

Citroflavonoid Anti-ageing Complex Fades Age Spots and Gives Skin Tone a Youthful Citrus Boost

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Abstract

Based on liposomal-encapsulated citroflavonoids, extracted from citrus fruits, this new cosmetic active fades age spots, brightens skin tone and increases overall skin luminosity. It has been shown to be safe and effective in both *in vivo* and *in vitro* studies.

Introduction

A decade ago anti-ageing products were concentrated mainly on achieving the reduction of wrinkles and the plumping-up of the skin to produce a younger appearance. However today, in addition to wrinkle reduction, there is much more focus on the evening out of the skin tone, which also gives skin its radiance and more youthful appearance. As skin ages it becomes less luminous and brown age spots begin to appear. Research has shown that the evening out of the skin tone has a significant effect on the estimated age of subjects^{1,2,3,4}. The citroflavonoid complex described here has been developed specifically to fade age spots, brighten skin tone and increase overall skin luminosity. The citroflavonoid complex used in this study is sold under the trade name Citrolumine 8™.

Skin pigmentation is caused by different levels of melanin in the skin, synthesised in melanosomes in the melanocyte cells by the action of tyrosinase, an enzyme which hydroxylates the amino acid tyrosine to dihydroxyphenylalanine (DOPA) and catalyses its oxidation to DOPA quinone. Raper⁵ originally elucidated the biosynthetic pathway of melanin, recently modified by Schallreuter et al⁶. Many products which aim to reduce skin pigmentation, target tyrosinase inhibition, as this is one of the key steps in pigment formation and can block other pigment-forming pathways^{7,8,9}.

The ideal candidate should have a good safety profile and skin tolerance, something many of the traditional skin lighteners such as hydroquinone do not have, having been associated in the past with many adverse effects^{9,10,11,12}. This has led to

a search for alternative plant-based skin lighteners which are both safe and effective^{13,14,15}.

Citroflavonoids

Citroflavonoids have been shown in the literature to have potent anti-inflammatory^{16,17,18,19,20,21,22} and antioxidant^{16,20,22,23,24,25} activity, additional properties which are also of interest for an anti-ageing cosmetic active. Flavonoids²⁶ and specifically citroflavonoids, both individually and as a mixture have been shown to inhibit tyrosinase^{27,28,29} and to have skin whitening properties^{22,30}.

Preliminary Analytical Screening

Unripe citrus fruits contain the highest concentration of flavonoids³¹. Citrus extracts from various citrus fruit species were analysed by HPLC for their content of naringin, narirutin, hesperidin and neohesperidin.

Figure 1 shows an HPLC trace of the chosen flavonoid mixture showing peaks for the major citroflavonoids present:

- Naringin (22%)
- Neohesperidin (5.3%)
- Narirutin (4.9%)
- Hesperidin (1.0%)

The chosen flavonoid mixture was then subjected to the following tests:

- *In vitro*: Mushroom tyrosinase inhibition test, cellular human epidermal melanocyte tyrosinase assay
- Preliminary safety tests (cytotoxicity, Ames, eye irritation)
- *In vivo*: The flavonoid mixture (0.4mg/ml) was liposomal-encapsulated (henceforth called citroflavonoid complex) and incorporated into a cosmetic lotion³² at 1% for *in vivo* tests and human patch testing

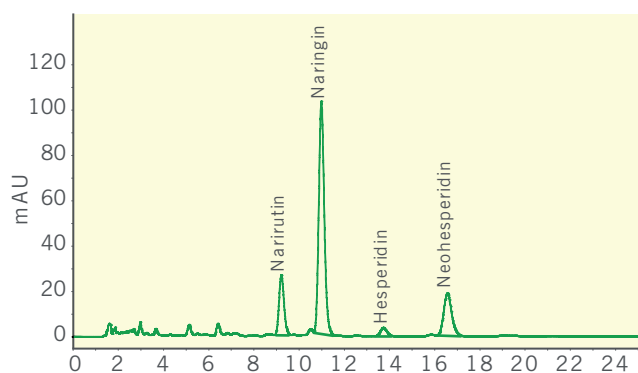


Figure 1. HPLC trace of flavonoid mixture showing major flavonoid peaks

Preliminary Safety Tests

Preliminary safety test including cytotoxicity, reverse mutation analysis (Ames), eye irritation (BCOP) and both single and repeat human patch test (HRIPT) were performed giving following results:

