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Effects of a Phenol-Enriched Purified Extract from Olive Mill Wastewater on Skin Cells

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Abstract: Olive trees (Olea europaea) and their processed products, such as olive oil, play a major role in the Mediterranean way of life. Their positive impact on human health is being intensely investigated. One research topic is the identification of new application areas of olive mill wastewater (OMWW). OMWW is characterized by the high content of polyphenols possessing many positive health effects. Thus, the phenol-enriched OMWW extract offers the potential for the treatment of skin disorders and for cosmetic application. The aim of the present study was to evaluate cell viability and proliferation, the anti-inflammatory and anti-oxidative properties of a phenol-enriched OMWW extract on an immortal keratinocyte cell line (HaCaT cells). Moreover, the influence on the growth of various microorganisms was investigated; furthermore, the effects on normal human epidermal keratinocytes (NHEK) and human melanoma cells (A375) were studied in a commercially available tumor invasion skin model. The phenol-enriched OMWW extract showed excellent antimicrobial activity. Moreover, a noticeable reduction in reactive oxygen species formation as well as Interleukin-8 release in HaCaT cells were observed. Finally, the inhibited growth of A375 melanoma nodules in the melanoma skin model could be shown. Our results indicate that the OMWW extract is a promising ingredient for dermal applications to improve skin health and skin protection as well as having a positive impact on skin ageing.

Keywords: HaCaT cells; NHEK cells; ROS-assay; tumor invasion skin model; IL-8; antimicrobial effects

1. Introduction

Olive trees (Olea europaea) and their processed products, such as olive oil, play a major role in the Mediterranean way of life. In Mediterranean countries, a significant decline in cancer risk has been shown in several studies [1]. This is associated with the Mediterranean diet and its associated phenol-rich nutrition. Olive fruits, especially olive oil and the by-products of the manufacturing process, are the focus of many research groups [2–5]. In addition to the oil phase, a large quantity of aqueous phase (olive mill wastewater, OMWW) and small quantities of pomace (plant fibers) are formed during the olive grinding process [4,6]. The disposal of these by-products is cost-intensive. Thus, the search for alternative applications is of great interest. In particular, OMWW seems to be very promising due to a high amount of polyphenols [7] which can further be enriched by extraction [8]. The quantity and the compositions of the phenols largely depend on the cultivation conditions of the olive trees, the manufacturing process of the olive oil, and the extraction method for the purification of OMWW [8]. These are essential for the biological activity of the final product. Polyphenols are attributed to many

health-promoting effects, such as anti-inflammatory [9], anti-microbiological [10], anti-oxidative [11], and potentially chemo-preventive properties [12]. Due to these properties, polyphenols are very promising for dermal applications. So far, though, only a few studies have been conducted with regard to OMWW application on the skin. Di Mauro et al. [13] investigated a sugar- and mineral-enriched fraction from OMWW for a cosmeceutical application and have shown its moisturizing properties in vivo. Therefore, the aim of the present study was to evaluate cell viability and proliferation, and the anti-inflammatory and anti-oxidative properties of a phenol-enriched OMWW extract on HaCaT cells. Furthermore, the influence on the growth of various microorganisms was investigated. Finally, the effects on normal human epidermal keratinocytes (NHEK) and human melanoma cells (A375) were studied in a tumor invasion skin model.

2. Materials and Methods

2.1. Preparation of the Olive Mill Wastewater (OMWW)

The olive mill wastewater (OMWW) used for the studies was provided by Agriturismo La Vialla (Castiglion Fibocchi (Arezzo), Italy). The phenol-enriched purified extract (OMWW extract) was obtained from Massimo and Daniele Pizzichini according to Patent formulation (Patent 8815815) [14]. The composition of the batch was provided by a certificate of analysis of Fattoria LaVialla (Table 1). The extract, originating from the aqueous part of olive oil production was filtered using a ceramic membrane, and concentrated by reverse osmosis [14]. Prior to use, the concentrate was centrifuged at 500× *g* for 20 s and filtrated (0.2 µm). Final dilutions were completed in a cell culture growth medium.

Table 1. Quantification of the phenolic ingredients in the olive mill wastewater (OMWW) extract, n.d. = not detected.

Phenolic Ingredient	Concentration/g·L^{-1}
hydroxytyrosol glycoside	2.01
hydroxytyrosol	2.52
tyrosol and derivates of glycosids	0.80
chlorogenic acid	0.10
caffeic acid	n.d.
β-hydroxy-verbascoside isomer 1	0.29
β-hydroxy-verbascoside isomer 2	0.26
rutin	n.d.
Verbascoside	1.04
luteolin-7-glucoside	n.d.
nüzhenide	n.d.
Isoverbascoside	n.d.
3,4-DHPEA-EDA (oleacein)	n.d.
oleuropein aglycon	0.71
p-coumaroyl-secologanoside	0.38
Oleocanthal	not analyzed
total absorption (280 nm)	14.67

