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(54) **Title:** A SKIN LIGHTENING COMPOSITION

(57) **Abstract:** The present invention relates to a skin lightening composition for topical application. The present invention provides for a composition that comprises specific fraction of peel of *Mangifera indica* comprising higher than a certain percentage of alkyl resorcinol and less than a certain percentage of urshols for enabling the skin lightening action.



A SKIN LIGHTENING COMPOSITION

Field of the invention

The present invention relates to a skin lightening composition for topical application
5 comprising selective fractions of natural materials having skin lightening efficacy.

Background of the invention

Highly pleasing skin appearance is one of the most desired expectations from personal care products from most consumers around the world. In tropical countries where
10 consumers generally have dark skin, there is a desire to have lighter skin appearance. In consumers who live far from the tropical countries e.g. the Caucasian people who generally have lighter skin, there is a need among such consumers to have an even tanned tone of their skin. Any exposure of the skin to sunlight, in such consumers often leads to blotchy skin, referred to as freckles and in some cases they experience
15 hyperpigmentation in localized areas of the skin. Most consumers experience blemishes on their skin after exposure to sun, on healing of wounds or after drying up of acne. Most people, all over the world, notice darker skin on their underarms (i.e in their axilia) as compared to other regions of their skin, although the underarms are rarely exposed to the sun. They prefer an even skin tone in their axilia as compared to other parts of the body.
20 In all of the above cases, consumers rely on cosmetic solutions to their skin appearance problems.

Thus, smooth, soft and glowing skin with even skin tone and colour is desired by all consumers who use personal care compositions for their skin. To provide this benefit, one
25 very commonly used approach is to include sunscreens or sunblocks in such cosmetic products. Another approach to controlling the colour, tone and appearance of the skin is the skin lightening approach where chemicals are added to personal care compositions which alter the formation of melanin in the skin through biochemical transformation in the stratum corneum thereby changing the colour and appearance of the skin. This approach
30 is capable of lightening the skin beyond the basic colour of skin. While this approach has been used successfully in many cosmetic products, researchers are still struggling to improve efficacy of skin lightening agents beyond a certain threshold.

One of the drawbacks of most skin lightening actives used so far is that they are usually
35 synthetically prepared chemical compounds. Synthetic chemicals have over time, taken a

negative connotation in the consumer's mind. Hence, many consumers are more and more, preferring actives originating from or extracted from natural sources to be used in such products.

5 In order to provide a solution to the several drawbacks in the art listed above, the present inventors have been working for many years on deriving actives from natural sources for various personal care benefits. In the present invention, the inventors have found that an extract of mango peel especially ripe mango peel, which is high in specific alkyl resorcinols at more than a minimum percentage exhibit skin lightening activity.

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JP2004-352658A discloses an external preparation for the skin, having both tyrosinase activity-inhibiting effects and cyclic AMP (cAMP) production-inhibiting effects, and also having excellent melanin production-inhibiting effects and bleaching effects.

The external preparation for the skin is obtained by formulating one or more kinds of
15 plants selected from *Dioscorea mexicana*, *Loranthus sp.*, *Symplocos racemosa*, *Pachyrrhizus erosus*, *Cedrus deodara*, *Quercus lusitanica*, *Woodfordia fruticosa*, *Mangifera indica* and *Terminalia arborea*, or a solvent extract thereof. The present inventors note that the 80/20 ethanol/ water extract of the plant disclosed in this publication is not capable of producing the extract with the desired amount of alkyl
20 resorcinol needed to get the skin lightening activity obtained by the present invention.

Indian Patent Application 2138/CHE/2007 (Cavinkare) discloses skin care agent and cosmetic and/or dermopharmaceutical composition obtained thereof and, in particular, to an anti-aging benefit agent for skin care. The anti-aging benefit agent of the invention is
25 preferably selectively obtained from skin and/or kernel of mango (fruit of *Mangifera indica* Linn.). The present inventors note that the method of preparing the extract disclosed therein does not clearly and unambiguously disclose the method of producing an extract with the desired amount of alkyl resorcinol needed to get the skin lightening activity obtained by the present invention.

30

Therefore there exists a need for a skin lightening composition comprising extracts from natural materials that is able to provide high skin lightening efficacy.

