 to 3.13).

When summarizing the three cell lines, a significant impairment of barrier formation can be seen with Myrtus/Oil 1:1,000 at days 3 and 4, there was also a trend of impairment at the other days. For the 1:5,000 dilution, there is a significant improvement of barrier formation at day 3, resulting in 20% higher calcium resistance (Figure 3.13).

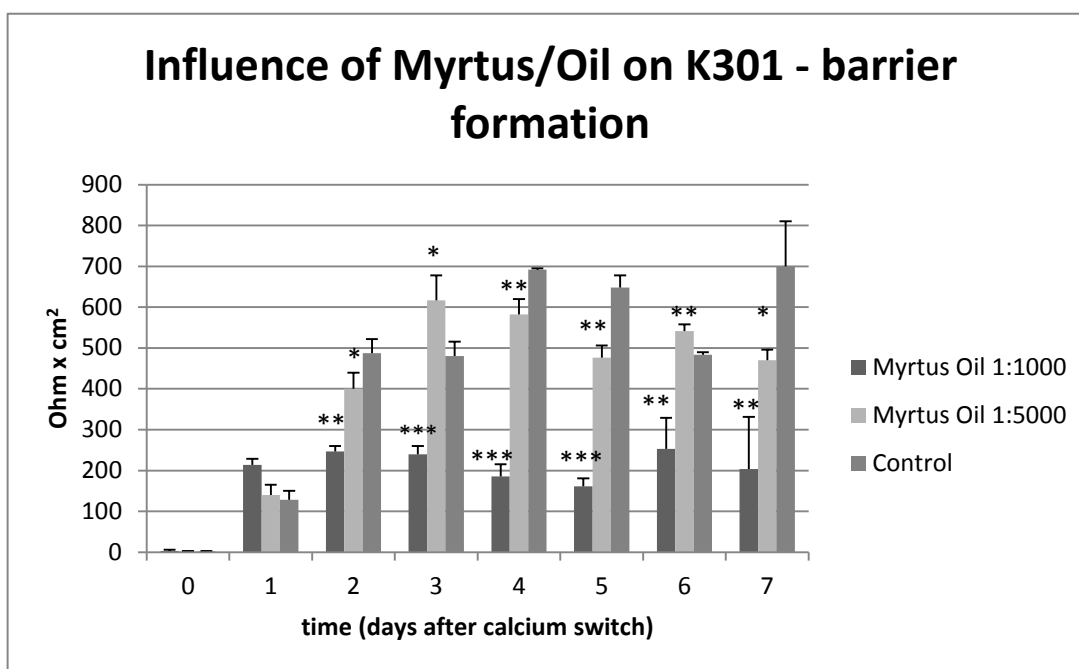


Figure 3.10 Influence of Myrtus/Oil on barrier formation of primary human keratinocytes (donor K301). Barrier formation was measured every 24 hours after the induction of barrier formation (0h). Myrtus/Oil was applied from 0h every 24 h. Mean \pm STD. N= 3 wells. Control: medium. Dilutions were performed in medium *: $p < 0.05$, **: $p < 0.01$, *** $p < 0.001$ compared to a control at each time point.

