PERGAMON

Phytochemistry 58 (2001) 949–958

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

High molecular compounds (polysaccharides and proanthocyanidins) from *Hamamelis virginiana* bark: influence on human skin keratinocyte proliferation and differentiation and influence on irritated skin

Alexandra Deters[a], Andreas Dauer[a], Esther Schnetz[b], Manige Fartasch[b], Andreas Hensel[a,*]

[a]*Hochschule Wädenswil, University of Applied Sciences, Pharmaceutical Biotechnology, Glycopharmacy Research Group, Gruental, CH-8820 Wädenswil, Switzerland*

[b]*Department of Dermatology, Glycopharmacy Research Group, University of Erlangen-Nürnberg, Hartmannstr. 43, D-91052 Erlangen, Germany*

Received 17 July 2001; received in revised form 10 August 2001

Abstract

Although extracts from *Hamamelis* bark have long been used in therapy of skin diseases and in cosmetic formulas there are only few pharmacological investigations verifying the activity of distinct *Hamamelis* bark constituents. Therefore two major classes of constituents, namely polymeric proanthocyanidins and polysaccharides were isolated from *Hamamelis* bark and tested concerning their influence on proliferation and differentiation of cultured human keratinocytes. While the polysaccharide fraction, consisting mainly of arabans and arabinogalactans, did not effect human keratinozytes, the proanthocyanidins strongly increased the proliferation of the cells, while the differentiation was not influenced significantly. Within a preliminary cumulative in vivo study on SLS-irritated skin, proanthocyanidins (ProcyanoPlus) were proven to reduce transepidermal water loss and erythema formation. Furthermore, a clinical scoring indicated that procyanidins can influence irritative processes significantly.

Keywords: *Hamamelis virginiana* L.; Polysaccharides; Araban; Arabinogalactan; Polysaccharides; Proanthocyanidins; Skin; Keratinocytes; Proliferation; Differentiation; Clinical study

1. Introduction

Extracts and destillates from *Hamamelis* bark and leaves are widely used as part of skin care products and in dermatological treatment of sun burn, irritated skin, atopic eczema (Korting et al., 1995) and to promote wound healing via anti-inflammatory effects (Korting et al., 1993). Several reports from traditional medicine indicate the efficacy of *Hamamelis* preparations in dermatology (Laux and Oschmann, 1993; Swoboda and Meurer, 1991). On the other side the clinical effects of this herbal drug in dermatology are proven only by very preliminary studies (Erdelmeier et al., 1996) while no information is available on pharmacological in vitro activities of *Hamamelis* ingredients on relevant skin cells to get a positive in vivo–in vitro correlation. However, the compounds responsible for the dermatological activity, are not identified clearly yet. It is assumed that the tannin fraction, consisting of hydrolysable and condensed tannins, which acounts up to 12% for *Hamamelis* bark (Hoffmann-Bohm et al., 1993) may contribute to the efficacy. Nevertheless, this was not proven unambigiously up to now.

During aqueous extraction of the plant material the presence of higher amounts of polysaccharides gets obvious. As some high-molecular carbohydrates are known to influence skin physiology in some cases quite extensively (Kitamura et al., 1997; Yagi et al., 1997; Chithra et al., 1998; Strickland et al., 1999; Eskelinin and Santalahti, 1992) the polysaccharide fraction of *Hamamelis* also may contribute to the dermatological activity.

* Corresponding author. Tel.: +41-1789-9670; fax: +41-1789-9950.

E-mail address: a.hensel@hswzfh.ch (A. Hensel).

0031-9422/01/$ - see front matter
PII: S0031-9422(01)00361-2

The aim of the present study was first to investigate the structural features of the *Hamamelis* bark polysaccharides and secondly to evaluate the dermatological potential of these polysaccharides and of high-molecular proanthocyanidin fraction on in vivo and in vitro skin proliferation and skin differentiation in keratinozyte in vitro culture.

A widely used pharmacological test system for such investigations are in vitro submerged cell cultures of human keratinocytes as a dynamic proliferating and differentiating system. This in vitro system represents the complex differentiation process of epidermal skin, leading to the anuclear cells filled with fibrous keratin and getting plasticized by hydration (Ponec and Kempenaar, 1995; Gibbs et al., 1997; van der Sandt et al., 1999). Beside toxic effects, different reaction patterns of keratinocytes towards exogenous test compounds can be detected using this system: compounds inducing cell differentiation can be separated from substances promoting the differentiation by increased formation of keratins K1 and K10. Therefore submerged keratinocytes allow detailed statements of potential mode of action of the test compounds.

2. Results

2.1. Isolation, fractionation and characterization of *Hamamelis* polysaccharides

Raw polysaccharides (RPS) with a carbohydrate content >99% and residual protein impurities of 0.4% were isolated from *Hamamelis* bark in a yield of 0.4%. A 1% solution of RPS showed kinematic viscosity of 1080 mPa s^{-1}, indicating that the mucilage consists of polymers with a low degree of polymerisation.

Fractionation of RPS by IEC resulted in one neutral and three acidic polymer fractions (Table 1). Each fraction was further separated by GPC on Sephacryl S-300 to yield, finally, 10 subfractions which were characterized concerning average molecular weight, uronic acid content, monosaccharide composition and linkage types of the respective monomers.

Neutral fractions H I.2 and H I.3 with quite low molecular weights between 4 and 13 kDa consisted mainly of arabinose. Linkage analysis of these fractions and GC–MS identification of the partially methylated alditol acetates indicated the presence of highly branched arabans with 1,3- and 1,3,5-linked arabinose residues (Table 2). This linkage pattern can be correlated to

Table 2
Linkage analysis of polysaccharide fractions H I and carboxyl-reduced fractions H II and III. Data indicate the respective molar composition

Carbohydrate	Linkage type	H I.1	H I.2	H I.3	H II.1	H III.1
Arabinose	1-		18	14	18	20
	1,2-	27	47	45	14	14
	1,3,5	15	31	33	4	3
0.5						
Xylose	1-	2				
	1,2-	2				
0.5						
Rhamnose	1,2,3-					2
	1,2,4-					7
0.5						
Glucose	1,4-	18	2	7	6	6
	1,4,6-	6	1	3		
0.5						
Galactose	1-				1	2
	1,3-	7			101	17
	1,6-				2	1
	1,3,6-	21			25	8
0.5						
Mannose	1,4-					
0.5						
Galacturonic acid[a]		2	1	1	18	20

[a] Determined as (1→4)-galactose.

