Potential of Cassia alata Leaf Extract in Inducing Differentiation and Migration of Mouse Melanoblasts


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Abstract  In vitiligo, active melanocytes in the epidermis are not present, whereas melanoblast cells in the outer root sheath of hair follicles are not affected. The existence of these inactive melanocytes provides a source for repigmentation of vitiligo. To evaluate the potential of herbal candidates in treatment of vitiligo, we studied the effect of Cassia alata leaf extract on the differentiation, proliferation and migration of melanoblast cells. Melanin content increased in melb-a melanoblast cells in response to this herb extract in a dose-dependent manner compared to control cells. In addition, it induced tyrosinase activity and altered melb-a cell morphology. A transwell migration assay showed the potential of this herbal candidate to induce direct migration of treated cells. To our knowledge, this is the first report on activity of this kind in Cassia alata. The findings of the present study are significant in the direction of developing safer strategies for vitiligo treatment.

Keywords: vitiligo, melanoblast, migration, repigmentation, Cassia alata

1. Introduction

Vitiligo is a medical condition in which irregular white patches appear on the skin because of loss of melanin, the pigment that determines skin, hair, and eye color. The clinical presentation of vitiligo is characterized by well circumscribed white cutaneous macules. The predominant histological finding in the depigmented areas of vitiligo is an absence of epidermal melanocytes [1,2]. The pathogenesis of vitiligo can be explained by autoimmune, neural, oxidative stress, autotoxic, viral, and melanocytes detachment mechanisms [3-5], but the precise pathogenesis of vitiligo remains elusive and is likely attributed to multiple factors [6]. About 0.5 to 1% of the world’s population currently suffers from vitiligo [6]. Vitiligo affects individuals of all ethnic origins and both sexes, but is more easily noticed on darker skin. Although vitiligo does not cause any physical discomfort or disability, it is associated with severe physiological and social consequences.

There are several treatment options available to vitiligo patients. Most treatments are intended to restore pigment to the skin either by stimulating the division and movement of existing melanocytes into depigmented areas or by stopping the underlying mechanism that causes the depigmentation [7]. The most widely prescribed therapies for vitiligo include the application of potent topical corticosteroids and the administration of phototherapy such as psoralen-UVA (PUVA) or, more recently, narrowband UVB [8]. However, concerns have been raised about side effects such as phototoxic reactions, blistering, and lack of data regarding long term skin cancer risk [9]. Surgical therapy can be very successful, but is very time-consuming and requires expensive facilities, thus limiting its widespread use [10]. Despite the availability of various medical treatments for vitiligo, a large percentage of patients fail to achieve satisfactory results. Considering the limitations and side effects of existing therapies, there has been an increase in interest by medical researchers, practitioners and also the...
general public in natural plants and herbs as sources of effective vitiligo treatment.

Ancient Egyptian and Indian writings depict psoralen-containing plants such as Ammi majus [11] and Psoralea corylifolia [12] being applied to pale macules and then exposed to sunlight. An Ayurvedic herb, Picrorhiza kurroa, has been reported to potentiate the effects of psoralens in the phototherapy of vitiligo [13]. Ginkgo biloba extract has been shown to have antioxidant and immunomodulatory properties, which possibly contribute towards treatment of vitiligo [14]. Recently, piperine, the major alkaloid found in the fruit of black pepper (Piper nigrum L.; Piperaceae) has been reported to stimulate the replication of melanocytes and induce the formation of melanocytic dendrites in vitro. The stimulatory effects of piperine on melanocyte proliferation and dendricity make it a potential treatment for vitiligo [15]. However, in vitiligo patients, active melanocytes in the epidermis are not present, whereas melanoblast cells in the outer root sheath of hair follicles are not affected [16]. These inactive melanocytes provide sources for repigmentation of vitiligo. So far, no herbal formulations have been reported to induce differentiation of melanoblasts. To the best of our knowledge, this study is the first investigation on the effects of an herbal extract on the differentiation and migration of melanoblast cells.

In a screening experiment to identify skin whitening agents from a total of 311 Philippine plants, we obtained 13 herbal candidates that stimulated melanogenesis in B16F10 mouse melanoma cells and studied their effects. Of these 13 candidates, Cassia alata was found to be capable of inducing differentiation, migration and tyrosinase induction in melanoblast cells without cytotoxicity. Cassia alata, a wild-growing shrub often cultivated as an ornamental plant, is indigenous to South America, but now widely distributed in the tropics. It is commonly known as candle bush, Guajava, Gelenggang (Malay), Akapulko (Philippines), ringworm bush, empress candle plant and seven golden candlesticks. Hydroxyanthracene derivatives were demonstrated as the active constituents in this plant [17]. Cassia alata has been recognized for centuries in traditional medicine for its role as a laxative as well as in the treatment of a variety of skin and respiratory diseases. To the best of our knowledge, this is the first investigation to evaluate the effects of Cassia alata leaf extract on the differentiation and migration of melanoblast cells.