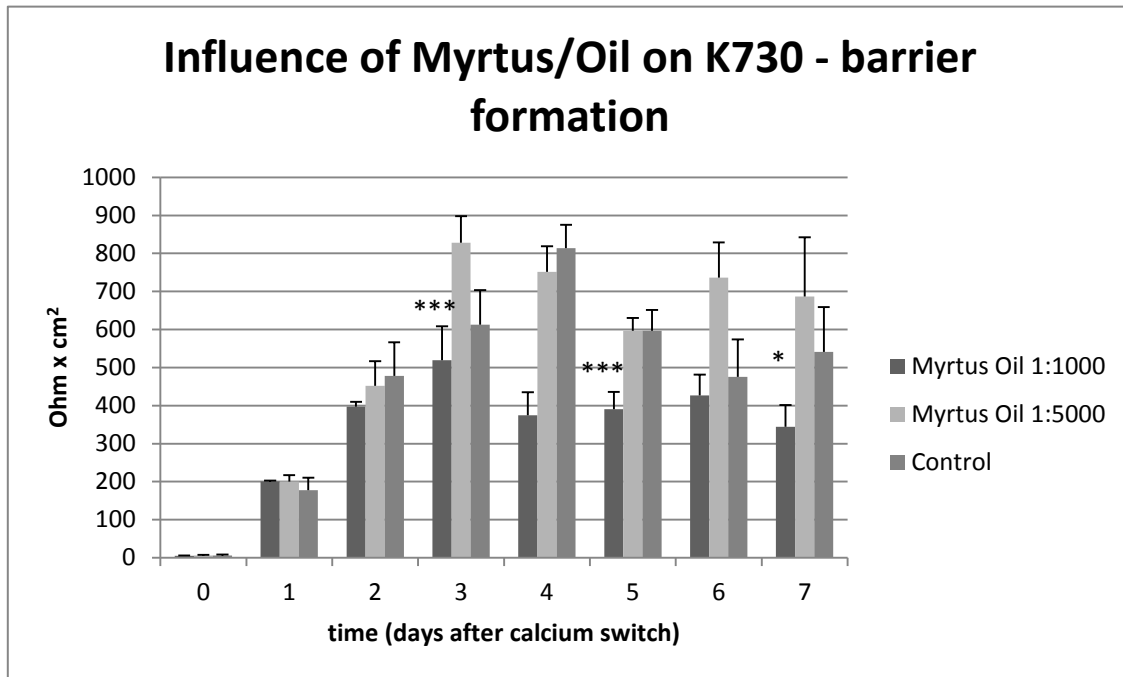


Figure 3.11 Influence of Myrtus/Oil on barrier formation of primary human keratinocytes (donor K730). Barrier formation was measured every 24 hours after induction of barrier formation (0h). Myrtus/Oil was applied from 0h every 24 h. Mean \pm STD. N= 3 wells. Control: medium. Dilutions were performed in medium *: $p<0.05$, **: $p<0.01$, *** $p<0.001$ compared to a control at each time point.

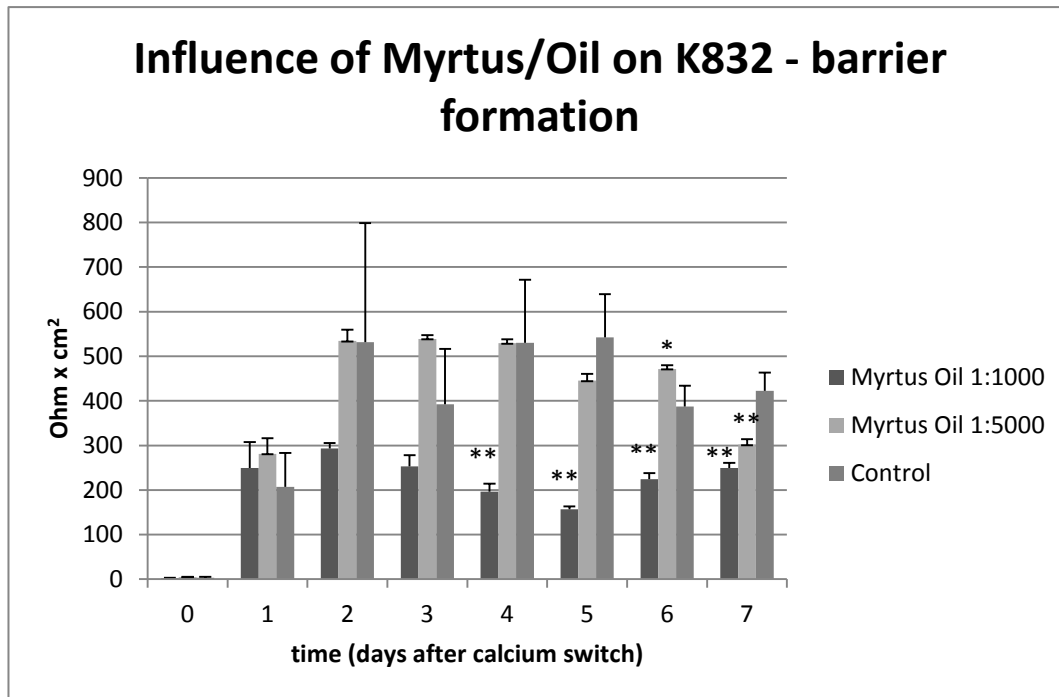


Figure 3.12 Influence of Myrtus/Oil on barrier formation of primary human keratinocytes (donor K832). Barrier formation was measured every 24 hours after induction of barrier formation (0h). Myrtus/Oil was applied from 0h every 24 h. Mean \pm STD. N= 3 wells. Control: medium *: $p<0.05$, **: $p<0.01$, *** $p<0.001$ compared to a control at each time point.

