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Skin-whitening effects of Mediterranean herbal extracts by *in vitro* and *in vivo* models

LUISA RIZZA, CLAUDIA BONINA, GIUSEPPINA FRASCA, and CARMELO PUGLIA, *Department of Pharmaceutical Science, School of Pharmacy, University of Catania, Viale A Doria 6, 95125 Catania, Italy.*

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Synopsis

Several plant extracts are able to protect skin against ultraviolet-light-induced damage and hyperpigmentation in a safe way. The anti-melanogenic effect of herbal extracts seems to be related to their antioxidant activity and their polyphenolic content. In this study, the skin-whitening effect of some Mediterranean species, already known for their strong antioxidant and radical scavenger activity, has been evaluated by *in vitro* and *in vivo* models. The results obtained showed that herbal extracts possessed an inhibitory effect on tyrosinase enzyme. Each extract showed a similar inhibiting activity even though it was less intensive than kojic acid and hydroquinone. Otherwise, a significant higher activity than kojic acid and hydroquinone was observed when the herbal extracts were combined. Furthermore, the anti-melanogenic activity and an evaluation of skin tolerance were affected by *in vivo* methods.

INTRODUCTION

Melanin is a physiological cutaneous "sunscreen" because it plays an essential role in the protection of skin from sunlight-induced damage. However, excessive production and accumulation of melanin can cause hyperpigmentation (melasma, freckles, age spot), and it can be associated with most serious skin disease such as melanoma (1–3). Melanin production can be inhibited by the reduction of ultraviolet (UV) exposure, by physical removal of corneal layer or inhibition of melanocyte biosynthesis and metabolism (4). Currently, topical application of melanin synthesis inhibitors is the less invasive procedure to avoid skin hyperpigmentation. Until now, traditional synthetic inhibitors of tyrosinase, such as hydroquinone and kojic acid, are considered the main active ingredients in cosmetic skin-whitening products (4–6). However, topical application of cosmetic and cosmeceutical products containing these active substances can raise several safety concerns especially for long-term treatments. Actually, some of their adverse effects, such as erythema, skin irritation, dermatitis, exogenous ochronosis, impaired wound healing, sclera and nail pigmentation, restricted their usage in the cosmetic industry (5,7,8).

In the search for novel depigmenting agents, the use of natural herbal extracts as new skin-care active ingredients has been recently emphasized. As reported by scientific works (5–10), several plant extracts are able to protect skin against UV-induced damage and to inhibit melanogenesis in a safe way, without cytotoxicity or mutagenicity effects. Nowadays, polyphenols are considered the main group of natural compounds responsible of these effects (11). The mechanism of action of polyphenols in human skin is not yet clearly understood, but it is supposed that their action is strictly correlated to reduction of cutaneous oxidative stress (11). In fact, it is well known that in melanocytes melanin synthesis occurs in a cascade of enzymatic reactions that converts tyrosine to melanin pigment. The key enzyme and rate-limiting reaction of this cascade is the tyrosinase that catalyzes three steps of melanin biosynthesis: hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), oxidation of DOPA to dopaquinone, and oxidation of 5,6-dihydroxyindole to indolequinone (5). Reactive intermediates produced are further oxidized to form melanin by a free radical-coupling pathway (4,9). Radical species, especially reactive oxygen ones, are considered to play an important role in regulating the melanogenesis and the proliferation of melanocytes (10). Antioxidants and inhibitors of radical production may affect melanogenesis events. Consequently, as reported by Panich *et al*. (3), the anti-tyrosinase effects of some plant extracts appeared to be correlated with their antioxidant potential. Finally, it was suggested that successful skin-whitening treatments could require the use of different anti-melanogenic ingredients to achieve a more intensive effect (12). Herbal extracts often induced more intensive effects, such as antioxidant and anti-melanogenic one, when used in combination (12–15).

In this study, the anti-melanogenic effects of herbal extracts obtained from typical Mediterranean species such as *Capparis spinosa*, *Citrus sinensis*, *Oryza sativa*, and *Olea europaea* have been studied. These plants have been chosen since characterized by an interesting antioxidant activity attributed to the high level of polyphenols (16–19). The anti-melanogenic effect of each extract and their combination was evaluated by *in vitro* models while *in vivo* experiments were performed to determine their skin-whitening action and skin tolerance (skin sensitivity to UVB irradiation).

