A Potent Tyrosinase Inhibitor from *Artocarpus Lakoocha* Heartwood Extract: Comparative Evaluation of Its Melanin-Reducing Efficacy in Guinea Pigs and Humans

Parkpoom Tengamnuay[1]*, Kumkwan Pengrungruangwong[1] and Kittisak Likhitwitayawuid[2]

[1]Department of Pharmacy and [2]Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand. Tel. 662-2188402, Fax. 662-2188401.

*To whom correspondence should be addressed (parkpoom.t@chula.ac.th)

Keywords: *Artocarpus lakoocha* Roxb., heartwood extract, tyrosinase inhibitor, melanin reduction, skin-whitening agent

Summary

The heartwood extract of *Artocarpus lakoocha* Roxb., which contains a potent tyrosinase inhibitor oxyresveratrol, was evaluated for its melanin-reducing efficacy in both guinea pigs and human volunteers. After 4 week-daily application of the extract dissolved in propylene glycol to the back of guinea pigs, significant reduction in melanin content was detected, with the effect greater than 3% kojic acid and solvent propylene glycol ($P < 0.05$). The extract was subsequently tested in female volunteers (3 groups of 20 subjects) using a parallel clinical trial with self-control. The first group received the *A. lakoocha* solution in propylene glycol whereas the second and the third group respectively received 0.25% licorice extract and 3% kojic acid in the same solvent. The subject in each group twice daily applied the test solution on one arm whereas the remaining arm was applied with only propylene glycol (self-control) for 12 weeks. The *A. lakoocha* extract was found to be the most effective agent, giving the shortest onset of significant whitening after only 4 weeks of application ($P < 0.05$), followed by 3% kojic acid (8 weeks) and 0.25% licorice extract (10 weeks). The whitening effect also increased with time, with the highest extent observed with *A. lakoocha* at week 12. The *in vitro* antityrosinase activity of *A. lakoocha* extract decreased with time upon storage at room temperature but could be stabilized by a combination of several antioxidants. In conclusion, the heartwood extract of *A. lakoocha* appeared to have promising potential for use as an effective and economical skin-whitening agent.

Introduction

In recent years, the number of Asian women aspiring for a whiter skin complexion has increased dramatically. This is due partly to the discovery of many effective skin whitening agents,

particularly those derived from natural sources such as arbutin, kojic acid and licorice extract. Promotion of these substances by the cosmetic industry in various media has played a critical role in the widespread popularity of skin whitening products among women, especially in the Fareast and Southeast Asia. Also, it is a traditional perception of the women in this region that a lighter skin can provide them the appearance of grace, nobility and youthfulness, as evidenced in many Asian poems and songs, which often describe the beauty of a lady in association with the fairness of her skin.

The skin color is mainly determined by the content of an epidermal pigment called melanin. Its major function is to provide protection against ultraviolet (UV) radiation. However, excessive melanin production is not desirable in many people since it may cause a darker or uneven skin color. The initial process of melanin production (melanogenesis) is controlled by tyrosinase, which is an enzyme catalyzing the hydroxylation of tyrosine, the precursor of melanin, into dihydroxyphenylalanine (DOPA) and other intermediates. Thus, inhibition of tyrosinase activity or its production can prevent melanogenesis [1]. Several plant extracts were found to exhibit strong antityrosinase activity *in vitro* such as those from *Areca catechu L.* [2], *Artocarpus incisus* [3], *Broussonetia spp.* (paper mulberry root bark extract) [4], *Glycyrrhiza glabra* (licorice extract) [5], *Prunus spp.* [6], and *Rheum officinale* [7]. Many of these extracts have been tested *in vivo* and commercially developed as skin whitening agents in cosmetic preparations such as kojic acid, licorice extract and *Morus alba* extract [8]. However, they are quite expensive and some of the users may develop skin hypersentivity upon application especially when using at high concentrations.

