
Citrus Flavonoids with Skin Lightening Effects – Safety and Efficacy Studies
Melanin, the substance responsible for the pigmentation of the skin, is produced to protect the DNA from harmful, mutagenic UV radiation. Age spots or solar lentigines are age-related and UV irradiation-induced pigmented spots that usually occur on sunlight-exposed areas such as the face, the back of the hands, and the upper chest. The rate-limiting enzyme in melanin formation is polyphenol oxidase tyrosinase (EC 1.14.18.1) which catalyses the oxidation of tyrosine to dopaquinone (4). The subsequent steps of melanin formation from dopaquinone can occur spontaneously at physiological pH. Dopaquinone is oxidized to dopachrome and through further oxidation reacts to melanin. Many of the currently used depigmenting agents are tyrosinase inhibitors, as it seems the most straightforward way to inhibit melanin formation. Although more recent in-depth knowledge of melanocyte biology and the processes underlying melanin formation has produced other interesting targets for the treatment of hyper-pigmentation, such as the inhibition of translation and maturation of tyrosinase, stimulation of degradation and acceleration of skin turnover. The best known skin whitening agent is hydroquinone, which has been used for over 50 years now although some controversy about it has come up in recent years. Long term use of high concentrations of hydroquinone can produce side effects like ochronosis, a thickening and darkening of skin, especially of dark-skinned individuals (5) and general safety concerns have been brought up by regulatory agencies all over the world. Therefore there is still a need for safe and effective skin lightening products and the hunt for natural skin lighteners is ongoing. Flavonoids form major constituents of the human diet as they contribute to the flavour and colour of many fruits and vegetables. Their beneficial antioxidant effects are thoroughly studied and established. The highest content of citrus flavonoids can be found in unripe citrus fruits (7).
These unripe fruits can be collected by thinning out, which is necessary for a good harvest of ripe fruits later on (8). Whole ground fruits are extracted with a water methanol mixture to yield the highest flavonoid content. The aim of this study was to find the ideal composition of flavonoids from various citrus fruit extracts to use as an effective skin lightening cosmetic ingredient.

Materials and Methods

All solvents from Merck, all other chemicals from Sigma Aldrich

Raw material
The various citrus extracts were bought from different extract manufacturers and analyzed by HPLC for their content of naringin, narirutin, hesperidin, and neohesperidin.

HPLC instrumentation
HPLC separation was carried out on a Surveyor HPLC system with degasser, binary high pressure mixing pump, column thermostat, and auto sampler. The HPLC was coupled to a DAD detector and data acquisition and processing was performed using ChromQuest Chromatography Data System (CDS) (all Thermo Fisher Scientific).

Separation of flavonoids
On a C18 column (Utisphere OBD, 120Å, 150 x 4.6 mm, 5um) an isocratic elution with 75% water, 10% methanol, 10% acetonitrile, and 5% acetic acid 99% was performed with a flow of 1 ml/min and subsequent UV detection at 285 nm.

Tyrosinase inhibition
Mushroom tyrosinase (333 U/ml, EC 1.14.18.1, Sigma Aldrich, Switzerland) was incubated in 18 mM phosphate buffer, extracts and tyrosine solution (final concentration of 1 mM) were added and absorbance was measured over 5 hours at 490 nm.

Cellular human tyrosinase activity test
Normal human epidermal melanocytes (NHEM, Bioalternatives, France) were incubated for 72 hours with test compound and control. After incubation, culture medium was removed and cells washed with PBS. The tyrosinase enzyme was extracted with Triton X in PBS and then incubated with 2 mM L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate. After incubation enzymatic activity was measured at 540 nm (ThermoMax micro plate reader, Molecular Devices).

Cytotoxicity
Normal Human Epidermal Melanocytes (NHEM, Bioalternatives, France) were incubated with concentrations from 0.0015 to 1.14 mg/ml of flavonoid mixture for 72 hours. Then a solution of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added, after incubation at 37 °C for 4 hours, the media was discarded and 100 ul DMSO was added to each well. The optical density of the dissolved residues was measured at 540 nm (Microplate reader, Molecular devices).

Liposomal encapsulation
The flavonoid mixture was mixed with glycerin, lecithin, tocopherol, sodium ascorbate, and homogenized with a high pressure homogenizer (Microfluidics, Lampertheim, Germany). Subsequently the liposome size was determined using a Zetasizer Nano ZS90S from Malvern Instruments (Worcestershire, United Kingdom).

Formulation
1% of the liposomal solution was incorporated into a lotion (Table 1) for application studies (patch tests and in vivo efficacy). The formulation was tested for stability towards temperature and time.

Patch tests
For both, single and repeated patch tests 25 healthy volunteers between 18 and 60 years were selected. The dorsal skin was cleaned with 70% alcohol before

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<th>Component</th>
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<td>Flavonoid mixture, Citrolumine 8™</td>
<td>Glycerin, Aqua, Lecithin, Citrus Paradisi (Grapefruit) Fruit Extract, Citrus Aurantium Amara (Bitter Orange) Fruit Extract, Sodium Ascorbate, Tocopherol, Heliantus Annus (Sunflower) Seed Oil</td>
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Table 1: Frame formulation for the liposomal encapsulated citrus flavonoid blend, as used in the application studies.
product application and then 1% of lipoosomal-encapsulated flavonoid mixture in a lotion was applied using a Finn Chamber (7 mm). The product was left in contact with the skin for 48 hours (single patch test) or 24 hours and reapplied 4 times (repeated patch test). The cutaneous reactions were evaluated 15 minutes, 1 hour, and 24 hours after removal of the product. One chamber with distilled water was used as negative control and for the single patch test only a positive control was applied with 0.5% sodium dodecyl sulphate (SDS).