2.2. Cell Viability Assay

The cell viability was determined with a calorimetric WST-1 assay (Roche Diagnostics Mannheim, Germany); the conditions were adapted to the respective issue. The cell viability of an immortalized human keratinocyte cell line (HaCaT cells, AddexBio, San Diego, CA, USA) was determined after 72 h to exclude negative effects in the anti-inflammatory assay. HaCaT cells were inoculated with 20,000 cells per well (96-well plate) in growth medium (DMEM low glucose with 10% FCS, Capricorn Scientific GmbH, Germany) and incubated for 24 h at 37 °C with 8.5% CO_2. After a visual inspection of the intact monolayer, the cells were washed twice with phosphate buffer, and 200 µL of the OMWW extract dilutions in the growth medium as well as growth medium in case of the untreated control

were applied for 72 h. At the end of incubation, the cells were washed twice with the phosphate buffer, and 100 µL of 5% WST-1 reagent in the growth medium was applied onto the cells and incubated for 4 h. Samples were measured at 450 and 630 nm (reference wavelength), using the Synergy HTX microplate reader (BioTek Instruments Inc., Germany). Cell viability was calculated in relation to the untreated control. The following changes in the protocol were done for the human malignant melanoma cell line A375 (Sigma-Aldrich, Taufkirchen, Germany) and the human epidermal keratinocytes neonatal NHEK (MatTek Corporation, Ashland, MA, USA) to estimate the application concentration for the tumor invasion model. The growth medium for A375 cells was DMEM with high glucose (4.5 g/L) (DMEM-HXA, 8.5% CO_2, Capricorn Scientific GmbH, Ebsdorfergrund, Germany) supplemented with 10% FCS, 2% glutamine (200 mM) and 1% sodium pyruvate (100 mM), respectively, and keratinocyte growth medium 2 (5% CO_2) delivered by PromoCell GmbH (Heidelberg, Germany) for NHEK cells. Both cell lines were inoculated with 40,000 cells per well (96-well plate), and sample incubation was done with 100 µL of the OMWW extract dilutions for 24 h. All experiments were carried out three times with at least six repetitions.

2.3. Anti-Inflammatory Assay In Vitro

HaCaT cells were cultured in a 24-well plate (20,000 cells per well) in 1 mL of growth medium overnight (24 h) at 37 °C and 8.5% CO_2. Prior to sample application (sample size: 1 mL in growth medium), the cells were washed twice with phosphate buffer. The inflammatory response was induced by the co-application of 10 ng/mL TNF-α (R&D Systems, Wiesbaden, Germany). The anti-inflammatory drug hydrocortisone (10^{-7} M) was used as a positive control. Cell-free supernatants were collected, and the Interleukin-8 (IL-8) concentration was determined by the commercially available ELISA kit (Life Technologies GmbH, Darmstadt, Germany). All experiments were carried out three times with at least three repetitions.

2.4. Assessment of the Antimicrobial Properties

For the testing of the antimicrobial properties of the OMWW concentrate, a time-modified assay was used, according to the European Pharmacopoeia (Ph. Eur.) method. Therefore, the inhibitory properties of the OMWW extract on the growth of various bacterial and mold organisms (Table 2) were determined by the suspension method, the punch hole method and the paper disk method. The cultivation and evaluation of the test organisms was based on the Pharmacopeia Europaea protocol [15]. Therefore, 10 mL of the OMWW extract was inoculated with the individual microorganism (MO), a bacterial concentration between 10^5 and 10^6 CFU/g and a mold concentration between 10^4 and 10^5 CFU/g. These samples were incubated at 25 ± 1 °C, and growth control was performed immediately after inoculation, i.e., after two days and one week of incubation with the dilution series determining the colony-forming units (CFU). Furthermore, the antimicrobial effectiveness was determined by the agar diffusion test. Two procedures were performed: the punch hole test and the paper disc method. With respect to the test organism, the medium was inoculated with 10^5 to 10^7 CFU per plate. Subsequently, a hole with a diameter of 11 mm was punched into the agar and filled with 0.5 mL of the OMWW extract (punch hole test), respectively, and an absorbent paper disc (∅ 9 mm) soaked with 250 µL of the OMWW extract was placed onto the agar (paper disc method). The plates were incubated as described in the Ph. Eur.: bacteria for 18 to 24 h at 35 °C (depending on the bacterium: aerobic/anaerobic), *Candida albicans* for 48 h at 25 °C, and *Aspergillus brasiliensis* for 5 days at 25 °C. The results were obtained by measuring the inhibition zone.

Table 2. Overview of the test organism and the used broth.

Test Organism, Strain ID	Broth
Staphylococcus aureus, WDCM 00035	Casein soya peptone agar
Escherichia coli, DSMZ 301	Casein soya peptone agar
Pseudomonas aeruginosa, WDCM 00026	Casein soya peptone agar
Candida albicans, WDCM 00054	Sabouraud dextrose agar without antibiotic supplements
Aspergillus brasiliensis, WDCM 00053	Sabouraud dextrose agar without antibiotic supplements
Staphylococcus epidermidis, WDCM 00036	Casein soya peptone agar
Propionibacterium acnes, DSMZ 1897	Casein soya peptone agar

2.5. Reactive Oxygen Species (ROS) Assay In Vitro

42,000 HaCaT cells per well (100 µL of growth medium without phenol red) were grown in a black 96-well plate with a clear bottom overnight (37 °C and 8.5% CO_2). The next day, the cell monolayer was washed twice with phosphate buffer, and 100 µL of the OMWW extract dilution or ascorbic acid (100 µM) plus 50 µM 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) sample per well were applied for 30 min. Then, the cells were washed with PBS buffer, and 100 µM H_2O_2 in phosphate buffer was added to the cells for 45 min (at 37 °C and 8.5% CO_2). A final washing step with phosphate buffer was done before measuring the fluorescence intensity (ex./em. 485/528 nm), using the microplate reader (100 µL of phosphate buffer, bottom read). To calculate the reduction of oxidative stress, the measurement signal of the control (cells treated with H_2O_2 only) is set to 100%. All experiments were done three times with at least five repetitions.

