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THE UV-B PROTECTIVE EFFECT OF HUMIC SUBSTANCES PROVIDE THE BASIS FOR THE DEVELOPMENT OF A PEAT LIPSTICK

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SUMMARY

Humic acids (HA) known for their antiviral and UV-B protecting effects are considered promising candidates for developing a photoprotective lipstick which should minimize or even prevent the risk of UV-induced herpes recurrences.
In this study, UV/VIS spectra of natural HA, synthetic HA-like substances and other lipstick components were analyzed to find out the most appropriate UV-absorbing ingredients for the product under development. The results show the expected high absorption degree of all HA in the UV-B range, but reveal certain differences in the UV-A range. Moreover, the contribution of matrix components to the total UV-B absorption of the lipstick was proved. The results 24 hours after UV exposition demonstrated significant dose-dependent UV-B protective effects of the tested HA similar to those of p-aminobenzoic acid (PABA) used as UV-B absorbing reference substance.

KEY WORDS: humic substances, UV-A absorption, UV-B absorption, UV-protective effect, U937 cells

INTRODUCTION

The sunlight that reaches the earth's surface has a wide spectrum consisting of 52 % visible light, 42 % infrared radiation, 5.6 % UV-A and 0.4 % UV-B radiation. UV-B radiation has both, positive and negative effects for human skin. On the one hand it is necessary for the activation of melanin generation in the skin and for the development of delayed pigmentation that protects like a natural sunshade the skin against UV light. On the other hand, prolonged exposure to UV-B radiation can cause skin erythema, sunburn and the formation of mutagenic DNA lesions capable of inducing skin cancer as a long term effect. UV-B radiation is also a major provocation factor for the reactivation of herpes labialis, a recurring viral infection caused by herpes simplex virus type 1.

Humic substances are known for their UV absorbing properties and show certain structural similarities to melanin. They have been proved to be effective against UV radiation in bacteria (Bitton *et al*., 1972; Muela *et al*., 2000) as well as in human cells (Hübner 2004; Klöcking *et al*., 2004; Kühn, 2005). Moreover, humic substances are antivirally active against various DNA and RNA viruses (Klöcking *et al*., 2006) and have anti-inflammatory effects (Klöcking *et al*., 1968).

The practical significance of the UV protecting effect of humic substances is currently studied in the BMBF supported research project 'Peat lipstick', which aims at humic substances as photoprotective agents in a lipstick to prevent herpes-simplex virus reactivation by UV-light.

MATERIALS AND METHODS

Test substances

The analyzed samples are natural humic acids extracted with 0.1 mol/l sodium hydroxide from Altteich Peat (North-Eastern Saxony). Furthermore we analysed synthetic humic substances prepared by oxidation of caffeic acid (→Na-KOP 466), hydrocaffeic acid (→Na-HYKOP), and 3,4-dihydroxyphenylalanin (→DOPA-OP) (Klöcking *et al.*, 2006). Humin Feed, the sodium salt of a brown coal humic acid (Humintech, Düsseldorf, Germany) and castor oil were also studied either individually or in combination with each other. Paraaminobenzoic acid (PABA) served as UV-B absorbing reference substance (Klöcking *et al.* 2004).

Determination of Absorption

Using 96-well microplates UV-Star® (Greiner Bio-One GmbH), the UV-Vis spectra were analysed with the spectrophotometer Synergy HAT Multimode reader for microplates (BioTek). All samples were measured at three concentrations (10, 100 and 1000 µg/ml) and at a layer thickness of 0.7 mm (100 µl).

Cell culture

The human cell line U937 was cultivated in RMPI 1640 medium containing 10% FBS and incubated at 37 °C in a 5% CO_2 containing, humid atmosphere. For determining the cell number, 100 µl of the cell suspension was counted in the Neubauer counting chamber. The live / dead staining with trypan blue shows living cells colorless, dead cells stained blue. The cell number was adjusted to 5 x 10^5 cells/ml by the addition of RPMI medium.

