行政院國家科學委員會專題研究計畫　成果報告

苎葉對皮膚黑色素細胞去色素化/過度色素化之研究

計畫類別：個別型計畫
計畫編號：NSC91-2320-B-041-019-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：嘉南藥理科技大學藥學系

計畫主持人：陳秋蘭

計畫參與人員：賴慧芬 賴慧娟 黃文良

報告類型：精簡報告

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中　華　民　國 92 年 10 月 6 日

行政院國家科學委員會補助專題研究計畫 ■成果報告 □期中進度報告

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一、中文摘要

在 1997 年初，台灣皮膚科門診發現，婦女為求美白而使用蒸過的荖葉來敷臉，結果臉部出現非常嚴重的黑白斑症之案例，而荖葉所造成的這種獨特現象之機轉究竟如何？還是一個未知數。荖葉是檳榔塊的組成之一，內含有許多酚類的物質，包括像丁香油酚、hydroxychavicol （HC）及 HC-diacetate (HC-dA) 等化合物。已知有一些酚類化合物，像是對苯二酚，具有去色素化的效果，而有些則會誘發發炎後的過度色素化的作用。究竟荖葉萃取液或其所含的酚類化合物是否會造成去色素化或過度色素化的作用，則是一個值得探討的一個課題。

酪氨酸是黑色素形成速率決定酵素，因此我們先以 L-dopa 當作受質來篩選荖葉萃取液或其中的酚類成份在體外對酪氨酸活，我們也利用 MTT 的方法以及錐蟲藍排除法來偵測荖葉萃取液或其中的酚類成份對小鼠的黑色素瘤 B16 細胞是否具細胞毒殺作用。最後，我們利用小鼠黑色素瘤 B16 細胞來探討荖葉萃取液或其酚類成份在細胞內對黑色素含量的影響，以黑色素在波長 415nm 呈現最大吸收的特性來加以偵測。

從我們的實驗結果得知：荖葉萃取液對於酪酶活性具有抑制的效果，但此抑制的效果並不具有劑量的相關性（0.1~100μg/ml）。同時，荖葉萃取液在 1~100μg/ml 的劑量下，是具有細胞的毒性作用且呈現劑量相關性。此外，這些試劑對於黑色素的產生並不具有明顯抑制的效果，反而在高劑量（100μg/ml）的處理之下，具有過度色素化的作用。但是，荖葉中的酚類化合物 HC、HC-dA 及丁香油酚在低劑量 (1μM) 時可降低黑色素的含量，其程度與正對照組的維他命 C 作用相當。因此，由我們的實驗結果可知，荖葉中的酚類化合物具有力。同時，使用蒸過的荖葉來敷臉造成黑白斑症的原因，可能是因為荖葉對黑色素細胞產生毒殺作用（白斑症）和過度色素化的作用（黑斑症）所致。

關鍵詞：荖葉、黑色素、酪胺酸

Abstract

In 1997, dermatologists documented a kind of severe facial leukomelanosis to the use of facial dressing with steamed *Piper betle* leaf (PBL) as a bleaching agent in Taiwan. The underlying mechanisms for this leukomelanosis have yet to be resolved. PBL is a component of areca quid and it contains many phenolic ingredients including eugenol, chavicol and hydroxychavicol. Phenolic derivatives are known for depigmentation, such as hydroquinone and sometimes even induce postinflammatory hyperpigmentation. In this study, we hypothesizes that PBL extracts induces leukomelanosis through inhibition of melanin synthesis and/or melanocytotoxicity. We first screened the inhibition potential of PBL on tyrosinase activity in the presence of L-Dopa as a substrate. We further determined the cytotoxicity of PBL extracts in mouse melanoma B16 cells by using tetrazolium salt and trypan blue exclusion assays. The effects of PBL extracts on melanin content in B16 cells were also measured by UV at OD_{405}. The data showed that PBL extracts were cytotoxic to B16 cells in a dose-dependent manner (10~100 μg/ml) and inhibited mushroom tyrosinase activity *in vitro*. These results demonstrate that PBL extracts induced leukomelanosis may through the inhibition of melanin synthesis and melanocytotoxicity.