Artocarpus lakoocha Roxb. (family Moraceae) is a tropical tree about 20-30 meters high commonly found throughout South and Southeast Asia. In Thailand, the local people have been using the dried aqueous extract of *A. lakoocha* heartwood in the traditional treatment of parasites such as tapeworms. The main constituent in the heartwood extract is 2,4,3',5'-tetrahydroxystilbene or oxyresveratrol [9]. Although the extract has long been characterized, its many other medicinal properties apart from anthelmintic are not widely known or studied.

Recently, Sritularak et al. [10] have screened a large number of plants for their *in vitro* antityrosinase activity and found that the heartwood extract of *A. lakoocha* exhibited the highest activity. Purification of the extract yielded two active components, namely, oxyresveratrol and resveratrol. However, oxyresveratrol was found to be 20-times more potent than resveratrol in inhibiting mushroom tyrosinase. In addition it was present in the extract in greater quantity (70 - 90% depending on the batch). Thus, the extract appeared to have a strong potential for use as a whitening agent in cosmetic preparations provided that its efficacy and safety have been tested. The main objective of this study was therefore to evaluate the *in vivo* skin whitening efficacy of *A. lakoocha* heartwood extract in guinea pigs and human volunteers. The results were subsequently compared with two tyrosinase inhibitors commonly used in whitening products (kojic acid and licorice extract). Furthermore, the stability of the extract in aqueous solution was assessed in the presence of various antioxidants.

MATERIALS AND METHODS

Materials

The extract of *Artocarpus lakoocha* was traditionally obtained by boiling small pieces of heartwood in water. After removing the remaining wood fragments and other insoluble residues, the aqueous extract was dried to give yellowish powder for use in the studies. The content of oxyresveratrol in the dried extract was determined to be 70% w/w [11]. The *in vitro* antityrosinase activity of the extract was also determined using tyrosinase (EC 1.14.18.1 from mushroom T-7755) and L-DOPA as enzyme and substrate, respectively (both were obtained from Sigma Chemicals Co., USA). Kojic acid was purchased from Nikko Chemicals Co., Japan, whereas licorice extract (PT-40) was from Maruzen Pharmaceutical Co., Japan. All other reagents were of analytical grade and used as received. The same batches of *A. lakoocha* extract, kojic acid and licorice extract were used in every study. All the test solutions were freshly prepared every 3 days except for those in the stability study.

Comparative Evaluation of Skin Whitening Activity in Guinea Pigs

The method was slightly modified from Imokawa et al. [12] and Jang et al. [4]. Eighteen black-skinned guinea pigs (weight 250 – 300 g) were shaved on their back and irradiated by a UVB lamp (290-320 nm, Philips TL 20 w/12) at an energy of 900 mJ/cm^2 per day (0.25 mW/cm^2 x 60 min per day at a distance of 17.5 cm from UVB lamp) for 3 consecutive days. Following the third UVB exposure, the animals were left for 18 days to allow for maximum darkening of the exposed skin. Then, the melanin content in the back of each guinea pig was measured using Mexameter MX 16 (Courage+Khazaka electronic, Germany). Shaving of the back of each animal was necessary before melanin measurements. The guinea pigs were then divided into three groups of six. The first and the second groups respectively received daily application of *A. lakoocha* extract and 3 % kojic acid dissolved in propylene glycol on their back whereas the third group received only propylene glycol solvent (control group). The application volume was fixed at 0.5 ml in all groups and thoroughly applied to the back of each animal for 4 weeks. The values of melanin were measured again at 2 and 4 weeks after application.

The value of % whitening relative to the initial melanin value was calculated for each guinea pig at 2 and 4 weeks according to the following formula:

$$\% \text{ Whitening} = [(X_0 - X_t)/X_0] \times 100\%$$

Where X_0 = initial melanin value measured at the start of the study (week 0 or immediately before sample application)

X_t = melanin value measured after t^{th} week of application, where t = 2 and 4 in guinea pig study and 2, 4, 6, 8, 10 and 12 in human study

The average values of % whitening were compared among the three groups using one-way ANOVA at 5% significance level. If significance was found, Duncan's new multiple range test was subsequently applied to rank the whitening effect of the three groups.

