Valorization Perspectives of Agro-Bio Waste in Pharmaceutics: Examples of Moso Bamboo Leaves and Rose Oil Distillation Waste Water

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Prof. Dr. V. Butterweck
Fakultätsverantwortliche

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Prof. Dr. Martin Spiess
Dekan

Thanatopsis

How doth the little busy cell
Tread the line ´twixt heav`n and hell?
Spinning threads of gene commands,
Repairing errors in the strands,
Cleaving daughters by mitosis
Without invoking apoptosis?
For life is but a tight-rope span,
A tenuous state for cell, or man:
With many a killer O_2 radical
From oxidations mitochondrial[1].

At least we now have consolation-
Death`s biocomic explanation –
Our time to shuffle from this scene`s
Dictated by gerontogenes[2].
And as we age `tis rather nice
To learn that dying`s put on ICE[3];
The senile scramblings of our brains
Are simply due to apopains`s
Aberrant acts, or morbid mutant[4]:
Morituri te salutant!

1. Kroemer, G. *et al.* (1995) FASEB J. 9, 1277-1287
2. Rattan, S. I. S. (1995) FASEB J. 9, 284-286
3. Kumar, S. (1995) Trends Biochem. Sci. 20, 198-202
4. Nicholson, D. W. *et al.* (1995) Nature 376, 37-43

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i. Abbreviations

AoROI	Area of Region of Interest
BrdU	5-bromo-2′-deoxyuridine
BLE	bamboo leaf extract
CLR	C-type lectin receptor
COD	chemical oxygen demand
COX-2	cyclooxygenase-2
CYP	cytochrome P450
CXCL8	(C-X-C motif) ligand 8
DAMP	damage-associated molecular pattern
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
dNTP	deoxynucleoside triphosphate
ELISA	enzyme-linked immunosorbent assay
EA	ellagic acid
ECM	extra cellular matrix
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinases
FCS	fetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein-coupled receptor
GSH	glutathione
HaCaT	human adult, low calcium, high temperature, skin keratinocytes
HIF	hypoxia inducible factor
H_2O_2	hydrogen peroxide
HUVEC	human umbilical vein endothelial cells
IF-γ	interferon γ
IGF-1	insulin-like growth factor-1
IL-1R	interleukin-1 receptor

IL-1Ra	IL-1 receptor antagonist
IL-1RII	IL-1 receptor type 2
iNOS	nitric oxide synthase
JNK	Jun N-terminal kinase
KGF	keratinocyte growth factor
LDH	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MMP	matrix metalloprotease
MTKI	multi-targeted kinase inhibitor
mTOR	mammalian target of Rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NGF	nerve growth factor
NLR	NOD-like receptor
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	nucleotide-binding oligomerization domain-containing protein
OMW	olive mill waste water
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PlGF	placenta growth factor
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PLA_2	phospholipase A_2
PRR	pattern recognition receptor
QQ	quercetin quinone
RANTES	regulated on activation, normal T cell expressed and secreted
RLR	(RIG)-l-like receptor

RODW	(polyphenol containing) rose oil distillation waste water
ROI	Region of Interest
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
TEAC	Trolox equivalent antioxidant capacity
TGF-β	tumor growth factor beta
TLR	Toll-like receptor
TMB	3,3',5,5''-tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
TRADD	TNF receptor-associated death domain
UV	ultra violet
VEGF	vascular endothelial growth factor

ii. Summary

Liquid and solid agro-bio waste consists of complex molecules, and depicts the residue of which the nutritionally valuable portion has been removed. Depending on its origin and constitution, the waste fraction can be pernicious for the environment. In cases of further processing of these residues to value-added products, it is possible to reduce or even avoid waste fractions and thereby reach a sustainable way of production. This relatively modern approach is referred to as valorization and regarded an environmentally friendly practice since it expands the whole production process and thereby increases the degree of utilization. The raw material for valorization-accessible matter predominantly is generated as by-products in the agricultural cultivation and the food manufacturing industry, which depict the first two steps in the food supply chain. Realizing valorization procedures without affecting the quality of the original product requires detailed knowledge about the composition of the waste fraction and other characteristics to assess its fields of application. Therein, inadequate biological stability, potentially pathogenic nature, high water content, potential for rapid auto-oxidation or a high level of enzymatic activity among others are limitation factors. Based on this concept, we investigated in pharmaceutical purposes of waste fractions of two different species: Moso bamboo (*Phyllostachys edulis*) and the Damask rose (*Rosa damascena*). In this context, particularly anti-inflammatory parameters, as well as wound-closure characteristics were of interest.

The first part of this work is elucidating moso bamboo (*Phyllostachys edulis*)-derived compounds. Moso bamboo wood is utilized in many different sections of predominantly Chinese handicrafts (see section 2.1). Furthermore, bamboo sprouts are edible and pose an important ingredient in far eastern cuisine. Due to the plant's fast growth of up to one meter per day, it depicts a potentially convenient resource for sustainable use of the wood. However, also tons of polyphenol containing leaves accrue during the harvest with no further pharmaceutical profit up-to-date. Since polyphenols are known for their multifaceted biological activities (see section 2.3), leaf extracts of *Phyllostachys edulis* were in the center of interest in terms of anti-inflammatory effects and wound closure activity on immortalized human keratinocytes and 3T3-swiss albino mouse fibroblasts. Therefore, levels of TNF-α-induced cytokines like IL-6, IL-8 and VEGF under influence of the extracts and its major single polyphenol isoorientin, respectively, were analyzed. Wound closure characteristics were evaluated employing a computerized modification of the classical scratch assay involving time lapse microscopy. It could be demonstrated an improvement of wound closure

activity under influence of bamboo leaf extract (BLE) at moderate concentrations. At higher concentrations, BLE inhibited wound closure without impairing cell viability. The effect of isoorientin was comparable. Furthermore, BLE reduced TNF-α-induced VEGF and IL-8 levels in HaCaT keratinocytes, isoorientin caused significant reduction of the cytokines in a dose-dependent manner. Generally, anti-inflammatory effects were determined moderately, since cytokine-levels of untreated cells could not be reached.

The second and main part of the project analyzes biological activities of by-products of the Bulgarian rose oil production. Rose oil is basically used for both as a food ingredient and in cosmetic formulations. As the world's main rose oil producer, Bulgaria cultivates the oil-bearing rose *Rosa damascena* in the Rose Valley producing more than 2000kg of the essential oil per year. Each kilogram of flowers requires the four-fold quantity of water for further processing (Fig. 4). The multi-step water steam distillation is meticulous, however, about 30.000 tons of polyphenol containing, bio-polluting waste water accumulate annually. This figure provides a first insight to recognize rose oil distillation waste water (RODW) as a substantial toxic factor. There is no effective post-production residue management in Bulgaria up-to-date.

Purification of RODW resulted in a polyphenol depleted and a polyphenol enriched fraction [(RF20-(SP-207)], of which the latter one was split into four sub-fractions F(I)-F(IV) depending on the analytical spectrum of polyphenolic compounds. In a dose-dependent fashion, RF20-(SP-207) revealed anti-proliferative properties in human keratinocytes determined by ELISA-based bioassays. Time-lapse microscopy detected a significant impairment of cell migration under influence of RF20-(SP-207) and F(IV), respectively. TNF-α induced VEGF-secretion was significantly reduced by both substances likewise. Since there is a close interplay between cell locomotion and inflammatory processes, the anti-inflammatory properties of both RF20-(SP-207) and F(IV) were elucidated utilizing reverse transcriptase polymerase chain reaction (RT-PCR). RF20-(SP-207) and F(IV) significantly decreased the gene expression and cellular protein secretion of IL-1β, IL-6, IL-8, RANTES and MCP-1 and therefore, markedly modified inflammatory parameters *in vitro.*

iii. Zusammenfassung

Flüssiger und fester Bio-Abfall besteht aus komplexen Verbindungen und stellt den Rückstand dar, von dem der nährstoffreiche Anteil abgetrennt wurde. Abhängig seines Ursprungs und seiner Zusammensetzung kann der Abfall einen umweltschädlichen Faktor darstellen. Im Falle einer Weiterverarbeitung dieser Rückstände zu aufgewerteten Produkten besteht die Möglichkeit Abfallanteile zu reduzieren oder gar zu vermeiden und dadurch eine nachhaltige Produktionsmethode zu erreichen. Dieser relativ moderne Ansatz ist auch als „valorization“ (deutsch: Aufwertung) bekannt und wird als umweltfreundliche Praxis betrachtet, da es den gesamten Produktionsprozess erweitert und damit den Nutzungsgrad erhöht. Die für die Aufwertung zugänglichen Rohstoffe werden hauptsächlich als Nebenprodukte in der Landwirtschaft und der Lebensmittelindustrie generiert, die die ersten beiden Stufen der Nahrungskette beschreiben. Das Umsetzen von Aufwertungstechniken ohne die Qualität des Originalprodukts zu beeinflussen setzt detaillierte Kenntnis der Zusammensetzung des Abfalls und andere Charakteristika voraus, um einen genauen Einsatzbereich des Nebenprodukts zu bestimmen. Dabei können eine verminderte biologische Stabilität, potentielle Pathogenität, ein hoher Wasseranteil, eine schnelle auto-Oxidation oder ein hohes Maß an enzymatischer Aktivität limitierende Faktoren sein. Auf diesem Konzept basierend haben wir pharmazeutische Verwendungen von Abfallprodukten von zwei verschiedenen Spezies untersucht: Moso Bambus (*Phyllostachys edulis*) und die Damaszener Rose (*Rosa damascena*). In diesem Kontext waren insbesondere antiinflammatorische Parameter, sowie Wundheilungscharakteristika von Interesse.

Der erste Teil des Projekts beleuchtet Verbindungen aus dem Moso Bambus (*Phyllostachys edulis*). Moso Bambus hat zahlreiche Verwendungen hauptsächlich im chinesischen Handwerk (siehe Kapitel 2.1). Zudem stellen Bambussprossen als essbarer Bestandteil eine wichtige Zutat in der fernöstlichen Küche dar. Durch ihr schnelles Wachstum von bis zu einem Meter pro Tag repräsentiert die Pflanze eine potentiell geeignete Quelle einer nachhaltigen Nutzung ihres Holzes. Jedoch fallen während der Ernte auch Tonnen von polyphenol-haltigen Blättern an, die bis dato keinen weiteren pharmazeutischen Nutzen innehaben. Polyphenole hingegen sind bekannt für ihre facettenreichen Bioaktivitäten (siehe Kapitel 2.3). Insofern stellt dieser Teil der Arbeit Blätterextrakte hinsichtlich ihrer anti-entzündlichen Aktivität auf immortalisierten humanen Keratinozyten und 3T3-swiss albino mouse Fibroblasten in den Mittelpunkt. Dafür wurden Spiegel von TNF-α-induzierten Zytokinen wie IL-6, IL-8 und VEGF unter Einfluss der Extrakte, bzw. seinem quantitativ

wichtigsten Polyphenol Isoorientin analysiert. Wundheilungscharakteristiken wurden unter Verwendung einer computerunterstützen Variante des klassischen „scratch assays“ mit Zeitrafferaufnahmen ermittelt.
Es konnte eine Verbesserung der Wundheilungsaktivität unter Einfluss von Bambusblatt-Extrakt (BLE) in moderaten Konzentrationen beobachtet werden. Im höheren Konzentrationsbereich inhibierte BLE den Wundschluss ohne die Zellviabilität zu beeinträchtigen. Der Effekt von Isoorientin war vergleichbar. Zudem reduzierte BLE TNF-α-induzierte IL-8 Spiegel in HaCaT Keratinozyten dosisabhängig, Isoorientin zeigte in dieser Hinsicht Signifikanz. Im Allgemeinen wurden die anti-entzündlichen Effekte als moderat bestimmt, da Zytokin-Spiegel von unbehandelten Zellen nicht erreicht wurden.

Der zweite Teil des Projekts analysiert biologische Aktivitäten von Nebenprodukten aus der bulgarischen Rosenölproduktion. Rosenöl wird hauptsächlich als Nahrungsbestandteil und in kosmetischen Formulierungen verwendet. Mit jährlich mehr als 2000kg des ätherischen Öls kultiviert Bulgarien als weltgrößter Rosenölproduzent die öl-bildende Rose *Rosa damascen*a im Rosental. Jedes Kilogramm Blüten benötigt dabei die vierfache Menge an Wasser für den weiteren Produktionsablauf (Fig. 2). Die mehrstufige Wasserdampfdestillation wird akribisch durchgeführt; allerdings akkumulieren dabei jährlich über 30.000 Tonnen polyphenolhaltiger, umweltschädlicher flüssiger Abfall. Dieser Wert erlaubt einen ersten Einblick, um „rose oil distillation waste water“ (RODW) als einen substantiellen umweltschädlichen Faktor zu erkennen. Unserem Wissensstand nach existiert bis dato noch keine effektive Rückstandsverwertung in Bulgarien.
Die Aufreinigung von RODW resultierte in einer Polyphenol-erschöpften und einer Polyphenol-angereicherten Fraktion [RF20-(SP-207)], von der letztere in vier Subfraktionen F(I)-F(IV) hinsichtlich ihres analytischen Spektrums an Polyphenol-Verbindungen aufgetrennt wurden. RF20-(SP-207) offenbarte dosisabhängig anti-proliferative Eigenschaften in humanen Keratinozyten, die auf Basis von ELISA Bio-Assays bestimmt wurden. Zeitraffer-Mikroskopie detektierte eine signifikante Beeinträchtigung von Zellmigration unter Einfluss von RF20-(SP-207), bzw. F(IV). TNF-α-induzierte VEGF-Sekretion zeigte sich signifikant reduziert durch beide Fraktionen gleichermaßen. Da ein enger Zusammenhang zwischen Zellbewegung und entzündlichen Prozessen besteht, wurden anti-entzündliche Eigenschaften von RF20-(SP-207) und F(IV) mittels Reverse Transkriptase Polymerase-Kettenreaktion (RT-PCR) beleuchtet. RF20-(SP-207) und F(IV) verursachten dabei sowohl eine signifikant verminderte Genexpression, als auch eine reduzierte zelluläre

Proteinsekretion von IL-1β, IL-6, IL-8, RANTES und MCP-1 und modifizierten somit deutlich entzündungsspezifische Parameter *in vitro*.

1. Aim of the Work

The world population is expected to grow from 6.7 billion in 2007 to 9.2 billion in 2050, which equals a rise of 37% [1]. Not only arable land, but also fresh water, air-quality and bio-diversity among others are essential to life. With putting pressure on them, the importance of an effective global resource management rises. There exists no exclusive tool to ensure environmental sustainability – in this context, research & development is one of several key areas.

Polyphenols as an important subgroup of phytochemicals are constituent parts in many natural resources and known for their multifaceted biological effects. To date, there exists a relatively broad data basis in the literature, which especially emphasizes on the antioxidant potential of the phytochemicals, but also on the strong metabolism when applied orally. This work analyzes polyphenol containing leaf extracts of *Phyllostachys edulis* and polyphenol-rich residues of rose oil distillation (*Rosa damascena*) on epidermal cells in terms of predominantly anti-inflammatory parameters, wound closure properties and inflammatory target gene expression using *in vitro* based settings.

The starting point was to establish a modification of the classical scratch assay including time lapse pictures to visually trace cell locomotion and proliferation of keratinocytes and fibroblasts over a longer period. The results then were compared with findings elucidating quantitatively cell proliferation, cell death and cytokine expression levels such as VEGF, IL-6, IL-8, RANTES and MCP-1. Finally, transcriptional activity of inflammatory genes was assessed. Thereby, it was possible to affirm the previous results by findings on a genetic level. Learning the major single compounds - isoorientin for *Phyllostachys edulis* and quercetin/ellagic acid/kaempferol for *Rosa damascena* - allowed to compare the observations with standard compounds and subsequently relate them to data in the literature.

With emphasis on *Rosa damascena*, distinct effects of the tested compounds on epidermal cell lines endorse a potential projection for *in vivo* experiments considering a possible treatment option of e.g. hyperproliferation and/or chronic inflammation involved skin diseases. In this tenor, to overcome issues like an insufficient bioavailability of polyphenols, a topical form of application would be imaginable.

Thereby, the work determines potentially pharmaceutical health benefits of two different types of natural products in the first instance, but also depicts a strategic approach to evaluate valorization options of originally process (agro-bio) waste.

[1] *Conféderation des industries agro-alimentaires de l'UE (CIAA)*, Managing Environmental Sustainability in the European Food & Drinks Industries, 2nd Edition http://www.fooddrinkeurope.eu/publication/managing-environmental-sustainability-in-the-european-food-drink-industries/, 2008 (accessed 11.10.2016)

2. Theoretical Background

2.1. The Concept of Biomass Waste Valorization

In every sort of industrial manufacturing, various types of waste accumulate. Regarding the food & drinks industry, especially organic waste mounts up. Agricultural raw material is the origin for all products of the food & drinks sector, but is also used for a long series of other branches like cosmetics and, of course pharmaceuticals among others. The partial overlapping of these branches is exemplified by neologisms like "cosmeceuticals" describing health benefits of a cosmetic or "foodaceuticals" addressing the well-being effect of certain foodstuff. In the following, it is not important which sector the final product is made for, but the composition of the waste fraction and its capacity for further benefit.

With 14,6% [1] of the market share within the European Union, the food & drink industry represents Europe's largest manufacturing sector in terms of turnover and, therefore, exceeds other large-scale industries like the automobile, chemical, machinery and equipment sectors [5]. The food supply chain includes agriculture, food manufacturing, transport, retail & distribution, and household & food services.

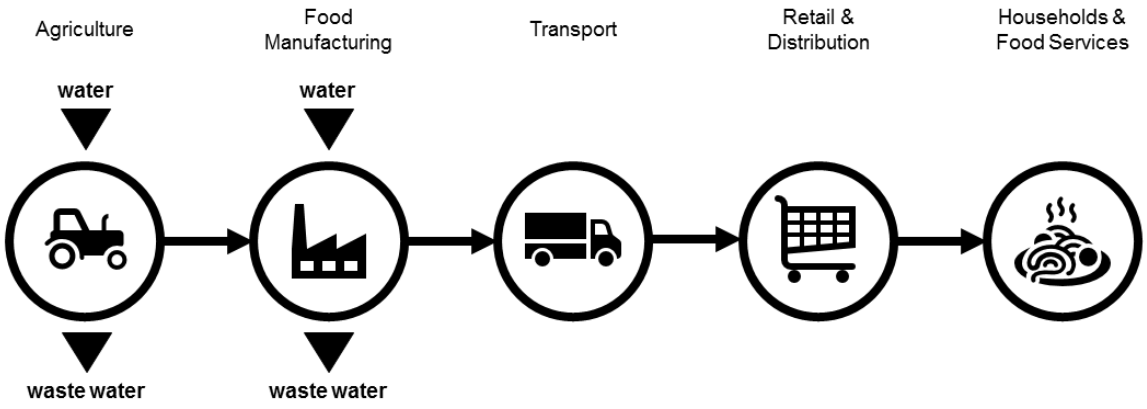

Fig. 1: The food supply chain starts with cultivation of natural resources and leads to the product for the end-consumer. Mainly in the agricultural sector and during food manufacturing, organic waste accumulates exemplified by the occurrence of waste water.

Each step again splits up in numerous single players, expanding the system to a highly diverse network. For instance, among the 32 million jobs in the European food & drinks sector, farmers represent the majority with a portion of 36% [5]. Since farming systems depict the starting point of the food supply chain, agricultural raw materials pose the basic substance of these life-cycle stages, wherein tons of organic agro-bio waste accumulate annually. Liquid and solid agro-bio waste consists of complex molecules, of which the nutritionally valuable portion has been extracted for further processing, but which is still available for use. In this

regard, the term "waste" can be irritating since it suggests a nugatory matter and should be replaced by the more neutral expression "by-product" if suitable for further usage. It accumulates predominantly during the agricultural cultivation, as well as in industrial processing in food manufacturing (Fig. 1). These are the two early stages in the food supply chain and depict the source of compounds that this work is dealing with. Due to the waste's diversity in terms of aggregate state, complexity of compounds and sheer quantity, it's impact affects economy, environment and society. In this regard, new strategies for proper energy use, greenhouse gas emissions, resource and waste management, water & wastewater management and packaging have emerged or is subject for improvement (e.g. by EU directives like ISO 14001 or the Sustainable Development Goals by the United Nations).

In brief, waste reduction is driven by strategies leading to "environmental sustainability", which requires an interaction of research, industry and policy makers of the food, cosmetic, pharmaceutical or agricultural sector, respectively. Regarding the modern consumerism lifestyle, it can be considered one of the major global challenges of the 21st century.
Every stage in the food supply chain has its own environmental repercussion from the farmer to the consumer. For example, reducing transport routes to save fuel costs and emission, exchanging plastic wrappings to bio-degradable materials etc. are popular approaches that mostly apply to latter stages in the food chain. However, attaining sustainability demands a shared responsibility among all players involved, but begins with the cultivation of raw material. Oftentimes, environmentally friendly practices are inflicted with additional costs and thus, are opposed by financial interests of the producers. Achieving the primary objective -the avoidance of waste accumulation in food manufacturing- is extremely difficult [6], but the establishment of waste treatment strategies on the food manufacturing level is one chief aspect in approaching sustainability. In waste management, the most simplistic form is waste disposal. Briefly, there are three basic methods depending on the water content: incineration, anaerobic fermentation, and composting [6]. For example, if the water content is <50% (m/m), incineration is the only feasible option. Wastes with a content > >50% (m/m) are applied to anaerobic fermentation producing methane and carbon dioxide (biogas). However, these forms can be described as an "end-of-pipe-abatement" [6] with no significant reduction or recycling options, because they do not affect the production process per se. A more favorable and relatively modern approach is a "zero-point-discharge", which implies the separation and selective recovery of compounds, and subsequent valorization of by-products to new value-added products [7]. The objective of this principle is multilayered such as harnessing natural resources, recycling of waste, et cetera. One example showing similarities

to the current situation of the Bulgarian rose oil production (see section 2.2) is the European olive oil production. As a main ingredient of the Mediterranean diet, virgin olive oil (from *Olea europaea*, also known as *Olea sylvestris*, Oleaceae) possesses a fairly positive image since this cuisine is associated with a lower risk of different forms of cancer, atherosclerosis, certain cardiovascular and neurodegenerative diseases [8-10]. Nevertheless, its industrial manufacturing generates problematic by-products in terms of environmental hazards. Typically, olive mill waste water (OMW) is a dark liquid due to its content of tannins and lignins (52.270-180.000 mg/L Pt/Co units) [11]. Its phenolic compounds and long-chain fatty acids are toxic to microorganisms and plants [12, 13]. Furthermore, phenols and sulfur dioxides generate emissions and thereby pollute the atmosphere [14]. Environmental toxicity, i.e. the organic load of waste waters can be specified by the chemical oxygen demand (COD), for instance. This parameter describes the total quantity of oxygen required for oxidation of organic compounds by a strong oxidizing reactant (e.g. potassium dichromate) under acidic conditions [15]. Usually, the value for COD is expressed in gram or milligram per liter. The literature reports COD-values for OMW between 40 and 220 g/L [13, 16, 17]. In this regard, it should be kept in mind that COD-values for OMW can vary, because of seasonal variances, different production methods, or different waste water treatments. As a comparison, the average COD of liquids processed by ordinary Swiss municipal waste water treatment plants is reported to be 155.6 mg/L before treatment [18], thus, incontrovertibly lower. Mainly in Greece, OMW is disposed of in specific evaporation ponds. There, the waste water transforms into an acidic sludge-type of liquid without further processing. This technique already poses a respectable achievement in terms of environmental protection, since OMW has been illegally disposed of on soil or in rivers for many years leading to fatal consequences for the environment in the Mediterranean area [16]. The major immediate drawbacks of evaporation pond disposals, however, are the occurrence of bad odor, soil infiltration and insect proliferation. This disposal method generally does not support the idea of a sustainable production. Studies about the pharmaceutical benefit of OMW among other valorization approaches exist (e.g. [19]). Nonetheless, no specific marketed product has emerged out of OMW, nor has an environmentally sound and cheap strategy for detoxification been established. Besides the liquid waste fraction, also (semi-)solid pomace accrues. Contrary to OMW, the pomace fraction is further utilized in forms such as soap, wood, biodiesel production, cosmetics and dietary products [20]. In terms of trade chains, the olive oil manufacturers typically resell accumulated olive cake to pomace treatment companies for

further processing. Therefore, the solid waste poses one example for a valorized, marketable by-product in agro-industrial olive oil production.

Tab. 1 gives an overview of major branches of the food & drink industry including their chief waste fractions, for which valorization options are not exhausted yet.

Branch	Type of waste
grain products	**bran, middlings, broken grains, seeds, shells, husks, fine dust, chaff, straw, ergot, brown rice waste, rice bran, rice flour, seedlings from malt, malt dust, grain separator waste**
noodles	**dough waste, eggshells**
potato processing industry	**potato peels**
coffee roaster	**silverskins**
sugar	**molasses, beet pulp, beet leaves and stems, carbonation sludge**
dairy processing industry	**whey, cheese residue, waste from milk production**
meat industry	**slaughterhouse waste**
egg products	**eggshells**
beer production	**malt dust, spent grains, break material, yeast, kieselguhr sludge**
wine cellars	**pomace, clarification sediment, yeast sediment**

Tab. 1: Overview of typical branches of the food industry producing various types of waste. Not for every sort of waste, valorization options have been assessed, which depend on biological stability, pathogenic nature, water content, enzymatic activity among others. Data adopted from [4].

However, the main complications regarding the valorization of agro-bio waste is a result of for example the inadequate biological stability, potentially pathogenic nature, high water content, potential for rapid auto-oxidation and high level of enzymatic activity among others [4]. This work is focusing on the examination of biomass waste-derived compounds for pharmaceutical purposes, which depicts one further approach of waste recovery.

2.2. Ecological Aspects of the Chinese Bamboo Cultivation and the Bulgarian Rose Oil Production

One of the natural resources this work has been used materials of is "bamboo". The term commonly describes a member of a taxonomic group of woody grasses (*Poaceae*), which belongs to the subfamily *Bambusoideae* [21]. More than 1300 species occur across the tropical and subtropical belt; except for Europe and Antarctica, bamboo is native to every continent [22]. China's bamboo population -that is referred to in the following- accounts for about 300 species growing on a land area of approximately 45.000 km^2 (Switzerland total area approximately 41.000 km^2), which relates to 2.8 % of the country's total forest area [23]. Covering 70 % of this forest area, moso bamboo (*Phyllostachys edulis*, also known as *Phyllostachys pubescens*) is the most prominent species, which primarily grows in the south of China.

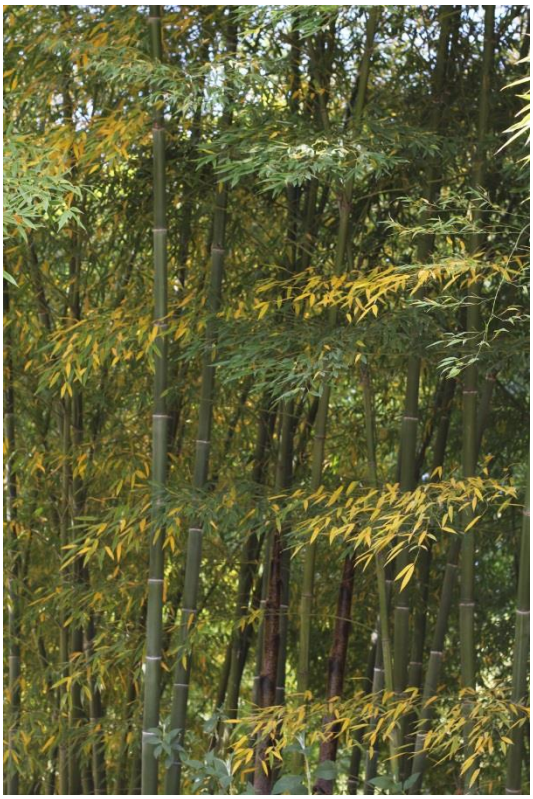

Fig. 2: Forest of Moso bamboo (*Phyllostachys edulis*). Picture adopted from http://bamboogarden.com

The graminaceous plant shows an above-average fast growth (up to one meter per day; Fig. 2) and can be considered notably versatile; over 40 % of the world's population utilize bamboo every day [24]. If used properly, bamboo forests can be an inexhaustible, sustainable source of raw material [25]. After cutting the culms, no replanting is needed. Due to its superior tensile strength, it is mostly used for handicrafts like furniture, paper, textiles, plywood boards and building material [24]. It is also harvested for its edible shoots and - in light of the climate change - bamboo gained considerable importance in carbon cycling [26, 27]. However, due to a lack of knowledge and equipment of the local farmers, forest management and production yield is still limited in many cases [28]. According to *Zhuang et al.* [21], the biomass proportion of leaves and twigs of an average moso bamboo tree accounts to 14 ± 4 %. Looking at the vast land areas of Chinese bamboo forests and the demanding needs of the world's population, bamboo leaves pose a by-product that accrues in tons. In the years 1990, 2000, and 2005, China's stock of bamboo resources was reported to be 96, 144, and 164

million tons, respectively [28]. As an organic material, the leaves of course do not pose an environmental hazard per se since the fall of leaves is part of the natural production cycle. Thus, an organic waste disposal in terms of composting would be possible, but this does not enable any recycling or recovering options and thus, does not support the idea of resource sustainability.
Nevertheless, the leaves have gained considerable attention in pharmaceutical research in recent years. Besides being a food source for cattle and wildlife animals [22], the leaves contain bio-active molecules (polyphenols among others) of potentially pharmaceutical value. These properties allow for various valorization options, of which further elucidation is needed. In fact, bamboo has attracted curiosity of western companies of late (e.g. Organic Bamboo Industries AG, St. Gallen, Switzerland), which focus on beauty- and health products made of bamboo leaf extracts.

With respect to the environmental issues from olive oil fabrication in Mediterranean countries, the situation of the industrial rose oil production in Bulgaria is comparable. For centuries, Bulgaria cultivates the oil-bearing *Rosa damascena* Mill. (Rosaceae) in the Valley of Roses ("Rozovata Dolina") near the city of Kazanlak (Fig. 3). Because of favorable climate conditions and centuries of experience, the rose oil (also known as rose otto or rose attar) is reckoned a product of higher quality and value compared to other producing countries. Since 2014, the European commission includes the essential oil in the list of protected geographical indications and designations of origin for agricultural product and foodstuffs under the name of "Bulgarsko rozovo maslo". The annual global consumption of pure rose otto is around 3000-4500 kg; of this, 80-90 % is produced by Bulgaria and Turkey [29]. In 2008, one kilogram of rose oil was sold at a price of 4600 €. For this reason, the industry has a respectable social impact as the product is mostly exported and, thus, supports local economy, tourism, research & development, generates jobs and overall creates a positive reputation of the nation. Nevertheless, its production method is afflicted with the accumulation of polyphenol containing rose oil distillation waste water (RODW).

