

Comparative *in vivo* study of the efficacy and tolerance of exfoliating agents using reflectance spectrophotometric methods

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Synopsis

The aim of the present study was to compare the effectiveness and the safety of different topical agents (glycolic acid, mandelic acid, and grape juice acid mixture) in skin exfoliation by objective instrumental methods. To evaluate the exfoliating effects of these substances, a new experimental *in vivo* protocol based on DHA (dihydroxyacetone)-induced skin pigmentation was used. Skin acceptability towards acid application was investigated by the evaluation of skin erythema induced by topical application of these substances at increased concentrations. Furthermore, their photosensitizing effects were evaluated by determining the increase in sensitivity to UV-light exposure in cutaneous sites previously treated with acids. These *in vivo* evaluations were monitored by reflectance spectrophotometry.

From the results obtained, we observed the differing capacities of the tested acids to increase the rate of skin regeneration, with a significant reduction in the time required to obtain skin renewal. The study pointed out that glycolic acid (10% w/w) induced a faster skin exfoliation, a more intense erythema, and a higher photosensitizing effect in comparison with the mandelic acid and grape juice acid mixtures. Further evidence showed that the mandelic acid and grape juice acid mixtures were able to induce a slower and safer peeling action in comparison with glycolic acid. Finally, our results suggest that the methodologies and protocols used in this study may help in choosing the most appropriate topical agents for skin exfoliating treatments.

INTRODUCTION

Despite new and emerging modalities in the field of dermatology, chemical peeling holds its own as an efficacious technique available for treatment of cutaneous diseases and conditions and for aesthetic improvement (1,2). Chemical peeling involves the application of one or more chemical exfoliating agents to the skin, resulting in a wound-healing process that can regenerate the epidermis and restore photodamaged, wrinkled, blemished, acne-scarred, or blotchy skin to its original appearance (3,4). A variety of chemical peeling agents are available, such as glycolic acid, trichloroacetic acid, salicylic acid, pyruvic acid, resorcinol preparations, and solid carbon dioxide (5), and new agents are being researched to create new ways of peeling (1).

Alpha-hydroxy acids (AHAs) are a class of compounds commonly used for chemical peeling, and glycolic acid is the most extensively studied of these acids (6). According to these studies, glycolic acid appears to induce an acid-dependent dis-cohesion of corneocytes, and when used for a long period of time in high concentration, it is able to increase cell proliferation of the basal epithelial cells in the epidermis, elastic fibers, and collagen (7,8). Many commercial skin-care products containing glycolic acid are proposed to counteract photoaging, decrease acne and pigmentary changes, or reduce stretch marks (8,9). As reported in several studies, better response is obtained only when glycolic acid is used at higher concentrations (50–70%) (10,11), increasing the risk of skin irritation.

The use of glycolic acids in chemical peeling is strictly correlated with some undesirable side effects such as persistent erythema and pruritus, burning, post-inflammatory hyper/hypopigmentation, hypertrophic scarring, and infectious complications (3,11,12). Moreover, recent experimental studies demonstrate that short-term application of glycolic acid sensitizes the skin to the damaging effects of UV light (13). To improve the safety of products, committees set up by associations of cosmetics manufacturers in Europe and in the USA recommend similar guidelines and, in particular, pH values higher than 3.5 and alpha-hydroxy acids contents lower than 7–10% (14).

Recently, in order to achieve a balance between performance and risks, many common organic acids and combinations of them, such as mandelic acid, lactic acid, and natural acids from fruits (such as tomatoes, lemons, grapefruits, oranges, and limes) have been used in commercial products.

The aim of the present study was to compare the effectiveness and the safety of different AHAs (glycolic acid, mandelic acid, and a blend of organic acids from grape juice) in skin exfoliation by objective instrumental methods. To evaluate the efficacy of the exfoliating agents, a new experimental *in vivo* protocol based on DHA (dihydroxyacetone)-induced skin pigmentation and a non-invasive instrumental method was used. DHA is a three-carbon sugar, formally a derivate of glycerol, and it is the most common and safe cosmetic ingredient used in sunless tanning products (15). The pigmentation produced by DHA is the result of the chemical reactions (Maillard reaction) between the DHA and the amino acids of the corneocytes in the upper layers of the stratum corneum, forming polymeric colored substances called melanoidins (15–17). Since DHA is bound in an irreversible way to the free amino groups, the resultant color lasts for several days on the skin and is only removed by natural skin renewal. Skin regeneration occurs by the continuous generation of new cells in the basal layer that rise through the epidermal layers of the skin until they reach the stratum corneum, where the skin cells die and eventually fall or slough off (18). Therefore, the color intensity is directly related to the amount of DHA bound in the skin, and the durability of the staining by DHA is strictly dependent on the rate of skin cell renewal.