Table 1
Fractionation of *Hamamelis* raw polysaccharides (RPS)

RPS									
Neutral fraction H I (13%)			0.1 M Eluate H II (41%)		0.25 M Eluate H III (26%)			0.5 M Eluate H IV (20%)	
H I.1	H I.2	H I.3	H II.1	H II.2	H III.1	H III.2	H III.3	H IV.1	H IV.1
6% of H I	38% of H I	56% of H I	62% of H II	38% of H II	55% of H III	29% of H III	16% of H III	15% of H IV	85% of H IV
126 kDa	13 kDa	4 kDa	158 kDa	50 kDa	79 kDa	16 kDa	5 kDa	126 kDa	4 kDa
			2% Rha	39% Rha	9% Rha	45% Rha		12% Rha	31% Rha
35% Ara	96% Ara	93% Ara	39% Ara	15% Ara	33% Ara	20% Ara	3% Ara	30% Ara	19% Ara
3% Xyl			tr.[b] Xyl	11% Xyl	tr. Xyl				
9% Man			4% Man				11% Man		
26% Gal			37% Gal	15% Gal	27% Gal	19% Gal		22% Gal	13% Gal
21% Glc	tr. Glc	4% Glc	6% Glc		8% Glc		45% Glc		
6% UA[a]	3% UA	3% UA	22% UA	20% UA	23% UA	16% UA	41% UA	36% UA	37% UA

[a] UA, uronic acids.
[b] tr., traces.

an araban with 1,3-backbone and side chains of 1,2-arabinose units, attached to the backbone via O-5 (Fig. 1).

The high molecular fraction H I.1 consisted mainly of arabinose and galactose in the ratio of about 3 to 2. The respective linkage pattern showed the existence of a typical neutral type II arabinogalactan with a 1,3-galactose backbone and branched arabinose side chains attached via O-6. The proportion of 1,2- and 1,4,6-linked glucose was determined to origin from starch.

All neutral fractions were contaminated with minor amounts of a glucan with 1,4- and 1,4,6-linked glucose. These carbohydrates may derive from α-linked starch, as the respective β-linked glucans would not be soluble by the aqueous extraction.

Acidic fractions consisted of a variety of heterogenous acidic arabinorhamnogalacturonans: the main chain of these polymers is build up by 1,3-linked galacturonic acid units; inserted 1,2-linked rhamnose units bear more or less branched arabinose-galactose side chains which are attched to the backbone via three-fold linked rhamnose units. These acidic polymers are the main compounds of fractions H II.2, H III.1, H III. 2, and H IV.1 and 2.

Fractions H II.1 and H III.3 additionally were composed by mannoglucans, typical reserve polysaccharides with β-1,4-mannose-glucose blocks, similar to that found in several *Digitalis* species (Hensel et al., 1998).

2.2. Influence of Hamamelis polysaccharides on keratinocytes

Raw polysaccharides (RPS) from *Hamamelis* bark were tested in concentrations of 0.1, 1, 10 and 50 μg/mL. After about three days of incubation it was obvious by mircroscopic observation that cells treated with 10 and 50 μg RPS showed nearly the same proliferation and differentiation behaviour as the untreated control groups.

Evaluation of proliferation rates by BrdU-incorporation indicated no significant influence of RPS after 10 days of incubation (Fig. 2A). The keratin K1 and K10

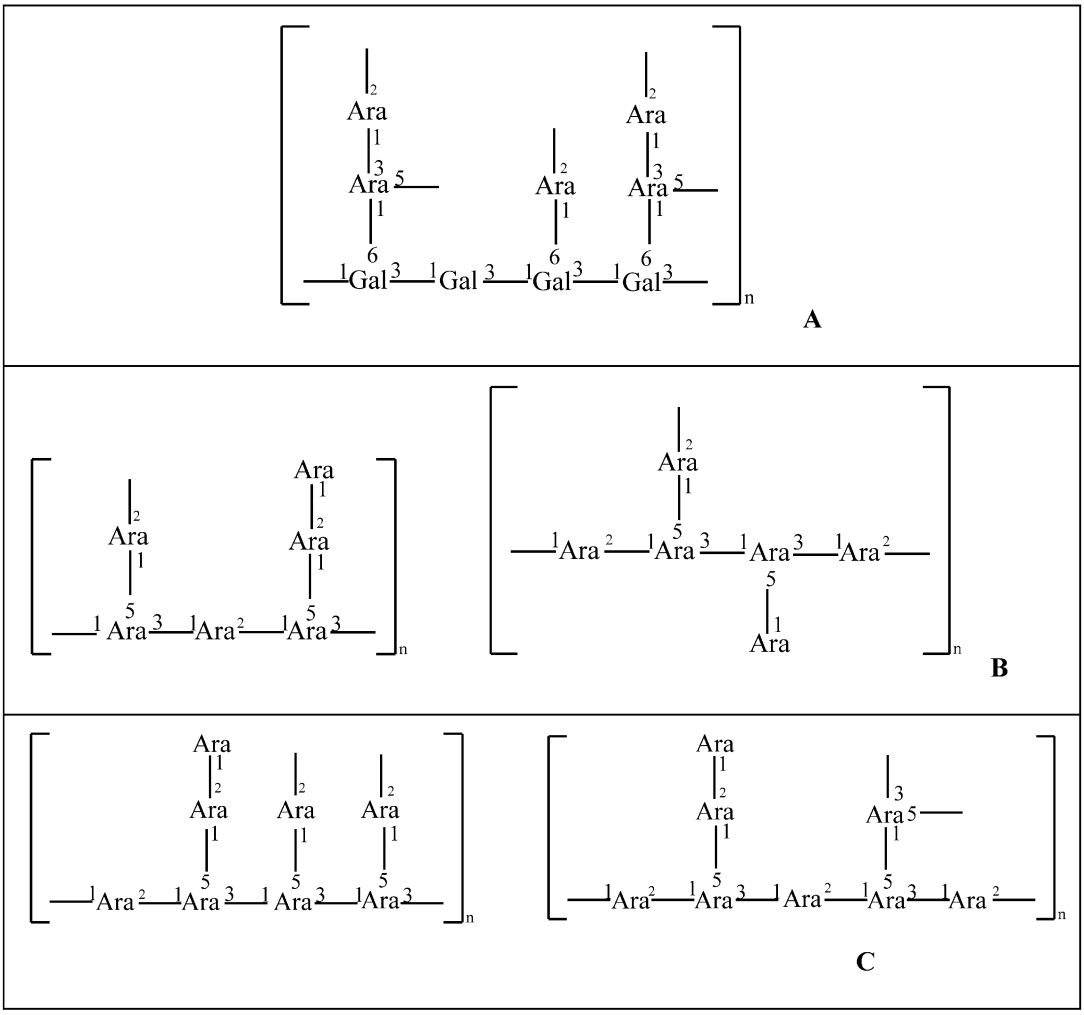

Fig. 1. Proposed structures of polysaccharides from *Hamamelis virginiana*: general structure of type II arabinogalactan H I.1 (A), neutral araban H I.2 (B), neutral araban H I.3 (C). The degree of substitution and length of side chains may vary among the polymers by heterodispersity.

formation also was not influenced significantly by RPS (Fig. 2B).