Fig. 3: Rose field in the Valley of Roses, Bulgaria. (*Rosa damascena*). Picture adopted from http://rosecosmetic.net

In brief, rose petals are typically harvested from May to June between sunrise and noon, when the oil content in the flower is at a maximum. Immediately after, the blossoms are put in the hydro-distillation still, where they are boiled for about two hours to release the essential oil. The water steam distillation of rose oil produces a valuable by-product called rose water next to the essential oil. The hydrosol contains 2-phenylethanol among others, which is partly water soluble [30] and mostly responsible for the distinct rose scent, because it is soluble in the essential oil and poses a major compound. By repeating distillation cycles manifold, 2-phenylethanol is transferred from the aqueous to the lipid phase and thereby the yield of the rose oil increases. Within this process, polyphenols that were originally contained by the flower, are now passed over to the waste water, of which large volumes accrue.
As an estimation, to obtain ten grams of the attar, about 100 kg of rose petals are necessary [31]. Depending on the size of the distillers, four to eight liters of water per kilogram rose material is added. In 2008, Bulgaria produced about 2000 kg of pure rose oil [29]. As a total, more than 30.000 tons of liquid waste accumulate per year, which provides a first insight to recognize RODW as a serious, substantial pollution factor.

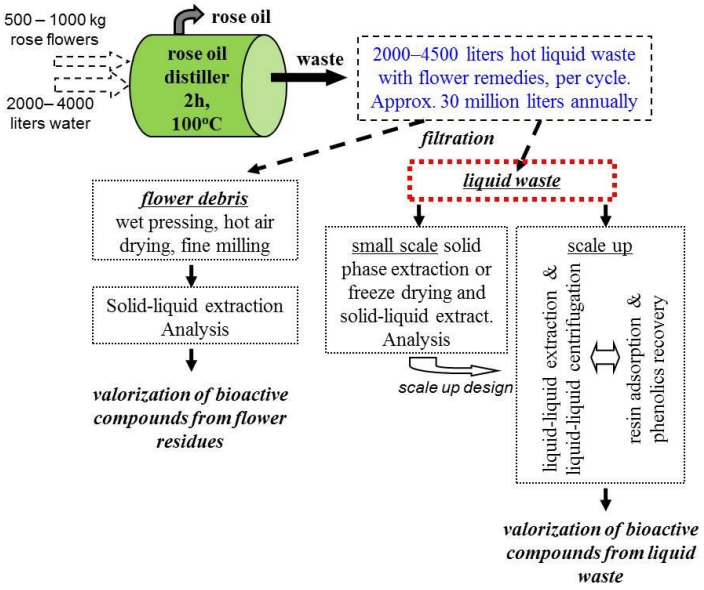

Fig. 4: Flow chart of the Bulgarian rose oil production including the liquid waste accumulation and compound purification.

The meticulous multi-distillation cycle process is referred to as cohobation [29] and describes the repeated return of the separated condensate to the still during the actual distillation process. This is necessary to increase the yield of the essential oil and enrich the hydrosol with intensive smelling compounds like 2-phenylethanol. Furthermore, this procedure results in relatively high levels of dissolved, non-volatile polyphenols in the remaining RODW, which has commonly been regarded as a low-quality by-product that is all too often disposed inappropriately. Fig. 4 shows a flow chart of the waste fractions accruing during the production process. Therein, only fractions out of the liquid residues were of interest.

Looking at the environmental toxicity of RODW, literature is scarce. *Avsar et al.* [32] reports a COD-value of 9500 mg/L, which refers to the rose attar production in Isparta, Turkey, but can be conjectured the same for the Valley of Roses. Up until today, there is no effective post-production waste water management in Bulgaria, when looking at the rose oil distillation. However, based on a sustainable way of resource utilization, RODW as a by-product could potentially pose an interesting valorization option for pharmaceutical purposes, which will be specified in the following.

2.3. Major Polyphenolic Compounds in Rosa damascena

One important aspect of waste water management is the analysis of its composition. Since polyphenols represent the pharmacologically relevant constituents herein, a short introduction shall be given.

Polyphenols describe a highly diverse group of predominantly natural, organic compounds encompassing simple monomers, oligomers to complex polymers [33]. Over 8000 different structures are identified, about half of them belong to the sub-group of flavonoids. There exist polyphenols such as anthocyanins, lignins or stilbenes that count as secondary metabolites, which are synthesized *in planta* as a defense strategy to deter herbivores [34], or in the case of flavonols and flavones act as UV filters [35]. Also, there are polyphenols, especially flavonoids, which attract organisms intending pollination or seed dispersal for survival and propagation. As dietary compounds, they mostly determine organoleptic properties in foodstuff and beverages. Despite sharing a common phenolic structure, they show fairly strong differences in their physicochemical properties. Particularly in case of the flavonoids, one of their most prominent feature is their antioxidant quality, which has been widely elucidated by the literature (e.g. [36-39]). What is more, bio-activities such as anti-histamine [40], anti-bacterial [41], anti-viral [42] and enzyme-inhibitory [43-45] among others have been described.

Encompassing more than 300 different chemical structures in total [29], both *Rusanov* [46] and *Schieber et al.* [47] have detected quercetin, kaempferol and their derivatives as prime phenolic constituents in RODW based on HPLC-ELSD and HPLC-LC/MS, respectively. These outcomes are specified through HPLC-ELSD guided analysis by *Solimine et al.* [45], who examined the substance utilized in the present work and detected quercetin, kaempferol and ellagic acid as the major constituents.

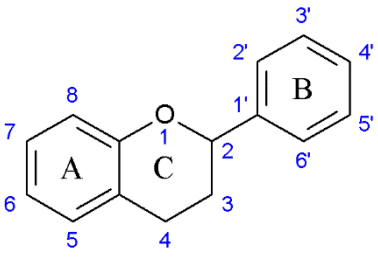

Fig. 5: C6-C3-C6 structure (flavan).

Quercetin and kaempferol are members of the flavonols, a sub-group of flavonoids (Fig. 6). The flavonol's backbone is a C6-C3-C6 structure (flavan; Fig. 5) in which the C6-units are of phenolic nature, while the C3-body as a chromane ring is subject to hydroxylation. With regards to quercetin and kaempferol, the B-ring is attached to the C2-position of the C-ring. Moreover, quercetin possesses two hydroxyl groups attached to the B-ring (C4' and C5'), whereas kaempferol has only one (C4').

In planta, most of the polyphenols exist as glycosides, giving quercetin and kaempferol a variety of more than 279 and 347 substitution patterns [35].

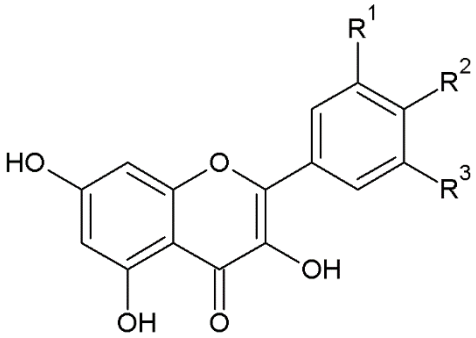

Fig. 6: basic structure of flavonols.
Quercetin R^1=H, R^2=OH, R^3=OH
Kaempferol R^1=H, R^2=OH, R^3=H

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is considered one of the most effective reactive oxygen species (ROS)-scavengers among all flavonoids [48], as well as a potent metal ion chelator. Anti-inflammatory effects in the form of symptom alleviation were observed using quercetin as treatment against chronic prostatitis [49]. *Begum and Terao* [50] reported about a quercetin-protective effect against aqueous cigarette-tar extract induced impairment of erythrocyte deformability. However, the variety of physiologic effects by quercetin also bears some unfavorable outcomes such as the quercetin paradox described by *Boots et al.* [51]. That is while scavenging free radicals formed by hydrogen peroxide as DNA-strand protection, quercetin converts into its oxidized form, primarily a semiquinone radical, which immediately forms to quercetin quinone (QQ) in a second reaction. QQ is thiol-reactive manifested by a preferential interaction with glutathione (GSH) ultimately leading to an increase in cytosolic calcium level and lactate dehydrogenase (LDH) leakage. Nonetheless, in 1999 the International Agency for Research on Cancer (IARC) has declared quercetin as non-carcinogen for humans [52].

Kaempferol (**3,4',5,7-tetrahydroxyflavone)** resembles the pharmacologic profile of quercetin in many aspects due to its structural similarity. However, the antioxidant potential, for instance, is lower because of the missing catechol structure in the B-ring. This structure-activity relationship is illustrated *in vitro* by the TEAC (Trolox equivalent antioxidant capacity) established by *Rice-Evans et al.* [38]. In brief, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) is a water-soluble vitamin E derivative, which scavenges 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$), a preformed radical cation chromophore, at pH 7.4. The TEAC is defined as the concentration of Trolox solution necessary to scavenge the same quantity of $ABTS^{\cdot+}$ like the antioxidant of interest in a concentration of 1 mM. Thus, the value permits a comparison of different antioxidants by expressing their scavenging potential and is typically used to characterize antioxidant capacities of polyphenol-containing solutions. For quercetin, a TEAC of 4.7 mM is reported, while kaempferol accounts for 1.34 mM [37].

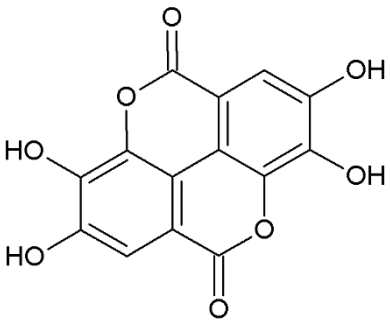

Fig. 7: ellagic acid is a dimer of gallic acid.

Pointing out the flavonol's beneficial effects on inflammation, kaempferol has been precisely reviewed by *Devi et al.* [53]. Ellagic acid (2,3,7,8-Tetrahydroxychromeno[5,4,3-cde]chromene-5,10-dione) is a gallic acid dimer, and therefore does not belong to the group of flavonoids (Fig. 7). *In planta*, ellagic acid exists in form of hydrolyzable tannins. Ellagitannins are esters of glucose and contribute to the structure of the cell wall and -membrane [54]. Naturally, ellagic acid (EA) occurs predominantly in nuts, berry fruits such as strawberries or raspberries, but, interestingly, also in distilled beverages like several types of whiskies, brandies and rum [55]. In the literature, ellagic acid oftentimes is associated with anticarcinogenic properties. *Zhang et al.* [56] suggests a pathway modulation of the tobacco specific carcinogen 4-(methylnitrosamino)-1-(3- pyridyl)-1-butanone reduction by ellagic acid-dependent inhibition of constitutive CYP-enzymes. *Vanella et al.* [57] hypothesize EA influencing apoptosis, for example by modifying the expression levels of proteins like TGF-β or IL-6, which are strongly involved in tumor growth and cell invasiveness of prostate cancer. In this context, numerous citations could be listed. However, besides anti-carcinogenic or -mutagenic effects, also subcellular pathway modulations are reported. *Edderkaoui et al.* [58] describe EA to stimulate apoptosis and inhibit proliferation of human pancreatic adenocarcinoma cells, an activation of the mitochondrial death pathway triggered by the release of cytochrome C and caspase-3 activation and a decreased activity of the transcription factor NF-κB. In higher concentrations, EA was found to inhibit proliferation of ovarian carcinoma in ES-2 and PA-1 cells by arresting both cell lines in the G_1-phase.
In contrast to the reportedly antioxidant activity of flavonoids and the physiological outcomes accordingly, ellagic acid has multifaceted effects on cells. Its numerously recited anticarcinogenic character is a result of direct (e.g. G_1-phase arrest) and indirect influence (subcellular pathway modulation) on the cell cycle, as well as on cell death determinants such as caspase-3 for instance. Above all, it must be kept in mind that most of these studies were conducted under *in vitro*-conditions. The question of the correlation between these data and studies in humans remains. One reason for this is, because *in vivo*-studies in humans are difficult to conduct due to obstacles such as environmental factors, protein bonding, colonic microflora, low bioavailability, complications in detection methods, rapidity, extent of metabolism and many others. However, few studies dealing with polyphenols used on animal models exist, for example by *Frei and Higdon* [59], who referred to the antioxidant activity of

tea polyphenols in animal models. Therein, they describe plasma and intracellular concentrations of tea catechins and polyphenols being 100 to 1000 times lower than physiological antioxidants like ascorbate, urate and GSH, which alludes to bioavailability issues of polyphenols. In contrast, oral feeding of a polyphenol-rich fraction of green tea to hairless SKH-1 mice with subsequent exposition to UVB radiation resulted in a lower COX-activity. This outcome was illustrated by a prevention of cutaneous edema and depletion of the antioxidant-defense system in epidermis demonstrated by *Agarwal et al.* [60]. Similarly, the uptake of green tea-containing drinking water stopped ethanol-induced GSH-level decrease in male rat livers [61]. A study addressing human plasma concentrations after consumption of epicatechin gallate, epigallocatechin, or epigallocatechin gallate has been executed by *Van Amelsvoort et al.* [62]. They found significant differences in the pharmacokinetic behavior of these substances among ten healthy volunteers and could not confirm a significant, stable increase of plasma antioxidant capacity. *Naumov et al.* [63] tested a formulation containing dihydroquercetin on burned rat skin and uncovered inhibition of factors inducing development of necrosis, tissue ischaemia, wound infections as well as facilitation of tissue regeneration. *Shubina and Shatalin* [64] tested various dihydroquercetin formulations on chemically burned rat skin and found protective properties including the re-establishment of intact hair follicles and sebaceous glands. Especially anti-inflammatory, immune modulating and anti-microbial functions among others have been ascribed to quercetin and ellagic acid by *Dzialo et al.* [3] reviewing plant phenolics in prevention of skin disorders (Fig. 8). Thus, disagreements exist in terms of bioavailability, bioactivity in target tissues and metabolism of dietary polyphenols, but it can be concluded that skin recovery effects by polyphenols have been observed. One reason for the differences between animal and human studies, for instance, could be animals generally receiving higher doses per kg bodyweight. Moreover, humans show wide genetic variations in response to

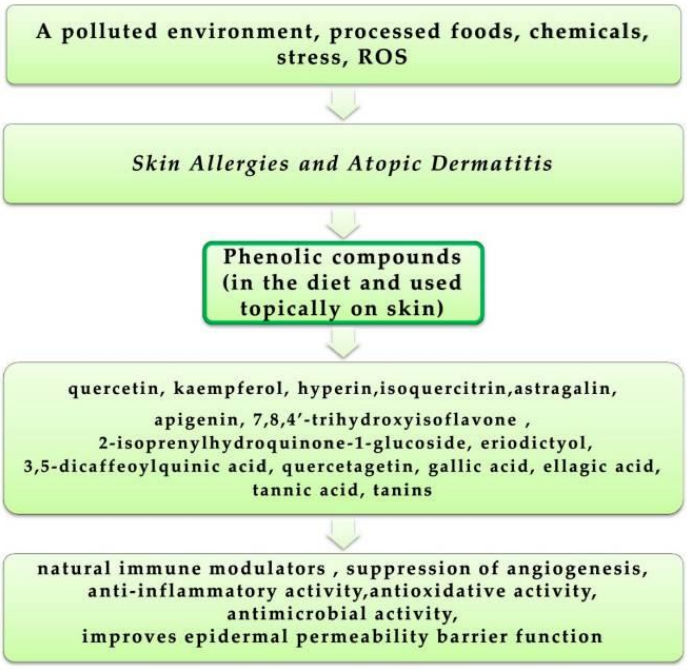

Fig. 8: Pharmacological properties of polyphenols. Figure adopted from *Dzialo et al.* [8].

oxidative stress and cancer, which can mask changes of biomarkers to some extent [59]. For reviews addressing this issue thoroughly, please refer to [65-67].

2.4. Inflammation

The previous chapter describes polyphenols, especially quercetin and ellagic acid as inflammation modulating substances. Since these compounds are key ingredients in *Rosa damascena*, and the fact that pro-inflammatory parameters play a crucial role in this work, it is important to outline the major processes during these types of skin insults.

The term "inflammation" is commonly used for the complex biological response of the mammalian organism triggered by any type of bodily insult such as pathogenic signals (e.g. UV- or X-ray irradiation, heat), abrupt anoxia, noxious chemicals (e.g. H_2O_2), infectious agents (viruses, bacteria, prions, fungi) or mechanical tissue damage. Regarding the surface of the body, the onset of the inflammatory response is clinically characterized by calor (heat; describing the increase of temperature in the affected tissue due to vasodilatation and thereby increased blood flow), dolor (pain), rubor (redness; evoked by the upregulated blood supply), tumor (swelling; body fluid accumulates because of increased tissue permeability) and sometimes functio laesia (loss of function; immobility) [68]. Since these symptoms are generic, it is apparent that the inflammatory response usually begins with the immediate activation of the innate immune system.

In brief, the inflammatory stimulus is typically detected by sensor cells such as macrophages, granulocytes, mast cells or dendritic cells through activation of innate pattern recognition receptors (PRR). According to *Rajamuthiah and Mylonakis* [69], PRR`s can be divided into four subgroups: Toll-like receptors (TLR's), C-type lectin receptors (CLR's), (RIG)-l-like receptors (RLR's) and NOD-like receptors (NLR's). They occur either at trans-membrane locations (TLR's, CLR's) or in the cytosol (RLR's, NLR's). These receptors individually recognize foreign pathogen-associated molecular patterns (PAMP's), which are mostly conserved, repetitive structures and unique to the specific stimulus (e.g. surface patterns of the gram-positive or gram-negative bacteria cell wall). Furthermore, PRR's can detect damage-associated molecular patterns (DAMP's), that is body's own cellular degradation products. Once activated, the sensor cells either act directly neutralizing the noxious agent (e.g. by phagocytosis) or indirectly by the release of mediators like cytokines and chemokines, which themselves entice other immune cells to the inflammatory site (chemotaxis) and thereby propagating the immune response [70]. Adjacent processes are governed by the adaptive immune system, which are highly specific depending on the insult and are directed to a long-lasting protection. Whereas the response of the innate immune system lasts a few days at most, the acquired part is active for days to weeks, because the organism creates memory T

and B cells to shield the body from a repeating insult. In any case, the adaptive immune response can only be initiated by the innate immune cascade [71].

The innate immune response takes effect minutes after injury or infection, but is limited in its effectivity due to a lack of specificity. From an evolutionary perspective, a self-limiting acute inflammation displays the first-line response of the organism and is crucial for survival. That is, considering a four to seven days-delay before the sophisticated adaptive immune system takes effect, it is of utmost importance for the organism to control the insult during this period. Without the tightly regulated cascade, the organism would eventually perish because of tissue disruption, sepsis etc.

Cytokines, Chemokines, Interleukins

To a small degree, the innate immune system can regulate its response. Depending on which PRR's are specifically activated, a certain subset of mediators is secreted by the sensor cells. One subgroup is represented by small proteins of about 25 kDa called cytokines. Their effects, which are briefly specified in the subchapters accordingly, can be autocrine, paracrine or -depending on their half-life in the blood and their bioavailability- endocrine. Generally, these effects are manifested in profound influence on leukocyte migration and function, hematopoietic cell numbers, temperature regulation, acute phase responses, tissue remodeling and cell survival. The way of production is a multi-step process and oftentimes happens by the activation of the key integrator of cell signaling and transcription factor NF-κB [72]. Using the example of IL-1β production by TLR activation, the cascade is illustrated in detail by *Takeda and Akira* [73]. *Keestra-Gounder et al.* [74] describe the NOD1 and NOD2 triggered IL-6 stimulation that also happens via the NF-κB cell signaling pathway.

Cytokines, which additionally attract and direct effector cells (chemotaxis) to the inflammatory site, are called chemokines (~8-14 kDa). The name has been introduced at the "Third International Symposium on Chemotactic Cytokines", which took place in Baden-bei-Wien, Austria, in 1992. The first and best characterized member of these type of proteins is CXCL8, better known by the name of IL-8. Responding cells are predominantly leukocytes, which in turn activate monocytes, neutrophils and other blood-derived effector cells. Furthermore, chemokines influence leukocytes of the adaptive immune system, too [70]. Compared to the variety of cytokines, there exists a relatively little number of receptors - showing a certain receptor promiscuity. A single chemokine can bind to several receptors,

while a single receptor can transduce signals to many chemokines. Mechanistically, their chemotactic effect is mediated by seven trans-membrane-domain receptors, which are part of the G-protein-coupled receptor (GPCR) superfamily [75]. Thus, their functional redundancy (see [75], Table 1) delivers a great beneficence in controlling the course of an inflammation. Structurally, chemokines can be divided into two major groups: the CC-chemokines present two vicinal cysteines, while in CXC-chemokines, the two cysteines are separated by one amino acid. Accordingly, CC-chemokines bind to CC-receptors, whereof nine different are known. CXC-chemokines on the contrary bind to CXC-receptors, whereof six are known up-to-date.

The term “interleukin” was originally introduced to simplify the nomenclature of proteins that either are secreted by or act on leukocytes. However, with progressing research, the complexity and number of this group of proteins increased rapidly. Although the “interleukin”-nomenclature is still popular, a structure-related designation (e.g. CXCL8 instead of IL-8) is considered more precise.

Generally, cytokines and chemokines function as potent immune modulators. They can be regarded as the interface between the innate and the adaptive immune system, since they take influence on them likewise. Just like in every form of communication, it takes a sender, a message and a recipient. Looking at this allegory, the activation of PRR´s poses as the sender that posts an encrypted message in form of the cytokine, which must be delivered to a cell containing the decrypter in form of the receptor transducing the signal [76]. In summary, four characteristic key features can be observed, that apply to almost the entire cytokine network: 1. pleiotropy (most cytokines cause several effects); 2. redundancy (one effect is caused by many different cytokines); 3. potency (most cytokines act in the nanomolar to femtomolar range); 4. action as part of a highly-regulated cascade.

Tumor Necrosis Factor Alpha

Originally introduced by the name of “cachectin” [77], tumor necrosis factor alpha (TNF-α) is a key mediator in the inflammatory response and secreted by many different cell types such as monocytes, macrophages, B/T cells, and fibroblasts. As a member of the acute-phase-proteins, the protein of 17 kDa can bind to two different receptors: TNF receptor type 1 (TNF-R1) and TNF receptor type 2 (TNF-R2) posing the TNF receptor superfamily. While the transduced signals of TNF-R2 are yet to be fully understood, TNF-R1 has been further

elucidated [78]. Predominantly, the receptor binding increases the gene expression of lots of pro-inflammatory genes by activation of NF-κB and thereby decisively influence the course of an inflammatory response. In addition, in some transformed cell types, the TNFR1-transduced signal realizes apoptosis and/or necrosis, e.g. by activation of caspase-8 through the adapter protein TNF receptor-associated death domain (TRADD) in the apoptotic pathway. As a third major characteristic, TNF-α can activate the mitogen-activated protein kinase (MAPK) cascades [79, 80], that is the Jun N-terminal kinase (JNK), the p38 MAP kinase [81] and to a smaller extend the extracellular signal-regulated kinases (ERK).

Its pleiotropy makes TNF-α a crucial factor in the regulation of inflammation, autoimmunity and neurodegeneration involved diseases such as rheumatoid arthritis, multiple sclerosis, Parkinson's disease, and Alzheimer's disease [82, 83]. Therefore, TNF-α has become one attractive target for drug development, for example in form of the biopharmaceutical Etanercept.

Interleukin-1 beta

One synonym of interleukin-1 beta (IL-1β) is "leukocytic pyrogen", which is presumably the first physiologic action it was related to. Its purification was originally described by *Dinarello et al.* in 1977 [84]. The interleukin-1 superfamily of ligands and receptors is probably associated with acute and chronic inflammation more than any other mediator in this context and fundamental for the innate immune system [85]. IL-1β (17.5 kDa) is preceded by its immature form pro-IL-1β (31 kDa), which must be cleaved intracellular by Interleukin-1 Converting Enzyme (ICE, also known as caspase-1) or extracellular by neutrophilic proteases to become responsive for the interleukin-1 receptor (IL-1R). Interestingly, the cytosolic segment of the ligand binding chain IL-1 receptor type 1 (IL-1RI) shares a homologous domain with the Toll protein in *Drosophila*. This explains some functional similarity of the two molecules in terms of TLR-activation and thus, can be considered the link to the nonspecific host defense in response to pathogenic invaders [85]. Pro-IL-1β is produced by macrophages and epithelial cells after exposition to PAMPs and DAMPs [86] (for a detailed review of the mechanism, see *Takeda and Akira* [73]). Downstream cytoplasmic signaling pathways modulate the expression of several genes including IL-1β itself, TNF-α, matrix metalloproteases (MMP's), nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), phospholipase A_2 (PLA_2) and others [87]. Surprisingly, despite its low specificity, IL-1β is

capable to trigger antigen-specific responses of murine T cells demonstrated by *Rosenwasser et al.* [88] and thereby, affecting the acquired immune system, too. However, IL-1β binds with greater affinity to the highly-conserved IL-1 receptor type 2 (IL-1RII). This receptor is mostly expressed on the cell surface of macrophages and B cells and thereby, poses a decoy receptor. The fact that glucocorticoids for instance induce the production of IL-1RII could partly explain the receptor's anti-inflammatory properties [85]. Studies suggest a relation between the level of IL-1β, IL-1 receptor antagonist (IL-1Ra) and IL-1RII in relation to disease severity. Therefore, IL-1β has also attracted the focus of drug development. One successful model in this regard is the biopharmaceutical anakinra, which is a recombinant version of the IL-1 Receptor antagonist (IL-1Ra) used in the treatment specifically for rheumatoid arthritis. It competitively binds to the IL-1RI (same affinity like IL-1β), but less to IL-1RII (compared to IL-1β) for which reason its potential is limited and considered inferior to TNF-α blockers [87, 89, 90]. Thus, research now tends to focus on blocking directly with higher inhibitory potency such as canakinumab (for detailed review, see [91, 92]).

Interleukin-8

Before establishing the general designation "Interleukin-8" (IL-8; also known by the systematic designation CXCL8), in various publications the protein was independently described as "neutrophil-activating factor" [93], "monocyte-derived neutrophil-activating peptide" [94], "monocyte-derived neutrophil chemotactic factor" [95] and others. Its activating properties in terms of neutrophil migration led to the assumption that it was neutrophil-specific. However, it subsequently was revealed that CXCL8 is actually produced by a diversity of cell types such as monocytes [93, 94, 96], T cells [97, 98], macrophages, keratinocytes [99], fibroblasts [100], epithelial cells, hepatocytes, synovial cells [101] and endothelial cells [102, 103]. CXCL8 is one out of eight members of the CXCL8 family, who all have an ELR (glutamic acid-leucine-arginine) motif in their structure in common [104, 105]. Moreover, the family shares the same receptors, that is CXCR1 and CXCR2, which are G-protein coupled and have different selectivity for their ligands making them interesting therapeutic targets for inflammation-related diseases (atherosclerosis, cancer, inflammatory bowel disease, ischemia, asthma, cystic fibrosis, multiple sclerosis, psoriasis, rheumatoid arthritis and many others). According to *Wolf et al.* [106], CXCL8 possesses a relative selectivity to CXCR1. However, both receptors are also produced by a great variety of cell types including keratinocytes [107], fibroblasts [108], endothelial cells [109], hepatocytes

[110] and others illustrating the ubiquity of the CXCL8 family. From a physiological perspective, CXCL8 has not only been related to chemotactic actions, but also to angiogenesis in human umbilical vein endothelial cells (HUVEC) [111], lung cancer cells [112] or L1.2 transfectants [113]. In summary, it can be said that *in vitro*-designed studies accentuate chemotactic effects of CXCL8, while *in vivo*-based studies prevalently emphasize on its pro-angiogenetic character, especially in diseases, which are related to chronic inflammation.

Monocyte Chemotactic Protein-1

In contrast to CXCL8, monocyte chemoattractant protein-1 (MCP-1/CCL2) does not belong to the CXC family, but to the CC family. Therefore, it does not share an ELR motif in its structure. For this reason, CCL2 predominantly chemo-attracts monocytes, memory T cells, and dendritic cells [114, 115], but poorly attracts neutrophils [116]. CCL2 was the first CC chemokine discovered in humans and originally isolated from mouse 3T3 fibroblasts [117], then soon thereafter from tumor cell lines [118, 119]. Triggers for the production of this protein in various cell types (e.g. epithelial, endothelial, smooth muscle, fibroblasts, astrocytes, monocytes, and microglial cells [120-122]), are detrimental factors like oxidative stress or other cytokines and growth factors such as platelet-derived growth factor (PDGF), interleukins IL-1 and IL-4, TNF-α, vascular endothelial growth factor (VEGF), bacterial lipopolysaccharide, and interferon γ (IF-γ) [123, 124]. CCL2 builds an axis with its prime receptor CCR2 expressed on the cell surface. Given the fact that this receptor also belongs to the G protein-coupled receptor superfamily, it is not a surprise that CCL2-elicited effects are pleiotropic [125] including a rise in intracellular calcium, induction of respiratory burst and gene expression [126, 127]. Because of the specific effects on mononuclear cell migration, it was suggested that CCL2 is a significant regulator of inflammatory diseases. In fact, this could be verified by several studies using genetically deficient mice, antibody- or inhibitor-mediated neutralization in mice (see e.g. [128-130]), and epidemiological studies in humans. The most extensive data in this regard probably exists for medical conditions such as atherosclerosis and multiple sclerosis. One aspect of the pathogenesis of arteriosclerotic vascular disease is described by monocytes penetrating subendothelial layers where they differentiate to macrophages, which chronically uptake cholesterol and become adherent to the arterial cell wall [131] resulting in the mature atherosclerotic plaque. CCL2 in this context is one major constituent to attract monocytes and therefore a decisive factor for the onset of the cascade [132]. Thus, a reduction of the number of circulating monocytes are associated

with the amelioration of the disease in general. In fact, this hypothesis could be validated by a number of studies using CCL2- or CCR2-deficient mice (e.g. see [128, 133, 134]).