On the basis of this assumption, in this study we tried to demonstrate that the use of DHA-induced pigmentation could be a valid method to estimate the activity of exfoliating agents in promoting skin regeneration. For an objective evaluation of DHA-induced pigmentation, the study was carried out by non-invasive instrumental reflectance spectrophotometry, and spectral data were used to quantify skin color intensity.

The safety profile of the AHAs versus skin was studied by two different *in vivo* studies: the evaluation of skin erythema induced by topical application of acids at different concentrations (10%, 30%, and 50% w/w) and the increase in sensitivity to UV light exposure

in cutaneous sites previously treated with AHAs. Both *in vivo* evaluations were monitored by reflectance spectrophotometry.

MATERIALS AND METHODS

SUBJECTS

In vivo experiments were performed on twenty healthy volunteers (females/males 14:6) of skin types II and III, aged 25–35 years. Between October 2006 and September 2007, the volunteers were recruited after medical screening that included filling in a health questionnaire and physical examination of the application sites. Subjects exhibiting such features as sunburn, suntan, burn marks, or any other active lesions that might interfere with evaluation were excluded from the study. After they were fully informed of the nature of the study, substances, and procedures involved, the subjects gave their written consent. For the period of the studies, *in vivo* experiments were carried out on the volar forearms of each volunteer. Each subject rested for 15 minutes before the experiments, and room conditions were set at $22^{\circ} \pm 2^{\circ}\text{C}$ and 40–50% relative humidity. Two research assistants were responsible for all recruitment and data collection.

TEST MATERIAL

Glycolic acid (70% cosmetic grade) and mandelic acid were supplied by A.C.E.F. (A.C.E.F. s.p.a., Fiorenzuola, Piacenza, Italy). Organic acids from white grape juice (a solution containing a blend of tartaric acid (18.5%), malic acid (12%), citric acid (3%), lactic acid (2.5%), gluconic acid (3.5%) and shikimic acid (0.5%)) were supplied by Bionap (Renegrade[®], Bionap s.r.l., Italy). Exfoliating gel formulations were prepared by gelification with xanthan gum (1% w/w) of aqueous solutions containing three different concentrations of glycolic acid (formulations labeled GLY), mandelic acid (formulations labeled MAN), and grape acids (formulations labeled GA). Standard samples of GLY, MAN, and GA were weighted and dissolved in water, obtaining solutions with a final concentration of 10%, 30%, and 50% w/w for each acid. The pH of the test materials was adjusted to 3.5 by using sodium hydroxide. The test materials were alphabetically coded by the manufacturer, and neither the research assistants nor the volunteers knew the content of the blinded formulations. A sunless tanning formulation containing 5% w/w dihydroxyacetone (A.C.E.F. s.p.a., Fiorenzuola, Piacenza, Italy) was prepared by mixing DHA in water and stirring for ten minutes.

INSTRUMENTS

Skin reflectance spectra were recorded using a reflectance visible spectrophotometer, X-Rite model 968 (XRite Inc. Grandville, MI), having 0° illumination and a 45° viewing angle, calibrated and controlled as previously reported (19). Reflectance spectra were obtained over the wavelength range of 400–700 nm using illuminant C and a 2° standard observer.

IN VIVO EVALUATION OF THE EXFOLIATING EFFECTS OF FORMULATIONS ON DHA-INDUCED SKIN PIGMENTATION

For each subject, four sites on the ventral surface of each forearm were defined using a circular template (1 cm²) and demarcated with permanent ink. Baseline skin assessment was performed by reflectance spectrophotometry on all sites. Each site was then treated with the 5% DHA formulation (200 µl) and kept under occlusive conditions with the use of Hill-Top Chambers (Hill Top Research Inc., Cincinnati, OH) for one hour, once daily for two consecutive days. After the removal of the chambers, the residual formulation was removed by gently wiping with cotton balls. One day after the second DHA application, the sites treated with DHA showed the development of a visually brownish coloration, and the skin reflectance spectrum of each site was recorded by reflectance spectrophotometry. Afterwards, three skin sites received a topical dose (200 µl) of 10% GLY, 10% MAN, or 10% GA formulation, applied by Hill-Top Chambers, once daily for 12 days. Application was completed within two minutes and terminated by cleaning the skin sites with cold water and neutralizing with 1% sodium bicarbonate solution. One site received no topical treatment (CONTR).

