Effective skin lightening with protective properties

ABSTRACT

A highly pure hexylresorcinol (>99% purity), designed for topical application, is shown to be a safe and effective skin lightener as demonstrated by *in vitro* and human clinical studies. Melanin inhibitory activity of hexylresorcinol is demonstrated to be due to the inhibition of various points in the melanogenesis pathway. Skin protective property of hexylresorcinol is shown to be due to its stimulatory effects on glutathione, glutathione peroxidase and glutathione reductase.

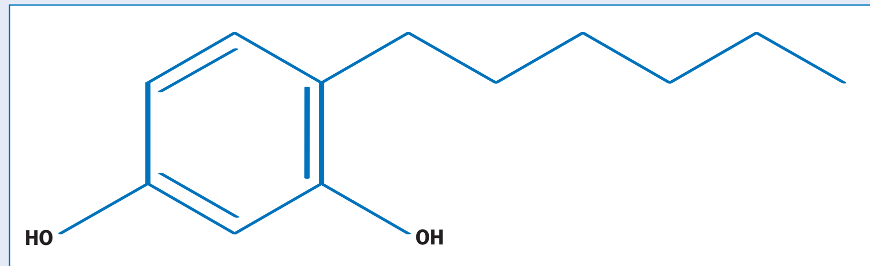

Figure 1: *Structure of 4-hexylresorcinol (HR).*

Alkylresorcinols (ARs) are amphiphilic phenolic lipids present in significant amounts in the bran fractions of rye and other cereals. ARs were reported to have antitumour, antibacterial, antifungal and antiparasitic activities.[1] These effects of ARs were attributed to membrane-modulating effects due to interactions of their alkyl tails with phospholipids and/or proteins, and to antioxidant effects of the phenolic hydrogen.[1] ARs are present in wheat and rye grains at levels of approximately 0.015% to 0.3% of whole kernel dry weight, thus representing a significant proportion of the phenolic compounds present in these cereals. ARs have been found to be bioactive in many *in vitro* models, and may be important in food and human nutrition.[2] 4-hexylresorcinol (HR, Fig. 1) is the most studied and well-known AR, which has an 80-year history of use and is reported to have anesthetic, antiseptic and anthelminitic properties.[3] It can be used topically on small skin infections, or as an ingredient in throat lozenges.[4] HR is an antiseptic that also has local anesthetic effects. Its antiseptic action kills the bacteria that may be associated with sore throats, while its anesthetic action helps relieve the pain. The action of sucking the lozenge allows the active ingredient to work in the area of the discomfort, and also helps to coat, lubricate and soothe a sore throat.

HR is reported to be an excellent inhibitor of polyphenol oxidase (PPO) which is responsible for enzymatic browning of fruits and vegetables. PPO (a mixture of monophenol oxidase and catechol oxidase enzymes) is present in nearly all plant tissues and can also be found in bacteria, animals and fungi.[5] In fact, browning by PPO is not always an undesirable reaction; the familiar brown colour of tea, coffee and cocoa is developed by PPO enzymatic browning during product processing. When used in combination with ascorbic acid, HR has been shown to be a very effective inhibitor of surface browning on many fresh-cut fruits, including apples and pears.[6,7] HR has a synergistic effect with ascorbic acid in the prevention of browning.[8,9] Ascorbic acid reduces quinones generated by polyphenoloxidase while HR specifically interacts with polyphenol oxidase, and renders it incapable of catalysing the enzymatic reaction. A post-cutting dip of HR, ascorbic acid and calcium lactate extended the shelf-life of pear slices from 15 to 30 days.[10] Red delicious apple slices treated with a combined anti-browning dip (HR, isoascorbic acid, N-acetyl cysteine and calcium propionate) and held at 5˚C maintained visual quality for five weeks.[11] HR is also reported to inhibit melanosis (black spots) in shrimp.[12] HR has a GRAS (Generally Recognized as Safe) status and is considered to be safe and effective in use as an anti-browning agent.[13]

