A NEW MELANOGENESIS INHIBITOR FROM *INGA ALBA* (SW.) WILLD.

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SUMMARY

By using sequentially efficacy tests based on tyrosinase, the key enzyme of melanogenesis, then a cell line of melanocytes cultured in vitro, we have been able to detect the whitening potential of a plant extract and then to develop a new whitening Active Ingredient whose the whitening potential was confirmed on cultured melanocytes.

Through a phytochemical approach, it seems that the whitening potential could be due to the "tannin" fraction of plant extract.

A complementary work is planned to explain more precisely which fractions are responsible for the whitening potential and a clinical test is in progress on 30 Asian skin type volunteers to show the whitening efficacy on human volunteers.

KEYWORDS

Melanogenesis, Whitener, Inga, Tanin, Tyrosinase

INTRODUCTION

Aim

Melanins are heterogeneous biopolymers produced by human skin and which involve two types of compounds, brown colored eumelanin and reddish-yellow colored phaeomelanin. Human skin phaeomelanin is not persistent, therefore only eumelanin determines the brown color of skin. It is well known that melanin synthesis is under the control of genetic and environmental factors. So, after a sun exposure, the melanin synthesis is increased and gives rise to cutaneous hyperpigmentation and during skin aging, to unaesthetic "age spots". Because in many regions the ideal of beauty is directly linked to a bright skin tone without age spots, the research of safe and efficient whitening products is a constant demand and needs the development of relevant efficacy tests.

In this aim, a collaboration with the French institute IRD for the research of new plants as source of cosmetic active ingredients was established and provided extracts from different medicinal plants of French Guyana in order to screen them for their properties of melanin synthesis inhibition.

Among the different plants studied, raw extracts from Inga sp. bark showed interesting activities both on tyrosinase and B16 murine melanocytes test models.

The different extracts obtained with solvent like water, ethanol and water-ethanol mixtures were active.

Based on these research results, Laboratoires Sérobiologiques have developed a new depigmenting Active Ingredient from *Inga alba (Sw.) Willd.* bark: named Active Ingredient in the text.

The melanin synthesis (or melanogenesis) (Figure 1)

Melanin pigments result of tyrosine metabolite polymerization. In fact melanin synthesis process is initiated by a specific rate-limiting enzyme known as tyrosinase. This enzyme of 70 kDa contains a copper ion in its active site and converts L-tyrosine to dihydroxy-phenyl alanine (DOPA) then to DOPA quinone [1].

In turn DOPA quinone can auto-oxidize spontaneously to DOPA chrome and then to 5,6 dihydroxy-indole (DHI) whose tyrosinase facilitates the oxidation and polymerization to DHI eumelanin [2].

However DOPA chrome tautomerase or DCT or TRP-2 (tyrosinase related protein 2) initiates another way of polymerization; TRP-2 tautomerizes DOPA chrome to 5,6 dihydroxy-indole-2-carboxylic-acid (or DHICA) which is oxidized and polymerized by TRP-1 (tyrosinase related protein 1) and Pmel17 protein to DHICA eumelanin [3, 4].

Moreover phaeomelanin results of polymerization of condensation product between DOPA and thiol derivatives such as glutathione (GSH) and cystein.