For each site, skin reflectance spectra were recorded over the monitoring period of two weeks that began at the conclusion of the 12 days. From the reflectance spectral data, the melanin index (M.I.) was obtained using the following equation (equation 1) (20):

$$\text{M.I.} = \left(\log \frac{1}{R_{650}} - \log \frac{1}{R_{700}} \right) + 0.015 \quad (1)$$

where the log of inverse reflectance values ($\log 1/R$) is the apparent absorbance at a specific wavelength (650 nm and 700 nm) and 0.015 is an adjusted instrumental factor. This index is calculated as the slope of the apparent absorbance levels from 650 nm to 700 nm and was used to measure both melanin and the melanogenic dose response. All the regions were measured in triplicate. After plotting M.I. values versus time, the time course of DHA-induced pigmentation disappearance was obtained for each site. The regeneration of the skin surface was obtained when the skin returned to the M.I. baseline, measured before dihydroxyacetone treatment, and the DHA-induced pigmentation disappeared. The time (days) required to obtain MI baseline indicated the rate of skin exfoliation and was expressed as the "recovery time" value (RT) for each skin site. The RT was inversely related to the cell turnover acceleration induced by topical application of the exfoliating formulations.

IN VIVO EVALUATION OF SKIN ERYTHEMA INDUCED BY TOPICAL APPLICATION OF THE FORMULATIONS

In vivo evaluation of skin erythema by reflectance spectrophotometry was used to determine the skin-irritant effect of the exfoliating agents after topical application. The experiments were performed on the same subjects as in the DHA-induced skin pigmentation protocol after a rest period of three months. Nine skin sites (defined as described above and distinct from the sites used in the first experiment) were treated with three

different concentrations (10%, 30%, and 50% w/w) of GLY, MAN, and GA formulations (200 μ l) under occlusion conditions by Hill-Top Chambers for 3–15 minutes depending on the subject's sensitivity. After the chambers' removal, the cutaneous sites were washed by means of cold water-soaked gauze pads. For each skin site the induced erythema was monitored for 50 hours by reflectance spectrophotometry. Since erythema is due to an increase in blood count in the subpapillary plexus of the skin, erythema index (E.I.) values were calculated by subtracting the logarithm of inverse reflectance ($\log 1/R$) values at 510 nm and 610 nm (mainly due to melanin absorption) from the sum of $\log 1/R$ values at 540 nm, 560 nm, and 580 nm, which represent the wavelengths of hemoglobin absorption peaks (equation 2) (19). All the regions were measured in triplicate.

$$\text{E.I.} = 100 \left[\log \frac{1}{R_{560}} + 1.5 \left(\log \frac{1}{R_{540}} + \log \frac{1}{R_{580}} \right) - 2 \left(\log \frac{1}{R_{510}} + \log \frac{1}{R_{610}} \right) \right] \quad (2)$$

To evaluate the time course of skin erythema, E.I. baseline values were subtracted from the E.I. values obtained after application of the formulations, to calculate Δ E.I. values. For each site, plotting Δ E.I. versus the time the area under the curve was computed using the trapezoidal rule to obtain AUC (area under curve) dimensionless index values directly related to the degree of skin erythema.

IN VIVO EVALUATION OF THE PHOTOSENSITIZING EFFECT INDUCED BY TOPICAL APPLICATION OF THE FORMULATIONS

In order to determinate the photosensitizing effect of the exfoliating agents, the skin erythema induced after UVB irradiation was evaluated in the same group of subjects participating in the previous studies, after a rest period of three months. For each subject, four skin test sites were defined on the ventral surface of each forearm. Three sites received 200 μ l of 10% GLY, 10% MAN, or 10% GA formulations, applied once daily for four consecutive weeks. As reported before, the acid applications were completed within two minutes and termination was performed by cleaning the skin sites with cold water and neutralizing with 1% sodium bicarbonate solution. One site was used as control (no topical treatment). At the end of the fourth week, all sites were exposed to a UVB irradiation dose, corresponding to the minimal erythema dose (MED), by using an ultraviolet lamp, model UVM-57 (UVP, San Gabriel, CA) that emitted radiation in the range of 290–320 nm, with an output peak at 302 nm.