2.6. Tumor Invasion in a Full-Thickness Skin Model In Vitro

The influence of the OMWW extract on tumor invasion was examined using the commercially available Melanoma skin model "MLNM-FT-A375" (MatTek Corporation, Ashland, USA). Based on the "Testing of Anti-Melanoma Drugs" protocol, provided by MatTek Corporation, the skin models were prepared for a long-time treatment (12 days) after delivery. The treatment of the skin samples with 1:400 dilutions of the OMWW extract (with cell culture medium) started on day 1 and was renewed every other day. As a positive control, the same number of skin samples was treated with the cell culture medium only. On day 3, 7 and 12 (plus day 0 for positive control), two skin samples per time point and treatment were prepared for a histology characterization: formalin fixation and Hematoxylin and Eosin (H&E) staining. Hematoxylin stains the cell nuclei violet/dark blue, while eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of pink/red.

2.7. Data Analysis and Statistics

Data are given as arithmetic mean values ± standard deviation. In order to verify differences, data were subjected to the nonparametric Kruskal–Wallis test, followed by the Dunn's multiple comparison test, in case of significance (GraphPad Prism® version 8.1.0(325), GraphPad Software, San Diego, CA, USA). Thus $p \leq 0.05$ is regarded to indicate a difference.

3. Results

3.1. Phenol-Enriched OMWW Extract Reduces Interleukin-8 Release in Epidermal Skin Cells (In Vitro)

First, the influence of the OMWW extract on HaCaT cell viability was investigated over 72 h. The decrease in HaCaT viability depends on the OMWW extract concentration. The OMWW extract dilution of 1:100 showed a slight decrease in cell viability (70.9% ± 10.2%) after 72 h (Figure 1). An OMWW extract dilution of 1:200 to 1:1000 showed no negative effect on HaCaT cell viability (viability ≥ 100%) compared to the untreated sample (Figure 1). Therefore, the anti-inflammatory assay was carried out with the OMWW extract dilutions of 1:200, 1:400 and 1:600.

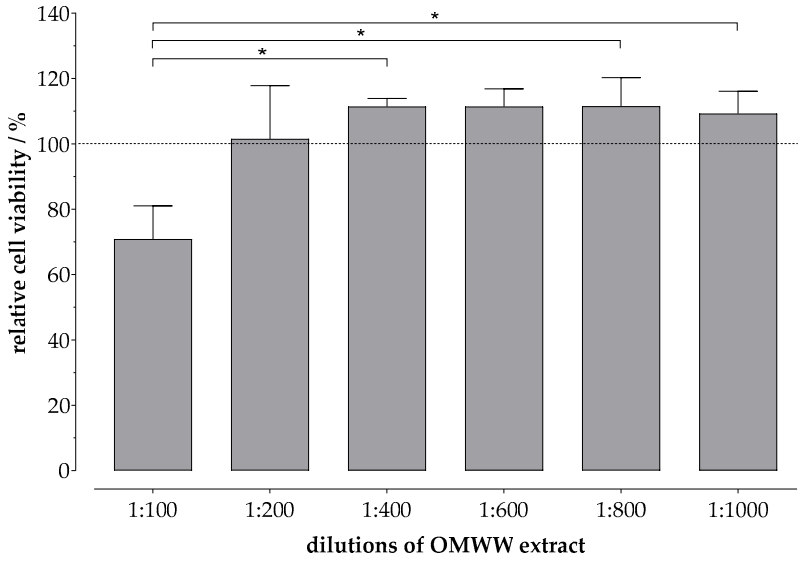


Figure 1. Viability of HaCaT cells (20,000 cells per well, 96-well plate) after treatment with the OMWW extract dilutions (1:100–1:1000) for 72 h, MW ± SD, $n = 3$, * $p \leq 0.05$ (each 6-fold). Indication of the viability is shown in relation to the untreated control (100% viability).

To study the possibility of an application of the OMWW extract in cases of inflammatory skin diseases, the modulation of Interleukin-8 (IL-8) release was studied in HaCaT cells. The stimulation of HaCaT cells with TNF-α (10 ng/mL) resulted in an increased release of IL-8 compared to the untreated control (Figure 2). Depending on the incubation period, the IL-8 concentration increased from 465.8 ± 31.0 pg/mL (24 h) to 2362.4 ± 35.9 pg/mL (72 h) after a TNF-α application. Furthermore, the sensitivity of the assay was controlled by the standard anti-inflammatory agent hydrocortisone (10^{-7} M), resulting in a reduction of 40% of IL-8 release compared to the TNF-α-treated sample after 72 h (Figure 2).