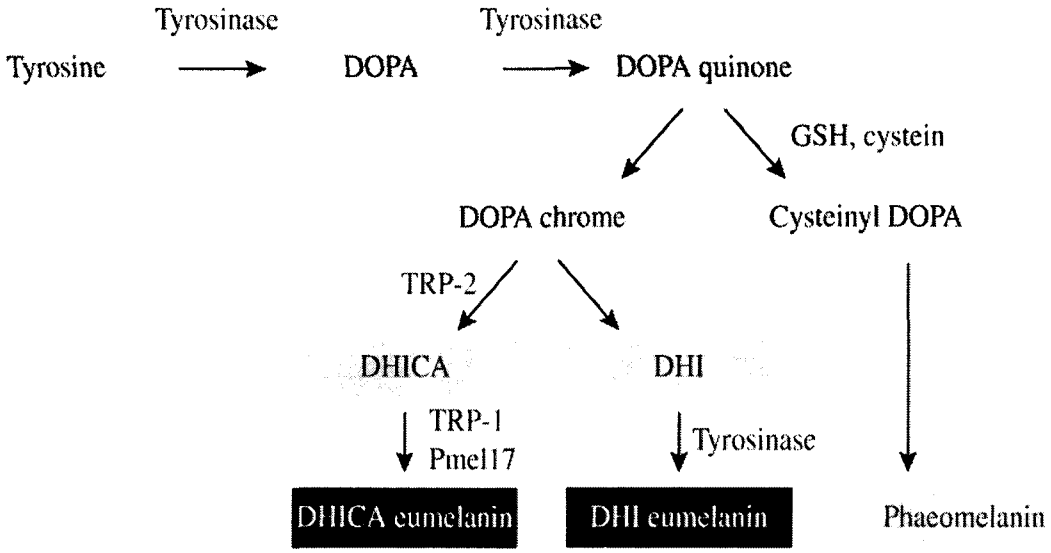

Figure 1: The melanin synthesis.

The melanin supplying (Figure 2)

The melanin synthesis occurs in specialized cells called melanocytes that exist as a minor population in the basal layer of epidermis. Melanocytes are cells with dendritic shape and contain specific organelles known as melanosomes where the melanin biopolymer is formed. Origin of melanosomes is from endoplasmic reticulum by assembling structural protein during stages I and II. Meanwhile the tyrosinase is released from endoplasmic reticulum and glycosylated via the vesicles of Golgi's apparatus. Afterwards the melanosomes are provided of maturated tyrosinase and can accumulate melanin during stages III and IV. Then fully melanized melanosomes migrate to the tip of melanocyte dendrites where they are engulfed by keratinocytes cells through a phagocytic mechanism, one melanocyte supplying around 36 surrounding keratinocytes.

In keratinocytes, melanosomes form a cap over the nucleus in order to lower the DNA damages by absorbing UV radiations [5].

Therefore melanosomes protect the genetic material of skin cells and consequently pigment the skin.

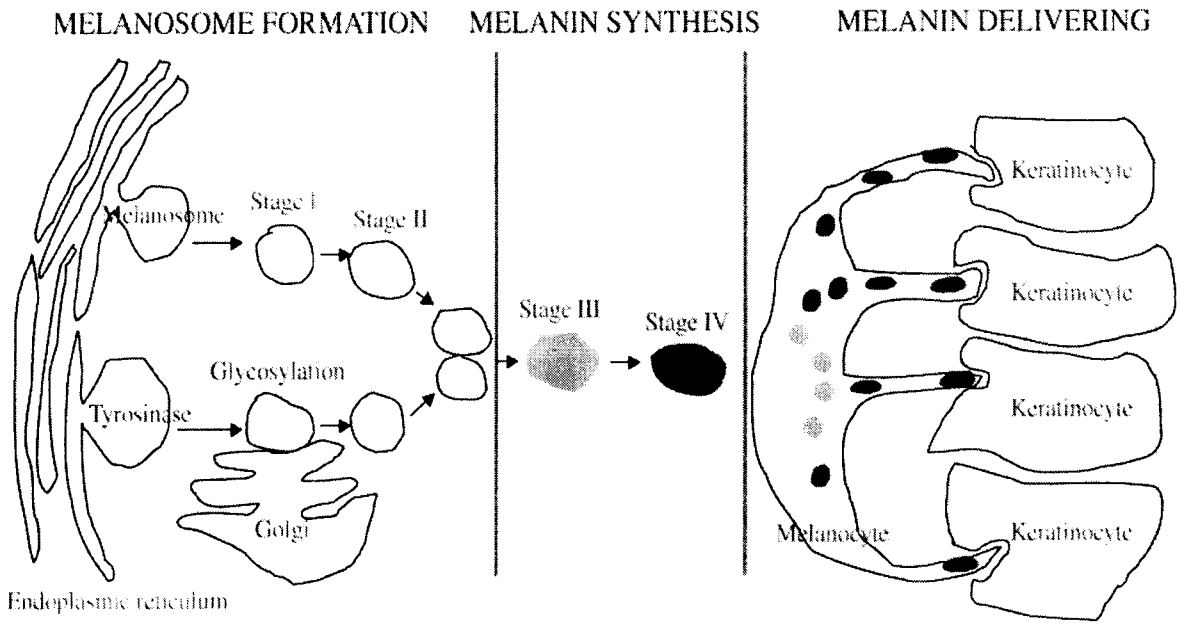

Figure 2: The melanin supplying.