DNA protection

Recently, the protective effects of resveratrol and HR against oxidative DNA damage in human lymphocytes induced by hydrogen peroxide were investigated.[14] The inhibition of oxidative damage in human lymphocytes by resveratrol and HR is shown to be due to the increase in the glutathione levels and the antioxidant enzymes – glutathione reductase and glutathione peroxidase.[14] Davydova *et al*[15] have shown long-term preservation of DNA in aqueous solution in the presence of methyl- and hexyl-resorcinols. The initial property of DNA is preserved to the greatest extent in the presence of HR. In another publication, Davydova *et al*[16] have shown that irradiating DNA with UV light in the presence of methyl- and hexyl-resorcinols results in comparatively insignificant DNA destruction as evidenced by the electrophoretic mobility pattern in agarose gel. Protective effect on DNA is more predominant with HR than with methylresorcinol.

Protein protection

HR has recently been reported to have an inhibitory effect against the formation of Maillard reaction product (*in vitro* using glucose and cysteine – anti-glycation effect).[17] Glycation is the result of a sugar molecule, such as fructose or glucose, bonding to a protein or lipid molecule without any enzyme. A glycation end product interferes with molecular and cellular functioning throughout the body. Damage by glycation results in stiffening of the collagen; collagen constitutes ~70% of skin proteins. These studies suggested that 4-hexylresorcinol might act as potential cancer chemo preventive agents in humans.

Although HR has shown many physiological properties, still very little is known about their biological effects on modulating the melanogenesis pathway. Recent publications have reported 4-alkylresorcinols to have excellent tyrosinase inhibitory activity.[18,19] Thus, the goal of the present work is three-fold:

- To investigate the effectiveness of a highly pure HR[20] (with 99% minimum purity having a residual resorcinol content of <0.1%) as a melanin inhibitor (*in vitro* and *in vivo*).
- To define its mode of melanin inhibitory activity.
- To show its applicability as a cosmetic ingredient to lighten and even-tone normal and hyperpigmented skin colour.

Materials

Skin lightening agents, enzymes and reagents were purchased from commercial sources. Skin lightening agents included are hexylresorcinol, abbreviated as HR (Synovea HR, Sytheon), hydroquinone (Eastman), glycyrrhiza glabra (licorice) root extract (Barnet Products) and kojic acid (Sigma-Aldrich).

Enzymes, cells and reagents used for the study were mushroom tyrosinase (Sigma-Aldrich), horseradish peroxidase (Sigma-Aldrich), B16 melanocyte cells (ATCC Catalog #CRL-6323), buffer solution prepared by combining KH_2PO_4 (VWR) (1362 mg) dissolved in distilled water (100 mL) and pH adjusted to 7.4 with a 2 M KOH solution (Sigma-Aldrich), L-DOPA (Sigma-Aldrich), L-tyrosine (Sigma-Aldrich), and hydrogen peroxide (Sigma-Aldrich).

Methods

***In vitro* melanin inhibitory activity:**
B16 melanocyte cells were cultured in DMEM supplemented with 10% cosmic calf serum. Cells were incubated with the test material for 72 hours. Afterwards, cultures were photographed. Cells were then counted and extracellular and intracellular melanin quantified by measuring absorbance at 490 nm, then standardised by cell number following the method described by Ando *et al*.[21]

Table 1a: L-DOPA substrate

Preparation	L-DOPA solution	Hydrogen peroxide solution	Buffer stock solution	Tyrosinase solution	Ingredient X solution	Buffer solution
A	0.5 mL	0.5 mL	1.0 mL	0.5 mL	0 mL	0.5 mL
B	0.5 mL	0.5 mL	1.0 mL	0.5 mL	0.1 mL	0.4 mL
C	0.5 mL	0.5 mL	1.0 mL	0.5 mL	0.2 mL	0.3 mL
D	0.5 mL	0.5 mL	1.0 mL	0.5 mL	0.3 mL	0.2 mL
E	0.5 mL	0.5 mL	1.0 mL	0.5 mL	0.4 mL	0.1 mL

