SCREENING FOR NEW ANTIOXIDATIVE COMPOUNDS FOR TOPICAL ADMINISTRATION USING SKIN LIPID MODEL SYSTEMS

Hagen Trommer and Reinhard H.H. Neubert

Institute of Pharmaceutics and Biopharmaceutics, School of Pharmacy, Martin-Luther-University Halle-Wittenberg, Wolfgang-Langenbeck-Straße 4, D-06120 Halle (Saale), Germany

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ABSTRACT

Purpose: The effects of forty seven different substances (drugs, plant extracts, plant ingredients and polysaccharides) on UV irradiation induced lipid peroxidation were investigated. Methods: Two lipid systems of different complexity were used as in vitro screening models. Iron ions were added as transition metal catalysts. A UV irradiation device was used to create high level radiation. The amount of lipid peroxidation secondary products was quantified by the thiobarbituric acid assay detecting malondialdehyde. Results: The screening for antioxidative compounds for topical administration resulted in new, interesting findings. In the drug testings amantadine, bufexamac, tryptophan, melatonin, propranolol and hyaluronic acid were found to act antioxidatively whereas for ascorbic acid pro-oxidative effects were determined. Buckwheat extract significantly reduced the level of irradiation induced lipid peroxidation as well as the extracts of St. John`s Wort, melissa and sage. The resistant starch novelose 330 and the samples of locust bean gum from a swing mill grinding series showed lipid protection after UV irradiation in the polysaccharide test rows. Conclusions: Human skin is constantly exposed to UV light and oxygen. Therefore, the administration of protectors in cosmetic formulations or sunscreens, as found in this study, may be helpful for the protection of the human skin against UV induced damage. In vivo experiments with substances found as protectors should follow to allow in vitro-in vivo correlation and clinical interpretation of the data.

Corresponding Author: Dr. H. Trommer, Bernhard-Kellermann-Str. 16, D - 04279 Leipzig, Germany. trommer@pharmazie.uni-halle.de

INTRODUCTION

Ultraviolet radiation may damage most important biomolecules, such as lipids, DNA, carbohydrates and proteins severely [1,2]. This is due to initiation of free radical reactions [3]. This process can be catalysed by transition metal ions as electron donors for molecular oxygen [4]. The reaction products of these reductions are highly reactive radical species, such as superoxide ion radicals and the most reactive hydroxyl radicals [5]. If the amount of these oxidative influences is higher than the amount of the components of the human antioxidative defense system a condition called oxidative stress is reached [6,7]. This imbalance and oxidative dysfunction can lead to oxidative damage of physiological structures in the entire organism [8].

The human skin is of particular interest, because it is constantly exposed to ultraviolet radiation, oxygen and other noxious environmental influences [9]. These factors can lead to premature skin ageing and mutation [10,11]. The outermost layer of the human skin, the stratum corneum, possesses a barrier function [12]. Peroxidation of the intercellular lipids of this layer, caused by UV radiation, results in damage to human skin. The decomposition of peroxides to a variety of carbonyl compounds may exert toxic action [13].

Liposomes are microscopic vesicles, usually composed of amphiphilic phospholipids. In aqueous solution, phospholipids spontaneously form these sphereshaped bilayer structures. Liposomes contain two compartments: one is the lipophilic compartment within the membrane, and the other is a hydrophilic compartment between the membranes [14]. They closely resemble the structures of biological membranes. Therefore, they are used as model systems for biological membranes in in vitro investigations [15].