The melanogenesis regulation

It was established that melanogenesis starts 3 or 4 days consequently to the UVB irradiation [6] that means that the melanogenesis is controlled through the activation of genes expression. In fact UVB irradiation induces the POMC (pro-opio-melanocortin) release from keratinocytes and POMC is cleaved into peptides such as ACTH (adrenocorticotropic hormone) and α-MSH (α-melanocyte stimulating hormone). Following their binding to the melanocortin receptor MC1-R [7], ACTH and α-MSH activate the melanocyte proliferation and the tyrosinase, TRP-1, TRP-2 and Pmel17 expressions through an induction of cAMP synthesis [8].

Therefore several pharmacological Active Ingredients such as forskolin and xanthic bases that enhance cAMP level, stimulate also the melanogenesis [9].

Moreover UVR stimulate also the IL1α, IL1β, endothelin and b-FGF release that increase the MC1-R expression on melanocytes [7]. b-FGF stimulates also the proliferation and melanogenesis of human melanocytes [10] and induces the release of endothelin-1 from keratinocytes. Endothelin-1 binds to a "G-protein" coupled receptor known as endothelin-B receptor on human melanocytes and activates the microtubule associated protein kinase (MAPK) and then the morphologic change of melanocyte to an activated form [11].

The UVB induced melanogenesis could be mediated through the release of thymidine dimers [12] or of the histamine which provokes morphologic change of melanocyte and activates the tyrosinase expression via a PKA (protein kinase cAMP dependent) stimulation [13], the PKA activation is consequent to an increase of cAMP level in melanocytes [14].

Many other factors such as prostaglandins PGE2, leukotriens LT-C4 and LT-B4, calcitonin gene related peptide (CGRP), the nerve growth factor (NGF) [15] and the stem cell factor (SCF or mast cell GF, or steel factor or KIT-ligand) [16] released from skin can induce also the melanogenesis.

The whitening Active Ingredients or "Whiteners"

Whiteners are common Active Ingredients for cosmetic applications to correct hyperpigmentation.

The first large group of whiteners involves Active Ingredients which interfere with tyrosinase activity: hydroquinone is an alternative substrate for tyrosinase which induces the release cytotoxic metabolites and then contributes to the skin melanocytes lost [17].

Other chemical agents interfering with tyrosinase activity are kojic acid, arbutin and ascorbic acid.

More recently, specific proteins were proposed to modulate melanogenesis: insulin reduces the tyrosinase activity [18] and the Agouti signaling protein acts as a competitor with α-MSH for binding to the MC1-R and therefore reduces the melanogenesis [9].

An other way to interfere with skin pigmentation is to inhibit the melanosome uptake by neighboring keratinocytes, so the protease-activated receptor (PAR-2) activation is correlated to keratinocytes phagocytosis resulting to melanosome ingestion [19] therefore serine protease inhibitor such as those isolated from soybean, blocks PAR-2 mechanism and reduces melanin storage [19].

The new melanogenesis inhibitor: botanical aspect and traditional uses

Inga alba *(Sw.) Willd.* is a widespread tropical tree extending from Southern Mexico to Panama and throughout South America to central Brazil and Bolivia. This species is present in both primary and secondary forest, usually on well drained land. This tree up to 40 m high and 1 m diameter has a reddish brown bark, leaves with 4-5 pairs of leaflets and axillary white inflorescence [20]. Its wood is a source of timber and bark is used in traditional medicine. Creoles use the decoction bark for dysentery and milled bark for dermatosis. Palikur tribe use the bark to cure leishmania. Inga trees are widely used for their fruits, and especially the pulp around the seeds which has a sweet taste [21, 22].

