嘉南藥理科技大學專題研究計畫成果報告

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一、中文摘要

在 1997 年，台灣的皮膚科醫生發現許多臉上患有嚴重黑白斑的病例，他們都使用蒸過的荖葉來作臉部的美白，為何會造成黑白斑的機轉不明。荖葉是檳榔塊的一個組成物，裡面含有許多酚類化合物，像丁香油酚等。已知有一些酚類化合物具有去色素的作用，有時也會引起發炎後過度色素化的反應。在此計畫中，我們就假設荖葉會誘發黑白班症是因為抑制黑色素的合成及/或細胞毒性的產生所致。我們首先藉由測試酪胺酸酵素的活性來篩選荖葉萃取液中是否具有抑制黑色素合成的成分，再以老鼠的黑色素瘤細胞來偵測荖葉萃取液的細胞毒性，最後再偵測荖葉萃取液是否可以降低黑色素的量。實驗結果發現荖葉萃取液在體外實驗可抑制酪胺酸酵素的活性，同時也具有細胞毒性且成劑量相關性，因此荖葉萃取液之所以造成黑白斑的現象可能就是因為抑制黑色素的產生及細胞毒性所致。

關鍵詞：荖葉、黑色素、酪胺酸酵素

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. *Piper betle* leaf (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 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.

Piper betle leaf, 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 preparation

Piper betle leaf will be purchased in a shop in Tainan. Piper betle leaves 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.

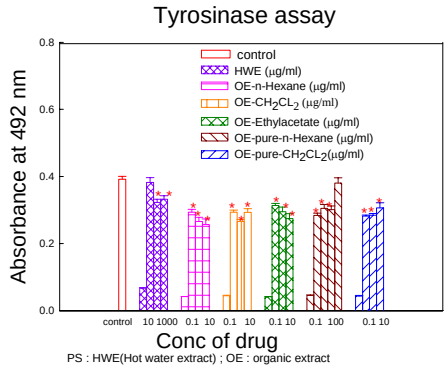
4. Melanin content assay

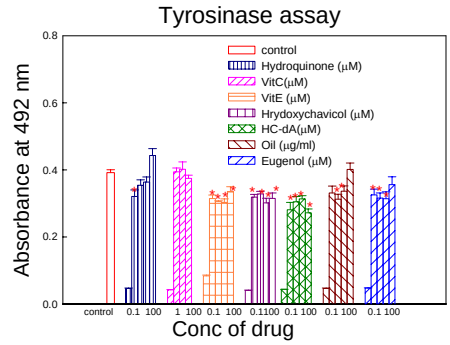
Cells are collected by trypsin/EDTA after treatment, and are counted with trypan blue exclusion method. The colors of cell pellets are evaluated visually, and pellets of 10^6 cells were solubilized in boiling 0.1N NaOH for 10 min. Spectrophotometric analysis of melanin content is performed at 400 nm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

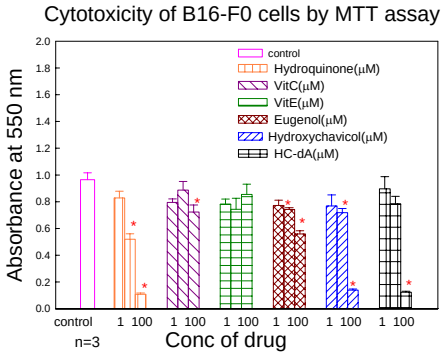
5. Tyrosinase assay

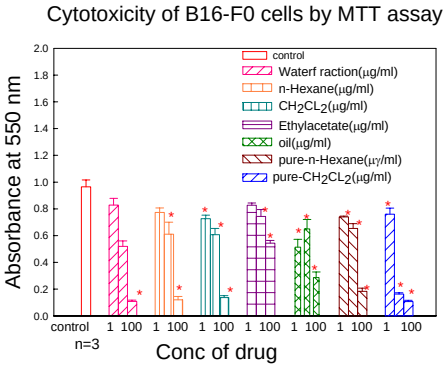
Cells pellets are lysed in 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 1 mM phenylmethylsuphonyl fluoride (PMSF), 10 μg/ml aprotinin and 10 μg/ml leupeptin. The radiometric determination of tyrosinase activity is performed as previously described. In brief, 0.09ml of each cell 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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嘉南藥理科技大學專題研究計畫成果報告

苎葉對皮膚黑色素細胞美白作用之探討

計畫類別：■個別型計畫　　□整合型計畫

計畫編號：CNP11-91-18

執行期間：91 年 1 月 1 日至 91 年 12 月 31 日

計畫主持人：陳秋蘭

共同主持人：

計畫參與人員：賴慧芬

執行單位：嘉南藥理科技大學藥學系

中華民國　　92 年　2 月　27 日