MATERIALS AND METHODS

TEST MATERIALS

Hydroquinone, kojic acid, and other cosmetic ingredients to produce topical formulation were supplied by A.C.E.F. s.p.a. (Fiorenzuola, Piacenza, Italy). Caper buds (*Capparis spinosa*), blood orange (*Citrus sinensis*), rice grains (*Oryza sativa*), and olive leaf (*Olea europaea*) solid extracts and their mixture were supplied by Bionap srl (Skin Moon; Catania, Italy). Briefly, plants (caper buds, rice grains, olive leaf) were chopped into small pieces and then extracted with ethanol/water solution (50:50) acidified to pH = 3 for citric acid for 12 h. Afterward, extracts and squeezed orange juice were filtered using 0.2 μm paper filter to remove any impurities and then were applied to a polystyrene/divinylbenzene XAD-16 column (Rohm and Haas, Philadelphia, PA). The resins were eluted with an ethanol/water solution (50:50), then ethanol was removed by evaporation and the aqueous residue was spray-dried. Herbal solid extracts showed to possess similar total phenol contents (% w/w)

by Folin–Ciocalteu method (20): 24% in *Capparis spinosa*, 25% in *Citrus sinensis,* 22% in *Oryza sativa,* 27% in *Olea europaea* extract, and 26% in their mixture.

Mushroom tyrosinase, L-DOPA, and other reagents were purchase from Sigma-Aldrich (Milan, Italy). Topical application of active compounds was performed by oil-in-water emulsions, adding skin-whitening ingredients, as reported in Table I. The emulsions were prepared by slowly adding the aqueous phase to the oily phase and a blend of surfactants under continuous agitation; both phases were kept at 70°C. This mixture was stirred until it was cool, thus forming emulsion formulations.

IN VITRO EVALUATION OF ANTI-TYROSINASE ACTIVITY

The *in vitro* tyrosinase inhibitory activity (IA) was determined by the dopachrome method. Herbal extracts and their combination were added at a concentration of 50 μg/ml to L-DOPA solution (10 m*M*, dissolved in a 5 m*M* acetate buffer, pH 5). Subsequently, tyrosinase enzyme was added to each solution to a final concentration of 4 U/ml. The amount of dopachrome in the reaction mixture was measured after incubation at 38°C for 60 min at 475 nm by UV-Vis spectrophotometer (UV-1700 PharmaSpec; SHIMADZU Milan, Italy). Kojic acid and hydroquinone, at the concentration of 50 μg/ml, were used as reference and a blank reaction was conducted without a sample. All measurements were performed in triplicate. The activity of each sample was expressed as the IA using equation 1:

$$\mathrm{IA}\ (\%) = \frac{\mathrm{OD}_{\mathrm{blank}} - \mathrm{OD}_{\mathrm{sample}}}{\mathrm{OD}_{\mathrm{blank}}} \times 100 \qquad (1)$$

where $\mathrm{OD}_{\mathrm{blank}}$ and $\mathrm{OD}_{\mathrm{sample}}$ are the optical density values of the blank and the solutions containing active ingredients, respectively.

IN VIVO EVALUATION OF SKIN-WHITENING ACTIVITY

In vivo experiments were performed on 15 healthy volunteers (females/males 10:4) of skin types II and III, aged 30–45 years. Volunteers were recruited after medical screening including the filling of a health questionnaire followed by physical examination of the application sites. Subjects exhibiting such features as sun-burn, suntan, burn marks, or any other active lesions, which might interfere with evaluation, were excluded from the study. After they were fully informed of the nature of the study, substances, and procedures involved,

Table I
Composition of Oil-in-Water Emulsions Used as Topical Skin-Whitening Formulations