Table 1b: L-tyrosine substrate

Preparation	L-tyrosine solution	Hydrogen peroxide solution	Buffer stock solution	Tyrosinase solution	Ingredient X solution	Buffer solution
A	1.0 mL	0.5 mL	0.5 mL	0.5 mL	0 mL	0.5 mL
B	1.0 mL	0.5 mL	0.5 mL	0.5 mL	0.1 mL	0.4 mL
C	1.0 mL	0.5 mL	0.5 mL	0.5 mL	0.2 mL	0.3 mL
D	1.0 mL	0.5 mL	0.5 mL	0.5 mL	0.3 mL	0.2 mL
E	1.0 mL	0.5 mL	0.5 mL	0.5 mL	0.4 mL	0.1 mL

Table 1c: L-DOPA substrate

Preparation	L-DOPA solution	Hydrogen peroxide solution	Buffer stock solution	Peroxidase solution	Ingredient X solution	Buffer solution
A	0.5 mL	0.5 mL	1.0 mL	0.5 mL	0 mL	0.5 mL
B	0.5 mL	0.5 mL	1.0 mL	0.5 mL	0.1 mL	0.4 mL
C	0.5 mL	0.5 mL	1.0 mL	0.5 mL	0.2 mL	0.3 mL
D	0.5 mL	0.5 mL	1.0 mL	0.5 mL	0.3 mL	0.2 mL
E	0.5 mL	0.5 mL	1.0 mL	0.5 mL	0.4 mL	0.1 mL

Enzyme inhibitory activity:
Preparation of stock solutions:

1. Buffer solution
   KH2PO4 99.8% & KOH 85+%
   (pH 7.4, 100 mM)
   Mol. Wt. 136.09,
   KH_2PO_4 (1362 mg) dissolved in distilled water (100 mL) and pH adjusted to 7.4 with a 2 M KOH solution
2. Mushroom tyrosinase solution
   (240 units per 1 mL)
   2440 units/mg
   Tyrosinase (2.5 mg) dissolved in 25 mL of distilled water
3. Horseradish peroxidase solution
   (124 units per 1 mL)
   124 units/mg
   Peroxidase (10 mg) dissolved in 10 mL of distilled water
4. L-DOPA solution (6.3 mM)
   Mol. Wt. 197
   33.1 mg dissolved in distilled water (25 mL)
5. L-tyrosine solution (1.3 mM)
   Mol. Wt. 181.2
   23.5 mg dissolved in distilled water (100 mL)
6. Hydrogen peroxide solution (5 mM)
   1/100 of 0.9/25 vol. dilution of a 50% wt. solution

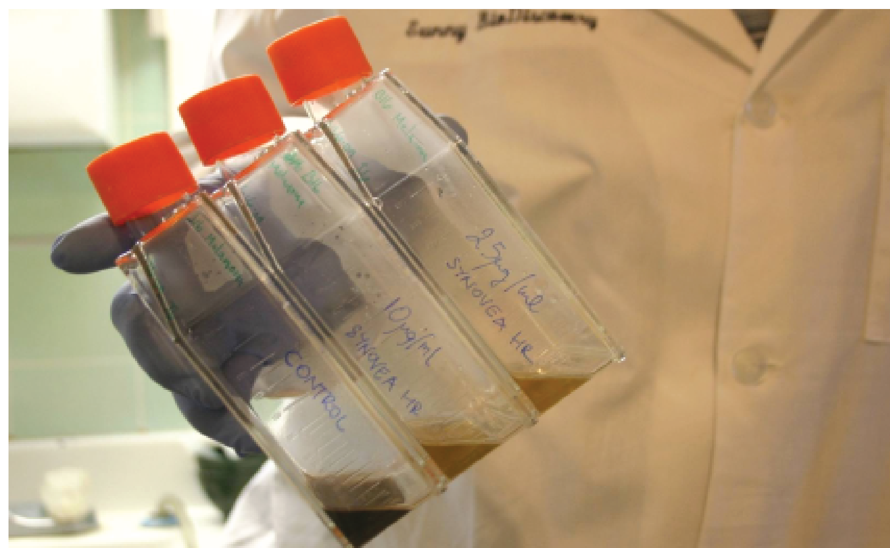

Figure 2: *Effect in the extracellular melanin production: control vs. heylresorcinol at 10 and 25 µg/mL.*