Vascular Endothelial Growth Factor

The vascular endothelial growth factor (VEGF) belongs to a family of seven homologs including the placenta growth factor (PIGF) 1 and 2, VEGF-B, VEGF-C and VEGF-D [135, 136]. Accordingly, there are three different receptors VEGFR-1, VEGFR-2 and VEGFR-3 with different ligand-binding affinities that belong to the receptor tyrosine kinases (RTK). However, the prototype among them is VEGF-A, which herein is referred to as VEGF below. Looking at its original designation "vascular permeability factor", two major attributes of this signal protein are apparent: permeability and vascularization. Generally, tissue permeability and angiogenesis are closely related, because neo-vascularization usually occurs under (re-) organization of endothelial cell architecture, e.g. by replacing cellular material. It had been a matter of debate, if these two actions are interdependent as one highly coordinated, sequential operation [137], which now has been resolved. In the healthy body, neo-vascularization as a permanent process is the result of pro- and anti-angiogenic factors and essential for the organism to repair damages like wounds, regulating the female reproduction cycle [138] or promoting embryogenesis, for instance [139]. To induce blood vessel growth, VEGF stimulates RTK`s expressed on the cell surface of endothelial cells as their primary target (there exist few other non-endothelial cell types, which also respond to VEGF such as retinal pigment epithelial cells [140], pancreatic duct cells [141] and Schwann cells [142]). Subsequently, a rapid increase of intracellular [Ca^{2+}] can be observed within seconds after exposure [143] as a first reaction. While *in vitro* studies demonstrated VEGF promoting endothelial cells to invade collagen gels and forming capillary-like structures [144, 145], *in vivo* models confirmed remarkable pro-angiogenic effects [146, 147] just like sprouting, proliferation, migration and tube formation in endothelial cells. Increased tissue permeability is an important component of the very early inflammatory response [148] allowing fluid to leave and enter the surrounding tissue. At this stage, VEGF is typically induced by the hypoxia inducible factor (HIF) regulated elements of the VEGF gene under hypoxic conditions. Moreover, it is considered the principal cytokine to induce a transient, reversible microvascular leakage by fenestrating capillaries [143, 149]. In addition, VEGF has been shown to cause vasodilatation *in vitro* [150], tachycardia, hypotension and a decrease in cardiac output in conscious rats [151]. Taking these facts into account, it is not surprising that

VEGF can play pivotal roles in certain pathologies related to cell proliferation and locomotion, including tumors, psoriasis, ischemic heart disease, diabetic neuropathies, age-related macular degeneration [152] and others. For some years, targeted anti-tumor therapies in form of anti-VEGF antibodies have gained attention in oncology. The first chemotherapy was established in 2004 by bevacizumab in the treatment of colon cancer. Soon thereafter, several multi-targeted kinase inhibitors (MTKI`s) followed. In the light of VEGF-related hypotension, one main side effect among this group of therapeutics is hypertension, which is observed in up to 80% of the patients and sometimes can limit the therapy [136].

2.5. Mammalian Wound Healing

Substances out of *Phyllostachys edulis* and *Rosa damascena* were tested employing a computer-assisted modification of the classical scratch assay. Therefore, epithelial cells under influence of these compounds were observed tracing cell locomotion by time lapse pictures. Thus, it is important to briefly specify major stages and processes during wound healing.
The skin poses the body`s outer sheath and can be regarded as the first line defense to protect the organism from the environment – a condition which is indispensable to life. Thus, every animal in existence has evolved repair mechanisms as a reaction to injuries [153]. As mentioned in the chapter "Inflammation", a mechanical damage, that is a cutaneous or subcutaneous wound, is one trigger to activate the inflammatory response, but inflammation is only a part of the entire wound healing process, which remains to be fully elucidated. In this regard, *Reinke and Sorg* [154] distinguish between the "regeneration" and the "reparation" of wounds. The first is characterized by a substitution of the tissue (e.g. of superficial epidermis), the latter is the predominant form in adults and happens in an unspecific fashion in which the healing process finally results in fibrosis for instance. However, depending on the acuteness (size, depth, cleanliness etc.) of the insult, a set of pathways can be activated governing blood coagulation, cell locomotion-governed processes of epithelial cells, blood vessel restoration, and of course certain immune system-related cascades. If the survival of the organism is secured, the distinct and immediate onset of all these actions must gradually fade, which is part of the recovery [2]. Typically, the physiological endpoint of reparation is a patch of cells (primarily fibroblasts) and extracellular matrix in abundance, commonly known as a scar. It restores the continuity of tissue, but does not behave like the surrounding tissue in regards of cohesion and performance. Once functional tissue never regains the former material stability; *Singer and Clark* report a 70%-breaking strength compared to normal skin [155]. Interestingly, some eukaryotic organisms are capable to fully regain tissue function after injury, for example humans during prenatal development [156]. The question of why this ability is lost with maturation to adulthood remains [157]. Unique to humans, there exist abnormal progressions, when wound healing drifts into a pathologic state, such as over-healing specified by the growth of keloids and hypertrophic scars, or under-healing manifested in chronic wounds with a retarded or defective closure mechanism. Taken together, these are the major biomedical challenges in wound healing [158]. In brief, the full course of reparation of injuries can be divided into three distinct, but overlapping stages: 1. homeostasis; 2. re-epithelialization; 3. remodeling.

Homeostasis and Inflammation Phase (I)

In the strict sense, the first stage of wound healing consists of two interdependent reactions: the vascular (homeostasis) and the cellular response (inflammation). Right after flushing the injury with blood and lymph fluid to remove microorganisms, antigens or cell debris [159], the organism`s priority is to establish a provisional wound matrix within only a few hours. Simultaneously with the activation of the extrinsic and intrinsic coagulation cascades, a transient vasoconstriction prevents further blood loss and supports a first wound closure by a blood clot. This "plug" serves as a reservoir for cytokines and growth factors and consists of structure-providing proteins like cross-linked fibrin, fibronectin, thrombospondins and other molecules, which are essential for enabling the migration of leukocytes, keratinocytes, fibroblasts and endothelial cells [154] (Fig. 9). Subsequently, the temporary vasoconstriction is followed by a sustained vasodilation ("rubor") to facilitate thrombocytes invade the matrix [160], which themselves release chemokines to attract white blood cells. Leukocytes in turn release cytokines including TNF-α, IL-1β, IL-6, IL-8, VEGF and other mediators to activate the inflammatory response, while the affected tissue typically shows an edematous, hypoxic picture.

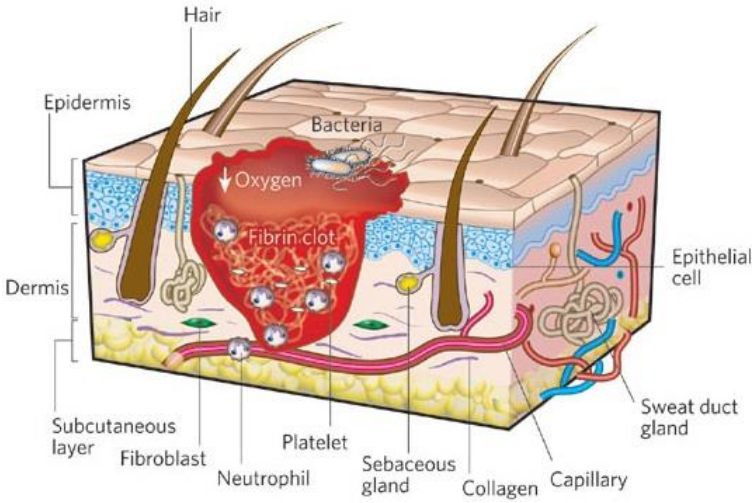

Fig. 9: Inflammation phase (I) - building of a blood clot. Figure adopted from *Gurtner et al.* [2].

The inflammation phase basically continues in a fashion described in chapter 2.4 "Inflammation" including the attraction of sensor cells and subsequent activation of immune reactions. Accounting for the time line, the total period of homeostasis/inflammation is strongly depending on the dimension of the wound and if it is encumbered by a bacterial infection. However, neutrophils responsible for bacterial degradation and attraction of other leukocytes arrive about 24h after injury and are present for circa two to five days (Fig. 9). According to *Eming et al.* [160], the activity of neutrophils ceases after a few days, while macrophages reach the site of injury within approximately two days after wounding. Not only responsible for host defense and phagocytosis of pathogens and cell debris [161],

macrophages also release their own pattern of cytokines/chemokines, which are essential for the continuance of the physiologic course of wound healing comprising the transition to the next stage: re-epithelialization [2].

Re-epithelialization and Granulation Phase (II)

While a successful first stage of wound healing is crucial for survival, the second stage concentrates on resurfacing of the wounded area, formation of granulation tissue and re-establishment of mechanical strength. Therefore, proliferation of fibroblasts is upregulated to provide enough cells to immigrate from the edges of the wound along the fibrin matrix.

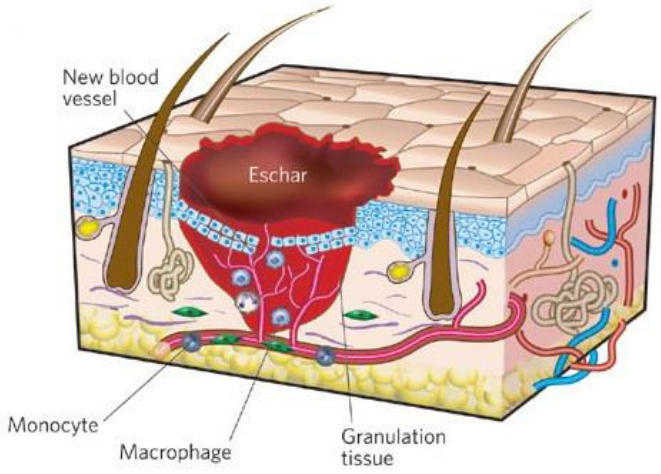

Fig. 10: Re-epithelialization phase (II) - re-surfacing the wound and establishing a vascular network. Picture adopted from *Gurtner et al.* [2].

Moreover, angiogenetic processes ensure a restoration of the vascular network [162, 163] (Fig. 10). Substances like collagen and fibronectin (ECM) fill out spaces and gaps and pose as the backbone for new connective tissue. As soon as the tissue loss is compensated, the re-epithelialization sets in by migration and maturation of keratinocytes to build up a surface layer above the granulation tissue on the one hand and on the other by epithelial stem cells derived from hair follicles or sweat glands [164-166]. Also, this process is boosted by numerous cytokines and growth factors, such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), insulin-like growth factor-1 (IGF-1) and nerve growth factor (NGF) [167]. Movement of cells is a complex, tightly regulated process, which involves cytoskeletal interaction with RhoGTPases including Rho, Rac and Cdc42 [168, 169]. These RhoGTPases primarily regulate the intracellular organization of fibers and thereby enable cell locomotion. Resurfacing is completed, when the epithelial cells on the leading edge of the wound touch each other, restoring the barrier function of the skin. Besides cell motility, newly formed tissue needs nutrients to survive. Initiated by several growth factors, neovascularization and angiogenesis ensure a proper blood supply. In brief, VEGF for instance binds to endothelial receptors of existing vessels and thereby activates certain signaling cascades accordingly. Subsequent endothelial secretion of proteolytic enzymes, which dissolve the basal lamina,

leads to proliferation and migration into the wound, forming a new tubular network of vessels. This action has been described as “sprouting” [154]. Angiogenesis is then completed by the re-establishment of blood perfusion. Finally, the formation of a transient granulation tissue replaces the provisional wound matrix and consists of dense fibroblasts, granulocytes, macrophages, capillaries and loosely organized collagen bundles. Granulation tissue is highly vascular, for which reason it appears with a classic redness and is characterized by a certain vulnerability. This point displays the transition to the third stage of wound healing, because remodeling already starts with forming and degrading of ECM [170, 171], as well as maturation of fibroblasts to myofibroblasts and consecutive apoptosis [172]. This second phase of wound healing is reported to last about 2-10 days [2].

Tissue Remodeling Phase (III)

The last phase of wound healing is mostly characterized by the change of collagen structure. During the previous proliferation phase, fibroblasts produced mostly disorganized collagen type III as a component of the granulation tissue. Collagen type III is now successively replaced by the stronger collagen type I, which is organized in small parallel bundles [173] (Fig. 11). The ratio of collagen type I and III is reported to be critical for proper wound healing and can be a determinant for scarring [174]. Additionally, angiogenesis and blood flow slowly declines in the newly formed tissue causing a termination in metabolic activity [154]. Myofibroblasts provoke a contractile response and thereby, decrease the surface of the scar [175]. Finally, most of the endothelial cells, macrophages and myofibroblasts undergo apoptosis or exit the wound. Epithelial-mesenchymal cross-talk regulates skin integrity and homeostasis [176]. After serious injury, subepidermal appendages including hair follicles or sweat glands neither heal, nor grow back. The last phase in wound healing can take up to 12 months, in some cases even longer [2].

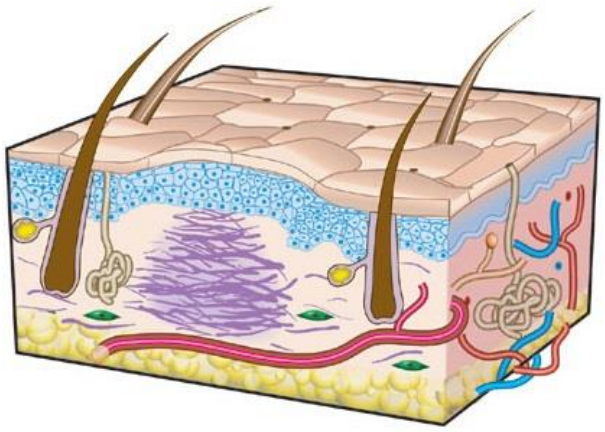

Fig. 11: Tissue remodeling phase (III) - the purple area represents disorganized collagen type III laid down by fibroblasts. Picture adopted from *Gurtner et al.* [2].

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3. Results and Discussion

3.1. In Vitro Anti-Inflammatory and Wound-Healing Potential of a Phyllostachys edulis Leaf Extract – Identification of Isoorientin as an Active Compound

The experimental part (except extract preparation and anti-inflammatory assays), the establishment of the scratch assay, the preparation of Fig. 3 and Fig.4, writing parts of the manuscript and the data analysis were my contributions to this publication. The manuscript was finalized by Prof. Dr. Butterweck.

-Jonas Wedler-

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In vitro antiinflammatory and wound healing potential of a *Phyllostachys edulis* leaf extract - identification of isoorientin as an active compound

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Abstract

Extracts prepared from the leaves of *Phyllostachys edulis* (bamboo) have received attention in pharmacological research due to their potent antitumor, anti-inflammatory, antimicrobial, and antiulcerogenic activities. In this study, anti-inflammatory effects of a bamboo leaf extract (BLE) on TNFα -induced overproduction of IL-8, VEGF and IL-6 in immortalized human keratinocytes (HaCaT) were investigated for the first time. In addition, wound healing effects were evaluated in 3T3-swiss albino mouse fibroblasts. BLE and isoorientin inhibited the TNF-α induced release of IL-8 and VEGF. Furthermore, isoorientin dose dependently reduced levels of IL-6 in TNF-α treated HaCaT cells. Wound-healing was evaluated using a modification of the classical scratch assay. For evaluation of the wound gap, a new computerized method based on time-lapse microscopy was developed. It was shown that BLE (10 µg/mL) improved wound closure by 28 % (12 h) and 54% (24 h), respectively. In concentrations of 50 µg/mL and above BLE inhibited cell migration without affecting cell viability. Isoorientin (10 µM) improved wound closure by 29 % (12 h) and 56 % (24 h). Comparable to BLE, higher concentrations of isoorientin prevented cell migration. It is suggested, that BLE as well as isoorientin exert a dual activity – in higher doses anti-inflammatory effects, and in lower concentrations antiangiogenic activities.

Keywords: *Phyllostachys edulis*, Poaceae, bamboo, wound healing, anti-inflammatory, time-lapse microscopy

Introduction

Wounds are defined as physical injuries that result in an opening or break of the skin that causes disturbance in the normal skin anatomy and function [1]. Wound healing can be grouped roughly in three different phases: the initial inflammation is followed by the granulation phase connected to re-epithelialization and in the end the long-term process of remodeling [2]. The inflammations' onset happens immediately after injury. It his highly regulated and depends upon pro-inflammatory mechanisms, which are gradually counteracted by various anti-inflammatory pathways mediated by factors like IL-10, hormones, and neurotransmitters [2]. If the anti-inflammatory response prevails, the process turns into the next stage of granulation and fibroblasts and keratinocytes migrate into the wound. Angiogenesis mediates capillaries to replace the fibrin matrix with granulation tissue, which depicts a substrate for keratinocyte migration at later stages. Maturation of the keratinocytes leads to a restoration of the epithelium's barrier function [3]. Contraction of the wound is done by fibroblasts located at the wound's edge that differentiated into myofibroblasts producing extracellular matrix (ECM) [3]. The final stage is specified by remodeling of the tissue's architecture [3].

In terms of wound healing, IL-6, IL-8 and VEGF are key players in these processes. IL-6 has both pro- and anti-inflammatory properties. TNF-α depicts a physiologic stimulus to IL-6 [4]. As a member of the chemokine family, IL-8 possesses chemotactic properties attracting neutrophils. Furthermore, it induces neutrophils to release lysosomal enzymes [5] and down-regulates collagen expression by fibroblasts [6]. Just like IL-6, its production is stimulated by the presence of TNF-α. Vascular endothelial growth factor (VEGF) is known to play a pivotal role in angiogenesis, but is also involved in acute inflammatory processes. It can be considered a mitogen, which regulates endothelial paracellular permeability and proliferation of mesenchymal cells. At the location of the wound and in endothelial cells of granulation tissue, VEGF and its receptor production are typically up-regulated [7].

Cells in unhealed wounds constantly produce inflammatory mediators in an uncoordinated and self-sustaining phase of inflammation that impairs the restoration of anatomic and functional integrity in the normal period of time [8, 9]. Thus, a prolonged inflammatory phase leads to delayed wound healing. Several wound healing strategies are therefore under development which target the major phases of cutaneous wound healing: inflammation, proliferation, and tissue remodeling. In this regard, medicinal plants have always been in the focus of several research programs to identify compounds with potential anti-inflammatory and wound healing properties [10].

Phyllostachys edulis (Carrière) J. Houz. (Poaceae) is one of the most important Chinese bamboo species economically [11]. While bamboo stems are widely used in furniture production the leaves are a byproduct during harvesting. Thus, research projects focusing on the valorization of bamboo leaf products towards utilization in the nutrition or cosmetic industry have gained considerable attention. It was shown that bamboo leaf extract exerts potent antitumor, anti-inflammatory, antimicrobial, and anti-ulcerogenic activities [12-16]. Recently, a special bamboo gauze coated with polymer and drug was developed as surgical bandage to facilitate faster wound healing [17]. It was the aim of the present study to investigate the anti-inflammatory and wound-healing potential of a *Phyllostachys edulis* leaf extracts in *in vitro* models in comparison to the flavonoid isoorientin which was identified as one of the major compounds.

Results

In order to determine potential cytotoxic effect of BLE (10-250 µg/mL), isoorientin (5 – 100 µM) and hydrocortisone (5-100 µM) in HaCaT cells, the MTT assay was used. Treatment with the different test compounds for 24 h at indicated concentrations had no significant cytotoxic effects on HaCaT cells. The cell viability for hydrocortisone ranged between 100 – 113%, for BLE between 100 – 103% and for isoorientin between 93 - 100% (Fig. 1S). Thus, extract concentration ranged from 25 – 250 µg/mL for subsequent experiments. Isoorientin (Fig. 1) was used in further experiments in concentrations ranging from 10-100 µM. Interestingly, hydrocortisone slightly increased cell viability in the used concentrations. However, no dose dependent effect was observed. For subsequent experiments, hydrocortisone was used in a concentration of 10 µM (or 3.6 µg/mL, correspondingly).

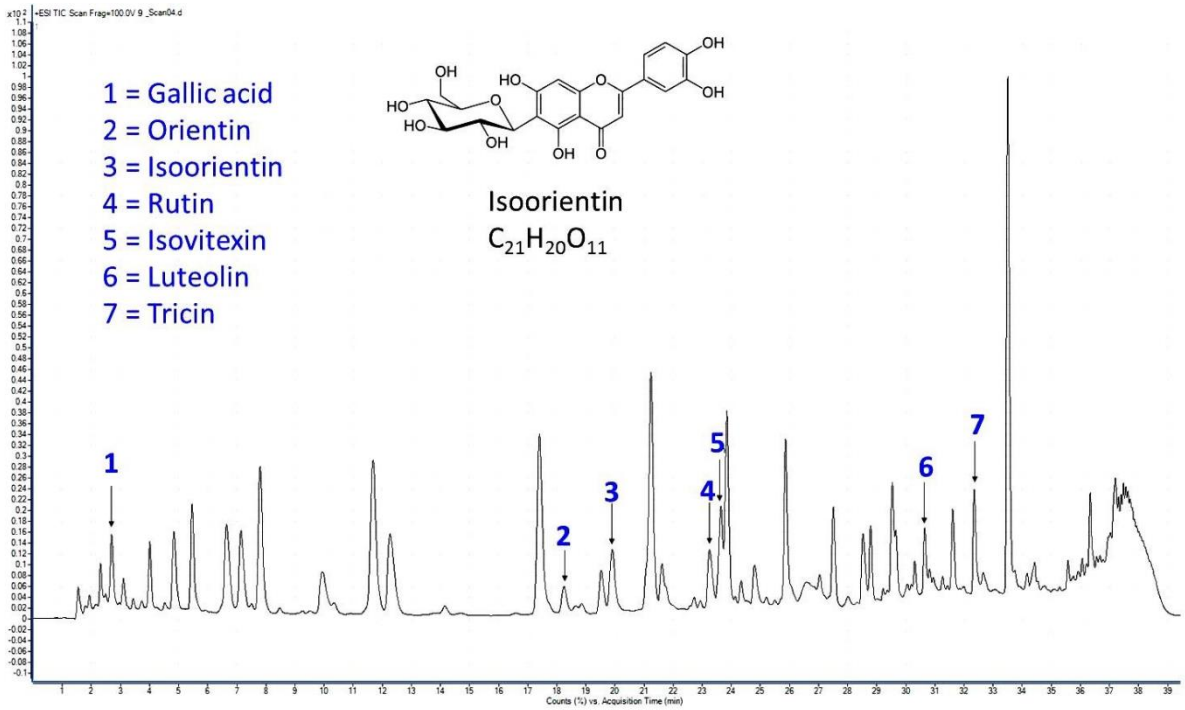

Figure 1: LC-MS chromatogram of a *P. edulis* leaf extract representing the total ion chromatogram (TIC) with only flavonoids annotated. (Color figure available only.)

To investigate whether BLE or isoorientin inhibit TNF-α-induced IL-6, IL-8 and VEGF expression, HaCaT cells were treated with TNF-α (20 ng/mL) after pre-incubation of cells for 6h with the various treatments. The results were compared to those of hydrocortisone (10 µM or 3.6 µg/mL, correspondingly). BLE had no effect on the TNF-α induced production of IL-6 (Fig. 2A). In contrast, isoorientin dose-dependently decreased IL-6 production in concentrations of 50 µM and 100 µM (Fig. 2B). As presented in Fig. 2C, upregulation of IL-8

by TNF-α treatment was dose-dependently reduced by BLE and isoorientin treatment (Fig. 2D). The effects on TNF-α induced production of VEGF are shown in Fig. 2E, F. BLE significantly decreased TNF-α stimulated VEGF production in HaCaT cells; isoorientin dose-dependently decreased TNF-α induced VEGF levels (Fig. 2F).

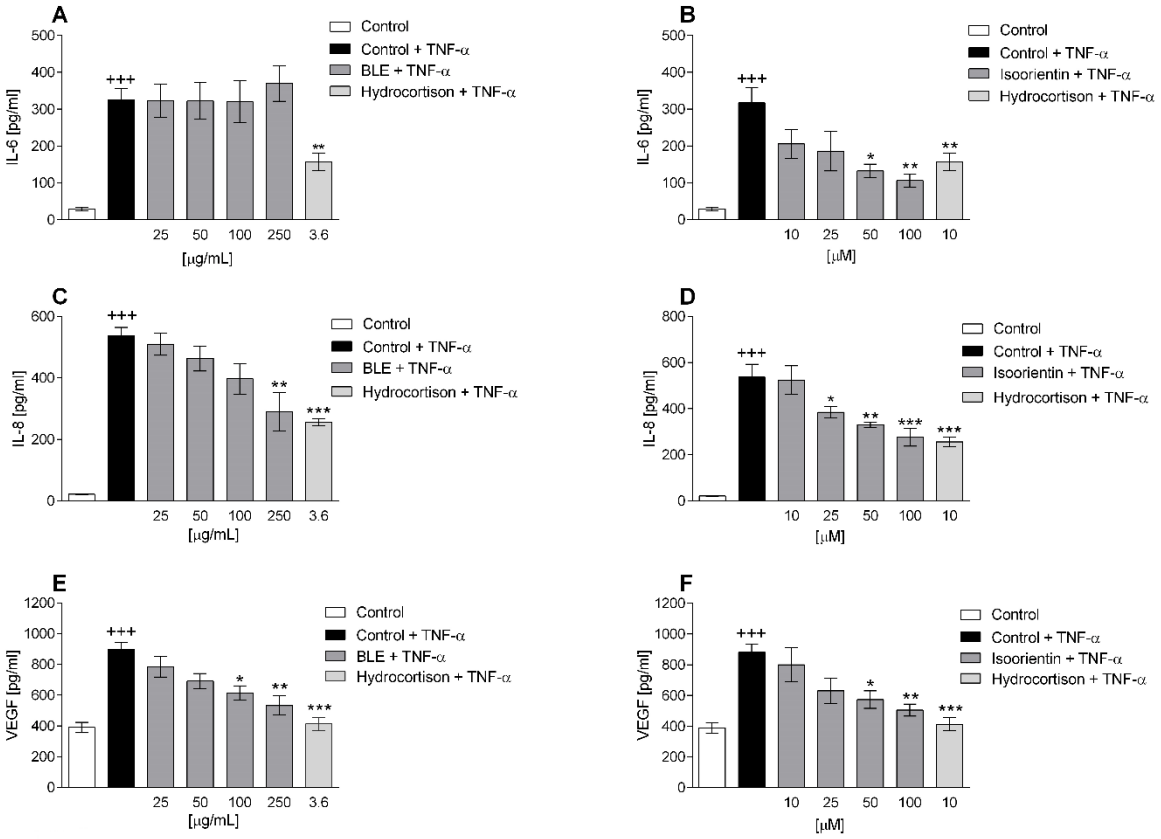

Figure 2: Effects of BLE, isoorientin and hydrocortisone on TNF-α (20 ng/mL) induced IL-6, IL-8 and VEGF secretion in HaCaT cells. IL-6, IL-8 and VEGF secretion were quantified by corresponding ELISA kits. Results are expressed as Mean ± SD of four independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs. TNF-α stimulated group; $^{+++}$p<0.001 vs. untreated control group.

Effects of BLE and isoorientin on the migration of 3T3 mouse fibroblasts were tested in an *in vitro* wound healing model, in which wounds were generated using silicon culture inserts. Cells were allowed to migrate across the rectangular region of interest ROI into the center of the wound gap (width 450 µm) for 24 h at 37 °C. A clear difference was observed between cells treated with DMEM (0% FCS) or with DMEM + 2% FCS (positive control) (Fig. 3A-D). Addition of 2% FCS to the medium significantly increased cell migration over a period of 24 h resulting in a 65% closed wound gap. In contrast, in cells treated with DMEM without any FCS supplementation the wound closure was approximately 20% after an observation period of 24h.

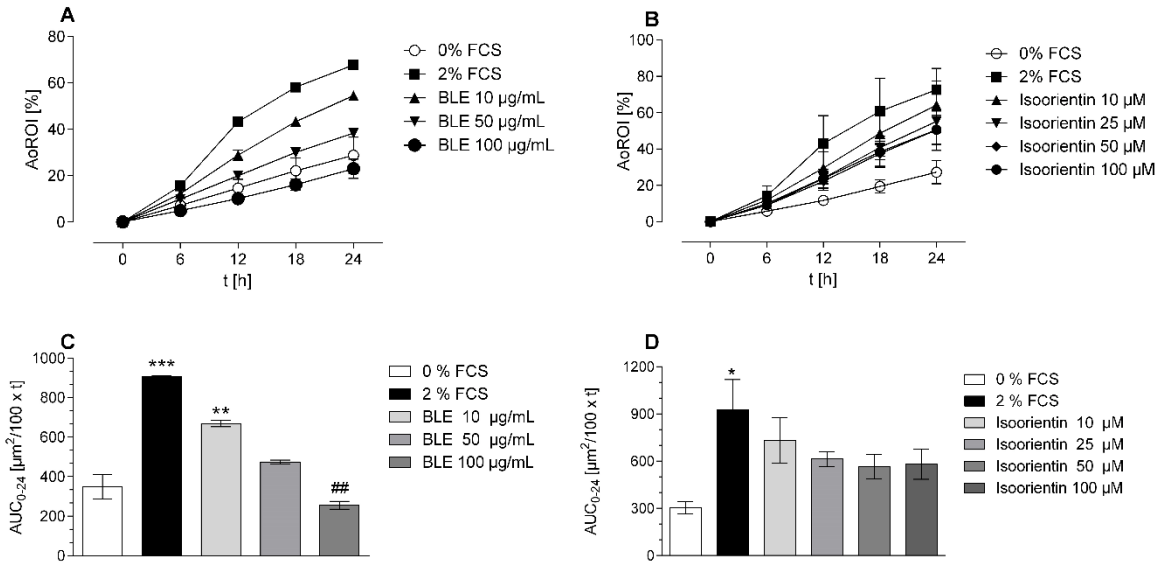

Figure 3: Effects of bamboo leaf extract and isoorientin on cell migration in 3T3 mouse fibroblasts. Graphs show the percentage of the predefined rectangle covered by cells over an observation period of 24 h (**A, B**) and the corresponding area under the curve (AUC) in µm^2 x t (**C, D**). Data represent the Mean ± SD of two independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs. 0% FCS control group; $^{\#\#}$p<0.01 vs. 50 µg/mL BLE.