UV irradiation and determination of UV-induced cell damage and UV protection

Cells were irradiated using the microprocessor-controlled device Bio-Sun (Vilber Lourmat). For this purpose, 100 µl of the cell suspension each was transferred into the wells of a 96-well flat-bottom microtitre plate. Before irradiation, a UV-transparent 96-well plate (UV-Star®), was placed on top of the primary (lower) plate. The wells were filled each with 100 µl humic substance dilution. The "doubleplate" was exposed to UV-B radiation at a wavelength maximum of 312 nm and a irradiation dose of 80 mJ/cm². After that, cell cultures were incubated at 5 % CO_2/37 °C for 24 hours. This was followed by determining the cell toxicity employing the XTT tetrazolium reduction assay (Roche Diagnostics) according to manufacturer's specifications. The optical density (OD) of formazan produced by mitochondrial dehydrogenases was measured after 3-h incubation at 37 °C at a wavelength of 450 nm (reference wavelength 620 nm). The UVB-protecting effect (PE) was calculated from the measured OD values by the following equation:

$PE_{UV-B} = \frac{x_i - x_0^*}{x_0 - x_0^*} \cdot 100\ \%$, where x_0 is the OD of *non-irradiated cell controls*, x_i is the OD of *irradiated cells treated with humic substances* and x_0^* is the OD of *irradiated untreated cells*.

RESULTS

Figure 1 shows the UV-VIS spectra of the analyzed humic acids and of *para*-aminobenzoic acid between 280 and 800 nm.

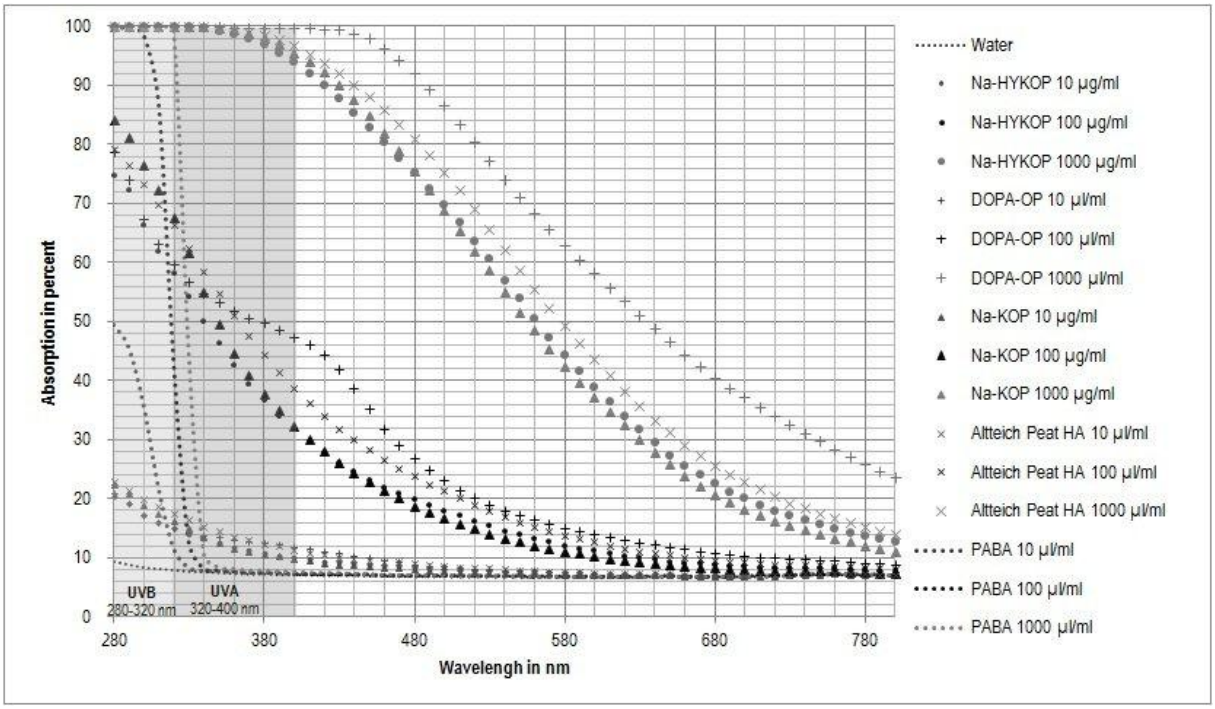

Fig. 1. Absorption spectra (280-800 nm) of HYKOP, DOPA-OP, Na-KOP 466, Altteich Peat HA and PABA; test substance concentrations: 10, 100 and 1000 µg/ml; layer thickness 0.7 mm.