Mitochondrial activity of RPS-treated keratinocytes was significant lower for the cells treated with 10 and 50 μg polysaccharides compared to the untreated control. This phenomenon may be attributed to an adhesive polymer layer on the outer cell surface with an inhibited energy flux into the cells.

LDH values were on control levels for all groups, indicating RPS to be non-toxic.

Summarizing *Hamamelis* polysaccharides have no influence on proliferation, differentiation and metabolic activity of human keratinocytes.

2.3. Influence of polymeric proanthocyanidins on human keratinocytes

Two fractions, B and C, of polymeric proanthocyanidins have been isolated from *Hamamelis* bark and characterized according to methods described in a recent study (Dauer et al., 1998; Dauer, 2000). These fractions with a high purity (>95% calculated as catechin) were characterized as proanthocyanidins consisting of 3-*O*-galloylated catechin- and 3-*O*-galloylated epicatechin units with 4⇒8-interflavan linkage. Galloylation occurred only at the *O*-3 position while all other positions were not esterified. The respective polymers showed average molecular weights of about 1500 Da (fraction B) and 3400 Da (fraction C) determined by GPC and thiolytic degradation.

Toxic effects of both tannin fractions against human keratinocytes were not observed in the dose range from 1 to 100 μg/ml as evaluated by the respective lactatdehydrogenase titers, which were on control level (<5 U/ml) over the complete time of incubation.

Treatment of keratinocytes with both fractions B and C for 9 days resulted in the formation of typical cell monolayers which differed from the untreated control groups by the degree of confluence: while the procyanidin-treated groups formed complete monolayers, the control groups showed only about 40 to 50% confluence at the same time.

As expected from these observations, a signficant increase of cell proliferation was found for the procyanidin-treated groups, as determined by bromo-deoxyuridine (BrdU)-incorporation into the keratinocyte DNA and subsequent quantification by anti-BrdU-antibodies (Fig. 3). After 9 days of incubation fraction B enhanced the cell proliferation rates in doses of 1 and 10 μg/ml significantly, while fraction C had no effects at 1 μg level, but exceeded significant proliferation rates at 10 μg/ml. All further studies were performed with fraction C because of the limited availability of fraction B.

When monitoring the cell proliferation rates during incubation procyanidin fraction C significant differences to the control groups occured after 6 days of incubation: a time-dependent increase of cell growth compared to the control group was observed. Differences were significant after an incubation period of 6 days (Fig. 4). This indicates that the procyanidins do not act when added to the incubation medium as an punctuell stimulus, but have to be applied continously over longer time periods to stimulate growth processes.

Mitochondrial activity of the keratinocytes was determined by MTT-test by feeding fraction C in doses

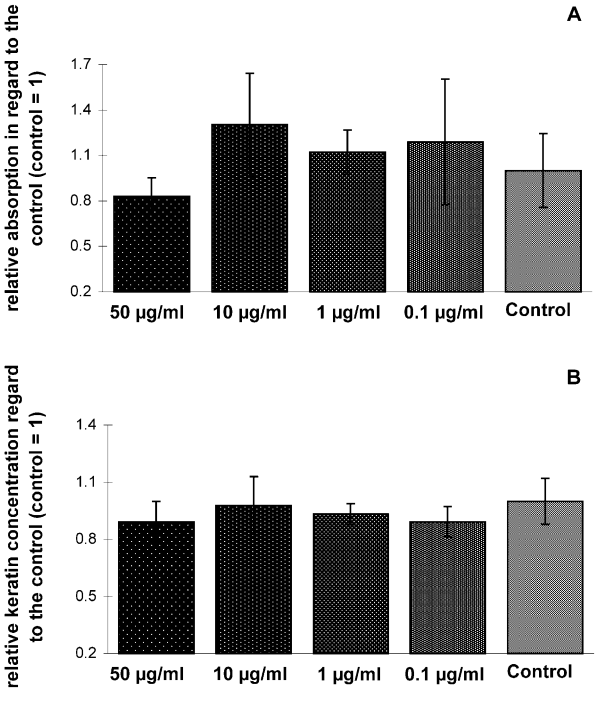

Fig. 2. Effects of raw polysaccharides from *Hamamelis virginiana* on cell proliferation (A) and differentiation (B) of normal human keratinocytes in different concentrations after 9 days of incubation. Results, determined by BrdU-incorporation in cell DNA and keratin K1/K10 determination (ELISA) are the mean±SD ($n=5$) and are not significant to the untreated control groups.

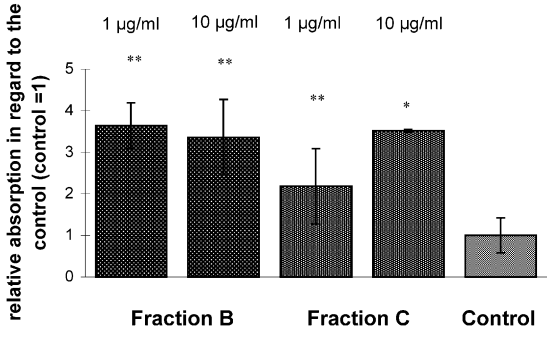

Fig. 3. Effects of oligomeric procyanidin fractions B and C (ProcyanoPlus) from *Hamamelis virginiana* on cell proliferation of normal human keratinocytes in concentrations of 1 and 10 μg/ml after 9 days of incubation. Results, determined by BrdU-incorporation in cell DNA, are the mean±SD ($n=10$) with $*P>0.1$ and $**P>0.05$ in comparison to the untreated control groups.