BLE in a concentration of 10 µg/mL significantly increased the cell migration rate if compared to the 0% FCS group (Fig. 3 A,C). A wound gap closure of approximately 55% was observed after 24 h. In concentrations of 50 µg/mL and 100 µg/mL of BLE, no statistically significant effects versus the 0% FCS control group were observed. However, multiple post-hoc comparisons between groups revealed a significant difference between the 50 µg/mL and 100 µg/mL BLE group ($p < 0.01$), indicating a stronger cell migration inhibitory effect in higher concentrations.

Isoorientin treated cells started to migrate into the region of interest at 6 h after removal of the silicon insert (Fig. 3B). Interestingly, the 24 h migration rate was slightly higher in a concentration of 10 µM than in higher concentrations and after 24 h approximately 55% of the wound gap were closed (Fig. 3B). In addition, treatment with 25, 50 and 100 µM of isoorientin could increase the overall migration rate within 24h although no difference between the various concentrations could be observed (Fig. 3D).

Since AUC and migration rate measurements showed only small statistical differences between the treatment groups, further image analyses were performed. It was of special interest to compare the effects of BLE and isoorientin in lower concentrations (Fig. 4). Using long term time-lapse imaging interval snap-shots of predefined positions in the gap during 0 h, 6 h, 12 h and 24h were taken (Fig. 4). Detailed image analysis showed that after 24 h BLE (10 µg/mL)

and isoorientin (10 µM) significantly increased cell migration into the region of interest if compared to the 0% FCS negative control group. If compared to the 2% FCS positive control group, BLE and isoorientin achieved a wound gap closure of 55% after 24h.

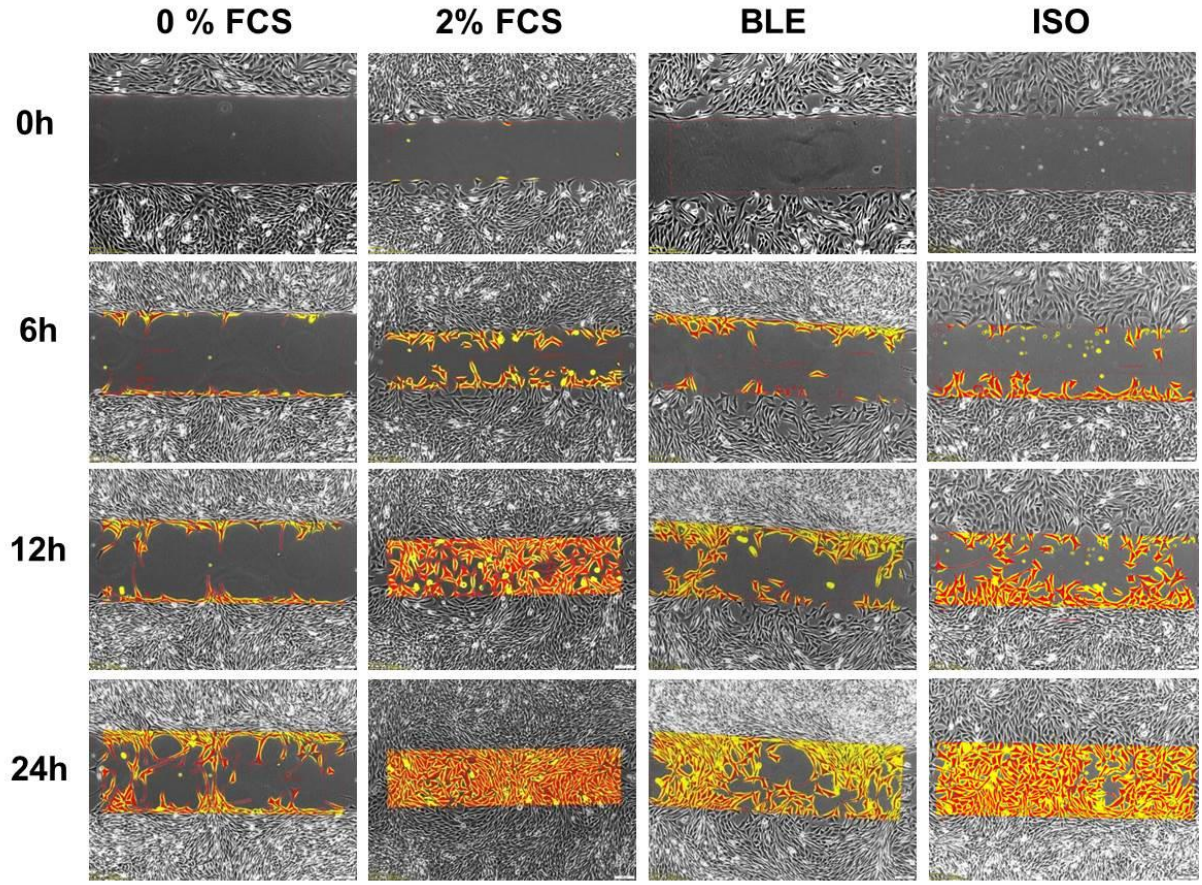

Figure 4: Cell migration in response to an artificial injury using long term time-lapse imaging interval snap-shots of predefined positions in the wound gap (450 µm) during 0 h, 6 h, 12 h and 24h (Fig. 4). Cells were treated with the negative control 0% FCS, the positive control 2% FCS as well as BLE (10 µg/mL), or ISO (isoorientin; 10 µM).

Discussion

Pro-inflammatory cytokines such as TNF-α and IL-6 are increased in the inflammatory phase of wound healing [2]. While TNF-α at low concentrations promotes wound healing by stimulation of inflammation, it has a destructive effect on wound repair in higher concentrations [2]. Interleukin-6 is produced in epidermal cells, fibroblasts and dermal endothelial cells under normal conditions but it is also synthesized by inflammatory cells infiltrating the skin in different pathological conditions [18]. Therefore, inhibition of pro-inflammatory mediators secreted from activated keratinocytes may be an effective therapeutic approach to regulate the progression of the wound healing process.

In the present study, the spontaneously immortalized human keratinocyte cell line HaCaT was used for the evaluation of anti-inflammatory activities. HaCaT cells maintain normal keratinocyte morphology and full epidermal differentiation capacity and remain non-tumorigenic [19]. Like human primary keratinocytes they produce cytokines and chemokines [20] which are involved in the development of inflammatory skin diseases.

Several plant extracts and natural compounds have been reported previously to have anti-inflammatory effects in activated keratinocytes [21-24]. In the present study, we found that BLE could not prevent the TNF-α mediated inflammatory response to IL-6 in HaCaT cells. It was demonstrated in a previous study that a hydroalcoholic extract prepared from the fresh leaves and branches of *Phyllostachys edulis* significantly reduced IL-6 overproduction under lipotoxic conditions in murine C2C12, 3T3-L1, and Hepa6 cells [15], most likely through an activation of NF-κB and AP-1 pathways. The different results of the two studies could be explained by either a different extract (fresh versus dry leaves) and/or different cell models.

When cells were pretreated with isoorientin (50 µM and 100 µM), the secretion of IL-6 was significantly reduced, comparable to that of the positive control hydrocortisone. IL-6-inhibition by isoorientin was not due to a general cytotoxic effect, since cell viability in all cultures remained constant throughout the incubation period in the presence of all compounds tested. Anti-inflammatory activities of isoorientin have been reported in previous studies. For example, Conforti et al. [25] reported that isoorientin exerts significant anti-inflammatory activity through inhibition of NO production in LPS stimulated mouse macrophage RAW264.7 cells, and Kupeli et al. [26] demonstrated an inhibition of carrageenan-induced hind paw edema model in mice. Further, our data on isoorientin are in good correlation with the data of Yuan et al. [27] who demonstrated that isoorientin significantly increased cell viability in mouse

microglial (BV-2) cells, blocked the protein expression of inducible nitric oxide synthase and cyclooxygenase-2, and decreased the production of nitric oxide, pro-inflammatory cytokines including TNF-α and interleukin-1β.

Vascular endothelial growth factor (VEGF) and the chemokine IL-8 play an important role in skin inflammation and are produced by activated keratinocytes [28, 29]. It has been demonstrated that TNF-α activates epidermal cells and induces the production of VEGF and IL-8 [30, 31]. Both mediators are overexpressed in skin diseases which are associated with aberrant angiogenesis [32-34]. In the present study, BLE could reduce VEGF and IL-8 levels induced by TNF-α in HaCaT cells. Isoorientin, which was identified as the main flavonoid in BLE, significantly reduced TNF-α induced increase in VEGF and IL-8 production. To our knowledge, no data were previously published on the effects of *Phyllostachys edulis* extracts and isoorientin on the secretion of VEGF and IL-8. Isoorientin is one of the major active compounds in *Phyllostachys edulis* which contributes to the moderate anti-inflammatory effects of the leaf extract by suppressing TNF-α induced production of proinflammatory cytokines (IL-6), chemokines (IL-8) and VEGF in HaCaT keratinocytes. Thus, isoorientin might have a potential as therapeutic agent for inflammatory skin diseases. However, it needs to be investigated in further studies in how far other polyphenols contribute to the overall activity of this plant and in how far interactions among fractions and compounds is important for the activity. Interestingly, extracts derived from either *Phyllostachys edulis* or other bamboo species have shown potential anti-inflammatory activities [12, 15, 35].

As mentioned previously, cutaneous tissue repair involves a complex reaction [36]. Fibroblasts play a critical role in normal wound healing. They are involved in key processes such as forming granulation tissue by proliferating and migrating and for creating new collagen structures to support the other cells associated with effective wound healing, as well as contracting the wound [37, 38]. Thus, it was of interest to investigate the effects of BLE as well as of isoorientin on this cell type to reveal if these compounds can contribute to the wound healing process. We performed a modification of the classical scratch assay by using cell seeding stoppers which were applied to the plate bottom. After the cells reached confluence the inserts were removed causing a gap of 450 μm. The cells then migrate across the created gap. Long term time-lapse imaging was performed by taking interval snap-shots of predefined positions in the gap during 24 h, thus minimizing error when compared with other methods that measure wound closure. This is an accurate and reproducible model to study wound healing by monitoring cell migration over an extended period.

Wound closure was improved by 28 % (12 h) and 54% (24 h), respectively, in the presence of 10 µg/mL BLE. Interestingly, higher concentration of the BLE inhibited cell migration without affecting cell viability. Isoorientin at a concentration of 10 µM improved wound closure by 29 % (12 h) and 56 % (24 h), respectively. Similar to BLE, higher concentrations of isoorientin prevented cell migration into the area of interest without considerable toxic effects. Based on these findings we hypothesize that the inhibition of cell migration of fibroblasts in a collagen matrix could be a result, in part, of the reduction of VEGF secretion. Our data show that TNF-α induced VEGF secretion was decreased at higher doses of BLE and isoorientin. We therefore suggest that BLE as well as isoorientin might have a dual activity – in higher doses (> 100 µg/mL extract or > 25 µM isoorientin) the compounds show an anti-inflammatory effect while in lower concentrations (≤ 10 µg/mL extract or ≤ 10 µM isoorientin) both compounds exert antiangiogenic activities by inhibiting migration possibly by prevention of VEGF secretion.

In conclusion, natural accelerators of cutaneous tissue repair with simultaneous anti-inflammatory activities are of great interest for a variety of dermatological disorders. Thus, treatment with *Phyllostachys edulis* leaf extract or isoorientin may be a potential therapeutic strategy to promote wound healing and to prevent inflammation in a persistent inflammatory condition. Further investigations of the precise mechanism by which *Phyllostachys edulis* and isoorientin reveal anti-inflammatory as well as wound healing properties are currently underway.

Materials and Methods

Materials

4′,6-Diamidino-2-phenylindole dihydrochloride, Dimethyl sulfoxide (DMSO), Acetic Acid 99,7 %, Sodium dodecyl sulfate (SDS), isoorientin (purity > 98 %), hydrocortisone (purity > 98 %), and **3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide ("MTT" purity 98%)** were purchased from Sigma-Aldrich. IL-6, IL-8 and VEGF human ELISA kits were purchased from LubioScience. Ibidi Culture-Inserts for Live Cell Analysis were obtained from Vitaris AG. Dulbecco's Phosphate buffered saline [-] $CaCl_2$; [-] $MgCl_2$ was purchased at Lubio science Invitrogen. Recombinant human TNF-alpha (purity > 97 %) was purchased at R&D Systems. Collagen Type I Rat Tail solution (purity > 90 %) was purchased at BD Biosciences.

Cell culture

Human HaCaT cells from histologically normal skin and Swiss 3T3 albino mouse fibroblasts (both from Cell Line Services) were maintained in Dulbecco's Modified Eagle's Medium supplemented with 1% Penicillin 10.000 U/mL / Streptomycin 10.000 µg/mL, and 10% fetal calf serum (FCS) (all from LubioScience) at 37 °C in a humidified atmosphere containing 5% CO_2 [19].

Plant material and extract preparation

The plant material of *P. edulis* was provided by Organic Bamboo Industries. It was identified by Mr. Liao Rong Qi, forest chief of Yangzhuang Town Station, WuYiShan, Fujian, China. Dried bamboo leaves (0.5 g) were grinded and extracted by Soxhlet extraction with water (30 mL) and finally lyophilized (final yield 100 mg). Voucher specimen of the corresponding extract (ICB_BLE2013_10) are deposited at the Institute for Chemistry and Bioanalytics, School of Life Sciences, University of Applied Sciences Northwestern Switzerland. The purified extract was separated on a Zorbax SB Phenyl column (3.0 x 150 mm, 1.8 µm) in gradient mode (25-95 B %, A water, B methanol plus 0.1 formic acid) with a flow rate of 0.4 mL/min at 35°C. Injection volume was set to 1 µL. Analysis was performed on an Agilent 6410 Triple Quadrupol Mass spectrometer with an electros spray ionization source operated in positive mode with an Agilent 1200SL HPLC System running under MassHunter B05.02. The

amounts of major flavonoids were quantified using a LC-MS/MS method. The quantification of individual flavonoids was performed by separate LC-MS/MS (SRM) experiments (data not shown). Isoorientin (Fig. 1) was detected as one of the main flavonoids with amounts of 5.32 g/kg. A flavonoid profile of a bamboo leaf aqueous extract is shown in Fig. 1.

Cell viability assay

To assess cell viability, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid-test (MTT-test) was conducted. Therefore, 200 µL of the HaCaT cell suspension was seeded into 96-well plates at a concentration of $6x10^4$ cells/well followed by an incubation period of 24 h (37 °C, 5% CO_2). After the incubation time the medium was discarded and the cells were washed with 150 µL PBS. The samples solved in DMEM were added in a dilution series. All sample solutions were dissolved in serum-free medium. The extract was tested in concentrations of 10, 25, 50, 100 and 250 µg/mL, hydrocortisone (positive control) and isoorientin were tested in concentrations of 5, 10, 25, 50 and 100 µM. Four independent experiments with triplicates were conducted. The controls consisted of wells with or without cells with pure DMEM or the solvent standard DMSO. After an incubation period of 24 h, 10 µL of MTT-solution (5 mg/mL PBS) was added and again incubated for 2h. The liquid was discarded before adding 100 µL of the cell-lysis buffer consisting of 99.4% DMSO, 0.6% Acetic Acid and SDS 0.1 g/mL. The optical density was read at 570 and 630 nm as a reference on a microplate reader (SpectraMax M2e). The viability was calculated according to the formula:

$$Viability\ [\%] = \frac{OD\ 570\ nm\ (sample) - OD\ 630\ nm\ (sample)}{mean\ OD\ 570\ nm\ (control) - mean\ OD\ 630\ nm\ (control)}$$

The experiments were executed utilizing cell culture passage numbers from 35 to 70.

Antiinflammatory activity

The experimental setup as well as the concentration of TNF-α for these experiments were chosen according to *Park et al.* [23] with some modifications. Briefly, HaCaT cells ($7x10^5$ cells per well, 12-well plates) were pre-incubated in a culture incubator for 6 h without or with the addition of different concentrations of BLE (25 – 250 µg/mL), isoorientin (10 – 100 µM) or with the positive control hydrocortisone (10 µM) before adding the pro-inflammatory cytokine TNF-α. After a pre-incubation time of 6h, TNFα (20 ng/mL) was added and the cells were incubated for further for 24 h. After the total incubation time of 30 h, VEGF, IL-8 and IL-6 were measured in cell supernatants by enzyme-linked immunosorbent assays kits as biomarkers of anti-inflammatory response according to manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader (SpectraMax M2e).

Cell migration assay (Wound healing scratch assay)

After positioning the cell culture inserts in the 12-well dishes, the 3T3 mouse fibroblasts were inoculated in a concentration of $4x10^5$ cells/mL into the collagen-coated wells. After an incubation time of 24 h, the cells reached confluence and the inserts were removed causing a gap of 450 µm. The subsequent washing with 1 mL PBS was followed by the manual addition of a scratch on the side of the well by a 100 µL pipette tip. In different concentrations accordingly, the wells we charged with the test compounds. BLE and isoorientin were dissolved in DMEM containing 0.2 % FCS. DMEM supplemented with 2% FCS was used as positive control, 0% FCS (DMEM without supplements) was used as negative control. Long term time-lapse imaging was performed using the Olympus IX83 automated inverted microscope platform for live cell imaging (Olympus). Interval snap-shots of predefined positions in the gap during 24 h were taken. Exact positions were defined in each gap of every well by using the Olympus software package cellSens Dimension 1.81 and the cells were observed for 24 h. BLE was in the concentrations 10, 50 and 100 µg/mL and isoorientin in concentrations of 10, 25, 50 and 100 µM. All experiments were conducted using passage numbers from 43 to 50.

Statistics

Data are shown as mean ± SD. All experiments were performed in triplicate and each experiment was repeated four times. Statistical analysis of data was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's and Tukey's multiple comparison test using the software package GraphPad Prism (version 5.01, GraphPad Software Inc.). In all cases differences were considered significant if $p < 0.05$.

The following computerized approach was used for evaluating cell migration: After selecting a rectangle (region of interest "ROI") in the cell-free gap by appropriate software tools, the area was calculated in [μm^2] automatically and set as 0 % μm^2 covered surface (defined as 'Area of region of interest, AoRoI'). Due to the migration of the cells into the ROI, the value for AoROI of the covered surface increased over time. The principle of measurement was based on a modified pixel counting by the software package. At t = 6, 12, 18 and 24 hours, the AoROI was measured and its value plotted in graphs. The area under the curve AUC_{24h} was computed by GraphPad Prism and depicts the magnitude of wound closure in one value.

Supporting information

Fig. 1S, showing the cell viability data, can be found as Supporting Information

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

In vitro antiinflammatory and wound healing potential of a *Phyllostachys edulis* leaf extract - identification of isoorientin as an active compound

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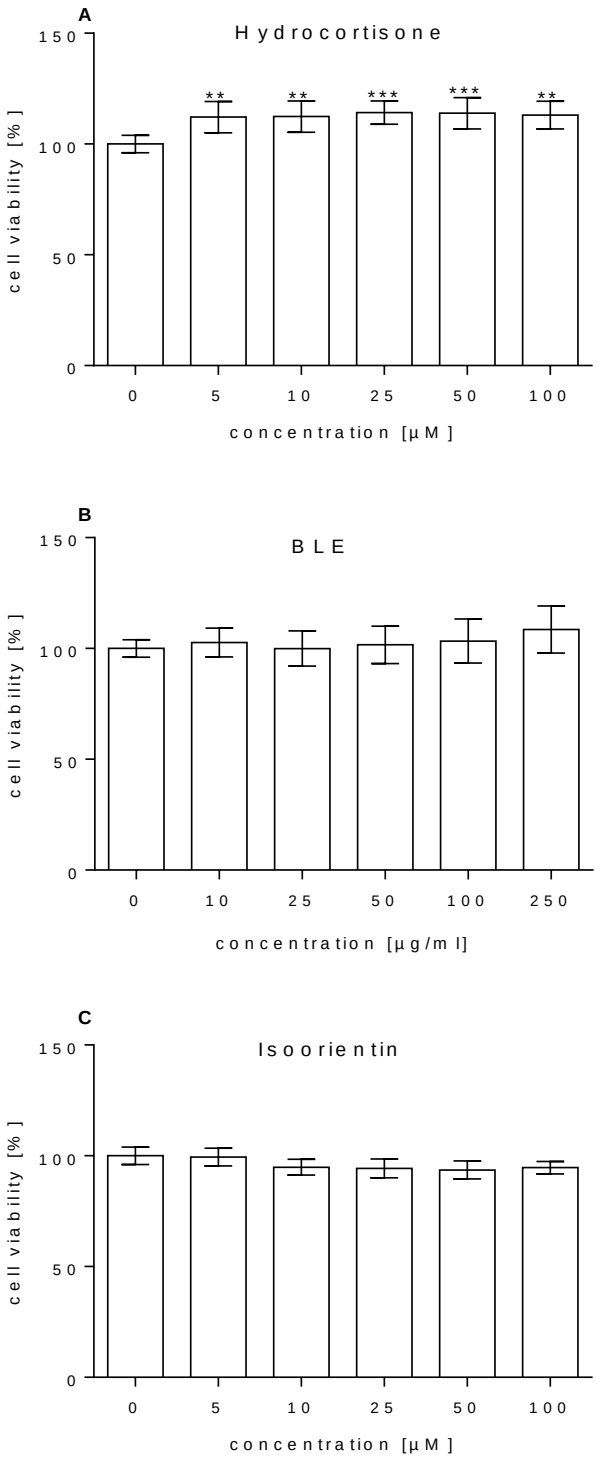

Figure 1S: Effect of BLE, isoorientin and hydrocortisone on cell viability in HaCaT cells. After pre-treatment of 24 h with the test compounds cell viability was measured using the MTT assay. Data are expressed as percentage and values represent the Mean ± SD of four independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs. untreated control group.

3.2. Polyphenol-Enriched Fraction of Rose Oil Distillation Wastewater Inhibits Cell Proliferation, Migration and TNF-α-Induced VEGF Secretion in Human Immortalized Keratinocytes

The experimental part (except extract preparation, see supporting information), the recording of time lapse pictures, the preparation of figures, data analysis including statistical evaluation and the writing of the first draft of the manuscript were my contributions to this publication.

-Jonas Wedler-

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A polyphenol-enriched fraction of rose oil distillation wastewater inhibits cell proliferation, migration and TNF-α-induced VEGF secretion in human immortalized keratinocytes

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Abstract

Water steam distillation of rose flowers separates the essential oil from the polyphenol containing rose oil distillation waste water (RODW). Recently, a strategy was developed to separate RODW into a polyphenol depleted water fraction and a polyphenol enriched fraction [RF20-(SP-207)]. Polyphenols are known to have a wide spectrum of biochemical and pharmacological effects. In particular, rose petals are known to contain compounds with potential anti-proliferative activity, such as flavonoids, gallic and protocatechuic acids, and tannins.

The objective of the present study was to investigate RF20-(SP-207) and a fraction F(IV) augmented in quercetin and ellagic acid for possible anti-proliferative effects in immortalized human keratinocytes (HaCaT).

The data demonstrated that RF20-(SP-207) revealed a dose dependent anti-proliferative activity (IC_{50} of 9.78 µg/mL). In a non-toxic concentration of 10 µg/mL, this effect was stronger than that of the two positive controls LY294002 (10 µM, PI3K-inhibitor, 30 % inhibition) and NVP-BEZ235 (100 nM, dual PI3K/mTOR-inhibitor, 30 % inhibition) and clearly exceeded the anti-proliferative action of quercetin (50 µM, approx. 25 % inhibition) and ellagic acid (1 µM, approx. 15% inhibition). Time lapse microscopy detected a significant impairment of cell migration under influence of RF20-(SP-207) and F(IV). At concentrations of 10 µg/mL of both, extract and fraction, cell migration was strongly suppressed (51% and 28% gap closure, respectively, compared to 95% gap closure 24 hours after control treatment). The suppression of cell migration was comparable to the positive controls LY294002 and NVP-BEZ235 and to quercetin. Furthermore, basal and TNF-α stimulated VEGF-secretion was significantly reduced by RF20-(SP-207) and F(IV) at 10 µg/mL (approx. 50 % compared to the untreated control).

In conclusion, RF20-(SP-207) has been shown to be very promising in terms of controlling cell proliferation and migration and could be developed as a supportive, local therapy against hyper-proliferation-involved skin diseases such as plaque psoriasis.

Keywords: *Rosa damascena*, Rosaceae, waste water, quercetin, ellagic acid, cell proliferation, cell migration, VEGF

Introduction

Rosa damascena Mill. f. trigintipetala Dieck (Rosaceae), commonly known as the oil bearing rose, is a plant rich in polyphenolic constituents with numerous biological activities such as anti-HIV, anti-bacterial, anti-oxidant, anti-tussive, anti-diabetic, anti-inflammatory and anti-plasmodial [1]. The flowers are annually harvested in the rose valley of Bulgaria in large quantities to extract its essential oil for the food and cosmetic industry. Rose oil production therefore poses a respectable economic factor. However, during steam distillation of the rose flowers the distillation water containing large amounts of polyphenolic compounds remains as the major part of the waste. During a single industrial distillation cycle approx. 4000 L of liquid waste are generated [2], thus causing a principal environmental problem in the area of the production. Currently, there is no common practice for rose oil distillation waste management. Following the completion of the distillation, the hot residue containing the flower debris and the distillation water is discharged in the drainage system or spread on the soil around the distillation factory. However, rose oil distillation wastewater (RODW) represents a serious environmental problem due to the high content of polyphenols which are difficult to decompose and which are considered bio-pollutants when discarded into the rivers. In a recent approach we developed a method to separate RODW into a polyphenol depleted water fraction and a polyphenol enriched fraction RF20-(SP-207) [3].

It is well known that polyphenols and especially flavonoids possess a wide range of pharmacological activities, such as anti-oxidant, free-radical scavenging, anti-cancerous, anti-inflammatory, anti-mutagenic, anti-proliferative and anti-depressive effects [4].

One of the major difficulties of elucidating the health effects of polyphenols is their unfavourable pharmacokinetic profile since it is well known that especially flavonoids are heavily metabolized by colonic microorganisms [5]. The active compounds may not be the native polyphenols found in food, which are most often tested in *in vitro* studies; they are more likely to be metabolites [6]. These considerations make the development of a value added polyphenol based dietary supplement for oral intake difficult. To circumvent issues related to the low oral bioavailability of polyphenols a topical application is the preferential form of treatment.

Taking these points into consideration, it was the initial focus of the present project to characterize RF20-(SP-207) for pharmacological effects such as anti-inflammatory, anti-proliferative and anti-migratory, since these activities of polyphenols from various natural

sources are well documented [7]. In a recent bioassay-guided approach, a further purified fraction augmented in quercetin and ellagic acid as well as the corresponding pure compounds were identified as major active compounds [8]. It was therefore of interest, to investigate their contribution to possible anti-proliferative and anti-migratory effects in addition.

Results

Investigating cell viability based on the MTT-assay, a significant decrease of cell viability at higher concentrations of RF20-(SP-207) was revealed. RF20-(SP-207) in concentrations of 100 and 200 µg/mL caused a significant decrease of conversion of the tetrazolium salt into the water-insoluble formazan compared to the untreated control (75 and 60%; Figure 1A). Albeit not significant, the 10 and 50 µg/mL treatment caused a reduction in cell viability by less than 20%. By comparing the four fractions F(I)-(IV) of RF20-(SP-207), there was no significant reduction determined (Figure 1B). Nevertheless, a decreased viability could be demonstrated for F(IV) in the concentration of 100 µg/mL (85% viability compared to control; Figure 1B). Further analysis of F(IV) in concentrations of 10, 50, and 100 µg/mL resulted in a significant decrease of cell viability at 50 and 100 µg/mL (80 and 70%, respectively; Figure 1C). The discrepancy between the two results of Figs. 1B and C can be ascribed to the normalization on two different controls and the limited intrinsic precision of this method.

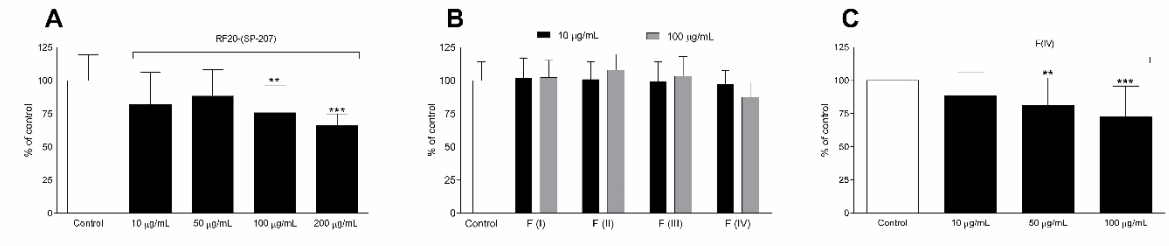

Figure 1: Effects on cell viability of **A** RF20-(SP-207), **B** F(I)-(IV) and **C** F(IV). Results expressed as Mean ± SD of three independent experiments. **p<0.01, ***p<0.001 vs. untreated control group.

The MTT assay is one of the standard techniques for assessment of cell viability. However, several studies have shown that some plant extracts and redox-active polyphenols interfere with the MTT assay since they directly reduce the tetrazolium salt even in absence of cells [9-11]. Because of inaccuracies inherent in the MTT-method, the ATP assay was chosen as an alternative to assess viability. Hence, HaCaT keratinocytes were treated with RF20-(SP-207) or F(IV) for 24 h. Subsequent measuring of the ATP levels provided information about the survival- and death-rate of the cells under influence of the corresponding substances. RF20-(SP-207) decreased cell viability of about 20% in the concentrations of 10 and 50 µg/mL compared to the untreated control while the concentration of 100 µg/mL caused a massive drop in cell viability to 45% (Figure 2).

The different relative values of the MTT and ATP assays are the results of the nature of the target that are measured. *Wang et. al.* [10] demonstrated that depending on the method, the curves are of certain steepness and thus, are not superimposable. F(IV) acted similar to RF20-(SP-207), albeit the lower concentration of 10 µg/mL did not influence the ATP level significantly. Based on these data, RF20-(SP-207) and F(IV) were used in a concentration of 10 µg/mL for subsequent experiments since it did not severely affect cell viability. Quercetin (50 µM) and ellagic acid (1 and 10 µM) also had no significant effect on cell viability (data not shown). The intracellular ATP-level is dependent on the phosphorylation of ADP by the ATP synthase. ATP is therefore a better marker for cell viability because of its presence in metabolically active cells. ATP levels decrease rapidly when the cells undergo necrosis or apoptosis. Mitochondrial ATP synthesis does not occur if the proton motive force is absent. Thus, the MTT assay can give false positive effects when uncoupling agents and polyphenolic compounds are used [12].