MATERIALS AND METHODS

Principle

The whitening potential was evaluated on tyrosinase activity and then on melanin synthesis from B16 melanocytes cultured in vitro.

In order to point out the class of compounds supporting the whitening activity, a phytochemical screening has been done to find out the phytochemical classes of the compounds present in the Inga bark extract. Among, the different classes investigated, the main compounds in the Inga bark extract seem to be the catechuic tannins.

Products to be tested

° Hydroquinone.

° Kojic acid.

° Arbutin.

° Ascorbic acid.

° Magnesium-Ascorbyl-Phosphate (Mg-Asc-P).

° Active Ingredient.

Protocol of efficacy tests

* Inhibition of tyrosinase activity [23]

Tyrosinase catalyzes the 2 first steps of melanin synthesis: oxidation of tyrosine in DOPA (dihydroxy-phenyl-alanine) and DOPA in DOPA chrome. Then DOPA chrome undergoes enzymatic oxidation and polymerization in melanin:

- L-DOPA is mixed with tyrosinase and product to be tested,

- recording of DOPA chrome optical density (OD) at 475 nm during 120 seconds (Figure 3),

- estimation of initial kinetic and of efficient concentration 50 (EC50) (Figure 4).

* Inhibition of melanin synthesis on B16 melanocytes [24, 25]

Melanocytes (B16 cell line) are inoculated in a standard medium of cell culture with fetal calf serum (or FCS). After an incubation of 3 days at 37°C and CO_2 = 5%, growth medium is exchanged for standard medium with a range of concentrations for each product to be tested. After an incubation of 3 days, the number of viable cells is determined by evaluation of the levels of cellular proteins (Bradford's method) and the level of synthesized melanin by recording the optical density at 475 nm of cell's homogenate.

The assay is carried out in triplicate and repeated 2 or 3 times and the results are expressed in % against control (cell culture medium without product) (Figure 5).

An index of activity referring to the ratio of: protein level / melanin level, is estimated.

Protocol of phytochemical analysis

To find out the polyphenols and the catechuic tannins in the Active Ingredient, a thin layer chromatography (TLC) of the product has been done with the following conditions.

Adsorbent: Silica gel TLC plate.

Polyphenols TLC:

- elution solvent: toluene, ethyl acetate, formic acid, water [20],

- detection: Neu + polyethyleneglycol reagent, UV 365 nm [26].

Catechuic tannins TLC:

- elution solvent: dichloromethane, methanol, water, acetic acid,

- detection: 4-(Dimethylamino)cinnamaldehyde, visible.

Then the catechuic tannins in the Active Ingredient have been assayed by spectrophotometry, with Bate Smith (1954), Porter (1986) modified reaction [27].

* Principle

This method involves the hydrochloric acid catalyzed depolymerization of condensed tannins in butanol to yield a red anthocyanin product that can be detected spectrophotometrically.

* Protocol

The reagent is prepared by adding ammonium iron III sulfate to hydrochloric acid (12N) solution and butanol solvent.

The reagent is added to the solubilized sample in a cap test tube and heated at 95°C for 1 hour. After cooling, absorbance is read at 550 nm and catechuic tannins content is evaluated against a standard curve of cyanidine chloride.

RESULTS

Inhibition of tyrosinase activity (Figures 3 and 4, and Table I)