- Ames Reverse Mutation Assay (OECD 471) using strains of *Salmonella typhimurium* and *Escherichia coli*: Non-mutagenic
- BCOP eye irritation (OECD 437): Non-irritant at 1%
- Single Patch Test: Non-irritating, dermatologically tested at concentration of 1%
- Human Repeat Insult Patch Test (HRIPT): Non-irritating and does not produce any sensitisation at concentration of 1%
- MTT cellular viability test showed no cytotoxic effects of the flavonoid mixture at 1% (0.1mg/ml) and at 4% (0.4mg/ml) viability was only reduced to 83% (Figure 2)

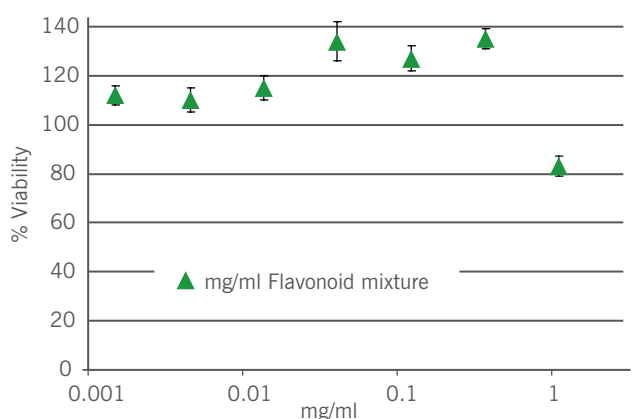


Figure 2. Cytotoxicity measurement with MTT reagent on primary epidermal melanocytes

In Vitro Activity

Tyrosinase Inhibition Tests

The flavonoid mixture was tested for its ability to inhibit tyrosinase using the mushroom tyrosinase inhibition test and the cellular human epidermal melanocyte assay

Mushroom Tyrosinase

- Mushroom tyrosinase was incubated with the test compounds and tyrosine and the absorbance measured over five hours at 490 nm

Cellular Human Epidermal Melanocyte Assay

- The tyrosinase enzyme was extracted from normal human epidermal melanocytes (NHEM) previously treated with the test compounds and was incubated with the substrate L-DOPA (dihydroxyphenylalanine) to determine their enzymatic activities

Results & Conclusion

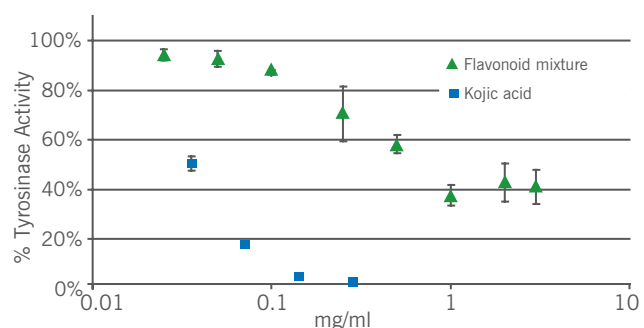


Figure 3. Inhibition of mushroom tyrosinase

- 0.4mg/ml of the flavonoid mixture reduced the mushroom tyrosinase activity by 42% and showed an estimated IC₅₀ of 0.75mg/ml (Figure 3). Kojic acid was used as a positive control in order to check the validity of the method

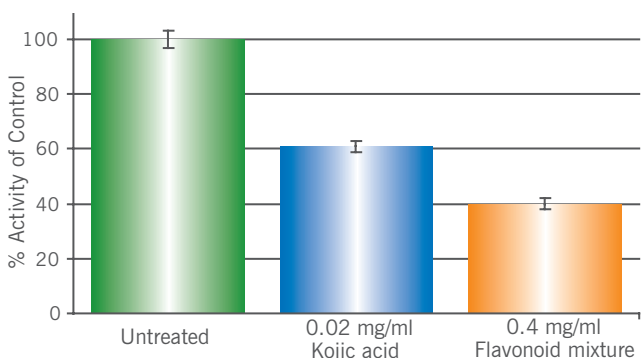


Figure 4. Cellular human epidermal melanocyte tyrosinase assay

- The flavonoid mixture at 0.4mg/ml reduced human cellular tyrosinase from primary epidermal melanocytes to 40% of untreated control and a reduction of 20% less than kojic acid (Figure 4)