It is therefore an object of the present invention to obviate at least some drawbacks of the prior art and provide a skin lightening composition comprising actives from natural sources.

5 SUMMARY OF THE INVENTION

According to the first aspect of the invention there is provided a skin lightening composition comprising

- (a) 0.1 to 10% on dry weight basis an extract from peel of ripe fruit of *Mangifera indica* which comprises higher than 1.5% alkyl resorcinol with a carbon chain length of 5 to 10 18 carbon atoms, by dry weight of the extract; and less than 0.01% urshiols by dry weight of the extract; and
- (b) a cosmetically acceptable base.

According to a preferred aspect, the extract of peel of ripe fruit of *Mangifera indica* 15 comprising higher than 1.5% alkyl resorcinol with a carbon chain length of 5 to 18 carbon atoms, on dry weight basis, and less than 0.01% urshiols by dry weight of the extract, is prepared, for use in the composition of the invention, using a process comprising the steps of

- (a) preparing an aqueous extract of the peel of the ripe fruit of *Mangifera Indica* ;
- 20 (b) passing the aqueous extract through MCI gel column; and
- (c) eluting the desired fraction with methanol, ethanol, acetone or mixtures thereof.

According to yet another aspect of the invention there is provided use of an extract of peel of ripe fruit of *Mangifera Indica* which comprises higher than 1.5% alkyl resorcinol, 25 with a carbon chain length of 5 to 18 carbon atoms, by dry weight of the extract as a skin lightening agent.

DETAILED DESCRIPTION OF THE INVENTION

These and other aspects, features and advantages will become apparent to those of 30 ordinary skill in the art from a reading of the following detailed description and the appended claims. For the avoidance of doubt, any feature of one aspect of the present invention may be utilized in any other aspect of the invention. The word "comprising" is intended to mean "including" but not necessarily "consisting of" or "composed of." In other words, the listed steps or options need not be exhaustive. It is noted that the 35 examples given in the description below are intended to clarify the invention and are not

intended to limit the invention to those examples per se. Similarly, all percentages are weight/weight percentages unless otherwise indicated. Except in the operating and comparative examples, or where otherwise explicitly indicated, all numbers in this description indicating amounts of material or conditions of reaction, physical properties of materials and/or use are to be understood as modified by the word "about". Numerical ranges expressed in the format "from x to y" are understood to include x and y. When for a specific feature multiple preferred ranges are described in the format "from x to y", it is understood that all ranges combining the different endpoints are also contemplated.

"Skin lightening Composition" as used herein, is meant to include a composition for topical application to skin of mammals, especially humans. Such a composition may be generally classified as leave-on or rinse off, and includes any product applied to a human body primarily for lightening skin but may also improve appearance, cleansing, odor control or general aesthetics. The composition is preferably a leave-on composition. The composition of the present invention can be in the form of a liquid, lotion, cream, foam, scrub, gel, soap bar or toner, or applied with an implement or via a face mask, pad or patch. Non-limiting examples of the compositions include leave-on skin lotions and creams, shampoos, conditioners, shower gels, toilet bars, antiperspirants, deodorants, depilatories, lipsticks, foundations, mascara, sunless tanners and sunscreen lotions.

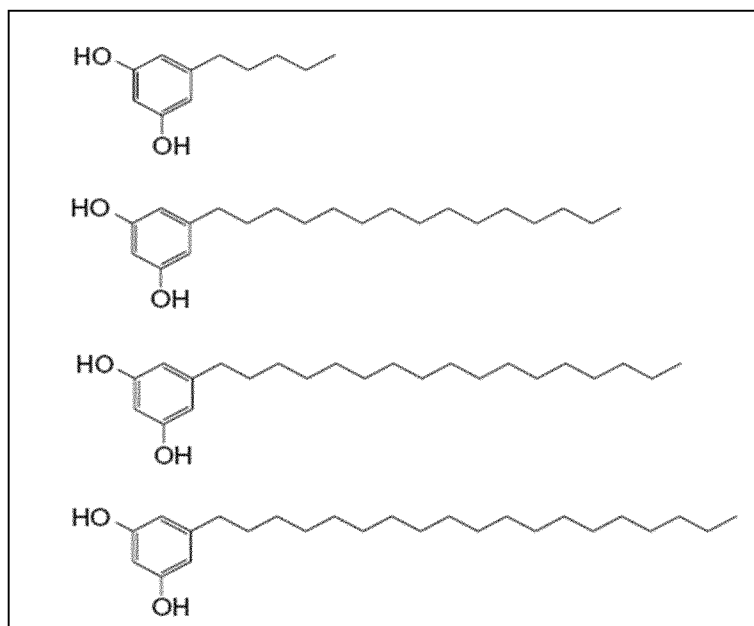
"Skin" as used herein is meant to include skin on the face and body (e.g., neck, chest, back, arms, underarms, hands, legs, buttocks and scalp).