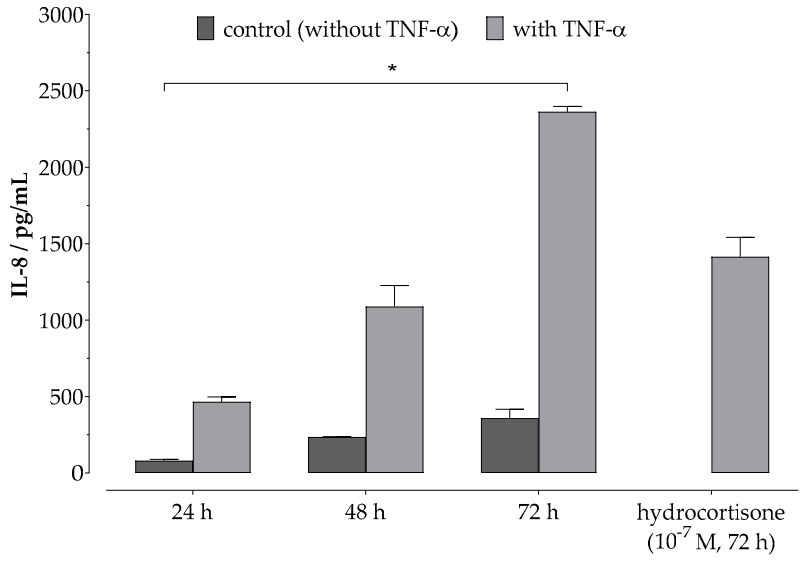


Figure 2. Pre-studies for Interleukin-8 (IL-8) release of HaCaT cells after treatment with 10 ng/mL TNF-α as a function of incubation time and treatment with hydrocortisone (10^{-7} M) for 72 h. MW ± SD, $n = 3$, * $p \leq 0.05$ (each in triplicates).

Stimulation of HaCaT cells with TNF-α resulted in a 4.8-fold higher release of IL-8 compared to the untreated control after 72 h (Figure 3). Co-administration of TNF-α and the OMWW extract

led to a reduction in IL-8 release up to 73.4 ± 10.4% (1:200) compared to the control with TNF-α. The reduction in IL-8 release depended on the concentration, 45.2 ± 5.8% (1:400), and 37.2 ± 8.4% (1:600) respectively. Already, the unstimulated samples showed a positive effect on IL-8 release in HaCaT cells. IL-8 production was reduced up to 72.4% (1:200) (Figure 3).

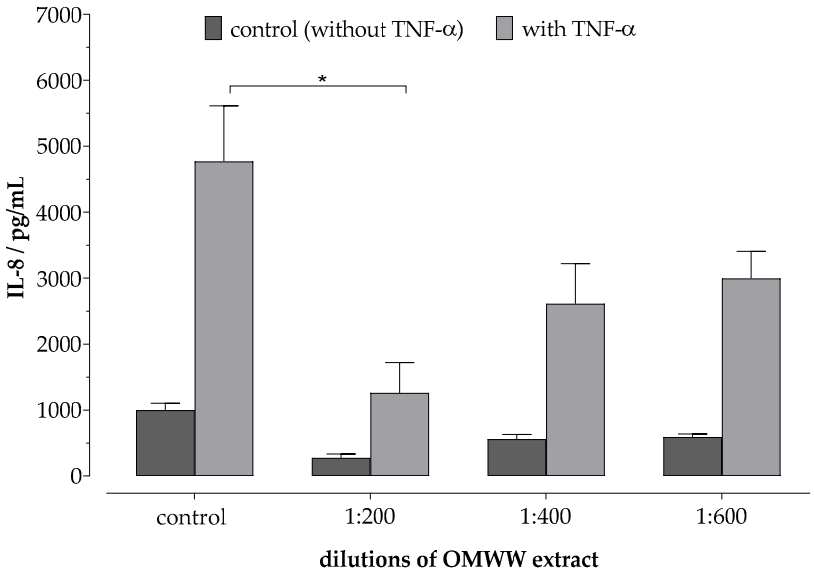


Figure 3. Determination of IL-8 release in HaCaT cells after treatment with 10 ng/mL TNF-α (controls without TNF-α) and incubation with the OMWW extract dilutions for 72 h. MW ± SD, $n = 3$, * $p \leq 0.05$ (each in triplicates).

The studies carried out on HaCaT cells showed that the OMWW extract positively influenced IL-8 release in vitro, thus demonstrating an anti-inflammatory effect on skin cells.

3.2. Phenol-Enriched OMWW Extract Inhibits the Growth of Selected Pathogenic Skin Organism

The influence of the OMWW extract on microbial growth was investigated by the suspension method (Table 3) and a determination of the inhibition zone (punch hole and paper disk) by the agar diffusion method (Tables 4 and 5). The OMWW extract showed excellent antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis* (both Gram-positive bacteria), *Escherichia coli*, *Pseudomonas aeruginosa* (both Gram-negative bacteria), *Candida albicans* (yeast), and *Propionibacterium acnes* (anaerobic Gram-positive bacteria). The antimicrobial activity against *Aspergillus brasiliensis* (mold) was less pronounced after 7 days of incubation, using the suspension method (Table 3).

Table 3. Studies on the antimicrobial effects of the OMWW extract on various microorganisms by the suspension method.