2. Materials and Methods

2.1. Materials and reagents

Murine FGF-basic (fibroblast growth factor) was purchased from PeproTech (Rocky Hill, NJ, USA). Dulbecco’s modified Eagle’s medium (DMEM), Phorbol 12,13-dibutyrate (PDBu), α-melanocyte stimulating hormone (α-MSH), 3,4-dihydroxy-L-phenylalanine (L-DOPA) and 3(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemicals (St Louis, MS, USA). RPMI-1640, fetal bovine serum (FBS), trypsin-EDTA, phosphate buffered saline (PBS) and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Cassia alata leaves were collected from the field of Baguio City, Philippines and were authenticated by Dr. G. A. Reyes of the College of Natural Sciences, Saint Louis University, Baguio City, Philippines. Dried leaves were extracted three times with 99.5% methanol for 4 h at 37°C. The resulting mixtures were filtrated and concentrated to dryness at 40°C under vacuum to produce a methanol extract.

2.2. Cell and culture conditions

B16F10 murine melanoma cells were purchased from ATCC (American Type Culture Collection) and cultured in DMEM medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO2. The cells were seeded into a 6-well plate (Falcon, USA) at a concentration of 0.3 × 10⁵ cells/mL and 96-well plate with 1.25 × 10⁵ cells/mL.

Melb-a cells were kindly provided by the Wellcome Trust, Functional Genomics Cell Bank (London, UK). Cells were grown in a humidified atmosphere with 10% CO2 at 37°C. Cells were routinely passed in complete RPMI 1640 medium supplemented with PDBu (20 nM), BFGF (1 ng/mL), fetal calf serum (5%), and L-Glutamine (2 nM). Cells were harvested by brief treatment with trypsin/ethylenediamine tetraacetic acid (EDTA) and then sedimented and resuspended in complete RPMI 1640 medium. Viable cells, determined by trypan blue exclusion, were counted in a hemocytometer, resuspended in an appropriate volume of complete RPMI 1640 medium, and seeded at 2 × 10⁴ cells per cm² in 10 cm cell culture dishes.

2.3. Assays

2.3.1. Cell differentiation and melanin assays

For the melanin assay, B16F10 cells were seeded at 2 × 10⁴ cells/plate in 12-well plates. After 24 h of cultivation, the cells were treated with samples at a concentration of 50 μg/mL for 48 h. The harvested cells were washed twice with PBS, resuspended in 1 N NaOH containing 10% DMSO and heated at 80°C for 1 h. The absorbance of extracted melanin was read at 405 nm using an ELISA microplate reader [18]. α-melanocyte stimulating hormone (α-MSH) (10 nM) which is known to stimulate differentiation, proliferation and migration in melanoblasts [19] was used as
the positive control in all the experiments, unless specified otherwise.

For the differentiation assay, melb-a cells were seeded at 6 × 10^4 cells/plate in 12-well plates. After allowing 1 day for cell attachment, the medium was changed and dried methanolic extracts of *Cassia alata*, dissolved in DMSO at different concentrations (from 6.25 to 25 μg/mL), were added to fresh complete RPMI 1640 medium. Equivalent volumes of the diluent (DMSO) were added to untreated samples as negative control. Culture media was replaced twice in 4 days. After 96 h, the cells were harvested by trypsinization. After washing twice with PBS, samples were dissolved in 1N NaOH containing 10% DMSO and then treated at 80°C for 1 h and cooled. The amount of melanin was determined spectrophotometrically (absorbance at 405 nm) and expressed as a percentage of the control. Treatment effects on melanoblast cell morphology were determined by phase contrast microscopy.

2.3.2. Cell migration assay
Cell migration was assessed in Transwell cell culture chambers (Costar 3422; Cambridge, MA, USA), in which polyvinylpyrrolidone-free polycarbonate filters with an 8.0 μm pore size were precoated with 1% gelatin. After incubating the cell insert for 1 h, melb-a cells (2 × 10^5/100 μL of cell suspension in serum free RPMI medium) were added to the upper chamber of the cell insert and 600 μL of RPMI serum free medium with treatment samples were added in the lower chamber. Transwell chambers were incubated for 24 h. At the end of incubation, the filters with gelatin intact were fixed with methanol, and then stained with hematoxylin and eosin. Cells on the upper surface of the filters were removed by wiping with a cotton swab, and cells that had migrated to the lower surface of the filters were counted under a microscope and photographed. Cells that migrated across the filters in 4 high-power fields (X40 objective) per insert were counted. For each migration condition, three identical replicates were performed.

