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Assessment of Anti-aging Potential of Nanostructured lipid carriers (NLCs) gel of *Myrica esculenta* & Coenzyme Q_{10} using UVA induced photo-toxicity model.

Prashant Kumar, and Anita Singh*

Department of Pharmaceutical Sciences, Kumaun University Bhimtal, Uttarkhand, India-263136

ABSTRACT

In this study, *Myrica esculenta* extract along with Coenzyme Q10 was entrapped in nano-structured lipid carriers (NLCs), incorporated into gel base to obtain ME nano-cosmeceutical gel and tested for anti-aging potential. Furthermore the methanolic extract (ME) with high antioxidant activity and Coenzyme Q10 were selected for loading in the stable NLCs. Then their characterization, stability study and *in-vivo* photo-protective study was carried out followed by the determination for skin irritation using albino wistar rat. The results demonstrated that the Coenzyme Q10 ME-NLCs showed good physical stability at 28 days after preparation. The optimized formulation F5 (G0) of Coenzyme Q10 ME-NLCs containing gel exhibited best release with no skin irritation in albino wistar rat. The antiaging parameters evaluated by UVA- UVB induced photo-protective potential and were significantly ($p<0.05$) with statistically optimized best formulation F5 (G0) Q10-ME NLC gel. The developed nano-cosmeceutical topical gel of Q10 ME extract from this study could be regarded as the effective anti-aging formulation.

Keywords: Nanostructured lipid carriers, ME extracts, Antioxidant activity, Anti-aging cosmetics.

Corresponding author

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INTRODUCTION

In modern age there is strong tendency in society to attain eternal youth with elegant facial manifestation as it is considered as the significant marker to the social relations. This tendency has developed a greater demand of cosmetic products in the cosmeceutical industry [1].

Skin aging is a gradual deterioration of physiological functions of skin leading to unappealing appearance. Skin aging is described using numerous theories out of which free radical theory based on oxidative stress is most exposed one [2].

This oxidative stress is further explained with the activity of reactive oxygen species (ROS. UV induces reactive oxygen species (ROS), such as superoxide anion (O_2-•), hydroxyl (•OH), peroxyl (RO_2•) and alkoxyl (RO•) attack our cell membranes which can damage DNA succeeding the disturbance in number of transcription factors inside nucleus. Furthermore it leads to decreased production and extensive destruction of collagen. AP, Activator protein; MMP, matrix metalloproteinase; NF, nuclear factor; TGF-B, transforming growth factor-b, the overall result is slow down of cellular functions as well as reducing the body's self-repair capabilities remarked by sagging and age spots [3,4].

There are strong evidence that plant extracts have been widely used as ingredients of topical agents for wound healing and antiagin [5-6]. Plant extracts containing polyphenols as tannins, galloylglucoses, flavonoids, phenolic acids. flavonoids and carotenoids counter these reactive oxygen species (ROS) to neutralize harmful effects of free radicals [7].

Further more specifically polyphenols has proven huge potential of protecting the skin against age-related diseases and ultraviolet-mediated oxidative damage and reinforce the endogenous protection system by topical application [8]. In skin, endogenous Q10 levels gradually decline with growing age [9]. Additionally, UV-irradiation, which leads to oxidative damage, drastically reduce skin's Q10 levels.

Moreover the topical bioavailability of CoQ10 is poor due to its lipophilic nature, as composed of 10 isoprene units in its chemical structure; hence the development of a practical topical formulation is highly desirable [10]. However, it is very difficult to develop a formulation that helps CoQ10 delivery to deeper layers of the skin because of its low aqueous solubility (log P >10) as well as the barrier function of stratum corneum [11].

To solve the above problem lipid based drug delivery systems are nowadays popular carriers due to their potential to increase solubility and improve bioavailability of poorly water soluble and/or lipophilic drugs [12]. Previously advanced generation, SLNs dragged a lot of interest as a drug delivery system as they offer the advantages of biocompatibility, drug targeting, modified release and ease of large scale production [13]. However, depending on the drug candidate, there are so many associated potential problems, such as drug leaking during storage and insufficient drug loading. Henceforth NLCs were introduced to overcome the general limitations associated with conventional lipid based formulations and solid lipid nanoparticles (SLNs) [14-15].

The major advantage of NLCs as carriers/ delivery systems is its ability to incorporate large quantities of drugs due to formation of a less ordered lipid matrix with many imperfections offering a close contact to the stratum corneum with unique lipid composition and smaller particle size, thus enhancing drug flux through the skin [16].

There are strong evidences that CoQ10 have been proved as nanostructure of lipid carriers (NLC-CoQ10) offering huge antioxidant potential through the skin. Further Co Q10-NLC, mainly lipophilic in structure, show a high solubility of substances, high stability, good penetration through the SC and low down skin irritation [17-18].

At present, there is a huge trend in the cosmeticeutical industry to develop multifunctional cosmetics with high antioxidant activity with high efficacy & greater safety concerns [19- 20].

In the present study researchers have made an attempt to enhance the antiaging potential of Coenzyme Q10 using the polyphenolic plant extract to achieve the higher efficacy and greater safety as desired by cosmeceutical industry as the need of time.

MATERIALS AND METHODS

Coenzyme Q10 was purchased from Sigma-Aldrich Chemicals, St. Louis, MO.

Precirol ATO 5 and Poloxamer 407 were provided as gift samples from Gattefossé Saint Priest, France & BASF Corporation, USA respectively. All other chemicals and reagents used were of analytical grade and procured from SD Fine Chem Limited, Mumbai.

