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Original Research Article

Auto-defense of skin and melanin regulation through glutathione reductase modulation: a new insight

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ABSTRACT

Background: The present study is to understand the Siddha cosmetic preparation - Eve fresh skinbrite cream/herbal extracts used in the cream in down-regulating the melanogenesis process and upregulating glutathione activity to offer skin lightening benefit.
Methods: The effect of certain herbal extracts or the combination in reducing tyrosinase activity and associated melanin formation was evaluated in B16F10 melanoma cells and also up regulating glutathione reductase activity leading to the conversion of a reduced form of glutathione (GSH) from GSSG.
Results: The extract of *Berberis aristata* exhibited even activity in inhibiting both tyrosinase and melanin in B16F10 melanoma cell lines. The extract combination also showed a greater effect on increasing GR activity.
Conclusions: The herbal ingredients individually and as a combination exhibited strong effects in reducing tyrosinase activity and also upregulating GR activity and thereby reducing skin pigmentation.

Keywords: Evefresh skinbrite, Glutathione reductase, GSSG, Tyrosinase inhibition, *Beriberis aristata*

INTRODUCTION

Skin is not just the biggest organ in human body from the point of view of its total size but is also the important organ to offer protection to the entire system.[1] With reference to its ability to regenerate and offering foundational protection to the total body by dealing several challenges of the environment which are either static or are versatile and dynamic, skin plays a big role. Therefore, any unusual or noticeable change in the skin, hair or nail must be considered as 'the earliest indicator/warning' of a systemic change, that can be disease related or age related or physiology or psychology related.[2]

A transient skin colour modification towards increased light absorption with the onset of photoperiodic shift in the environment where sun overhead tropic of cancer or the equator, is quite natural but if such modified colour effect of skin persist, such an effect also may indicate the possible photo-vulnerability of the skin and so are the complications associated with the above change, example – skin cancer.[3]

Therefore, modified skin colour change towards increased light absorption should not be seen and treated just as a cosmetic problem but must be seen essentially in the larger medical complications that may be in-waiting, silently.[4]

The most abundant bio-chemical molecule in the body to execute the cellular and nuclear defence is glutathione (GSH). The abundance of glutathione can be equated to the level of abundance of glucose or cholesterol in our body. The cellular energy demand is constantly met by the mitochondria present in the cell which regularly synthesise and supply adenosine triphosphate (ATP). Mitochondria also has its own deoxyribonucleic acid (DNA) so as to be

totally and completely empowered and self-reliant. The glutathione is the bio-chemical entity that provide armour of protection and security to mitochondria from wide range of oxidative damaging substances.[5]

Effort to treat or modify the pigmentary changes of the skin obviously due to increased level of pigment formation by focusing melanogenesis as the sole target although may offer the desired benefit but at the same time also may make the skin extremely vulnerable to the signal that may cascaded the above changes initially, especially the same level of exposure is if unavoidable. The remedial measure must include impairing pigment formation and simultaneously cellular and nuclear protection. The second demand of the skin, the most critical and essential is often neglected, overlooked or is understood poorly.

The innate defence mediation offered by glutathione at sub-cellular level provide an easy scope to us to enhance the role of the same through a formulation that is aimed for skin lightening effect. Due to the abundance of glutathione in the body, it is always assumed that it would perform its role without any trigger or elicitation. The subsequent skin research has taught us, mere abundance of glutathione cannot be assumed to offer great cellular and nuclear defence as the abundant glutathione that we notice is always in the ground state and not at the functional state. The ground state of glutathione is the oxidized form which is expressed as GSSG and the functional form of the same is reduced glutathione, GSH. Glutathione is tripeptide composed of three amino acids such as cysteine, glycine and glutamic acid.