Test Organism	CFU/g (Start)	2 d		7 d	
		CFU/g Sample	Reduction in %	CFU/g Sample	Reduction in %
S. aureus	4.30×10^5	<10	>99.99	<10	>99.99
E. coli	1.06×10^6	<10	>99.99	<10	>99.99
P. aeruginosa	8.70×10^5	<10	>99.99	<10	>99.99
C. albicans	3.20×10^5	<10	>99.99	<10	>99.99
A. brasiliensis	8.00×10^4	3500	95.63	not determined	not determined
S. epidermidis	1.16×10^6	<10	>99.99	<10	>99.99
P. acnes	1.20×10^6	<10	>99.99	<10	>99.99

Table 4. Studies on the antimicrobial effects of the OMWW extract on various microorganisms by detection of the inhibition zone, by the punch hole and the paper disk method. The dashed line identifies the inhibition zone.

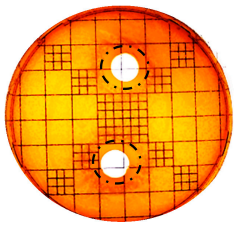
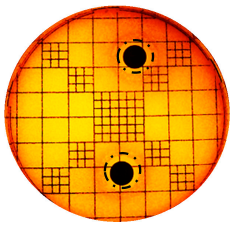
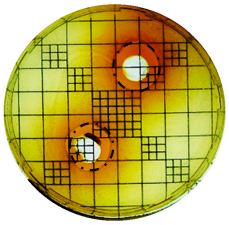
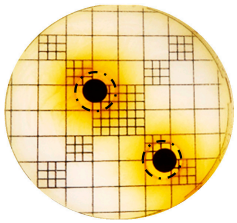
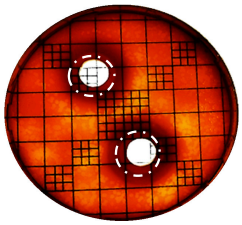
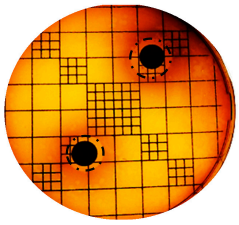
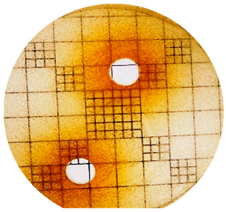
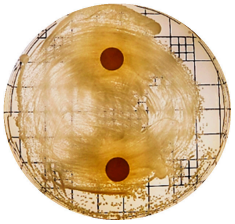
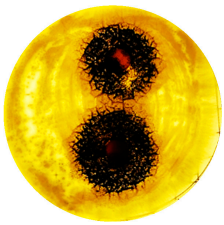
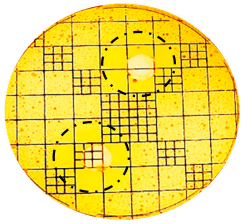
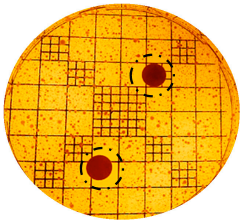
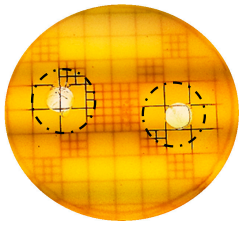
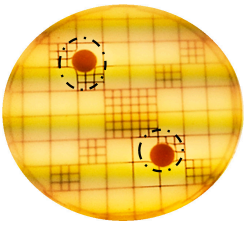
	Punch Hole Method	Paper Disk Method
S. aureus:		
E. coli:		
P. aeruginosa:		
C. albicans:		
A. brasiliensis:		
S.epidermidis:		
P. acnes:		

Table 5. Quantification of the inhibition zone formed, depending on the microorganism and its inoculation density for the punch hole method and paper disc method.

Organism	Inoculation CFU/g Sample	Inhibition Zone/mm (Punch Hole Method)	Inhibition Zone/mm (Paper Disc Method)
S. aureus	4.30×10^6	3.0	2.0
E. coli	1.06×10^7	4.0	2.5
P. aeruginosa	8.70×10^6	3.5	3.0
C. albicans	3.20×10^6	0	0
A. brasiliensis	8.00×10^5	0	0
S. epidermidis	1.16×10^7	7.0	3.1
P. acnes	1.20×10^6	9.0	7.0

In more detail, the variable potency in growth-inhibiting efficacy of the OMWW extract was observed by the detection of the inhibition zone (Tables 4 and 5). The OMWW extract showed an antibiotic effect against all the bacterial strains tested and was most effective against *S. epidermidis* and *P. acnes*. No growth-inhibiting properties could be detected against *C. albicans* and *A. brasiliensis*.

Considering these results, the OMWW extract revealed very good antimicrobial activity and a moderate effectiveness against the yeast and the mold.

3.3. Phenol-Enriched OMWW Extract Reduce the Formation of Free Radicals In Vitro

The anti-oxidative property was studied by the ability of the OMWW extract to reduce the formation of free radicals in HaCaTs cells. Reactive oxygen species (ROS) generation in HaCaT cells was induced by the application of H_2O_2 (100 µM). Based on Rossi et al. [14], the OMWW extract was applied in a concentration of 1:250 and 1:500. In the presence of the OMWW extract, ROS generation was clearly reduced and even more effective than the application of 100 µM ascorbic acid (Figure 4). Thus, the OMWW extract showed anti-oxidative properties in vitro.

