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Cell and tissue culture-based *in vitro* test systems for evaluation of natural skin care product ingredients

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Abstract

Ingredients of natural origin are increasingly used for cosmetic and personal care product formulations. Characteristics of new natural and organic brand cosmetic products need to be determined, including safety profile and efficacy potential. A number of *in vitro* tests can be considered suitable to provide estimation of the safe and effective concentration range for biologically active ingredients from established and novel sources. Since animal testing of cosmetic ingredients and final compositions is now banned in the European Union, *in vitro* cell and tissue culture based methods have to provide an alternative. Various skin cell monoculture test systems and more complex models such as cell co-cultures and three-dimensional organotypic tissue cultures are reviewed. In the present paper references to study reports and protocols that have been used to characterize a variety of natural origin ingredients using *in vitro* cell and tissue test systems are summarized.

Key words: cell culture, cosmetic products, natural ingredients, *in vitro* test, co-culture, organotypic tissue.
Abbreviations: CSC, constitutive skin colour; DED, de-epidermized dermis; ECM, extracellular matrix; EU, the European Union; HDMVEC, human dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; MMPs, matrix metalloproteases; ROS, reactive oxygen species; TIMPs, tissue inhibitors of MMPs; UVR, ultraviolet radiation; 3D, three-dimensional.

Introduction

There is an increasing demand for cosmetic and personal care products that are based on substances of natural origin and exclude organic chemically derived synthetic compounds. Novel natural sources for ingredients have been identified and there are new extracts that need to be characterised. Different extraction methods and starting material differences result in natural ingredients with the same name but different composition and biological activity. While natural substances contain biologically active compounds of considerable concentration and potency, their safe and efficacious concentration ranges may remain untested. Consequently, manufacturers of natural and organic brand cosmetics claim safety and efficacy of their products in the absence of experimental supportive evidence (Nohynek et al. 2010; Antignac et al. 2011). Regulations for cosmetics address safety of the products to a certain extent, e.g. in the European Union (EU), by provision of a list of ingredients that are prohibited or limited to certain cosmetic products, microbiological quality standards and other general safety requirements. Regulation (EC) No 1223/2009 contains a list of substances that are prohibited in the composition of cosmetic products (Annex II) and a list of substances that are subject to restrictions or specific conditions of use (Annex III). According to the Regulation, the safety of finished cosmetic products can already be ensured on the basis of knowledge of the safety of the ingredients that they contain, which can be a theoretical assessment. Besides assessment of available published data the application of experimental test methods is encouraged, in particular for small and medium-sized enterprises. Natural and organic brand cosmetic consumers have become better informed and express interest in questions related to product claims and whether these are substantiated with experimental data.

In the EU Regulation, "cosmetic products" are defined as substances or preparations intended to be placed in contact with various external parts of the human body for cleaning, perfuming, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition. In the US, cosmetic products are usually referred to as personal care products (EU 2003; Morganti, Paglialunga 2008; Antignac et al. 2011). In the EU, safety assessment guidelines of cosmetic products and their ingredients have been provided by the Scientific Committee on Consumers Products and guidance for testing of cosmetic products has been published by Scientific Committee on Consumer Safety (SCCP 2006; SCCS 2012). Safety of ingredients and final formulations should be assessed using *in vitro* tests that replace animal testing with alternative test methods. Currently the EU legislation for cosmetics bans all kinds of animal testing for cosmetic ingredients, final compositions and marketing of animal-

tested cosmetics (EU 2003; 2006; Morganti, Paglialunga 2008; EU 2009; Pauwels, Rogiers 2010). With regard to animal-based repeated-dose toxicity tests, reproductive toxicity tests, and toxicokinetics, the marketing prohibition applied from March 2013. This prohibition is applicable regardless of the availability of alternative test methods.

Requirements for documentation of efficacy claims are only fragmentary and the present regulatory framework is limited. Cosmetics manufacturers in Europe have to substantiate claims made on cosmetic products to protect consumers from being misled. The EU Cosmetics regulation placed a label obligation on marketers of cosmetics to have supporting evidence available for product claims. Texts, names, trademarks, pictures and figurative or other signs should not be used to imply that cosmetic products have characteristics or functions that they do not have (Serup 2001; EU 2009).

In vitro testing can be applied for ingredient and final product safety assessment and justification of efficiency claims. Currently, *in vitro* testing is mostly applied for safety assessment and *in vivo* testing with volunteers for efficacy assessment. However, use of *in vitro* tests for efficacy of cosmetic ingredients provides certain advantages because it is safe during early phases of new product development, results are obtained considerably faster, and the experimental setting is more cost-effective and allows to perform formulation and ingredient characteristic screening within ranges that would not be feasible with volunteers. Natural ingredients used in cosmetic products may contain a variety of preparations or isolated active substances, and the physicochemical properties of the substance can limit choice of the test method. Preparations for testing can include plant juices, extracts, tinctures, oil, lipids, polysaccharides, and essential oils. Purified active substances include vitamins, antioxidants, sterols and other ingredients. A wide range of wild and cultivated plants, fungi, herbs and microorganisms have been investigated or used as potential sources of cosmetic ingredients. (Pieroni et al. 2004; Lintner et al. 2009; Antignac et al. 2011; Mukherjee et al. 2011; Raut et al. 2012; Yingngam, Rungseevijitprapa 2012). Although different biological effects can be tested in cell free systems or using models as isolated membranes, liposomes, model organisms like yeasts, this review focuses on use of mamalian cell and tissue cultures. Investigations may involve different systems on cellular or tissue-like structural levels. Use of primary human skin cells, cell lines, reconstructed full thickness skin models and human cadaver skin de-epidermized dermis (DED) based organotypic tissue is well documented in the scientific literature.

Cell types for *in vitro* tests

Keratinocytes

Epidermis is a stratified squamous epithelium on the skin surface. It is composed predominantly of keratinocytes. The proliferative compartment is located in the innermost layer, transiently amplifying keratinocytes are attached to the basal membrane of extracellular matrix. In self-renewing tissue basal cells periodically withdraw from the cell cycle and activate a programme of terminal differentiation. They lose contact with basal membrane and move up towards the skin surface through three different stages: spinous, granular and squamous (Fuchs 1990; Feliciani et al. 1996). Epidermis is at the border to the environment and is highly dependent on balance between proliferation, differentiation, desquamation and apoptosis of keratinocytes. Balance is impaired during ageing, due to decreased proliferation and differentiation of keratinocytes, and reduced responsiveness to stimuli like growth factors and hormones (Berge et al. 2008).