The Cinderella that maintains the ratio of GSH/GSSG is glutathione reductase and therefore enhancing the activity of the above enzyme is essential to lower the load of GSSG and higher the load of GSH so that the protection is offered while the same remedial measure is tailored to down regulate melanogenesis.[6]

The present study reports the effect of a Siddha cosmetic preparation Evefresh skinbrite cream in upregulating glutathione activity while exerting suppressing action on tyrosinase enzyme and melanin synthesis. Details are presented in the article.

METHODS

Description of Evefresh skinbrite cream

Evefresh skinbrite cream is composed of the following herbal extracts such as *Curcuma zedoaria, Berberis aristata, Glycyrrhiza glabra, Curcuma zedoaria*, aloe vera in a cream base that also contains avacado oil and vitamin E acetate.

Culture of B16F10 murine melanoma cells

Eagles minimal essential medium supplemented with 10% heat inactivated fetal bovine serum and 2 mM L-glutamine at 37ºC in a humidified atmosphere containing 5% CO2 was used for the above purpose. Different concentrations extract combination and individual extracts- 10, 20, 30 µg/ml were added to the culture after the cells being seeded and incubated for 48 hrs under cell culture condition.[7]

Tyrosinase assay

Tyrosinase activity was performed through DOPA oxidase activity. Approximately 10^7 cells were pelleted and then washed twice with phosphate buffered saline. After centrifugation, the supernatant was decanted. The cell pellet was dissolved in 1.0 ml of 0.5% sodium deoxycholate in distilled water and allowed to stand at 0°C for 15 minutes. Tyrosinase activity was assayed spectrophotometrically by following the oxidation of DOPA to dopachrome at 475 nm. The reaction mixture consisting of 3 ml of 0.1% L-DOPA in 0.1 M phosphate buffer, pH6.8 was mixed with cell lysate. Assay was performed at 37°C in a spectrophotometer.[8]

Melanin measurement

Melanin content was measured by using standard procedure. Approximately 10^7 cells were pelleted by centrifugation at 1000 g for 5 minutes and then washed twice with phosphate buffered saline. After further centrifugation, the supernatant was decanted, the precipitated cells were re-suspended in 200 µl of distilled water, and 1 ml of ethanol-ether 1:1 was added to remove opaque substances other than melanin. The mixture was stored and suspended at room temperature for 15 minutes. After further centrifugation at 3000 g for 5 minutes, the precipitate was solubilized by treatment with 1 ml 1N NaOH/10% dimethyl sulfoxide at 80°C for 30 minutes in a capped tube. The absorbance was measured at 400 nm and the melanin content per cell was calculated and expressed as percentage of control (=100%).[9]

Cell culture assay using HepG2 cells

The effect of the extract combination and the individual extracts of each herb were assessed for glutathione reductase (GR) up or down regulation using HepG2 cells.[10]

The cells were grown in Roswell Park Memorial Institute medium (RPMI 1640) that contained glucose (2 g/l), supplemented with 10% foetal calf serum, 10000 U/ml penicillin, 10 mg/ml streptomycin, 1% glutamine, 1% Hepes buffer solution, pH 7.4, and maintained in a humidified atmosphere of 95% O_2: 5% CO_2, at 37°C.

The cells when attained 80% confluence, cells were trypsinized, centrifuged (1700 rpm for 5 minutes at room temperature), resuspended in fresh medium, and plated in microtiter wells (2×104 cells/well) or in six-well dishes (106 cells/well). After attachment, they were incubated in serum-free medium, to which 10, 20, 30 µg/ml of the extract combination or the in extracts were added and kept for further 24 hours at 37°C, then the tested for GR.