Comparative Evaluation of Skin Whitening Activity in Human Volunteers

The protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. Sixty female volunteers, age between 20 – 48 years with normal healthy skin, were recruited in the study. . They were divided into three groups of 20. The initial melanin values were taken from both the left and right upper arms of each subject using Mexameter prior to application of the test substances. Each volunteer in the first group was then applied twice daily with five drops of *A. lakoocha* extract in propylene glycol on the outer surface of one of her upper arms, whereas the remaining arm was applied with the same quantity of only propylene glycol (the self-control arm). The daily application continued for twelve weeks and the melanin content was measured at a two-week interval to observe for any changes in the melanin content and calculated as % whitening similar to the guinea pig study. Statistical comparison of % whitening at various application times was then made between the arm treated with *A. lakoocha* and the self-control (propylene glycol-treated) arm of the same subject to detect for any significant difference using paired student's t-test at 5% level. The procedure was similar for the subjects in the second and the third groups in which the *A. lakoocha* test solution was respectively replaced with 0.25 % licorice extract and 3 % kojic acid dissolved in propylene glycol. All subjects were required to cover their upper arms with appropriate clothing at all time to avoid interference from the sunlight and external environments.

Stability of *A. lakoocha* Solutions

Stability of *A. lakoocha* extract in aqueous solution was evaluated with respect to its antityrosinase activity. Solutions of *A. lakoocha* extract in the mixture of 20 % propylene glycol and 80 % water were prepared with and without antioxidants (sodium metabisulfite, butylated hydroxyanisole (BHA), EDTA and their combinations. All the solutions were kept at ambient temperature (28 °C) for 24 weeks and their tyrosinase inhibitory activity was periodically determined by the DOPAchrome method using L-DOPA as the substrate [7].

Results and Discusion

Guinea Pig Study

FIGURE 1 is a histogram showing the values of % whitening after application of propylene glycol, kojic acid and *A. lakoocha* extract to the back of guinea pigs. Since all the animals had been pre-exposed to UVB to stimulate the melanin production, natural whitening process also occurred to restore the skin color back to the original condition. As seen in the control group which received only the solvent propylene glycol, natural whitening process resulted in a slight reduction of the melanin content from the starting value, giving an average % whitening (mean $\pm$ s.d.) at week 2 of about 0.90 $\pm$ 2.34 %, which increased to 3.26 $\pm$ 1.15 % at week 4. On the other hand, application of kojic acid (3% in propylene glycol) induced a much greater reduction in melanin content, giving a mean % whitening of 4.84 $\pm$ 1.71 % at week 2 and further increased to 5.38 $\pm$ 1.55 % at week 4. Also, application of *A. lakoocha* extract in propylene glycol resulted in an even

more pronounced reduction of melanin content, with the mean % whitening values of 4.82 $\pm$ 1.99 % and 7.59 $\pm$ 1.40 % at week 2 and 4, respectively.

Statistical comparison of % whitening among the three groups, either at 2 or 4 weeks, revealed that both kojic acid and *A. lakoocha* extract were more effective than the control group in reducing the melanin content ($P < 0.05$, ANOVA and Duncan's new multiple range test). In addition the extract of *A. lakoocha* was significantly the most effective after 4 weeks of application ($P < 0.05$).

The initial *in vivo* data thus indicated that the extract of *A. lakoocha* heartwood exhibited an effective skin whitening action in the guinea pig model. The promising results from the guinea pig study had led to the decision to confirm its whitening efficacy and safety in human volunteers.

Human Study

FIGURES 2, 3 and 4 are respective plots of % whitening versus time following topical application of *A. lakoocha* extract, kojic acid and licorice extract. From FIGURE 2 it can be seen that the average % whitening of the upper arms treated with *A. lakoocha* continuously increased with application time, i.e., from 0.18 % at week 2 to 2.78 % at week 12. However, the values for the arms treated with propylene glycol (self-control) increased only slightly, from 0.00 % at week 2 to 0.65 % at week 12. When paired student's t-test was applied to the whitening data at various weeks, significant difference was found in the mean % whitening values between the arms treated with *A. lakoocha* and the self-control arms after 4 weeks of application. After week 4, the significance became even more pronounced ($P << 0.05$) as the difference between the two arms continued to increase until the end of the study (week 12), indicating a greater whitening efficacy of *A. lakoocha* over the control (propylene glycol).