Cultivation of keratinocytes has been extensively studied in the previous decades, resulting in standardized and accepted isolation and propagation protocols and accumulation of knowledge about keratinocyte behaviour *in vitro*. Keratinocytes can either be maintained under feeder layer-dependent conditions or under defined conditions in serum-free, media with a low calcium concentration (Rheinwald, Green 1975; Boyce, Ham 1983; Ura et al. 2004). Both methods have certain advantages and disadvantages, and users have to decide about the used culture strategy, based on the specific demands of their application. Serum containing culture medium significantly increases the amount of undesired cells (e.g. fibroblasts and melanocytes) while decreasing the amount of attaching keratinocytes. Advantages of serum- and feeder-based techniques include higher resistance to apoptosis, e.g. after adenoviral infection. It has also to be taken into account that it is possible to switch to serum-free culture conditions at any time point while changing from serum-free medium to serum-based conditions is not recommended (Aasen, Belmonte 2010). Compared to some cell types like fibroblasts, keratinocytes require more care and avoiding apoptosis in low density cultures, and differentiation and senescence when reaching confluence is difficult. For detailed understanding of the mechanisms present in skin, the cellular responses of basal keratinocytes to UV radiation (UVR) can be determined with the help of skin equivalents comprising a dermis and a differentiated epidermis. The basal keratinocytes can be divided into two subgroups, one of which expresses high levels of β1 integrin, the other expressing low levels of β1 integrin. Both populations react differently to UV irradiation with respect to their proliferation and thus represent different subgroups of keratinocytes (Hendrix et al. 1998).

Immortalized keratinocyte lines have been established with the HaCaT line being the best characterized and most frequently used in research. The HaCaT cell line originally was described as an obviously immortalized cell line which maintains full epidermal differentiation capacity and proliferation capacity at more than 140 passages (Boukamp et al. 1988; Boukamp et al. 1997).

Dermal fibroblasts

Fibroblasts are a heterogeneous cell population and the most common connective tissue cells in the human body. Skin fibroblasts are spindle-shaped cells with long cytoplasmic prolongations derived from multipotent mesenchymal cells, which are closely linked to fibres responsible for dermal architecture and resilience. Dermal fibroblasts synthesize various growth factors and cytokines that are important in regeneration processes. They also regulate differentiation of keratinocytes and deposit dermal extracellular matrix consisting of collagens type I, III, IV, laminin, and elastin (Mazlyzam et al. 2008; Vedrenne et al. 2012). Fibroblasts play a major role in deposition of extracellular matrix (ECM). Extracellular matrix production is important as it serves as structural support for cells and provides a transport system for nutrients and waste products (Eble, Niland 2009). ECM components produced by fibroblasts include collagens, elastin, glycoproteins, such as fibronectin, and glycosaminoglycans. Fibroblasts are able to organize a complex ECM network, which provides skin with its property of water retention and remarkable biomechanical properties. Fibroblasts in skin, along with endothelial cells, keratinocytes and macrophages secrete matrix metalloproteases (MMPs) and their tissue inhibitors (TIMPs). Balance between these is important in skin homeostasis and regeneration processes (Vedrenne et al. 2012).

The above mentioned functions of fibroblasts in skin highlight target mechanisms for natural origin substances to be tested in cell cultures. These include promotion of cell proliferation, stimulation or suppression of secretion of specific cytokines or growth factors, and stimulation of production of ECM components. Fibroblasts are also suitable for testing effect of natural compounds on cellular senescence and ability to protect them from unfavourable environmental conditions (Bae et al. 2009; Kim et al. 2011; Chiang et al. 2012).

Melanocytes

Melanocytes are dendritic cells located in the *stratum basale* layer of epidermis and epidermal appendages, i.e. sebaceous glands and hair follicles. These cells are derived from the neural crest and migrate into epidermis early in embryonic life. Once in the epidermis, melanocytes extend dendrites from the cell body and create contact with the surrounding keratinocytes. This close anatomical relationship between these two cell types is called the "epidermal melanin unit". Melanocytes produce the pigment melanin and transfer it via their dendritic processes to keratinocytes. This is responsible for skin colour and has an important role in protection against UVR. Melanin synthesis takes place in the melanosome, a highly specialised organelle which is unique to pigment cells. Melanin production in mammalian melanosomes is a process involving a series of enzymatic and biochemical events. The initial steps in the biosynthesis of both types of melanin are under the control of the enzyme tyrosinase (EC 1.14.18.1). An absence of tyrosinase in humans results in oculocutaneous albinism, which is characterised by complete absence of melanin in tissues. Melanin is the major source of human skin colour and ethnic variations in constitutive skin colour (CSC) result mainly from differences in the amount of melanin (as well as type of melanin) within the epidermis. Skin pigmentation can be enhanced by exposure to UVR and by hormones. These increases in pigmentation are termed facultative skin colour as they are reversible and tend to decline toward CSC when stimulation is discontinued. Based on the epidermal melanin content of unexposed skin and on the genetic capacity of the skin to tan in response to UVR, a classification of six sun-reactive skin phototypes in humans has been introduced, reviewed by Fitzpatrick (1988).

Melanocyte and melanoma monocultures, co-cultures and organotypic tissue models have been used to study skin pigmentation mechanisms and also to test cosmetic formulations that target skin pigmentation. Their main mechanism of action is reduction of tyrosinase activity via transcriptional or post-translational regulatory mechanisms (see reviews by Solano et al. 2006; Chang 2009; Gillbro, Olsson 2011). The rate of melanogenesis and melanin production ultimately depends on the level of enzymatic activity of tyrosinase, the initial and the rate limiting enzyme for pigment melanin synthesis.

Endothelial cells

Endothelial cells line all blood vessels in the human body and are involved in different physiological processes, including the control of vasomotor tone, maintenance of blood fluidity and permeability, secreting of various cytokines and chemokines. They are important players in innate immunity, as well as in formation of new blood vessels from existing ones, angiogenesis. However endothelial cell morphology, functions and gene expression may vary between organs, blood vessel type and even the different place in the same blood vessel.

Capillaries, the smallest blood vessels in human body, are located also in the dermal layer of the skin and provide nutrition and waste product removal from surrounding tissue. Different environmental factors, pathological changes or aging can cause blood vessel disorders, such as blood vessel weakness, which can lead to broken blood vessels, or on the contrary, hyperplasia. For example, exposure to UVR may cause erythema (Kripke 1994), vascular hyperpermeability, epidermal hyperplasia and other skin conditions (Pearse et al. 1987). Therefore, it is very important not only to combat skin problems already existing, but to take preventive measures regarding strengthening of capillary walls, active substances can induce or reduce new blood vessel formation, angiogenesis.

Angiogenesis is a process consisting of different

events, such as cell migration, adhesion, proliferation, differentiation and cell organization in tube-like structures. This process is tightly regulated, as it plays an important role in wide range of processes, for example, in wound healing, formation of varicose veins and tumour growth. Angiogenesis plays an important role in different skin conditions. Suppression of angiogenesis may be important in cases of psoriasis, as it is believed that hyperproliferation of keratinocytes leads to extensive secretion of VEGF (vascular endothelial growth factor), which leads to development of too permeable neovessels (Armstrong et al. 2011). Angiogenesis is very important in wound healing as well, because blood supply provides nutrients and waste removal from the site of injury.