The invention provides for a skin lightening composition comprising 0.1 to 10 % on weight dry basis, of an extract of peel of ripe fruit of *Mangifera indica*, the extract comprising more than 1.5% alkyl resorcinol by weight of the extract, on dry weight basis and less than 0.01% urshiols by dry weight of the extract. The alkyl resorcinol in the extract comprises alkyl chains with a carbon chain length of 5 to 18 carbon atoms. The extract is present in 0.1 to 10%, preferably 0.1 to 5%, more preferably 0.1 to 2.5% by weight of the composition.

The mango is a fleshy stone fruit belonging to the genus *Mangifera*, consisting of numerous tropical fruiting trees in the flowering plant family Anacardiaceae. The mango is native to the Indian subcontinent from where it was distributed worldwide to become one of the most cultivated fruits in the tropics. While other *Mangifera* species (e.g. horse mango, *M. foetida*) are also grown on a more localized basis, *Mangifera indica* – the

'common mango' or 'Indian mango' – is the mango tree commonly cultivated in many tropical and subtropical regions. It is the national fruit of India, Philippines and Pakistan.

The chemical structures of alkyl resorcinols, commonly present in extract of mango peel is given below



Structure of alkyl resorcinols commonly present in mango peel

The peel of ripe mangoes are used for preparing the extract for use in the composition of the invention. It has been determined by the present inventors that the peels of unripe mangoes are not suitable for extraction of the actives desired for obtaining the benefits of the present invention. Also, several other members of the fabaceae family are not suitable for the purposes of the present invention.

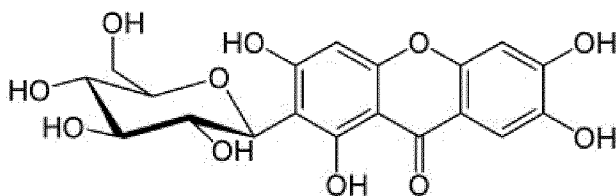
Fruits of *Mangifera indica* L. (Anacardiaceae) as well as other parts of the plant have widely been used in traditional medicine (Khare, 2004). The fruits are also used as a nutrient and for the industrial production of products like juice, jams and purees. The fruit peels are discarded as a waste. The members of the family Anacardiaceae are known to contain alkyl or alkenyl derivatives of phenol, catechol, and resorcinol (Kozubek and Tyman, 2005).

It is preferred that the composition of the invention comprises less than 0.5% mangiferin, more preferably less than 0.2% mangiferin. It has been observed by the inventors that

mangiferin at the amount present in mango peel extract when included in the composition of the invention does not contribute to skin lightening efficacy and is preferably removed during the extraction process.

5 Mangiferin has the structure as given below.

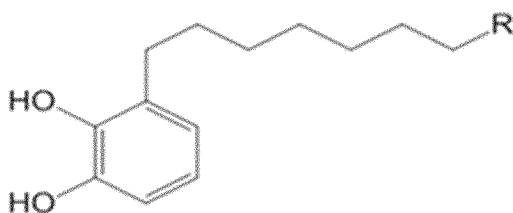
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The composition of the invention comprises less than 0.01% urshiols. It has been observed by the inventors that urshiols at the amount present in mango peel extract when included in the composition of the invention does not contribute to skin lightening efficacy. In fact, the present inventors have determined that unless the extract is so prepared that it comprises less than 0.01% urshiols the extract is unsuitable for use on topical surfaces since the presence of higher amounts of urshiols causes irritation on the skin.

20

Urshiol has the structure as given below.