It is interesting to note that the control arms (propylene glycol-treated) also showed some degree of whitening relative to the initial melanin contents. This was due to the fact that all the volunteers were required to cover their upper arms with proper attires and avoid direct exposure to sunlight throughout the entire study period, thereby resulting in the natural whitening of the control arms. However, this process occurs at a much lower rate and extent since the addition of *A. lakoocha* extract to the solvent propylene glycol evidently induced much more effective whitening.

The application of kojic acid and licorice extract also resulted in a significant whitening when compared to their respective control (FIGURES 3 and 4). The values of % whitening also gradually increased from week 2 through week 12. However, the rate and extent of significant whitening effect was lower than that caused by *A. lakoocha* extract. For example, the volunteers treated with kojic acid showed a mean maximum whitening at week 12 of 2.23 %, which was somewhat lower than that of *A. lakoocha* at the same period (2.78 %). In addition, significant difference in % whitening was detected after a longer period of application (at week 8 and afterwards), indicating a slower onset of efficacy than the *A. lakoocha* extract (FIGURES 2 and 3). The volunteers treated with licorice extract also showed a lower whitening efficacy since the maximum % whitening was only 1.70 % at week 12 and significant whitening was detected only at week 10 and 12 of application (FIGURE 4).

Thus, the data from the current human study firmly supported the previous findings from the guinea pig study that the heartwood extract of *A. lakoocha* appeared to be more effective than 3 % kojic acid and 0.25 % licorice extract, particularly with respect to the rate of whitening. Its high efficacy could be related to its strong *in vitro* inhibitory activity on the mushroom tyrosinase enzyme. The concentration to produce 50 % inhibition of tyrosinase (IC_{50}) was reported to be only 1.5 μM or 0.37 μg/ml for oxyresveratrol, the active consitutuent of *A. lakoocha* heartwood [11]. This value was much lower than that of kojic acid (5.8 μg/ml) and the licorice extract (12.9 μg/ml), both of which are widely used whitening agents in many cosmetic preparations and served as positive controls in this study [8,13]. The greater antityrosinase activity of oxyresveratrol was also reported by other researchers [14], who found that oxyresveratrol was a potent inhibitor of mushroom tyrosinase with IC_{50} of 1.2 μM, a value 32-fold stronger than kojic acid. They also postulated that the depigmenting effect of oxyresveratrol was through reversible inhibition of tyrosinase activity rather than suppression of the expression and synthesis of the enzyme.

Regarding the side effects, no skin rashes or serious skin disorders were observed in all subjects receiving *A. lakoocha* or licorice extract. On the other hand, one subject receiving kojic acid developed a skin rash and had to withdraw from the study after 6 weeks. The symptom disappeared after treatment with topical antihistamine. Thus, the heartwood extract of *A. lakoocha* appeared to be well tolerated and could be as safe as the licorice extract. However, since skin hypersensitivity is unpredictable, the extract should be further evaluated along with other longer-term skin toxicity.

Stability Study

Initial stability data showed that the tyrosinase inhibitory activity of *A. lakoocha* extract in aqueous solution decreased with time upon storage at room temperature. As seen from TABLE I, the activity dropped to about 50 % of the initial value after 24 weeks. The activity could be stabilized by addition of various antioxidants. Combination of two or more antioxidants was found to stabilize the extract better than the single component. In particular, the mixture of sodium metabisulfite and butylated hydroxyanisole (BHA) appeared to provide the best stabilization, giving the antityrosinase activity of about 80 % of the initial value after 24-week storage.

Conclusions

The results of this study clearly demonstrated that the heartwood extract of *A. lakoocha* was able to reduce melanin formation in both guinea pigs and human volunteers. Comparing to other tyrosinase inhibitors commonly used in whitening products like kojic acid and licorice extract, the readily available and less expensive *A. lakoocha* extract showed the fastest onset of efficacy, requiring only 4 weeks of application for significant whitening effect. Considering the potent antityrosinase activity of oxyresveratrol, its major constituent, the extract appears to have a very promising potential for use as a safe, effective and economical whitening agent in the cosmetic industry. More studies are being carried out to determine the optimal concentrations, stability as well as safety and efficacy of *A. lakoocha* extract in different formulations.

