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Effect of the combination of different depigmenting agents *in vitro*

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Synopsis

Melanin plays a key role in our skin, protecting us against ultraviolet radiation, but there are situations in which its anomalous accumulation can lead to either aesthetic problems or diseases like melasma. For this reason, it is important to find agents that are able to decrease the skin pigmentation. It has been demonstrated that the melanin synthesis pathway can be inhibited at different levels by different mechanisms of action. The aim of this project is to combine some of these agents with different mechanisms of action on this pathway in order to find synergistic effects in the inhibition of tyrosinase and melanin synthesis. Kojic acid + α-lipoic acid combination are the only ones that have shown a synergistic effect over mushroom tyrosinase. However, this effect is not seen in melanin synthesis inhibition, although this combination is the most effective one. A potentiation effect is seen in arbutin + α-lipoic acid and kojic acid + azelaic acid combination. Kojic acid and α-lipoic acid combination might prove a good approach as treatment for hyperpigmentation disorders.

INTRODUCTION

Melanin is a pigment present in most living organisms. In animals, it derives from the amino acid tyrosine, and it is synthesized in melanocytes, which are located in the epidermis. More specifically, inside these cells, melanin is synthesized in specialized organelles called melanosomes. Melanin has different functions, among which the most important is its function as a protecting pigment against ultraviolet radiation of the sun (1–3).

Despite its beneficial role in sunlight protection, there are diseases in which an anomalous accumulation of melanin happens (hyperpigmentation), such as melasma or post-inflammatory hyperpigmentation (4). However, sometimes it is simply an aesthetic matter, where the patient wants to eliminate non-malignant dark spots. Hydroquinone is the depigmenting agent of reference and one of the most effective up to now, but it is cytotoxic (causing long-term adverse effects). Thus, hydroquinone has been banned in the European Union (24th Dir 2000/6/EC) as a skin-lightening agent because of its toxicity, although it can still be

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obtained on prescription for the treatment of melasma and other hyperpigmentary disorders (5–7). For this reason, there is an increasing concern about developing and finding new depigmenting agents that are effective and not cytotoxic so they can be used as an alternative to hydroquinone (6). The use of synergistic inhibition would be of interest to increase the inhibition and to reduce the dose needed to produce the desired effect too.

Melanin synthesis can be inhibited at different levels of the pathway. This has led to the idea that compounds with different mechanisms of action can be combined to obtain synergistic effects, so we can get a greater reduction of the melanin synthesis. Regarding the background in the combination of some of these agents, the one that has proved to be the most effective is a standard triple combination cream, which contains hydroquinone (which inhibits tyrosinase activity), an exfoliating agent (which stimulates the epidermal turnover and reduces the oxidation caused by hydroquinone), and a corticosteroid to reduce inflammation. Clinical trials have been performed with this cream and it has shown a good effectiveness.

Arbutin, a derivative compound from hydroquinone, inhibits tyrosinase in a competitive way (as an alternative substrate of tyrosine or L-3,4-dihydroxyphenylalanine [L-DOPA]), without affecting the expression of the enzyme (3,8–11). Kojic acid, which is a fungal metabolite obtained from *Aspergillus* or *Penicillium*, also inhibits tyrosinase activity by chelating copper atoms, which are essential for its function, in the active site of the enzyme. It also has antioxidant properties, preventing the conversion of o-quinone to L-DOPA, which will form melanin at the end (8,10,12–14). Azelaic acid, synthesized by the fungus *Pityrosporum ovale*, acts as a competitive inhibitor of tyrosinase, besides affecting the mitochondrial metabolism and having an antioxidant effect neutralizing free radicals. It also has a certain anti-proliferative and cytotoxic effect for melanocytes by inhibiting enzymes involved in DNA synthesis (2,10,15,16). α-Lipoic acid (or thioctic acid) also has a double action, as azelaic acid and kojic acid. On one hand, it inhibits the expression of microphthalmia-associated transcription factor (MITF), thus inhibiting the expression of the melanogenic enzymes (Tyrosinase, TYRP-1, TYRP-2). On the other hand, it has antioxidant properties in its oxidized form, although it is more antioxidant in its reduced form (dihydropholic acid) (17–20).