Glutathione reductase assay

Glutathione reductase (GR), catalyzes the reduction of GSSG, the oxidised form of glutathione, to GSH, which is nothing but reduced form of glutathione. The reduced form of glutathione is essential for the glutathione redox cycle in order to maintain adequate levels of reduced cellular GSH. During the reduction of GSSG by glutathione reductase, it is estimated that one molecule of NADPH is consumed for each molecule of GSSG reduced. Therefore, the reduction of GSSG by GR can be determined by the measurement of the consumption of NADPH.[11] In brief, the GR assay was done in a cuvette with 1 M tris-HCl buffer + 5 mM EDTA (pH 8.0), 0.033 M GSSG, 2 mM NADPH, and varying concentrations of the test sample adjusted to final volume of test mixture to 1.0 ml. The decrease in absorbance, which reflects the oxidation of NADPH during reduction of GSSG by GR was read spectrophotometrically at 340 nm. Results were expressed as units of GR activity/mg cell protein. The experiment was repeated 5 times to confirm the result.

Assay of photo-protection

The effect of extract combination and individual extracts in offering UV protection to 3T3- fibroblast cell line in the presence and absence of GSSG+GR was studied. UVB irradiation was given with an intensity of 2 and 5 mJ/cm^2 to the cells in a microplate. 30 µg/ml either the extract combination or the individual extracts was used for the study. In the case of GSSG + GR or GSSG alone or GSH, we have used 10µg of each for the study. Post UV exposure the cells were trypsonized, washed and then studied by MTT to determine the percentage of cell death. The formazan formed was measured by spectrophotometer.[12]

RESULTS

Tyrosinase and melanin inhibition

The extract of *Berberis aristata* exhibited even activity in inhibiting both tyrosinase and melanin in B16F10 melanoma cell lines which was comparable with extract combination (Table 1).

GR assay

The extract combination showed greater effect in increasing GR activity than any of the individual herb (Table 2).

Table 1: Tyrosinase and melanin inhibition.

Sample	% inhibition/concentration of sample in µg/ml					
	Melanin			Tyrosinase		
	10	20	30	10	20	30
Berberis aristata	32	55	72	28	48	62
Glycyrrhiza glabra	24	43	51	12	22	34
Curcuma zeodaria	22	30	35	20	22	25
Aloe vera	10	17	21	9	11	12
Extract combination	45	67	88	22	48	79

Table 2: GR assay.

S. no.	Sample details	Activity of GR (m U/mg protein) and µg of herb treatment		
		10	20	30
1	*Berberis aristata*	18	25	34
2	*Glycyrrhiza glabra*	20	32	35
3	*Curcuma zeodaria*	15	21	29
4	*Aloe vera*	11	12	10
5	Extract combination	35	42	48
6	Untreated control	10		

Assay of photo-protection

The extract of *Berberis aristata* exhibited reasonable level of UV protection to the cells however when the extract combination combined to GSSG+GR showed greater UV protection over GSSG + GR clearly suggest the effect of extract combination in increasing GSH which in turn results in greater UV protection (Table 3).

Table 3: Assay of photo-protection.

Sample (30 µg/ml)	UV treatment/time/% of cell death	
	2 mJ/cm^2 /5 min	5 mJ/cm^2 /5 min
Berberis aristata	32	44
Glycyrrhiza glabra	51	72
Curcuma zeodaria	80	86
Aloe vera	72	81
Extract combination	60	72
GSSG	52	56
GSH	48	51
GSSG+GR	34	42
GSSG + GR + extract combination	12	15

DISCUSSION

Pigmentary changes in the skin is not just a cosmetic issue but may indicate the increasing photo-vulnerability of the skin due to various underlying immunological/ pathological factors besides indiscriminate exposure to sun and skin aging.[13] In the beginning such photo-change that occur in the skin may offer protection to the nucleus in the skin cells from UVA and UVB but such protection is only transient if the underlying condition prevails. More often, without much understanding of the underlying conditions that trigger such pigmentary change of the skin, remedial approach is sought to downregulate the skin pigment to make the skin look/appear normal. Such an approach would produce harmful effect in the long run which is seldom given any due importance or significance.