Acknowledgments

The authors wish to thank the Departments of Pharmacy and Pharmacognosy of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, for providing research facilities and Ouiheng Import Co., Ltd., for providing financial support.

References

1. Prota, G., Melanin and melanogenesis, *Cosmetic and Toiletries*, **111** (1996) 44-45.
2. Lee, K.K., and Choi, J.D., The effect of *Areca catechu* L.extract on anti-inflammatory and antimelanogenesis, *Int. J. Cosmet. Sci.*, **21** (1999) 275-284.
3. Shimizu, K., Kondo, R., Sakai, K., Lee, S.H., and Sato, H., The inhibitory components from *Artocarpus incisus* on melanin biosynthesis, *Planta Med.*, **64** (1998) 408-412.
4. Jang, D.I., Lee, B.G., Jeon, C.O., Jo, N.S., Park, J.H., Cho, S.Y., Lee, H., and Koh, J.S., Melanogenesis inhibitor from paper mulberry, *Cosmetic and Toiletries*, **112** (1997) 59-62.
5. Yokota, T., Nishio, H., and Kubota, Y., The inhibitory effect of glabridin from licorice extracts on melanogenesis and inflammation, *Pigment Cell Res.*, **11** (1998) 335-361.
6. Matsuda, H., Nakamura, S., and Kubo, M., Studies of cuticle drugs from natural sources. II. Inhibitory effects of prunus plants on melanin biosynthesis, *Biol. Pharm. Bull.*, **17** (1994) 1417-1420.
7. Iida, K., Hase, K., Shimomura, K., Sudo, S., Kadota, S., and Namba, T., Potent inhibitors of tyrosinase activity and melanin biosynthesis from *Rheum officinale*, *Planta Med.*, **61** (1995) 425-428.
8. Kim, J.H., and Lee, K.T., Inhibitory effects of *Morus alba* extracts on melanogenesis, *Cosmetic and Toiletries*, **113** (1998) 65-70.
9. Mongkolsuk, S., Robertson, A., and Towers, R., 2,4,3',5'-tetrahydroxystilbene from *Artocarpus lakoocha*, *J. Chem. Soc.*, (1957) 2231-2233.
10. Sritularak, B., De-Eknamkul, W., and Likhitwitayawuid, K., Tyrosinase inhibitors from *Artocarpus lakoocha*, *Thai J. Pharm. Sci.*, **22** (1998) 149-155.
11. Sritularak, B., Chemical Constituents of *Artocarpus lakoocha* and *A. gomezianus*, Master's Thesis, Department of Pharmacognosy, Graduate School, Chulalongkorn University, Bangkok, Thailand, 1998.
12. Imokawa, G., Kawai, M., Mishima, Y., and Motogi, I., Differential analysis of experimental hypermelanosis induced by UVB, PUVA and allergic contact dermatitis using a Brownish Guinea Pig model, *Arch. Dermatol. Res.*, **278** (1986) 352-362.
13. Likhitwitayawuid, K., Sritularak, B., and De-Eknamkul, W., Tyrosinase inhibitors from *Artocarpus gomezianus, Planta Med.*, **66** (2000) 275-277.
14. Kim, Y.M., Yun, J., Lee, C.K., Lee, H., Min K.R., and Kim, Y., Oxyresveratrol and hydroxystilbene compounds. Inhibitory effect on tyrosinase and mechanism of action, *J. Biol. Chem.*, **277** (2002) 16340-16344.