In vitro models

Monocultures

Monoculture models of skin are characterized by culture of one cell type, which has previously been extracted from a skin biopsy or derived from an immortalized cell line. Such continuous cell lines are derived from malignant tissue of a particular cell type (e.g. melanoma), or more often have been transformed *in vitro*. Primary keratinocytes, fibroblasts, melanocytes and endothelial cells can be used for testing of natural ingredients. Use of monocultures in *in vitro* testing is advantageous as it allows observation of biological effect of compound on a particular cell type. A disadvantage of this system is that it is difficult to assess impact of active substances on interaction of different cell types. Testing of active substances in monocultures involves cell cultivation in presence of the test substance either under normal conditions or in presence of stress factors, and monitoring of changes in cell morphology and functionality.

The combination of knowledge on keratinocyte isolation and cultivation and their role in skin integrity and ageing processes renders keratinocyte monocultures a valuable test system for screening of active substances. The tests performed using primary keratinocyte cultures (normal human keratinocytes) or immortalized human keratinocyte cell line HaCaT have focused on assessment of proliferation and migration, cytokine and growth factor production and release, and resistance to environmental stress (UV irradiation, heat, chemicals). As many natural extracts for cosmetic use are rich in antioxidants, keratinocyte monocultures are frequently employed to test their efficiency to protect skin cells from UV irradiation. Different approaches are used to test antiradical efficacy of a natural component in keratinocyte monocultures. Protective characteristics might be evaluated by applying exogenous stress to keratinocytes after stimulation by an active substance, thus allowing to determine if pre-treated skin is more stress resistant. In contrast active substances could be added to the cell culture after exposure to stress. This would indicate if the active substance possesses regenerative potential. Additionally production of specific stress related metabolites, including MMPs, heat shock proteins, prostaglandins, cyclooxygenase, differentiation specific markers, as involucrin, could be assessed in keratinocyte cultures. (Engel et al. 2007; Molinari et al. 2013; Rodrigues et al. 2013). Studies and protocols of natural active substances and compounds tested on human keratinocyte cultures are summarized in Table 1.

Primary human skin fibroblasts are easy to extract from small biopsies or post-operative material, which can be expanded in large amounts as there are well established isolation and cultivation protocols (Takashima 2001). Fibroblast monocultures are frequently used for testing of skin active natural compounds. Decrease of cell proliferation is characteristic of skin ageing and leads to reduced synthesis of ECM components. Because of this strategy of anti-ageing, cosmetic ingredient testing usually include monitoring of fibroblast cell proliferation and migration, changes in collagen and elastin production both at gene expression and protein level, and accumulation of senescence markers (Dimri et al. 1995). As environmental factors play a significant roles in skin ageing, skin fibroblast cell cultures are used to test effects of oxidative stress and efficiency of natural compounds to reverse stress induced cellular senescence. As reactive oxygen species (ROS) affect also production and degradation of ECM, synthesis of MMPs and TIMPs might also be done in parallel with cell proliferation and ROS accumulation measurements (Binic et al. 2013). Different molecular and cell biology techniques including qPCR, ELISA, immunoblotting and immunocytochemistry have been applied to detect changes in skin cell monocultures. Studies and protocols of natural active substances tested on human skin fibroblast cultures are summarized in Table 2.

Monoculture models are useful for studying human skin pigmentation mechanisms, melanocyte physiology, and for testing ingredients that target the melanin production pathway. Characterisation of the mechanisms that regulate human skin pigmentation has highlighted tyrosinase as an essential enzyme for melanin production. Overall, studies have demonstrated that monolayer models allow to test effects of natural ingredients on tyrosinase activity and melanin formation, which can represent skin pigmentation. Primary melanocytes have been used to study skin pigmentation and also to test cosmetic formulations. The majority of cosmetic products in this category aim to reduce the pigmentation level of human skin, and include ingredients referred to as skin whitening or lightening agents. However, primary melanocyte cell culture is technically difficult to establish and it has a short *in vitro* replication span, unless strong synthetic mitotic stimulants are used in tissue culture media. These mitotic stimulants render melanocytes irresponsive to most melanogenesis regulating factors; therefore cell

Table 1. Plant derived extracts and isolated active substances that have been tested in keratinocytes

Plant name (family)	Part used (main active substances)	Cell type	Aim of the testing	Test method	Results / possible mechanism of action	References
Harpephyllum caffrum (Anacardiaceae)	Leaves (isolated phenolics)	HaCaT (German Cancer Research Centre)	Leaf extract in association with its antioxidant and capability to protect keratinocytes from UV	Determination of radical scavenging activity by DPPH and ORAC assay; Il-6 and Il-8 measured using cytokine human 10plex bead based assay	Diminished UV phototoxic reaction of keratinocytes; isolated kaempferol sulphatogalactopyranoside did not interact with UVB-triggered IL-6 production of HaCaT keratinocytes	Nawwar et al. 2011
Hamamelis virginiana (Hamamelidaceae)	Bark (polymeric proanthocyanidins and polysaccharides)	Obtained either from commercial available cultures (Cell Systems, St Katharinen) or isolated from human skin	Influence on proliferation and differentiation of cultured human keratinocytes	Growth rates were determined using trypan-blue; mitochondrial activity by the MTT test; lactate dehydrogenase quantification – LDHassay; cell proliferation was determined by BrdUassay; quantification of keratins K1 and K10 by ELISA	Polysaccharide fraction did not affect human keratinocytes; proanthocyanidins strongly increased the proliferation of the cells, while the differentiation was not significantly influenced	Deters et al. 2001
Opuntia ficus-indica (Cactaceae) *Mesembryanthemum crystallinum* (Aizoaceae)	Cactus pear (polysaccharides: cold water soluble, NwPS; non-swelling pectin, NPec) Whole plant	HaCaT-keratinocytes (DKFZ, Heidelberg, Germany)	Effect of pressed juices on cell physiology	Cell proliferation by BrdU incorporation assay; reduction of tetrazolium salts to determine metabolic activity (MTT); necrotic effects assessed by LDH-release	Juice from *M. crystallinum* significantly increased proliferation; proliferation was not significantly influenced by low concentrations of cactus pear polysaccharides; however, it was inhibited by 100 mg mL^{-1} NPec; NwPS significantly enhanced metabolic activity of HaCaTs	Deters et al. 2012
Mauritia flexuosa (Arecaceae)	Fruit (buriti oil)	HaCaT	Possible cytotoxic effects of topical creams and lotions produced with buriti oil	Neutral red release assay to evaluate the cytotoxicity of the emulsions	Presented low cytotoxicity to cells at high concentrations and the addition of vitamin E increased cell viability	Zanatta et al. 2008
Mauritia flexuosa (Arecaceae)	Fruit (buriti oil)	HaCaT	Whether cosmetic, produced with buriti oil and commercial surfactants, can exert photoprotective effect against UVA and UVB	NRU assay for the estimation of cell viability/cytotoxicity	Emulsions prepared with sorbitan monooleate and PEG-40 castor oil and containing panthenol as active ingredient, were able to reduce the damages caused by radiation	Zanatta et al. 2010