The skin lightening preparations and preparations to deal hyper-pigmentary changes of the skin are largely studied and tested for their action on the key rate-limiting enzyme in the gamut of skin pigmentation process – melanogenesis, called tyrosinase and its effect on melanocytes with respect to inhibiting the biochemical reaction of melanin synthesis. Melanin offer protection to the cell in toto and to the nucleus in particular. Besides melanin, yet another bio-chemical molecule that offer protection to the genetic material of the cell and even to the mitochondrial DNA is the reduced form of glutathione which is often expressed as GSH. GSH is the produced from the oxidized form of glutathione – GSSG though the action of an enzyme called GR.

Like cholesterol and glucose, GSSG is abundant in our body but the conversion of GSH from GSSG does not occur freely and therefore the DNA and mtDNA protection cannot be assumed automatically due to such an abundance of GSSG.[14] If the skin lightening/managing hyper pigmentation formulation have a positive effect on GR and thereby could increase the conversion of the reduced form of glutathione from the ground form which is the oxidized form will have huge positive effect to the skin besides such formulation offering the much needed benefit of reduced skin pigmentation.

But so far, not much attempt has been made to develop a formulation that would reduce tyrosinase enzyme, reduce melanogenesis and simultaneously also would positively affect GR and GSH formation.

In the present study we have tested the effect of certain herbal extracts and their combo in reducing the activity of tyrosinase enzyme, melanogensis using B16F10 melanoma cells along with positive effect on GR activity and the final effect of the possible conversion of GSH in providing photoprotection to the cells under in vitro condition.

The herbal ingredients individually and as combo exhibited strong effect in reducing tyrosinase activity and the resultant melanin formation. When the above experiment was repeated with the presence of GSSG + GR and GSH, we found that the extracts and the combo, GSSG + GR + treatment could offer protection to the cells from UVB possibly due to GSH conversion as the herbal extracts or the combo when tested alone did not exhibit such UVB protection effect.

Interestingly the UVB exposed cells to the extracts or the combo did not show higher production of melanin as the results were comparable with the treated cells without UVB exposure. The above possibility could be due to the monolayer of cells that we used or could be due to the effect of herbal extracts or combo on melanogenesis. The above findings suggest that the extracts and the extract combo could exhibit activity to downregulate the pigmentary changes which is observed to be independent and stable from the effect of the extracts and the combo in upregulating GR activity and possible formation of GSH. The benefit plurality of herbal extracts is well known due to the presence of many phyto-constituents present in the herbal extracts. Several di-similar pharmacological activities have been reported from same herbal extract by several previous workers.

In the present study we did observe not just an allied benefit but much needed and less studied/reported benefit from herbal extracts that are vastly used in skin lightening preparations. Skin lightening benefit is largely a cosmetic need whereas the DNA protection is not just medical but a genetic need. Always when we go after the former benefit in excess, the consequence due to the above is seldom weighed or understood until the consequence warrants further treatment intervention.

From outside of the scope of skin lightening benefit, the findings of the present study also strongly indicate the wide spectrum of benefit of the herbal extracts or the combo to the skin by increasing the availability of GSH. GSH is a very powerful antioxidant that protect the skin in general besides offering specific protection to the genetic material. Therefore, the above benefit would make the skin appear youthful, radiant, blemish free and wrinkle free, the desire and dream of everyone. Herbal extracts therefore can also offer many other benefits to the skin besides their usefulness skin lightening benefit.

We have not studied the extent of GR activation and the extent of GSH formation. Further, we have also not studied the extent of DNA protection rather than total cell protection. Although the above limitations we admit in the study, but such limitations no way impedes the scientific merit and larger interpretation of findings where the extracts and the combo having strong effect in reducing the skin pigmentation and increasing GSH availability and associated photo-protection to the genetic component of the cells. The allied and much needed benefit of the extracts and the combo reported by us may begin a new chapter in the science of skin lightening formulations and their expanding market.

CONCLUSION

Evefresh skinbrite cream/extract combination is useful in treating hyperpigmentation, it's established by inhibition/ down regulating the tyrosinase enzyme, melanin synthesis and also the extract combination increases the GR activity and thereby offers protection to the cells.

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