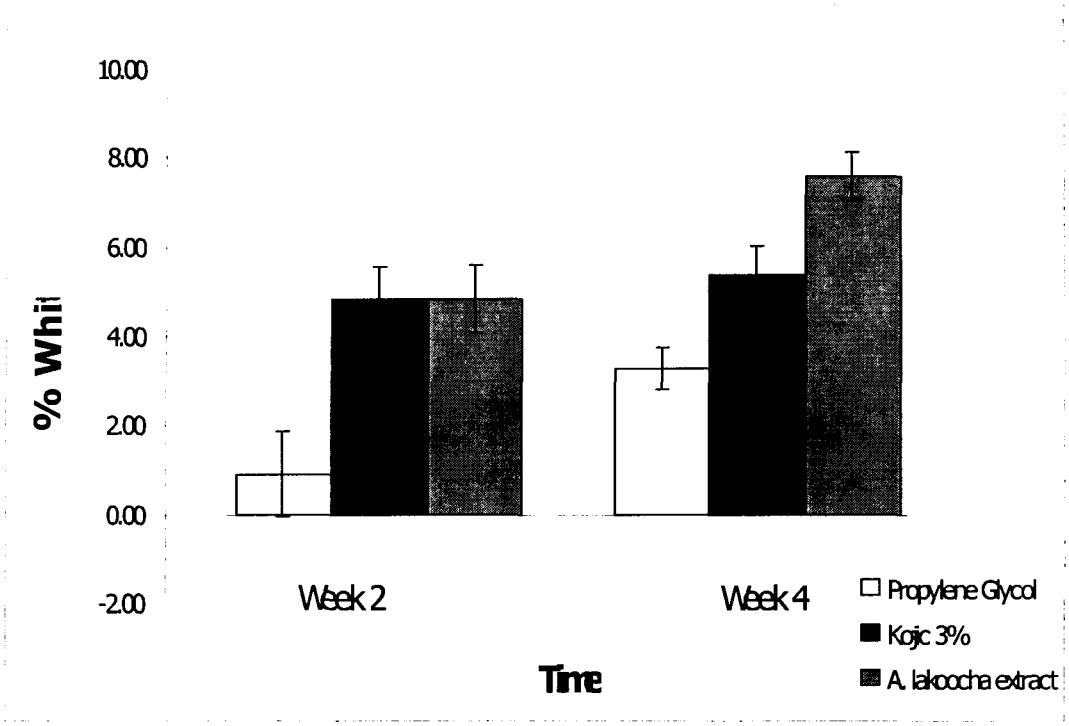

FIGURE 1. Histogram comparing % whitening after application of *A. lakoocha* extract, kojic acid and propylene glycol to guinea pigs for 2 and 4 weeks. Data = mean $\pm$ s.e.m. (n = 6).