Urushiol I : $R = -(CH_2)_7-CH_3$

Urushiol II : $R = -CH=CH-(CH_2)_5-CH_3$

Urushiol III : $R = -CH=CH-CH_2-CH=CH-(CH_2)_2-CH_3$

Urushiol IV : $R = -CH=CH-CH_2-CH=CH-CH=CH-CH_3$

Urushiol V : $R = -CH=CH-CH_2-CH=CH-CH_2-CH=CH_2$

The composition of the invention comprises a cosmetically acceptable base. The cosmetically acceptable base is preferably a cream, lotion, gel or emulsion.

5 Personal care compositions may be prepared using different cosmetically acceptable emulsifying or non-emulsifying systems and vehicles. Preferred cosmetically acceptable bases comprise 1 to 25% fatty acid. A further preferred aspect provides for inclusion of 0.1 to 10% soap, which may be in addition to the 1 to 25% fatty acid. A highly suitable base is a cream. Vanishing creams are especially preferred. Vanishing cream bases
10 generally comprise 5 to 25% w/w fatty acid and 0.1 to 10% w/w soap. Vanishing cream base gives a highly appreciated matt feel to the skin. C₁₂ to C₂₀ fatty acids are especially preferred in vanishing cream bases, further more preferred being C₁₄ to C₁₈ fatty acids. The most preferred fatty acid is stearic acid. The fatty acid may also be a mixture of palmitic and stearic acid. The fatty acid in the composition is more preferably present in
15 an amount in the range of 5 to 20% w/w of the composition. Soaps in the vanishing cream base include alkali metal salt of fatty acids, like sodium or potassium salts, most preferred being potassium stearate. The soap in the vanishing cream base is generally present in an amount in the range of 0.1 to 10%, more preferably 0.1 to 3% w/w of the composition. Generally the vanishing cream base in cosmetic compositions is prepared
20 by taking a desired amount of total fatty matter and mixing with potassium hydroxide in desired amounts. The soap is usually formed in-situ during the mixing. The personal care composition when formulated as a vanishing cream preferably comprises 60 to 85%, more preferably 65 to 80% w/w water. Water is generally present in many compositions prepared as per the invention and is generally present in 40 to 85% by weight of the
25 composition.

The composition of the invention may comprise another skin lightening agent other than the extract of the invention. This additional skin lightening agent is preferably chosen from a vitamin B3 compound or its derivative e.g. niacin, nicotinic acid, niacinamide or any
30 other well known skin lightening agent. Most preferred additional skin lightening agent is niacinamide. Niacinamide, when used, is preferably present in an amount in the range of 0.1 to 10%, more preferably 0.2 to 5% w/w of the composition.

The personal care composition may preferably additionally comprise one or more UV
35 sunscreens. The UV sunscreens may be inorganic or organic. A wide variety of organic

sunscreen agents are suitable for use in combination with the essential ingredients of this invention. Most suitable organic sunscreen are 2-ethylhexyl-p-methoxycinnamate (as a UVB sunscreen agent) and/ or butylmethoxydibenzoylmethane (as a UVA sunscreen agent).

5

A safe and effective amount of sunscreen may be used in the compositions of the present invention. The composition preferably comprises from about 0.1% to about 10%, more preferably from about 0.1% to about 5% w/w of a sunscreen agent.

10 Useful inorganic sun-blocks are also preferably used in the present invention. These include, for example, zinc oxide iron oxide, silica, such as fumed silica, and titanium dioxide.

Ultrafine titanium dioxide in either of its two forms, namely water-dispersible titanium
15 dioxide and oil-dispersible titanium dioxide is especially suitable for the invention. Water-dispersible titanium dioxide is ultra-fine titanium dioxide, the particles of which are non-coated or which are coated with a material to impart a hydrophilic surface property to the particles. Examples of such materials include aluminium oxide and aluminium silicate. Oil-dispersible titanium dioxide is ultrafine titanium dioxide, the particles of which exhibit a
20 hydrophobic surface property, and which, for this purpose, can be coated with metal soaps such as aluminium stearate, aluminium laurate or zinc stearate, or with organosilicone compounds.

By "ultrafine titanium dioxide" is meant particles of titanium dioxide having an average
25 particle size of less than 100 nm, preferably 70 nm or less, more preferably from 10 to 40 nm and most preferably from 15 to 25 nm. The total amount of inorganic sun block that is preferably incorporated in the composition according to the invention is from 0.1 to 5% w/w of the composition.