In this study, lipid model systems of different complexity were used as in vitro counterparts of the intercellular lipid matrix of the stratum corneum [16]. For the ultraviolet irradiation experiments an irradiation chamber, allowing selective well-defined UV exposure, was used. For the evaluation of the amount of oxidative damage, the thiobarbituric acid (TBA) assay detecting malondialdehyde (MDA) as a classic lipid peroxidation secondary product was employed [17,18]. It is the most frequently used method for the quantification of peroxidative lipid damage [19]. Other approaches for investigating the consequences of free radical attack on lipids, include, for example, the detection of conjugated dienes by UV spectroscopy or high-performance
liquid chromatography (HPLC), assays of lipid hydroperoxides, analysis of aldehydic degradation products by 2,4-dinitrophenylhydrazine derivatisation or by reaction with 1,3-cyclohexanedione [20].

Transition metal catalysts play a key role in UV induced lipid peroxidation and skin damage. Therefore, iron ions were added to each sample before irradiation. Transition metal ions may act as electron donors for the reduction of molecular oxygen. The reaction products are reactive oxygen species (ROS) such as superoxide ion radicals and the most aggressive hydroxyl radicals. These species can severely damage most skin biomolecules. Furthermore, lipid hydroperoxides are unstable in the presence of transition metal ions. They break down to lipid alkoxyl radicals and lipid peroxyl radicals, which is important for two reasons. Firstly, it generates lipids for the propagation of lipid peroxidation. Secondly, it generates non-radical fragmentation products with biological activity [21].

UV radiation can significantly increase the skin pool of non-heme iron (Fe^{2+}) in dermis and epidermis. Researchers have measured 18 ppm/dry weight of unexposed body parts (buttocks, thigh) versus 53 ppm/dry weight in epidermis biopsies of exposed parts such as cheek, forehead and neck [22]. This is an indirect pathway leading to the formation of dangerous oxygen radicals in addition to the direct damage of biomolecules by UV. Furthermore, Green et al. [23] demonstrated that human skin plays a significant role in iron ion excretion. In a collaborative study a total iron content in the epidermal skin of 22.5 (+/−17.8) mg was calculated from material obtained from eccrine areas. The involvement of iron ions in UV irradiation induced free radical formation in the skin was confirmed by Buettner and Jurkiewicz [24]. The treatment of skin samples with the iron chelator Desferal® resulted in a significant reduction of a spin adduct after irradiation giving evidence to support the key role of iron in UV mediated free radical formation.

Forty seven different substances, polysaccharides, plant ingredients and extracts were tested in this study for their properties to prevent stratum corneum lipids from UV induced oxidative damage in the in vitro lipid model screening systems.

**Material and Methods**

**Reagents**

α-Linolenic acid (LLA), L-α-Dipalmitoylphosphatidylcholine (DPPC), Cholesterol (Chol), Ferrous sulfate, Malondialdehyde-bis-(dimethylacetal), 2-Thiobarbituric acid and Trichloroacetic acid for the thiobarbituric acid (TBA) assay as well as all the drug substances (unless stated otherwise) tested in the screening (all of analytical grade) were obtained from Sigma (Deisenhofen, Germany). Cyclosporin A was kindly provided by Novartis Pharma (Basel, Switzerland). Bufexamac was supplied by Caeio (Hilden, Germany). Hyaluronic acid was a gift from the Institute of Experimental Microbiology Jena (Jena, Germany). The other polysaccharides and swimming samples were provided by the Department of Food Chemistry and Preventive Nutrition (German Institute of Human Nutrition, Potsdam-Rehbrücke, Germany). The extracts of St. John’s Wort, melissa and sage were purchased from Finzelberg (Andernach, Germany), the buckwheat extract came from Phytochem (Ichenhausen, Germany). The plant extracts used in this study were described by the suppliers as tested according to the procedures of Pharm Eur 1997 or comparable guides and complied with the specifications. Methanol of HPLC grade and chloroform (LiChrosolv®) were purchased from Merck (Darmstadt, Germany). Table 1 shows an overview of the substances tested in this study.

**Sample preparation**

Chol, LLA and DPPC as a liposome generator were used as constituents of the lipid model systems. The simple system was an oil-in-water dispersion of LLA. The samples were obtained by shaking the system for 120 minutes using a laboratory flask shaker GFL 3006 (Gesellschaft für Labortechnik, Burgwedel, Germany).

