Inhibition of Mushroom-Tyrosinase by *Aloe* Extract

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Abstract: Inhibition by *Aloe* extracts of L-dopa oxidation by mushroom-tyrosinase was examined. 2″-*O*-Feruloylaloesin and aloesin at concentrations of 0.4 μM showed inhibition of 27 and 30 %, respectively. Lineweaver-Burk plots of the concentration of L-dopa in the absence and presence of 2″-*O*- feruloylaloesin, 0.4 and 0.8 μM, showed that this compound inhibits mushroom-tyrosinase noncompetitively. The K_i value obtained was 8.5×10^{-5} M. 2″-*O*-Feruloylaloesin and aloesin contents were analyzed by a reversed-phase HPLC, and their seasonal variations were observed.

Introduction

One of the causes of hyperpigmentation is the over-production of dermal melanin pigment which is synthesized within the melanocyte on the melanosome by the action of tyrosinase, which converts tyrosine to L-dopa, dopaquinone and subsequent autopolymerization to melanin (1). The inhibition of tyrosinase by natural products, such as hydroquinones (2) and catechols (3), was examined for the purpose of treating hyperpigmentation corresponding to melanin formation, such as melasma and ephelides (3). Hinokitiol was found to be one of the inhibitors of mushroom-tyrosinase (4). The current increases in the use of *Aloe* extract as a skin cosmetic prompted us to identify the active component (5). In this report, the isolation from fresh *Aloe* leaf of the inhibitor of L-dopa oxidation by mushroom-tyrosinase and its analysis by high performance liquid chromatography (HPLC) are presented.

Materials and Methods

General

Thin layer chromatography (TLC) was performed on silica gel plates (F_{254}, DC-Aufolieen Kieselgel 60, Merck) with the following solvent systems: EtOAc : MeOH : H_2O (20 : 3 : 2) and $CHCl_3$: EtOH : H_2O (30 : 7 : 3). Blue fluorescence under UV-light (or UV-detection after diluted sulfuric acid-spray followed by heating) was used for detection.

Plant material

Fresh leaf of *Aloe arborescens* var. *natalensis* Berger, harvested in September 1984 in the herb garden of the University, where a specimen is available for inspection.

Separation

Fresh leaves (1.4 kg) were homogenized, followed by centrifugation at 9,000 g for 20 min. The supernatant was dialyzed in a dialysis membrane (Visking tubing, Union Carbide, Co.) against distilled water for 48 h, and the outer phase was lyophilized to give a pale yellow powder (14 g). This powder (0.4 g), dissolved in distilled water (10 ml), was chromatographed over a column of styrene-divinylbenzene resin (Amberlite XAD-2, 2.5 × 14 cm, Rohm and Haas Co.). The elution was with distilled water (1 l) followed by MeOH (100 ml). The eluates were evaporated to dryness yielding colorless (310 mg) and yellow powders (42 mg), respectively. The pooled yellow powder (20 g) was chromatographed over a silica gel column (2.5 × 40 cm) and eluted with EtOAc (1 l), EtOAc : acetone (4 : 1, 4 l), EtOAc : acetone (3 : 1, 2 l), and acetone (2 l), successively. From the EtOAc eluate yellow powder (0.02 g) containing chrysophanol and aloe-emodin was obtained. From the EtOAc : acetone (4 : 1) eluate, a dark yellow powder (7.8 g) was obtained. This powder was rechromatographed over a silica gel column followed by recrystallization from actone-hexane (1 : 1) to give a colorless powder, 2″-*O*-feruloylaloesin (40 mg), m.p. 153–156 °C. From the mother liquid, barbaloin (14 mg), m.p. 137–140 °C, was obtained. From the EtOAc : acetone (3 : 1) eluate, aloenin (0.2 g) m.p. 135 °C, was obtained and from the acetone eluate, colorless amorphous aloesin (3 mg), m.p. 142–144 °C, was obtained. The inner solution in the dialysis was lyophilized to give a colorless polymer fraction (2 g).