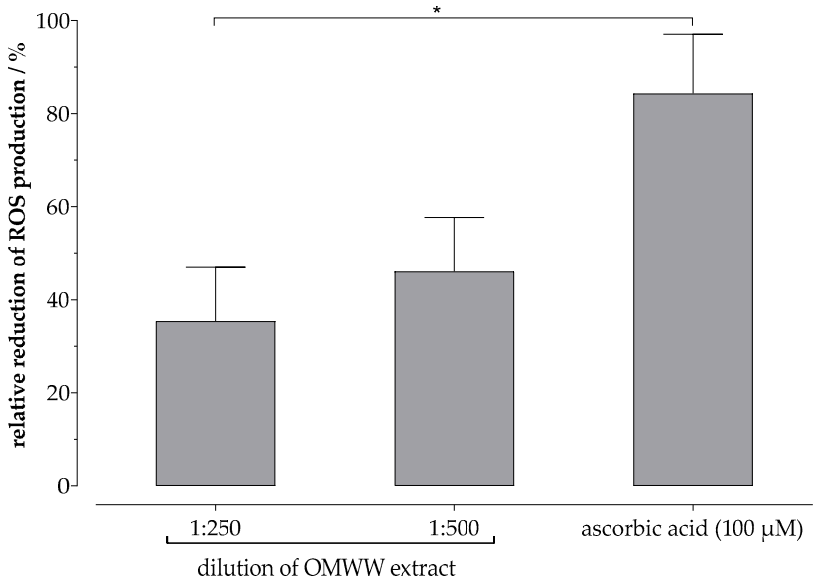


Figure 4. Treatment (30 min) of HaCaT cells with each OMWW extract dilution (1:250 and 1:500) and ascorbic acid (100 µM) after ROS induction by H_2O_2 (100 µM) for 15 min; MW ± SD, $n \geq 3$, * $p \leq 0.05$ (with five replications). HaCaT cells, only treated with H_2O_2, represent 100% of the generated reactive oxygen species (ROS).

3.4. The Phenol-Enriched OMWW Extract Modulates the Invasion of Tumor Cells in the Skin In Vitro

The melanoma skin model is a tool to study the influence of substances on the growth of A375 cells, human metastatic melanoma cells, within a full-thickness skin model consisting of normal human-derived epidermal keratinocytes (NHEK) and normal human-derived dermal fibroblasts. Skin models are more physiologically relevant and predictive than 2D cultures (monolayer) [16,17]. First, we investigated the influence of the OMWW extract on the viability of A375 and NHEK cells. The results showed a concentration-related effect of the OMWW extract on the cell viability of NHEK and A375 cells. NHEK cells treated with the OMWW extract showed clear increased cell proliferation, up to 189.1% with the 1:200 OMWW compared to the untreated control (Figure 5, dark grey bars). In comparison, the viability of A375 cells was approximately 90.1% (1:100) to 115.7% (1:400) after an OMWW extract treatment and, thus, comparable to the untreated control (Figure 5, light grey bars).

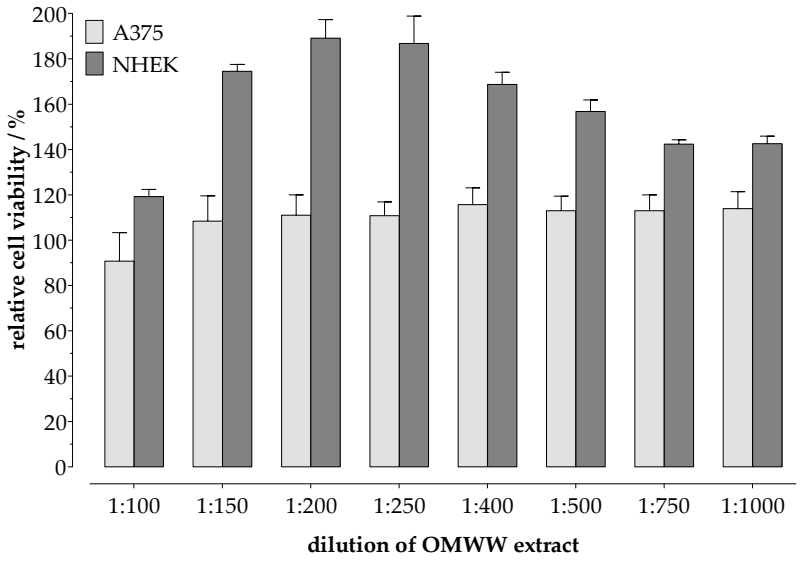


Figure 5. Viability of NHEK and A375 cells (40,000 cells per well, 96-well plate) depending on treatment with the OMWW extract dilutions (1:100–1:1000) after 24 h. MW ± SD, n = 3 (with six replications). An indication of the viability is shown in relation to the untreated control (=100% viability).

The melanoma skin model can be used to study the invasion of tumor cells (A375) into the dermis. In brief, the cultivation of an untreated skin sample results in the development of nodes by the incorporated metastatic melanoma cell line (A375) within 12 days (Figure 6). By applying potent substances, modulation in tumor invasion can be detected. The control of day 0 (Figure 6 left) shows the different layers of the skin (dermis with fibroblasts, epidermis with keratinocytes and the *stratum corneum*) and the membrane of the tissue culture inserts. Arrows indicate the melanoma cell clusters at the epidermal–dermal junction. On day 12 (Figure 6 right), the melanoma nodes are large and clearly visible in the control skin sample.