The spectra of humic acids show the expected typical curves. They are characterized by high absorption in the higher-energy (shorter wavelength) areas of the spectrum and by increasingly less absorption in the lower-energy areas (long wavelengths). The three measured humic substance concentrations are mirrored by three well recognizable curve groups. By contrast, PABA has a significant UV absorption with a maximum at about 280 nm, but does hardly absorb UVA radiation. Humic substances at concentrations of 100 µg/ml absorb between 60 (320 nm) and 84 (280 nm) percent UV-B and about 50 (360 nm) percent UVA. At the highest tested concentration (1000 µg/ml) the test humic substances provide a total filtering of UV-B light and absorb over 95 percent of UVA whereby DOPA-OP proved to be most effective.

Figure 2 presents the spectra of two experimentally used lipstick ingredients (Humin Feed and castor oil) as well as of three combinations of both. Castor oil is one of the basic matrix components of lipsticks that contain usually 50 to 70 percent of this oil. It is characterized by a relatively high UV absorption with a maximum at 269 nm, but does hardly absorb visible light. The question was, whether castor oil due to its high concentration in the stick does influence the UV absorption of the lipstick matrix as a whole. In contrast to humic substances, the oil was measured as pure, undiluted fluid.

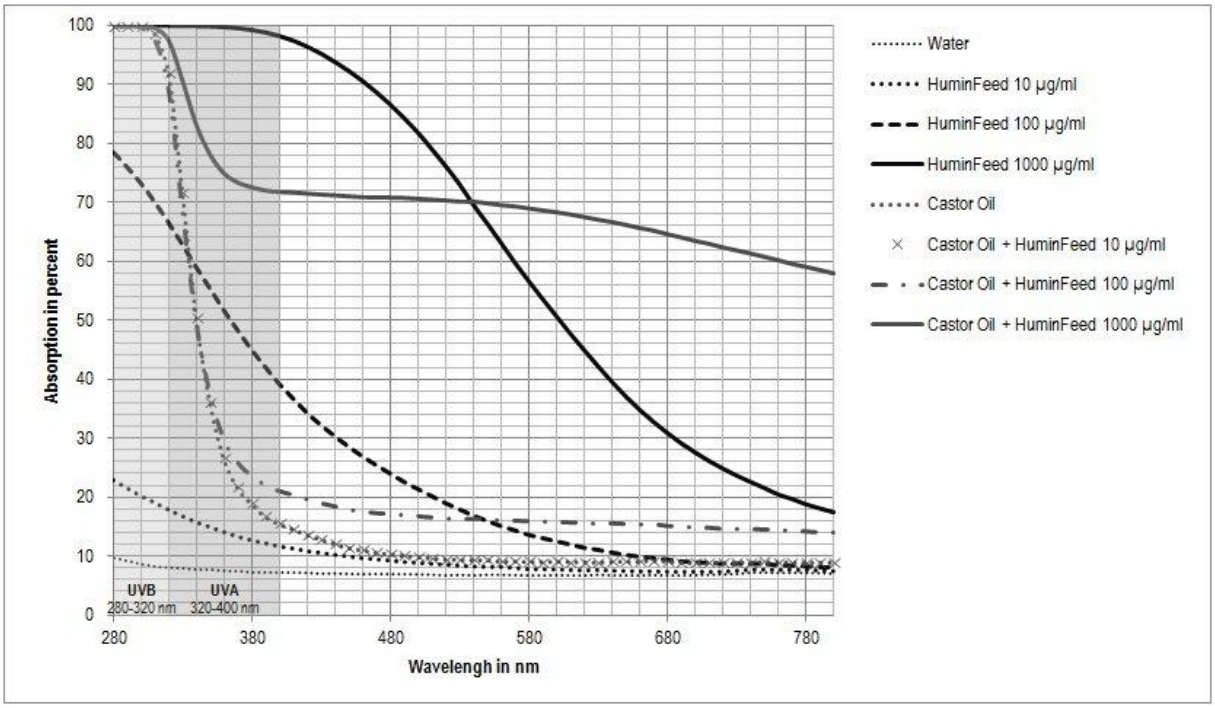

Fig. 2. Absorption spectra (280-800 nm) of Humin Feed, Castor Oil and various mixtures of Humin Feed and Castor Oil; Humin Feed concentrations: 10, 100 and 1000 µg/ml; layyer thickness 0.7 mm.

As shown in Figure 2, the absorption curves of the brown coal humic acid Humin Feed correspond largely with those of the Altteich peat humic acid in Figure 1. The data from the combination of Humin Feed with castor oil, however, show a surprising picture. The UV-A absorption of 100 and 1000 µg/ml Humin Feed is clearly diminished whereas the absorption at wavelengths >560 nm is clearly enhanced. This may indicate the formation of new, previously unknown compounds as a result of the reaction of castor oil with humic acids. A more detailed analysis of interactions between UV-absorbing substances and matrix ingredients is of high interest and will be the issue of future studies.