Procedure

The stock solutions were added in an orderly manner with quantities according to Table 1 to obtain a final total volume of 3.0 mL for each preparation. First, the substrate (L-DOPA or L-tyrosine) was mixed with the hydrogen peroxide and the buffer solutions. Proportions are different due to solubility reasons and details are given in Tables 1a and 1b. This mixture was incubated for 10 minutes at 25˚C. Then, 2 mL of this mixture was added to 1 mL of mixture solution containing tyrosinase or peroxidase, ingredient X and buffer solution according to Table 1. After 15 minutes of reaction time, the spectra were recorded between 300 nm and 700 nm and the optical density at 475 nm (Dopachrome) measured.

***In vivo* melanin inhibitory activity (human clinical study):**

A skin lightening composition was prepared having the formula set forth in Table 2. The final skin lightening composition with HR was found to have a pH value of 6.2 at 25˚C. A second skin lightening composition was prepared in the same manner with the same formulation except that the highly pure HR was replaced with 2% hydroquinone (Eastman) and water adjusted accordingly.

Each of the skin lightening compositions was applied to different test sites on the forearms of 15 individuals. The compositions were applied twice a day, morning and evening, for eight weeks. The test sites, as well as untreated sites on each forearm, were evaluated by a trained clinical evaluator for brightness of skin, evenness of skin tone, appearance of skin, dryness of skin and radiance of skin using a 10 point scale prior to the first application and following week four and week eight. Each test site was evaluated for skin colour change as represented by the change in L values and ITA˚ (Individual Topology Angle – COLIPA SPF test method) as measured by chromometric measurement. ITA˚ was calculated using the formula:

$ITA^{\circ} = [\text{Arc Tangent}(L^{*}-50)/b^{*})]180/3.1416$

Wherein, L^{*} value - lightness and b^{*} – colour in blue-yellow axis. The results of the testing were presented in Table 3, wherein the delta represents the percent change in skin colour from the baseline colouration of the untreated skin.

As seen from Table 3, even a 0.5% purified hexylresorcinol composition provided a comparable, statistically significant change in skin colour to that attained with like composition containing 2% hydroquinone: perhaps the most effective known skin whitening agent. Such results are consistent with a "lightening" of the skin. No adverse effects were noted for either composition over the test period.

Table 2: Skin lightening composition.

Ingredient	Trade Name/Supplier	Wt %
Phase A-1		
Water (demineralised)		74.70
Disodium EDTA		0.10
Glycerin		3.00
Phase A-2		
Acrylates/C10-30 Alkyl Acrylate Copolymer	Carbopol Ultrez 21/BF Goodrich	0.20
Phase B		
Caprylic/Capric Triglycerides	Myritol 318/Cognis	4.00
Cetearyl Isononaoate	Cetiol SN/Cognis	4.00
Glyceryl Stearate, PEG-100 Stearate	Arlacel 165/Uniquema	2.00
Sorbitan Stearate	Arlacel 60/Uniquema	0.50
Dimethicone	DC, 200/100CST	1.00
Gransil GCM	Cyclomethicone, Polysilicone-11, Petrolatum/Grant Industries	5.00
Cetyl Esters	Crodamol SS/Croda	1.00
Phase C		
Hydroxyethyl Acrylate/Sodium Acryloyldimethyl Taurate Copolymer/Squalane/Polysorbate 60	Simugel NS/Seppic	0.75
Phase D		
Ethoxydiglycol	Transcutol/Gattefosse	2.00
Hexylresorcinol	Synovea HR/Sytheon	0.50
Phase E		
AMP-95 (aminomethyl propanol)	to pH 6.20	0.25
Phase F		
Phenoxyethanol, Methylparaben, Propylparaben	Phenonip XB/Clariant	1.00

Table 3: Skin colour change.