The flux rate measured at the skin surface was 0.80 mW cm^{-2} . For each skin site the induced erythema was measured by reflectance spectrophotometry (equation 2) twenty-four hours after the irradiation exposure, and the photosensitivity was expressed as the percentage calculated from erythema index values using equation 3:

$$\text{Photosensitivity \%} = \frac{\text{E.I.}_T - \text{E.I.}_C}{\text{E.I.}_C} \times 100 \quad (3)$$

where E.I._C is the erythema index of the no-treatment skin site and E.I._T is the erythema index of the sites treated with the formulations.

DATA ANALYSIS

All data obtained were submitted to a statistical analysis. All statistical comparisons in instrumental assessment were evaluated using repeat-measure analysis of variance (ANOVA) followed by the Bonferroni-Dunn post-hoc pair-wise comparison procedure. A p value of less than 0.05 was considered significant.

RESULTS

IN VIVO EVALUATION OF THE EXFOLIATING EFFECTS OF THE FORMULATIONS ON DHA-INDUCED SKIN PIGMENTATION

The effects of 10% GLY, 10% MAN, or 10% GA formulations on skin exfoliation rates were evaluated by objective instrumental observation of *in vivo* DHA-induced skin pigmentation disappearance. DHA-induced skin pigmentation was determined as a melanin index by reflectance spectrophotometry. For each subject, skin reflectance spectra were recorded and melanin indices were calculated over the monitoring period of the study. The trends of skin pigmentation disappearance are reported in Figure 1 by plotting M.I. values versus time (days). As can be seen, after DHA-pigmentation, the formulations showed different trends in M.I. value reductions over time. Moreover, to better compare the skin exfoliation rate, the “recovery time” (RT) values are given in Table I for each subject admitted into the study. The results showed that 10% GLY, 10% MAN, and 10% GA formulations were effective in inducing skin M.I. reduction in comparison with the non-treated site (CONTR) ($p < 0.05$). An RT value of 10% GLY was significantly lower than an RT value of 10% GA ($p < 0.05$). Furthermore, a significant difference was found between the RT value of 10% MAN and that of the 10% GA formulation ($p < 0.05$).

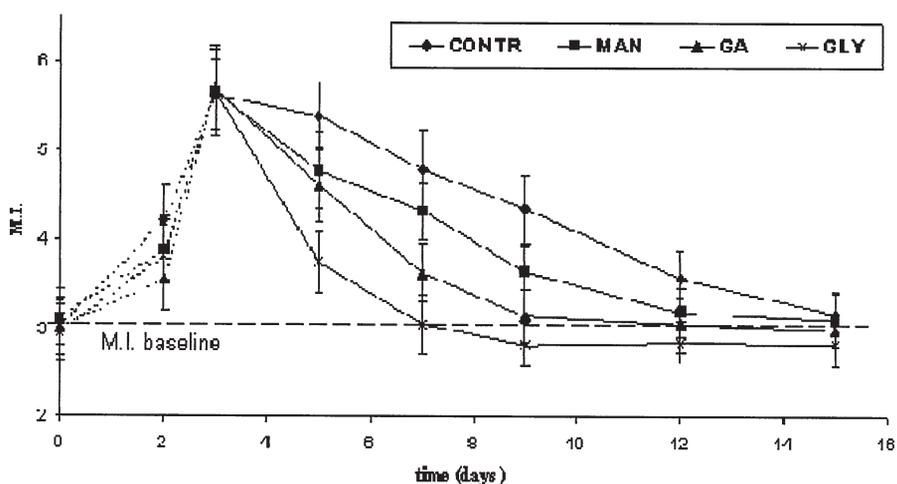


Figure 1. Trends of the melanin index (M.I.) vs time (days) for subjects recruited in the CONTR (no topical treatment), 10% GLY (glycolic acid), 10% MAN (mandelic acid), and 10% GA (grape acids) groups.