The complex systems were prepared as liposomes consisting of Chol, LLA and DPPC. For the preparation of multilamellar vesicles (MLVs) with a small size distribution, the thin layer method with consecutive extrusion was applied. The lipids were dissolved in 0.5 ml chloroform. The solvent was removed by use of a rotation vaporizer Labo-Rota C-311 (Resona Technics, Switzerland) and vacuum pump PIZ 100 Mini-Tower-MPC 050-Z (Saskia Hochvakuum- und Labortechnik, Ilmenau, Germany). To ensure a thin and homogeneous lipid film a fast rotation speed was chosen (150 rpm). The remaining lipid film was dispersed in 0.5 ml chloroform. The solvent was removed by use of a rotation vaporizer Labo-Rota C-311 (Resona Technics, Switzerland) and vacuum pump PIZ 100 Mini-Tower-MPC 050-Z (Saskia Hochvakuum- und Labortechnik, Ilmenau, Germany). To ensure a thin and homogeneous lipid film a fast rotation speed was chosen (150 rpm). The remaining lipid film was dispersed in 20.0 ml of double distilled water by shaking intensively for 3 hours. To achieve the final concentration, the remaining double distilled water was added. The concentrations of the ingredients in the liposome stock solutions were: DPPC 1.0 mM; Chol, LLA 0.5 mM.
Table 1. Substances tested in the screening study

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Table 1 A</th>
<th>Table 1 B</th>
<th>Table 1 C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probenecide</td>
<td>Diphenhydramine</td>
<td>L-Dopa</td>
<td>Uric acid</td>
</tr>
<tr>
<td>Maprotiline</td>
<td>Ascorbic acid</td>
<td>L-Tyrosine</td>
<td>L-Tryptophan</td>
</tr>
<tr>
<td>Piracetame</td>
<td>Taurine</td>
<td>L-Tryptophan</td>
<td>Propafenone</td>
</tr>
<tr>
<td>Amantadine</td>
<td>Beta-Carotene</td>
<td>Uric acid</td>
<td>p-Aminobenzophenone</td>
</tr>
<tr>
<td>Q10 (Ubiquinone)</td>
<td>L-Tyrosine</td>
<td>L-Tryptophan</td>
<td>Dibenzoylmethane</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>L-Tryptophan</td>
<td>Propafenone</td>
<td>7H-Benzimidazol</td>
</tr>
<tr>
<td>Bufexamac</td>
<td>Beta-Carotene</td>
<td>Uric acid</td>
<td>Ethyl-p-Aminobenzoat</td>
</tr>
<tr>
<td>Melatonin</td>
<td>Melanin</td>
<td>Propranolol</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>Melanin</td>
<td>Melanin</td>
<td>Propafenone</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>Propranolol</td>
<td>Melanin</td>
<td>Melanin</td>
<td>Melanin</td>
</tr>
<tr>
<td>Plant ingredients/Analgesics</td>
<td>Analgesics</td>
<td>Analgesics</td>
<td>Analgesics</td>
</tr>
<tr>
<td>Silibinin</td>
<td>ASS</td>
<td>Fenoprofen</td>
<td>ASS</td>
</tr>
<tr>
<td>S-Ketoprofen</td>
<td>Salicylic acid</td>
<td>Indometacin</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>R-Ketoprofen</td>
<td>S-Ketoprofen</td>
<td>Diclofenac</td>
<td>S-Ketoprofen</td>
</tr>
<tr>
<td>Plant extracts</td>
<td>Plant extracts</td>
<td>Plant extracts</td>
<td>Plant extracts</td>
</tr>
<tr>
<td>Quercetin</td>
<td>St. John’s Wort</td>
<td>St. John’s Wort</td>
<td>St. John’s Wort</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>Melissa</td>
<td>Melissa</td>
<td>Melissa</td>
</tr>
<tr>
<td>Rutin</td>
<td>Sage</td>
<td>Sage</td>
<td>Sage</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>Polysaccharides</td>
<td>Polysaccharides</td>
<td>Polysaccharides</td>
</tr>
<tr>
<td>Acacia gum</td>
<td>Xanthan</td>
<td>Xanthan</td>
<td>Xanthan</td>
</tr>
<tr>
<td>Guar gum</td>
<td>Novelose 330</td>
<td>Locust bean gum</td>
<td>Novelose 330</td>
</tr>
<tr>
<td>Agar agar</td>
<td>Pectin</td>
<td>Pectin</td>
<td>Pectin</td>
</tr>
</tbody>
</table>