In the next step of investigations we examined the UV-protective effect of HYKOP, DOPA-OP, Na-KOP 466, Altteich Peat HA and PABA in human U937 cells. According to Kühn (2005) we used a test arrangement where cells and test substances were placed in two separate plates which enabled us to evaluate the UV filtering effect independent of possible interactions with the test substance. The results are shown in figure 3.

At low concentrations (10 µg/ml) Na-KOP and Altteich Peat HA have 24 h after irradiation only a little protecting effect on the cells. In contrast, all humic substances at concentration of 100 and 1000 µg/ml protect the U937 cells by more than 60 percent. PABA at 100 µg/ml is able to completely protect cells against UV-induced damage. A nearly complete UV protection is also provided by humic acids at the highest test concentration of 1000 µg/ml.

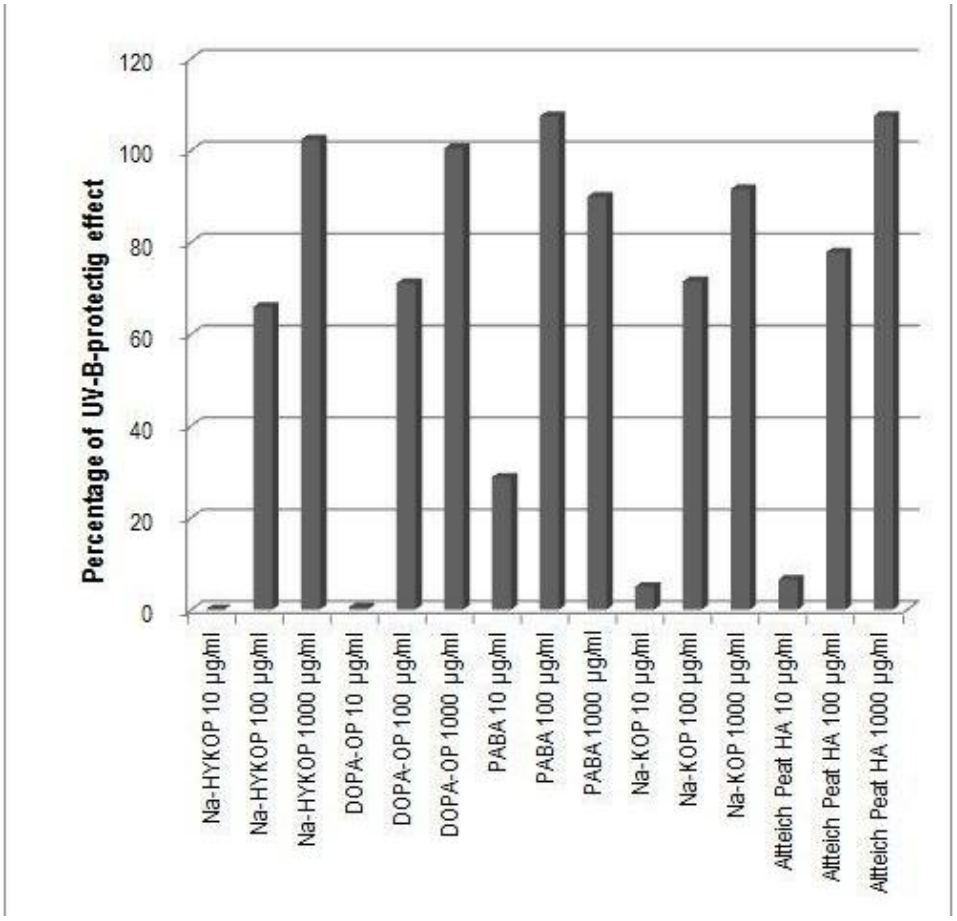

Fig. 3. UV-B protective effect of HYKOP, DOPA-OP, Na-KOP 466, Altteich Peat HA and PABA in U937 cells; XTT test 24 h after irradiation;

The brown coal humic acid Humin Feed signals a similar behaviour (Fig. 4). Whereas the UV protecting effect is negligible at 10 g/ml, it amounts to about 75% at 100 µg/ml and reaches 100 % at the highest test substance concentration (1000 µg/ml).