Oil-in-water emulsion	Oil phase (PPG-15 stearyl ether-8 g, isohexadecane\PPG-15 stearyl ether-4 g); Aqueous phase (Distilled water-73.7 g); Surfactants and structurizing agents (Steareth 2–3.5 g, steareth 2.1–2.5 g, stearic acid-2.5 g, cetylstearylic acid-2.1 g, xanthan gum-0.3 g, ethylene glycolphenyl undecylether *p*-hydroxybenzoate-0.4 g)
Formulation A	Hydroquinone-3 g
Formulation B	Kojic acid-3 g
Formulation C	Mixture of herbal extracts-3 g

they gave their written consent. Two research assistants were responsible for all recruitment and data collection. The *in vivo* experiments were carried out on the volar forearms of each volunteer, and each subject was rested for 15 min before the experiments and room conditions were set at 22 ± 2 °C and 40–50% relative humidity. Four sites on the ventral surface of each forearm were defined using a circular template (1 cm^2) and demarcated with permanent ink. During the first week of experiments, skin tanning was induced in each site by exposition of a lamp that simulate sunlight (Helios Italquartz srl, Milan, Italia), which emitted in a range of 300–400 nm (6.5 mW cm^{-2}), for 2–8 min depending on the minimal erythema dose (MED) of each subject. The initial melanin value and the melanin content obtained during the monitoring period were performed by reflectance visible spectrophotometer X-Rite model 968 (X-Rite Inc., Grandville, MI) as previously reported (21). The variation of skin melanin values was monitored for a total period of 4 weeks. To avoid induced and interfering skin erythema events, exposition to a lamp was not conducted on the third and the sixth day of the first week. For each forearm, three skin sites were treated with tested formulations and the one not treated was considered as control. Tested formulations were applied after each lamp treatment for the first week and once daily in the second, third, and fourth week of the study. To evaluate the time course of skin pigmentation, melanin index (MI) baseline values were subtracted from the MI values to calculate mean MI (ΔMI) values. For each site, plotting ΔMI vs. time, the area under the curve was computed using the trapezoidal rule to obtain area under curve (AUC) dimensionless index values directly related to the degree of skin pigmentation. From the AUC values obtained, the skin-whitening effect of each formulation was expressed as the inhibition of skin pigmentation (PI) using the following equation:

$$\mathrm{PI}\,(\%) = \frac{\mathrm{AUC}_C - \mathrm{AUC}_S}{\mathrm{AUC}_C} \times 100 \tag{2}$$

where AUC_C is the AUC value of no treated skin site and AUC_S is the AUC value obtained from treated sites.

IN VIVO EVALUATION OF PHOTOSENSITIZING EFFECT OF SKIN-WHITENING AGENTS

The skin tolerance of skin-whitening agents was investigated by an *in vivo* model previously reported (21). After a rest period of 6 months, the same subjects participating in the previous study were enrolled to evaluate if skin-whitening application had increased the skin sensitivity to UVB irradiation. For each subject, four skin test sites were defined on the ventral surface of each forearm. Three sites were treated with formulations once daily for 4 consecutive weeks and one site was used as control (no topical treatment). At the end of the fourth week, all sites were exposed to UVB irradiation dose, corresponding to the MED by using the lamp previously described to simulate sunlight. The induced erythema was measured, after 24 h from the skin site exposure, by reflectance spectrophotometry and the registered photosensitivity was expressed as a percentage calculated from erythema index values using equation 3:

$$\text{Photosensitivity}\,(\%) = \frac{\mathrm{EI}_T - \mathrm{EI}_C}{\mathrm{EI}_C} \times 100 \tag{3}$$

where EI_C is the erythema index of control sites and EI_T is the erythema index of sites treated with formulations.

STATISTICAL ANALYSIS

All data obtained were submitted to statistical analysis. Evaluation of the statistical significances was performed by Student's *t*-test for *in vitro* data. All statistical comparisons of *in vivo* data were evaluated using repeat measure analysis of variance followed by the Bonferroni–Dunn post hoc pair-wise comparison procedure (21). A p value of less than 0.05 was considered significantly different.