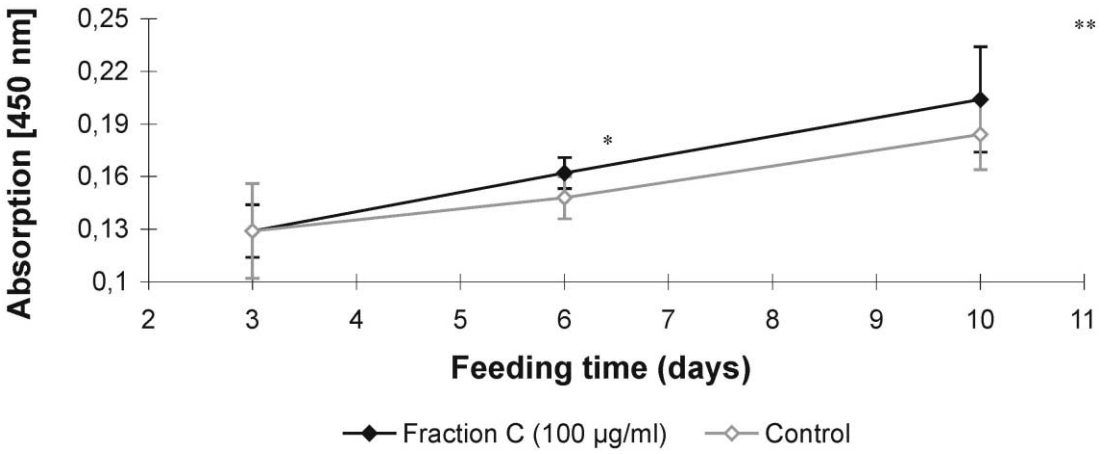

Fig. 4. Effect of oligomeric procyanidin fraction C (ProcyanoPlus) on cell proliferation of normal human keratinozytes over a 10 days incubation period as dertermined by BrdU-incorporation test (*$P > 0.1$ and **$P > 0.05$ in comparison to the untreated control groups).

of 1 and 10 µg/ml (Fig. 5). Significant increase of mitochondrial activity were observed with both concentrations and indicated an induction of metabolic activity by contact of the cells with fraction C.

In addition to these parameters, typical differentiation-specific keratins K1 and K10 (Muzarelli et al., 1999; Lane et al., 1985) were surveyed in the cells treated with fractions B and C with high-dose calcium as positive control. At doses of 1 and 10 µg/ml no significant increase of keratin formation was observed in procyanidin-treated groups (Fig. 6). This clearly indicates that the condensed tannins only induce keratinocyte proliferation, but do not act on the differentiation towards cornified cells.

2.4. Influence of procyanidins on irritated skin

For preliminary in vivo evaluation of *Hamamelis* procyanidins on irritated skin a semi-solid formulation containing 1% of *Hamamelis* procyanidin (ProcyanoPlus) was tested on its efficacy in preventing an sodium lauryl sulfate (SLS) induced irritant contact dermatitis in a standardized repeated short-time occlusive irritation test (ROIT) (Fartasch et al., 1999; Schnetz et al., 2000).

Over a three day period, defined skin areas of healthy volunteers were irritated using 0.5% SLS and with procyanidins in aqueous Eucerin®. To evaluate the influence of the pure cream formulation aqueous "placebo" Eucerin® without procyanidins was used additionally. One test site (positive control) received the irritant alone, two further test sites served as control (remained untreated or water).

The results of the TEWL correlated well with the results of the clinical score and the colorimetry. In all cases an irritation with SLS—with or without test product—had led to an increase of the TEWL values in comparison to the untreated area. This shows clearly that a local barrier disruption had occurred. By the pretreatment with ProcyanoPlus in aqueous eucerine and by pretreatment with eucerine alone the intensity of

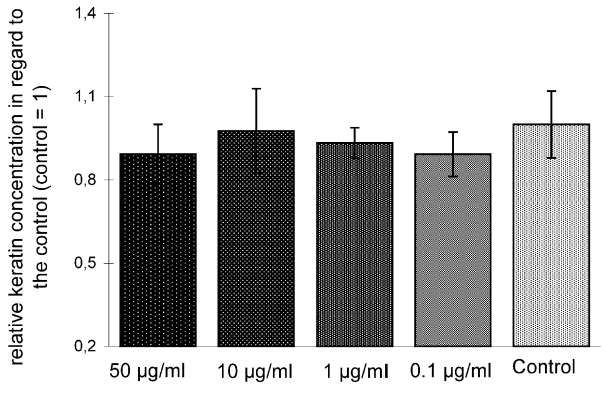

Fig. 5. Effects of oligomeric procyanidin fractions B and C (ProcyanoPlus) from *Hamamelis virginiana* on cell differentiation of normal human keratinocytes in concentrations of 1 and 10 µg/ml after 9 days of incubation. Results, determined by quantification of keratin K1, K10 concentrations (ELISA), are not significant to the untreated control groups.

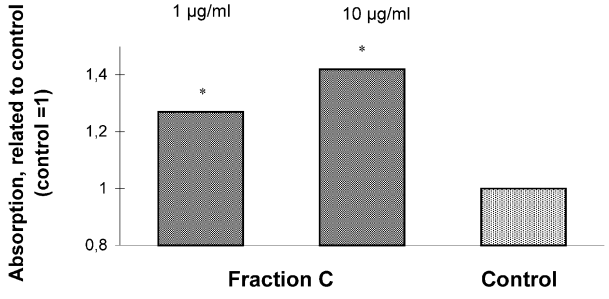

Fig. 6. Effects of oligomeric procyanidin fraction C (ProcyanoPlus) from *Hamamelis virginiana* on mitochondrial activity of natural human keratinocytes in concentrations at 1 and 10 µg/ml level after 9 days of incubation. Results, determined by MTT-test, are the mean ± SD ($n = 10$) with *$P > 0.1$ in comparison to the untreated control groups.

the reaction was diminished (Fig. 7). This effect was clearly more pronounced when ProcyanoPlus was applied. Treatment of the SLS-irritated skin areas with "placebo" aqueous Eucerin® reduced TEWL-increase for 45%, in comparison to SLS induced TEWL enhancement. Treatment with the procyanidin-containing formulation decreased TEWL significantly by 53%.

2.4.1. Clinical score

Treatment with 0.5% SLS caused an increase in the clinical scoring as a measure of dermal inflammation (Fig. 8). The clinical signs of an inflammation were clearly reduced by treatment with either aqueous Eucerine of ProcyanoPlus. But the effect of ProcyanoPlus was significantly better compared to pure eucerine.

Similar results were obtained by colorimetry measurements (Fig. 9), assessing skin color as a sign for erythema formation: irritation with SLS leads to strongly increased values, which are reduced when the irritated skin areas are treated with procyanidins.

3. Discussion

Information on pharmacological effects of high molecular compounds from *Hamamelis* bark is limited to only few reports due to the complexicity of the chemical structures of these compounds. Erdelmeier et al. (1996) reported on antiviral and antiphlogistic activities of high molecular weight proanthocyanidins from *Hamamelis* bark.