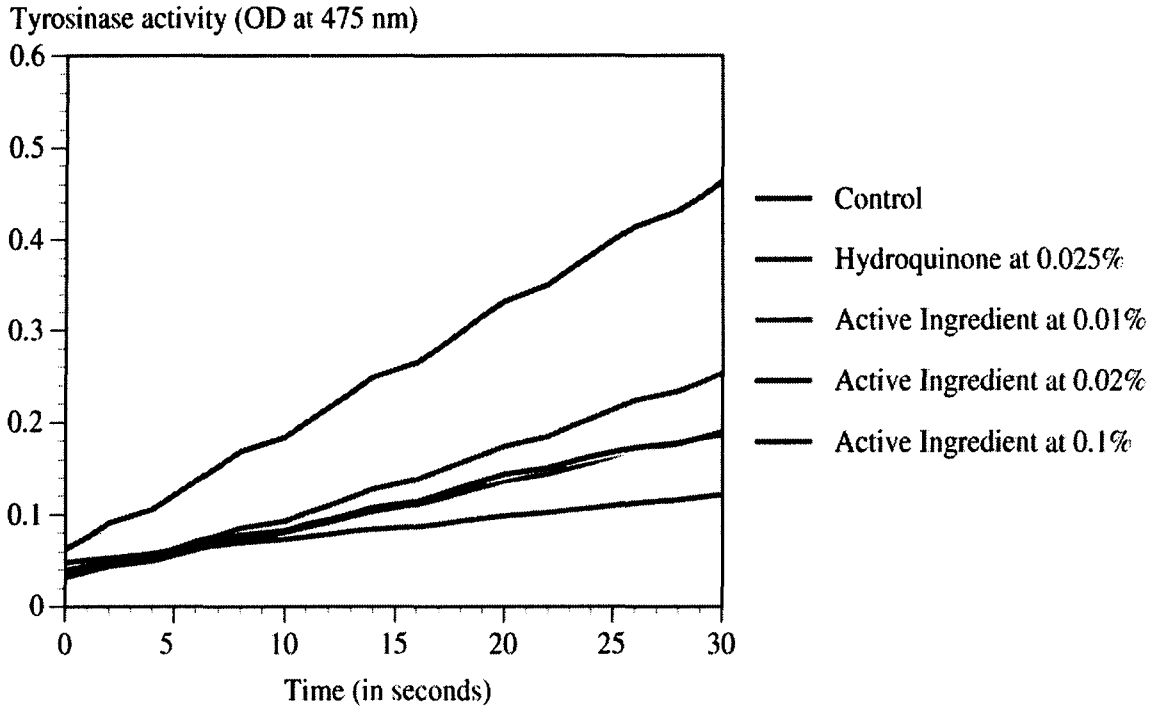

Figure 3: Kinetic curves of tyrosinase activity.

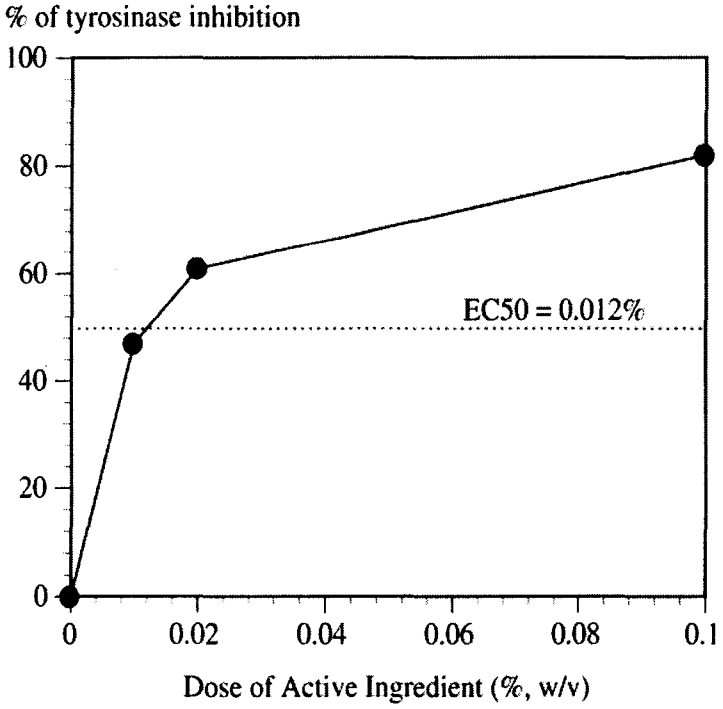

Figure 4: Determination for tyrosinase inhibition.

Table I: EC50 for inhibition of tyrosinase activity.

Products	EC50 (in %, w/v)
Hydroquinone	0.025
Active Ingredient	0.012

These data demonstrate that the Active Ingredient distinctly decreased the tyrosinase activity and presents a whitener potential better than hydroquinone.