In this work, some widely used depigmenting cosmetic agents, which are arbutin, kojic acid, azelaic acid, and α-lipoic acid (see Figure 1), will be evaluated individually and in different combinations to look for synergistic effects between them. Mechanisms of action will be studied with a cell-free mushroom tyrosinase assay, although for some of them it has been already suggested (1,8,9). Pair combinations will be done to assess synergistic effects over mushroom tyrosinase and melanin content. For the determination of melanin content, maximum non-cytotoxic concentrations will be obtained for these compounds.

METHODS

MATERIALS

Arbutin, kojic acid, azelaic acid, α-lipoic acid, L-DOPA, synthetic melanin, mushroom tyrosinase, α-melanocyte-stimulating hormone (α-MSH) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide (NaOH) was purchased from Panreac (Barcelona, Spain).

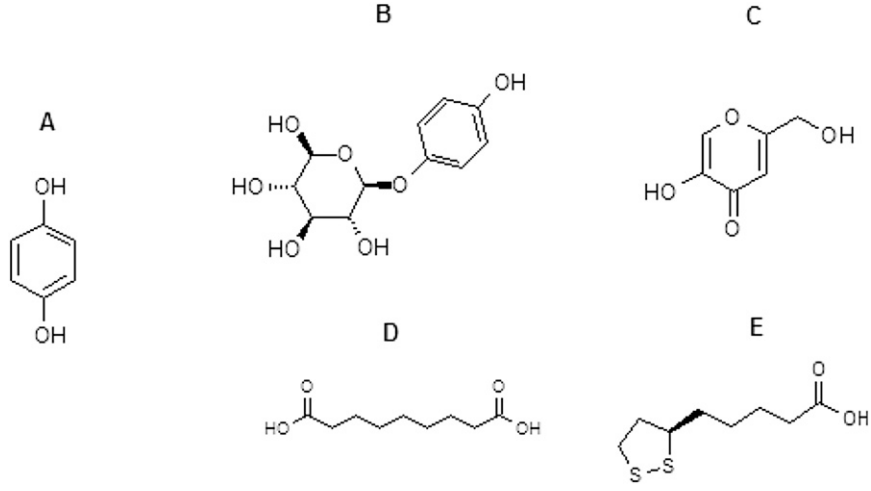

Figure 1. Chemical structure of (A) hydroquinone, (B) arbutin, (C) kojic acid, (D) azelaic acid, and (E) α-lipoic acid.

CELL CULTURE

Human skin melanocytes (HSM; ATCC, ATCC number: CRL-2208) in passes 8 to 11 were cultured with Dermal Cell Basal Medium (ATCC, Primary Cell Solutions, ATCC Number: PCS-200-030) supplemented with insulin (5 mg/ml), ascorbic acid (50 mg/ml), glutamine (6 m*M*), epinephrin (1 m*M*), calcium chloride (1.5 m*M*), peptide growth factor, M8 supplement, penicillin (10 units/ml), and streptomycin (10 units/ml). They were incubated at 37°C and 5% of CO_2 and culture medium was changed every 2 to 3 days.

CELL VIABILITY ASSAY

WST-1 assay (WST-1, Cat. No. 11 644 807 001, Roche, Mannheim, Germany) was performed to determine cell viability. HSMs ($1–2 \times 10^5$ cells/ml) were cultured in 96-well microplates for 24 h. The next day, different concentrations (1:10 dilutions from 1 mg/ml solution or 5 mg/ml for the less cytotoxic ones) for each compound were added and cells were cultured for 72 h. After treatment, the cells were treated with 10 µl of WST-1 and incubated at 37°C for 4 h. Cell viability was determined by measuring the optical density at 450 nm. Cells in culture medium without inhibitors were used as a control. Experiments were run in triplicate.

MUSHROOM TYROSINASE KINETICS ANALYSIS

Kinetic analysis of mushroom tyrosinase inhibition was performed as previously described with some modifications (24). Fifty microliters of mushroom tyrosinase (1000 units), various concentrations of L-DOPA (0.5, 1, 2, and 3 m*M*) as a substrate and

phosphate-buffered saline (PBS) (pH = 6.8) with or without inhibitor were added to a 96-well plate in a total volume assay of 150 µl. Using a microplate reader, the initial rate of dopachrome formation was determined by the increase of absorbance at 450 nm (OD450/min). Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) of the tyrosinase activity were determined by the Lineweaver–Burk's plot.