Up to now, no information is available about effects of *Hamamelis* plant metabolites on normal human keratinocytes. Furthermore, to our knowledge this is the first time that structural data about polysaccharides

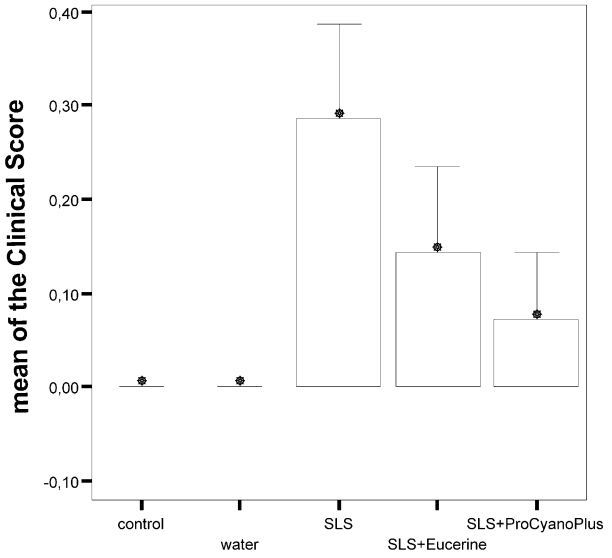

Fig. 8. Clinical scoring after 3 days cumulative irritation of skin by SLS 0.5% and treatment with aqueous Eucerin® or aqueous Eucerin® containing 1% fraction C (ProcyanoPlus). Bars represent the mean of the clinical score, error bars represent the standard error of the mean.

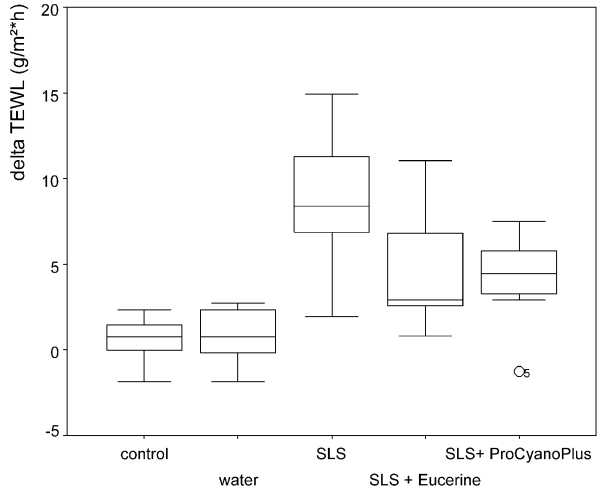

Fig. 7. Transepidermal water loss (TEWL) after a three days cumulative irritation of the skin by SLS 0.5% and treatment with aqueous Eucerin® or aqueous Eucerin® containing 1% fraction C (ProcyanoPlus). The distribution of data are presented as box plots. Box plots show the median, the 25th percentile, the 75th percentile, and values that are far removed from the rest. The lower boundary of the box is the 25th percentile, the upper boundary the 75th percentile; the line inside the box represents the median. Lines are drawn from the ends of the box to the largest and smallest observed values that are not outliers. Cases with values that are between 1.5 and 3 box lengths from the upper or lower edge of the box are called outliers (designated with the letter o.

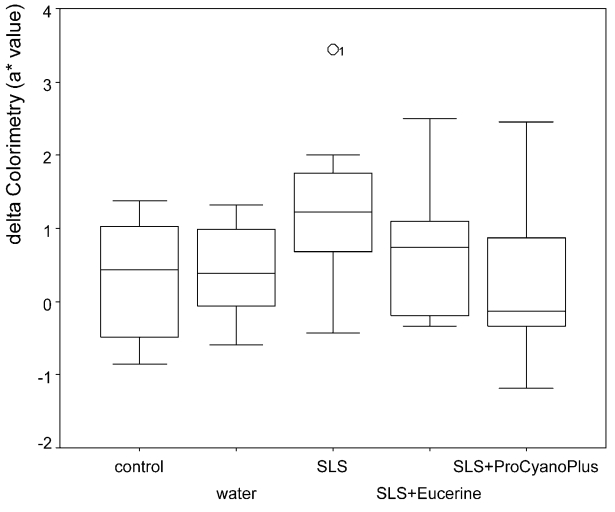

Fig. 9. Colorimetric data after a 3 day cumulative irritation of skin by SLS 0.5% and treatment with aqueous Eucerin® or aqueous Eucerin® containing 1% fraction C (ProcyanoPlus). The distribution of data are presented as box plots. Box plots show the median, the 25th percentile, the 75th percentile, and values that are far removed from the rest. The lower boundary of the box is the 25th percentile, the upper boundary the 75th percentile; the line inside the box represents the median. Lines are drawn from the ends of the box to the largest and smallest observed values that are not outliers. Cases with values that are between 1.5 and 3 box lengths from the upper or lower edge of the box are called outliers (designated with the letter o).

occurring in the bark of *Hamamelis virginiana* L. are provided.

This study focused on the effects of polymeric proanthocyanidins and polysaccharides isolated from the bark of *Hamamelis virginiana* on normal human keratinocytes.

Additionally, polysaccharides were included in this study, due to the fact that carbohydrates—also polysaccharides—are getting more and more into the focus of skin research because of antiphlogistic and proliferation enhancing effects as well as for stimulation of skin immune response (Berthod et al., 1994; Kitamura et al., 1997; Muzarelli et al., 1999).

For this reason, water-soluble polysaccharides were isolated in good yields from *Hamamelis* bark, fractionated and structurally characterized. Molecular weights, monomer composition and linkage types were determined. Principally, three different types of polysaccharides were identified: low molecular 1,3-linked arabans were the major polymers of the neutral fraction. While arabans are commonly 1,5-linked polymers the *Hamamelis* arabans represent quite unusual structures. Minor amounts of type II arabinogalactans, commonly occuring as cell structural components, were found additionally to the arabans in the neutral fraction of the mucilage. The heterogeneous acidic fractions consisted almost exclusively of rhamnogalacturonans with a high portion of neutral pentoses, mainly arabinose.

Testing of these polysaccharides on a dynamic keratinocyte cell system with the specific properties of forming differentiated, cornified cells after adequate stimuli did not indicate any positive carbohydate-mediated activity. Thus, polysaccharides cannot significantly contribute to the skin activity of *Hamamelis* bark.

In contrast, two different polymeric procyanidin fractions exhibited strong and dose-dependent induction of cell proliferation and mitochondrial activity of keratinocytes. Interestingly, these compounds did not influence the state of differentiation of keratinocytes. These results indicate, that *Hamamelis* proanthocyanidins only stimulate cell growth extensively, but do not influence the formation of cross-linked cornified envelopes (Thatcher and Rice, 1985). Thus, polymeric proanthocyanidins act as good proliferation stimulants for skin cells, leading probably to enhanced cell growth. As the effects were observed at low doses, likely to be reached under in vivo conditions in the skin epidermis the results may be of relevance for the respective in vivo situation. Similar inductors of cell proliferation, without induction to keratinized, differentiated cells are part of classical skin care products, as for example *Aloe vera* gel. From our point of view, it is promising that the tannins tested are not toxic towards the cells. The adstringent properties of the tannins do not have any negative influence on the cells within the dose-range tested.