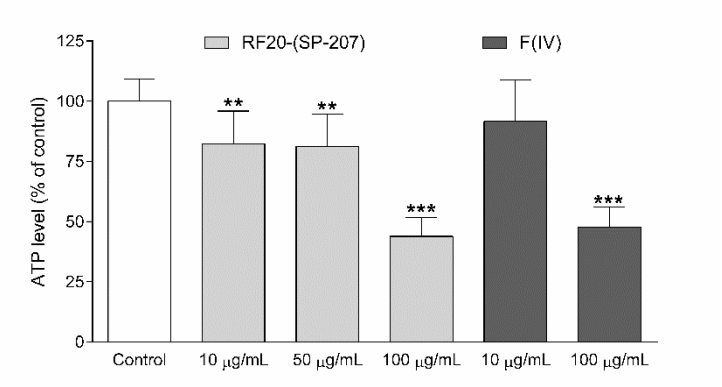

Figure 2: Effects of RF20-(SP-207) and F(IV) on A) the cellular ATP-level. Results expressed as Mean ± SD of three independent experiments. **p<0.01, ***p<0.001 vs. untreated control group or lysis buffer, respectively.

The keratinocytes' proliferation rate was determined by the BrdU-assay. Additionally to RF20-(SP-207), the PI3K/mTOR-inhibitor NVP-BEZ235 (100 nM) and the PI3K-inhibitor LY294002 (10 µM), as well as quercetin (50 µM) and ellagic acid (1 µM) were applied and their outcomes compared to an untreated control. RF20-(SP-207) significantly decreased BrdU incorporation at 5 and 10 µg/mL (63% vs. control) while NVP-BEZ235 and LY294002 resulted in a DNA-synthesis inhibition of 56% and 57% (Figure 3A). The IC_{50} of RF20-(SP-207) was determined at 9.78 µg/mL (Figure 3B). Comparing these results with those of F(IV), inhibition of thymidine incorporation was similar; 10 µg/mL provoked a proliferation decrease of 28 % correlating to 10 µg/mL of RF20-(SP-207) (Figure 3C). Ellagic acid (1 µM) reduced the BrdU incorporation by 14 %, followed by the significant effect of quercetin (50 µM) with a drop of 25 % (Figure 3A/D).

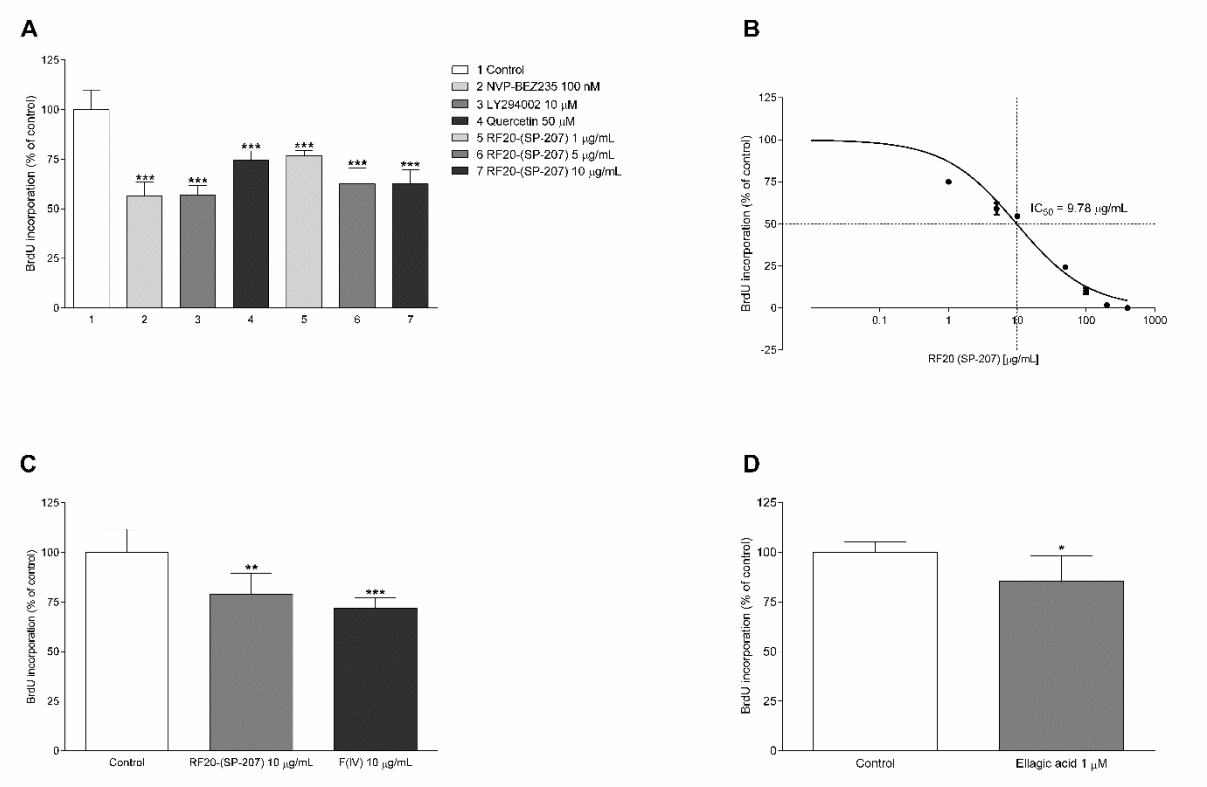

Figure 3: A Inhibition of the BrdU incorporation in de novo synthesized cellular DNA by RF20-(SP-207); **B** IC_{50} determination of the BrdU inhibition by RF20-(SP-207); **C** comparison of RF20-(SP-207) and F(IV) on BrdU inhibition. **D** Inhibition of the BrdU incorporation in de novo synthesized cellular DNA by ellagic acid at a concentration of 1 μM. Results expressed as Mean ± SD of three independent experiments. **p<0.01, ***p<0.001 vs. untreated control.

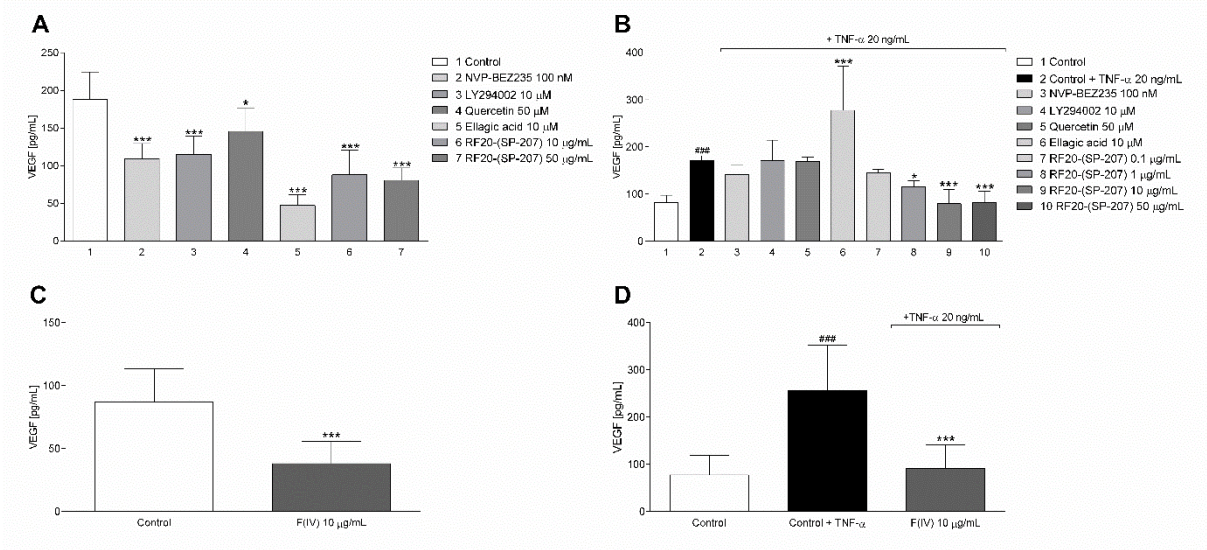

Figure 4: Effects of RF20-(SP-207), quercetin, ellagic acid, NVP-BEZ235 and LY294002 on the cellular VEGF production level in **A** unstimulated HaCaT keratinocytes and **B** TNF-α (20 ng/mL) stimulated keratinocytes for 24 h; effects of F(IV) in **C** unstimulated HaCaT keratinocytes and **D** TNF-α (20 ng/mL) stimulated keratinocytes for 24 h at 20 ng/mL. Results are expressed as Mean ± SD of three independent experiments. *p<0.1, **p<0.01, ***p<0.001 vs. TNF-α stimulated control<, ###p<0.001 vs unstimulated control.

Treatment of HaCaT cells with RF20-(SP-207) significantly decreased VEGF secretion in concentrations of 10 and 50 µg/mL (Figure 4A). The effect was comparable to that of the positive controls LY294002 (10 µM) and NVP-BEZ235 (100 nM). While quercetin (50 µM) only had a moderate effect on VEGF secretion, a significant suppression of VEGF release was observed for ellagic acid (1 µM). Stimulation of HaCaT cells with TNF-α (20 ng/mL) significantly triggered release of VEGF 2-fold compared to control cells (Figure 4B). Pretreatment with RF20-(SP-207) significantly inhibited TNF-α stimulated VEGF secretion dose dependently. Interestingly, ellagic acid, quercetin, NVP-BEZ235, and LY294002 could not reduce VEGF-secretion after TNF-α stimulation (Figure 4B). For F(IV) experiments were repeated using the same setup. In case of the unstimulated setup, F(IV) at 10 µg/mL could significantly decrease VEGF of the untreated control (Figure 4C). Similar effects were observed in the stimulated setup where F(IV) at 10 µg/mL could significantly decrease VEGF secretion compared to the stimulated control.

To further substantiate anti-proliferative effects of RF20-(SP-207) and F(IV), the Cell Death Detection ELISA^{PLUS} photometric enzyme immunoassay was used to detect internucleosomal degradation of genomic DNA during apoptosis (Figure 5). Cells were treated with the positive control anisomycin (10 µM), NVP-BEZ235 (100 nM), LY294002 (10 µM), RF20-(SP-207), F(IV) (both at 10 µg/mL), quercetin (50 µM), ellagic acid (1 µM) and compared to an untreated control. After 24 hours, the enrichment of histone-complexed DNA fragments in the cells cytoplasm was determined spectrophotometrically. Anisomycin inhibits the peptidyl transferase of the 80s ribosome system and thereby induces apoptosis. Treatment with this substance at 10 µM yielded a 16-fold increase of cell death rate in HaCaT cells. NVP-BEZ235, LY294002 and ellagic acid failed to induce significant DNA damage at the used doses. Non-toxic doses of quercetin and RF20-(SP-207) increased DNA fragmentation by 11-fold; F(IV) by 7-fold, respectively.

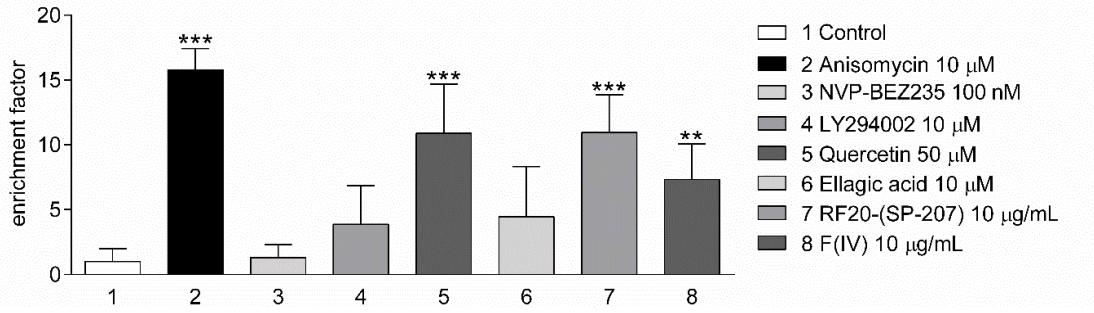

Figure 5: Quantification of DNA-histone-complexes expressed as the enrichment factor (equation of NVP-BEZ235, LY294002, quercetin, ellagic acid, RF20-(SP-207) and F(IV) compared to an untreated control serving as a (-)-control and anisomycin posing a (+)-control. Results expressed as Mean ± SD of two independent experiments. **p<0.01, ***p<0.001 vs. untreated control.

Time lapse cell migration assays were performed by placing silicone inserts into 12-well plates, thereby inducing a gap in the monolayer. The migration process started after subsequent removal of the inserts. Cell migration was observed for 24 hours and pictures were taken every 30 minutes. For better data presentation, the 24 h observation period was divided into five time points (0; 6; 12; 18; 24h) to demonstrate closure of a 450 µm cell gap (Figure 6 and Table 1).

Figure 6 shows representative images and gap closure values only from a single experiment.

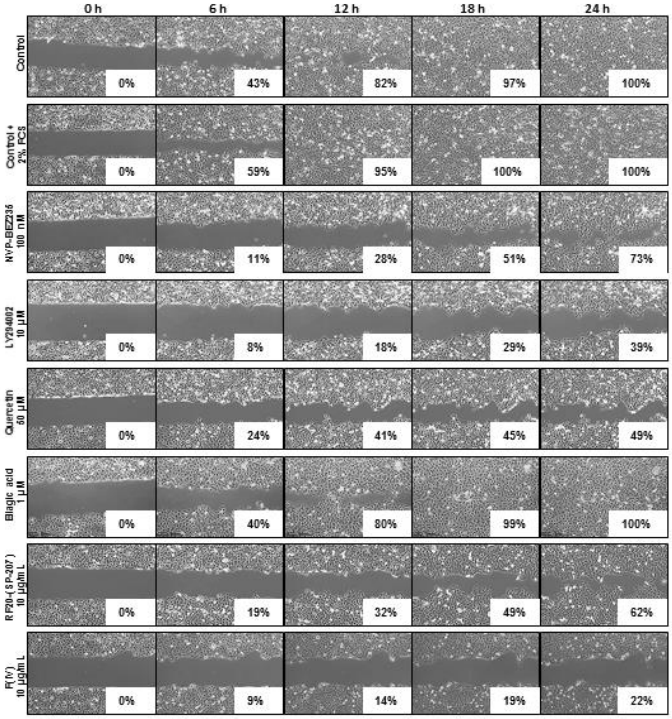

Figure 6: Cell migration in response to an artificial injury using long term, time-lapse imaging interval snap-shots of predefined positions in the wound gap (450 µm). Pictures show the momentum at 0 h, 6 h, 12 h, and 24h. HaCaT cells were treated with the control 0% FCS, the positive control 2% FCS as well as RF20-(SP-207) (10 µg/mL), F(IV) (10 µg/mL), quercetin (50 µM), ellagic acid (1 µM), LY294002 (10 µM) and NVP-BEZ235 (100 nM).

All values presented in Table 1 are the outcomes of at least three independent experiments and presented as Mean ± SD. At t = 24 h, a gap closure of 93.29 ± 12.86% was reached for the non-supplemented control (Table 1). Medium supplemented with 2% FCS was used as positive control. In contrast to the non-supplemented control, the gap was closed almost to 100% already after 12 h (95.18 ± 9.63%). RF20-(SP-207) exerted distinct inhibitory effects on cell migration (at t = 24h 51 ± 17.57%) in comparison to the untreated control. F(IV) provoked even stronger effects. At 10 µg/mL the migration speed slowed down clearly (18.62 ± 4.20 % closure at t = 24h). NVP-BEZ235 (100 nM) and LY294002 (10µM) were selected to include migration-modulators of different modes of action [13, 14]. The two reference compounds NVP-BEZ235 and LY294002 generated a clear impairment on cell migration versus the untreated control (approx. 64% and 39% closure at t = 24h, respectively; Figure 6; Table 1). The phosphatidylinositol-3-kinase inhibitor LY294002 was the strongest among the references in terms of inhibition of cell migration. The effects of RF20-(SP-207) and F(IV) on cell migration were more comparable to those of LY294002. Further, the effects of quercetin and ellagic acid

as major constituents of F(IV) were examined. Both compounds had different effects on cell migration. While quercetin in a concentration of 50 µM showed a strong anti-migratory effect (44.66 ± 19.62% gap closure after 24h), ellagic acid in a concentration of 1 µM did not markedly affect cell migration and remained on the control level.

gap closure % [mean ± SD**]**

	0h	6h	12h	18h	24h
Control	**0**	**43.27 ± 14.26**	**74.85 ± 21.37**	**89.56 ± 16.62**	**93.29 ± 12.86**
Control 2% FCS	**0**	**62.52 ± 26.96**	**95.18 ± 9.63**	**100.00**	**100.00**
NVP-BEZ235 100 nM	**0**	**13.73 ± 2.75**	**26.12 ± 2.78**	**45.60 ± 7.60**	**63.82 ± 13.52**
LY294002 10 µM	**0**	**11.90 ±4.00**	**20.78 ± 2.83**	**28.06 ± 7.00**	**38.69 ± 2.71**
Quercetin 50 µM	**0**	**17.24 ± 5.84**	**34.10 ± 9.33**	**41.00 ± 16.20**	**44.66 ± 19.62**
Ellagic acid 1 µM	**0**	**31.75 ± 11.74**	**69.90 ± 16.75**	**96.93 ± 4.44**	**99.10 ± 1.91**
RF20-(SP-207) 10 µg/mL	**0**	**17.85 ±1.87**	**29.86 ± 6.81**	**41.44 ± 12.31**	**51.00 ± 17.57**
F(IV) 10 µg/mL	**0**	**8.54 ± 2.13**	**12.29 ± 2.20**	**16.55 ± 3.00**	**18.62 ± 4.20**

Table 1: Cell migration in response to an artificial injury. Time-lapse microscopy was performed and snapshots were taken every 30 min. during 24h, whereof values for statistical calculation were used from images at t = 0h, 6h, 12h, 18h, and 24h. Gap size was calculated by utilizing the software Image J including the wound healing tool-macro. Detected spaces were converted into percent-ages; 0% refers to no movement of cells, 100% depicts a total gap closure. Values represent the mean ± SD of the closed area in percent of the primordial cell gap induced by an insert calculated by at least three independent experiments.

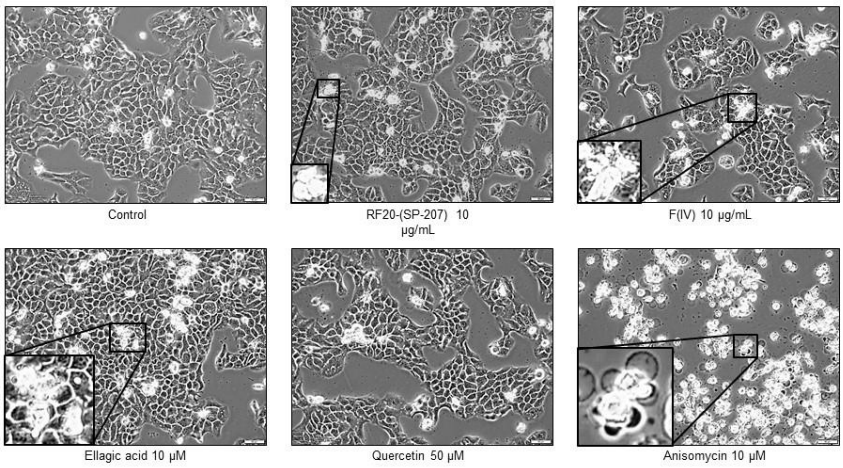

Figure 7: Morphology of HaCaT cells treated with the control, RF20-(SP-207) (10 µg/mL), F(IV) (10 µg/mL), ellagic acid (1 µM), quercetin (50 µM), and anisomycin (positive control, 10 µM) for 24 h under phase contrast microscopy (20× objective).

To further determine the effects of RF20-(SP-207) and F(IV) on growth inhibition, morphological changes were examined under a phase-contrast microscope. Cells treated with anisomycin clearly showed apoptotic morphology (fragmented nuclei, membrane blebbing) (Figure 7). The images also show obvious morphological changes when HaCaT cells were treated with RF20-(SP-207) and F(IV) with visible apoptotic vesicles although the effects were less pronounced compared to the anisomycin-treated cells.

Discussion

In the present study, the spontaneously immortalized human keratinocyte cell line HaCaT was used to examine potential anti-proliferative activities of RF20-(SP-207) and its concentrated fraction F(IV). Despite omitting a detailed cell cycle analysis by flow cytometry, it was demonstrated that RF20-(SP-207) and F(IV) effectively inhibited proliferation and migration of human keratinocytes as shown by the results of the BrdU incorporation assay and the time lapse microscopy data. The effects of RF20-(SP-207) and F(IV) can be explained by presence of quercetin and partly by ellagic acid since both compounds are the major compounds thereof. In addition, it was shown that RF20-(SP-207) and F(IV) significantly inhibited basal and TNF-α induced stimulation of vascular endothelial growth factor (VEGF) in a concentration range that did not affect normal cell viability. The observed effects are further comparable to the PI3K-inhibitor LY294002 and the dual PI3K/mTOR-inhibitor NVP-BEZ235 which both were used as positive controls.

In the epidermal layer of the skin, keratinocytes represent the major cell type. In response to endocrine and immune triggers, keratinocytes can release various cytokines such as IL-6 or IL-8 but also release VEGF [15]. When the balance between growth, differentiation and death is altered in keratinocytes, they undergo hyper-proliferation, abnormal differentiation and inflammatory infiltration. This will result in the pathological manifestation of psoriasis, which is a chronic, inflammatory skin disease, characterized by hyper-proliferation and aberrant differentiation of keratinocytes [16]. Approximately 1-3% of the world population is affected by this chronic skin disease. An improved understanding of the molecular basis underlying psoriasis in recent years has led to the introduction of anti-proliferative and TNF-α blocking biologicals (e.g. etanercept, adalimumab) as an effective treatment alternative for this disease [16]. Although these therapies are highly effective, many patients experience serious side effects, most prominently a higher risk of infections [17]. In addition, these compounds require invasive administration such as subcutaneous or intravenous injections. However, for patients with mild symptoms a less invasive approach is desired. Therefore, a topical regimen acting locally by influencing cell fate, viability and proliferation, is a desirable treatment approach.

In the present study, it was shown that the polyphenol enriched fraction of rose oil distillation water RF20-(SP-207) and a purified fraction of F(IV) not only inhibited cell proliferation but also significantly suppressed basal and TNF-α stimulated VEGF secretion. TNF-α is a pro-inflammatory cytokine that plays a key role in the pathogenesis of psoriasis [18]. TNF-α levels are elevated in psoriasis and play an important part in T-cell proliferation and disease

pathogenesis [19]. It stimulates keratinocytes to release VEGF, which is a potent stimulator of cell proliferation, migration, and survival [20]. VEGF is not only a key regulator of cutaneous angiogenesis, where it contributes to several physiological and pathogenic processes in the skin. These include for instance cancer development, hair growth, psoriasis, and wound healing. The signal protein also increases the tissue permeability during the onset of an inflammation process and therefore executes a multimode function (for review see [21]). Increasing experimental evidence has shown the effectiveness of anti-VEGF strategy for the treatment of psoriasis [22]. Many existing psoriasis therapies influence the production or function of VEGF. However, the clinical use of current VEGF inhibitors is limited due to a number of potentially serious side effects [23].

Our findings that RF20-(SP-207) and F(IV) significantly reduce basal and TNF-α stimulated VEGF secretion in human keratinocytes represent therefore a promising treatment option that is worth being further investigated. Since the effects of F(IV) resemble that of RF20-(SP-207) it can be speculated that ingredients only found in this fraction are responsible for the observed effects. As mentioned earlier, F(IV) contains quercetin and ellagic acid as major constituents.

The anti-proliferative and cell migration inhibitory properties of both compounds have been studied in a number of cell types [24, 25]. In particular, it has been shown that ellagic acid inhibits VEGF-induced phosphorylation of VEGFR-2 in endothelial cell as well as PDGF-induced phosphorylation of PDGFR in smooth muscle cells [25]. Furthermore, ellagic acid inhibited VEGF-induced migration of endothelial cells. The authors demonstrated that ellagic acid presented a greater selectivity for normal cells than for tumor cells and concluded that the anti-angiogenic effects of ellagic acid can be explained by dual inhibition of VEGF and PDGF receptors [25].

Wang et al. [26] reported that ellagic acid significantly inhibited a series of VEGF-induced angiogenesis processes including proliferation, migration, and tube formation of endothelial cells. Ellagic acid further inhibited VEGFR-2 tyrosine kinase activity and downstream signaling pathways such MAPK and PI3K/Akt in endothelial cells [26].

In the present study ellagic acid significantly inhibited VEGF secretion in unstimulated keratinocytes. However, after stimulation with TNF-α no reduction of VEGF release was observed. Only very few literature data are available that report effects of ellagic acid in keratinocytes and they focus on either the anti-inflammatory or anti-cancer activities [27, 28]. The observed anti-angiogenic properties of ellagic acid in keratinocytes in the present study are

in line with published data observed in various cancer cells [25, 29]. To our knowledge, this is the first time that effects of ellagic acid in TNF-α stimulated keratinocytes were investigated. In the present study, ellagic acid - like quercetin - could not reduce VEGF levels induced by TNF-α in HaCaT cells. Interestingly, F(IV) which contains both, quercetin and ellagic acid, significantly decreased VEGF secretion in TNF-α stimulated cells. It is speculated, that both compounds might act synergistically under the chosen experimental conditions. Synergistic effects of quercetin and ellagic acid have been reported previously [30]. The authors found that a combination of both compounds synergistically induced apoptosis, inhibited cell proliferation and reduced cell growth in human leukemia cells.

The antiangiogenic effects of quercetin targeting VEGF pathways have been investigated comprehensively in the literature [31, 32]. In the present study, quercetin lightly affected basal VEGF secretion, but did not alter TNF-α stimulated VEGF secretion compared to the stimulated control. It has to be emphasized again that in most studies reporting the anti-angiogenic effects of quercetin cancer cell lines were used, whereas only a few studies with quercetin and keratinocytes exist. Redondo et al. [33] demonstrated that quercetin suppressed the incorporation of ^{3}H-thymidine into human keratinocytes and significantly inhibited VEGF secretion in basal and EGF- or PMA-treated human keratinocytes. However, the contradicting results between the study of Redondo et al. [33] and the current study could possibly be explained by different experimental settings – we used TNF-α to stimulate VEGF secretion while in the Redondo study VEGF was added to the cells. Although not directly related, Weng et al. [34] recently found that luteolin could inhibit TNF-α stimulated VEGF secretion in HaCaT cells. The experimental setting in this study was comparable to the one used in the present investigation. In a recent study using the same experimental conditions we could show that a bamboo leaf extract significantly decreased TNF-α stimulated VEGF production in HaCaT cells. Isoorientin was identified as major active substance and dose-dependently decreased TNF-α induced VEGF levels [35].

Interestingly, F(IV) which contains besides other polyphenols mainly ellagic acid and quercetin [8] reduced VEGF secretion under basal and TNF-α stimulated conditions. Since this effect resembles that of RF20-(SP-207), it is assumed that the activity is located in this fraction. Recent data of our group support this assumption [8]. We further speculate that the overall effect on basal and TNF-α stimulated VEGF secretion can be explained by a synergistic effect of quercetin and ellagic acid. As mentioned above, such effects have been described in the literature and underline the observations in the present study [30].

Cell migration is an extraordinary complex mechanism mostly driven by a dynamic interplay of random motion, chemokinesis and chemotaxis [36]. It was shown in the present study that RF20-(SP-207) and F(IV) have been similar in their impact on cell migration at 10 µg/mL, provoking a severely retarded gap closure comparable to the reference compound LY294002. As mentioned above, the major constituents of RF20-(SP-207) and F(IV) are quercetin and ellagic acid. Both compounds had different outcomes on cell migration in the present experiments with quercetin showing more pronounced effects. Cao et al. [31] recently reported that quercetin at a concentration of 40 µM strongly inhibited A375, A2058, and B16F10 cell migration. Similar results were observed by Prateeshkumar et al. [37] who showed that quercetin significantly inhibited VEGF induced migration of HUVEC cells in a dose dependent manner with maximum inhibition of cell migration at 40 µM. Several other studies also reported a suppression of cell migration by quercetin in various concentration and various cell lines [24, 38, 39] and our results together with the decreased proliferation as shown in the BrdU incorporation assay are in line with these published data.

The anti-invasive effects of ellagic acid are also well described [25, 29]. Ellagic acid did not have a significant effect on cell migration in our study, although the compound reduced basal VEGF levels. The slightly different effects between the published results and our data could be explained by different treatment responses between cancer cell lines and normal cells. The physiological processes of cell migration are different in tumor cell lines because they are activated by several pro-migratory factors without stop signals causing an imbalance of signals. Due to this imbalance, cancer cell lines can modify their migration mechanisms in response to different conditions [40]. It is therefore not unlikely that compounds may differently affect pathways between normal and cancer cell lines [41]. Furthermore, ellagic acid is hardly soluble in pure aqueous media. DMSO as a solubilizer was used in a max. concentration of 0.1 %, which is nontoxic for HaCaT keratinocytes. Taking these issues into account, it was not possible to test ellagic acid concentrations higher than 10 µM.

Another important finding of the present study is that RF20-(SP-207), F(IV) and quercetin showed pro-apoptotic effects in addition to the pronounced anti-proliferative activities. It is assumed that the anti-proliferative effects of RF20-(SP-207) and F(IV) are a result from a cytotoxic effect, and an induction of apoptosis. A similar mechanism has been suggested for topical corticosteroids, which are often used in dermatology for their anti-proliferative effects [42]. Skin diseases such as psoriasis are characterized by hyper-proliferation and decreased apoptosis of epidermal keratinocytes [43]. Thus, RF20-(SP-207) could be developed as a novel

non-steroidal therapy directed towards inhibition of cell proliferation and enhancement of apoptotic process in psoriasis.

In summary, RF20-207, a polyphenol-enriched fraction of RODW, demonstrated potent inhibitory effects on cell proliferation and migration. Fraction (IV), which contains quercetin and ellagic acid in significant amounts, resembles the activity of RF20-(SP-207). The effects of the individually tested compounds quercetin and ellagic acid, could partly explain the potent anti-proliferative and anti-migratory effects of RF20-(SP-207) and F(IV). Their effect could be additive or synergistic and more experiments are necessary to clarify the exact mechanism of compound interaction. There is an increasing interest in plant-derived compounds for the treatment of hyper-proliferative skin diseases because they present safer alternatives when compared with synthetic medicines. Although a large number of plant based inhibitors of cell proliferation have been reported in the literature they are typically not commercially available. Their further clinical evaluation as active ingredients for dermatological purposes is often limited, since large amounts are usually needed for incorporation in topical formulations. Our approach therefore could close a gap in the raw material supply chain since large quantities of this highly active 'bioproduct' of rose oil distillation water is easily accessible. Appropriate topical formulations have to be developed and the effectiveness and safety has to be further evaluated.