Inhibition of melanin synthesis on B16 melanocytes (Figure 5 and Table II)

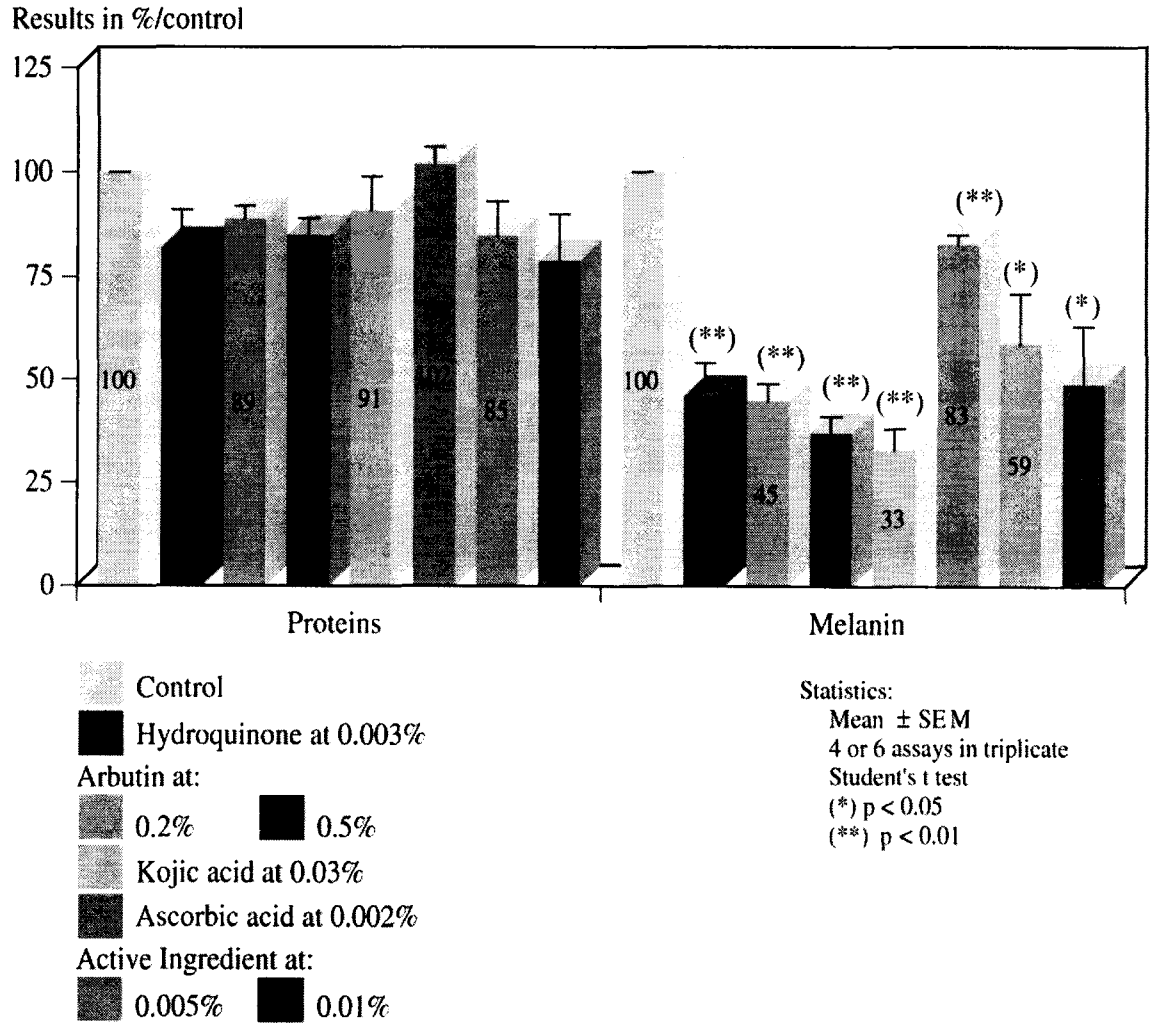

Figure 5: Results for the standard references and the Active Ingredient.

Table II: Inhibition of melanogenesis on B16 melanocytes: index I of activity.