Tyrosinase assay

Tyrosinase was assayed by a modified method of Pomerantz (6). A sample (0.4 μM) was dissolved in 10 % dimethyl sulfoxide (1 ml) with sonication. When crude extract and unkown samples were tested, 0.2 mg of each sample was dissolved in 10 % dimethyl sulfoxide (1 ml) with sonication, and tested, because of the poor solubility. To the test solution (1 ml), 1 ml of 1/15 M phosphate buffer, pH 6.8, 0.5 ml of mushroom-tyrosinase (96 U/ml, Sigma Chemical Co.), and 0.5 ml of L-dopa (1 μM) were added, and the reaction mixture was allowed to stand for 2 min at 25 °C. The amount of dopachrome in the reaction mixture was measured as absorbance at 475 nm. The change in absorbance at 475 nm with or without sample was linear with time for 2 min. The percent inhibition of the tyrosinase reaction was calculated as follow:

$$\% \text{ inhibition} = \frac{(A-B)-(C-D)}{(A-B)} \times 100$$

A: Absorbance at 475 nm without test sample after incubation.
B: Absorbance at 475 nm without test sample before incubation.
C: Absorbance at 475 nm with test sample after incubation.
D: Absorbance at 475 nm with test sample before incubation.
The molar absorbancy coefficient of dopachrome at 475 nm is 3.7×10^3.

HPLC analysis

Quantitation was carried out according to a modified method of Graf (7). The yellow powder (3 mg) which was obtained from the dialysis followed by the column chromatography on Amberlite XAD-2 (0.5 × 10 cm, Rohm and Haas Co.), was dissolved in 50 % MeOH (1 ml) and filtered through reversed-phase C_{18} cartridges (Toyo Pak, Toyo Soda Mfg. Co. Ltd.). The filtered samples were quantitated by a reversed-phase HPLC. A reversed-phase HPLC was carried out on a column (TSK-Gel ODS 120 A column (4.6 mm I.D. × 25 cm), Toyo Soda. Mfg. Co. Ltd.) at a flow rate of 1.0 ml/min in a solvent system of MeOH : H_2O (1 : 1, v/v) with a HPLC system (655 Hitachi HPLC). The

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optical density of the column effluents was monitored with a UV-monitor at 254 nm (Hitachi variable wave length UV-monitor), and peak areas were calculated with a computing integrator (System I, Spectra-Physics). The following retention times (min) were obtained: aloesin (3.36), aloenin (5.03), 2"-*O*-feruloylaloesin (8.35), isobarbaloin (14.81), and barbaloin (16.56) (8).

Results

A linear rate of increase in absorbancy was achieved for the first 2 min, when L-dopa (1 μM) was used as the substrate. However, in case of L-tyrosine (1 μM), a linear rate of increase in absorbancy was achieved only following a lag period of 10 min. This may be tentatively explained on the basis of mutual inhibition of L-dopa and L-tyrosine. L-Dopa inhibition of L-tyrosine conversion to melanin has been demonstrated by Kim and Tchen (9) with mushroom-tyrosinase. Accordingly, the inhibition study of *Aloe* extracts for L-dopa oxidation with mushroom-tyrosinase was carried out. Table I summarizes the inhibition of *Aloe* extracts for L-dopa oxidation with mushroom-tyrosinase. Because of the insolubility in water, all samples were dissolved in 10 % dimethyl sulfoxide (1 ml) with sonication at a concentration of 0.4 μM and tested. The dialyzable fraction of the supernatant showed 5 % inhibition, whereas the non-dialyzable fraction, having a high molecular mass, did not show any activity. The dialyzable fraction, containing carbohydrate, carboxylic acid, amino acid, and phenolic components, was chromatographed over a column of Amberlite XAD-2 with water and MeOH.