Table I

RT (recovery time) Values for Each Subject, Obtained in Treating Skin Sites with 10% GLY (glycolic acid), 10% MAN (mandelic acid), and 10% GA (grape acids) Formulations or without Treatment (CONTR)

Subjects	CONTR	10% MAN	10% GA	10% GLY
1	15	12	7	6
2	15	9	7	7
3	15	12	10	8
4	15	10	10	5
5	14	10	10	9
6	14	10	6	6
7	14	9	8	8
8	15	10	9	6
9	11	15	10	7
10	15	14	7	8
11	15	13	9	9
12	13	15	10	7
13	15	9	9	4
14	14	11	8	5
15	15	9	7	7
16	13	14	11	6
17	15	13	7	7
18	14	10	7	5
19	15	12	11	4
20	14	10	8	6
Mean	14.30	11.35	8.55	6.50
S.D	1.03	2.06	1.54	1.47

The "recovery time" value (RT) was evaluated as the time (days) required to obtained an M.I. baseline value (before dihydroxyacetone treatment) for each site.

IN VIVO EVALUATION OF SKIN ERYTHEMA INDUCED BY TOPICAL APPLICATION OF THE FORMULATIONS

The skin erythema induced by topical application of three different concentrations (% w/w) of glycolic, mandelic, and grape acids was evaluated by reflectance spectrophotometric measures. Spectral data were recorded for each site treated with the formulations over a monitoring period of 50 hours. The AUC values calculated over the monitoring time are shown in Figure 2, and the trends in Δ E.I. versus time are reported in Figure 3 (curves a, b, and c) at 10%, 30%, and 50% w/w. A significant increase in skin erythema was observed from the lower to the higher concentrations for GLY, MAN, and GA. Furthermore, at all three concentrations, GLY showed higher values of AUC than MAN and GA ($p < 0.05$). No statistical differences were found between MAN and GA at 10% w/w of concentration ($p > 0.05$). However, MAN induced a significantly higher AUC than GA at 30% and 50% w/w ($p < 0.05$).

IN VIVO EVALUATION OF THE PHOTOSENSITIZING EFFECTS INDUCED BY TOPICAL APPLICATION OF THE FORMULATIONS

The increase in skin sensitivity to UV light, induced by topical application of the 10% GLY, MAN, and GA formulations, was expressed as the photosensitivity percentage calculated using the erythema index values obtained by the reflectance spectrophotometric method.

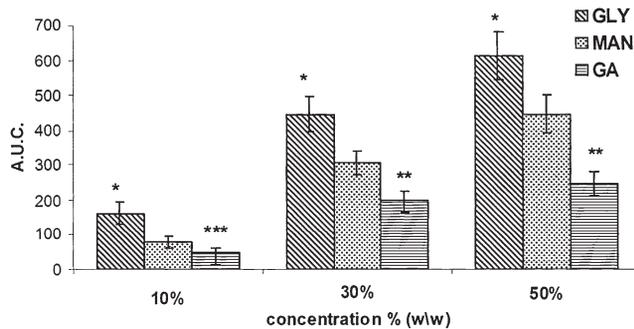


Figure 2. Mean area under curve (AUC) values (\pm SD) obtained by applying GLY (glycolic acid), MAN (mandelic acid), and GA (grape acids) formulations at three different concentrations (10%, 30% and 50% w/w) to skin sites of subjects admitted into the study. AUC values were directly related to the degree of skin erythema induced by topical application of the formulations. * $p < 0.05$ (significantly different) vs MAN and GA; ** $p < 0.05$ (significantly different) vs MAN; *** $p > 0.05$ (no significantly different) vs MAN.

As reported in Figure 4, topical application of the GLY formulation for four weeks induced a higher value of photosensitivity % than the MAN and GA formulations ($p < 0.05$), whereas the GA formulation induced a lower degree of skin erythema after UVB irradiation than the GLY and MAN formulations ($p < 0.05$).

DISCUSSION

Recently, many common alpha-hydroxy acids (AHAs) and combinations of them, such as mandelic acid, lactic acid, and natural acids present in fruits, wine, and milk have been extensively used as chemical peeling agents in cosmetic dermatological products. Known beneficial effects of chemical exfoliation include improvement in several cutaneous diseases and conditions such as acne vulgaris, melasma, scarring, and photodamage. It was previously reported that to evaluate the effects of AHAs, it was essential to identify the type, the pH of the formulations, and above all the concentration employed (6). Therapeutic/cosmetic peels required fairly high concentrations of AHAs (20–70%). However, the greatest impact of AHAs has been in skin-care and beauty products in which AHAs are employed at lower concentrations (4–10%).