MEASUREMENT OF CELL-FREE TYROSINASE ACTIVITY

Cell-free tyrosinase activity was measured as previously described with some modifications (22). Fifty microliters of L-DOPA 2 m*M*, 50 µl of mushroom tyrosinase (200 U/ml), and 50 µl of each compound (arbutin 500 µg/ml, kojic acid 10 µg/ml, azelaic acid 100 µg/ml, and α-lipoic acid 50 µg/ml) were added to a 96-well microplate. Everything was dissolved in PBS pH 6.8. After 15 min at 37°C, absorbance at 450 nm was measured to determine the amount of dopachrome produced in the reaction mixture. Sample consisting of L-DOPA and tyrosinase was used as a control. Experiments were run in triplicate. Percentage of inhibition was calculated following the formula:

$$\% \text{ Inhibition} = (A_{sample}\ (450\ nm)/A_{control}\ (450\ nm)) \times 100$$

To determine the type of interaction between the agents, dose-effect curves were obtained for each agent and in combination. This data was used to run the CompuSyn software, which uses the method of Chou and Talalay for drug combination analysis (23). Combination index (CI) indicates synergism (<1), where the effect of the combination is higher than the sum of the individual effects, additive effect (=1), where the effect of the combination is the sum of the individual effects, or antagonism (>1), where the effect of the combination is smaller than the sum of the individual effects.

DETERMINATION OF MELANIN CONTENT

Melanin content was measured as previously described with some modifications (21). HSM ($1–2 \times 10^5$ cells/ml) were seeded into 6-well culture plates and cultured for 24 h. The next day, cells were added 0.1 m*M* IBMX and 0.1 µm α-MSH plus the different compounds, each one at their maximum non-cytotoxic concentrations, and incubated for 72 h. After treatment, cells were trypsinized and centrifuged 5 min at 1200 rpm. Melanin pellets were dissolved in 200 µl of 1N NaOH at 100°C for 30 min. Absorbance at 405 nm was measured, and the melanin content was calculated against a known standard of synthetic melanin. Experiments were run in triplicate.

STATISTICAL ANALYSIS

Statistical significance of experimental data was determined by one-way ANOVA/Dunnett's multiple comparison test and Student *t*-test. Differences were considered statistically significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

RESULTS

EFFECT OF ARBUTIN, KOJIC ACID, AZELAIC ACID, AND α-LIPOIC ACID ON CELL VIABILITY

To find the maximum concentration at which these agents were not cytotoxic, WST-1 assay was performed. The maximum non-cytotoxic concentrations obtained were 1000 µg/ml for arbutin, 100 µg/ml for kojic acid, and azelaic acid and 10 µg/ml for lipoic acid, which indicates that the four agents are less cytotoxic than hydroquinone (5 µg/ml) (Figure 2). Besides, significant proliferative effect was observed in all the agents.

These maximum non-cytotoxic concentrations were used to perform the same assay doing combinations of two agents. This was done to ensure that the agents, used in a