Table 1. /continued/

Plant name (family)	Part used (main active substances)	Cell type	Aim of the testing	Test method	Results / possible mechanism of action	References
Prunella vulgaris (Lamiaceae)	Aerial parts	HaCaT (Institute of Biophysics, Academy of Science of the Czech Republic)	Protective effects of *P. vulgaris* and its main phenolic acid component, rosmarinic acid, against UVA-induced changes	Cell viability monitored by neutral red assay (NRA) and by LDH release into medium; cell proliferation (BrdU) assay; Comet assay to monitore DNA damage; caspase-3/actin expression by Western blotting	Reduced UVA-caused decrease in cell viability; suppressed UVA-induced ROS production; post-treatment reduced DNA damage; inhibited UVA-induced activation of caspase-3	Psotova et al. 2006
Citrus bergamia (Rutaceae)	Fruit (juice)	NCTC 2544 provided by Interlab Cell Line Collection (Genoa, Italy)	Anti-inflammatory/antioxidant activity on human keratinocytes treated with interferon-gamma (IFN-γ) and histamine (H)	Anti-inflammatory/antioxidant ability was determined through evaluation of ICAM-1 and iNOS expression by Western blot; production of NO with Griess reagent and concentration of ROS by fluorescent quantitative analysis with DCFH-DA; cell viability by MTT assay	Extract did not inhibit cell proliferation and significantly reduced dose-dependently ICAM-1, iNOS, NO, ROS and GAG production in cells exposed to IFN-γ and H	Graziano et al. 2012
Oryza sativa (Poaceae)	Rice bran, enzymatic extract form rice bran (EERB)	Foreskin keratinocytes and reconstructed human epidermis	Potential cytotoxicity of EERB and its and possible protection against free radicals produced by UVR	Cell viability assessment (MTT assay) and MDA (malondialdehyde) production	EERB did not induce cytotoxic effect for concentrations inferior or equal to 100 l g mL^{-1}; human keratinocyte monolayers were protected of irradiation preventing 33% the lipid peroxidation process at concentration of 10 μg mL^{-1} of EEBR; in reconstructed human epidermis, 100 μg mL^{-1} decreased lipid peroxidation process by 44%	Santa-Maria et al. 2010
Gynura procumbens (Asteraceae)	Leaves	HaCaT	Protective activity of extract on skin photoaging and elucidate its mode of action	Detection of ROS production; characterization of cytokines by ELISA	Extract markedly reduced the production of ROS; inhibitory effect on releasing pro-inflammatory cytokines (IL-6 and IL-8)	Kim et al. 2011
Astragalus membranaceus (Fabaceae)	Root; non-fermented (HQNB) and fermented preparations (HQB)	HaCaT, Cell Lines Service (Germany) and HEK, Cascade Biologics (Portland, OR)	Effects HQNB and HQB on hyaluronic acid (HA) production	Cell viability MTT assay; hyaluronic acid content was determined using ELISA; RT-PCR to evaluate hyaluronan synthase gene expression	Growth-stimulating effect of HQNB; HQB markedly and dose-dependently increased expression of hyaluronan synthase3 and hyaluronan synthase2 mRNA in cells	Hsu, Chiang 2009

Table 1. /continued/

Plant name (family)	Part used (main active substances)	Cell type	Aim of the testing	Test method	Results / possible mechanism of action	References
Myristica fragrans (Myristicaceae)	Rhizomes (macelignan)	HaCaT	Protective effects of macelignan on HaCaT against UVB damage. Inhibitory effects on UVB-induced MMP-9 and COX-2 and investigate the molecular mechanism	Secretion of MMP-9 was measured by gelatin zymography; expression of COX-2,mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase/Akt (PI3K/Akt), c-Fos, c-Jun, and CREB were assayed by western analysis	Macelignan at a concentration of 0.1–1 mM increased the viability of HaCaT cells and inhibited MMP-9 secretion and COX-2 expression in a concentration-dependent manner. Macelignan treatment reduced the activation of UVB-induced MAPKs, PI3K/Akt, and their downstream transcription factors	Anggakusuma et al. 2010
Usnea barbata (Parmeliaceae)	Thallus (major constituent usnic acid)	HaCaT	Anti-inflammatory properties of U. barbata extract (UBE) containing 4% usnic acid in UVB model with HaCaT keratinocytes	UVB-induced COX-2 expression was assayed by Western analysis; high sensitivity PGE2 EIA for determination of UVB-induced PGE2 production; cytotoxicity of UBE was assessed with an ATP assay	UBE inhibited PGE2 production at a half-maximal concentration of 60 μg mL^{-1} (2.4 μg mL^{-1} usnic acid) that did not affect the UVB-induced up regulation of COX-2, suggesting an effect on enzyme activity rather than on protein expression. The inhibition of PGE2 production by UBE was not due to cytotoxicity	Engel et al. 2007
Platycodon grandiflorum (Campanulaceae)	Root	HaCaT (German Cancer Research, Germany)	Protective effect of P. grandiflorum (CKS) against UVA damage on HaCaT. Inhibitory effects of CKS on UVA-induced MMP-1 and investigate the molecular mechanism	Measurement of cell viability using MTT and LDH leakage assay; detection of ROS production using the redox-sensitive fluorescent dye H2DCFDA; MMPs activity assay using an EnzoLyte MMP-1 fluorometric assay kit; MMP-9 activity by gelatin zymography and Western blot analysis; IL1-b, IL-6 and MMP-1 mRNA expression by RT-PCR and qPCR in case of MMP-1	CKS increased cell viability and inhibited ROS production. Pretreatment with CKS inhibited UVA-induced production of MMP-1 and MMP-9. CKS decreased UVA-induced expression of the inflammatory cytokines IL-1b and IL-6. CKS markedly suppressed the enhancement of collagen degradation. CKS also suppressed UVA-induced activation of NF-jB or c-Jun and c-Fos, and phosphorylation of MAPKs	(Hwang et al. 2011)