In a preliminary clinical pilot study the procyanidins were tested within a cumulative irritation model on skin of healthy volunteers. All test parameters, including clinical scoring, transepidermal water loss and measurement of the skin colour, indicated clearly that the procyanidins positively influenced SLS induced irritation and skin barrier disruption.

Summarizing, high-molecular proanthocyanidins from the bark of *Hamamelis virginiana* L. can be assessed to be at least partly responsible for the medicinal use of this plant in dermatology and cosmetics. If and to what extent the hydrolysable and low-molecular condensed tannins, which are also present in the material contribute to the skin activity has to be evaluated in further studies.

Summarizing also, the results of this study demonstrate that polymeric proanthocyanidins from *Hamamelis virginiana* can be assessed to be at least partly responsible for the medicinal use of tannin-containing *Hamamelis*-preparations in dermatology and cosmetics by influencing keratinocyte proliferation.

4. Experimental

4.1. General

Heat-sterilization was performed either at 121 °C/1 h in an autoclave or at 175 °C/2 h in an oven. Statistical analysis was done using an unpaired student *t*-test.

Hamamelis polysaccharides were extracted from a batch of *Hamamelis viriginiana* bark, obtained in 1998 from Abtswinder Naturheilmittel, Abtswind, Germany. A voucher specimen is retained in the documentation center of the Institute of Pharmaceutical Biology, University of Erlangen.

Proanthocyanidins (patent pending) were obtained under the trade name ProcyanoPlus by DermoPlus-Hensel (Schlossbergstrasse 53, CH-8820 Wädenswil, Switzerland).

4.2. Isolation and fractionation of polysaccharides and procyanidins

Polysaccharide isolation: 200 g of powdered material were pre-extracted for 20 h in a Soxhlet system with acetone and subsequently for 30 h with methanol. The eluates were discarded. The residue was dried at 40 °C (154 g) and after addition of 30 g polyvinylpyrrolidone extracted three times with 770 ml of water. The combined extracts were concentrated to a 150 ml volume and slowly added into 600 ml 2-propanol. The precipitated raw polysaccharides (RPS) were centrifuged at 3600 *g*, dialysed and lyophilized to yield a nearly white powder (0.51% related to the dried starting material).

Fractionation of RPS was performed by IEC on DEAE-Sephacel (Pharmacia, Freiburg). Conditions: gel in the phosphate form, column dimension 20×2 cm, flow rate 80 ml/h, fraction size 5 ml, mobile phases: water, then step gradient with sodium phosphate buffers pH 6.0 with ion strength 0.1 mol/l, 0.25 mol/l, 0.5 mol/l. Carbohydrate-containing fractions were pooled, dialysed and lyophilized.

All fractions obtained from IEC were further fractionated by GPC on Sephacryl HR S-300 (Pharmacia, Freiburg). Conditions: column dimensions 1.9×77 cm, flow rate 25 ml/h, fraction size 2 ml, mobile phase: water. Carbohydrate-containing fractions were pooled, dialysed and lyophilized.

Isolation of proanthocyanidins (Dauer et al., 1998): the powdered *Hamamelis* bark (400 g) was percolated using acetone–water mixture in the ratio 7:3. The acetone was removed from the extract in vacuo and the aqueous residue was first extracted extensively with petroleum ether (rejected) and following with ethyl acetate (5×300 ml, rejected). The aqueous phase was freeze-dried to yield 47 g solid, brownish product. Of this 6.0 g were chromatographed on a Sephadex LH-20 column (Amersham-Pharmacia, Upsala) with ethanol (fraction A, yield 46%), methanol (fraction B, yield 26%) and acetone–water mixture (fraction C, yield 11%).

4.3. Polysaccharide analysis

Total carbohydrates were assayed with the phenol–sulphuric test (Dubois et al., 1956) and by resorcinol-test (Monsigny et al., 1988). Determination of uronic acids was performed using m-hydroxydiphenyl and galacturonic acid as reference material (Blumenkrantz and Asboe-Hansen, 1973). Carbohydrate-containing solutions were concentrated under reduced pressure at temperatures below 40 °C. Cellulose membranes with cut-off of 3500 Da were used for dialysis. The molecular weight distribution of polysaccharides was determined by GPC using standard dextrans (Sigma, Steinheim, Germany). Polysaccharides were hydrolysed with trifluoracetic acid 2 mol at 121 °C for 60 min. Alditol acetates of neutral sugars were prepared according to Blakeney et al. (1983). GLC was performed on a Hewlett-Packard HP 6890 system with mass selective detector at 220 °C with helium as carrier gas (pressure 1.3 bar) on a HP-225 fused silica capillary column (30 m×0.25 mm i.d., film thickness 0.25 μm).

Reduction of acidic polysaccharides to the carboxyl-reduced polymers was accomplished in the presence of carbodiimide and $NaBD_4$ as described by Taylor and Conrad (1972). Methylation analysis was performed according to the Hakamori method, modified by Harris et al. (1984).

4.4. Submerged keratinocyte culture

Keratinocytes were obtained either from commercial available cultures (Cell Systems, St Katharinen) or isolated from human skin, obtained by operative resection. Therefore the skin was decontaminated using a solution of fungizone (0.2%), gentamycin (5.9 μg/ml), sodium hydrogen carbonate (7.5%) in DMEM medium (Gibco, Karlsruhe). Tissue was dissected mechanically and after incubation of the tissue in dispase solution, dermis and epidermis were separated. The epidermis was trypsinated for 15 min at 34 °C with trypsin 0.05% and sodium edetate 0.53 mM. Reaction was stopped by the addition of TNS (Cell Systems, St Katharinen). Keratinocytes were centrifuged at 463 *g* for 5 min, resuspended in PBS, centrifuged again and resuspended in KGM-2 incubation medium in 96-well-tissue plates. Incubation was perfomed at 34 °C in a humidified 5% carbon dioxide atmosphere.

Secondary cultures were obtained at 60 to 70% confluence after trypsinisation. All cultures were free from contaminants of fibroblasts or melanocytes and were confluent after about 9 to 14 days of culture.

Feeding of the test substances to the keratinocytes was done after dissolving the compounds in KGM–2/DMSO mixtures. Positive controls were obtained from high-calcium media (Ca^{2+}, 1.5 mM), negative controls incubated in medium with no supplements.