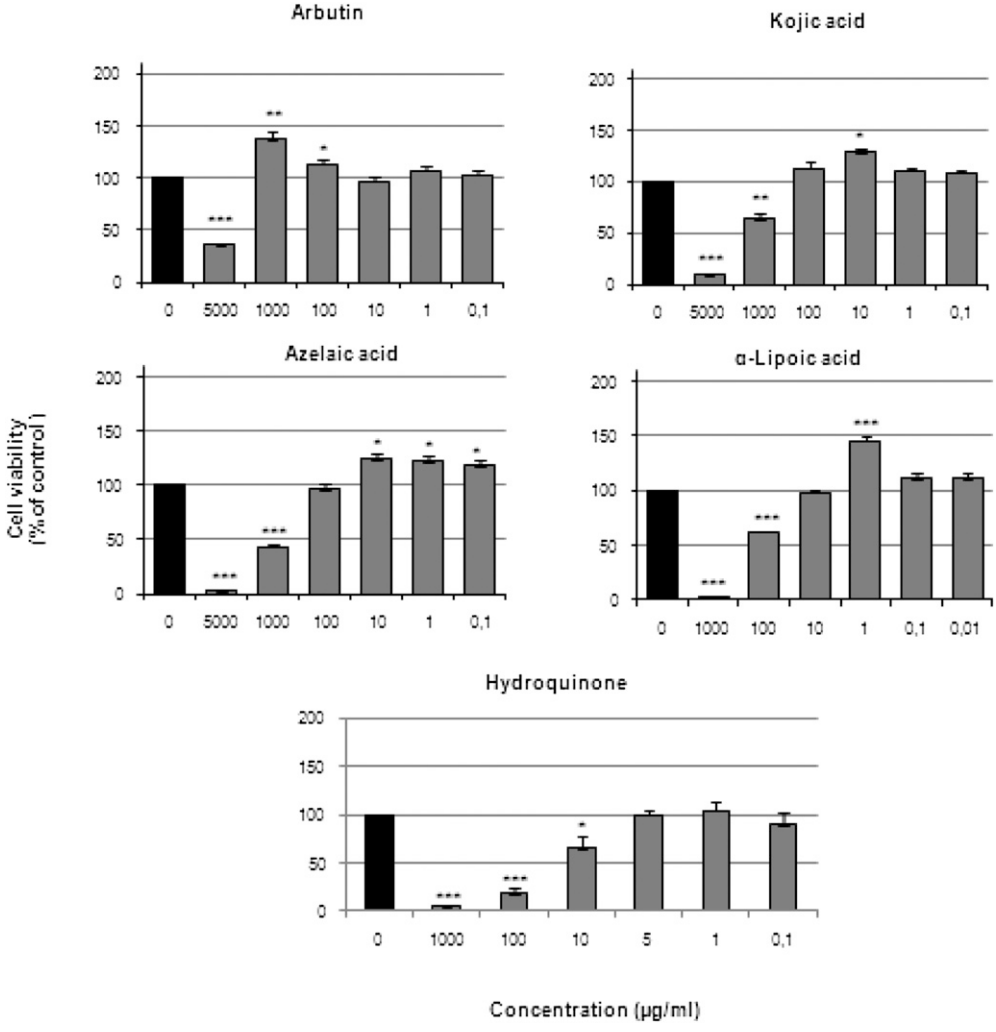

Figure 2. Human skin melanocytes viability with different concentrations of the agents cultured individually. Results are expressed as percentage of cell viability relative to control. Values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) are considered significantly different.

non-cytotoxic concentration, were still not cytotoxic in combination with others. As shown in Figure 3, only arbutin + α-lipoic acid combination had a significantly cytotoxic effect on HSM proliferation at their maximum non-cytotoxic concentration individually. Besides, as it happened with the individual treatment, arbutin + azelaic acid, arbutin + kojic acid, and arbutin + α-lipoic acid combinations showed a significant proliferative effect on HSM cells.

ARBUTIN, KOJIC ACID, AZELAIC ACID, AND α-LIPOIC ACID MUSHROOM TYROSINASE KINETIC ANALYSIS

To find the type of inhibition of these agents over mushroom tyrosinase, different concentrations of substrate (L-DOPA) in the presence of the inhibitors were used. Low concentrations of inhibitor were used to see possible synergistic effects between them. Lineweaver–Burk plots of 1/v versus 1/[L-DOPA] were made and showed that arbutin, kojic acid, α-lipoic acid, and azelaic acid are competitive, mixed, competitive, and competitive inhibitors, respectively, on diphenolase activity of mushroom tyrosinase (Figure 4).