Table 1. /continued/

Plant name (family)	Part used (main active substances)	Cell type	Aim of the testing	Test method	Results / possible mechanism of action	References
Codium fragile (Codiaceae)	Whole plant and a single compound, clerosterol	HaCaT	Anti-inflammatory potential of *C. fragile* using buthanol and ethylacetate fractions of 80% methanol extract (CFB or CFE) and a single compound, clerosterol (CLS)	Cell viability measured by MTT assay; protein concentration determined by BCA protein assay kit; optimal induction time for expression of COX-2 and iNOS protein by UVB irradiation determined based on Western blot analysis; quantitative analysis of PGE2 by ELISA	Induced cytotoxicity and reduced expression of pro-inflammatory proteins including COX-2, iNOS, and TNF-α. Effectively suppressed UVB-induced production of pro-inflammatory mediators such as prostaglandin E2 and nitric oxide	Lee et al. 2013
Lithospermum erythrorhizon (Boraginaceae)	Root (lithospermic acid and two derivative esters, 9”-methyl lithospermate and 9’-methyl lithospermate)	HaCaT	Whether lithospermic acid and its derivatives might improve permeability barrier of skin by stimulating protein level of serine palmitoyltransferase (SPT)	Cell viability measured by MTT assay; change in mRNA expression of SPT by RT-TCR assay; Western blot analysis to examine the protein levels	All compounds significantly increased SPT expressions in the relative quantity of SPT1 mRNA as well as SPT2 mRNA. Raised level of SPT protein in a dose-dependent manner, with the increased level of SPT protein in HaCaT cells of 55, 23, and 81% at the concentration of 100 μg mL^{-1}	Thuong et al. 2009
Pinus massoniana (Pinaceae)	Bark (contains taxifolin)	HaCaT	Effect of *P. massoniana* bark extract (PMBE) and taxifolin on intercellular adhesion molecule-1 (ICAM-1) expression	Growth inhibition measured by a CCK-8 assay; effect of PMBE and taxifolin on ICAM-1 protein expression of HaCaT cells assayed using flow cytometric assay, immunohistochemical analysis, Western blot analysis and RT-PCR of ICAM-1 mRNA expression	PMBE pre-treatment significantly inhibited IFN-γ-induced ICAM-1 expression. PMBE-mediated inhibition of ICAM-1 mRNA and protein expression was greater than taxifolin mediated-inhibition, and the front on inhibition of ICAM-1 protein expression was 2.24–2.30-fold of the latter	Wu et al. 2009
Citrus sinensis (Rutaceae)	Fruit (anthocyanins, flavanones and hydroxycinnamic acids) and ascorbic acid	HaCaT	Efficacy of red orange extract in modulating cellular responses to UVB in HaCaT	Lactate dehydrogenase leakage assay and trypan blue exclusion assay for cytotoxicity testing; electrophoretic mobility shift assay (EMSA) and Western blotting for protein analysis	NF-kB and AP-1 translocation and procaspase-3 cleavage	Cimino et al. 2007

Table 2. Plant derived extracts and active substances that have been tested in dermal fibroblast monolayers

Plant name (family)	Part used (main active substances)	Cell type	Aim of the testing	Test method	Results / possible mechanism of action	References
Emblica officinalis (Euphorbiaceae)	Fruit	NB1RGB (Riken Cell Bank)	Effect of E. officinalis extract for production of procollagen and matrix metalloproteinases (MMPs)	Mitochondrial activity by WST-8 assay; protein quantification by immunoassay	Decreased production of MMP-1, increased TIMP-1 level	Fujii et al. 2008
Emblica officinalis (Euphorbiaceae)	Fruit	HS68 cell (ATCC CRL 1635) (Rockville, MD, USA)	Efficacy of E. officinalis (EO) to inhibit UVB-induced photoaging in human skin fibroblasts	Mitochondrial activity by MTT-assay; quantifications by immunoassay techniques; hyaluronidase inhibition assay; cell cycle analysis	Inhibited UVB-induced MMP-1 in skin fibroblasts; EO exhibited inhibitory activity of hyaluronidase; EO prevented UVB disturbed cell cycle to normal phase	Adil et al. 2010
Campsis grandiflora (Bignoniaceae)	Flower	Primary skin fibroblasts provided by Chinese Academy of Medical Science	Antioxidative and anti-inflammatory activities	MTT assay for cell viability; LDH assay for lactate dehydrogenase release; DNA fragmentation assay	Protected cell survival from H2O2-induced toxicity; inhibited the H2O2-induced leakage of lactate dehydrogenase enzyme release and DNA fragmentation; scavenging activities of radicals and ROS	Cui et al. 2006
Gynura procumbens (Asteraceae)	Leaves	Fibroblasts obtained from neonatal foreskins	Protective activity of the extract on skin photoaging, its mode of action	MMP-1 expression level by ELISA and Western blot analysis; zymography for evaluating the enzymatic activity of MMP-9	Inhibited MMP-1 expression and enzymatic activity of MMP-9	Kim et al. 2011
Labisia pumila (Myrsinaceae)	Root	Fibroblasts obtained from neonatal foreskins	Anti-photoaging effects	Cell viability assay; sircol collagen assay to evaluate collagen synthesis; ELISA and Western blot of pro-inflammatory cytokines	Inhibited MMP-1 expression; decreased collagen synthesis of human fibroblasts by UVB was restored back to normal level after treatment with L. pumila extract	Choi et al. 2010
Michelia alba (Magnoliaceae)	Leaves	Foreskin fibroblasts (Hs68)	Effect on expression and activity of matrix metalloproteinases (MMPs) UVB exposure	MTT assay fo cell viability test; quantitative determination of hyaluronic acid by ELISA; Western blotting for MMPs, MAP kinase, and type I procollagen; zymography for MMP-9; measurement of total collagen synthesis by Sircol™ soluble collagen assay	Inhibited collagenase and elastase activities; exhibited antioxidant activity, elevated hyaluronic acid content and inhibited UVB-induced MMP-1, MMP-3 and MMP-9 expression and inhibited MMP-9 activity; inhibited UVB-induced ERK and JNK kinase	Chiang et al. 2012

Table 2. /continued/

Plant name (family)	Part used (main active substances)	Cell type	Aim of the testing	Test method	Results / possible mechanism of action	References
Opuntia ficus-indica (Cactaceae) *Mesembryanthemum crystallinum* (Aizoaceae)	Cactus pear (polysaccharides: cold water soluble, NwPS; non-swelling pectin, NPec); whole plant	Fibroblasts isolated from skin grafts	Effect of pressed juices on cell physiology	Cell proliferation by BrdU incorporation assay; reduction of tetrazolium salts to determine metabolic activity (MTT); necrotic effects assessed by LDH-release	Juice from M. crystallinum significantly increased proliferation; no influence on metabolic activity; 100 mg mL^{-1} of NwPS and 1 mg mL^{-1} NPec stimulated proliferation of fibroblasts; metabolic activity was not affected by NPec or NwPS	Deters et al. 2012
Astragalus membranaceus (Fabaceae)	Roots, non-fermented (HQNB) and fermented preparation (HQB)	Adult skin fibroblasts (Cascade Biologics (Portland, OR)	Effects of HQNB and HQB of A. membranaceus on hyaluronic acid (HA) production	Cell viability MTT assay; hyaluronic acid content was determined using ELISA; RT-PCR to evaluate hyaluronan synthase gene expression	Growth-stimulating effect of HQNB; HQB markedly and dose-dependently increased the expression of hyaluronan synthase3 and hyaluronan synthase 2 mRNA	Hsu, Chiang 2009
Camellia japonica (Theaceae)	Seeds (oil)	Fibroblasts, neonatal foreskin (Amore-Pacific Corporation R&D Center, Korea)	*C. japonica* oil as an anit-wrinkle agent	Quantitative detection of type I collagen – ELISA; quantification of MMP-1 activity by the Biotrak MMP-1 activity assay system; cytotoxicity (MTT) assay	Induced type-1 procollagen synthesis and inhibited MMP-1 activity	Jung et al. 2007
Terminalia chebula (Combretaceae)	Fruits	Fibroblasts from adult skin	*In vitro* anti-aging activities of the extracts that have been traditionally used for longevity	Extracts tested for antioxidative and tyrosinase inhibition activity as well as the proliferative and MMP-2 inhibition activity	Stimulated normal human fibroblast proliferation more than ascorbic acid; tyrosinase and MMP-2 inhibition	Manosroi et al. 2010
Vaccinium uliginosum (Ericaceae)	Fruits (anthocyanins)	Dermal fibroblasts (Clonetics,San Diego, CA)	Capacity of anthocyanin-rich extract to inhibit photoaging	Immunocytochemistry for type 1 procollagen; Western blot analysis for expression level of procollagen; analysis of RT-PCR in case of MMP-1 and procollagen type 1; ELISA assay for secretion of TNF-a, interleukin (IL)-8, IL-6, and IL-1b	Removal of reactive oxygen species; diminished UV-B augmented-release of inflammatory interleukin IL-6 and IL-8	Bae et al. 2009
Asclepias tuberosa (Apocynaceae)	Root (fraction contained 21 pregnane glycosides)	Human skin fibroblasts (NB1RGB cells) cell bank of the RIKEN BioResource Center (Japan)	Anti-aging phytochemicals, based on proliferation of human skin fibroblasts	To evaluate proliferation using Alamar Blue	8,12;8,20-diepoxy-8,14-secopregnane glycosides increased proliferation of NB1RGB cells in a dose-dependent manner, from 117 to 147% at 100 μg mL^{-1}, compared to untreated fibroblasts	Warashina et al. 2011