Only the MeOH eluate, containing phenolic components, showed a 5 % inhibition. Chromatographic separations of the MeOH eluate were done on a silica gel column according to an earlier paper (10), and a mixture of chrysophanol and aloe-emodin, barbaloin, 2"-*O*-feruloylaloesin, and aloesin showed 0, 10, 27, and 30 % inhibition, respectively, whereas L-ascorbic acid, a positive control, showed 23 % inhibition. During these experiments, it was found that aglycone of aloenin having an α-pyrone skeleton showed 8 % inhibition, although this compound was not detected in the fresh leaf. HPLC analysis showed that samples harvested in Nov. 1985, Feb. 1986, June 1986, and Aug. 1986, contained 2"-*O*-feruloylaloesin (18.4; 43.0; 44.6; 60.4 w/w %) and aloesin (1.4; 2.0; 1.0; 2.9 w/w %).

Table I. Inhibition effect of *Aloe* extract on mushroom-tyrosinase[a].

Compound	Inhibition (%)
Barbaloin	10.33 ± 0.67*
A mixture of chrysophanol and aloe-emodin	0
2"-*O*-Feruloylaloesin	27.00 ± 0.57*
Aloesin	29.67 ± 0.56*
Aloenin	0
Aglycone of aloenin[b]	8.33 ± 0.40*
L-Ascorbic acid	22.67 ± 0.33

[a] Enzyme assay was carried out under the conditions given in Material and Methods. The values are mean ± s.e. of three measurements.
* statistically significance from L-ascorbic acid at $p < 0.05$. Data were analyzed using F-test to determine the significance difference between the means.
[b] Aglycone of aloenin was obtained by acid hydrolysis followed by chromatographic separation (12).

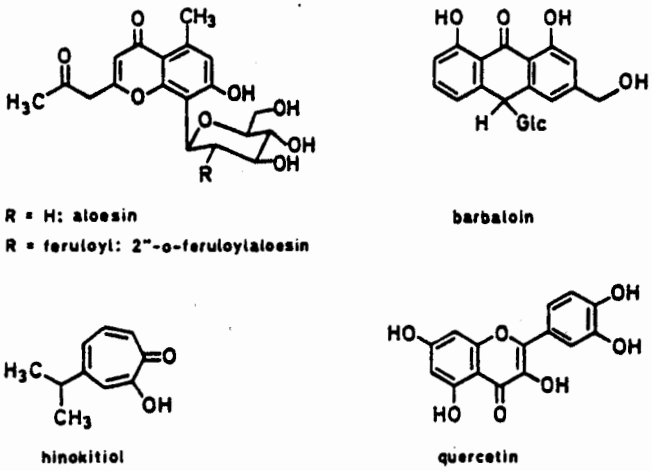

Chart 1. Structure of inhibitors for mushroom-tyrosinase

Discussion

Tyrosinase inhibitor

Barbaloin has been shown to be an active inhibitor in *Aloe* extract (11). However, in the present assay barbaloin showed only 10 % inhibition. Among the naturally occurring components, flavonoids and troponoids containing an α-ketol skeleton have been reported to be mushroom-tyrosinase inhibitors. The present experiments revealed the occurrence of other inhibitors, 2"-*O*-feruloylaloesin and aloesin, containing a γ-pyrone skeleton, in *Aloe* extract, although the aloesin content was very small.

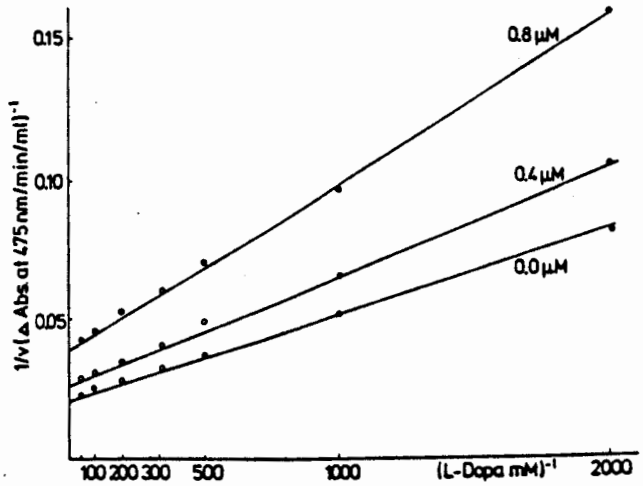

Fig. 1. Inhibition of dopachrome formation by 2"-*O*-feruloylaloesin at 0.4 and 0.8 μM.