The stock solution was passed through a 400 nm polycarbonate filter (Costar, Cambridge, UK) at 20°C under nitrogen using an extruder device (Lipex Biomembranes, Vancouver, BC, Canada) to obtain vesicles of a uniform size. This step was repeated five times. To control the vesicle size distribution and ensure the success of extrusion, the particle diameters were determined via photone correlation spectroscopy using a Malvern Instruments Autosizer 2c equipped with a series 7032 Multi-8 Correlator (Malvern, Worcester, UK).

The exact composition of the samples as well as an overview of the manufacturing processes are shown in Table 2. The concentrations of the test substance stock solutions were as follows: 1 mM for the drugs and analgesics, 1% for the plant ingredients and 0,01 % for the polysaccharides tested. The irradiation of the 5 ml samples was started after 15 min incubation time. An ethanol-water mixture (60:40) was used as a solvent for all the plant extract experiments as well as for the control samples of the plant extract tests.

The final concentrations used in this study for the test substances are given in the relevant figures provided. Ferrous sulfate (10 μM) was added to the samples as an electron donor and catalyst for the Haber-Weiss reaction to initiate ROS generation via a Fenton type reaction. Previous investigations have shown that there is no effect when irradiating lipid model systems without transition metal catalysts [15].

All the liposome suspensions, fatty acid dispersions and test substance solutions were freshly prepared just before use.

UV Irradiation

UV-B irradiation experiments were carried out using a UV irradiation chamber (Dr. Gröbel UV-Elektronik, Ettlingen, Germany) enabling a selective exposure to UV-B due to the special lamp F15/T8 15 W with a main emission range of 290 – 320 nm (Sankyo Co, Tokyo, Japan).

Prior to irradiation, 5.0 ml of each sample (concentrations given in Table 1) were transferred to 55 mm open glass dishes. The optical pathlength
Table 2. The systems used for screening (additionally 10 µM FeSO₄ were added to each sample)

<table>
<thead>
<tr>
<th>System</th>
<th>Lipids</th>
<th>Concentration of lipid (µM)</th>
<th>Manufacturing Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Simple system (LLA)</td>
<td>α-Linolenic acid</td>
<td>100</td>
<td>Shaking for 120 min</td>
</tr>
<tr>
<td>2. Complex system (LLA/CHOL/DPPC)</td>
<td>α-Linolenic acid</td>
<td>100</td>
<td>Liposomes (MLVs)</td>
</tr>
<tr>
<td></td>
<td>DPPC</td>
<td>200</td>
<td>prepared by the thin film method with consecutive extrusion (400 nm membrane)</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

was 2.1 mm and a homogeneous exposure was assumed in spite of light scattering.

The samples were treated with an UV-B dose of 0.25 J/cm² which corresponds approximately with the 10 fold of the minimal erythema dose (MED) for normal pigmented (type II in the skin type classification) people [25]. This high dosage was required to provoke stress conditions.

Thiobarbituric acid assay
The thiobarbituric acid test is a quantitative assay for the detection of MDA, and is the most frequently used technique to determine lipid peroxidation products.