cultures derived are suboptimal for ingredient screening tests. Alternatively, some non-standardized biological cell culture media supplements can be used, e.g. bovine or porcine pituitary extracts. This significantly increases the costs for experimentation, and a biological additive is associated with much variations and uncertainty for result interpretation. Failure to establish primary melanocyte culture or to increase cell numbers required for the experiment is not uncommon. Since the rate of melanin production ultimately depends on the level of enzymatic activity of tyrosinase, the initial and rate limiting enzyme, immortalized pigment cells that express tyrosinase are most often used for *in vitro* tests. These include melanoma (malignant melanocyte) cultures and some artificially immortalized melanocyte lines. Historically, the first and still most widely used model for pigmentation and tyrosinase activity related tests is the murine melanoma line B16, which is particularly easy to handle, proliferates rapidly and does not require complex protocol and expensive tissue culture media. Activity of tyrosinase is a target for the majority of ingredients developed for skin pigmentation altering cosmetic formulations, and for well established study protocols and screening methods. While regulation of the transcription rate of tyrosinase is important for melanogenesis, an increasing amount of evidence indicates that post-translational regulatory mechanisms that determine tyrosinase activity *in situ* are essential. Extensive reviews of test systems for natural active substance effect on tyrosinase activity and melanin production are available (Solano et al. 2006; Chang 2009; Smit et al. 2009; Kim et al. 2012; d'Ischia et al. 2013).

Endothelial cell monocultures have been widely used to evaluate antiradical potency of natural skin active ingredients. The endothelial cells that line blood vessels are very sensitive to injury caused by oxidative stress. Endothelial cells play an important role in physiologic hemostasis, blood vessel permeability, and response of blood vessel to other physiologic and pathologic stimuli. Any abnormality in endothelial cell structure and function may contribute significantly to blood vessel diseases such as thrombosis, atherosclerosis, and vasculitis. Procedures of endothelial cell isolation and cultivation have been established and several commercially available well characterized cell lines are available. HUVEC cells are among the most frequently used ones. Past studies have used HUVECs for *in vitro* experiments related to vascular dysfunction. They have played a major role as a model system for the study of the regulation of endothelial cell function and the role of the endothelium in the response to biologically active substances and environmental factors. There are several other endothelial cell lines available for *in vitro* testing purposes. Human dermal microvascular endothelial cells (HDMVECs) are a widely used endothelial cell type. These cells have been used *in vitro* to investigate different active substances and their effect on angiogenesis (Bagchi et al. 2003). In 1983 Edgell with colleagues established a permanent human cell line by fusing HUVEC cells with the permanent human pulmonary adenocarcinoma cell line A549 (Edgell et al. 1983). Since then, cell line EA.hy926 has been used for *in vitro* tests to investigate active ingredient effects on endothelial cells (Habtemariam 2002). Studies and test protocols of compounds of natural origin tested on endothelial cell cultures and immortalized cell lines are summarized in Table 3.

Co-cultures

Co-cultures in context of *in vitro* testing are defined as simultaneous cultivation of two to three different cell types, either spatially separated or non-separated. Use of co-cultures as test system allows assessing simultaneous stimulation of several cell types and evaluating changes in their interaction. Use of keratinocyte-fibroblast co-cultures in testing is of physiological importance, since dermal-epidermal interplay has a significant role in skin tissue homeostasis and regeneration. Use of a co-culture system with cell types being spatially separated or in direct contact is a question of experimental design. Some studies suggest that direct cell-cell interactions are required while others indicate that dominantly soluble factors play role in cell interaction.

Two-chamber type co-culture models involve culturing fibroblasts and keratinocytes in two separate chambers separated by a semi-permeable membrane. Such models have long been used to study paracrine signalling between keratinocytes and fibroblasts. In this model, keratinocytes are usually grown on a semi-permeable membrane insert while fibroblasts are grown in a tissue culture dish. Soluble factors secreted by the fibroblasts are able to pass through the membrane and affect growth and differentiation of keratinocytes and vice versa (Mazzalupo et al. 2002). Effects of natural active components studied in co-cultures mainly include assessment of growth factor secretion and production of structural components. Reports of plant-derived active components studies in co-cultures are summarized in Table 4.

Organotypic cultures

Typical 3D organotypic cultures involve seeding and culturing of keratinocytes on dermis equivalents, which are created by mixing fibroblasts with type I collagen and allowing the collagen to solidify. Keratinocytes are seeded onto the dermis equivalent, allowed to attach, and are exposed to the air–liquid interface (Bell et al. 1981; Loo, Halliwell 2012). Tissue engineered skin organotypic cultures have been produced for clinical and research applications. Organotypic cultures can be advantageous for safety and efficacy testing of active substances in cases when cell monolayers or simple co-cultures do not provide relevant experimental data. Organotypic 3D tissue cultures allow to mimic closer *in vivo* skin tissue and allows to study tissue

Table 3. *In vitro* effects of plant and fungi derived extracts and isolated active compounds on endothelial cells