Enzyme kinetics

Lineweaver-Burk plots of the concentrations of L-dopa in the absence and the presence of 2"-*O*-feruloylaloesin, 0.4 and 0.8 μM, showed that this compound noncompetitively inhibits mushroom-tyrosinase. The K_i value of this compound was 8.5×10^{-5} M (v_{max} = 50 μM/min/ml; $K_m = 1.5 \times 10^{-3}$ M).

HPLC studies

HPLC analysis showed a seasonal variation in 2"-*O*-feruloylaloesin and aloesin content in *Aloe* extract. A high content of 2"-*O*- feruloylaloesin was observed in the growth season of *Aloe*.

General conclusions

Flavonoids and troponoids containing an α-ketol group are known to inhibit mushroom-tyrosinase in competition to L-dopa. This may be explained in terms of the similarity in the functional groups between the dihydroxyphenyl group in L-dopa and the α-ketol group in flavonoids and troponoids. On the other hand, 2"-*O*-feruloylaloesin, in which the aloesin skeleton plays an important role, inhibited mushroom-tyrosinase in noncompetition to L-dopa. This finding presents a new type of the inhibitor from natural origin.

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cohol used was found to be without any effect on the experiments. The spasmolytic effect of angelicin, in most of the tests, was compared with papaverine hydrochloride.

Smooth muscle relaxant activity in vitro

Guinea pig ileum

A 2–3 cm long piece of ileum from a freshly killed guinea pig was suspended in an organ bath containing aerated Tyrode solution (pH 7.4) at 34 °C. Isotonic contractions were recorded on a kymograph through a frontal writing lever. Spasmolytic activity of the compound was assessed by its ability to prevent the contractions induced by a submaximal concentration (g/ml) of acetylcholine (2.5×10^{-6}), histamine (3×10^{-6}), serotonin (5-HT, 5×10^{-7}), or barium chloride (2×10^{-3}). In all the isolated preparations, graded doses of the spasmolytic compound were tested against the submaximal concentration of the spasmogen and the IC_{50} (50 % inhibitory concentration) was calculated graphically in each experiment. The mean IC_{50} values were then determined from at least 3 experiments with SE.

Rabbit jejunum

A piece of 3–4 cm long, spontaneously contracting jejunum of a freshly killed rabbit was suspended in an organ bath as above and the contractions recorded likewise. Graded doses of the spasmolytic agent were tested for inhibition of the tone and the motility of the jejunum.

Guinea pig tracheal chain

The trachea was removed from a freshly killed guinea pig. Rings were connected in series (8) and suspended in an organ bath containing Ringer solution (pH 6.1) saturated with oxygen/carbon dioxide (95 % O_2 + 5 % CO_2) at 34 °C. The contractions induced by 50 µg/ml of acetylcholine and histamine were recorded as above and the effect of graded concentrations of the spasmolytic agents was tested on these contractions.

Cat aortic strip

A 2 cm long spiral strip of the descending aorta of a freshly killed cat was suspended in an organ bath containing Krebs-Henseleit (pH 7.4) solution saturated with oxygen/carbon dioxide at 35 °C as described by Furchgott and Bhadrakom for the rabbit (9). The contractions induced by adrenaline (10 µg/ml) were recorded on a polygraph through an isometric transducer (Grass FT 03 C).