Material and Methods

Chemicals and Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT, purity >98%), adenosine 5'-triphosphate (purity >99%), sodium dodecyl sulfate (SDS), acetic acid (purity >99.7%), anisomycin (purity >98%), ellagic acid (EA, purity >95%) and dimethyl sulfoxide (purity >99.5%; DMSO) were purchased from Sigma-Aldrich (Buchs, CH). 5-bromo-2'-deoxyuridine-(BrdU), human VEGF ELISA kits, Dulbecco's Phosphate buffered saline [-] $CaCl_2$; [-] $MgCl_2$, Trypsin-EDTA 0.5% (10x), LY294002 (PI3K-inhibitor) and NVP-BEZ235 (dual PI3K/mTOR-inhibitor) were provided by LubioScience (Luzern, CH). *E. coli*-derived recombinant human TNF-α (purity >97%) was purchased from R&D Systems (Abingdon, UK). Quercetin dehydrate (purity >99%) was purchased from Extrasynthese (Genay, FR). CellTiter-Glo Luminescent Cell Viability Assay was obtained from Promega (WI, USA). Rat tail collagen type I was provided by Trevigen Inc. (Gaithersburg, MD, USA). Cell Death Detection ELISA^{PLUS} Kit was from Roche Diagnostics (Rotkreuz, CH).

Cell culture

Human non-tumorigenic HaCaT keratinocytes (Cell Line Services, Eppelheim, Germany) were maintained in Dulbecco's Modified Eagle's Medium high glucose 1x supplemented with 1% (v/v) Penicillin 10.000 U/mL / Streptomycin 10.000 µg/mL and 10% (v/v) heat inactivated fetal calf serum (FCS) (LubioScience, Luzern, Switzerland) at 37 °C in a humidified atmosphere containing 5% CO_2. Experiments were routinely conducted at approximately 80-90% confluency [44]. All experiments were carried out between passages 32 and 70. Periodical tests on mycoplasma contaminations were negative. All compounds were used in a final DMSO concentration of max. 0.1% (v/v). Concentrations of LY294002 (10 µM), NVP-BEZ235 (100 nM) and anisomycin (10 µM) which were used as reference compounds were chosen according to published data [13, 45, 46].The concentration of quercetin was also selected based on published data [30, 37]. Ellagic acid could not be used in concentrations higher than 10 µM because of solubility issues (a final concentration of 0.1% DMSO would have been exceeded which led to cell toxicity).

Plant material and extract preparation

Wastewater obtained after distillation of full-blown *R. damascena* flowers was obtained from the distillery of the Institute of Roses, Essential and Medicinal Crops (IREMC) in Kazanlak, Bulgaria, in June 2013. Wastewater was filtered through a cheese-cloth to afford RODW which was stored at +4°C in 10 L plastic bottles. RF20-(SP-207) was prepared from RODW as described in [3]. Fractions (I)-(IV) were obtained and phytochemically characterized as described in [8]. An extensive phytochemical description of RF20-(SP-207) and F(IV) is published in [3]. RF20-(SP-207) mainly consisted of phenolic compounds, such as isoquercitrin, kaempferol, quercitrin, rutin, astragalin, multiflorin A, ellagic acid, and several kaempferol and quercetin glycosides [3]. F(IV) mainly consisted of ellagic acid, quercetin, and kaempferol [8]. A voucher specimen of RODW_June2013 is stored at -20°C at the Institute for Pharma Technology, School of Life Sciences, FHNW.

Cell viability assay

Cell viability was assessed by conducting the MTT-test. 200 µL of a HaCaT cell suspension was seeded into sterile 96-well plates at a concentration of $1.5x10^5$ cells/mL. After an incubation time of 24 hours (37 °C, 5% CO_2), the medium containing 10% FCS was discarded and the plate was washed with 200 µL/well phosphate buffer saline (PBS). RF20-(SP-207) was tested in concentrations of 10, 50, 100 and 200 µg/mL. Fractions (I)-(IV) were tested in concentrations of 10 and 100 µg/mL, respectively. After an incubation period of 24 hours and a washing step with PBS, 10 µL of MTT-solution (5 mg/mL PBS) dissolved in 100 µL DMEM was added and incubated for another 2 hours. The liquid was aspirated before adding 100 µL of the cell-lysis buffer consisting of 99.4% DMSO, 0.6% acetic acid and SDS 0.1 g/mL. The optical density was read at 500 nm on a microplate reader (SpectraMax M2e, Molecular Devices, Sunnyvale, U.S.A.). The viability was calculated according to the equation:

$$(1)\quad viability\ [\%] = 100 * \frac{OD_{500nm}(sample) - OD_{500nm}(NoCells)}{OD_{500nm}(control_{mean}) - OD_{500nm}(NoCells)}$$

ATP Assay

The CellTiter-Glo Luminescent Cell Viability assay was carried out according to the manufacturer's protocol. The following alterations were made: cells were grown in a transparent, sterile 96-well plate at a density of $1.5x10^5$ cells/mL. After an incubation period of 24h (37 °C, 5% CO_2), cells were washed with PBS, subsequently treated accordingly with 100 µL solution and incubated for another 24h. 100 µL of CellTiter-Glo reagent was then added to each well resulting in a 1:1 dilution. The sample plates were agitated for 2 min by an orbital shaker and incubated for 10 min. 100 µL of each well was then transferred to a white, opaque sterile 96-well plate and immediately read by a microplate reader (SpectraMax L, Molecular Devices, Sunnyvale, U.S.A.).

Cell proliferation assay (BrdU)

Samples for the 5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay were prepared similar to the MTT-test. The assay was conducted according to the manufacturer's protocol. Two hours before the end of the corresponding period, BrdU reagent (10 µM, 10µL) was added into all wells except the unstained control. After discarding the medium, 200 µL of a fixing/denaturing solution was added and incubated for another 30 min. Subsequently, the liquid was exchanged by the primary anti-BrdU monoclonal antibody and incubated for 1h. Subsequently, the unbound antibody was washed off and horseradish peroxidase-conjugated goat anti-mouse IgG was added, which binds to the detector antibody. Finally, 50 µL of 3,3',5,5''-tetramethylbenzidine (TMB) substrate was added and converted by the peroxidase conjugate and the reaction was stopped by 2.5 N sulfuric acid stop solution before the plate was read at 450 nm wavelength using a plate reader (Spectramax i3x Multimode detection platform, Molecular Devices, Sunnyvale, U.S.A.). The IC_{50}, i.e. the concentration of the extract resulting in 50% inhibition of BrdU incorporation was calculated from dose-response curves by non-linear regression analysis using GraphPad Prism version 6.01 (GraphPad software Inc., USA).

Measurement of vascular endothelial growth factor secretion

The experimental setup including the TNF-α concentration was chosen according to Park et al. [47], with some modifications. For unstimulated samples, cells were seeded into 12-well plates at a density of $1.5x10^5$ cells/mL. After an incubation time of 24 hours (37 °C, 5% CO_2), DMEM

was discarded, cells were washed with 1mL/well PBS. Samples were prepared as described above. To assess the basal secretion of VEGF in keratinocytes, DMEM with 0% FCS was applied as a control. In contrast, all wells of stimulated cells were supplemented with TNF-α 20 ng/mL and substances accordingly and incubated for 24 hours. Supernatants were stored in sterile micro centrifuge tubes and deep-frozen (-20 °C) immediately. The VEGF-ELISA was exerted according to manufacturers' instructions. Absorbance was measured at 450 nm using a microplate reader (Spectramax i3x Multimode detection platform, Molecular Devices).

Cell migration assay

After placing the cell culture inserts in the 12-well dishes, the HaCaT keratinocytes were seeded at a concentration of $1.5x10^5$ cells/mL into collagen-coated wells and bred for 24 h (37 °C, 5% CO_2), whilst the cells reached confluence. The inserts were removed causing a gap of ca. 450 µm. After washing the cells with 1 mL PBS, the wells were charged accordingly with the test compounds in different concentrations. Medium supplemented with 2% FCS served as a positive control. Long-term, time-lapse imaging was performed using the Olympus IX83 automated inverted microscope platform for live cell imaging (Olympus Corporation). Interval snapshots (every 30 min.) of predefined positions in the gap during 24h were taken using the Olympus software package cellSens Dimension 1.81. All stacks of recorded images consisted of 49 pictures, whereof 5 pictures were extracted to display the momentum at t= 0, 6, 12, 18 and 24 h. To calculate the size of the gaps over time, post-analysis of the snapshots was conducted by utilizing the software Image J (version 1.49q) including the wound healing tool-macro© (written by Volker Baecker 2010-2012, Nathalie Cahuzac and Virginie Georget). Therefore, detected spaces were converted into percentages; 0% refers to no movement of cells, 100% depicts a total gap closure.

Cell morphology and DNA fragmentation

The Cell Death Detection ELISA^{PLUS} photometric enzyme immunoassay was used for the relative quantification of cytoplasmic histone-complexed DNA fragments (mono- and oligonucleosomes) after induction of apoptosis or when released from necrotic cells. The assay was performed according to the manufacturer's instructions. Therefore, 1.5×10^5 cells/mL were seeded into a 96-well plate and incubated for 24 hours. Subsequently, the supernatant was discarded and the cells were lysed to obtain the cytoplasmic fraction. 20 µL of this fraction was

transferred to a streptavidin coated 96-well plate and 80 µL of an immunoreagent provided by the kit was added. After an incubation period of 2 hours, the wells were washed with 300 µL of incubation buffer per well. Finally, absorbance was measured at 490 nm (Spectramax i3x Multimode detection platform, Molecular Devices). The rate of apoptosis is reflected by the enrichment (fold increase) of mono- and oligonucleosomes accumulated in the cytoplasm and was calculated according to the formula [14]:

$$(2)\quad EF_{Cell\ Death\ Elisa} = \frac{O.D._{sample}}{\overline{O.D.}_{control}}$$

Results are expressed as mean ± SD values of triplicates of two independent experiments. For the determination of cell morphology, cells were placed at a density of 1.5 x 10^5 cells/mL into a 12-well plate and then RF20-(SP-207), F(IV) (both at 10 µg/ml, respectively), ellagic acid (1 µM), quercetin (50 µM) and anisomycin (positive control, 10 µM) were added to the wells and incubated for 24 h. Cells in each well were examined and photographed under a phase-contrast microscope by using a 20 x objective.

Data analysis

Data are shown as mean ± SD. All experiments were performed minimum in triplicates, and each experiment was repeated at least three times. Statistical analysis of data was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using the software package GraphPad Prism (version 6.01, GraphPad Software Inc). In all cases, differences were considered significant if $p < 0.05$. The IC_{50} of RF20-(SP-207) was calculated from dose-response curves by non-linear regression analysis using GraphPad Prism software.

Supporting Information

Detailed information about the preparation and characterization of the investigated compounds can be found in the supporting information, which is an excerpt from [8] and [3].

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

A polyphenol-enriched fraction of rose oil distillation wastewater inhibits cell proliferation, migration and TNFα-induced VEGF secretion in human immortalized keratinocytes

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Preparation and characterization of the investigated compounds

1. Column chromatography and TLC analysis

   Column chromatography was performed on a Sephadex® LH-20 column (100 x 5.5 cm I.D.) using 90% methanol as mobile phase, at a flow rate of 2 ml/min. A portion of the RF-SP207 resin extract (6.8 g) was dissolved in 60 ml 80% methanol and loaded into the column. Fractions were collected every 10 min using a SuperFrac autosampler (GE Healthcare). A total of 300 fractions were collection in glass tubes and combined to 12 main fractions based on TLC patterns. Four additional late eluting fractions were collected in flasks giving rise to a total of 16 main fractions: F1 (1-74, 1.32g), F2 (75-88, 0.22g), F3 (89-104, 0.40g), F4 (105-110, 0.17g), F5 (111-122, 0.64g), F6 (123-130, 0.31g), F7 (131-136, 0.28g), F8 (137-152, 0.42g), F9 (153-174, 0.26g), F10 (175-194, 0.23g), F11 (195-226, 0.45g), F12 (227-300, 0.56g), F13 (0.19g), F14 (0.47g), F15 (0.07g) and F16 (0.07g). TLC analysis of fractions was performed on precoated silica gel 60 F_{254} plates (Merck) with a mobile phase composed of ethyl acetate/formic acid/glacial acetic acid/water (100/11/11/26). Detection was at UV 254 and 366 nm. Spots were visualized at UV-366 nm after spraying 1% methanolic Naturstoffreagenz A followed by 5% ethanolic PEG-4000. Fractions F1 to F16 were combined based on HPLC and TLC data to give four pool fractions I to IV in order to simplify pharmacological testing. Fractions F1 and F2 were combined in pool “I”, fractions F3, F4 and F5 in pool “II”, fractions F6, F7, and F8 in pool “III”, fractions F9 to F16 in pool “IV” (Figure 1S).

2. Compound purification

   Compounds **1** to **14** were purified from Sephadex® LH-20 fractions F1 to F16 by preparative HPLC. The prep HPLC system consisted of a SCL-10VP controller, LC-8A binary pumps, a UV-Vis SPD-M10A VP detector and Class-VP 6.12 software (all Shimadzu). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) with the exception of fraction 12 where 0.1% formic acid was added to both solvents. The flow was set to 20 ml/min. Equilibration time between the injections was 10 min. UV spectra were recorded from 200 to 400 nm. Separations were performed on a SunFire Prep C_{18} OBD (30 × 150 mm, 5 µm, Waters) column equipped with a SunFire Prep guard column (19 x 10 mm, 5 µm, Waters). Preparative HPLC was performed under following isocratic conditions: 20% ACN for F1, 22% ACN for F3, 25% ACN for F6 and F8, 30% ACN for F4 and 22% + 0.1% formic acid for F12 and compounds were isolated as previously

described [3]. Quercetin (**16**) and Kaempferol (**15**) were major peaks of F13 and F12 respectively and their identity was confirmed by co-chromatography of reference compounds as previously described [3].

3. HPLC-ELSD-DAD-MS analysis

   Analytical HPLC analyses of fraction I-IV were performed on a LC-20AD instrument system (Shimadzu) equipped with a SPD-M20A PDA detector, an evaporative light scattering detector (ELSD) serie 3300 (Alltech) and a LCMS-8030 detector (Shimadzu). For the ELSD, N_2 flow was 2.5 L/min, and evaporation temperature was 60 °C. The mobile phase consisted of 0.1% formic acid (solvent A) and ACN + 0.1% formic acid (solvent B) and the flow was set to 0.4 ml/min. Separations were performed on a C_{18} SunFire^{TM} column (3.0 x 150mm, 3.5 µm, Waters) equipped with a guard column (3.0 x 20 mm, 3.5 µm, Waters) which were thermostatted at 40 °C. Each sample was prepared at a concentration of 3.5 mg/ml and 10 µl was injected. The following gradient was used; 10%B isocratic for 4 min, gradient 4-5min to 17% B, 5-26min to 27%B, 26-27min to 50%B, 27-35min 50%B isocratic, 35-36min to 100%B, 36-41min 100%B isocratic, 41-42min to 10%B. All HPLC-ELSD chromatograms are shown in **Figure 2S.**

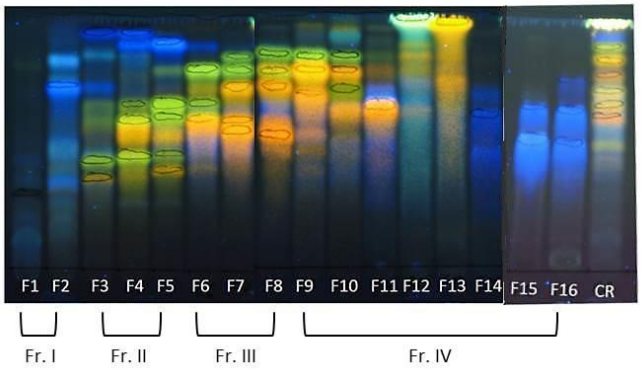

Figure 1S: Based on HPLC and TLC data, pools of four fractions were created. Fractions F1 and F2 were combined in pool "I", fractions 3, 4 and 5 in pool "II", fractions 6,7, and 8 in pool "III", fractions 9,10,11,12,13,14,15 and 16 in pool "IV".

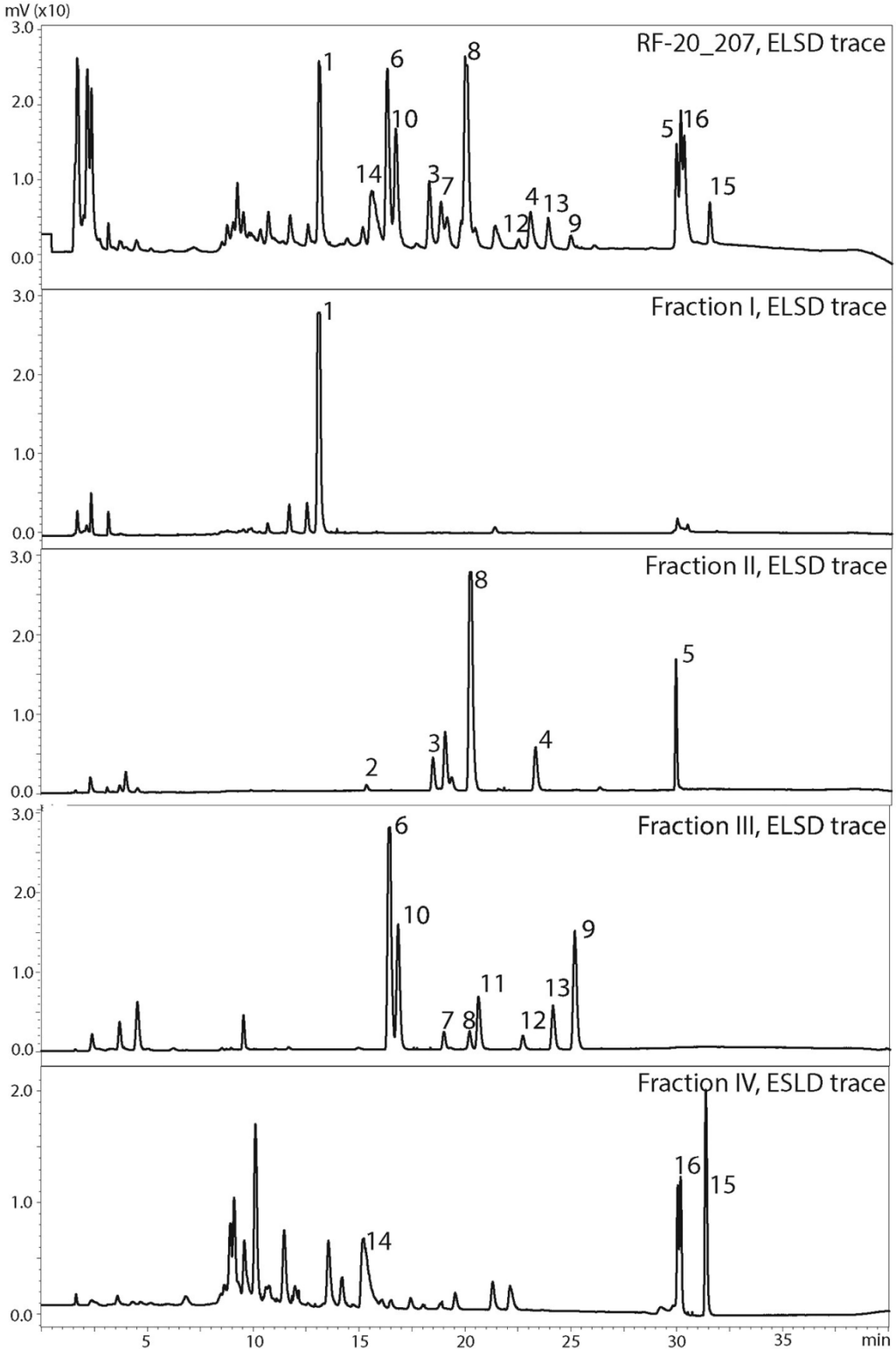

Figure 2S: HPLC-ELSD chromatogram of the LH-20 pooled fractions FI to FIV and crude polyphenol fraction RF20-207.

Peak No.	Compound	Rt	ESI+	Sephadex LH20 Fraction	Combined Fraction
1	Phenylethyl-glucopyranoside	13.8	285.5	1	I
2	Quercetin-3-O-rhamnosylglucoside (Rutin)	15.8	611.2	3	II
3	Kaempferol 3-O-rutinoside	18.7	595.2	3	II
4	Kaempferol-3-O-glucosylrhamnoside (Multiflorin B)	23.4	595.2	4	II
5	Kaempferol-3-O-acetylglucosylrhamnoside (Multiflorin A)	30.3	637.2	4	II
6	Quercetin-3-O-glucoside (Isoquercitrin)	17.1	465.2	6	III
7	Kaempferol-3-O-galactoside	19.2	449.1	6	III
8	Kaempferol-3-O-glucoside (Astragalin)	20.4	449.1	6	III
9	Kaempferol-3-O-rhamnoside	26.3	433.1	6	III
10	Quercetin-3-O-galactoside (Hyperoside)	16.8	465.2	8	III
11	Quercetin-3-O-rhamnoside (Quercitrin)	20.8	449.1	8	III
12	Kaempferol-3-O-xyloside	22.8	419.1	8	III
13	Kaempferol-3-O-arabinoside	24.3	419.1	8	III
14	Ellagic acid	16.3	303.5	12	IV
15	Kaempferol	31.9	287.2	12	IV
16	Quercetin	30.8	303.3	13	IV

Table 1S: Major compounds identified in Fraction I-IV [3]. Identity of quercetin and kaempferol were confirmed by co-chromatography of reference compounds. All other compounds were isolated and identity confirmed by NMR as previously described [3].

3.3. In Vitro Modulation of Inflammatory Target Gene Expression by a Polyphenol-Enriched Fraction of Rose Oil Distillation Waste Water

The RNA and Real Time Quantitative RT-PCR under supervision of Anna Weston, the bioassays, the preparation of figures and tables, and the writing of the manuscript were my contributions to this publication. The method of RT-PCR data analysis was elaborated in collaboration of Anna Weston, Julia Rausenberger and me.

-Jonas Wedler-

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In vitro modulation of inflammatory target gene expression by a polyphenol-enriched fraction of rose oil distillation waste water

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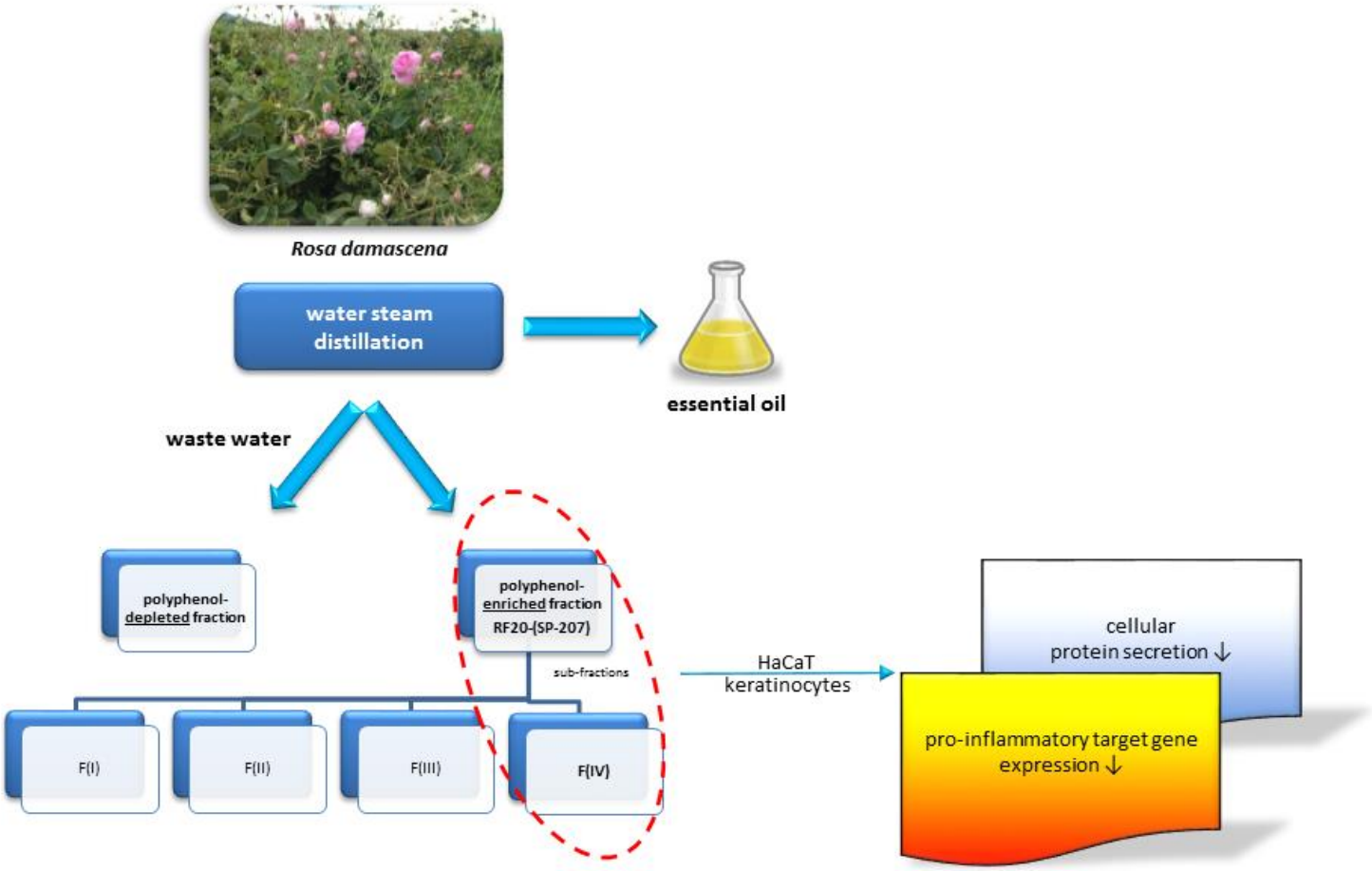
Abstract

Classical production of rose oil is based on water steam distillation from the flowers of *Rosa damascena*. During this process, large quantities of waste water accrue which are discharged to the environment, causing severe pollution of both, groundwater and surface water due to a high content of polyphenols. We recently developed a strategy to purify the waste water into a polyphenol-depleted and a polyphenol-enriched fraction RF20-(SP-207). RF20-(SP-207) and sub-fraction F(IV) significantly inhibited cell proliferation and migration of HaCaT cells. Since there is a close interplay between these actions and inflammatory processes, here we focused on the fractions' influence on pro-inflammatory biomarkers. HaCaT keratinocytes were treated with RF20-(SP-207), F(IV) (both at 50 µg/mL) and ellagic acid (10 µM) for 24 h under TNF-α (20 ng/mL) stimulated and non-stimulated conditions. Gene expression of IL-1β, IL-6, IL-8, RANTES and MCP-1 was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) and cellular protein secretion of IL-8, RANTES and MCP-1 was determined by ELISA based assays. RF20-(SP-207) and F(IV) significantly decreased the expression and cellular protein secretion of IL-1β, IL-6, IL-8, RANTES and MCP-1. The diminishing effects on inflammatory target gene expression were slightly less pronounced under TNF-α stimulated conditions. In conclusion, the recovered polyphenol fraction RF20-(SP-207) from rose oil distillation waste water markedly modified inflammatory target gene expression *in vitro*, and, therefore, could be further developed as alternative treatment of acute and chronic inflammation.

Keywords: *Rosa damascena*, waste water, anti-inflammatory, RT-PCR, ELISA

Graphical Abstract



1. Introduction

While acute inflammation can be triggered by either foreign microbial invaders or mechanical damage of the skin, chronic inflammation is a result of habitual or environmental factors. By the inflammatory response, the organism tries to defeat the trigger by removing necrotic cells and tissues damaged from the original insult, and thus, to initiate tissue repair [1]. However, in some cases the inflammatory reaction damages the host more than the trigger. Thus, drugs like (non-) steroidal anti-inflammatory drugs (NSAID) have been developed to control the course of the inflammatory response. Considering its complex cascade, not only a cellular crosstalk is crucial, the organism also instantly provides various chemokines and growth factors among others [2]. Some pro-inflammatory constituents of this mixture are the tumor necrosis factor alpha (TNF-α), IL-1β, IL-6, IL-8, Regulated on Activation Normal T-cell Expressed and Secreted (RANTES) and, Monocyte Chemotactic Protein 1 (MCP-1). Taking TNF-α into account, its pivotal role attracted much attention to successfully develop several potent biosimilars [3]. IL-6 promotes inflammation by inducing neovascularization and hyperplasia, which is especially the case for chronic developments [4]. IL-8 is a potent chemoattractant for T lymphocytes, neutrophils and eosinophils [5]. IL-1β levels are usually elevated manifold during progression of auto-immune diseases in particular [6]. As members of the c-c motif family, both RANTES and MCP-1 depict mediators, potent chemo-attractants for macrophages and leukocyte activators [7, 8]. Attenuating or deactivating these agents among others as alternative or improvement to standard drugs is a desirable objective in the treatment of inflammation. In this context, plant derived extracts have gained considerable scientific attention.

Rosa damascena Mill. f. *trigintipetala* Dieck (Rosaceae) is known for containing a wide spectrum of polyphenols and anthocyanidins. While the oil exerts anti-microbial effects for instance, polyphenols by the majority are responsible for the medicinal properties such as anti-HIV, anti-oxidant, anti-tussive, anti-diabetic or anti-plasmodia activity [9]. Nowadays, the flowers are annually harvested to extract the essential oil for the cosmetic and food industry [10], whereof about 50% of the global share is produced in Bulgaria [11]. Because of its low content of essential oils (0.02 % according to Baser et. al. [12]), in each distillation cycle, between 500 and 1000 kilogram of rose petals is used, accruing the four-fold quantity of liquid waste [11]. The volume of production amounts to approximately 1000-2000 kilogram pure rose oil each year. Hence, it not only poses a respectable economic factor, but also a serious environmental damage. Rose oil distillation waste water (RODW) contains hydrophilic polyphenols, which are considered bio-pollutants [13] and thus, is difficult to decompose.