In this study, the Buege-Aust method of the TBA assay was applied [26]. Two millitres of a stock TBA reagent containing 15 % (w/v) trichloroacetic acid in 0.25 M HCl and 0.37 % (w/v) thiobarbituric acid in 0.25 M HCl were added to 1.0 ml of the UV-B treated sample. After heating at 90° C for 15 minutes and cooling , the red TBA:MDA-complex (2:1) appears and allows fluorescence measurement. An HPLC system (Merck-Hitachi, Darmstadt, Germany) equipped with an autosampler AS-4000A, interface D-6000A, pump L-6200A, UV-VIS-Detector L-4250 and a fluorescence detector F-1080 was used to quantify the pigment. A reversed phase column (LiChrospher® 100, RP 8, particle size 5 µm) was used with a mobile phase of methanol/water (30/70). The excitation wavelength was 515 nm and the emission measurement was performed at 555 nm.

A calibration curve was generated using MDA which was formed from malondialdehydebis-(dimethylacetal) under acidic conditions.

Statistical analysis
All data shown represent the mean values ± SD of the measurements (n = 6). Statistical analysis of the effects of the different test substances or extracts on the TBA-RP concentration after UV-B irradiation was performed using a one-way ANOVA.

In all cases, post-hoc comparisons of the means of individual groups were performed using Dunnett’s multiple comparison test. A significance level of P < 0.05 (*) between groups was accepted as being statistically significant. All calculations were performed using GraphPad Prism 2.0 (GraphPad Software Inc., San Diego, CA, USA).

Results and discussion

Development of suitable model systems for an antioxidative screening
In search for novel, unknown antioxidants for topical application, a suitable model was required. There were two standards which these model systems should meet. Firstly, these systems should be simple to avoid an overlapping of effects which would make data interpretation difficult or impossible. Secondly, the main properties of the stratum corneum intercellular lipid matrix should be present to assure similarity to the horny layer of human skin. A simple system containing only one stratum corneum lipid was used for the experiments. Furthermore, a complex system which is similar to the real horny layer of the skin was generated by stratum corneum lipid addition.

Substances belonging to the physiological horny layer lipid matrix were chosen as model lipids for the experiments. Chol, LLA and DPPC as a liposome generator were used for system modelling.
The test substances had to have at least one of the following properties to be included in the screening study:

1. The exact mechanism of action of the substance is not currently known in detail.
2. The substance is present in skin physiologically.
3. The substance may act as an antioxidant due to its structural properties.
4. The incorporation into modern semisolid formulations for topical application is possible from a pharmaceutical and technological point of view.
5. Although the substance has another main effect, antioxidative behaviour may be assumed and therefore screening is worthwhile.

**Thiobarbituric acid assay**

**Drugs**

Figure 1 depicts the results of the thiobarbituric acid assay of the simple stratum corneum lipid model system when adding different drugs as test substances. The effects of 100 µM screening substance on a LLA dispersion are shown. Figure 1A shows surprising effects for ascorbic acid and amantadine. When adding ascorbic acid the secondary lipid peroxidation products (measured as malondialdehyde units) are significantly increased. Conflicting data concerning the interactions of vitamin C with ROS has lead to controversy in the literature. It is reported that ascorbic acid may have both antioxidant and pro-oxidant properties [27,28]. Genotoxic effects were found and this was suggested to be a result of the ability of ascorbic acid to decompose lipid hydroperoxides to DNA damaging secondary products [29].

Recently, we have shown that ascorbic acid exhibits concentration dependent pro-oxidative effects on lipid model systems of differing complexity. The molecular mechanism of ascorbic acid degradation after its lipid damaging action has been demonstrated by mass spectrometry and detailed studies regarding the redox properties of ascorbic acid were carried out by EPR investigations [16]. Firstly, ascorbic acid reduces Fe^{III} ions. More Fe^{II} ions are available and the Fenton reaction can run at a higher level leading to more peroxidized lipids. Following the results of Lee et al. [29], more substrate for ascorbic acid to decompose is in the system. The amount of cell toxic aldehydes is increased which may be the reason for the increased levels of the TBA assay.