Skin lightening active	Week	L value	% improvement based on L value	ITA˚	% improvement based on ITA˚
Hexylresorcinol – 0.5%	0	56.88		19.09	
	4	58.82	3.4	24.34	27.5 (*p*-value 0.027)
	8	59.54	4.7 (*p*-value 0.006)	26.26	37.6 (*p*-value 0.008)
Hydroquinone – 2%	0	57.10		20.72	
	4	58.95	3.2	24.67	27.7 (*p*-value 0.001)
	8	59.54	4.3 (*p*-value 0.003)	27.22	38.4 (*p*-value 0.001)

Safety

HR has a GRAS status[24] and has a long history of human use as a topical skin and mucosal disinfectant for treatment of superficial wounds, and as a component of soaps, hand washes and skin cleaners. HR is considered to be safe[22] and effective for use in throat lozenges and as an anti-browning agent.[13] HR is included in the pharmacopoeias of Australia, US, UK, Finland, Netherlands and Ireland for its use as an antiseptic. European Commission, Health & Consumer Protection Directorate-General, considers 4-hexylresorcinol as toxicologically acceptable for the prevention of melanosis in shrimps.[23]

In order to determine the safety profile of highly pure HR for topical use, Sytheon has carried out human repeat insult patch (HRIPT) and skin scarification tests. HRIPT (1%, 2% & 5% in corn oil, total subjects 240) demonstrates it to be a non-primary irritant and a non-primary sensitiser. Skin-irritating propensities using 0.1% and 0.5% level on scarified human skin also showed it to have a low irritating potential and the data compares well with saline control.

Results and discussion

Melanin inhibitory activity of hexylresorcinol

Visible changes in melanin content were observed in cell cultures incubated with HR (Fig 2). The growth rate of cultured B16 melanoma cells was not significantly

altered in the presence of HR during the 72 hour incubation period, indicating that the HR-induced regulatory effects on melanogenesis of melanoma cells occurred without affecting cell proliferation. At 10 µg/mL use level, HR had inhibitory effects on extracellular and intracellular melanin production by 75% and 36%, respectively, when compared with placebo (Fig. 3). Intercellular melanin production was found to be inhibited by HR by 36% at 10 µg/mL level.

Results for tyrosinase inhibitory activities are shown in Table 4 for L-tyrosine substrate and L-DOPA substrate. The results demonstrate that HR has an excellent inhibitory activity against tyrosinase using both substrates (DOPA and tyrosine). Licorice shows good inhibitory activity as well but it is a plant extract; hence consistency could be a problem. The intense yellow colour of licorice makes it difficult to make aesthetically pleasing formulations. Kojic acid is also effective, but is known to be muatagenic[24] and causes contact dermatitis and erythema on long-term use.[25] Hydroquinone showed good inhibitory activity when tyrosine was used as a substrate, but is less effective with DOPA. Hydroquinone suffers from its toxicity problems and its use is banned in many countries.[26]

Results for peroxidase inhibitory activities are shown in Table 5 using DOPA as a substrate. HR has an excellent inhibitory activity against peroxidase followed by kojic acid and hydroquinone, whereas licorice is least effective.

Hydrogen peroxide, a potent apoptosis-inducing agent, can freely cross biological membranes and it is believed to cause DNA strand breakage by generation of the hydroxyl radical close to DNA molecule, via the Fenton reaction.[27,28] HR was shown to inhibit DNA damage in human lymphocytes induced by H_2O_2.[14] Literature shows that fragments of nucleic acids can stimulate melanin synthesis.[29] This indicates yet another mechanism by which HR reduces melanin synthesis by protecting against DNA damage.

It is known that glutathione dose dependently inhibits melanin synthesis in the reaction of tyrosinase and L-DOPA.[30] The inhibition of melanin synthesis is shown to be recovered by increasing the concentration of L-DOPA, but not recovered by increasing tyrosinase. Glutathione inhibited the binding between tyrosinase and L-DOPA. Although the synthesised melanin was aggregated within one hour, the aggregation was inhibited by the addition of glutathione. These results indicate that glutathione inhibits the synthesis and agglutination of melanin by interrupting the function of L-DOPA. Since HR induced an increase in glutathione level in a concentration-dependent manner as compared to the control group,[14] it is yet another mechanism by which HR interrupts melanin synthesis. Based on our work and literature information, melanin inhibitory activity of HR on various points in the melanogenesis pathway is summarised in Figure 4.