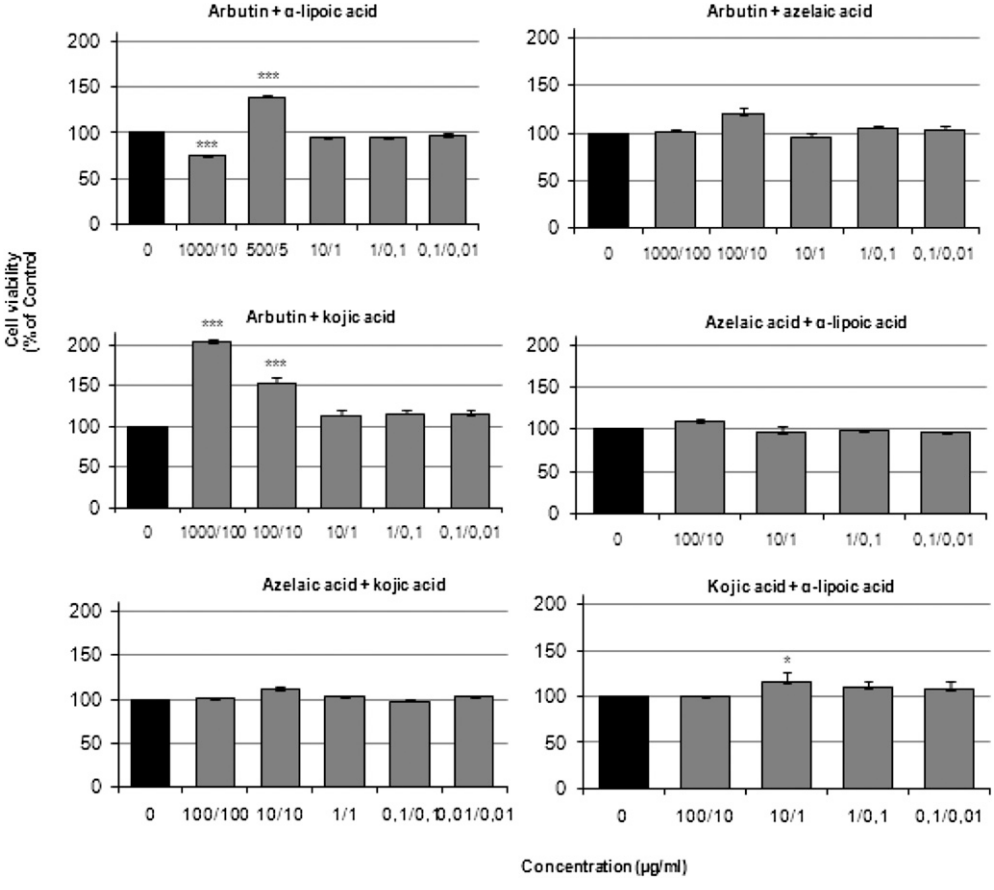

Figure 3. Human skin melanocytes viability with the maximum non-cytotoxic concentrations of the agents combined and its dilutions. Concentrations are expressed as concentration of the first agent/concentration of the second agent. Results are expressed as percentage of cell viability relative to control. Values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) are considered significantly different.