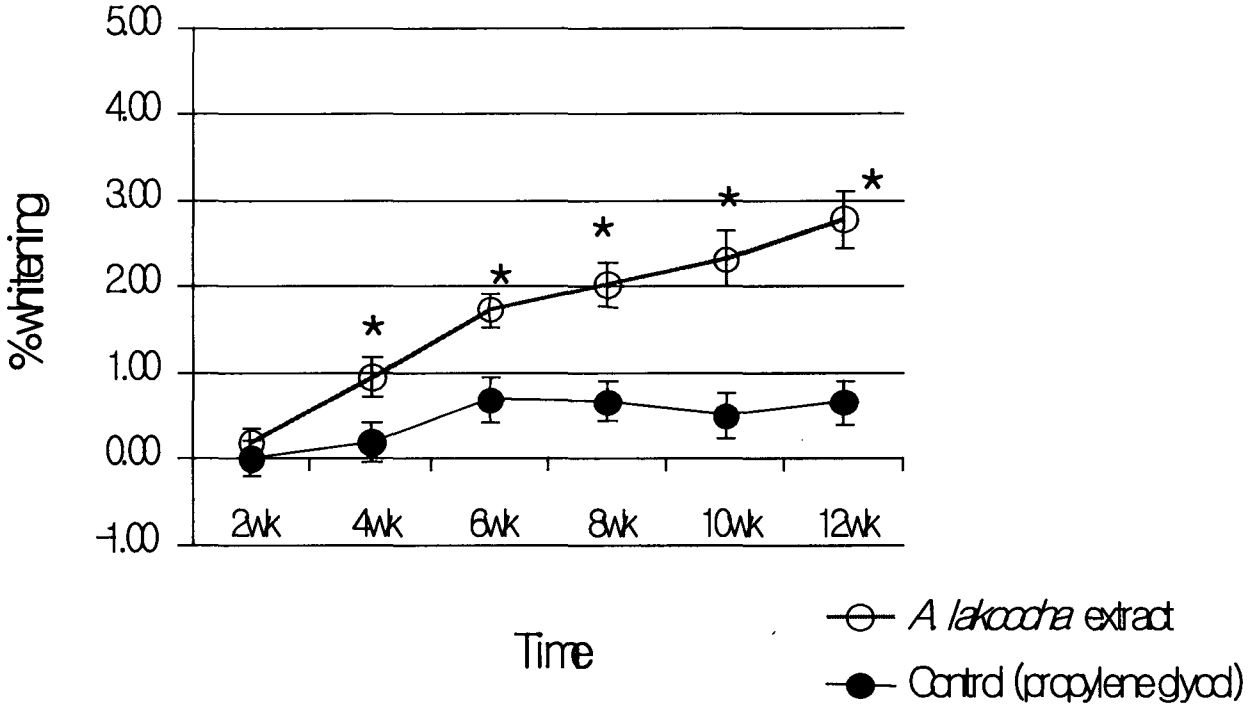

FIGURE 2. Whitening effect of *A. lakoocha* heartwood extract in propylene glycol in comparison with control (propylene glycol alone) during daily application to the upper arms of female volunteers for 12 weeks. Data = means ± s.e.m. (n = 20).

* Significant from control ($P < 0.05$) at the same time point.

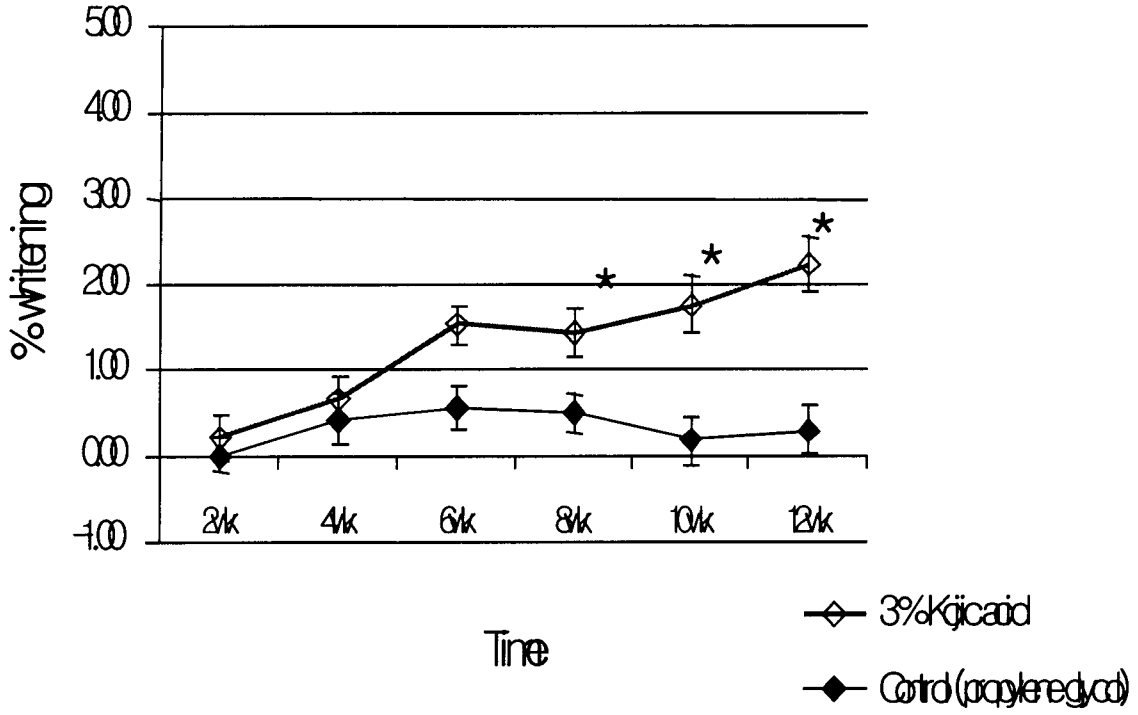

FIGURE 3. Whitening effect of kojic acid (3 % in propylene glycol) during daily application in volunteers for 12 weeks. Data = mean ± s.e.m. (n = 20).