The following pictures (Figure 7) show the results of the treatment of the Melanoma skin model with an OMWW extract dilution (1:400) compared to the untreated control of 12 days. Each treatment was done in duplicate, and the representative section from each treatment is presented (10× magnification). The OMWW extract apparently affects the growth of melanoma cells and the formation of melanoma nodes within the three-dimensional skin models, respectively. The A375 cell clusters were smaller in size. These results suggest that the OMWW extracts reduce the growth of A375 melanoma nodules in vitro compared to the untreated samples as determined by a reduction in cell cluster size.

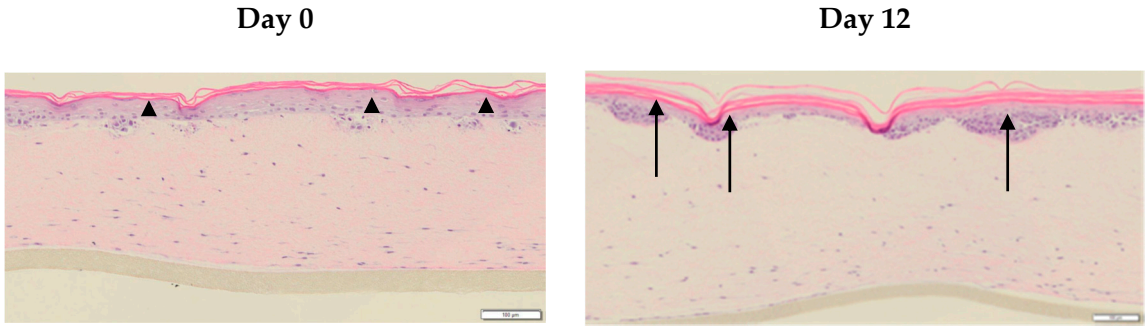


Figure 6. MatTek Melanoma skin model (MLNM-FT-A375) cultivation without treatment. Day 0 (**left**) and day 12 (**right**) of cultivation in our labs. Short arrows indicate the incorporated A375 cells; long arrows indicate some of the melanoma cell clusters at the epidermal–dermal junction, H&E staining, 10× magnification; scale bar 100 µm.

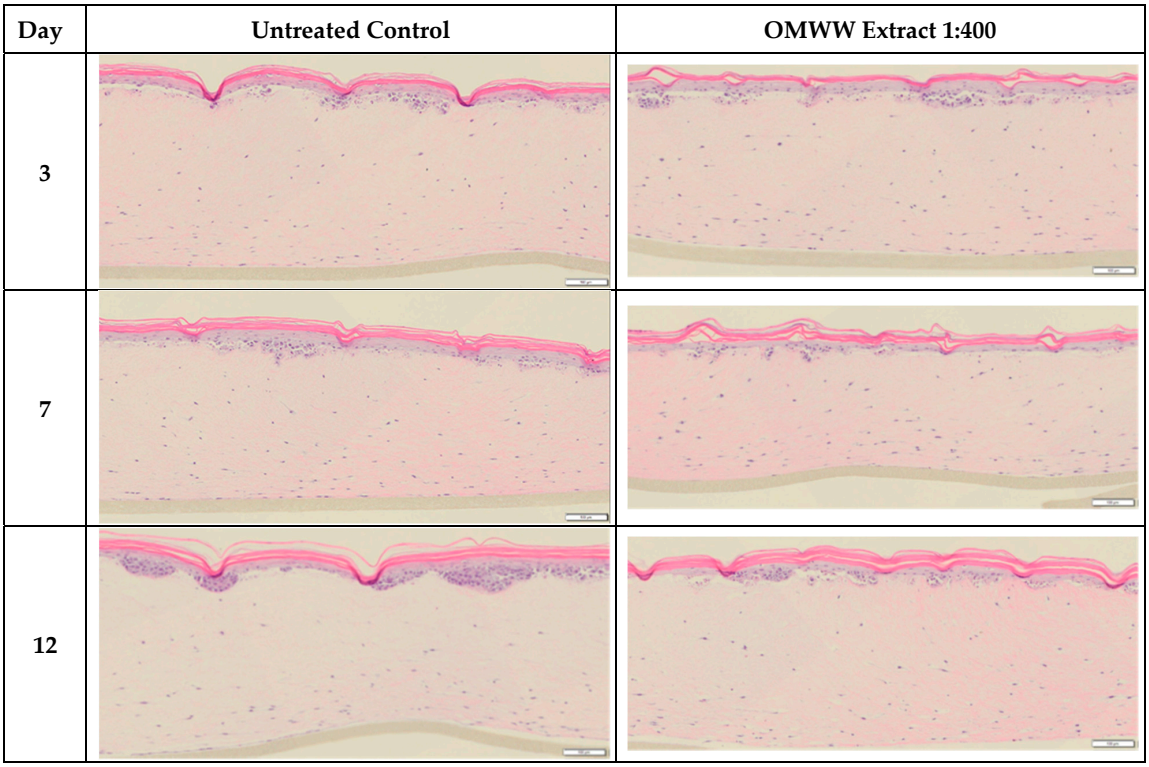


Figure 7. Results of the tumor invasion investigation of the MatTek Melanoma skin model. Skin section with H&E staining, 10× magnification; scale bar 100 µm. Two skin samples per time point and treatment were prepared for histology characterization; a representative cross section is shown.