Application study
Caucasian skin: Six healthy female volunteers of Caucasian skin type (all Fitzpatrick skin types II) were recruited for application of the lotion or a control (formulation without active ingredient). The cream was applied twice a day during 8 weeks on the back of the hand and the face. Melanin Index was measured on the back of the hand and the face with a Skin Pigment Analyzer, SPA99 (Courage & Khazaka, Köln, Germany) and photographs were taken from the face and back of the hand with VisioFace Quick (Courage & Khazaka). Asian skin: Six healthy female volunteers of Asian skin type were recruited for application of the lotion on the external forearm. The lotion was applied twice daily during 8 weeks. Skin color was measured with a Chromameter CR300 (Konica Minolta, Dietikon, Switzerland). All volunteers were measured and photographed 3 times; at day 0 (baseline), day 28, and day 56.

Results

Raw material
The various citrus extracts were tested by chromatographic separation for their content of narirutin, naringin, hesperidin, and neohesperidin. Two extracts were chosen, one from unripe citrus paradisi (grapefruit) and one from unripe citrus aurantium amarum (bitter orange) fruit and mixed. The resulting mixture of raw materials resulted in the following flavonoid content of 4.9% narirutin, 22.0% naringin, 1.0% hesperidin, and 5.3% neohesperidin (Fig. 1).

Tyrosinase inhibition (mushroom tyrosinase)
Inhibition of mushroom tyrosinase was tested in a concentration range from 0.025 to 3 mg/ml. Up to 1 mg/ml inhibition increased dose dependently up to 40% of untreated control, in concentration higher than 1 mg/ml there was no further increase possible (Fig. 2). The flavonoid mixture showed an estimated IC50 of 0.75 mg/ml.

Cellular human Tyrosinase activity test
Tyrosinase activity was tested after incubation of NHEM with 0.004 to 0.4 mg/ml flavonoid mixture for 72 hours and subsequent tyrosinase extraction of the cells. As a control 0.02 mg/ml of kojic acid was used. A concentration of 0.004 mg/ml of flavonoid mixture reduced the tyrosinase activity to 66%, 0.04 mg/ml to 65%, and 0.4 mg/ml to 40%, whereas kojic acid reduced tyrosinase activity to 61% at 0.02 mg/ml (Fig. 3).

Viability/ Cytotoxicity
Up to 0.4 mg/ml flavonoid mixture viability was increased up to 130%, at 1.12 mg/ml a reduction of viability to 83% was measured (Fig. 4). A concentration of 3.3 mg/ml resulted in no remaining viability (data not visible).
Safety tests
Ames reverse mutation assay (OECD No. 471) using *Salmonella typhimurium* and *Escherichia coli* showed no mutagenic potential of the liposomal-encapsulated flavonoid mixture. The bovine corneal opacity and permeability assay (BCOP, OECD No. 437) of the liposomal-encapsulated flavonoid mixture revealed no eye irritation potential of the product.

Liposomal encapsulation
Liposomes showed an average particle size of 120 nm which was stable over several production batches. They formed a transparent, stable solution and no preservation was necessary to keep the solution from microbiological contamination. A Pharmacopoeia Europea 6 preservation test for topical products was performed and received a grade B evaluation.

Stability of frame formulation
The formulation for the application study was stable for 6 month at room temperature, at 40°C, and at 2-8°C in the refrigerator.

Patch tests
The single and repeated human patch tests showed no irritation and sensitization potential of the liposomal-encapsulated flavonoid mixture in a lotion at 1%, which corresponds to 0.4 mg/ml of flavonoid mixture.

Application Study

Caucasian Skin
The application study on Caucasian skin (n=6) showed skin lightening effects and age spots brightening after 28 and 56 days of application compared to baseline. The effects on age spot brightening were slightly more apparent than the lightening effects on skin tone. Both on hand (Fig. 5) and face (Fig. 6) the resulting brightening effects were in the same range. Fig. 7 shows the spot-fading and skin brightening effects of a VisoFace Quick picture of the back of the hand on day 0 and day 56.
Asian Skin
After promising results on Caucasian skin an additional study on the external forearm of Asian volunteers (n=3) was performed and showed a lightening effect compared to baseline (day 0), which was significantly present after 56 days (Fig. 8).

Discussion
The flavonoid mixture was effective in vitro. Good inhibition has been shown in the mushroom tyrosinase enzyme inhibition test with an estimated IC50 of about 0.75 mg/ml. The cellular human tyrosinase inhibition assay resulted in stronger tyrosinase inhibition, there the estimated IC50 was 0.24 mg/ml of flavonoid mixture. Originally the potential lightening effect of citrus flavonoids was discovered by mushroom tyrosinase inhibition assay (9).

A range of tests showed that the citrus flavonoid blend is safe for application on skin. No cytotoxicity has been observed in concentrations that were applied. In the single and repeated patch tests no irritation or sensitisation potential was found and also no mutagenic potential (as tested by Ames Test, data not shown) and no eye irritation was detectable (BCOP test, data not shown). These findings show the excellent safety profile of the flavonoid blend for use as a cosmetic ingredient.

In vivo studies showed that it was very effective in brightening the skin tone and had an even stronger effect on the fading of age-spots, resulting in a more even skin tone. To get more accurate data with lower standard deviations a bigger study with at least 20 volunteers would be necessary. The high standard deviation was due to the small test groups (n=6 for Caucasian and n=3 for Asian Skin). After 28 days effects were already visible and were enhanced after 56 days, thus implying that stronger brightening effects can be obtained after longer application. These data suggest that the liposomal-encapsulated citrus flavonoid blend (Citrolumine 8™ from Cosmetochem AG, Switzerland) can be used at 1% in a cosmetic lotion as a very safe and effective skin lightening ingredient.
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References

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