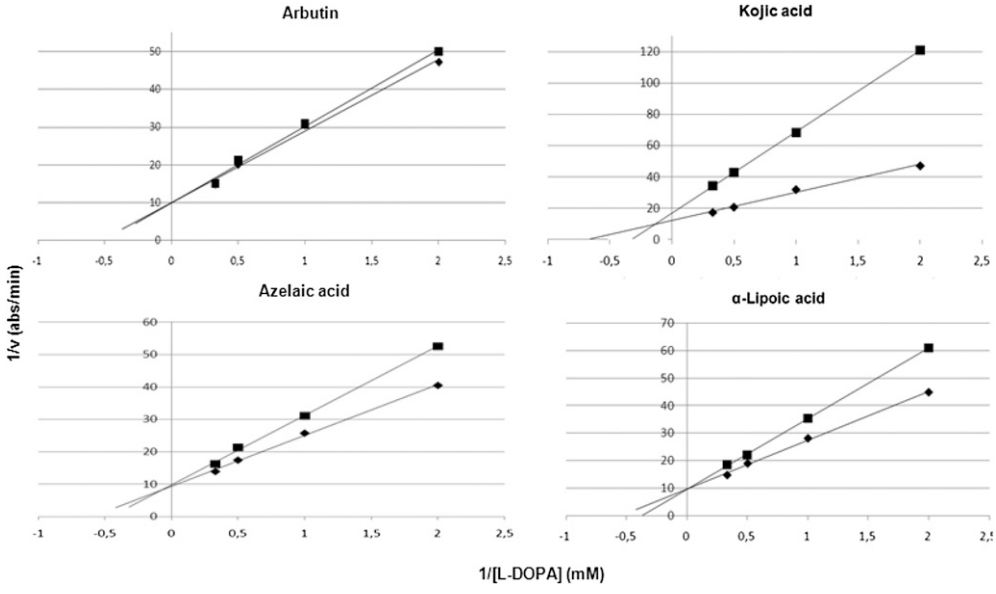

Figure 4. Lineweaver–Burk plots of mushroom tyrosinase. Arbutin (500 µg/ml), kojic acid (10 µg/ml), azelaic acid (100 µg/ml), and α- lipoic acid (50 µg/ml) were incubated with 0.5, 1, 2, and 3 m*M* L-DOPA.

EFFECT OF ARBUTIN, KOJIC ACID, AZELAIC ACID, AND α-LIPOIC ACID AND THEIR COMBINATIONS ON MUSHROOM TYROSINASE ACTIVITY

To investigate the inhibition of arbutin, kojic acid, azelaic acid, and α-lipoic acid on tyrosinase, mushroom tyrosinase assay was performed. Dose/effect curves were obtained for each agent and combination. Kojic acid plus each one of the other three agents were the combinations used here due to the difference in the inhibition type (mixed vs. competitive). α-Lipoic acid + azelaic acid combination was used as a control because they share the type of inhibition over tyrosinase (competitive). CIs at the IC50 are shown in Table I. The CI at this fraction affected (50%) was chosen because the variability is greater at low-effect levels and at high-effect levels. These results indicate that kojic acid + α-lipoic acid have a synergistic effect on mushroom tyrosinase, whereas kojic acid + arbutin and kojic acid + azelaic acid have an antagonistic effect on mushroom tyrosinase. α-Lipoic acid + azelaic acid combination, both of them being competitive inhibitors, have an additive effect on mushroom tyrosinase.

Table I
Combination Index of Kojic Acid + α-Lipoic Acid, Kojic Acid + Azelaic Acid, Kojic Acid + Arbutin and α-Lipoic Acid + Azelaic Acid, Obtained from the Output of the CompuSyn software. Next to the Names Is the Ratio of Concentration for Each Combination

	Combination index (IC50)	Type of interaction
Kojic acid + α-lipoic acid (ratio 1:5)	0.70	Synergism
Kojic acid + azelaic acid (ratio 1:10)	1.33	Antagonism
Kojic acid + arbutin (ratio 1:50)	1.29	Antagonism
α-Lipoic acid + azelaic acid (ratio 1:2)	1.12	Additive

To assess the effect of these agents and their combinations on melanogenesis, the inhibition of melanin production in HSMs was examined. The concentrations used were the maximum non-cytotoxic for each agent and combination. As shown in Figure 5, arbutin, kojic acid, azelaic acid, and α-lipoic acid produced an inhibition of 27%, 16%, 3%, and 46% at their maximum non-cytotoxic concentrations, respectively. Arbutin + azelaic acid, arbutin + kojic acid, arbutin + α-lipoic acid, azelaic acid + α-lipoic acid, kojic acid + α-lipoic acid, and azelaic acid + kojic acid produced an inhibition of 25%, 33%, 35%, 41%, 47%, and 22%, respectively. Hydroquinone inhibition on melanin synthesis was used as a reference compound. These results suggest that there is not an observable synergistic effect between these agents. However, a potentiation effect is observed in arbutin + α-lipoic acid and azelaic acid + kojic acid combinations in which one of the agents has no effect individually, but the combination effect is higher than the effect of the other agent.

DISCUSSION

In this study, combination effects between arbutin, kojic acid, azelaic acid, and α-lipoic acid were investigated. In the complex synthesis pathway of melanin, the key enzyme is tyrosinase, which regulates the first two steps of the pathway and is a common target of depigmenting agents. However, depigmenting agents can act at different levels in the production of melanin. This is why the most common classification for these agents is based on their mechanism of action. Because of these different ways to inhibit melanin synthesis, it has been proposed that combining these agents can lead to an increase in the inhibition of melanin synthesis, making the effect of the combination higher than the sum of the two agents individually, which is called a synergistic effect.