RESULTS

The anti-tyrosinase activity of kojic acid, hydroquinone, herbal extracts from caper buds, blood orange, rice grains, and olive leaf, and their combination was evaluated by *in vitro* dopachrome method. As reported in Table II, all the active ingredients possessed an inhibitory effect on tyrosinase enzyme. Each extract showed a similar inhibiting activity even though it was less intensive than kojic acid and hydroquinone. Otherwise, a significant higher activity than kojic acid and hydroquinone was observed when the herbal extracts were combined. The results of *in vivo* studies corroborated the *in vitro* findings. In fact, the topical application of the formulations containing hydroquinone, kojic acid, and the mixture of herbal ingredients induced a significant reduction of the skin melanin index. The trends in mean MI variation (ΔMI) vs. time for subjects are reported in Figure 1. After the first week of sunlamp treatment, a significant increase of skin pigmentation was observed in the control skin sites (not treated), whereas treated sites showed lower MI values, as evidenced by AUC values reported in Figure 2. Finally, from the inhibition of skin pigmentation (PI) values showed in Figure 3, it was observed that hydroquinone (PI = 69%) and the mixture of herbal extracts (PI = 75.9%) were more effective to inhibit skin pigmentation than kojic acid (PI = 52.3%).

The photosensitizing effect of each skin-whitening agent was also evaluated by *in vivo* study. As reported in Figure 4, hydroquinone and kojic acid (photosensitivity of 48% and 32% respectively) induced comparable skin sensitivity to UV light after topical application (4 weeks) but more intensive than mixture of herbal agents (photosensitivity of 17%).

Table II
Inhibitory Activity (IA) Percentage of Tyrosinase Obtained from Kojic acid, Hydroquinone, Caper Buds, Blood Orange, Rice Grains, and Olive Leaf Extracts and Their Mixture at the Concentration of 50 μg/ml.

Sample	IA %
Kojic acid	43.7 ± 3.8
Hydroquinone	30.2 ± 3.1
Caper buds (*Capparis spinosa*) extract	18.2 ± 2.7[a]
Blood orange (*Citrus sinensis*) extract	22.0 ± 2.9[a]
Rice grains (*Oryza sativa*) extract	17.9 ± 2.3[a]
Olive leaf *(Olea europaea)* extract	23.1 ± 2.5[a]
Mixture of herbal extracts	60.2 ± 4.1[a]

[a]Significantly different compared to kojic acid and hydroquinone ($p < 0.05$).

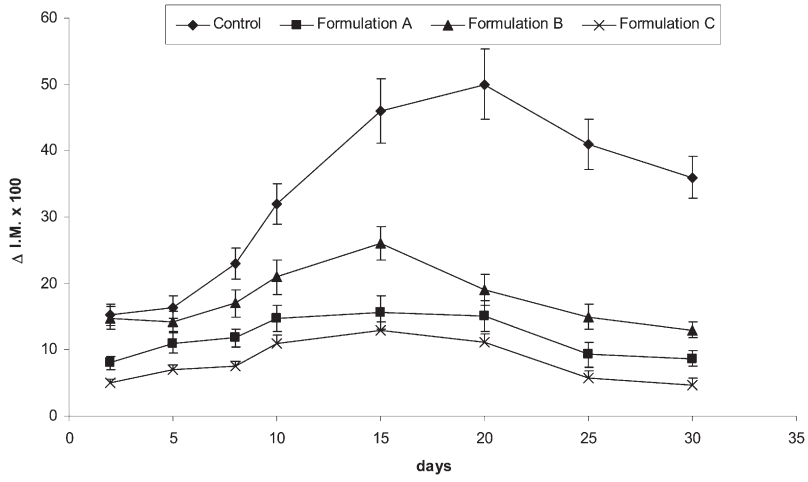

Figure 1. Trend of skin melanin index (ΔMI) vs. time (days) over the monitoring period of 4 weeks for skin sites treated with formulation A (hydroquinone), formulation B (kojic acid), and formulation C (mixture of herbal extracts) or no treated (Control).