In the present study, we compared the effects of three organic acids employed at 10% w/w of concentration (glycolic acid, mandelic acid, and a blend of organic acids from grape juice) as exfoliating agents by topical application. To this purpose we used a new *in vivo* method of evaluation.

For many decades, the fluorescent dansyl chloride (DC) has been used as a marker on skin to assess stratum corneum turnover time and the exfoliation rate *in vivo* (21). However, the level of DC fluorescence is often difficult to evaluate and the hazard of the dansyl chloride test is not negligible (22). Recently, dihydroxyacetone (DHA) has been introduced as a safe DC substitute. Since DHA can reach only the upper layers of the stratum granulosum, it is considered to be nontoxic. Although somewhat chemically distinct from melanins, the DHA-skin complex melanoids are very similar to melanins both spectroscopically and physically (21,23). In this study, we introduce the use of the melanin index obtained by reflectance spectrophotometric data to evaluate DHA-induced

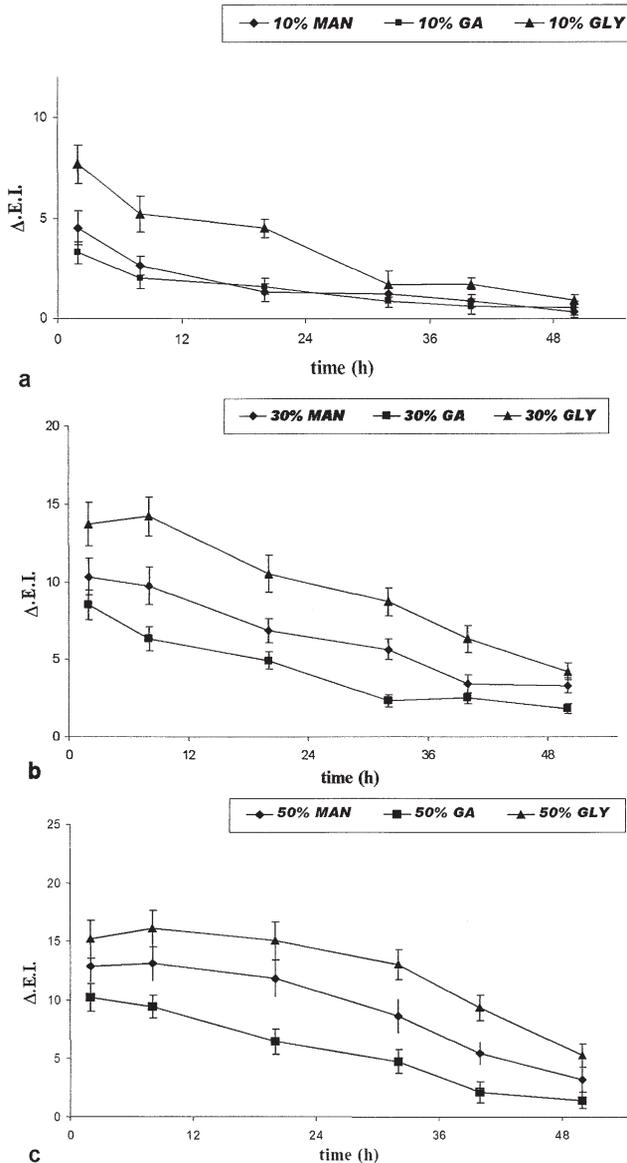


Figure 3. Trends of the erythema index (Δ .E.I.) vs time (hours) for GLY (glycolic acid), MAN (mandelic acid), and GA (grape acids) formulations at three different concentration, (a) 10%, (b) 30%, and (c) 50% w/w, recorded after topical application over the monitoring period of 50 hours.

pigmentation disappearance and to investigate the efficacy of exfoliating agents in inducing skin regeneration. Through the use of this method, we observed the different capacities of the tested acids to increase the rate of skin regeneration, with a significant reduction in the time required to obtain skin renewal. Topically applied at a 10% w/w concentration, grape acids showed an exfoliating effect significantly different from that of mandelic acid but were less active in comparison with glycolic acid. It is believed that at this concentration AHAs decrease corneocyte cohesion and enhance skin desquamation by