4. Discussion

Olive mill wastewater is a by-product of olive oil production. Due to the high content of polyphenol in OMWW, the potential health properties are widely discussed and investigated [5,18]. The main polyphenols present in our purified and phenol-enriched OMWW extract are hydroxytyrosol, hydroxytyrosol glycoside, verbascosides, tyrosol, and oleuropein aglycon [Table 1]. The composition of the OMWW extract is essential for health-promoting effects, and strongly depends on the cultivation conditions of olive trees as well as on the applied preparation techniques [8]. Previous studies using this phenol-enriched OMWW extract revealed the anti-angiogenic and angiopreventive activity in endothelial cells [14] and the potential chemo-preventive properties in colon cancer [12]. Polyphenols are associated with several positive effects on cell viability and proliferation, inflammatory processes, microbiological growth and the formation of reactive

oxygen species [18]. Therefore, the phenolic-enriched OMWW extract has great potential for dermal application. Based on the experimental conditions of the bacterial count reduction assay by the European Pharmacopoeia [15], a good antibacterial effect against Gram-negative (*E. coli*, *P. aeruginosa*) and Gram-positive bacteria (*S. aureus*, *S. epidermidis*, *P. acnes*) was detected. Regarding the inhibition zone determination, the Gram-negative bacteria were less susceptible to an application of the OMWW extract. This is well in accordance with other published data [19] and might be due to the outer membrane layer of Gram-negative bacteria, which serves as an additional barrier hindering the entrance of antibiotics [19]. The anti-fungal activity against *C. albicans* (yeast) and *A. brasiliensis* (mold) was less pronounced, and an inhibition of growth could be detected using the suspension method. Mahmoudi et al. [19] have already shown the less pronounced anti-microbiological effect of *C. albicans* and *A. brasiliensis* compared to Gram-positive and Gram-negative bacteria with the leaf extract of *Ficus carica*. The difference between the suspension and agar plate method is probably due to the dilution of the OMWW extract by diffusion into the agar medium. The excellent antibacterial activity and moderate anti-fungal activity is based on the high content of hydroxytyrosol and oleuropein in our OMWW extract [3,20]. Due to this antimicrobial effect, the OMWW extract can act as a booster for preservatives in dermal formulations to prevent contamination. Furthermore, this effect may have a positive impact on pathogenic germs on the skin, which are involved in various skin diseases, such as psoriasis or atopic dermatitis. Skin cells are constantly exposed to free radicals and other reactive oxygen species (ROS) caused by normal metabolic processes in the human body or from external sources like UV-radiation, cigarette smoke and air pollutants. An undesirable excess of ROS, called oxidative stress, is involved in inflammatory processes, skin aging and alteration of DNA resulting in skin cancer. Hydroxytyrosol, the major component of the phenol-enriched OMWW extract, is well known for anti-oxidant, anti-inflammatory and anti-tumor effects [18]. Thus, we verified these properties in vitro on human skin cells. The application of the OMWW extract clearly reduced H_2O_2-induced ROS formation in HaCaT cells in a dose-dependent manner; this is in accordance with the results in human umbilical vein endothelial cells by Rossi et al. [14]. Rossi et al. [14] have demonstrated that this phenol-enriched OMWW extract is even more effective in inhibiting ROS formation compared to the polyphenol hydroxytyrosol alone. Among others, inflammatory processes are involved in skin wounds; an excessive or permanent immune reaction can lead to chronic wounds [21]. The regulation of inflammatory mediators such as IL-8 may facilitate the healing process of these skin defects. The TNF-α-induced formation of IL-8 in HaCaT cells was down-regulated by the OMWW extract in a dose-dependent manner. This result is in accordance with the reported down-regulation of IL-8 in colon cancer cells by OMWW and hydroxytyrosol [12]. Several studies reported the anti-tumor activity of polyphenols [14,22,23] and discussed different chemo-preventive mechanisms [24]. Therefore, we investigated the influence of the OMWW extract in a commercially available melanoma skin model (Mattek MLNM FT A375) containing keratinocyte and fibroblast layers with melanoma nodules [25]. The growth and migration of A375, a metastatic melanoma cell line, were restricted during the 12 days. The OMWW extract seems to selectively eliminate the melanoma cells in the full-thickness skin model. Studies on the influence of the OMWW extract on cell viability (monolayer) revealed enhanced NHEK proliferation in contrast to A375 cells after 24 h. Comparable effects have been shown for polyphenols of black tea [26] as well as an induction of apoptosis in several cancer cells by the polyphenol epigallocatechin-3-gallate [27]. Among others, oleuropin and hydroxytyrosol are responsible for this antitumoral effect [28,29].

5. Conclusions

We presented a purified and phenol-enriched OMWW extract, a natural source of polyphenols, as a promising compound for dermal application. Due to its considerable anti-microbial, anti-inflammatory, anti-oxidative and anti-tumor activity, several fields of application are possible. This study evaluated the application of the OMWW extract in a melanoma 3D skin model for the first time. The superiority of

the complex polyphenol mixture over the pure substances should be the subject of further investigations with skin cells.

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