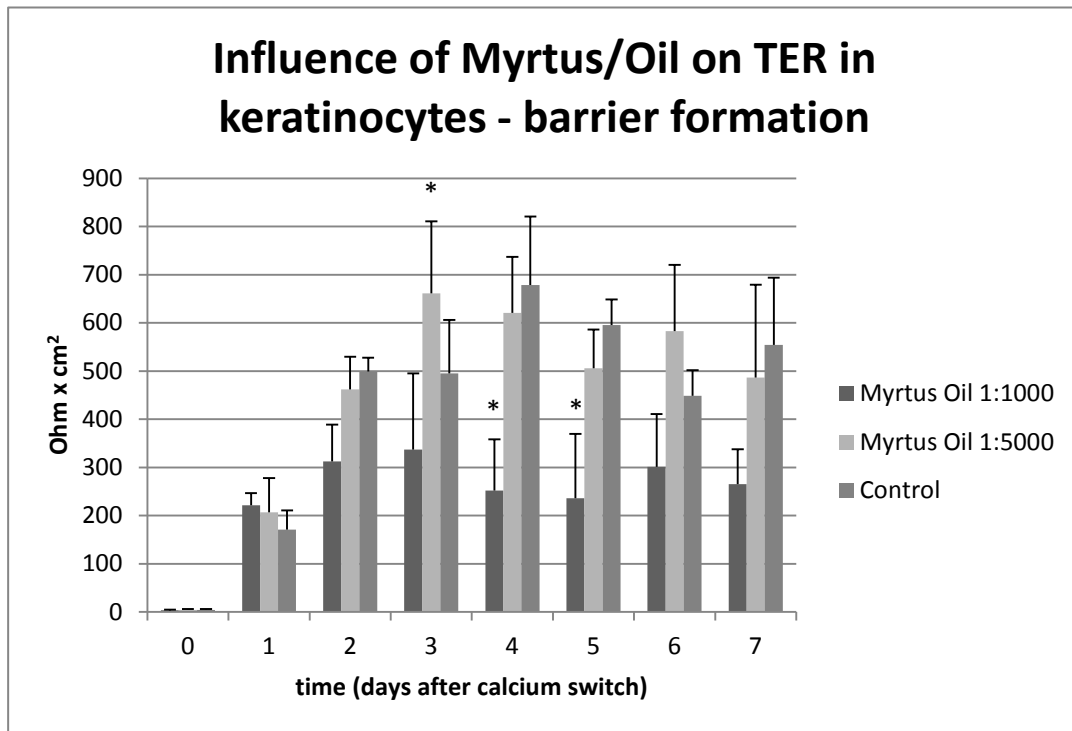


Figure 3.13 Influence of Myrtus/Oil on barrier formation of primary human keratinocytes (summary of donors K301, K730 and K832). Barrier formation was measured every 24 hours after induction of barrier formation (0h). Myrtus/Oil was applied from 0h every 24 h. Mean \pm STD. N= 3 wells. Control: medium. Dilutions were performed in medium *: $p < 0.05$, **: $p < 0.01$, *** $p < 0.001$ compared to a control at each time point.

3.4 Effect of Myrtus/Oil on barrier function of primary keratinocytes in a preformed barrier

To test the effect of Myrtus/Oil on barrier function in primary keratinocytes with a preformed barrier, Myrtus/Oil was applied to the cells after the formation of a robust TER ($\geq 400 \text{ Ohm} \times \text{cm}^2$ for 72 h).

Using primary keratinocytes from three different donors we showed that Myrtus/Oil at 1:5,000 dilution improved barrier function at all time points. This was significant at days 6 and 8 (24h and 72h after the start of Myrtus/Oil application) for cells from donor K301 (Figure 3.14), at days 6-10 (24h – 5 days after start of Myrtus/Oil application) for cells from donor 730 (Figure 3.15) and at days 6-9 (24h – 4 days after the start of Myrtus/Oil application) for cells from donor 832 (Figure 3.16). When summarizing the three cell cultures, a significant improvement with Myrtus/Oil 1:5,000 was observed at days 6-8 (24h – 72h after the start of the Myrtus/Oil application). There was a mean increase of 77% (day 6), 55% (day 7), and 67% (day 8) (Figure 3.17). Myrtus/Oil at 1:1,000 dilution showed no influence in cells from donor K301 (Figure 3.14), but improved the barrier significantly at days 6-10 in cells from donor K730 (Figure 3.15) and at days 6 and 9 in cells from donor K832 (Figure 3.16). When summarizing all three cell cultures, an improvement of barrier function after Myrtus/Oil 1:1,000 application was observed at day 6 (24h after start of application). There was a mean increase of 45% (Figure 3.17).