4.5. Characterization of keratinocyte physiology

Growth rates were determined after trypsinisation using trypan-blue, mitochondrial activity by the MTT-test according to Mosmann (1983).

Lactat dehydrogenase (Amador et al., 1963) was quantified from the incubation supernatant using LDH-assay kit (Sigma, Steinheim).

Cell proliferation was determined by BrdU-kit (Boehringer, Mannheim) according to Porstmann et al., 1985.

For quantification of keratins K1 and K10 the cells were washed with PBS and keratins solubilized by adding 150 μl urea 8 M, β-mercaptoethanol 0.2 M and Tris 1.5 mM. Fifty microlitres of the solubilisate was transferred to a 96-well microtiter plate with 100 μl sodium carbonate buffer 0.05 M. The mixture was incubated for 18 h at 4 °C. After removal of excess of the keratin antigen and 1 h incubation with block medium (milk powder 7% in PBS with Tween 20, 0.05%) 100 μl of a monoclonal mouse-anti-human-keratin-antibody (Dako, Hamburg, diluted 1:400 in block medium) was added. The mixture was incubated at 37 °C for 60 min. After washing three times with 150 μl PBS containing Tween 20 0.05%, 150 μl POD-conjugated goat-anti mouse-IgG-antibody (Sigma, Steinheim, 1:2000 in block medium) was added and the mixture incubated at 37 °C for 60 min. After 3 washing steps, 100 μl of substrate solution (phenylendiamine 0.4 mg/ml in sodium

phosphate buffer 0.2 M, pH 5.0 and 11 μl hydrogen peroxide 30%) was added, the mixture reacted 10 min at room temperature and the reaction stopped by addition of 100 μl sulfuric acid 2.5 M. The absorbance was determined in an ELISA-reader at 492 nm. Calibration was performed using standard keratins.

4.6. Clinical testing

4.6.1. Test protocol (ROIT)

Fifty microliters of the irritant (aqueous SLS, 0.5%, purity of SLS >99%, Merck, Darmstadt, Germany) was applied twice daily under patch occlusion for 30 min, with an interval of 3–3.5 h. This schedule was followed for 3 consecutive days. Application was performed by means of a flat aluminum chamber (Large Finn Chambers®, inner diameter: 12 mm) with filter paper discs, fixed on Scanpor® tape. The middle part of both volar forearms was used as the test site with a total of 2 patches on each arm. One test site received the irritant alone, two other test sites were pretreated with the test products (ProcyanoPlus and aqueous Eucerine, 0.05 ml/2 cm^2 skin) 10 min before the irritant was applied. Two further test sites served as control sites (remained untreated or water). The procedure was repeated every day during the experiment. The placing of the products was changed from person to person according to a system of rotation (randomization).

4.6.2. Evaluation

All measurements were carried out in air-conditioned rooms (room temperature 20–22 °C, relative humidity between 30–45%, room temperature and humidity data being recorded during the measurements, applying the guidelines of the Standardization Group of the European Society of Contact Dermatitis (Pinnagoda et al., 1990; Fullerton et al., 1996). All measurements were performed after a rest of 30 min for equilibration. Measurements were performed in the morning of day 1 and in the afternoon of day 3.

4.6.3. Transepidermal water loss (TEWL) measurements

The quantitative measurements of TEWL were carried out using the Tewameter TM 210 (Courage and Khazaka, Cologne, Germany). The TEWL was calculated automatically and expressed in g/m^2 h. The Tewameter probe was used in a holding device to avoid heating of the probe. The probe was rested on the skin and TEWL was continuously recorded for a period of 3 min. Mean values were obtained from two successive recordings for every test site.

4.6.4. Skin temperature

Skin temperature was measured prior to TEWL measurements using a Digimed H11S device (ttw, Waldkirch, Germany) and had to range between 28–32 °C (Mathias et al., 1981).

4.6.5. Clinical examination

Visual scoring was performed according to the following scale: 0, no reaction; 0.5, slight scaling or very weak erythema; smooth surface; 1, weak erythema, possibly slight infiltration, slight roughness, slight scaling, mild edema, fine fissures; 2, erythema, more roughness, scaling and edema, fissures; 3, pronounced erythema, extensive scaling, pronounced edema, possibly vesicles, bullae, pustules and/ or pronounced crusting.

4.6.6. Colorimetry

For colorimetry, the measuring head of a Chroma-Meter CR 300 (Minolta, Ahrensburg, Germany) was applied to the skin avoiding pressure. Measurements were conducted in triplicate. For the measurements the L^* a^* b^* system was chosen, for this coordinates most closely reflects the color of human skin (Ale et al., 1996); a^* represents the green ($-a^*$)–red ($+a^*$) axis, therefore we used this coordinate for evaluation.

4.6.7. Volunteers

Seven healthy caucasian subjects (2 male, 5 female; age 22 to 38 years, mean age 29.8 years) with no history of atopic eczema or other skin diseases. Written informed consent was obtained from all of them. The study was approved by the ethical committee.

References

Ale, I., Laugier, J.P.K., Maibach, H.I., 1996. Spacial variability of basal skin chromametry on the ventral forearm of healthy volunteers. Arch. Derm. Res. 288, 774–777.

Amador, E., Dorfman, L.E., Wacker, W.E.C., 1963. Serum lactic dehydrogenase, an analytical assessment of current assays. Clin. Chem. 9, 391–395.

Berthod, F., Saintigny, F., Chretioen, D., Hayek, C., Collombel, C., Damou, O., 1944. Optimization of thickness, pore size and mechanical properties of a biomaterial designed for deep burn coverage. Clinical Mater. 15, 259–265.

Blakeney, A.B., Harris, P.J., Henry, R.J., Stone, B.A., 1983. A simple and rapid preparation of alditol acetats for monosaccharid analysis. Carbohydr. Res. 113, 291–299.

Blumenkrantz, N., Asboe-Hansen, G., 1973. New method for quantitative determination of uronic acids. Anal. Biochem. 54, 484–489.

Chithra, P., Sajithal, G.B., Chandrakasan, G., 1998. Influence of *Aloe vera* on the glucosaminoglycans in the matrix of healing dermal wounds in rats. J. Ethnopharmacol 59, 179–186.

Dauer, A., Metzner, P., Schimmer, O., 1998. Proanthocyanidines from the bark of *Hamamelis virginiana* exhibit antimutagenic properties against nitroaromatic componds. Planta Medica 64, 324–327.