Plant name (family)	Part used (main active substances)	Cell type	Aim of the testing	Test method	Results / possible mechanism of action	References
Hamamelis virginiana	Root, hamamelitannin	Human EAhy926 endothelial cell line	Cytoprotective effect on endothelial cells	Cytoprotection by MTT assay; cell adhesion by endothelial/monocyte adhesion test	Hamamelitannin inhibited cytotoxic effects of TNF without altering its effect on endothelial adhesiveness	Habtemariam 2002
Aesculus hippocastanum	Seed; β-escin sodium from Pekining University Third Hospital	HUVEC; ECV304 cells	Direct effect of β-escin sodium on endothelial cells	Cell proliferation by SRB (sulforhodamine B) assay; apoptosis by cell staining with annexine; migration by migration assay; motility by staining with rhodamine-phalloidin	β-escin sodium inhibited angiogenesis by depressing ECs proliferation and migration, and by inducing EC apoptosis	Wang et al. 2008
Antrodia cinnamomea	Mycelia	HUVEC	Immunomodulatory effect on the anti-angiogenesis	Tube-like structure formation by cultivation on ECM gel; cytokine secretion by ELISA	Polysaccharides from A. cinnamomea with MW>100kDa were strongly anti-angiogenic	Yang et al. 2009
Wild blueberry, bilberry, cranberry, elderberry, raspberry, strawberry	Fruit	HDMVEC	Anti-angiogenic properties	Tube-like structure formation by cultivation on Matrigel	Combination of extracts from six types of berries significantly impaired angiogenesis	Bagchi et al. 2003
Ganoderma lucidum	Fruiting body, polysaccharides	HUVEC	Potential anti-angiogenesis effect	Cell proliferation by MTT assay; cytotoxicity by LDH (lactate dehydrogenase activity) assay; apoptosis by staining with annexin	Anti-angiogenic activity of G. lucidum polysaccharides is achieved through direct inhibition of vascular endothelial cell proliferation	Cao, Lin 2006
Undaria pinnatifida	Sporophyll, fucoidan	HUVEC (Cell Bank of the Chinese Academy)	Anti-angiogenic effect of fucoidan, to examine the mechanism of action	Cell proliferation by MTT assay; migration by migration assay; tube-like structure formation by ECM gel; gene expression by RT-PCR	Fucoidan significantly inhibited angiogenesis	Liu et al. 2012
Vitis vinifera	Seeds, grape seed proanthocyanids (GSPs)	HDMVEC	GSPs modulate multiple signalling pathways and exhibit antiangiogenic effects	Cell viability by SRB method; migration by migration assay; enzymatic activity of MMP-2 and MMP-9 by gelatin zymography; tube formation by cultivation on ECM gel	GSPs exhibited antiangiogenic effects and it is attributed to the inhibition of VEGF and Ang-1 signalling	Huang et al. 2012
Panax ginseng, Panax quinquefolium, Panax notoginseng	Not mentioned	HUVEC	Effect of the extracts on angiogenesis	Invasion by Matrigel-coated transwell assay; tube-like structure formation by cultivation on ECM gel	Showed concentration-dependent angiogenesis stimulating effect	Sengupta et al. 2004

Table 3. /continued/

Plant name (family)	Part used (main active substances)	Cell type	Aim of the testing	Test method	Results / possible mechanism of action	References
Cordyceps militaris	Fruiting body	HUVEC (Clonetic)	Effect on angiogenesis	Proliferation by SRB assay; gene expression by RT-PCR; tube-like structure formation by cultivation on ECM gel; wound healing by scratch assay	Extract had antiangiogenic properties realized through reducing bFGF expression, preventing tube formation and inhibiting wound healing in dose-dependent manner	Yoo et al. 2004
Phellinus linteus	Fruiting body methanol extracts (PLME)	HUVEC (Lonza)	Effect of methanol extracts on angiogenesis	Proliferation assay (Promega); migration and invasion by scratch assay; tube-like structure formation by cultivating on Matrigel	PLME treatment had antiangiogenic effect by inhibition of HUVEC cell proliferation, migration and assembly to tube-like structures	Lee et al. 2010
Salix spp.	Bark from young branches or dried pieces of current-year twigs	HUVEC	Ability of willow bark extract to prevent oxidative-stress-induced death	Cell viability by MTT assay; expression of mRNAs by RT-PCR; protective effect by intracellular GSH (glutathione) measurement	Active ingredients in the extract, other than salicin, contributed to increase in antioxidant enzymes and prevention of oxidative stress	Ishikado et al. 2012
Patrinia villosa	Aqueous extract from whole herb	HUVEC	I*n vitro* effect on various cellular activities of HUVECs	Cell proliferation by WST-1 assay; cell migration by scratch assay; capillary-like structure formation on ECM gel	Extract significantly induced angiogenesis	Jeon et al. 2010
Salvia desoleana	Leaves	HUVEC; ECV304	Effects of extract on cytoprotection against ROS-induced oxidative EC death	Antioxidant effect by CyQUANT NF assay	Extract provided protection against H2O2-induced oxidative stress in both endothelial cell types	Posadino et al. 2012
Panax ginseng	Roots	HUVEC	Effect of ginseng extract on angiogenesis	Cell proliferation by MTT assay; chemotaxis by chemotaxis assay; tubular-like structure formation by incubation on ECM gel; mRNA expression by RT-PCR	Ginseng extract induced angiogenesis	Kim et al. 2007
Salvia miltiorrhiza	Roots	HUVEC-derived EA.hy 926 endothelial cells	Whether *Salvia miltiorrhiza* resemble the action profile of endothelium-derived nitric oxide (NO)	Expression of eNOS mRNA by RNase protection assay; mRNA expression by RT-PCR; eNOS proteins by Western blot	Extract from Salvia miltiorrhiza incresed eNOS promoter activity, eNOS mRNA and protein expression and NO production in cells	Steinkamp-Fenske et al. 2007
Kaempferia parviflora	Rhizomes, ethanol extract	HUVEC	Effect of ethanol extract from Kaempferia parviflora on endothelial function	NO production by diazotization reaction; mRNA expression by RT-PCR; protein production by Western blot	Improved endothelial function by activation of NO production and eNOS mRNA and protein expression but not iNOS expression	Wattanapitayakul et al. 2007

Table 4. Cell co-cultures used for *in vitro* testing

Cell types included in co-culture	Mechanisms investigated	References
Keratinocytes & melanocytes	Melanocyte dendricity, tyrosinase activity, melanin formation and melanosome transfer *in vitro*; substances that affect melanogenesis and melanin transfer to keratinocytes that forms skin pigmentation	Berens et al. 2005; Eves et al. 2005; Regnier et al. 2005; Ma et al. 2010; Ando et al. 2011
Fibroblasts & keratinocytes	Wound healing; keloid scarring; spatial distribution and self-organisation of skin cells	Wang et al. 2003; Stark et al. 2004; Sun et al. 2006; Werner et al. 2007; Butler et al. 2008; Shariati et al. 2009; Loo, Halliwell 2012; Wang et al. 2012
Endothelial cells & fibroblasts	Tubular-like structure development (vasculogenesis); angiogenic sprouting; wound healing; capillary lumen formation	Nakatsu et al. 2003; Oberringer et al. 2007; Sukmana, Vermette 2010; Eckermann et al. 2011; Liu et al. 2013

architecture and cell-cell, cell-ECM interactions (MacNeil 2007; Bernerd, Asselineau 2008). Models containing a collagen matrix are representative of human skin, and can be considered for the study of cosmetic ingredients. However, it has to be taken into account that collagen models may not be useful for testing all substances as some are absorbed by the collagen and a considerably higher quantity of may be required. This would interfere with interpretation of results and may not be a rational approach in cases where absorbance by collagen is significant. One of the alternatives is use of de-epidermized dermis (DED) as substrate that allows the construction of a skin equivalent. DED is prepared from cadaver skin samples, with epidermis removed and dermis incubated in saline solution or which has undergone freezing-thawing cycles to disintegrate cells. Unlike collagen gels, this method has a native extracellular matrix and a basal membrane that facilitates cell adhesion.