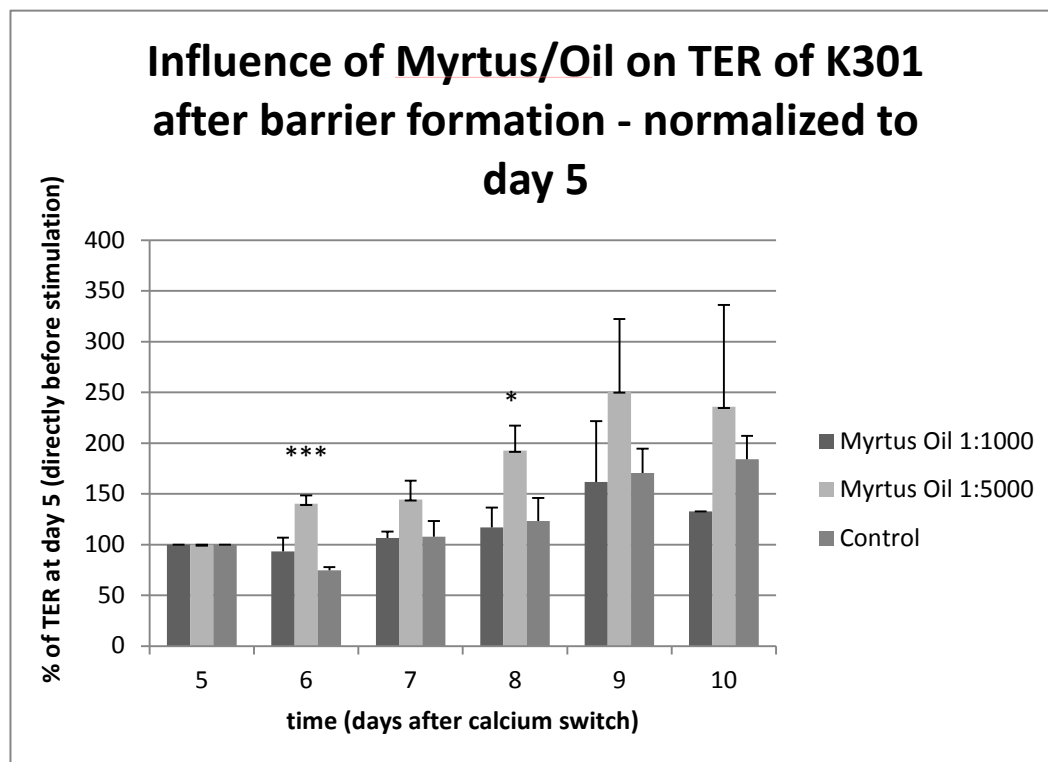


Figure 3.14 Influence of Myrtus/Oil on barrier function of primary human keratinocytes with a preformed barrier (donor K301). Barrier function was measured every 24 hours after induction of barrier formation (0 days). Myrtus/Oil was applied from day 5 every 24h. TER values are normalized to the respective value at day 5 before Myrtus/Oil application. Mean +/- STD. n= 3 wells. Control: medium. Dilutions were performed in medium *: $p<0.05$, **: $p<0.01$, * $p<0.001$ compared to a control at each time point.**

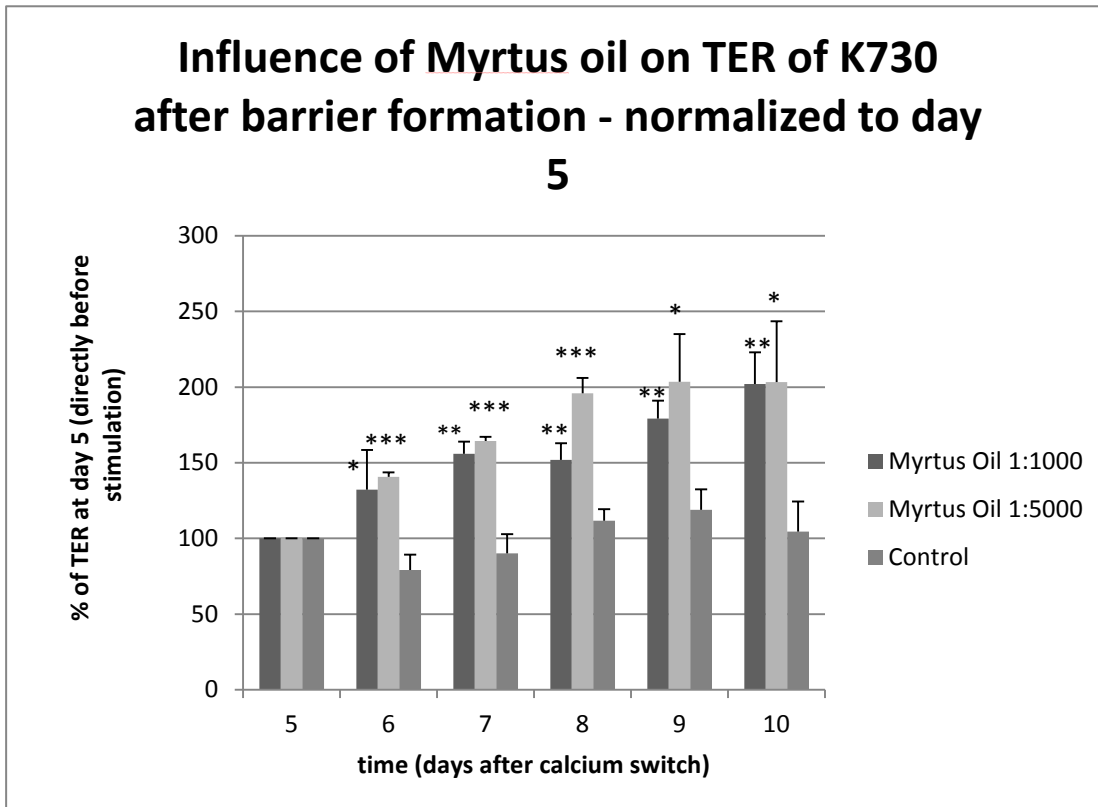


Figure 3.15 Influence of Myrtus/Oil on barrier function of primary human keratinocytes with a preformed barrier (donor K730). Barrier function was measured every 24 hours after induction of barrier formation (0 days). Myrtus/Oil was applied from day 5 every 24h. TER values are normalized to the respective value at day 5 before Myrtus/Oil application. Mean +/- STD. n= 3 wells. Control: medium. Dilutions were performed in medium *: p<0.05, **: p<0.01, * p<0.001.**