30 The composition according to the invention may also comprise other diluents. The diluents act as a dispersant or carrier for other materials present in the composition, so as to facilitate their distribution when the composition is applied to the skin.

Diluents other than water can include liquid or solid emollients, solvents, humectants,
35 thickeners and powders.

The cosmetically acceptable base is usually from 10 to 99.9%, preferably from 50 to 99% w/w of the composition, and can, in the absence of other cosmetic adjuncts, form the balance of the composition. The composition of the invention may comprise water.

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The composition of the invention may comprise a conventional deodorant base as the cosmetically acceptable carrier. By a deodorant is meant a product in the stick, roll-on, or propellant medium which is used for personal deodorant benefit e.g. application in the under-arm area which may or may not contain anti-perspirant actives.

10

Deodorant compositions can generally be in the form of firm solids, soft solids, gels, creams, and liquids and are dispensed using applicators appropriate to the physical characteristics of the composition.

15 Deodorant compositions which are delivered through roll-ons generally comprise a liquid carrier. Such liquid carrier can be hydrophobic or comprise a mixture of both hydrophilic and hydrophobic liquids. They may be in the form of an emulsion or a microemulsion. The liquid carrier or mixture of carriers often constitutes from 30 to 95% and in many instances from 40 to 80% w/w of the composition.

20

Hydrophobic liquid carriers commonly can comprise one or more materials selected within the chemical classes of siloxanes, hydrocarbons, branched aliphatic alcohols, esters and ethers that have a melting point not higher than 25°C and a boiling point of at least 100°C.

25 Hydrophilic carrier liquids that can be employed in compositions herein commonly comprise water and/or a mono or polyhydric alcohol or water-miscible homologue. Monohydric alcohols often are short chain, by which is meant that they contain up to 6 carbons, and in practice are most often ethanol or sometimes iso-propanol. Polyhydric alcohols commonly comprise ethylene or propylene glycol, or a homologue can be
30 employed such as diethylene glycol.

The compositions that remain in liquid form can be applied employing conventional applicators such as a roll-on or by being pumped or squeezed through a spray-generating orifice. Such compositions may be thickened, for example using one or more thickeners
35 described subsequently herein.

When a deodorant composition employed herein comprises an aerosol composition, it contains a propellant in addition to a base composition as described herein above, commonly in a weight ratio of from 95:5 to 40:60, and in many formulations, the weight ratio is from 90:10 to 50:50.

The propellant is conveniently a low boiling point material, typically boiling below -5°C , for example an alkane such as propane, butane or isobutane, and possibly containing a fraction of pentane or isopentane, or a hydrofluorocarbon or fluorocarbon of similar carbon content. During filling of the aerosol canister, the propellant gas is liquified by virtue of the elevated pressure that is generated therein.

The compositions of the present invention can comprise a wide range of other optional components. The CTFA Cosmetic Ingredient Handbook, Second Edition, 1992, which is incorporated by reference herein in its entirety, describes a wide variety of non-limiting cosmetic and pharmaceutical ingredients commonly used in the skin care industry, which are suitable for use in the compositions of the present invention. Examples include: antioxidants, binders, biological additives, buffering agents, colorants, thickeners, polymers, astringents, fragrance, humectants, opacifying agents, conditioners, exfoliating agents, pH adjusters, preservatives, natural extracts, essential oils, skin sensates, skin soothing agents, and skin healing agents.

The composition is formulated in any known format, more preferred formats being creams or lotions.

Process

The invention also provides for a process to prepare the extract comprising higher than 1.5% alkyl resorcinol having carbon chain length of 5 to 18 carbon atoms, on dry weight basis, for use in the composition of the invention, the process comprising the steps of

- (a) preparing an aqueous extract of the peel of ripe fruit of *Mangifera Indica* ;
- (b) passing the aqueous extract through MCI gel column; and
- (c) eluting the desired fraction with methanol, ethanol, acetone or mixture thereof.

The details of the process which is most preferred for extraction is given below:

Most preferred method of preparation of aqueous extract:

- (1) Dry mango peel powder is extracted with distilled water at about 90°C for about 30 minutes.
- (2) After cooling down the extract to about 35 - 40°C, the extract is filtered to get a clear solution.
- 5 (3) The solution is then concentrated to dryness under reduced pressure using a rotary evaporator.
- (4) The dried aqueous extract is then stored at a low temperature (of about 4°C).