The drug amantadine, used for Parkinson disease treatment, was included in the screening experiments as well. The lipid protective effect of amantadine in this study can be explained by a Schiff base reaction where the amino group of amantadine reacts with the carbonyl group of malondialdehyde resulting in the formation of azomethine. Hence, the analyte is masked and cannot be quantified properly by the TBA assay. This corresponds with the results of Albrecht-Goepfert et al. [30]. Several aminoadamantane derivatives have been tested for their influences on ROS. Neither radical scavenging nor singlet oxygen quenching was observed. On the other hand, topical application of amantadine could be of advantage because skin damaging carbonyls can be neutralised this way.

Figure 1B depicts the protective effects of the non-steroidal antiphlogistic drug, bufexamac and the amino acid tryptophan. While antioxidant effects of the melatonin precursor, tryptophan, have already been described in the literature [31], those properties of bufexamac are a new finding. Recently, we have shown that bufexamac exhibits antioxidative effects on both lipid model systems and HaCaT keratinocytes. EPR spectroscopy was used for detailed studies regarding the radical scavenging properties of bufexamac. The molecular mechanism of the bufexamac fragmentation and degradation after UV exposure has been demonstrated by mass spectrometry [18].

Some of the often described naturally-occurring antioxidants, such as beta-carotene, ubiquinone and uric acid [32], surprisingly showed no effects in this study (Figure 1B).

The significant lipid protective effects of melatonin, melanin, propranolol and hyaluronic acid on the LLA dispersion after UV irradiation are demonstrated in Figure 1C. The results for melanin may be due to its filtering properties of UV radiation. This is because the complex tyrosine derivative, after its in vivo synthesis in the melanocytes of the skin, is responsible for the protection of the DNA. It is also considered to be a radical scavenger [33]. A very interesting result is the inability of substances usually utilised in sunscreens to protect the lipids from UV induced peroxidation. The substances which were used to represent these UV filter molecules were the following: dibenzoylmethane, 7H benzimidazol and ethyl-p-aminobenzoate. All three chemicals were ineffective in providing significant lipid protection after UV irradiation in this study.

Antioxidative properties of propranolol [34] and melatonin [35,36] can be found in the literature. Melatonin has been found to protect human skin from UV induced erythema in an in vivo study when topically used on its own or applied in combination with vitamin E and C [37].
Figure 1. Concentration of thiobarbituric acid reaction products (TBA-RP Conc.) and influence of ultraviolet irradiation and different test drugs (100 µM each) in the LLA screening system.

The antioxidative properties of hyaluronic acid were recently shown to be due to transition metal scavenging. The fragments of hyaluronan with smaller molecular weights resulting from enzymatic digestion showed antioxidant properties as well [17].

**Analgesics**
Due to the bufexamac results more analgesics were tested showing protective effects of diclofenac [38] and ibuprofen [39] together with pro-oxidative influences of both ketoprofen enantiomers (Figure 2A). The structures of all analgesics tested are depicted in Figure 2B. The properties of ketoprofen should be known because the instruction leaflet of ointments containing this substance warns about sunlight exposure and other UV treatment of the skin when using the formulation [40]. The phototoxicity of the ketone after UV irradiation of the molecule has been explained by the generation of hydrogen peroxide determined by capillary electrophoresis experiments [41]. Its analogue fenoprofen showed no effects on the amount of MDA after UV irradiation and for ibuprofen lipid protective effects were measured. Thereby, the phototoxic properties of ketoprofen must be connected with its benzophenone structure (Figure 2B). Acetyl-salicylic acid and salicylic acid failed to protect lipids from oxidative damage and the addition of indometacin lead to a slight augmentation of the MDA amount after UV stressing.