To evaluate these possible synergistic effects, we first performed a WST-1 assay to find the maximum non-cytotoxic concentrations of each inhibitor and combination between them, which were used after in the following experiments. To study the mechanism of action of these agents, we performed a kinetic analysis on the inhibition of mushroom tyrosinase. Afterwards, we evaluated possible synergies between different combinations in mushroom tyrosinase using the Chou–Talalay method (23). Finally, we tested the

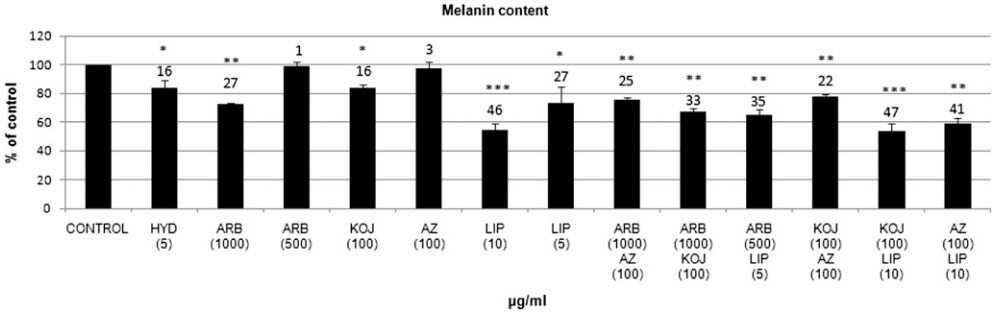

Figure 5. Effect of arbutin, kojic acid, azelaic acid, α-lipoic acid, and their combinations on melanin synthesis in human skin melanocytes. Cells were incubated at the maximum non-cytotoxic concentrations for 72 h. Results are expressed as percentage relative to control.

inhibition of melanin production of these agents and combinations in human melanocytes to detect possible synergistic or increased effects.

In the WST-1 assay, we saw that arbutin, kojic acid, azelaic acid, and α-lipoic acid were not cytotoxic up to 1000, 100, 100, and 10 μg/ml, respectively. As observed, all these agents are less cytotoxic than hydroquinone, as it has been previously described (1,2,30). Moreover, we observed that arbutin (1000 μg/ml), kojic acid (100 μg/ml), and α-lipoic acid (10 μg ml) showed a very significant increase in cell viability ($p < 0{,}001$), and azelaic acid was also but with less significance ($p < 0.05$). A cytoprotective and antioxidant activity of arbutin has been reported by Seyfizadeh *et al*. and Takebayashi *et al*. (25,26), although they used liver cells and fibroblasts, respectively, instead of melanocytes. Antioxidant effect of kojic acid, azelaic acid, and α-lipoic acid has also been reported (10,12,19,20). So, it might explain this proliferative effect (25). This effect is also seen in some of the combinations between agents. Regarding the combinations tested, hydroquinone was not included because as mentioned in the introduction, it is very cytotoxic and has been forbidden in cosmetics.

Mushroom tyrosinase inhibition kinetics was studied to find out the mechanism of inhibition of these agents over mushroom tyrosinase. Our results confirm what is proposed by other authors: arbutin and azelaic acid are competitive and kojic acid is a mixed-type inhibitor (1,8,9). Besides, in this project, we have studied the type of inhibition of α-lipoic acid, which as far as we know has not been described before. According to our results, α-lipoic acid seems to be a competitive inhibitor of diphenolase activity of mushroom tyrosinase.

Mushroom tyrosinase assay was performed to study synergistic effects of agent combinations on tyrosinase activity. Dose/effect curves for each compound and combination were performed to obtain the CI, which indicates synergism (<1), additive effect (=1), or antagonism (>1). As expected, an additive effect was observed between α-lipoic acid and azelaic acid as they have the same type of inhibition (competitive) on mushroom tyrosinase. Kojic acid is a mixed-type inhibitor of mushroom tyrosinase, so synergistic effects with the other agents could be expected. Indeed, kojic acid + α-lipoic acid combination showed to be synergistic (CI = 0.70). However, kojic acid with arbutin or azelaic acid combinations showed to have an antagonistic relationship. This might be due to the fact that although arbutin, azelaic acid, and α-lipoic acid bind to the same site of the enzyme (same type of inhibition), their binding can be directed to different mechanistic forms (different enzyme–substrate complexes) (27). So this could explain why there is a synergy between α-lipoic acid and kojic acid and an antagonistic effect between arbutin or azelaic acid and kojic acid.