Most preferred method of preparation of MCI gel fractions:

- 10 (1) Dry mango peel powder is extracted with distilled water at about 90°C for about 30 minutes.
- (2) After cooling down to about 35 - 40°C, the extract is filtered to get a clear solution.
- (3) The clear solution is then passed through an MCI gel column.
- (4) The eluent is then collected and the column was washed further with sufficient
15 amount of water.
- (5) The MCI gel column was then eluted with 300 ml of methanol or ethanol or acetone and the eluent was concentrated to dryness to obtain the desired MCI gel adsorbed fraction.
- 20 According to yet another aspect of the invention the extract of peel of the ripe fruit of *Mangifera Indica* for use in the composition of the invention is prepared using a process comprising the steps of
- (a) preparing an aqueous extract of the peel of the ripe fruit of *Mangifera Indica*;
- (b) passing the aqueous extract through MCI gel column; and
- 25 (c) eluting the desired fraction with methanol or ethanol or acetone.

According to yet another aspect of the invention there is provided use of an extract of peel of ripe fruit of *Mangifera Indica* fruit which comprises higher than 1.5% alkyl resorcinol, having carbon chain length of 5 to 18 carbon atoms, by dry weight of the
30 extract, as a skin lightening agent.

The use is preferably non-therapeutic.

The invention is now further described by way of the following non-limiting examples.

Examples

Examples A, B and 1: Efficacy of composition as per the invention as compared to those outside the invention as measured using tyrosinase inhibition assay.

5 Example A: Ethyl resorcinol, a highly effective skin lightening active was used.

Example B: An aqueous extract of mango peel was prepared as per the following procedure

25 grams of dried mango peel powder was taken and extracted with 200 ml of distilled water at 90 °C for about 30 minutes at 800 mb vacuum. The liquor was separated using
10 decantation and vacuum filtration to obtain about 184 ml of liquor. About 84 ml of this liquor was dried at 70 °C and 30 mb vacuum to obtain about 2.8 grams of dried aqueous extract.

Example 1: An extract as per the invention was prepared as follows.

15 25 grams of dried mango peel powder was taken and extracted with 200 ml of distilled water at 90 °C for about 30 minutes at 800 mb vacuum. The liquor was separated using decantation and vacuum filtration to obtain about 184 ml of liquor. About 100 ml of this liquor was passed through MCI gel. The adsorbed phase was eluted with acetone and the acetone liquor was dried under vacuum to obtain about 1.7 g of the desired extract.

20 The samples B and 1 were analysed for % alkyl resorcinol.

Colorimetric method for alkyl resorcinol quantification using of Fast Blue RR salt:

A stock solution of 0.05% Fast Blue RR salt was prepared in methanol. Fresh working solution of Fast Blue RR reagent was prepared by mixing 1 part stock reagent with 5
25 parts of methanol.

The alkyl resorcinol was expressed as % olivetol equivalent. A stock solution of olivetol was prepared in methanol at concentration of 10 mg/ml. A calibration curve was drawn using varying concentrations of stock solution (2µg-10 µg) in assay tube and made up to 200 µl with methanol. Stock solution of mango peel samples was prepared in water at
30 concentration of 10 mg/ml. 100µl from the stock solution was used for assay and the volume was made up to 200µl with methanol. Then, 2 ml of working solution of Fast Blue B salt was added to each assay tube. 10µl of a basifying reagent (10% potassium carbonate) was also added. Absorbance of the reaction mixture was measured after 30 min at 480 nm.

(Ref: D.A. Sampietro *, M.A. Vattuone, C.A.N. Catalán, Food Chemistry 115 (2009) 1170–1174)

5 Samples prepared as per Example A, B and 1 were subjected to tyrosinase inhibition assay (as described below) to study the extent of skin lightening that can be expected.

Tyrosinase inhibition assay:

10 Reference: Duthie G., Crozier A., Curr. Opin. Nutr. Metab. Care, 3, 447-451 (2000).

Stock Solutions: The following solution were first prepared:

0.1M KH_2PO_4 (pH: 6.5):- 1.361gms of KH_2PO_4 dissolved in 100ml of milli Q water and the pH is adjusted with 2M KOH to 6.5, then the solution is filtered and stored at 4°C.