**Plant ingredients and plant extracts**
The properties of flavonoids to act as naturally occurring antioxidants in food and their potential role in the prevention of chronic diseases are well-known and have been reviewed [42,43]. The antioxidative behaviour of these polyphenolic plant secondary metabolites is attributed to reductive, radical scavenging, singlet oxygen quenching and transition metal chelating properties of flavonoids. Recently Kessler et al. [44] published a detailed study of the relationship between the structure and the radical scavenging potential of the flavonoids and used this data to explain the anti- and pro-oxidant activity measured during their experiments with rutin and other quercetin derivatives.

Among the four flavonoids tested in our study only the St. Mary’s thistle ingredient silibinin and the flavone quercetin showed a lipid protecting property when added to the samples before UV irradiation. Hesperetin and rutin did not act antioxidatively in this study (Figure 3A).
The benzodioxane derivative silibinin is used as an hepatoprotective substance and lipid peroxidation and prostaglandine synthesis inhibiting properties have been previously described [45,46]. Figure 3B shows the structures of the flavonoids tested in this study. The results here support the statements of Boehm et al. [43] that glycosylations of the oxygen in position 3 of the C ring and 3’ or 4’ of the B ring clearly reduce the radical scavenging properties of the molecules. This was observed when comparing the effects of quercetin and its glycoside derivative rutin.

The methylated flavanone hesperetin also failed in protecting lipids from UV induced damage. This may have been due to the decrease of antioxidative power of hesperetin compared to quercetin as there are not enough hydroxyl groups present in the hesperetin molecule. Boehm et al. [43] considered 4 to 6 hydroxyl groups as an optimal requirement for radical scavenging with no increased effects by compounds with more than 6 hydroxyls. Our results are in accordance with this. We found antioxidative properties for silibinin and quercetin which contain 5 hydroxyl groups.
**Figure 3.** A) Concentration of thiobarbituric acid reaction products (TBA-RP Conc.) and influence of ultraviolet irradiation, flavonoids (100 µM each) and plant extracts (0.2 % each) in the LLA screening system. B) Structures of the flavonoids tested. C) Concentration of thiobarbituric acid reaction products (TBA-RP Conc.) and influence of ultraviolet irradiation and buckwheat extract (0.1 %, 0.2 %, 0.5 %) in the simple and complex screening system.
All the plant extracts used in this antioxidant screening showed helpful effects in decreasing the amount of UV irradiation induced lipid peroxidation. The extracts of St. John’s Wort, melissa and sage (Figure 3A) as well as buckwheat extract acted as protectors. (Figure 3C). The properties of the St. John’s Wort extract are due to its flavanoid and phenolic acid content [47] as well as the antioxidative behaviour of the sage extract [48]. The performance of melissa extract is explained by the polyphenols which the plant synthesises i.e. rosmarinic acid, chlorogenic acid, ferulic acid and caffeic acid [49,50]. The buckwheat extract effects on the lipids are demonstrated by showing the results of both simple and complex screening systems. The flavonoid fraction of Fagopyrum esculentum contains several flavonoids such as the aglycone quercetin which showed an antioxidative effect when tested as a single flavanol. The antioxidative properties of buckwheat extract towards oxidative stress has been shown in various in vivo and in vitro systems [51].

Remarkably the TBA-RP concentration levels after irradiation were significantly below the measured value of the non UV irradiated control using St.John’s Wort and the buckwheat samples (0.1%. 0.2%, respectively). This means that these extracts are able to prevent UV induced lipid peroxidation and furthermore have the ability to reduce the content of already existing aldehydic lipid peroxidation secondary products. This may be explained by a UV catalysed carbonyl reaction of MDA with some of the other plant extract ingredients, such as tanning agents, bitter constituents or essential oils.