Table 4: Tyrosinase inhibitory activities.

IC_{50} concentrations for reactions of tyrosinase inhibition of skin lightening ingredients

Skin lightening ingredients	IC_{50} (L-tyrosine) (µg/mL)	IC_{50} (L-DOPA) (µg/mL)
Hexylresorcinol (HR)	1	25
Licorice extract	2	30
Kojic acid	8	35
Hydroquinone	10	230

Table 5: Peroxidase inhibitory activities.

IC_{50} concentrations for reactions of peroxidase inhibition of skin lightening ingredients

Skin lightening ingredients	IC_{50} (L-DOPA) (µg/mL)
Hexylresorcinol (HR)	130
Licorice extract	500
Kojic acid	230
Hydroquinone	225

Skin protective property of hexylresorcinol

Melanin synthesis is an oxygen-dependent process that acts as a potential source of reactive oxygen species (ROS) inside pigment-forming cells. Glutathione not only inhibits melanin synthesis, but also protects cells from oxidative injury due to its antioxidant property.[31] Glutathione reduces any disulfide bond formed within cytoplasmic proteins to cysteines by acting as an electron donor. In the process, glutathione is converted to its oxidised form, glutathione disulfide (GSSG). Glutathione reductase is an enzyme that reduces glutathione disulfide (GSSG) back to glutathione. Yen *et al*[14] has also reported stimulation of glutathione reductase and glutathione peroxidase[32] by HR. The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. Glutathione has multiple functions.[33,34] Larsson *et al* has reported recently that UVA irradiation induces immediate loss of reduced glutathione in both melanocytes and keratinocytes, whereas UVB irradiation of keratinocytes caused instant reduction of reduced GSH and impaired plasma membrane stability.[35] This result clearly indicates inclusion of glutathione stimulatory substances, like HR, in sunscreen products.

Following information is obtained from www.wikipedia.org:

Glutathione is the major endogenous antioxidant produced by the cells, participating directly in the neutralisation of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms.

Through direct conjugation, Glutathione detoxifies many xenobiotics (foreign compounds) and carcinogens, both organic and inorganic.

Glutathione is essential for the immune system to exert its full potential, for example:

1. *Modulating antigen presentation to lymphocytes, thereby influencing*

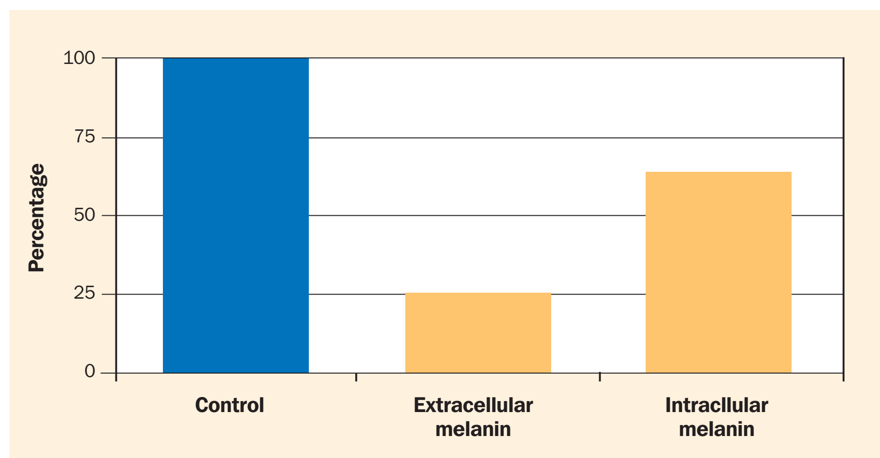

Figure 3: *Effects of hexylresorcinol in the extracellular and intracellular melanin production at 10 µg/mL.*