3.5mM L-DOPA:- 6.985 mg of L-DOPA is dissolved in 10ml of buffer and is stored at 4°C covered with aluminum foil. (Prepared freshly).

1mg/ml Mushroom Tyrosinase (prepared freshly)

3mg/ml herbal extract (for water soluble samples)

1mg/ml herbal extract (for DMSO soluble samples)

Samples	Buffer (μl)	Inhibitor (μl)	Enzyme (μl)	DOPA (μl)	Total (μl)
Blank	200	-	-	-	200
Positive control	106	-	8	86	200
Negative control	114	-	-	86	200
Water control	46	60(water)	8	86	200
DMSO control	98	8(DMSO)	8	86	200
Color control	140(in water) 192(in DMSO)	60(in water) 8(in DMSO)	-	-	200
Inhibitor sample	46(in water) 98(in DMSO)	60(in water) 8(in DMSO)	8	86	200

20 Sequence of addition is: water-buffer-inhibitor-enzyme-5min incubation-L-dopa.

The assay was performed in a flat bottom 96 well plate.

After 30 min of addition of DOPA, absorbance was measured at 450nm for Dopachrome formation using TECAN Reader.

The data is summarized in Table -1 below.

5 Table – 1

Example	% Alkyl resorcinol: alkyl chain length of 5 to 18 carbon atoms	Conc. Of active used(ppm)	% tyrosinase Inhibition
A	-	40	90
B	1.0	900	62
1	1.8	40	78

The higher the %tyrosinase inhibition value the greater is the potential of being an effective skin lightening agent.

10 The data in Table -1 indicates that with the extract as per the invention (Example -1) provides for vastly superior tyrosinase inhibition as compared to an aqueous extract (Example – B). The data is comparable to ethyl resorcinol which is one of the best skin lightening agents available.

Melanin Content inhibition assay

15 The samples of Example B and 1 were subjected to melanin content inhibition assay which is another one of the pathways responsible for skin lightening.

Cell types used for assay - HeMnDP (darkly pigmenting neonatal foreskin melanocytes, Cascade Biologics, used between P50-70).

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Procedure for set up of co-cultures:

1. Seed 4×10^4 HaCaT and 4×10^4 HeMnDP (1:1) per well (single wells / treatment) in 12-well Nunc plates on Day 0 so as to establish a co-culture system.
2. The volume of media per well should be 1mL of a 1:1 mix of Melanocyte Growth Media and Keratinocyte Growth Media
- 25 3. 24 hours later, add fresh media containing actives. Again use 1mL of a 1:1 mix of MGM and KGM for active addition). Ensure that appropriate vehicle controls are included in the assay (Final concentrations of DMSO should not exceed 0.1% in both the treatments and the vehicle control, 4-ER is tested at $10 \mu\text{M}$).
- 30 4. Incubate plates for 72 hours in a 37°C CO_2 incubator.

5. At the end of the time point, progress the plates for Calcein assay followed by melanin content estimation.

Calcein-AM viability assay

- 5 1. Remove spent media from all wells of a 12 well plate and rinse once with 400µl of 1xPBS-Ca-Mg (see preparation below), including wells without cells (control wells).
2. Prepare 1µM of Calcein-AM (working stock) in 1xPBS-Ca-Mg from 1mM stock (e.g. 10µl of 1mM Calcein stock in 100% DMSO in 10ml of 1xPBS-Ca-Mg). Add 400µl of above Calcein-AM working stock solution to each well including controls cells.
- 10 3. Cover the plate with aluminum foil completely and incubate for 30 minutes in the regular CO₂ incubator. In case of multiple plates, stagger Calcein addition with a gap of at least 10 minutes between plates, so as to not exceed 30 minutes incubation with Calcein AM in any one plate.
4. In the meantime, start TECAN instrument (in measurement lab) and after 30min of dye incubation, measure fluorescence (excitation at 490nm and emission at 520nm).
- 15 5. After reading the plate, remove buffer containing Calcein and proceed for melanin content assay.