* Significant from control ($P < 0.05$) at the same time point.

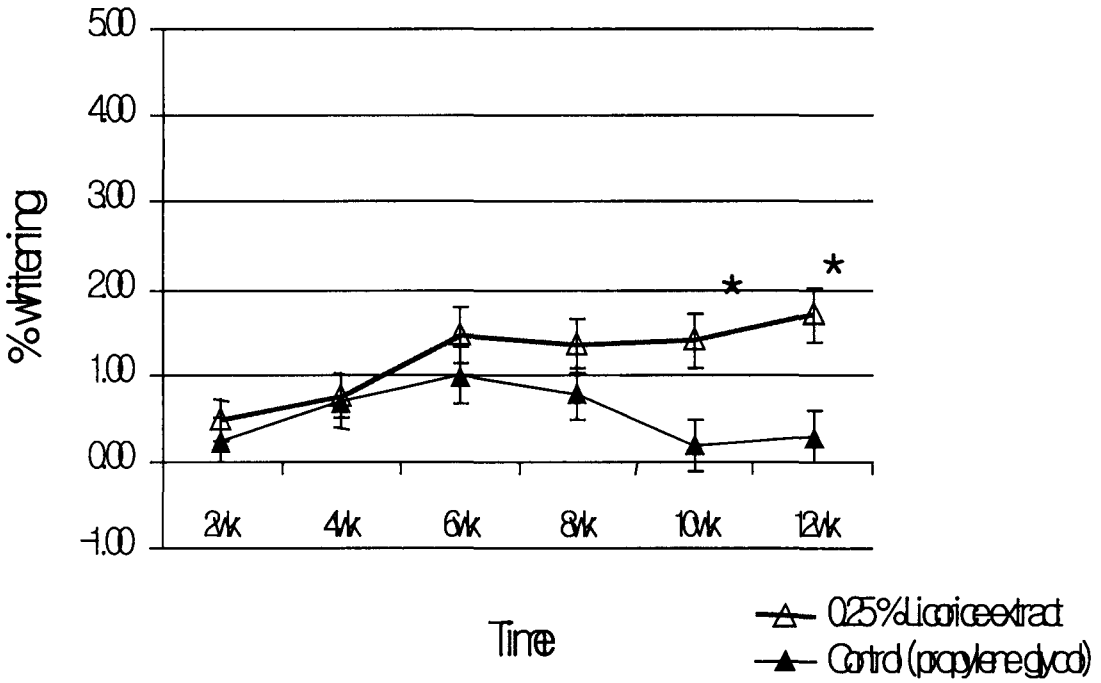

FIGURE 4. Whitening effect of licorice extract (0.25 % in propylene glycol) during daily application in volunteers for 12 weeks. Data = mean ± s.e.m. (n = 20).

* Significant from control ($P < 0.05$) at the same time point.

TABLE I. Stability at room temperature of *A. lakoocha* heartwood extract in propylene glycol-water (20:80) measured as percent inhibitory activity on mushroom tyrosinase relative to the initial value. Data = means of 3 replicates (s.d. not shown).

Time	% Relative tyrosinase inhibitory activity							
(week)	P	P+A1	P+A2	P+A3	P+A4	P+A5	P+A6	P+A7
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
4	82.88	96.51	95.95	92.62	97.35	103.11	98.51	100.05
8	71.41	92.57	92.05	89.35	96.35	86.97	85.74	93.16
12	65.30	87.81	84.04	81.62	94.33	82.64	82.49	87.85
16	68.90	78.15	84.82	82.89	91.25	83.58	85.07	81.38
24	50.34	62.87	68.46	58.40	80.78	69.74	75.68	69.14

P = *A. lakoocha* extract without antioxidants; A1 = Sodium metabisulfite; A2 = BHA; A3 = EDTA; A4 = A1 + A2; A5 = A1 + A3; A6 = A2 + A3; A7 = A1 + A2 + A3