2.3.3. Viability assay
Melb-a cells were cultured at 4.0 × 10^3 cells/mL in a 96-well plate. After 24 h, the cells were treated with extract of *Cassia alata* at different concentrations (from 6.25 to 25 μg/mL). After 96 h of incubation with the test compound (herbal extract), 100 μL of MTT (5 mg/mL in phosphate buffered saline) solution was added to the wells. After 4 h of incubation, the medium was removed and 100 mL of DMSO was added to dissolve the formazan produced in the cells. The absorbance of each well was then read at 540 nm by using an ELISA microplate reader. The optical density of formazan formed by control cells was used as a reference (assumed to be 100%) [18].

B16F110 cells were cultured at 1.25 × 10^4 cells/mL in a 96-well plate. After 24 h, the cells were treated with samples at various concentrations for 48 h. After incubation with MTT solution for 4 h, the optical density of each well was read at 540 nm in an ELISA microplate reader.

2.3.4. Cellular tyrosinase assay
Cellular tyrosinase activity was assayed as DOPA oxidase activity. Melb-a cells were seeded in 60 cm dishes at a density of 2 × 10^4 cells/dish and cultured for 24 h. After being treated with samples for 96 h, the cells were harvested with cold PBS and lysed with 100 μL of lysis buffer (0.1 M phosphate buffer, pH 6.8, containing 1% (w/v) Triton X-100) supplemented with protease inhibitors. The cells were disrupted by sonication at 40°C for 30 min and lysates were clarified by centrifugation at 13,000 rpm for 20 min. After determining the protein content with a protein assay kit (Bio-Rad, USA), lysates were adjusted with lysis buffer to contain equal amounts of protein. These lysates were added to a 96-well plate containing 2.5 mM L-DOPA in 0.1 M phosphate buffer (pH 6.8). After incubation at 37°C for 1 h, the absorbance of samples was measured at 475 nm using an ELISA reader.

2.3.5. Statistical analysis
All determinations were run in triplicate on at least two different experiments, and the results were reported as the mean and standard deviation. The statistical significant differences from the control were analyzed by the Student's t-test.

![Fig. 1. Effect of *Cassia alata* leaf extract on melanin content and cell viability in cultured B16F10 melanoma cells. Data is expressed as the change of melanin content and cell viability relative to untreated control. Each determination was made in triplicate and data shown are means ± S.D. *p < 0.05, **p < 0.01: Statistically significant vs. the value of the control group.](image-url)
3. Results and Discussion

Based on experiments conducted in B16F10 mouse melanoma cells, *Cassia alata* was initially selected as a hyperpigmenting candidate. Fig. 1 shows the effect of *Cassia alata* in melanogenesis and cell viability in B16F10 cells. Based on this finding, we decided to investigate the effect of this herb in mouse melanoblast cells.

First, we examined whether exposure to the plant extract would affect the cell viability of melb-a melanoblasts. As illustrated in Fig. 2, treatment with *Cassia alata* leaf extract neither affected cell viability or the significant stimulation of melanoblast proliferation under the concentrations tested, showing its possible clinical usefulness. Since melanoblasts are unpigmented cells which lack functional tyrosinase catalytic activity, cell differentiation was measured on the basis of melanin content in the treated cells. The untreated cells remained white and showed 0% melanin induction while treatment with 10 nM α-MSH (positive control) showed 170% melanin induction. The melanin content was found to be increased following treatment by *Cassia alata* leaf extract in a dose-dependent manner as shown in Fig. 3. Cell pellets also clearly demonstrated the effect of treatment on melb-a differentiation. Maximum stimulation of melanin synthesis was observed at 25 μg/mL. Hence this extract concentration was used for subsequent experiments. To further investigate the effect of treatment on tyrosinase induction, cellular tyrosinase assays

![Graph showing cell viability and melanin content](image1)

**Fig. 2.** Effects of *Cassia alata* leaf extracts on cell viability in cultured melb-a melanoblasts. Data is expressed as percentage change of cell viability relative to untreated control. Each determination was made in triplicate and data shown are means ± S.D. *p < 0.01: Statistically significant vs. the value of the control group.