Melanin content measurement was carried out to study synergistic effects on melanin synthesis. The individual values are in concordance with that of Lajis *et al*. (13), Tai *et al*. (28), and Lee *et al*. (29). Hydroquinone inhibition value was very low because the concentration used was smaller than the other compounds due to its high cytotoxicity. Arbutin + azelaic acid combination had similar values than arbutin alone (27% vs. 25%), indicating that there is neither synergy nor antagonism between them. The same happens with the azelaic acid + α-lipoic acid combination (41% of inhibition in combination and 45% of inhibition by α-lipoic acid alone) and kojic acid + α-lipoic acid combination (47% of inhibition in combination and 46% by α-lipoic acid alone). However, kojic acid + α-lipoic acid combination showed to be synergistic over mushroom tyrosinase. This mismatch between mushroom tyrosinase inhibition and melanin synthesis inhibition

results can be due to the fact that there is not always a perfect correspondence between these values because mushroom tyrosinase and human tyrosinase are different in some aspects (7,16). Also, when evaluating melanin synthesis inhibition, we are using cells, which are a more complex system than an *in vitro* enzymatic assay, and thus other mechanisms of action performed by these inhibitors might influence in the final melanin content. Arbutin + kojic acid combination exerted an additive effect on melanin inhibition in human melanocytes, as the combination value is higher than both individual values but not enough to produce synergy (33% vs. 27% and 16%). Besides, a slight potentiation effect can be seen in the arbutin + α-lipoic acid combination, as there is not inhibition of arbutin at 500 μg/ml, and its combination with α-lipoic acid has a greater effect than the individual inhibition of α-lipoic acid (35% vs. 27%). A similar potentiation effect is observed in the kojic acid + azelaic acid combination, where azelaic acid barely inhibits individually melanin synthesis, but the combination with kojic acid is higher than the individual inhibition by kojic acid (22% vs. 16%). This effect can be explained because arbutin and azelaic acid are probably facilitating α-lipoic acid and kojic acid inhibition, respectively, and thus increasing the melanin inhibition.

Despite the fact that arbutin, kojic acid, azelaic acid, and α-lipoic acid have different inhibition mechanism over tyrosinase and melanin synthesis, it does not seem to be strong enough to produce a synergistic effect on melanin inhibition when combining them. It may be possible that the fact some of them act by themselves at different steps in the melanin pathway makes it difficult to cause or to observe a synergistic effect, that is, α-lipoic acid is an inhibitor of tyrosinase and the expression of MITF.

To sum up, kinetic analysis on mushroom tyrosinase was done to study the type of inhibition of these agents, and afterward see if differences in this inhibition were able to cause synergistic effects on tyrosinase inhibition and melanin synthesis. Interestingly, kojic acid + α-lipoic acid combination induced a synergistic effect on mushroom tyrosinase, whereas kojic acid + arbutin and kojic acid + azelaic acid combination showed to be antagonistic. When evaluating these combinations on human melanocytes, arbutin + kojic acid had an additive effect on melanin synthesis, and a potentiation effect was observed in the arbutin + α-lipoic acid and kojic acid + azelaic acid combination. However, the most effective combination was kojic acid + α-lipoic acid (47% of inhibition on melanin synthesis). Kojic acid + α-lipoic acid might be a good approach as treatment for hyperpigmentation disorders.

Further research in this project will include the measurement of human tyrosinase inhibition and the impact on other proteins involved in the melanin pathway by these agents to confirm that they are acting on different levels of melanogenesis. Besides, it will include the research of other agents that are acting on other steps of melanin synthesis. so it can be combined to produce a synergistic effect and so a greater depigmenting effect. Furthermore, combinations of three or more different inhibitors will be performed.

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