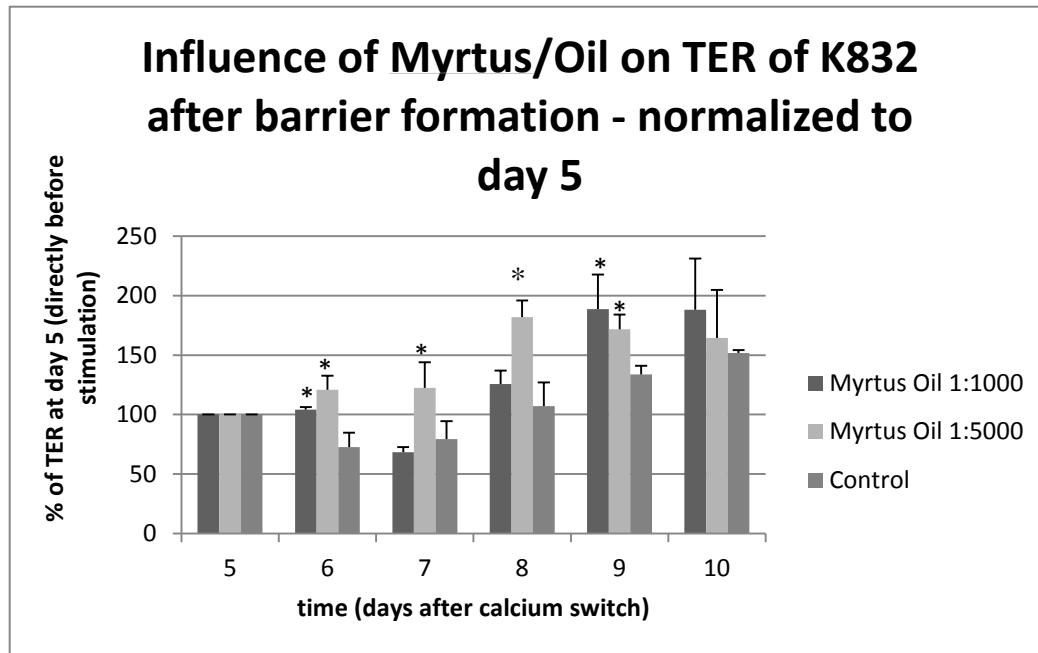


Figure 3.16 Influence of Myrtus/Oil on barrier function of primary human keratinocytes with a preformed barrier (donors K832). Barrier function was measured every 24 hours after induction of barrier formation (0 days). Myrtus/Oil was applied from day 5 every 24h. TER values are normalized to the respective value at day 5 before Myrtus/Oil application. Mean +/- STD. n= 3 wells. Control: medium. Dilutions were performed in medium *: $p<0.05$, **: $p<0.01$, * $p<0.001$.**

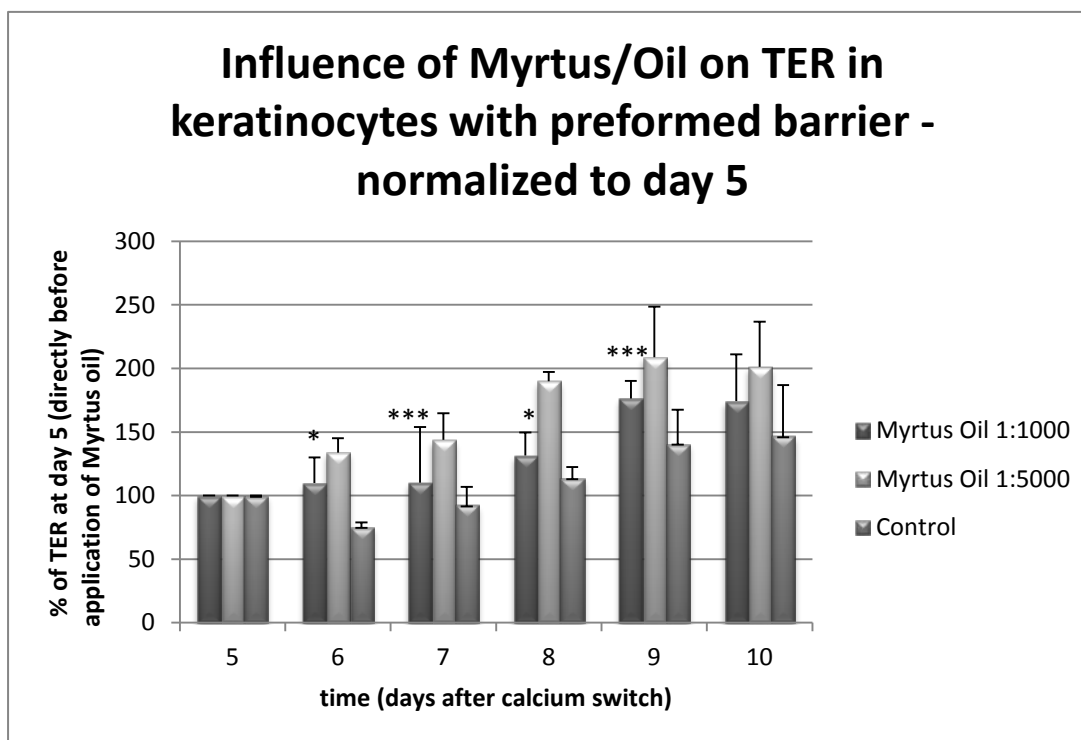


Figure 3.17 Influence of Myrtus/Oil on barrier function of primary human keratinocytes with a preformed barrier (summary of donors K301, K730 and K832). Barrier function was measured every 24 hours after induction of barrier formation (0 days). Myrtus/Oil was applied from day 5 every 24h. TER values are normalized to the respective value at day 5 before Myrtus/Oil application. Mean +/- STD. n= 3 cell lines. Control: medium. Dilutions were performed in medium *: p<0.05, **: p<0.01, * p<0.001.**

3.5 Effect of Myrtus/Oil on cell viability under “TER” conditions

Because TER measurement cells had to be treated differently to the “normal” cell viability assays, that meant that they had to be incubated with a different medium (Epilife) and Myrtus/Oil application was performed every 24h, we also performed viability assays under “TER” conditions. Figure 3.18 shows that there was no influence of Myrtus/Oil on cell viability under these conditions.