Dauer, A., 2001. Untersuchungen zur antimutagenen Wirkung von Gerbstoffen aus der Rinde von *Hamamelis virginiana* L. in *Salmonella typhimurium* und menschlichen Hepatomzellen (HepG2). Dissertation, Universität Erlangen-Nürnberg.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–355.

Erdelmeier, C.A., Cinatl, J. Jr., Rabenau, H., Doerr, H.W., Biber, A., Koch, E., 1996. Antiviral and antiphlogistic activities of *Hamamelis virginiana* bark. Planta Medica 62, 241–245.

Eskelinin, A., Santalahti, J., 1992. Special natural cartilage polysaccharides for the treatment of sun-damaged skin in femals. J. Int. Med. Res. 20, 99–105.

Fartasch, M., Schnetz, E., Ennen, J., Lanzendörfer, G., Diepgen, T.L., 1999. Characterization of barrier alteration by repetitve irritation—effect of barrier cream. Zeitschrift für Hautkrankheiten 74, 1–6.

Fullerton, A., Fischer, T., Lauti, A., Wilhelm, K.P., Takiwaki, H., Serup, J., 1996. Guidelines for the measurement of skin colour and erythema. Contact Dermatitis 35, 1–10.

Gibbs, S., Vicanová, J., Bouwstra, J., Valstar, D., Kempenaar, J., Ponec, M., 1997. Culture of reconstructed epidermis in a defined medium at 33 °C shows a delayed epidermal maturation, prolonged lifespan and improved stratum corneum. Arch. Derm. Res. 289, 585–595.

Harris, P.J., Henry, R.J., Blakeney, A.B., Stone, A.B., 1984. A simple and rapid preparation of alditol acetats for monosaccharide analysis. Carbohydr. Res. 127, 59–73.

Hensel, A., Schmidgall, J., Kreis, W., 1998. The plant cell wall- a potential source for pharmacologically active polysaccharides. Helv. Chim. Acta 73, 37.

Hoffmann-Bohm, K., Ferstel, W., Aye, R.D., 1993. *Hamamelis*. In: Hänsel, R., Keller, G., Rimpler, H., Schneider, G. (Eds.), Hagers Handbuch der Pharmazeutischen Praxis, Vol. 5. Springer Verlag, Berlin, New York, pp. 368–384.

Kitamura, N., Ota, Y., Haratake, A., Ikemoto, T., Tanno, O., Horikoshi, T., 1997. Effects of ethyl-a-D-glycosid on skin barrier disruption. Skin Pharmacol. 10, 153–159.

Korting, H.C., Schäfer-Korting, M., Hart, H., Laux, P., Schmid, M., 1993. Anti-inflammatory activity of *Hamamelis* distillate applied topically to the skin. Influence of vehicle and dose. Eur. J. Clin. Pharmacol. 44, 315–318.

Korting, H.C., Schäfer-Korting, M., Klovekorn, W., Klovekorn, G., Martin, C., Laux, P., 1995. Comperative efficiacy of *Hamamelis* distillate and hydrocortisone cream in atopic eczema. Eur. J. Clin. Pharmacol. 48, 461–465.

Lane, E.B., Bartek, J., Purlcis, P.-F., Leigh, I.M., 1985. Keratin antigens in differentiating skin. Ann. NY Acad. Sci. 455, 241–258.

Laux, P., Oschmann, R., 1993. Die Zaubernuss *Hamamelis virginiana* L. Zeitschrift für Phytotherapie 14, 155–166.

Mathias, T., Wilson, D., Maibach, H.I., 1981. Transepidermal water loss as a function of skin temperature. J. Invest. Dermatol 77, 219–220.

Monsigny, M., Petit, C., Roche, A.-C., 1988. Colorimetric determination of neutral sugars by a resorcinol sulfuric acid mikromethod. Anal. Biochem. 175, 525–530.

Mosmann, M., 1983. Rapid colorimetric assay for cellular growth and survival: applications to proliferation and cytotoxicity assays. J. Immun. Methods 65, 55–163.

Muzarelli, R.A., Mattioli-Belmonte, M., Pugnaloni, A., Biagini, G., 1999. Biochemistry, histology and clinical uses of chitins and chitosans in wound healing. EXS 87, 251–264.

Pinnagoda, J., Tupker, R.A., Agner, T., Serup, J., 1990. Guidelines for transepidermal water loss (TEWL) measurement. Contact Dermatitis 22, 164–178.

Ponec, M., Kempenaar, J., 1995. Use of human skin rekombinants as an in vitro model for testing the irritation potential of cutanous irritants. Skin Pharmacol. 8, 49–59.

Porstmann, T., Ternyck, T., Avrameas, S., 1985. Quantitation of 5-bromo-2-deoxyuridine into DNA an enzyme immunoassay for the assesment of the lymphoid cell proliferative response. J. Immunol. Methods 82, 169–179.

Schnetz, E., Diepgen, T.L., Elsner, P., Frosch, P.J., Klotz, A.J., Kresken, J., Kuss, O., Merk, H., Schwanitz, H.J., Wigger-Alberti, W., Fartasch, M., 2000. Multicentre study for the development of an in vivo model to evaluate the influence of topical formulations on irritation. Contact Dermatitis 42, 336–343.

Strickland, F.M., Darvill, A., Albersheim, P., Eberhard, S., Pauly, M., Pelley, R.P., 1999. Inhibition of UV-induced immun suppression and interleukin-10 production by plant oligosaccharides and polysaccharides. Photochem. hotobiol 69, 141–147.

Swoboda, M., Meurer, J., 1991. Therapie von Neurodermitis mit *Hamamelis virginiana*-Destillat in Salbenform. Zeitschrift für Phytotherapie 12, 114–117.

Taylor, R.T., Conrad, H.E., 1972. Stochiometric depolymerization of polyuronides and glycosaminoglycans to monosaccharids following reduction of their carbodiimid-activated carboxyl groups. Biochem. 11, 1383–1388.

Thatcher, S.M., Rice, R.H., 1985. Keratinocyte-specific transglutaminase of cultured human epidermal cells: Relation to cross-linked envelope formation and terminal differentiation. Cell 40, 685–695.

van der Sandt, J., Roguet, R., Cohen, C., Esdaile, D., Ponec, M., Cosini, E., Barker, C., Fusenig, N., Liebsch, M., Benford, D., de Brugerolle de Fraissinette, A., Fartasch, M., 1999. The use of keratinocytes and human skin models for predicting skin irritations. ATLA—Alternatives to laboratory animal 27, 723–755.

Yagi, A., Egusa, T., Arase, M., Tanabe, M., Tsuji, H., 1997. Isolation and characterization of the glycoprotein fraction with a proliferation-promoting activity on human and hamster cells in vitro from Aloe vera gel. Planta Med. 63, 18–21.