Cat ureter

A 2–3 cm long piece of ureter from a freshly killed cat was suspended in an organ bath containing aerated Tyrode solution (pH 7.4) at 35 °C. Contractions were recorded as described for the aortic strip. Smooth muscle relaxant activity of angelicin was seen against KCl (100 µg/ml) induced contractions of the ureter.

Guinea pig bile duct

The contraction of the common bile duct removed from a freshly killed guinea pig was recorded as above. The spasmolytic activity of the compound was assessed by its ability to inhibit the contraction induced by 50 µg/ml of acetylcholine and histamine.

Monkey gall-bladder

A strip (3 mm wide 3 cm long) of the gall-bladder of a freshly killed monkey was suspended in an organ bath containing oxygenated Tyrode solution (pH 7.4) at 34 °C. The contractions were recorded on a polygraph as described above. The smooth muscle relaxant effect of angelicin was tested against acetylcholine and histamine (6 µg/ml) induced contractions.

Rat uterus

Female virgin rats were pretreated with 1 mg/kg *s.c.* of stilboesterol 24 h before sacrifice. One of the uterine horns of the rat was suspended in an organ bath containing de Jalon's solution (pH 6.8) bubbled with oxygen/carbon dioxide at 30 °C. Contractions were recorded on a kymograph. The anti-spasmodic effect of the compound was assessed by its ability to inhibit oxytocin (0.1 mU/ml) and 5-HT (2.5×10^{-7} g/ml) induced contractions.

Smooth muscle relaxant activity in vivo

Intestinal movement in cat

Cats were anaesthetised with pentobarbitone sodium (40 mg/kg *i.p.*). After laparotomy the intestinal motility was recorded through a Jackson's enterograph on a kymograph (4). The blood pressure was recorded through a cannulated common carotid artery via a mercury manometer. A femoral vein was cannulated for *i.v.* injection and a rubber catheter was put into the duodenal lumen for intraduodenal administration of drug.

Gastrointestinal propulsion of charcoal meal in mouse

Mice in groups of 5 each and fasted for 24 h were used. A charcoal meal (10 % charcoal powder in 2 % gum acacia in water) was administered orally in a dose of 1 ml/100 g. The test compound was administered orally 1 h prior to the administration of charcoal meal. The control group received an equal volume of normal saline. Half an hour after the meal, the animals were killed by decapitation and the percentage length of intestine traversed by the charcoal meal from pylorus to caecum was determined.

Other pharmacological effects

Isolated guinea pig atria

The atria of a freshly killed guinea pig were suspended in an organ bath containing oxygenated Locke's solution (pH 7.4) at 36 °C. The contractions were recorded on a kymograph through a Starling-heart lever. The effect of angelicin on the atrial contraction was seen.

Skeletal muscle contraction in rat

The isolated phrenic nerve diaphragm preparation was made according to the method of Bülbring (10) and suspended in an organ bath containing oxygenated Kreb's solution (pH 7.4) at 36 °C. The phrenic nerve was stimulated supramaximally (10–15 V, 1 msec, 0.1 Hz) with a Grass S_4 stimulator to produce contractions of the diaphragm muscle which were recorded through a spring lever on a kymograph.

Local anaestetic activity

The surface anaestetic activity was seen on rabbit cornea and the infiltration anaesthetic activity was observed by intradermal injection in guinea pig according to the methods described by Bülbring and Wajda (11).

Results

Spasmolytic activity in vitro

The IC_{50} values of angelicin and papaverine in various preparations are given in Table I.

Guinea pig ileum

Angelicin (5–20 µg/ml) and papaverine (1.5–5 µg/ml) produced a concentration dependent inhibition of the contractions induced by acetylcholine, 5-HT, histamine, and $BaCl_2$. The activity against all the spasmogens was almost equal and the effect was reversible on washing (Fig. 2). Papaverine was found to be about 2.5–4.5 times more potent than angelicin (Table I) depending upon the spasmogen, the lowest ratio being obtained against $BaCl_2$ and highest against 5-HT induced contractions.