In Vivo Activity

Chromameter and High Resolution Imaging

Studies on Skin Tone & Age-Spots

Test Protocol

- Skin colour or the melanin index (MI) was measured using a Skin Pigment Analyzer SPA99 (Caucasian) and a Chromameter CR300 (Asian) and high resolution imaging studies. A two month evaluation of the brightening and anti-ageing effects of 1% of the citroflavonoid complex, in a cosmetic lotion³² applied twice daily, was carried out on three Asian and six Caucasian female volunteers in a pilot study
- Both faces and the back of the hands were evaluated for general brightening effects on skin tone and specific effects on age-spots

Results & Conclusion: Caucasian Skin

Facial Skin

- The citroflavonoid complex, when used at 1% in a cosmetic lotion³², faded pigmentation of age spots and in addition brightened the skin tone (Figure 5)

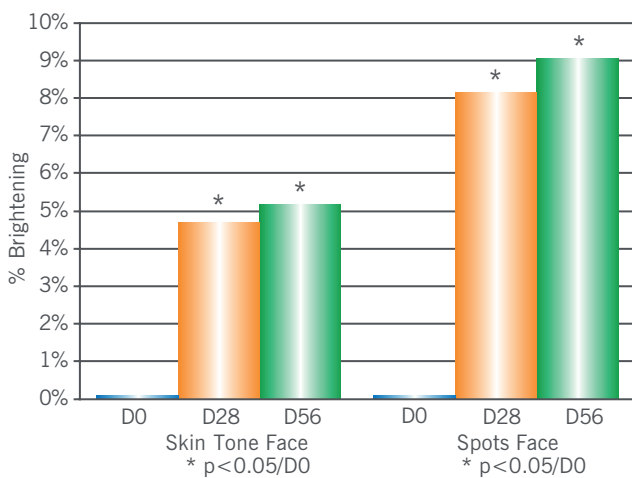


Figure 5. Brightening effect of 1% citroflavonoid complex on Caucasian facial skin

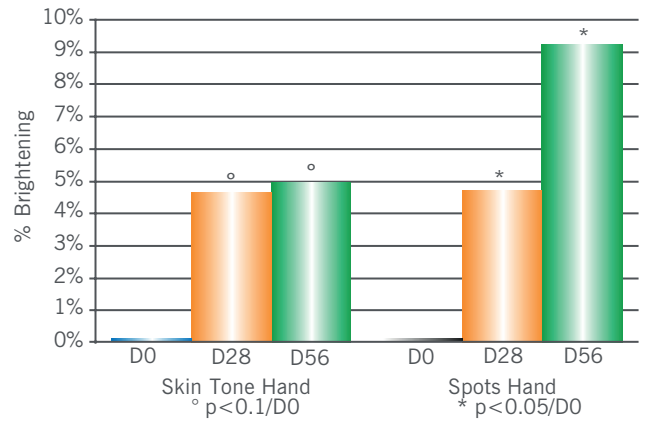


Figure 6. Brightening effect of 1% citroflavonoid complex on Caucasian skin on backs of hands

By Day 56 there was:

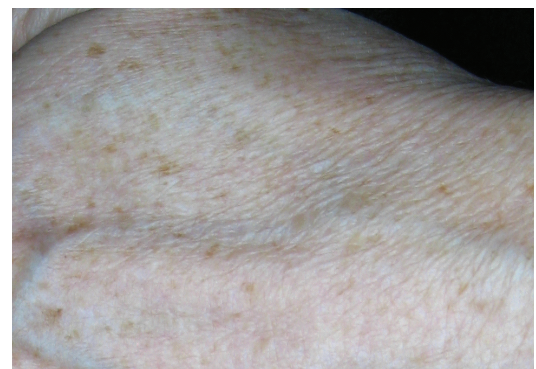
- 9.2% increase in the lightening of the age spots
- 5.0% increase in brightening of the skin tone