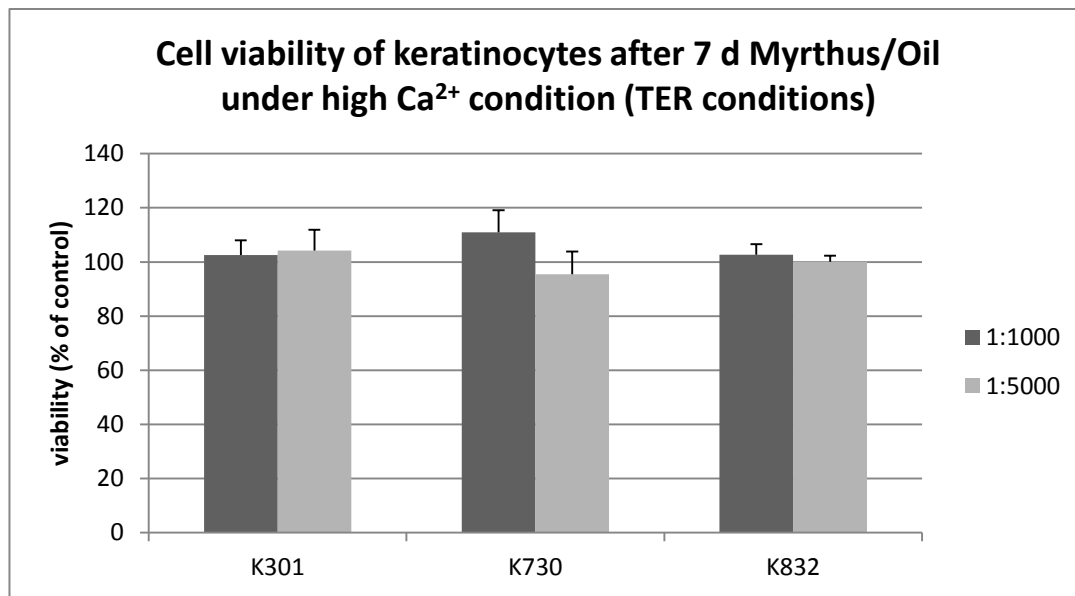


Figure 3.18 Viability of keratinocytes incubated with different doses of Myrtus/Oil after 7 days of incubation under high Ca^{2+} -condition in transwell filters with daily application of Myrtus/Oil. Means \pm STD. Experiments were performed in triplicate. K301, K730 and K832 denote different keratinocyte donors. Control: Medium. Dilutions were performed in medium *: $p < 0.05$, **: $p < 0.01$, * $p < 0.001$ compared to controls.**

Chapter 4

Discussion

4. Discussion

This study investigated the short-term (incubation for 20 hours) and long-term (incubation for 5 days) effects of two different Myrtus extracts (Myrtus/EtOH and Myrtus/Oil) in different concentrations on keratinocyte cell viability in low calcium (undifferentiated) and high calcium (differentiated) cells. Furthermore, it elucidated the effect of Myrtus/Oil on barrier function in primary keratinocytes during calcium-dependent formation of a (TJ) barrier and after the formation of a (TJ) barrier.

Given previous reports concerning the useful effects of Myrtus extract on burns and infections since ancient times (Eskandari et al. 2007), as well as recent studies on the performance of tight junctions (TJ) as skin defense barriers (Ohnemus et al. 2007, Kuo et al. 2012), the hypothesis proposed in this study was whether the positive effects seen for *M. communis* on skin and mucosa infections might also be mediated by a keratinocyte (TJ) barrier strengthening activity which would lead to a decrease in the uptake of pathogens. This study was conducted in order to find answers to this question.

4.1 Effect on cell viability

In a first step to characterize the two extracts which were available for this study, short- and long-term cell viability assays were performed at low (undifferentiated) and high (differentiated) calcium conditions.

The Myrtus/Oil extract used in this study is a commercially available Myrtus extract with essential oils dissolved in ethanol (Myrtlx®), which is already used undiluted for the treatment of minor aphthosis and herpes simplex. We could show that it is toxic to keratinocytes when applied undiluted (100 µl/ml of the extract) and at 1:100 dilution (1 µl/ml). Thus, besides the positive toxic effect on viruses/bacteria, the ready to use product with 10% extract may have a negative effect on the skin and also on mucosa cells at these concentrations. Therefore, it might be beneficial to further dilute the extract in order to avoid any negative effects on the viability of keratinocytes. Of course it will have to be tested whether the positive antiviral/antibacterial effects of this extract are preserved at higher dilutions. However, it has to be taken into account that

when applied onto skin, the extract first has to pass the stratum corneum before coming into contact with viable cells. After penetration of the stratum corneum the effective dose might be much lower than the one applied. This also applies to some extent for some areas of the oral cavity.

Gursay et al. tested essential oil from *M. communis* from Turkey. They found no cytotoxicity for HaCaT cells with 1 μ l/ml *M. communis* essential oil after 24h of incubation. At 5 μ l/ml they also did not observe cytotoxicity, although there was detachment of 10% of the keratinocytes (Gursay et al. 2009). Another publication of the same group also using *M. communis* from Turkey described the same results (Zeidan-Chulia et al. 2012). The clear cytotoxicity for 1 μ l/ml of *M. communis* oil found in this thesis could be explained by a higher sensitivity of primary keratinocytes compared to the cell line HaCaT. In addition, essential oil from *M. communis* from Turkey may have a slightly different composition compared to *M. communis* from Iran. Variability in the composition of *M. communis* essential oil was even shown in samples from different parts of Algeria (Bouzabata et al. 2010). Bouzabata et al. showed that essential oil from *Myrtus nivellei* showed no cytotoxic effects on HaCaT keratinocytes in concentrations up to 1.25 μ l/ml when applied for 24 hours (Bouzabata et al. 2013). At 2.5 μ l/ml they found a reduction of cell viability to calcium 30%. However, the composition of *Myrtus nivellei* oil differs from that of *M. communis* and again, HaCaT cells were used, therefore a direct comparison of these results is difficult.