However, up-to-date, the by-product is discharged into the drainage system or spread on local soil [14]. For this reason, primarily valorization of a by-product in terms of pharmaceutical beneficence attended by a development of a strategy for wastewater management is one interesting aspect.

Agents dissolved in rose oil distillation waste water are compounds like kaempferol, quercetin, and ellagic acid and their glycoside derivatives [13, 15-17]. We recently established a strategy to separate RODW into a polyphenol depleted and a polyphenol enriched fraction RF20-(SP-207) [13]. Subsequently, RF20-(SP-207) was submitted to a bioassay-guided fractionation using Sephadex LH-20 and was split into four sub-fractions F(I)-(IV) according to their major compounds [17]. Based on our previous results [14], we chose F(IV) for further investigation.

In a former study [14], we reported significant inhibiting effects of RF20-(SP-207) and F(IV) on human keratinocytes in terms of proliferation and migration. Since there exists a close interplay between these cellular processes and events of inflammation [18], the influence on exclusively pro- inflammatory biomarkers was of interest. Hence, the focus of this study was to explore the effect of RF20-(SP-207) and F(IV) on transcriptional activity of five different key targets in comparison to one of their major single compounds ellagic acid.

2. Experimental

2.1 Chemicals and Reagents

Ellagic acid (EA, purity >95%) and dimethyl sulfoxide (purity >99.5%; DMSO) were purchased from Sigma-Aldrich (Buchs, CH). Human IL-8 ELISA kit, Dulbecco's Modified Eagle Medium high glucose (1x), Dulbecco's Phosphate buffered saline without $CaCl_2$ and $MgCl_2$, Trypsin-EDTA 0.5% (10x), 10.000 Units/mL Penicillin and 10.000 µg/mL Streptomycin and heat inactivated calf serum (FCS) were provided by LubioScience (Luzern, CH). *E. coli*-derived recombinant human TNF-alpha (purity >98%), human MCP-1 and RANTES ELISA kit were purchased from BioLegend (San Diego, USA).

2.2 Cell Culture

Human non-tumorigenic HaCaT keratinocytes (Cell Line Services, Eppelheim, Germany) were maintained in Dulbecco's Modified Eagle's Medium high glucose 1x supplemented with 1% (v/v) Penicillin 10.000 U/mL / Streptomycin 10.000 µg/mL and 10% (v/v) heat inactivated fetal calf serum (FCS) at 37 °C in a humidified atmosphere containing 5% CO_2. Experiments were routinely conducted at approximately 80-90% confluency [19] and carried out between passages 55 and 67. Both, RF20-(SP-207) and F(IV) were tested in a concentration of 50 µg/mL, while ellagic acid was used at 10 µM. Additionally, the substances were individually applied in the same concentrations with a supplementation of 20 ng/mL TNF-α. This set-up was used to simulate an *in vitro* inflammatory condition. The results of each experiment were compared to an untreated control signifying keratinocytes exposed to medium with 0% serum, and a treated control composed of medium with 20 ng/mL TNF-α. Except ellagic acid (DMSO concentration 0.125%), all compounds were used in a final DMSO concentration of max. 0.1% (v/v), which has been proven harmless for this cell line (data not shown).

2.3 Plant Material and Extract Preparation

Wastewater obtained after distillation of full-blown *R. damascena* flowers was obtained from the distillery of the Institute of Roses, Essential and Medicinal Crops (IREMC) in Kazanlak, Bulgaria, in June 2013. RF20-(SP-207) was prepared from RODW as described previously [13]. Briefly, the concentration process includes a filtration process to eliminate suspended solids in the wastewater, followed by adsorption of phenolic compounds from RODW onto

adsorption resins (XAD and SP). Finally, desorption of the polyphenol fraction from the resin matrix was achieved using ethanol and/or aqueous ethanol. The result of the process was a wastewater low in soluble organic compounds and an enriched polyphenol fraction (RF20-(SP-207)). The profile of RF20-(SP-207) was similar to that of RODW, and showed the presence of flavonols such as quercetin and kaempferol glycosides as major metabolites [13]. Fractions (I)-(IV) were obtained and phytochemical characterized as described in [17]. Extensive phytochemical descriptions of RF20-(SP-207) and F(IV) is published in [13, 17]. RF20-(SP-207) mainly consisted of phenolic compounds, such as isoquercitrin, kaempferol, quercitrin, rutin, astragalin, multiflorin A, ellagic acid and several kaempferol and quercetin glycosides [13]. F(IV) mainly consisted of ellagic acid, quercetin and kaempferol [17]. A voucher specimen of rose oil distillation waste water (#RODW_June2013) is stored at -20°C at the Institute for Pharma Technology, School of Life Sciences, FHNW.

2.4 RNA and Real Time Quantitative RT-PCR

After cell treatment and incubation in 12-well plates, mRNA was extracted using the RNEasy Mini Kit by Qiagen (Valencia, USA). Subsequently, mRNA concentration was measured at 260 nm using the NanoDROP 2000c Spectrometer (Thermo Fischer Scientific) and samples were diluted accordingly to a final concentration of 6 ng/µL. Reverse transcription was done in two steps utilizing the Biometra T3000 Thermocycler (Göttingen). First, 66 ng of RNA were charged with 80 ng Oligo-dT primers/reaction (Qiagen) and incubated for 5 min at 70 °C. Immediately after adding a solution containing 200 units of M-MLV reverse transcriptase 1x buffer (Promega) and 0.5 mM of each dNTP (Solis Biodyne), reverse transcription was continued by incubation for one hour at 37 °C. After retrieval of the cDNA, control and samples were mixed with the TaqMan Gene Expression assay probes (Thermo Fischer Scientific) and Faststart TaqMan Probe Master Mix (Roche Diagnostics) according to the manufacturer's instructions. cDNA was amplified in a Corbett Rotor-Gene 6000 (Qiagen). An initial denaturation of 10 min at 95 °C was followed by 40 cycles of 15 sec at 95 °C and one min. at 60 °C. Fluorescence was detected at 510 nm and data were collected using the "Rotor Gene Q – Pure Detection" Software (v. 2.3.1). All values were normalized to the expression of the housekeeping gene Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH).

2.5 Measurement of IL-8, RANTES, MCP-1 Secretion

The experimental setup including the TNF-α concentration was chosen according to Park et al. [20], with some modifications. For the samples and controls, cells were seeded into 12-well plates at a density of $1.5x10^5$ cells/mL. After an incubation time of 24 hours (37 °C, 5% CO2), DMEM with 10% (v/v) serum was discarded, cells were washed with one mL/well PBS and medium was exchanged to DMEM containing 0% FCS or the test solutions plus 20 ng/mL TNF-α. The second 24-hour period happened under the same conditions. Protein levels obtained from the untreated control are regarded as the basal secretion of the designated target in keratinocytes. In the end, supernatants were aspirated, stored in sterile micro centrifuge tubes and deep-frozen (-20 °C) immediately. Absorbance was measured at 450 nm using a microplate reader (Spectramax i3x Multimode detection platform, Molecular Devices).

2.6 Statistical Analysis

Data are shown as mean ± standard deviation (SD). ELISA experiments were performed in triplicates; each experiment was repeated at least three times (on different days). The protein levels were then calculated according to a standard curve. PCR experiments were executed in duplicates, a sample and one technical replicate; experiments were repeated at least five times (on different days). Results were processed according to the comparative C_T Method ($\Delta\Delta C_T$-Method;
http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf) by the following equations using Microsoft Excel 2013 :

(1) $\Delta C_T = C_{T\,Target} - C_{T\,Reference}$

(2) $\Delta\Delta C_T = \Delta C_{T\,test\,sample} - \Delta C_{T\,calibrator\,sample}$

(3) $SD\ (\Delta C_T) = SD\ (\Delta\Delta C_T)$

(4) $fold\ change = 2^{-\Delta\Delta C_T}$

(5) $fold\ change\ range = 2^{-(\Delta\Delta C_T \pm s)}$

To process the results by the comparative C_T Method, the replication efficiency of the housekeeping gene and the targets have to be equal. The manufacturer of the TaqMan gene expression assays (Applied Biosciences) guarantees an amplification efficiency close to 100% (http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_040377.pdf). The term "down-regulation" refers to a gene expression level below the untreated control (<1), whereas "up-regulation" describes a level >1. Further statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using the software package GraphPad Prism (version 6.01, GraphPad Software Inc.). In all cases, differences were considered significant if p was below 0.05.

3. Results

3.1 Gene Expression Inhibitory Activities of RF20-(SP-207), F(IV) and Ellagic Acid

As a first step, the target-specific C_T-values of cells treated by RF20-(SP-207), F(IV) (both 50 µg/mL) and ellagic acid at 10 µM were detected, calculated to fold changes and compared to the control group (Figure 1A, C, E). In a second step, the target-specific C_T-values of a sample set of the same substances each stimulated with 20 ng/mL TNF-α was obtained (Figure 1B, D, F). This set-up was used to simulate an inflammation state scenario *in vitro*.

Figure 1A reveals a significant ($p<0.001$), 4.8-fold IL-1β-gene expression of the TNF-α stimulated control compared to the control group. However, there was no significant difference between the control group and the groups treated with the test substances. Figure 1B shows that all substances significantly ($p<0.001$) reduced the IL-1β-gene expression level ranging between 20-fold for RF20-(SP-207) and 10-fold for ellagic acid compared to 90-fold of the stimulated control when they were concomitantly applied with 20 ng/mL TNF-α.

Figure 1C displays that addition of TNF-α significantly increased IL-8 gene expression while the expression of IL-8 for RF20-(SP-207), F(IV) and ellagic acid remained on control level. When all test substances where concomitantly applied with TNF-α, the gene expression of IL-8 was significantly downregulated if compared to the TNF-α group alone. (Figure 1D). It was of further interest to determine if RF20-(SP-207) or F(IV) have an effect on the transcriptional activity of IL-6. No treatment revealed a significant divergence compared to the unstimulated control (Figure 1E). When cells were stimulated with TNF-α, a 5-fold increase in IL-6 gene expression was observed (Figure 1 F). Interestingly, this increase could not be blocked by RF20-(SP-207). It has to be mentioned that the relative stimulation of the IL-6 transcription by the stimulated control was not as strong as the fold changes of stimulated controls tested for the other targets. The fold-change of ellagic acid exceeded the control clearly (9-fold vs. 5-fold), whilst only F(IV) caused a significant ($p<0.1$) reducing effect in gene expression to untreated control-level (Figure 1F).

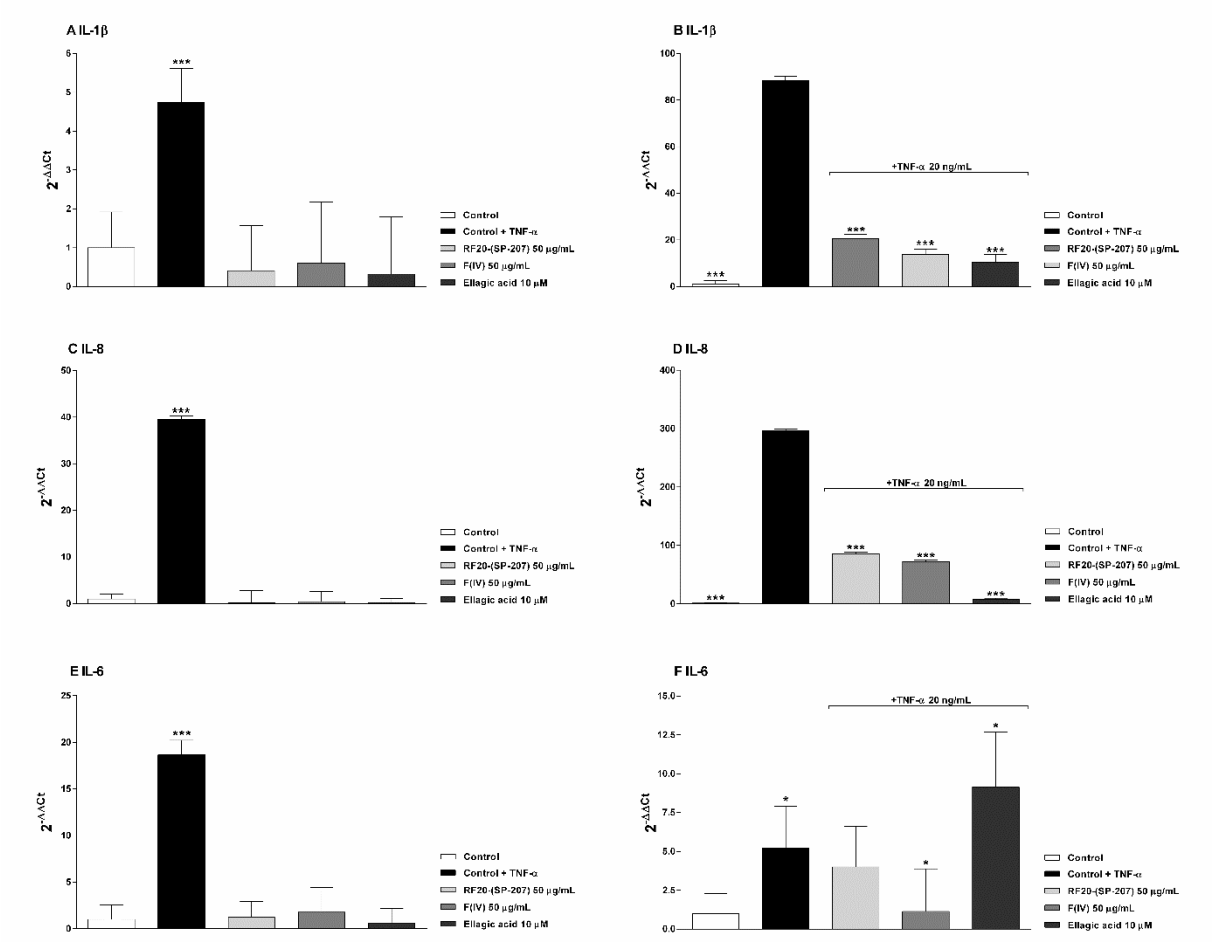


Figure 1: Transcriptional activity of selected chemokines in HaCaT keratinocytes under influence of RF20-(SP-207) (50 µg/mL), F(IV) (50 µg/mL) and ellagic acid (10 µM). **A** fold change of IL-1β expression; **B** fold change of IL-1β with substances supplemented with TNF-α (20 ng/mL); **C** fold change of IL-8 expression; **D** fold change of IL-8 expression with substances supplemented with TNF-α (20 ng/mL); **E** fold change of IL-6 expression; **F** fold change of IL-6 expression with substances supplemented with TNF-α (20 ng/mL). Data expressed as Mean ± SD of $2^{-\Delta\Delta C_T}$. N ≥ 3 independent experiments. **A, C, E** *** p<0.001 vs. Control; **B, D, F** *p<0.1, ***p<0.001 vs. Control + TNF-α.

Considering RANTES, the TNF-α-boosted control caused a significant (p<0.001) stimulation in gene expression (14.5-fold, Figure 2 A), while this was not the case for the unstimulated agents. The $2^{-\Delta\Delta CT}$-values of RF20-(SP-207) and F(IV) remained at 1.5-fold and 1.6-fold, respectively. Merely the value of the ellagic acid-treated sample was slightly higher with 2.6 in fold change, however, not significantly different to the unstimulated control. This ratio is comparable to the TNF-α-stimulated set-up (Figure 2 B). In contrast to a 50-fold up-regulation of the boosted control, the extract and its fraction decreased the RANTES-gene-expression to 6.6- and 5.6-fold, respectively. This diminishing effect was weaker under influence of ellagic acid (18-fold expression). Nevertheless, all stimulated substances caused significant (p<0.001) reducing effects.

Containing a c-c motif, MCP-1 belongs to the same family like RANTES. In both experimental set-ups, an intensive, significant ($p<0.001$) up-regulation of the stimulated control could be detected (Figure 2 C and D; 100 and 417-fold, respectively). Ellagic acid and F(IV) were determined at a 0.7- and 0.8-fold change and therefore generated a weak down-regulation (Figure 2 C). With a fold-change of 1, RF20-(SP-207) did not alter transcriptional activity compared to the untreated control. In contrast, fold-changes in the inflamed set-up were detected elevated (Figure 2 D). Nonetheless, $2^{-\Delta\Delta CT}$-values of the three substances were discovered to be significantly ($p<0.001$) reduced compared to the stimulated control with a 417-fold expression increase. Among the three sample agents, the expression-rate of F(IV) were detected at 58-fold, ellagic acid at 97-fold and RF20-(SP-207) at 149-fold.

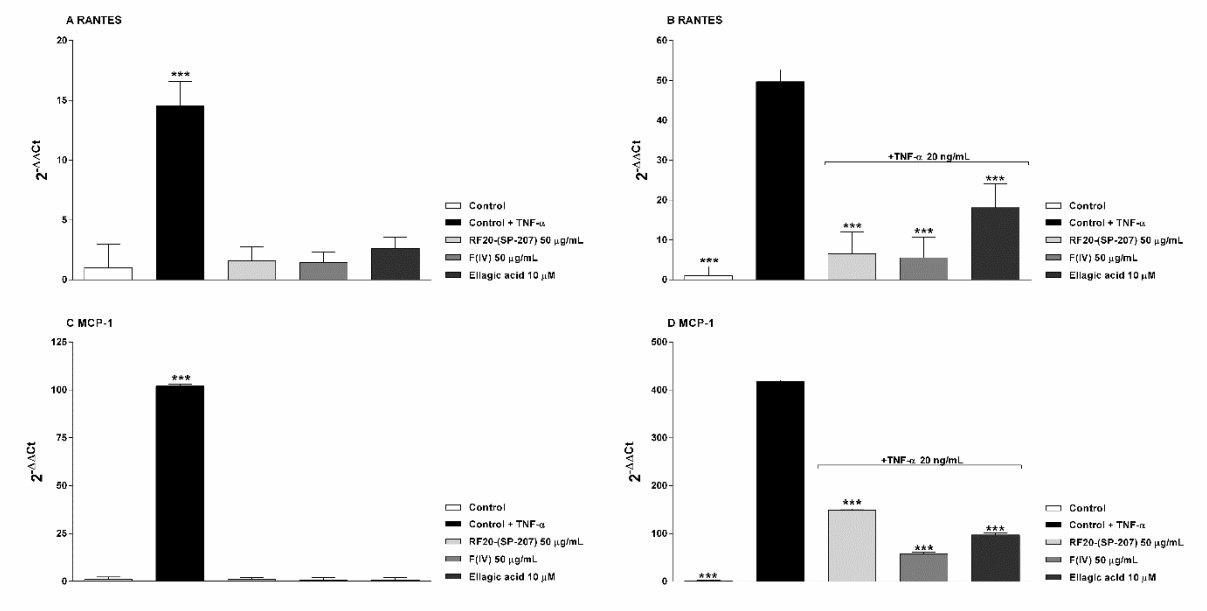


Figure 2: Transcriptional activity of selected chemokines in HaCaT keratinocyte under influence of RF20-(SP-207) (50 µg/mL), F(IV) (50 µg/mL) and ellagic acid (10 µM). **A** fold change of RANTES expression; **B** fold change of RANTES expression with substances supplemented with TNF-α (20 ng/mL); **D** fold change of MCP-1 expression; **E** fold change of MCP-1 expression with substances supplemented with TNF-α (20 ng/mL). Data expressed as Mean ± SD of $2^{-\Delta\Delta C_T}$. $N \geq 3$ independent experiments. **A, B** ***$p<0.001$ vs. Control; **C, D** ***$p<0.001$ vs. Control + TNF-α.

3.2 Protein Secretion of IL-8, RANTES and MCP-1

To investigate potential correlations of gene expression with protein secretion, IL-8, RANTES and MCP-1 were selected as biomarkers for ELISA-based assays. Comparability was ensured by choosing the same setting (unstimulated/stimulated) like in previous experiments.

With 543 pg/mL, the IL-8 production in HaCaT keratinocytes was significantly ($p<0.001$) increased in the TNF-α-stimulated control (Figure 3 A). The levels of unstimulated samples ranged between 2.6 pg/mL for F(IV) and 7.8 pg/mL for ellagic acid. Yielding 8.3 pg/mL, the unstimulated control was negligibly higher. Cells under influence of TNF-α plus the particular substance secreted higher amounts of IL-8 with exception of F(IV). The addition of 50 µg/mL RF20-(SP-207) resulted in a protein level of 48 pg/mL, which relates to less than 10% of the level secreted by the stimulated control. Ellagic acid +TNF-α caused a secretion level of 175 pg/mL relating to 32 % protein concentration of the stimulated control. However, all tested agents were capable to decrease the protein secretion significantly ($p<0.001$).

By executing the ELISA assay detecting the RANTES release, it could be demonstrated that all tested agents possessed significant ($p<0.001$) decreasing properties on protein secretion compared to the stimulated control except ellagic acid supplemented with 20 ng/mL TNF-α (Figure 3 B). While all levels were in line with the unstimulated control (approx. 4 pg/mL RANTES), ellagic acid +TNF-α generated a protein raise up to 25.9 pg/mL, which was slightly higher than the stimulated control (23.4 pg/mL).

The characteristics of RF20-(SP-207), F(IV) and ellagic acid were finalized by conducting the ELISA assay indicating MCP-1 as a biomarker (Figure 3C). In the unstimulated set-up, the concentrations under influence of each treatment accordingly did not alter significantly from the unstimulated control (approx. 10 pg/mL MCP-1). However, the inflamed scenario displayed a different picture. While treatment with 50 µg/mL of F(IV) caused a reduction of the protein level to 24 pg/mL MCP-1, RF20-(SP-207) reduced to an average of only 211 pg/mL. This value correlates to 9.5 % of the stimulated control and therefore also results in a significant ($p<0.001$) decrease. Exceptionally the treatment of ellagic acid + TNF-α did not decrease the MCP-1 level. Interestingly, yielding 2490 pg/mL in average, it mildly outperformed the stimulated control.

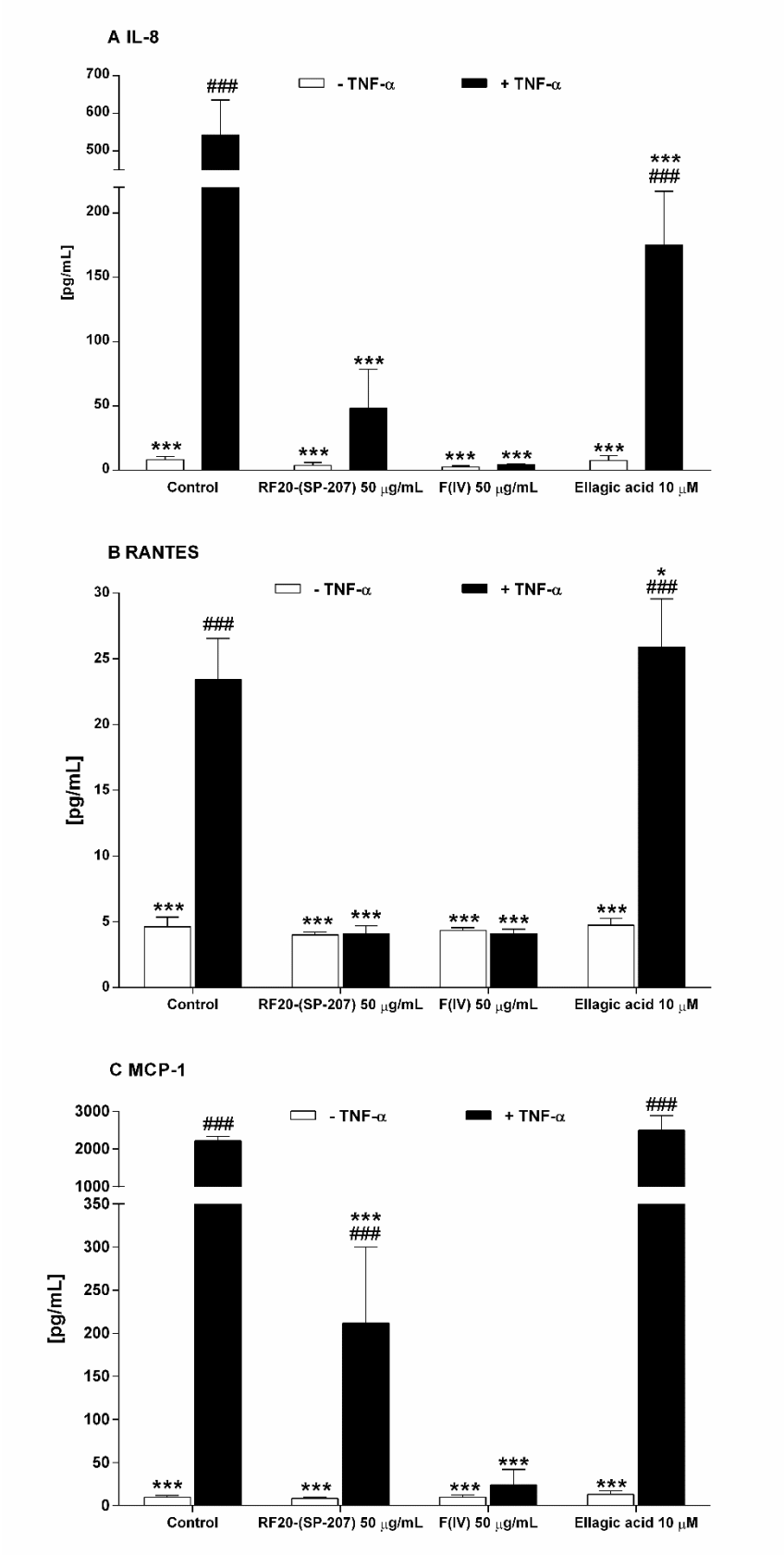


Figure 3: Differential regulation of ELISA-based protein secretion in HaCaT keratinocytes under influence of RF20-(SP-207) (50 µg/mL), F(IV) (50 µg/mL) and ellagic acid (10 µM) with and without supplementation of TNF-α (20 ng/mL). **A** IL-8 secretion; **B** RANTES secretion; **C** MCP-1 secretion. Data expressed as Mean ± SD. N ≥ 3 independent experiments. ***p<0.001 vs. Control + TNF-α. ###p<0.001 vs. Control.

4. Discussion

In a previous study [14], we discovered a pronounced inhibitory effect of RF20-(SP-207) and F(IV) on human keratinocytes regarding proliferation and migration. Cellular reproduction and locomotive actions are closely related to events of inflammation [18]. Thus, the focus of this study was to explore the effect of RF20-(SP-207) and F(IV) on transcriptional activity of five different pro-inflammatory key targets in comparison to ellagic acid. As a follow-up investigation, three of these targets were examined in respect of their protein secretion.

With IL-1β, IL-6, and IL-8 playing pivotal roles in events of inflammation [21-23], their differential gene modulation under influence of polyphenols were of interest. In this regard, numerous publications highlight the suppressive effects of flavonoids. For instance, *Cho et. al.* [24] reported a down-regulation of transcriptional activity of IL-1ß and IL-6 by treating RAW 264.7 cells with various concentrations of quercetin in a dose- and time-dependent fashion. Min et. al. [25] exposed HMC-1 cells to quercetin and observed down-regulation of the gene expression of IL-1ß, IL-6 and IL-8. Albeit research is currently ongoing, one mechanism of these effects is discussed to occur by quercetin blocking the transcription factor NF-κB [26-28] and other serine/threonine kinases like the p38 mitogen activated protein kinase [25, 29]. Quercetin, kaempferol, ellagic acid and their derivatives are major compounds in RF20-(SP-207), as well as in its sub-fraction (for compound analysis, see [17]). Despite differences in the cell line and mode of stimulation, the findings of the present study are widely in accordance with the literature within the meaning of IL-1ß, IL-6 and IL-8 gene expression modulation. However, the detection of a down-regulation of IL-1β and IL-8 gene expression by ellagic acid + TNF-α compared to an increased protein secretion for the same targets was not as uniform as results obtained for RF20-(SP-207) and F(IV) for these targets. *Kemp et. al.* [30] analyzed cytokine expression levels in epithelial cells of human cervical tissue and found homogenous results for IL-8, but heterogeneous values for IL-6, which indicates a natural variance of the target. Nevertheless, our results of both, the transcriptional activity and the protein secretion, correlate for RF20-(SP-207) and F(IV) on IL-8, RANTES and MCP-1.

RANTES and MCP-1 are chemokines with a chemotactic mode of action for various cell types and therefore, they are related in their mechanism [31]. For example, as a c-c motif member, RANTES recruits leukocytes to the site of action under the conditions of chronic inflammation [32]. Less extensive literature exists about the gene modulation of these two biomarkers under influence of polyphenols. Fukushima et al. [33] conducted a DNA microarray on HMC-1 human mast cells after exposition of ellagic acid/quercetin containing *Drosera* species and demonstrated a down-regulation of these two biomarkers, IL-8 and IL-6 among others. For

instance, testing extracts of *D. spatulata* (2.63 % ± 0.31 ellagic acid; 0.41 % ± 0.09 quercetin), *D. tokaiensis* (1.09 % ± 0.17 ellagic acid; 0.53 % ± 0.07 quercetin) and *D. rotundifolia* (0.17 % ± 0.04 ellagic acid; 0.45 % ± 0.05 quercetin) on human mast cells stimulated by activated T-cell membranes, a gene modulation of MCP-1 resulted in 16.2 ± 5.2, 1.1 ± 0.5 and 1.7 ± 0.4-fold, respectively, while the stimulated control was detected at 3012.3 ± 1443.9-fold. RANTES gene expression revealed a 2.9 ± 1.0 (*D. spatulata*), 0.8 ± 0.2 (*D. tokaiensis*) and a 1.2 ± 0.4-fold change (*D. rotundifolia*) with a stimulated control at 88.0 ± 9.9-fold change. Interestingly, *D. spatulata* containing the highest amount of ellagic acid and the lowest amount of quercetin induced the highest value of fold change. The same accounts for IL-6 and IL-8. With a 1.8- and 208.4-fold change, respectively, *D. spatulata* revealed the highest value among the three species. Taking these facts into account, our data correlate with published data. Thus, an involvement of the individual flavonoids present in RF20-(SP-207) on differential gene modulation can be assumed. This interpretation also accounts for the following observations.
Regarding the ELISA-based results, the commonality of all of them is the surprisingly elevated protein level under influence of 10 µM ellagic acid supplemented with 20 ng/mL TNF-α. Since the level of the IL-8 assessment resulted in not more than 32 % of the stimulated control, a direct interaction between the polyphenol and the stimulant as well as a cross reactivity is excluded. Polyphenols, particularly flavonoids are known to have synergistic properties [34-38]. Ellagic acid as a pure compound in combination with TNF-α revealed weak anti-inflammatory potential on our selection of targets, whereas concomitant application of TNF-α and RF20-(SP-207) and its sub-fraction F(IV) possessed clear anti-inflammatory properties. Thus, in light of the aforementioned literature and the present data, a synergism of polyphenolic compounds in the fraction RF20-(SP-207), as well as in the sub-fraction F(IV) is assumed.