Skin on Back of Hands

- There was a significant lightening effect on the age spots of the backs of the hands (Figures 6 and 7)



Day 0



Day 56

Figure 7. Visioface Quick photos of brightening effect of 1% citroflavonoid complex on age spots on Caucasian skin

Results and Conclusion on Asian Skin Forearm Skin

- Following significant results on the fading of age spots on Caucasian skin, preliminary tests on Asian skin also showed a positive lightening of skin tone (Figure 8)
- The citroflavonoid complex at 1% caused a marked lightening of Asian skin (external forearm) after 56 days (Figure 8).

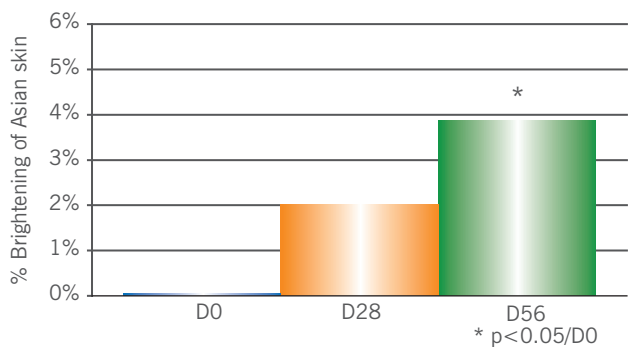


Figure 8. Brightening effect of 1% citroflavonoid complex on Asian forearm skin

Conclusion

Results described in this paper show that the liposomal-encapsulated citroflavonoid complex when used at 1% in a cosmetic lotion, is a safe, plant-derived cosmetic active which fades age spots, brightens skin tone, increasing overall skin luminosity and has been shown to be effective in both *in vitro* and *in vivo* studies. In addition citroflavonoids are also known to have potent antioxidant and anti-inflammatory properties which adds to the attraction of this ingredient as a cosmetic anti-ageing active.

References

1. Fink, B. et al. (2006) Visible skin colour distribution plays a role in the perception of age, attractiveness, and health in female faces *Evol. Hum. Behav.* 27, 433-42.
2. Fink, B. & Matts, P.J. (2007) The effects of skin colour distribution and topography cues on the perception of female facial age and health *J. Eur. Acad. Dermatol. Venereol.* 22, 493-498.
3. Matts, P.J. et al. (2007) Colour homogeneity and visual perception of age, health, and attractiveness of female facial skin *J. Am. Acad. Dermatol.* 57 (6): 977-84.
4. Gunn, D.A. et al. (2009) Why some women look young for their age *PLoS One* 4 (12): e8021. doi:10.1371/journal.pone0008021.
5. Raper, H.S. (1928) The anaerobic oxidases *Physiol. Rev.* 8, 245-82.
6. Schallreuter, K.U. et al. (2008) Regulation of melanogenesis – controversies and new concepts *Exp. Dermatol.* 17, 395-404.
7. Ando, H. et al. (2007) Approaches to identify inhibitors of melanin biosynthesis via the quality control of tyrosinase *J. Invest. Dermatol.* 127, 751-61.
8. Chang, T-S. (2009) An updated review of tyrosinase inhibitors *Int. J. Mol. Sci.* 10, 2440-75.
9. Parvez, S. et al. (2006) Survey and mechanism of skin depigmenting and