Figure 4 shows that castor oil alone protects the cells against UV-B irradiation by about 73.5 %. The effect is increased by adding a little amount of Humin Feed (10 µg/ml) to castor oil up to about 80 %. At the next concentration step (100 µg/ml Humin Feed) the mixture provides almost 95 % UV protection. Complete UV protection was reached by 1000 µg/ml Humin Feed with and without castor oil. From this it follows that the degree of UV protection may benefit especially at low and mean humic substance concentrations from the presence of UV-B absorbing oils.

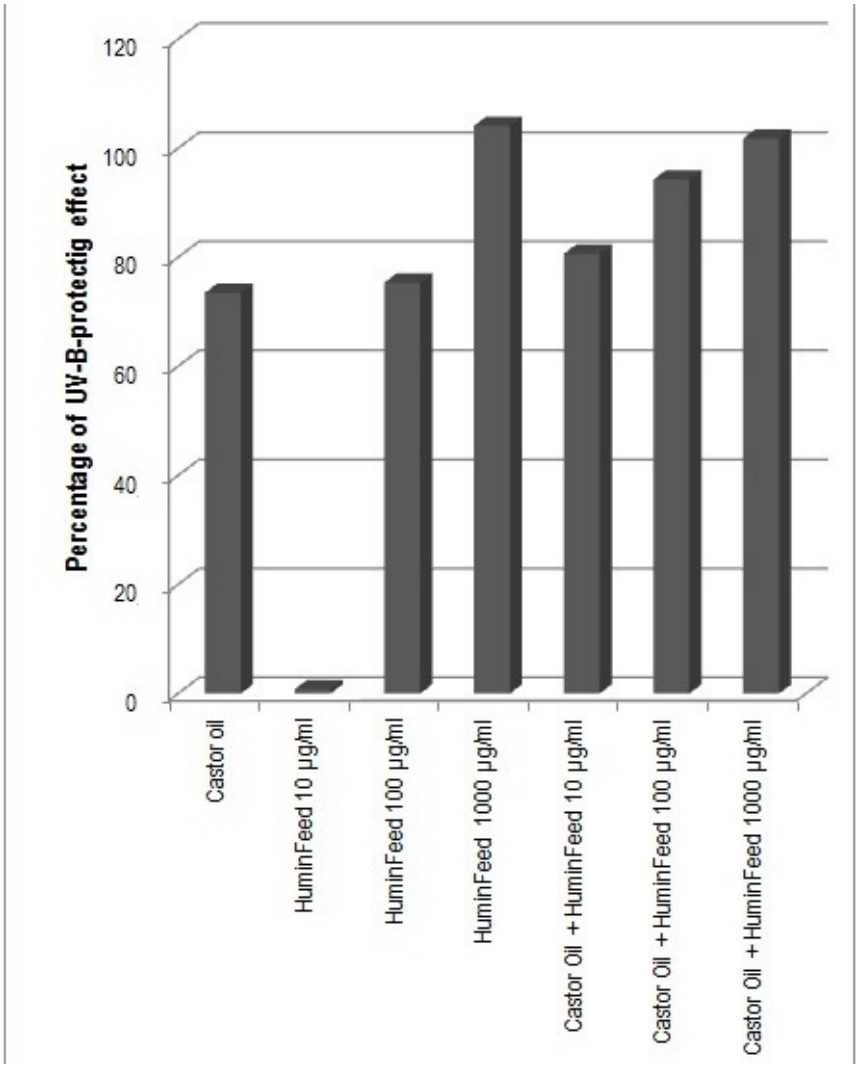

Fig. 4. UV-B protective effect of Humin Feed and HuminFeed-Castor Oil Mixture, 24 h after irradiation length of optical path in the wells of the upper plate 0.7 mm through 0.7 mm substance.

CONCLUSIONS

Aiming at the development of a UV-protecting peat lipstick, we studied two natural humic acids and three synthetic humic acid-like substances for their UV-Vis absorption as well as for UV-protecting effects in human cell cultures. The results of these studies are consistent with the following conclusions.

First, all humic substances tested are capable of strongly absorbing UV-B and, to some extent, also UV-A radiation.

Second, the obtained UV absorption spectra correlate well with the UV-B protective effect of the appropriate humic substances in cell cultures.

Third, pure castor oil as a usual lipstick component absorbs UV-B radiation to a higher degree and exerts also a UV-protective effect to human cells. In combination with the browncoal humic acid Humin Feed castor oil supports the UV-B absorption of humic acids, but decreases the absorption of UV-A. It will be an aim of future studies to elucidate possibly underlying molecular interactions between individual lipstick components.

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