Myrtus/EtOH cannot be used for long term cultures (5 days) at concentrations up to 1:5,000, which means 20 μ g/ml because of its cytotoxic effect. There was no difference between low and high calcium conditions. In short-term experiments (20h), *Myrtus*/EtOH reduces cell viability to less than 15% at 1:100 dilution and 30% at 1:500 dilution under low calcium conditions. Under high calcium conditions this effect was even more pronounced with a reduction to less than 15% at 1:500 dilution. In general, elevation of calcium levels reduces cell proliferation (Alberts et al. 1995, Furuse et al. 2010) and increases differentiation of cells. This seems to make them more vulnerable to *Myrtus*/EtOH.

At 1:1,000 and 1:5,000 dilution there was no clear effect on cell viability at low and high calcium conditions in short term experiments. To our knowledge, there has only been one previous report which also used an ethanolic extract of *M. communis*. This extract was derived from *M. communis* from Greece (Myrtacine ®) (Fiorini-Puybaret et al. 2011). It showed an inhibition of HaCaT cell proliferation after incubation for 72 hours with 1 and 3 µg/ml extracts by 27% and 76%, respectively. There was no effect with 0.3 and 0.1 µg/ml. However, extracts from different *M. communis* plants vary, and the extraction protocol for this ethanolic extract is not the same as the one used in our project. Moreover, we investigated cell viability in primary keratinocytes, while in the publication, cell proliferation was investigated in HaCaT cells. Therefore, it is difficult to compare the results of the two studies. However, it hints at an impairing effect of the ethanolic extract on keratinocytes at higher concentrations.

Of note, we saw slight differences between keratinocytes derived from different donors concerning the effect of *Myrtus* extracts. This clearly indicated that different individuals are more or less susceptible to the effects of *Myrtus* extracts and underscores the importance of different donors

Even though *Myrtus*/EtOH seems on first sight in short- and especially in long-term experiments more toxic than *Myrtus*/Oil, it is difficult to compare the respective dilutions of the preparations. *Myrtus*/EtOH extract is based on 10% weight/volume of the extract in solvent, *Myrtus*/Oil extract on 10% volume/volume. Thus e.g. 1:100 dilution of the *Myrtus*/EtOH means 1 mg/ml of the extract, while for *Myrtus*/Oil it means 1 µl/ml. This could make a difference in the final concentration of the various compounds. Unfortunately, we could not gain access to a more detailed description of the concentrations of the components of *Myrtus*/EtOH. From qualitative analysis we know that the composition of both extracts is different, even though some ingredients overlap. They already differ on a visual basis, as *Myrtus*/EtOH has an intense brown color, while *Myrtus*/Oil is clear.

4.2 Effect on keratinocyte barrier function

Interestingly, the results of this study showed that Myrtus/Oil at 1:5,000 dilution (0.02 µl/ml) improves keratinocyte barrier formation and reinforces the keratinocyte barrier that has already formed. Kirschner et al. (2013) have shown that transepithelial resistance which was used as a parameter for barrier function in this study mainly reflects the paracellular resistance of keratinocytes due to functional TJs and transcellular resistance due to membranes only to a minor degree. Thus, it is likely that the effect seen here is mediated by changes of TJ functionality. However, it cannot be excluded that Myrtle extract also affects cell membranes and might therefore also improve transcellular barrier function. This should be investigated in future experiments.

At 1:1,000 dilution, Myrtus/Oil significantly impairs barrier formation but shows no effect on the preformed barrier. One explanation for this result could be the cytotoxic effect of the extract during barrier formation which would, independent from the functionality of TJs, reduce TER. However, MTT assays performed at the end of the TER experiments showed no negative influence of the 1:1,000 dilution on cell viability. Thus, it seems to be a specific effect of Myrtus/Oil on TJs which should be investigated in more detail in future.

It is generally supposed that Myrtus/Oil could be used dose-dependently to reduce/enhance TJ formation. In the preformed barrier, it might be interesting to investigate a concentration between 1:500 and 1:1,000 which might be able to open TJs without showing cytotoxicity.

Again, as for cell viability, the different susceptibility of cells from different donors on the effects of Myrtus/Oil could be observed.

This is to our knowledge, the first description of the effect of Myrtus/Oil extracts on the keratinocyte barrier. Thus, concerning the mechanism of how Myrtus/Oil affects the keratinocyte/TJ barrier nothing has been known up to now. The next steps would be to investigate the influence of Myrtus/Oil on TJ mRNA and protein expression, in addition to TJ protein localization.

Because of the known influence of Myrtus/Oil on inflammation (Feisst et al. 2005) and the effects of inflammatory cytokines such as IL1-β and TNF-α, on

TJ protein expression and TER (Kirschner et al. 2009), the involvement of cytokines might be possible and should be taken into account.