Preparation of PBS-Ca-Mg

- 20 20x PBS-Ca-Mg stock buffers (for 1 litre solution)

Solution A:

348mM Na₂HPO₄ (anhydrous): 49.4 g

70mM NaH₂PO₄ (dihydrate): 10.92 g

- 25 Solution B:

18mM CaCl₂: 2.6g

70mM KCl: 5.22 g

18mM MgCl₂ (hexahydrate): 3.66 g

2740mM NaCl: 160.3 g

- 30

Prepare 50 ml of each of Solutions A and B in autoclaved distilled water and store at room temperature (storing at 4°C can lead to precipitation).

1X PBS-Ca-Mg working stock

Take 5ml each of solutions A and B, add 90 ml of water, to make the working stock.
Filter just before use and do not refrigerate.

5 Preparation of 1mM Calcein-AM stock

1. Add 500µl of 100% DMSO to 1mg of Calcein AM (Fluka / Sigma Aldrich, CAS No 148504-34-1).
2. Mix well and add another 500µl of 100% DMSO.
3. Mix well, aliquot (20 µl), cover with Al foil and store at -20°C.
- 10 4. 10 µl from above aliquot can be appropriately diluted as mentioned in Step 2 above (do not expose to light even during this step) to yield 1µM working stock and the remaining unused Calcein stock can be moved back to -20°C.

Melanin Content assay

- 15 Preparation of MCA reagent (10% DMSO in 1N NaOH)-

Volume of MCA reagent (mL)	Volume of DMSO (100%) (mL)	Volume of 10N NaOH (mL)	Volume of water (mL)
10	1	1	8

1. After reading for Calcein-AM, remove buffer containing Calcein-AM from all the wells.
2. Add 150µl of above MCA reagent to each well.
- 20 3. Foil the plates completely and incubate for it for 1hr at 60°C with gentle shaking.
4. Transfer 120µl of solution from each well into a 384 well plate.
5. Measure the absorbance in the Lab-5 Tecan at 405nm.
6. For non-cytotoxic concs (based on Calcein cut-off of $\pm 15\%$ of control), calculate Avg MCA/Avg Calcein) and report MCA as %control, followed by % inhibition.

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The higher the %melanin content inhibition, the greater is the potential as a skin lightening active.

The data is summarized in table – 2.

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Table – 2

Example	Conc. Of active used	% melanin content inhibition
B	10 ppm	0.8
1	10 ppm	29.3

The data in Table – 2 indicates that composition as per the invention (Example -1) is superior to Example – B when measured using melanin content inhibition assay.

Claims

1. A skin lightening composition comprising
 - (a) 0.1 to 10% on dry weight basis an extract from peel of ripe fruit of *Mangifera indica* which comprises higher than 1.5% alkyl resorcinol with a carbon chain length of 5 to 18 carbon atoms, by dry weight of the extract and less than 0.01% urshiols by dry weight of the extract; and
 - (b) a cosmetically acceptable base.
2. A composition as claimed in claim 1 comprising less than 0.5% wt% mangiferin on dry weight basis.
3. A composition as claimed in any one of the preceding claims wherein said extract of peel of the ripe fruit of *Mangifera Indica* is prepared using a process comprising the steps of
 - (a) preparing an aqueous extract of the peel of the ripe fruit of *Mangifera Indica* ;
 - (b) passing the aqueous extract through MCI gel column; and
 - (c) eluting the desired fraction with methanol, ethanol, acetone or mixtures thereof.
4. A composition as claimed in claim 3 wherein the peel of the *Mangifera Indica* fruit has been dried and powdered before the step of preparing the aqueous extract.
5. A composition as claimed in claim 3 or 4 wherein the aqueous extract is prepared using water at a temperature range of 70 to 100 °C.
6. A process to prepare an extract of peel of ripe fruit of *Mangifera indica* comprising higher than 1.5% alkyl resorcinol with a carbon chain length of 5 to 18 carbon atoms, on dry weight basis, and less than 0.01% urshiols by dry weight of the extract, for use in the composition of the invention, the process comprising the steps of
 - (a) preparing an aqueous extract of the peel of the *Mangifera Indica* fruit ;
 - (b) passing the aqueous extract through MCI gel column; and
 - (c) eluting the desired fraction with methanol, ethanol, acetone or mixtures thereof.

7. Use of an extract of peel of ripe fruit of *Mangifera Indica* fruit which comprises higher than 1.5% alkyl resorcinol, with a carbon chain length of 5 to 18 carbon atoms, by dry weight of the extract as a skin lightening agent.