	Index I (= ratio of protein level / melanin level)
Hydroquinone at 0.003%	1.8
Arbutin at 0.2%	2.0
Kojicacid at 0.03%	2.8
Ascorbic acid at 0.002%	1.2
Active Ingredient at 0.01%	1.6

These data demonstrate that the Active Ingredient distinctly lowered the melanin synthesis in B16 melanocytes within a dose-dependent way, the Active Ingredient activity is less high than kojic acid but with a lesser concentration.

Moreover the Active Ingredient presents a whitener potential comparable to arbutin and hydroquinone and better than ascorbic acid.

Results of phytochemical analysis (Figure 6)

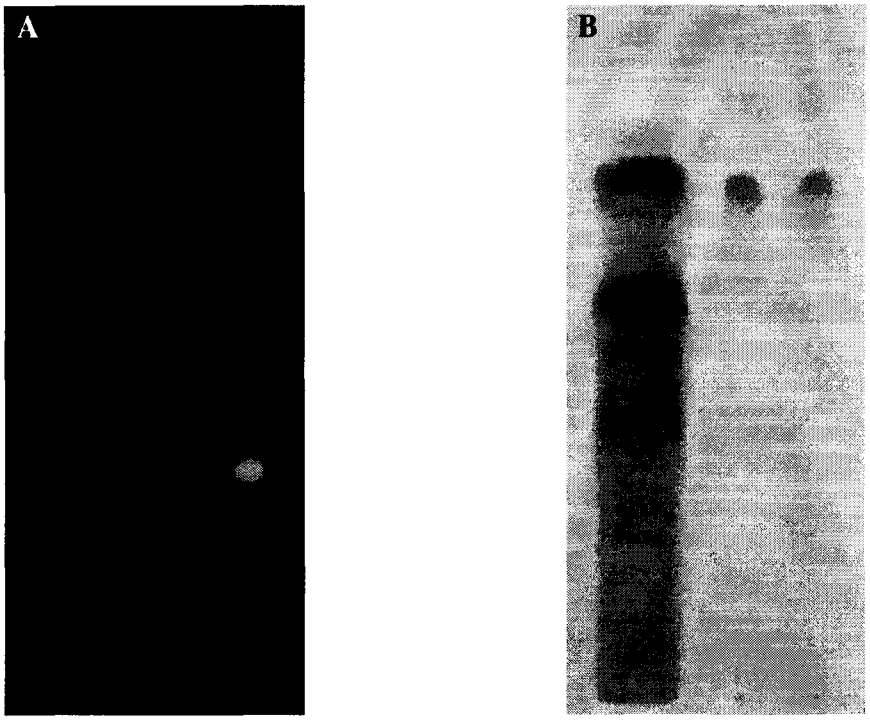

Figure 6: Polyphenols fingerprint of the catechuic tannins fingerprint of the Active Ingredient by thin layer chromatography (TLC).
A & B: 1 - Ethyl acetate extract of the Active Ingredient
A: 2 and 3 - Standards: rutin, chlorogenic acid
B: 2 and 3 - Standards: catechin, epicatechin

Result of spectrophotometry assay of catechuic tannins

The TLC fingerprints clearly showed at last three catechuic tannins. The spectrophotometry assay confirmed the result and indicated a catechuic tannin concentration of 8.49 % dry matter.

CONCLUSION

The Active Ingredient has shown a good potential of tyrosinase inhibition, even better than hydroquinone.

The Active Ingredient has shown a good potential of inhibition of melanin synthesis into B16 melanocytes cultured in vitro: I* = 1.6 at 0.01%.

However some standard references have shown a better activity than the Active Ingredient (kojic acid I = 2.8 versus 1.6) but at higher concentrations (kojic acid at 0.03% versus 0.01%).

While the Active Ingredient activity is at the same level as hydroquinone (I = 1.8 at 0.003%), arbutin (I = 2.0 at 0.2%) and ascorbic acid is less active (I = 1.2 at 0.002%).

* Index I of activity = highest ratio of [protein level / melanin level] for a non toxic concentration.

To conclude, the Active Ingredient has shown a good potential to inhibit melanin synthesis, this potential was mediated by the tyrosinase inhibition and catechuic tannins might be responsible for this effect. Further studies are on track.

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