5. Conclusion

Research on anti-inflammatory properties of flavonoids and their mechanism has been extensively reviewed in the literature. One favored approach is the analysis of the polyphenols' effect on transcription factors like NF-κB or serine/threonine proteases, thereof possible downstream effects are then inferred. By focusing on very specific downstream targets that play pivotal roles in the direct inflammatory response, we illustrated the anti-inflammatory potential of RF20-(SP-207) and suggest this polyphenol-enriched fraction as a promising candidate for the treatment of acute and chronic inflammation. By valorization of a by-product of the rose oil

production, these findings also might be relevant for the establishment of a waste water management.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

To provide detailed C_T-values of the targets IL-1β, IL-6, IL-8, RANTES and MCP-1, ten tables are attached to this work displaying the means ± standard deviations for each biomarker. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.fitote.2016.08.019.

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Supporting Information

In vitro modulation of inflammatory target gene expression by a polyphenol-enriched fraction of rose oil distillation waste water

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Sample	IL-1ß Average C_T	GAPDH Average C_T	ΔC_T IL-1ß-GAPDH	$\Delta\Delta C_T$	fold change $= 2^{-\Delta\Delta CT}$
Untreated	**30.95 ± 0.50**	**19.70 ± 0.77**	**11.25 ± 0.92**	**0 ± 0.92**	**1** **(0.53 – 1.90)**
TNF-α [20 ng/mL]	**27.62 ± 0.75**	**18.61 ± 0.44**	**9.01 ± 0.87**	**-2.25 ± 0.87**	**4.8** **(2.60 – 8.68)**
RF20-(SP-207) [50 µg/mL]	**31.92 ± 1.05**	**19.37 ± 0.45**	**12.55 ± 1.16**	**1.30 ± 1.16**	**0.4** **(0.18 – 0.91)**
F(IV) [50 µg/mL]	**30.97 ± 1.42**	**19.00 ± 0.66**	**11.97 ± 1.56**	**0.72 ± 1.56**	**0.6** **(0.21 – 1.80)**
Ellagic acid [10 µM]	**31.75 ± 1.23**	**18.86 ± 0.81**	**12.88 ± 1.47**	**1.06 ± 1.47**	**0.3** **(0.14 – 1.67)**

Tab. 1: Values for target IL-1β (non-stimulated). Results express the Mean ± SD, N ≥ 3 independent experiments in duplicates.

Sample	IL-1ß Average C_T	GAPDH Average C_T	ΔC_T IL-1ß-GAPDH	$\Delta\Delta C_T$	fold change $= 2^{-\Delta\Delta CT}$
Untreated	**37.02 ± 1.58**	**22.95 ± 0.22**	**14.07 ± 1.59**	**0 ± 1.59**	**1** **(0.33 – 3.02)**
TNF-α [20 ng/mL]	**29.75 ± 1.12**	**22.15 ± 1.24**	**7.60 ± 1.67**	**-6.47 ± 1.67**	**88.4** **(27.71 – 282.16)**
RF20-(SP-207) [50 µg/mL]	**32.30 ± 1.74**	**22.60 ± 0.43**	**9.71 ± 1.79**	**-4.36 ± 1.79**	**20.6** **(5.94 – 71.14)**
F(IV) [50 µg/mL]	**31.92 ± 1.85**	**21.63 ± 1.50**	**10.29 ± 2.38**	**-3.78 ± 2.38**	**13.8** **(2.64 – 71.75)**
Ellagic acid [10 µM]	**33.74 ± 3.34**	**23.04 ± 0.26**	**10.69 ± 3.35**	**-3.38 ± 3.35**	**10.4** **(1.02 – 105.99)**

Tab. 2: Values for target IL-1β + TNF-α 20 ng/mL (stimulated). Results express the Mean ± SD, N ≥ 3 independent experiments in duplicates.

Sample	IL-8 Average C_T	GAPDH Average C_T	ΔC_T IL-8-GAPDH	$\Delta\Delta C_T$	fold change $= 2^{-\Delta\Delta CT}$
Untreated	**32.74 ± 0.65**	**19.70 ± 0.77**	**13.04 ± 1.01**	**0 ± 1.01**	**1** **(0.50 – 2.01)**
TNF-α [20 ng/mL]	**26.35 ± 0.57**	**18.61 ± 0.44**	**7.74 ± 0.72**	**-5.31 ± 0.72**	**39.5** **(24.04 – 65.01)**
RF20-(SP-207) [50 µg/mL]	**34.73 ± 2.52**	**19.37 ± 0.50**	**15.36 ± 2.57**	**2.32 ± 2.57**	**0.2** **(0.03 – 1.19)**
F(IV) [50 µg/mL]	**33.15 ± 2.02**	**19.00 ± 0.66**	**14.16 ± 2.12**	**1.11 ± 2.12**	**0.5** **(0.11 – 2.01)**
Ellagic acid [10 µM]	**33.88 ± 0.17**	**18.86 ± 0.81**	**15.02 ± 0.82**	**1.98 ± 0.82**	**0.3** **(0.14 – 0.45)**

Tab. 3: Values for target IL-8 (non-stimulated). Results express the Mean ± SD, N ≥ 3 independent experiments in duplicates.

Sample	IL-8 Average C_T	GAPDH Average C_T	ΔC_T IL-8-GAPDH	$\Delta\Delta C_T$	fold change $= 2^{-\Delta\Delta CT}$
Untreated	**39.05 ± 1.26**	**22.95 ± 0.22**	**16.10 ± 1.27**	**0 ± 1.27**	**1** **(0.41 – 2.42)**
TNF-α [20 ng/mL]	**30.03 ± 2.42**	**22.15 ± 1.24**	**7.89 ± 2.72**	**-8.21 ± 2.72**	**296.1** **(44.95 – 1950.52)**
RF20-(SP-207) [50 µg/mL]	**32.28 ± 2.58**	**22.60 ± 0.43**	**9.68 ± 2.62**	**-6.41 ± 2.62**	**85.3** **(13.89 – 523.95)**
F(IV) [50 µg/mL]	**31.55 ± 1.84**	**21.63 ± 1.50**	**9.92 ± 2.37**	**-6.18 ± 2.37**	**72.3** **(14.00 – 373.73)**
Ellagic acid [10 µM]	**36.30 ± 2.24**	**23.04 ± 0.26**	**13.25 ± 2.25**	**-2.84 ± 2.25**	**7.2** **(1.51 – 34.09)**

Tab. 4: Values for target IL-8 + TNF-α 20 ng/mL (stimulated). Results express the Mean ± SD, N ≥ 3 independent experiments in duplicates.

Sample	IL-6 Average C_T	GAPDH Average C_T	ΔC_T IL-6 - GAPDH	$\Delta\Delta C_T$	fold change $= 2^{-\Delta\Delta CT}$
Untreated	**37.60 ± 1.32**	**19.70 ± 0.77**	**17.89 ± 1.53**	**0 ± 1.53**	**1** **(0.35 – 2.89)**
TNF-α [20 ng/mL]	**32.29 ± 1.51**	**18.61 ± 0.44**	**13.67 ± 1.57**	**-4.22 ± 1.57**	**18.7** **(6.29 – 55.36)**
RF20-(SP-207) [50 µg/mL]	**36.98 ± 1.67**	**19.37 ± 0.50**	**17.62 ± 1.74**	**-0.28 ± 1.74**	**1.2** **(0.36 – 4.04)**
F(IV) [50 µg/mL]	**36.08 ± 2.56**	**19.00 ± 0.66**	**17.08 ± 2.65**	**-0.81 ± 2.65**	**1.8** **(0.28 – 11.00)**
Ellagic acid [10 µM]	**37.60 ± 1.39**	**18.86 ± 0.81**	**18.74 ± 1.61**	**0.84 ± 1.61**	**0.6** **(0.18 – 1.70)**

Tab. 5: Values for target IL-6 (non-stimulated). Results express the Mean ± SD, N ≥ 3 independent experiments in duplicates.

Sample	IL-6 Average C_T	GAPDH Average C_T	ΔC_T IL-6 - GAPDH	$\Delta\Delta C_T$	fold change $= 2^{-\Delta\Delta CT}$
Untreated	**39.16 ± 1.29**	**22.95 ± 0.22**	**16.20 ± 1.30**	**0 ± 1.30**	**1** **(0.41 – 2.47)**
TNF-α [20 ng/mL]	**35.97 ± 2.42**	**22.15 ± 1.24**	**13.83 ± 2.72**	**-2.38 ± 2.72**	**5.2** **(0.79 – 34.21)**
RF20-(SP-207) [50 µg/mL]	**36.80 ± 2.58**	**22.60 ± 0.43**	**14.20 ± 2.62**	**-2.00 ± 2.62**	**4.0** **(0.65 – 24.54)**
F(IV) [50 µg/mL]	**37.65 ± 2.30**	**21.63 ± 1.50**	**16.02 ± 2.75**	**-0.18 ± 2.75**	**1.1** **(0.17 – 7.60)**
Ellagic acid [10 µM]	**36.06 ± 3.54**	**23.04 ± 0.26**	**13.01 ± 3.55**	**-3.19 ± 3.55**	**9.1** **(0.78 – 106.64)**

Tab. 6: Values for target IL-6 + TNF-α 20 ng/mL (stimulated). Results express the Mean ± SD, N ≥ 3 independent experiments in duplicates.

Sample	RANTES Average C_T	GAPDH Average C_T	ΔC_T RANTES - GAPDH	$\Delta\Delta C_T$	fold change $= 2^{-\Delta\Delta CT}$
Untreated	**32.52 ± 1.84**	**19.70 ± 0.77**	**12.82 ± 2.00**	**0 ± 2.00**	**1 (0.25 – 4.00)**
TNF-α [20 ng/mL]	**27.57 ± 2.01**	**18.61 ± 0.44**	**8.96 ± 2.06**	**-3.86 ± 2.06**	**14.5 (3.48 – 60.52)**
RF20-(SP-207) [50 µg/mL]	**31.51 ± 1.06**	**19.37 ± 0.50**	**12.14 ± 1.17**	**-0.68 ± 1.17**	**1.6 (0.71 – 3.60)**
F(IV) [50 µg/mL]	**31.28 ± 0.60**	**19.00 ± 0.66**	**12.28 ± 0.88**	**-0.54 ± 0.88**	**1.5 (0.79 – 2.68)**
Ellagic acid [10 µM]	**30.28 ± 0.46**	**18.86 ± 0.81**	**11.42 ± 0.93**	**-1.40 ± 0.93**	**2.6 (1.39 – 5.02)**

Tab. 7: Values for target RANTES (non-stimulated). Results express the Mean ± SD, N ≥ 3 independent experiments in duplicates.

Sample	RANTES Average C_T	GAPDH Average C_T	ΔC_T RANTES - GAPDH	$\Delta\Delta C_T$	fold change $= 2^{-\Delta\Delta CT}$
Untreated	**39.05 ± 2.33**	**22.95 ± 0.22**	**16.10 ± 2.34**	**0 ± 2.34**	**1 (0.20 – 5.06)**
TNF-α [20 ng/mL]	**32.61 ± 2.76**	**22.15 ± 1.24**	**10.46 ± 3.03**	**-5.64 ± 3.03**	**49.7 (6.09 – 405.63)**
RF20-(SP-207) [50 µg/mL]	**35.97 ± 5.50**	**22.60 ± 0.43**	**13.38 ± 5.51**	**-2.72 ± 5.51**	**6.6 (0.14 – 300.26)**
F(IV) [50 µg/mL]	**35.25 ± 4.85**	**21.63 ± 1.50**	**13.62 ± 5.07**	**-2.47 ± 5.07**	**5.6 (0.17 – 187.00)**
Ellagic acid [10 µM]	**34.96 ± 6.05**	**23.04 ± 0.26**	**11.92 ± 6.05**	**-4.18 ± 6.05**	**18.1 (0.27 – 1200.72)**

Tab. 8: Values for target RANTES + TNF-α 20 ng/mL (stimulated). Results express the Mean ± SD, N ≥ 3 independent experiments in duplicates.

Sample	MCP-1 Average C_T	GAPDH Average C_T	ΔC_T MCP-1 - GAPDH	$\Delta\Delta C_T$	fold change $= 2^{-\Delta\Delta CT}$
Untreated	**31.16 ± 1.06**	**19.70 ± 0.77**	**11.46 ± 1.31**	**0 ± 1.31**	**1 (0.40 – 2.48)**
TNF-α [20 ng/mL]	**23.40 ± 0.98**	**18.61 ± 0.44**	**4.78 ± 1.07**	**-6.67 ± 1.07**	**102.0 (48.52 – 214.29)**
RF20-(SP-207) [50 µg/mL]	**30.70 ± 0.57**	**19.37 ± 0.50**	**11.33 ± 0.76**	**-0.13 ± 0.76**	**1.1 (0.65 – 1.85)**
F(IV) [50 µg/mL]	**30.95 ± 1.03**	**19.00 ± 0.66**	**11.95 ± 1.22**	**0.50 ± 1.22**	**0.7 (0.30 – 1.65)**
Ellagic acid [10 µM]	**30.63 ± 0.72**	**18.86 ± 0.81**	**11.77 ± 1.08**	**0.31 ± 1.08**	**0.8 (0.38 – 1.70)**

Tab. 9: Values for target MCP-1 (non-stimulated). Results express the Mean ± SD, N ≥ 3 independent experiments in duplicates.

Sample	MCP-1 Average C_T	GAPDH Average C_T	ΔC_T MCP-1 - GAPDH	$\Delta\Delta C_T$	fold change $= 2^{-\Delta\Delta CT}$
Untreated	**36.75 ± 2.12**	**22.96 ± 0.22**	**13.80 ± 2.13**	**0 ± 2.13**	**1 (0.23 – 4.39)**
TNF-α [20 ng/mL]	**27.24 ± 2.46**	**22.15 ± 1.24**	**5.09 ± 2.76**	**-8.71 ± 2.76**	**417.6 (61.77 – 2823.54)**
RF20-(SP-207) [50 µg/mL]	**29.18 ± 2.81**	**22.60 ± 0.43**	**6.58 ± 2.85**	**-7.22 ± 2.85**	**148.8 (20.69 – 1069.45)**
F(IV) [50 µg/mL]	**29.57 ± 2.33**	**21.63 ± 1.50**	**7.94 ± 2.77**	**-5.86 ± 2.77**	**57.9 (8.49 – 394.46)**
Ellagic acid [10 µM]	**30.24 ± 3.84**	**23.04 ± 0.26**	**7.19 ± 3.85**	**-6.60 ± 3.85**	**97.3 (6.77 – 1398.91)**

Tab. 10: Values for target MCP-1 + TNF-α 20 ng/mL (stimulated). Results express the Mean ± SD, N ≥ 3 independent experiments in duplicates.

4. Conclusion and Outlook

This work selected parts of *Phyllostachys edulis* (Carrière) J. Houz. (Poaceae) and *Rosa damascena* Mill. (Rosaceae) for further analyzation because of their high contents of polyphenols, which are known for their diverse biological effects (chapter 2.3). Moreover, both plants are utilized in the industrial fabrication of typical products of their country of origin the Republic of Bulgaria and the People's Republic of China, respectively. Essentially, inflammation key targets as well as wound closure characteristics were of interest from a pharmaceutical point of view. Due to the accumulation of tons of waste fractions within the production line, valorization options of the by-products of *Phyllostachys edulis* and *Rosa damascena* was also one aspect from an ecological point of view. Because of this, herein it was investigated in anti-inflammatory, proliferative, and cell locomotive properties of phenol-rich resources of the two species.

In planta, polyphenols can have defensive functions, for example to deter herbivores, or act as an attractant like flavonoids attract organisms intending pollination or seed dispersal for survival and propagation (see chapter 2.3). The literature has extensively described pleiotropic actions in terms of pharmacological effects, especially outcomes based on *in vitro*-conditions. Among them, anti-oxidant and radical scavenging characteristics are two of the most prominent ones. Nevertheless, results of both *in vitro*- and *in vivo*-studies are discussed controversially because of certain difficulties in the realization of these experiments as described in chapter 2.3.

Anti-inflammatory and migrative/proliferative effects of Phyllostachys edulis

Phyllostachys edulis, also known as moso bamboo, is cultivated widely on the Asian continent and its wood is utilized for lots of different purposes in Chinese culture (see chapter 2.2). During the harvest, tons of polyphenol-containing leaves accumulate, which have no noteworthy further use for pharmaceutical purposes up-to-date. Therefore, the leaves were subjected to aqueous Soxhlet extraction and subsequent lyophilization to obtain the bamboo leave extract (BLE) for further processing. HPLC analysis with subsequent LC-MS/MS identified isoorientin as one of the main flavonoids with amounts of 5,32 g/kg and thus, was selected for subsequent experimentation. Pretreatment of HaCaT keratinocytes with isoorientin at 50 µM and 100 µM caused a significant reduction of cellular IL-6 secretion comparable to the positive control hydrocortisone (10 µM), while cell viability was not impaired throughout the incubation time. On the contrary, BLE could not exert a significant

reduction of cellular IL-6 secretion, which emphasizes on the specific capability of isoorientin to reduce IL-6 levels. Presumably, the concentration of isoorientin in BLE was too low to cause such effect. Furthermore, isoorientin also significantly decreased TNF-α-induced VEGF- and IL-8 secretion in a dose dependent fashion. These results stand in good accordance with reports in the literature (see 3.1 Discussion). Moreover, wound closure effects of 3T3-swiss albino mouse fibroblasts have been assessed utilizing a computerized method of the classical scratch assay with support of time lapse microscopy. In this context, fibroblasts as well as keratinocytes as epidermal cell lines play a crucial role in the healing of tissue damages (see chapter 2.5). 12- and 24-hour observations caused comparable outcomes of approximately 28% and 55% gap closure by the extract and isoorientin at 10 µg/mL and 10 µM, respectively. In higher concentrations of both agents individually, cell migration was inhibited without considerable toxic effects on cell viability. A hypothesis for this circumstance is partly the reduction of cellular VEGF secretion, which is necessary for a proper creation of ECM and cutaneous angiogenesis.

Anti-inflammatory and migrative/proliferative effects of Rosa damascena

Bulgaria is the world's biggest rose oil producer, which is essentially used as a food ingredient or in cosmetic formulations. Farmers cultivate *Rosa damascena* on vast fields in the heartland of Bulgaria. Employing a certain method of water steam distillation in which for each kilogram of flowers the four-fold quantity of water is necessary, phenol-rich liquid waste residues (RODW) accumulate resulting in about 30.000 tons annually with no further use up-to-date (see chapter 2.2). Purification of RODW resulted in a polyphenol depleted and a polyphenol enriched fraction [(RF20-(SP-207)], of which the latter one was split into four sub-fractions F(I)-F(IV) depending on the phytochemical spectrum of polyphenolic compounds. Both for RF20-(SP-207) and F(IV), anti-proliferative effects were detected, which was concluded by significantly decreased BrdU-values in *de novo* synthesized DNA, as well as reduced cellular VEGF-expression levels. Since cellular reproduction and locomotive actions are interrelated with inflammatory processes, wound closure properties were of substantial interest. 24h-time lapse microscopy detected a retarded wound closure of human keratinocytes under influence of RF20-(SP-207) at 10 µg/mL, while F(IV) at 10 µg/mL almost caused an abrogation. By comparison with morphologic changes of the cell structure under influence of anisomycin at 10 µM, an elevated rate of apoptosis is assumed by both compounds individually at the same concentrations as used before. A follow up-study (see chapter 3.3) investigated in the transcriptional activity of the pro-inflammatory key targets IL-

1β, IL-8, IL-6, RANTES and MCP-1 under influence of RF20-(SP-207) and F(IV) by utilizing real time quantitative RT-PCR. Concomitant application of TNF-α and RF20-(SP-207) or TNF-α and F(IV), respectively, uncovered comparable results suggesting an inflammatory downregulation. Further analyzation in terms of cellular protein secretion of IL-8, RANTES and MCP-1 has been done subsequently. Therein, significantly reduced secretion levels were detected.

Limitations in the comparison of Phyllostachys edulis and Rosa damascena as polyphenol containing species

The previously described results create a picture of polyphenol containing plants with distinct profiles of pharmacological *in vitro*-effects. In this context, it must be mentioned that the experimental focus of the work predominantly was put on the rose flower, for which reason investigation in this species has been more profound.
As a similarity, it can be said that the utilized substances of both plants exert anti-inflammatory effects based on the regulation of cellular secretion of pro-inflammatory cytokines. The exact mechanisms exerted by the two species remain to be elucidated, however, it can be assumed to have different pathways involved: first because of the individual composition of compounds and second because of the sheer intensity of the detected anti-inflammatory properties. For instance, with isoorientin decreasing significantly cellular IL-6 levels, but not BLE, and effects caused by substances from the rose flower being substantially different than those of the bamboo leaves, it can be noticed that the structure-activity relationship is a complex matter for such multicomponent systems.

Clear differences accounting for locomotive actions were observed in time lapse experiments. In brief, while bamboo enhanced cell migration, the rose flower impaired it. However, it must be kept in mind that these findings were generated by using two different cell lines: 3T3-swiss albino mouse fibroblasts in the first case and HaCaT keratinocytes in the second (because of substance- and hardware-specific compatibility). Different cell lines have individual migration speeds, reproduction cycles, sensitivities regarding compound tolerance etc. Although both are epidermal cell lines, the comparability thus is limited. Above all, *in vitro* cell cultures are imperfect systems when simulating *in vivo* conditions, especially mono-cultures. There exist multi-cultures, but their partly intricated cultivation methods are not payed back by the findings in most cases, for which reason animal models might be preferred as a next step. Additionally, because of previously obtained results and time and budget reasons, RT-PCR experiments have been exclusively conducted using the fraction from

RODW. Thus, a more detailed picture can be drawn of the pharmacological effects exerted by *Rosa damascena*. Albeit biological effects of prominent single polyphenols like quercetin, kaempferol, catechins and lots more are well described in the literature, the complexity of such multicomponent mixtures hinder a reliable prediction regarding their pharmacology. Not only there are interspecific differences between the species, but also intraspecific variations due to the intensity of the sun, soil quality, rainfall etc. Hence, detailed research of every candidate is indispensable for the analysis of pharmacological effects.

Future perspectives

Different to by-products of the damask rose, there already exist few health/beauty related lifestyle products made of bamboo plants on the market (for example www.organicbamboo.org). To our knowledge, this is the first study analyzing the effects of moso bamboo on specific pro-inflammatory targets of epidermal cells. Deeper investigation on specific mechanisms of a broader range of targets with involvement of subsequent *in vivo*-studies would be necessary to extend the knowledge about the pharmacological profile. The previously described results might trigger an impulse for further elucidation. Based on these findings and on the limited bioavailability of polyphenols including strong first-pass effects, the substance could pose an interesting treatment option to promote wound healing and decrease inflammations in a topical application form such as semisolids (gels, cremes, ointments etc.) or bio-degradable solid forms (transdermal patch) to enable a local therapy. Moreover, chemical synthesis of flavonoids is cost-intensive and complex to achieve and therefore not a feasible option, at least not in a large-scale production. Therefore, a natural source of raw material is of high importance.

The pharmacological *in vitro*-effects of the damask rose, like compromising sequences in the cell cycle among others, offer several interesting treatment options of principally hyperproliferation- and/or chronic inflammation-involved skin diseases such as psoriasis for instance. Therefore, again *in vivo*-studies are indispensable, however, a topical application form because of bioavailability-/toxicity-/solubility-related issues for a local therapy is conceivable. Additionally, *in vivo*-related parameters like stability, penetration characteristics and of course skin tolerance etc. must be assessed.

Considering the rising pressure on natural resources at present time, sustainability gains importance. In this context, the question of cost-effective up-scaling options to industrial standards of the polyphenol extraction of the damask rose is crucial (Fig. 4, chapter 2.2).

Rusanov et al. [1] used activated absorption resins to efficiently obtain polyphenol-enriched RODW and therein suggest a medium-scale system. This could serve as a foundation for further optimization regarding the efficiency of extraction and cost reduction. In any case, it is of utmost relevance for the world's population to remember the respect for clean air, water and food, which are the basic biological needs for every creature. To secure them, it is necessary to optimize the utility of resources, which starts in the very beginning of the food chain and other production lines. Within this work, pharmacological *in vitro*-effects of two natural resources were successfully characterized, which could pose an interesting option for a beneficial treatment method. As a follow-up study, it would be fascinating to assess *in vivo* effects in animal and human skin models, as well as to assess to which extent the existing *in vitro* results stand in accordance with them. Moreover, technological examinations are necessary to incorporate the compounds -or a certain fraction- into an appropriate form of application. Subsequently, just like a further benefit of bamboo leaves, waste water of the rose oil production could be collected for further processing and thereby converted into an uncritical state for disposal. Altogether, the previously reported results offer the basis for potentially new strategies to valorize an original waste fraction to a value-added product.

[1] *K. Rusanov, E. Garo, M. Rusanova, O. Fertig, M. Hamburger, I. Atanassov, V. Butterweck,* Recovery of polyphenols from rose oil distillation wastewater using adsorption resins-a pilot study, Planta Medica 80(17) (2014) 1657-64.

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6. Appendix

PUBLICATIONS

This doctoral thesis is essentially based on three publications. The fourth publication listed below provides further chemical information about the substance utilized in the present work. The experimental part of Jessica Solimine was done under my practical support:

1. **Jonas Wedler**, Tony Daubitz, Götz Schlotterbeck, Veronika Butterweck
   In Vitro Anti-Inflammatory and Wound-Healing Potential of a Phyllostachys edulis Leaf Extract – Identification of Isoorientin as an Active Compound
   Planta Medica (2014) 80(18):1678-1684

2. **Jonas Wedler**, Krasimir Rusanov, Ivan Atanassov, Veronika Butterweck
   *A Polyphenol-Enriched Fraction of Rose Oil Distillation Wastewater Inhibits Cell Proliferation, Migration, and TNF-α-Induced VEGF Secretion in Human Immortalized Keratinocytes**
   Planta Medica (2016) 82(11/12):1000-1008

3. **Jonas Wedler**, Anna Weston, Julia Rausenberger, Veronika Butterweck
   In Vitro Modulation of Inflammatory Target Gene Expression by a Polyphenol-Enriched Fraction of Rose Oil Distillation Waste Water
   Fitoterapia (2016) 114:56-62

4. Jessica Solimine, Eliane Garo, **Jonas Wedler**, Krasimir Rusanov, Orlando Fertig, Matthias Hamburger, Ivan Atanassov, Veronika Butterweck
   Tyrosinase Inhibitory Constituents from a Polyphenol Enriched Fraction of Rose Oil Distillation Wastewater
   Fitoterapia (2015) 108:13-19

POSTERS

1. T. Daubitz, **J. Wedler**, W. Riedl, G. Schlotterbeck, K. Nieber, V. Butterweck: *Phyllostachys Edulis* Leaf Extract Reduces TNFα-Induced Release of VEGF and IL-8 in Immortalized HaCaT Cells, 6th Swiss Pharma Science Day 2013, Bern, Switzerland, August 2013
2. **J. Wedler**, G. Schlotterbeck, V. Butterweck: Wound healing effects of *Phyllostachys edulis* leaf extracts and isoorientin in 3T3-swiss albino fibroblasts, Annual Research Meeting, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland, February 2014
3. **J. Wedler**, G. Schlotterbeck, V. Butterweck: Wound healing effects of *Phyllostachys edulis* leaf extracts and isoorientin in 3T3-swiss albino fibroblasts, 29. Schweizerische Jahrestagung für Phytotherapie, Winterthur, Switzerland, June 2014
4. **J. Wedler**, V. Butterweck: A Polyphenol Enriched Fraction of Rose Oil Distillation Waste Water Inhibits Proliferation in Immortalized Human Keratinocytes and Promotes Apoptosis, 7th Swiss Pharma Science Day 2014, Bern, Switzerland, August 2014
5. **J. Wedler**, E. Garo, K. Rusanov, M. Hamburger, I. Atanassov, V. Butterweck: *In vitro* inhibition of migration and proliferation by a polyphenol enriched fraction of rose oil distillation waste water and its purified fractions on immortalized human keratinocytes, Annual Research Meeting, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland, February 2015
6. **J. Wedler**, E. Garo, K. Rusanov, M. Hamburger, I. Atanassov, V. Butterweck: A Polyphenol Enriched Fraction of Rose Oil Distillation Water Inhibits Proliferation in Immortalized Human Keratinocytes and Induces Apoptosis, 8th Swiss Pharma Science Day 2015, Bern, Switzerland, August 2015
7. **J. Wedler**, E. Garo, K. Rusanov, M. Hamburger, I. Atanassov, V. Butterweck: A Polyphenol Enriched Fraction of Rose Oil Distillation Water Inhibits Proliferation in Immortalized Human Keratinocytes and Induces Apoptosis, 63rd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (Gesellschaft für Arzneipflanzen- und Naturstoff-Forschung e.V.), Budapest, Hungary, August 2015

SHORT LECTURES

1. **J. Wedler** Sustainability Meets Medicine - The Rose On The Rise, Überfachliche Kompetenzen Mündliche Präsentationen: Methoden und Selbstvertrauen, Matuschek Consulting, Basel, Switzerland, October 2014

2. **J. Wedler** Ebola: Portrait d'un Serial Killer, Sprachenzentrum Universität Basel, Basel, Switzerland, December 2014

3. **J. Wedler** Rose Oil Distillation Waste Water Valorization, Seminarreihe Forschung der Hochschule für Life Sciences, Fachhochschule Nordwestschweiz, Muttenz, Switzerland, January 2015

4. **J. Wedler** A Polyphenol Enriched Fraction of Rose Oil Distillation Water Inhibits Proliferation in Immortalized Human Keratinocytes and Induces Apoptosis, Young Researchers Workshop, 63rd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (Gesellschaft für Arzneipflanzen- und Naturstoff-Forschung e.V.), Budapest, Hungary, August 2015