Results of this study may provide a starting point for other studies in the skin field. Studies by Kirschner et al. on psoriasis (Kirschner et al. 2009), Feldmeyer et al. on NISCH syndrome (Feldmeyer et al. 2006), Ohnemus et al. on infections due to *Staphylococcus aureus* (Ohnemus et al. 2007) and DeBenedetto et al. on atopic dermatitis (De Benedetto et al. 2011), are indicative of skin barrier and TJ damage. Therefore, further studies on the effects of Myrtus extract on these diseases can be recommended.

5. Summary

M. communis extracts were described to have beneficial effects in the treatment of infectious diseases. The question arises, whether these effects are only due to the previously described antibacterial, antiviral and antifungal effects of the extracts, or whether they might also have a barrier strengthening effect, thus preventing the uptake of pathogens. To investigate this question, an ethanolic and an oily extract of Myrtus leaves from Iran were investigated. In the first step, the influence of both extracts on cell viability was tested. Because a 10% solution of the oily extract is already used for the treatment of aphthae and herpes simplex, this concentration was the starting point of the experiments.

Both extracts were toxic when applied as a 10% solution as well as 1:100 dilutions of this solution in short term (20h) and long term (5d) experiments, as well as in low (undifferentiated) and high (differentiated) calcium conditions. At 1:500 dilution ethanol extract was toxic in all conditions, while Myrtus/Oil was only toxic in long term experiments. Myrtus/EtOH was also toxic at 1:1,000, and 1:5,000 dilutions in long term, but not in short term experiments. There were no negative effects of Myrus/Oil in short-term and long-term experiments at high calcium conditions, while there was a negative effect at low calcium conditions.

Subsequently, Myrtus/Oil extract in dilutions 1:1,000 and 1:5,000 were studied concerning keratinocyte barrier performance (transepithelial electrical resistance (TER) during calcium-dependent formation of TJs and after the

formation of TJs. During TJ formation Myrtus/Oil at 1:1,000 dilution impaired barrier formation and at 1:5,000 dilution it improved barrier formation in primary keratinocytes (72h after application). After the formation of TJs, this extract at 1:1,000 dilution resulted in a slightly improved barrier function in keratinocytes 24h after application and at 1:5,000 dilution, and it clearly improved barrier function in keratinocytes 24h, 48h and 72h after application.

In summary, this study showed that Myrtus/Oil extract has a dose-dependent positive or negative effect on the formation of the keratinocyte barrier. On the preformed barrier, there was a positive effect at 1:5,000 dilution. Higher concentrations of the extract resulted in cell toxicity and therefore it has a negative effect. It might be beneficial to dilute the extract that is already currently used in disease management, on account of its antiviral effects, in order to avoid any negative effects on the viability of surrounding keratinocytes.

6. Recommendations for future studies

- Examining the mechanism of Myrtus extract effect on TJ proteins
- Examining Myrtus extract effect on ichthyosis disorders
- Examining Myrtus extract effect on psoriasis
- Examining Myrtus extract effect on skin infections
- Analysis of Myrtus extract and its effective constituents
- Examining Myrtus extract effect on cosmetic therapies (repairing UV damage and maintaining elasticity of the skin)
- Examining Myrtus extract effect on inflammatory factors like interleukin and TNF- α
- Examining Myrtus extract effect on special factors involved in covering skin cell proliferation and differentiation

7. List of abbreviation

- °C Centigrade
- Ca²⁺ Calcium
- CaCl₂ Calcium chloride
- Cldn Claudin
- Cm² Square centimeter
- CO₂ Carbon dioxide
- d Day
- EDTA Ethylene diamine tetraacetic acid
- ELISA Enzyme linked immunosorbent assay
- EtOH Ethanol
- FCS Fetal calf serum
- g Gravity (acceleration to gravity)
- h hour
- IL1-β Interleukin-1 beta
- JAM Junctional adhesion molecule
- HaCaT Human adult low calcium high temperature
- K Keratinocytes
- m² Square meter
- Malp-2 Mycoplasma macrophage-activating lipopeptide-2
- M. communis Myrtus communis
- mg Milligram
- ml Milliliter
- mM Millimol
- mm Millimeter
- μg Microgram
- μl Microliter
- μm Micrometer
- mRNA Messenger RNA
- MTT Methylthiazole tetrazolium
- MUPP-1 Multi-PDZ domain protein
- NISCH Neonatal ichthyosis-sclerosing cholangitis

- nm Nanometer
- PBS Phosphate-buffered saline
- PKC Protein kinase-c
- ppm Parts per million
- Rh Rhesus Factor
- R.Solani Rhizoctonia solani
- RT Room temperature
- Ω Ohm
- S. aureus Staphylococcus aureus
- S. epidermidis Staphylococcus epidermidis
- Sb Stratum basale
- SC Stratum corneum
- SG Stratum granulosum
- Ssp Stratum spinosum
- STD Standard deviation
- TAMPs TJ associated mARVEL proteins
- TER Transepithelial electrical resistance
- TGF Transforming Growth Factor
- TJ Tight Junction
- TLR2 Toll-like receptor 2
- TNF α Tumor necrosis Factor α
- UV Ultraviolet
- ZO-1 Zonula Occludens Protein 1

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