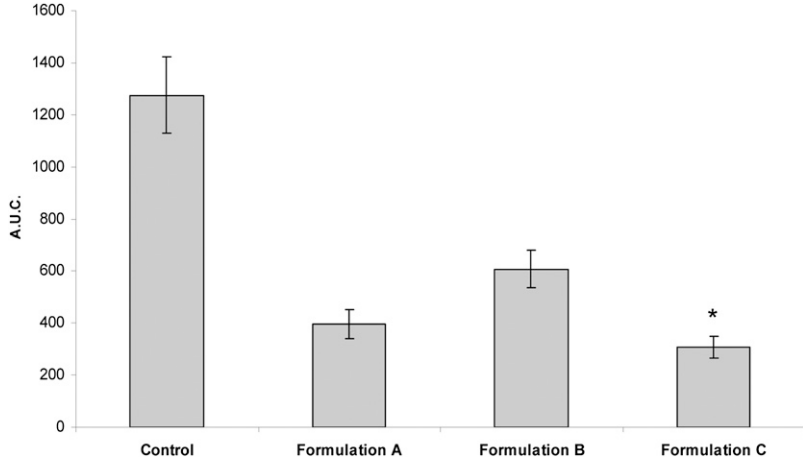

Figure 2. Mean area under curve values (AUC ± DS) obtained from skin sites treated with formulation A (hydroquinone), formulation B (kojic acid), and formulation C (mixture of herbal extracts) or no treated (control) over the monitoring period of 4 weeks; $*p > 0.05$ (no significantly different) vs. formulation A.

DISCUSSION

The *in vitro* data obtained in this study showed that the active compounds contained in typical Mediterranean plants (*Capparis spinosa*, *Citrus sinensis*, *Olea europaea*, and *Oryza sativa*) possessed anti-tyrosinase activity and they could be considered natural skin-whitening ingredients. Moreover, their effects were increased and potentiated when herbal extracts of each plant were mixed. The *in vivo* models used in this work validated the skin-whitening effect and the skin tolerance of this combination in comparison with kojic acid and hydroquinone which are well-known active ingredients used in cosmetic field.

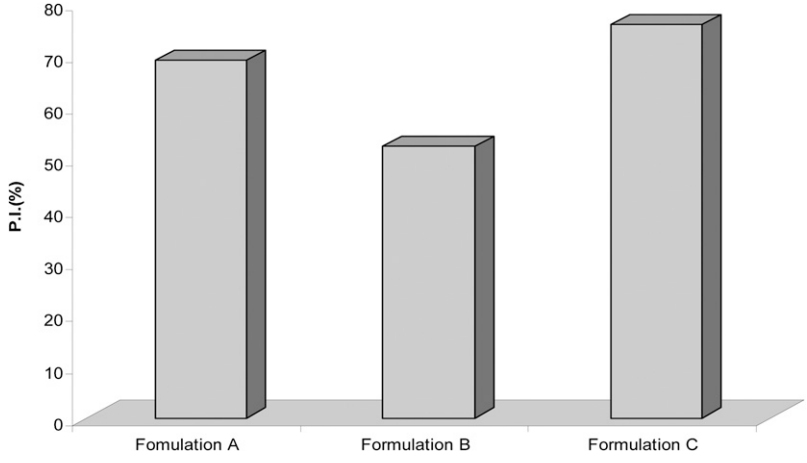

Figure 3. Percentage of Inhibition of skin pigmentation (PI) of formulation A (hydroquinone), formulation B (kojic acid), and formulation C (mixture of herbal extracts) obtained by *in vivo* study over the monitoring period of 4 weeks.