Keywords: *Piper betle* leaf, melanin, tyrosinase

I. Introduction

Betel quid (BQ) chewing is a common habit in some Asian countries, including Taiwan. BQ is generally composed of areca nut, *Piper betle* leaf (PBL) or inflorescence, lime and additives such as tobacco. However, the composition of BQ varies in different geographic locations. *Piper betle* inflorescence is used in the preparation of BQ in Taiwan and Papua New Guinea,

whereas the leaf of *Piper betle* Linn. is used in almost all of the BQ chewing countries. Chewing BQ has been associated with oral submucous fibrosis, leukoplakia, and oral squamous cell carcinoma (OSCC). In Taiwan, tobacco is not included in the preparation of BQ, however, epidemiological studies showed that BQ chewing is still the main cause of OSCC (1).

An unexpected disease related to the use of BQ component appeared in Taiwan recently. After the advertisement by a local newspaper that steamed betel leaves can serve as a facial bleaching remedy, this has gained popularity in some local female groups. In one report indicated that the bleaching effect occurred within 1 week in 8 of 15 patients (2). Among them, 4 claimed that significant bleaching occurred within 3 days after nightly use of steamed betel leaves before bedtime. The rapidity of this bleaching effect is faster than any of the commercially available bleaching agents (3). However, many patients developed severe facial leukomelanosis after prolonged use of this home remedy. The underlying mechanism for this leukomelanosis has remained elusive.

PBL, the mature green leaves of *Piper betle* vines, has been used in the preparation of BQ since ancient times (4). The Working Group of IARC concluded that the data are inadequate to allow an evaluation of the carcinogenicity of betel leaf to experimental animals (4). Besides having been used as a part of BQ together with areca nuts and slaked lime for centuries, *Piper betle* leaves have been found to possess diverse biologic effects including antifungal, antiseptic, and antihelmintic effects (5).

Piper betle leaves contain volatile oils, nitrate, and small quantities of sugar, starch and tannin. The most important constituents of betel leaves may be the various chemicals in the essential oils, especially eugenol and hydroxychavicol (6,7). We hypothesize that the essential oils in betel leaves may be responsible for this leukomelanosis, perhaps through **inhibition of melanin synthesis or melanocytotoxicity.**

Melanogenesis is the process of the production and subsequent distribution of melanin by melanocytes within the skin and hair follicles (8,9). The copper- containing enzyme **tyrosinase** catalyzes the first two rate-limiting reactions, the oxidation of tyrosine into dopa and subsequently the corresponding *ortho*-quinone (dopaquinone). Quinones are chemically reactive compounds that are potentially harmful, but in melanocytes the normal process of melanogenesis is not usually associated with significant toxicity due to the compartmentation of the reaction within membrane-limited organelles (melanosomes) and because of the rapid cyclization of the intermediate quinone. Such formed melanin has many biological functions including the scavenge of oxidative free radicals (10,11). To date, research on the regulation of melanogenesis has focused on factors which affect **tyrosinase**, the rate-limiting enzyme in the melanogenic pathway, by searching for chemicals which competitively inhibit tyrosinase function.

Various dermatologic disorders result in the accumulation of excessive levels of epidermal pigmentation. However, a global market demand has developed recently for skin-lightening agents as vanity cosmeceutical products, because lighter skin color is preferred by some dark-skinned individuals in many countries and races (12). Unfortunately, several purportedly active agents (e.g. arbutin and kojic acid, among others) have not been demonstrated yet to be clinically efficacious when critically analyzed in carefully controlled studies. The U.S. FDA-approved pharmaceutical products containing 2-4% hydroquinone (HQ) are moderately efficacious, but HQ is considered to be cytotoxic to melanocytes and potentially mutagenic to mammalian cells (12,13). **Desirable skin-whitening agents** inhibit the synthesis of melanin in melanosomes by acting specifically to **reduce the synthesis or activity of tyrosinase, exhibit low cytotoxicity, and are non-mutagenic.**

II. Material and Methods

1. Piper betle leaf extract (PBLE) preparation

PBL will be purchased in a shop in Tainan. PBL will be minced and warmed with hot water for 3 hours. When it is cold,

the filtrate is then frozen immediately and lyophilized.

2. Cell culture and treatment

The pigmented human melanoma cell line RPMI 7951 is cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and appropriate amounts of antibiotics and fungizone. The test agents are added to the cell cultures for various times.