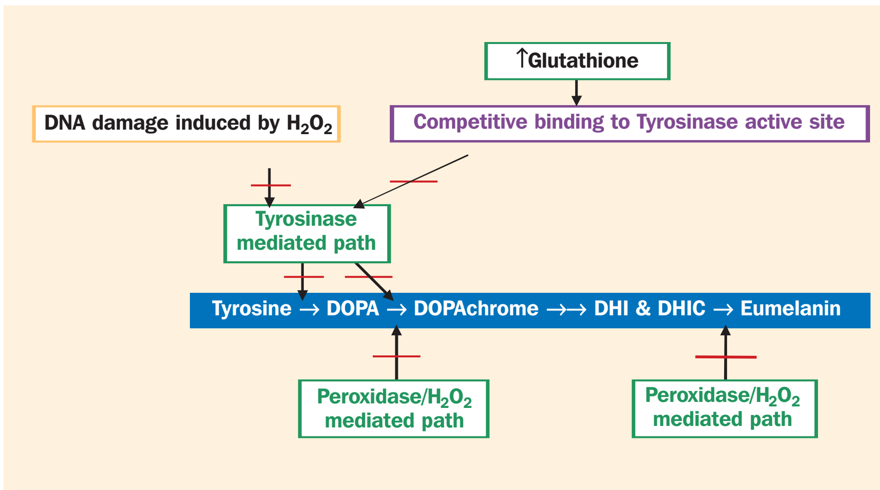

Figure 4: *Various points in the melanogenesis pathway inhibited by hexylresorcinol.*

cytokine production and type of response (cellular or humoral) that develops.

2. *Enhancing proliferation of lymphocytes thereby increasing magnitude of response.*
3. *Enhancing killing activity of cytotoxic T cells and NK cells.*
4. *Regulating apoptosis, thereby maintaining control of the immune response.*

Glutathione plays a fundamental role in numerous metabolic and biochemical reactions such as DNA synthesis and repair, protein synthesis, prostaglandin synthesis, amino acid transport and enzyme activation. Thus, every system in the body can be affected by the state of the glutathione system.

HR has been reported to have an inhibitory effect against the formation of Maillard reaction product (*in vitro* using glucose and cysteine) – anti-glycation effect.[17] Glycation is the result of a sugar molecule, such as fructose or glucose, bonding to a protein or lipid molecule without any enzyme. Glycation leads to advanced glycation end products which interfere with molecular and cellular functioning throughout the body. This causes the release of highly-oxidising side products such as hydrogen peroxide. Damage by glycation causes stiffening of the collagen.

Conclusion

Melanogenesis is the process of production and subsequent distribution of melanin by melanocytes and is controlled by many factors. Highly purified hexylresorcinol has been shown to be a safe and effective skin lightener as demonstrated by *in vitro* and human clinical studies. The skin lightening effect is due to its inhibitory activity against tyrosinase and peroxidase/H_2O_2 enzymes as well as its stimulatory effect on glutathione synthesis. Observing that fragments of nucleic acids can stimulate melanin synthesis; it seems that the reduction of DNA damage by HR is also responsible for inhibition of melanin synthesis.

HR is shown to have a protective effect against oxidative DNA damage in human lymphocytes induced by hydrogen peroxide. Among a wide variety of reactive oxygen species (ROS), H_2O_2 plays a pivotal role because it is generated from nearly all sources of oxidative stress and oxygen radicals and can diffuse freely in and out of cells and tissues. Both non-mitochondrial- and mitochondrial-derived $O2^{+\cdot}$, and H_2O_2 are toxic to cells by direct attack at the molecular level or indirectly by generating secondary reactive species such as hydroxyl radical in presence of Fe^{2+}. The protective effect of HR is attributed to its stimulatory effects on glutathione production, and modulation of antioxidant defense enzymes – glutathione peroxidase and glutathione reductase. Also, HR has an inhibitory activity against the formation of Maillard reaction product (anti-glycation effect). The skin lightening effect and skin protection benefits of HR make it an ideal choice for a variety of skin care products targeting young and matured skin alike. PC

● *A part of this work was presented at the Society of Cosmetic Chemists of South Africa annual meeting held in Midrand, September 2007.*

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