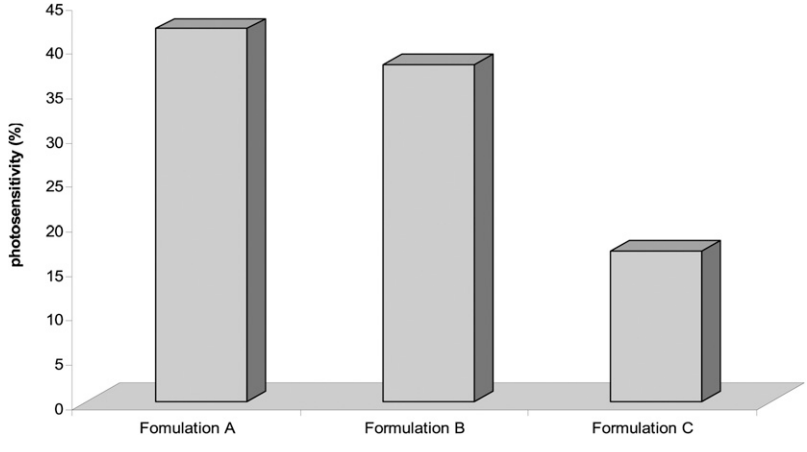

Figure 4. Increase in skin sensitivity to UVB irradiation expressed by photosensitivity percentage after 6 weeks of treatment with formulation A (hydroquinone), formulation B (kojic acid), and formulation C (mixture of herbal extracts) vs. control (no topical treatment).

In our study, caper buds, blood orange, rice grains, and olive leaf can be considered important natural sources of anti-melanogenic substances for the high level of polyphenolic content. In fact, it is possible to suppose that skin-whitening effect of herbal combination may be attributed to polyphenols and their association (8, 22–25).

It is known that oxidative species are involved in skin UV-induced pigmentation and antioxidant substances such as polyphenols can affect melanogenesis processes by improving skin oxidative stress defense (8). Moreover, from the data obtained in the *in vitro* study, we observed a direct inhibition of tyrosinase enzyme activity by herbal extracts. Our results are in agreement with data reported in several studies that attributed interesting anti-melanogenic activity to some group of polyphenolic compounds commonly contained in these plants. Flavonoids such as hesperidin, naringenin, and eriodictyol are considered the most abundant flavanones contained in citrus fruits (8). The effects of

these flavonoids on melanin synthesis have been already evaluated (8,9). Hesperidin and naringenin have a chemical structure similar to hydroquinone and it was supposed that they can act as a substrate competitor for tyrosinase (26). Hesperidin showed to induce inhibition of tyrosinase activity in human primary melanocytes in a dose-dependent manner and to protect fibroblasts and collagen against oxidative and UVA-induced damages (8,27). Moreover, flavonoids such as quercetin, kaempferol, and their derivates, typically contained in *Capparis spinosa* extract (25,28), showed to possess a strong tyrosinase inhibitory activity (23). Further investigation provided that the anti-melanogenic effect of quercetin and kaempferol was due to different mechanism of actions. Quercetin was able to decrease the intracellular tyrosinase activity not only by inhibition of enzyme activity but also by reduction of its protein expression (29), and kaempferol could act by copper chelation of tyrosinase enzyme as long as its 3-hydroxyl group was free (23). Similar activity was associated to hydroxycinnamic acid group, such as ferulic acid and caffeic acid, mainly contained in *Oryza sativa* extracts, by inactivation of tyrosinase effect (30,31). Finally, the skin protective action against UV light of olive leaf extract and its polyphenols, such as oleuropein and luteolin, was already known (22,24,32).

In addition, one of the main drawbacks of skin-whitening agents, such as hydroquinone and kojic acid, is the skin-sensitizing effect to the sunlight exposure due to the repeated application of these substances. To simplify treatment regimen and reduce the risk of side effects, application of skin-whitening ingredients usually needs to avoid sun exposure and to use protective ingredients, such as sunscreens (32–36). The *in vivo* model used in this work can predict the skin photosensitizing effect induced by cosmetic ingredients after repeated topical applications (21). Results showed a safer activity of the natural mixture than hydroquinone and kojic acid. As previously reported in literature (11), we can suppose that polyphenols could act as cutaneous sunscreen and they can protect the skin against the radiation-induced inflammation, oxidative stress, DNA damage, and other diseases induced by UV-light exposure.

In conclusion, in this study we evaluated the anti-melanogenic effects and the safety of herbal extracts obtained from typical Mediterranean species such as *Capparis spinosa*, *Citrus sinensis*, *Oryza sativa*, and *Olea europaea* by *in vitro* and *in vivo* models. Further studies could be useful to better understand the mechanisms of action and the future application of these polyphenols as skin-whitening agents.

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