![Graph showing cellular tyrosinase activity](image2)

**Fig. 3.** Effects of *Cassia alata* leaf extracts on melanin content in cultured melb-a melanoblasts. Data is expressed as percentage change of cell viability relative to untreated control. Each determination was made in triplicate and data shown are means ± S.D. *p < 0.05, **p < 0.01: Statistically significant vs. the value of the control group.

![Image of cell pellets](image3)

**Fig. 4.** Effects of *Cassia alata* leaf extracts on cellular tyrosinase induction compared to the negative control without treatment. Data is expressed as percentage change of cell viability relative to untreated control. Each determination was made in triplicate and data shown are means ± S.D. *p < 0.01: Statistically significant vs. the value of the control group.

![Micrographs showing dendrigoisenscence](image4)

**Fig. 5.** Effect of treatment on melb-morphology. Phase contrast micrograph showing dendrigoisenscence in cultures treated with αMSH and *Cassia alata*. (A) Untreated melanoblasts, (B) culture treated with αMSH, (C) culture treated with *Cassia alata* leaf extract (25 μg/mL).
were carried out. Treatment with *Cassia alata* leaf extracts significantly stimulated cellular tyrosinase activity in melb-a cells whereas in untreated melb-a cells we could not detect any tyrosinase activity (Fig. 4).

Cell expansion, dendritogenesis and pigmentation were stimulated in cells treated with α-MSH as well as with the *Cassia alata* extract. In cultures treated with α-MSH and *Cassia alata* extract, a large number of differentiated melanoblasts were observed (Fig. 5). This result suggests that *Cassia alata* extracts can stimulate differentiation, dendritogenesis and pigmentation of melanoblasts in a similar way to α-MSH.

To determine the efficiency of *Cassia alata* leaf extract to induce melanoblast migration, a Transwell migration assay was performed using filters precoated with 1% gelatin. Treatment with the plant extract resulted in a 5 fold increase in the number of melanoblasts migrating to the lower surface of the filters while treatment with α-MSH resulted in an 8 fold increase compared to the untreated control (Fig. 6). This result shows that *Cassia alata* leaf extracts can directly stimulate the migration of melanoblast cells.

Through various forms of therapy, it has been reported that melanocytes could be recruited from the outer root sheath of the hair follicle to repigment vitiligo affected skin [20]. Vitiligo patients with localized areas of hypopigmentation are often treated successfully with 8-methoxypsoralen (8MOP) and ultraviolet (UV) A radiation (PUVA therapy). Repigmentation in such cases is initiated by inducing inactive melanoblasts residing in the hair follicles, followed by their upward migration to the epidermis to differentiate and form perifollicular pigment islands [21]. Currently, effective approaches to activate melanoblasts are not available, but could be promising to treat vitiligo patients.

Sviderskaya *et al.* [22] successfully produced a cloned line of immortal melanocyte precursors, termed melb-a melanoblasts. The characteristic phenotype of these cells includes a lack of expression of tyrosinase and no ability to oxidize DOPA under standard culture conditions. However, as melanocytes precursors, they do express other melanocyte specific proteins such as Tyrp1 and Det. Thus, melb-a melanoblasts provide an ideal model to study the differentiation and migration of melanoblasts and to characterize the mechanisms underlying this.

Our results suggest that *Cassia alata* leaf extracts can
induce melanoblast differentiation in media that maintains melanoblasts in normal circumstances. Care was taken to adjust the seeding concentration to maintain cell confluency below 8 \times 10^4 \text{cells/mL} to avoid spontaneous differentiation by the end of the incubation [22]. Therefore, the differentiation observed after treatment cannot be attributed to spontaneous differentiation. Since melb-a cells lack tyrosinase expression, the cellular tyrosinase assay is a direct method to prove differentiation. In differentiated melanoblasts, tyrosinase activity suggested DOPA reactivity in melanoblast cells which might have occurred through protein activation or maturation. Morphological alterations in melb-a cells with more dendrites after treatment with Cassia alata extracts also reveals its effects on melanoblast differentiation. Our investigation on the effect of Cassia alata on the migration of melb-a cells indicates that it has a chemo attractant factor, which appears to stimulate melanoblast migration and differentiation.

4. Conclusion

In this study we showed that Cassia alata leaf extracts induced differentiation and migration in melb-a melanoblast cells. At this stage we do not know the underlying mechanism by which the candidate stimulates these melanocyte precursors. Further studies are in progress to isolate the active compound from the plant extract and to characterize the mechanism involved in melanoblast activation. These studies would be important for designing preclinical and clinical studies on the efficacy of Cassia alata as a stimulant for skin repigmentation in vitiligo.

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