- lightening agents *Phytother. Res.* 20 (11): 921-34.
10. Draeos, Z.D. (2007) Skin lightening preparations and the hydroquinone controversy *Dermatol. Ther.* 20 (5): 308-13.
11. Tse, T.W. (2009) Hydroquinone for skin lightening: Safety profile, duration of use and when should we stop? *J. Dermatolog. Treat.* November 1, (epub ahead of print).
12. Dadzie, O.E. & Petit, A. (2009) Skin bleaching: highlighting the misuse of cutaneous depigmenting agents *J. Eur. Acad. Dermatol. Venereol.* 23 (7): 741-50.
13. Parvez et al. (2007) Naturally occurring tyrosinase inhibitors: mechanism and applications in skin health, cosmetics and agricultural industries *Phytother. Res.* 21, 805-16.
14. Zhu, W. & Gao, J. (2008) The use of botanical extracts as topical skin-lightening agents for the improvement of skin pigmentation disorders *J. Invest. Dermatol. Symp. Proceed.* 13, 20-4.
15. Gupta, S. (2010) Plant-based skin whitening cosmetics <http://www.insidecosmeceuticals.com/articles/2010/03/plant-based-skin-whitening-cosmetics.aspx>
16. Kanaze, F.I. et al. (2007) Pharmacokinetics of the citrus flavone aglycones hesperetin and naringenin after single oral administration in human subjects *Eur. J. Clin. Nutr.* 61 (4): 472-7.
17. Benevente-García, O. & Castillo, J. (2008) Update on uses and properties of Citrus flavonoids: new findings in anti-cancer, cardiovascular and anti-inflammatory activity *J. Agric. Food Chem.* 56, 6185-205.
18. Valfeiadou, K. et al. (2009) The citrus flavone naringenin inhibits inflammatory signalling in glial cells and protects against neuroinflammatory injury *Arch. Biochem. Biophys.* 484 (1): 100-9.
19. Giménez-Bastida, J.A. et al. (2009) A citrus extract containing flavanones represses plasminogen activator inhibitor-1 (PAI-1) expression and regulates multiple inflammatory, tissue repair, and fibrosis genes in human colon fibroblasts *J. Agric. Food Chem.* 57 (19): 9305-15.
20. Trombetta, D. et al. (2010) *In vitro* protective effects of two extracts from bergamot peels on human endothelial cells exposed to tumour necrosis factor-alpha (TNF-alpha) *J. Agric. Food Chem.* 58 (14): 8430-6.
21. Fang, F. et al. (2010) A novel regulatory mechanism of naringenin through inhibition of T-lymphocyte function in contact hypersensitivity suppression *Biochem. Biophys. Res. Commun.* 397 (2): 163-9.
22. Tsai, Y.H. et al. (2010) *In vitro* permeation and *in vivo* whitening effect of topical hesperetin microemulsion delivery system *Int. J. Pharm.* 388, (1-2): 2547-62.
23. Zieli ska-Przyjemka, M. & Ignatowicz, E. (2008) Citrus fruit flavonoids influence on neutrophil apoptosis and oxidative metabolism *Phytother. Res.* 22 (12): 1557-62.
24. Yoo, K.M. et al. (2009) major phytochemical composition of 3 native Korean citrus varieties and bioactive activity on V79-4 cells induced by oxidative stress *J. Food Sci.* 74 (6): C462-8.
25. Guimarães, R. et al. (2010) Targeting excessive free radicals with peels and juices of citrus fruits: grapefruit, lemon, lime and orange *Food Chem. Toxicol.* 48 (1): 99-106.
26. Gao, H. et al. (2007) Inhibitory effects of 5,6,7-trihydroxyflavones on tyrosinase *Molecules* 12, 86-97.
27. Sasaki, K. & Yoshizaki, F. (2002) Nobiletin as a tyrosinase inhibitor from the peel of Citrus fruit *Biol. Pharm. Bull.* 25 (6): 806-8.
28. Zhang, C. et al. (2007) Tyrosinase inhibitory effects and inhibition mechanisms of nobiletin and hesperidin from citrus peel crude extracts *J. Enzyme Inhib. Med. Chem.* 22 (1): 83-90.
29. Itoh, K. et al. (2009) Inhibitory effects of Citrus hassaku extract and its flavanone glycosides on melanogenesis *Bio. Pharm. Bull.* 32 (3): 410-5.
30. Huang, Y.B. et al. (2010) The effect of component of cream for topical delivery of hesperetin *Chem. Pharm. Bull. (Tokyo)* 58 (5): 611-4.
31. Kubo, M. et al. (2004) Seasonal variation in anti-allergic activity of citrus fruit and flavone glycoside content *Nat. Med.* 58 (6): 284-94 (in Chinese with English abstract).
32. Cosmotechem frame formulation (2010) ref. f 1343e Skin Lotion Citrolumine 8™.

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