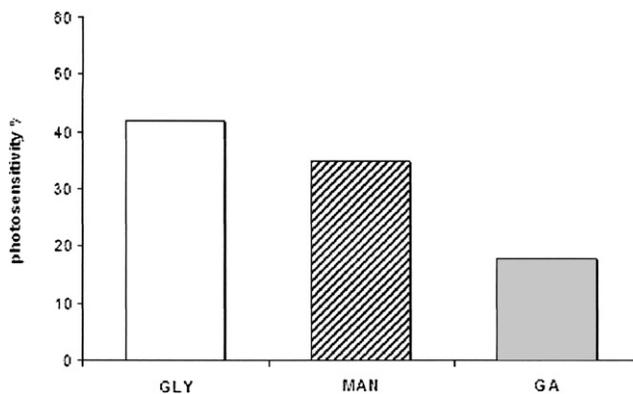


Figure 4. Increase in skin sensitivity to UVB irradiation expressed by photosensitivity percentage after short-time treatment (four weeks) with GLY (glycolic acid), MAN (mandelic acid), and GA (grape acids) formulations containing 10% of acids vs control (no topical treatment). The photosensitivity % values were calculated from the erythema index obtained 24 hours after UVB exposure for each subject participating in the study.

acidification of polar domains present within the hydrophilic lipid bilayers or by activation of acid protease crucial for desmosomal degradation (6). Furthermore, it has been reported that AHAs with a small molecular size are more active because they penetrate the skin more deeply (11). Glycolic acid is the simplest AHA, and it has the smallest molecular weight and size, followed by lactic, malic, tartaric, and citric acids, etc. This may explain the fact that glycolic acids tend to better penetrate the skin and accelerate skin regeneration. Mandelic acid and the organic acids contained in grape juice (tartaric acid, malic acid, citric acid, lactic acid, gluconic acid, and shikimic acid) have a greater molecular size and have more difficulty in penetrating the skin.

On the other hand, since the chemical peeling produces an insult to the skin, several side effects, such as erythema and redness, develop after treatment with exfoliating agents (3,9). Moreover, recent studies report that short-term dermal exposure with low concentrations of exfoliating agents results in increased photosensitivity to UV light, measured as increased erythema and tanning (13,24).

Skin tolerance to the exfoliating treatment is usually assessed by a visual and subjective record system. However, when objective and quantitative data are required, an instrumental and non-invasive method is preferred for more accurate evaluations of the adverse skin effects. To this end, the skin tolerance and the photosensitizing effects of exfoliating acids were investigated by reflectance spectrophotometric *in vivo* evaluation of skin erythema induced by topical application and after UV-light exposure. Since erythema is due to an increment in blood count in the subpapillary plexus of the skin and none of the main skin chromophores (hemoglobin and melanin) absorb in narrow bands, the erythema index is not exclusively a linear function of hemoglobin content, but is affected by skin melanin content (25). On the basis of these assumptions, the skin reflectance spectra, obtained by recording information on the optical spectrum of visible light ranging from 400 nm to 700 nm, is regarded as an accurate and reliable evaluation of the skin hemoglobin amount. Thereafter, the skin reflectance spectral values permit calculation of the erythema index by subtracting the main melanin absorption peaks (510 and 610 nm) from the hemoglobin absorption peaks at wavelengths of 540 nm, 560 nm, and 580 nm

(19). To better evaluate the skin tolerance of the tested acids, three different concentrations commonly used in light, medium, and deep peeling were topically applied. The study pointed out that skin erythema induced by topical application of the test organic acids increases with higher concentrations. Moreover, the spectrophotometric reflectance approach is also able to evaluate slight changes in mild erythema induced by UV exposure.

Recent experimental studies have demonstrated that subclinical irritation may be associated with topical exposure to glycolic acid. It is conceivable that the pro-inflammatory mediators released in irritated skin can affect events leading to UV-light sensitivity (13), increasing the risks of acute and chronic skin reactions. However, it is important to note that the rapid penetration of glycolic acid induces a more rapid skin exfoliation but causes a more intense skin redness and irritation. Thus, it is possible to infer that because of their molecular size, mandelic acid and grape acids are absorbed at a slower rate than glycolic acid, thereby causing less skin irritation. In addition, our results suggest that the spectrophotometric reflectance approach used in this study represents a sensitive method for monitoring and comparing the effectiveness and the safety of different topical agents in skin exfoliating treatments. The methodologies and protocols used in this study may help in choosing the most appropriate topical agents for short/long term and mild/strong skin-exfoliating treatments.

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