Considering topical administration of the substances found to be lipid protective one has to bear in mind the phototoxic potential of the St. John’s Wort ingredient, hypericin. Apparently, for the antidepressive action of St. John’s Wort formulations all the plant ingredients are necessary. For topical administration further investigation is required to ascertain whether the antioxidative properties remain after having hypericin isolated in order to find out which of the St. John’s Wort ingredients explicitly account for the antioxidative properties. The advantages of a topical application of melissa and sage extract are the abilities of caffeic acid and ferulic acid to penetrate through the stratum corneum into the deeper cutaneous layers and provide protection of human skin there. This was shown as a vehicle pH independent process in vitro and in vivo by Saija et al. [49].

**Polysaccharides**

More polysaccharides have been tested for their antioxidative properties in the LLA screening system because of the protective effects of hyaluronic acid (Figure 1C). In Figure 4A the resistant starch novelose 330, a nutrition fibre, significantly decreased the amount of MDA after UV irradiation. Furthermore, acacia gum, agar agar, alginic acid, guar gum and xanthan have been tested without displaying any protective effects.

Figure 4B shows the results of the TBA assay of pectin and locust bean gum ground by a swing mill for different time intervals. Whereas pectin showed pro-oxidative effects, the locust bean gum was able to reduce the amount of lipid peroxidation secondary products after UV radiation treatment. A correlation between protective behaviour and milling time was not observed. Antioxidant activity of carob tree (Ceratonia siliqua) plant material has been shown [52]. Polyphenols were extracted from carob pods and the in vitro antioxidant activity of the crude polyphenol fraction was evaluated.

Lipid peroxidation reducing effects for glycosaminoglycans have been described [53]. The mechanism of these lipid protecting effects of these polysaccharides seems to be the chelation of transition metal ions. Sipos and co-workers suggested complexes of the kind L[Fe(OH)3]n where L is the polysaccharide monomer [54]. This chelation can lead to anti- and pro-oxidative effects and therefore, has to be tested in every isolated case [55]. Recently the effects of different metal ions including Zn, Co, Mn etc. on the oxidative damage and the antioxidant capacity of hyaluronan have been investigated and compared with iron [56].

Further investigations need to test the compounds found as protective in this study for their in vivo influences, for example, on the MED after topical application on subjects. This is of importance for in vivo evaluation of the generated data and its clinical interpretation. It is also timely to assess the stability of the antioxidants in several formulations using the preformulation approach [57] and examine the penetration patterns of the tested substances [58].

**CONCLUSION**

Forty seven substances and extracts were screened for antioxidative effects using an in vitro lipid model system. For amantadine, bufexamac, tryptophan, melatonin, melanin, propranolol, diclofenac, ibuprofen and hyaluronic acid, lipid protective effects were measured using the TBA
assay. The flavanoids silibinin and quercetin and the plant extracts of St. John’s Wort, melissa, sage and buckwheat were able to reduce the amount of secondary lipid peroxidation products as well. St. John’s Wort, sage and buckwheat extracts were capable of lowering the TBA assay levels of the UV irradiated test samples below the value measured for the non-irradiated control. Among the polysaccharides tested, the resistant starch, novelose 330 and the different samples of locust bean gum showed antioxidative properties. On the other hand, pro-oxidative effects were measured for ascorbic acid, ketoprofen and for the pectin samples.

These results demonstrate a new approach for skin protection by topical application of substances with antioxidative potency.

Considering human skin as the outermost organ of the human body and its constant exposure to UV light and oxygen, combined with an increased iron ion content of the UV exposed skin, topical administration of some of the substances found in this study, when used in cosmetic and pharmaceutical semisolid formulations, could be protective for the lipids in human skin. The use of substances with small molecular weights which are easy to handle or of molecules naturally occurring in human skin may be of special advantage.

REFERENCES


[51] Mukoda, T., Sun, B. and Ishiguro, A., Antioxidant activities of buckwheat hull extract toward various oxidative stress in vitro