3. Cytotoxicity

The cytotoxicity of PBL or HC is determined by assay for the reduction of tetrazolium-based compound MTT. Melanocytes are plated at a density of 10^4 cells/well into 96-well tissue culture plates. Cells are treated with the indicated concentrations of PBL or HC for various times, the medium was then removed and 0.5 mg/ml MTT in medium is added to each well. Following a 2 h-incubation period the medium is removed, and 100 µl DMSO is added to each well. The viable cells can be calculated from the A570 values determined with a microtiter plate reader. PBLE- or HC-induced cytotoxicity is also determined by counting the living cell exclusion of trypan blue dye.

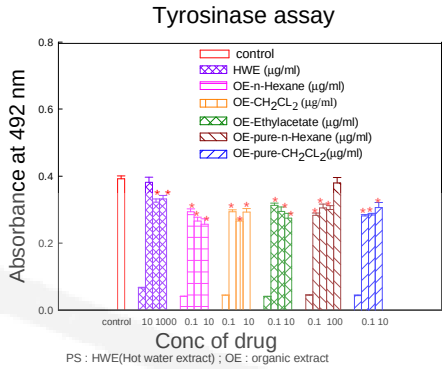
4. Melanin content assay

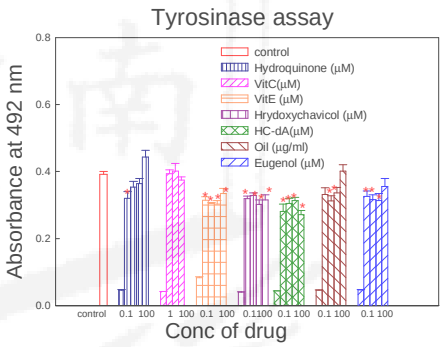
Cells are collected by trypsin/EDTA after treatment, and are counted with trypan blue exclusion method. The colors of cell pellets are evaluated visually, and pellets of 10^6 cells were solubilized in boiling 0.1N NaOH for 10 min. Spectrophotometric analysis of melanin content is performed at 400 nm absorbance (14,15).

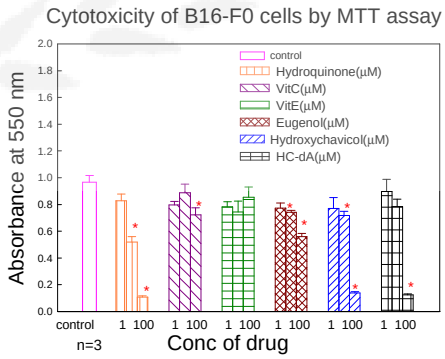
5. Tyrosinase assay

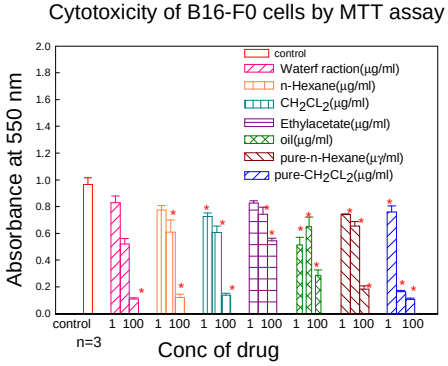
Cells pellets are lysed in 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 1 mM phenylmethylsuphonyl fluoride (PMSF), 10 µg/ml aprotinin and 10 µg/ml leupeptin. The radiometric determination of tyrosinase activity is performed as previously described. In brief, 0.09ml of each cell extract (20 µg protein content) is incubated for 60 min at 37^0C with 0.01 ml sodium phosphate buffer containing 1 µCi of L-[ring-3,5-^{3}H]tyrosine, 5 µg of L-dopa and 1% Triton X-100. One milliliter of activated charcoal (10% w/v) in 0.1 M citric acid is then added and specimens are centrifuged for 10 min at 2000g at 4^0C. The supernatants are applied to 0.2 ml columns of Dowex-50 (Bio-Rad Laboratories, CA, USA), equilibrated in 0.1 M citric acid, washed with 0.5 ml of 0.1 M citric acid, and the effluents are counted by scintillation spectrometry for the formation of ^3H_2O (14).

III. Results & Conclusions









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IV. References

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