Currently bioengineered skin tissue cultures serve as model systems to identify corrosive, irritant and toxic characteristics of substances that come in contact with skin, and to assess regenerative potential of different active substances. Organotypic skin substitutes used for *in vitro* testing can be divided in two types: the first consists of keratinocytes seeded on matrix, thus simulating only epidermis; the second type consists of an epidermal layer and dermal layer where fibroblasts are seeded in a 3D matrix. Different types of natural and synthetic polymers can be used to produce matrix for the dermal layer, including collagen, fibrin, lactic and glycolic acid polymers (Robinson et al. 1999; Groeber et al. 2011).

The more recent bioengineered organotypic cultures have been improved further to mimic *in vivo* tissue by introducing additional cell types and to meet requirements of different testing strategies. To assess effects on chemical and active substances on microvasculature, endothelial cells are added to a dermal layer to form vascular like structures. Testing of active substances with skin whitening potential requires introduction of melanocytes in tissue culture. In more complex models, immune cells can be added, for example when sensitizing potential should be assessed. Dendritic cells might be used as an alternative to animal testing of immune-sensitizing compounds. A disadvantage of dendritic cells as a test system is that it is a single cell model and does not allow testing of water insoluble compounds. Many current tissue models do contain keratinocytes, fibroblasts and melanocytes but cannot be used in testing of sensitizing compounds, as they do not contain immunologically active cells. For example, the KDF-Skin model contains keratinocytes, fibroblasts and dendritic cells. A modified version of this model, VG-KDF-Skin is produced using collagen vitrigel instead of conventional collagen gel (Uchino et al. 2009). Models for different skin conditions (acne, psoriasis) can be produced as bioengineered skin tissue test systems.

Commercial organotypic *in vitro* skin models have been developed to replace animal models and, in the case of validated products, to improve test reliability. For cosmetic product manufacturers, these models allow testing of safety of their products and their pharmaceutical effects on complete human epidermis and dermis. Several companies produce organotypic skin models, including pigmented skin models that contain primary human melanocytes from different CSC types and are useful for testing skin lightening products. Commercial organotypic skin will be the least cost-effective *in vitro* experimental setting, and non-validated models may present manufacturing process batch-to-batch variability. List of commercial organotypic *in vitro* skin models is provided in Table 5.

Conclusions

Relatively simple one cell type monolayer based test methods present a good and cost-efficient starting point to test for safe concentrations and to substantiate biological effect claims. Most cosmetic formulation ingredients will come in contact with skin keratinocytes and possibly fibroblasts; therefore both cell types have been established as common *in vitro* monolayer test systems. As shown in

Table 5. Commercially available human skin equivalents for *in vitro* applications

Name	Cell type and product description	Manufacturer	Applications
EpiDermFT	Fibroblast-containing dermis with keratinocyte-containing epidermis	MatTek	General skin research, photo-aging/ photo-damage, UV radiation, skin wound healing
Advanced Skin Test 2000 (AST2000)	Dermal equivalent with embedded fibroblasts as a basis and epidermal layer of keratinocytes on top	CellSystems Biotechnologie GmbH	To measure effects of skin-active substances
SkinEthic Rhe (Reconstructed Human Epidermis)	Human keratinocytes; cells seeded on polycarbonate filter	SkinEthic	Skin corrosion an irritation testing; epidermal differentiation, percutaneous absorption; effects of UVA and UVB irradiation; UVB protection
EpiSkin	Normal human keratinocytes cultured on a collagen matrix at the air-liquid interface	SkinEthic	Skin corrosion; skin irritation; photoxicity; percutaneous absorption; effects of UVA and UVB exposure; UVB protection
Epiderm	Neonatal human-derived epidermal keratinocyte culture formed multi-layered, highly differentiated model of human epidermis; cells seeded on a collagen gel	MatTek	Skin irritation; toxicology research
StrataTest	Near-diploid human keratinocyte cell line in full thickness skin model	StrataTech	Wound healing assays; phototoxicity; acute toxicity; irritancy
Epidermal Skin Test 1000 (EST1000)	Reconstructed epidermal model made from primary human keratinocytes	CellSystems Biotechnologie GmbH	Skin corrosivity
Reconstituted Human Epidermis : RHE – EPI/001	Human keratinocytes from different donors; cells seeded on a polycarbonate filter	StratiCell	Safety and cosmetic product activity tests, ingredient penetration and absorption studies
Reconstituted Human Pigmented Epidermis: RHE – MEL/001	Human melanocytes and keratinocytes from different donors formed multi-layered model of pigmented human epidermis; cells seeded on a polycarbonate filter; melanocytes from Black or Caucasian donors available	StratiCell	Skin pigmentation, mlanin transfer and nuclear capping of keratinocytes;, validation of new active ingredients acting on melanogenesis; evaluation of photo-protection (sunscreens).
MelanoDerm	Human melanocytes and neonatal human epidermal keratinocyte culture formed multi-layered, highly differentiated model of pigmented human epidermis; cells seeded on a collagen gel; melanocytes from Asian, Black or Caucasian donors available	MatTek	Allows topical application of cosmetic formulations; evaluation of cosmetic and pharmaceutical ingredients for skin pigmentation modulation.
SkinEthic RHPE	Human melanocytes and keratinocytes formed multi-layered model of pigmented human epidermis; cells seeded on polycarbonate filter; melanocytes of CSC phototypes II (Caucasian), IV (Asian) and VI (Black) available	SkinEthic	The phototype VI model is used to evaluate the whitening potential of skin care formulations and phototypes II and IV models to assess the induction of pigmentation by UV irradiation and/or by chemical modulators.

the review, many of natural substances have been tested with keratinocytes and fibroblasts, which has provided evidence of their efficacy and aided to clarify mechanisms of action. More specific biological effects can be tested with appropriate pigment and endothelial cell monolayer test systems. The effectiveness of *in vitro* tests can be enhanced by combination of different methods and test strategies. For assessment or confirmation of effects identified in monolayer cell culture systems, co-cultures of different skin cell types and organotypic 3D tissue test models can be used. A number of parameters can be recorded and safety profile established for cosmetic formulations with organotypic skin models, but they do not functionally substitute *in vivo* tests with volunteers. Overall, *in vitro* cell and tissue tests are advisable for manufacturers as a cost-effective solution for new cosmetic product formulation development, in order to ensure consumer safety and to substantiate product claims.

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