The plant specimen, i.e. dried leaves of *M. esculenta* were collected from northern himalayan region of Garhwal Uttarakhand, India in the month of March 2015 from the height of 1685.53 Meters N 29.8688° N, 78.8383° E.. The plants were identified and authenticated in BSI, Allahabad, India. A voucher specimen has been submitted in the Institute's herbarium. Extraction and antioxidant activity was determined as per the DPPH assay method. This is published in our previous paper. [21]

These results encouraged us to develop a NLC based topical drug delivery system which could be utilized for antiaging potential. To develop and optimize the formulation 2^3 factorial design was utilized. NLCs were prepared through solvent injection technique [22].

In our previous study the standardization of plant extract was carried out through HPLC method followed by quantification of gallic acid in *M. esculenta* extract using RP-HPLC. Optimized formulations were further subjected for development of NLC gels followed by assessment of antiaging potential as well as skin irritation study. In the present work the researchers have mainly focussed on UVA induced photo protective potential as well as skin irritation study of the finally prepared NLC gel formulations.

Selection of NLC for hydrogel

The criterion for the selection of NLCs for the preparation of the hydrogel was based on the particle size and % entrapment efficiency suitable for the administration by the topical route.

Preparation of the gels

Coenzyme Q10 ME-NLC loaded hydrogels were prepared by dispersion method. In this method the Gel base was prepared by dispersing Carbopol 934 in distilled water. The polymer was hydrated in distilled water for 2 hr and then stirred to obtain a homogeneous gel base of 1% w/w. For the preparation of Coenzyme Q10 ME-NLC loaded gel, the NLC dispersion was then centrifuged at 2000 rpm for 20 min and the pellets obtained were incorporated into the prepared gel base to get 1%w/w Coenzyme Q10 ME-NLC in the gel base (G1- G4). Furthermore coenzyme Q10 reference formulation was prepared by triturating Coenzyme Q10 with carbopol 934 gel base (G0).

Characterization of NLC gel

The basic physicochemical parameters were studied for the developed formulation.

Physical Appearance: The prepared gel formulations were evaluated visually for their color, clarity, homogeneity and appearance. As shown in table 1 [23].

Table 1: Physical parameters

S. No.	Parameters	Control (G0)	F1 (G1)	F2 (G2)	F5 (G3)	F6 (G4)
1.	Color	White	Fade white	Fade white	Fade white	Fade white
2.	Clarity	Clear	Translucent	Translucent	Translucent	Translucent
3.	Homogeneity	Achieved	Achieved	Achieved	Achieved	Achieved

pH and rheological measurements

The pH of the NLC loaded gel was determined by using Digital pH meter & the viscosity of gel was measured by using Brookfield viscometer using T-bar spindle (spindle-C, S-95) at 50 rpm 25^{0}C. The spindle was kept perpendicularly into the gel placed in a beaker in such a way that the spindle does not touch the bottom of beaker. The spindle was rotated at a speed of 50 rpm and the readings were recorded after 30 sec when the level of gel was stabilized.

Spreadibility

The Spreadibility of the prepared gel was evaluated by using slides method. In this method initially 1gm of gel was kept between the two slides. Then previously weighted plate was kept above the gel & more weights were added on the plate until the gel stops spreading. The measurement of final cumulative weight and the total time taken by the gel to spread was performed and noted. Then total weight applied and mass of the gel were compared by the time as shown in table 4 [24].

Spreadibility= Mass x Length/ Time (1.0)

***Ex-vivo* permeation study**

Albino wistar rats weighing 180–220 g of age about 2–3 months were selected for *in vitro* permeation study. All the investigations were performed after approval by the Institutional ethical committee (IAEC KUDOPS/19) and in accordance with the disciplinary principles and guidelines of the committee for the purpose of control and supervision of experiments on animals. The hair of the rats were carefully trimmed and the abdominal skin was excised from the connective tissue and was placed on aluminium foil. The presence of any adhering fats or subcutaneous tissue was teased off carefully.

The prepared skin sample was carefully mounted on the Franz diffusion cell in such a way so that the dermal side of the skin was facing the receptor compartment. The test gel (2 mg/cm^2) was applied as a thin film on the epidermal side with an effective diffusional area of 2.2 cm^2 and receptor volume of 11 ml. The receptor compartment contained 11 ml of phosphate buffer pH 6.8 stirred at 200rpm and maintained at 32 ± 1°C. One ml sample was withdrawn through the sampling port of the diffusion cell at 1, 2, 3, 4, 6, 8, 10 and 12 hr time intervals and analyzed using HPLC. An equal volume of fresh phosphate buffer pH 6.8 was replaced into the receptor compartment after each sampling.

The study was done in triplicate and average values were calculated. The statistical test was performed at 5% level of significance by using ANOVA to determine significance. The cumulative amount of penetrant that permeated the skin per unit surface area was plotted against time. The linear portion of the plot was taken as being the steady-state flux, (J_s) and was determined by using formula

$$J_s = V / A \times dc/dt$$

Where, V is the receptor volume, A is surface area, c is concentration of Q10-ME in the receptor phase and t is the time.

Evaluation of photoprotection potential of Co Q10-ME loaded gel formulations with respect to gallic acid against UVA irradiation

Efficacy study of gel formulations as a photoprotective agent against UVA exposure

Photo-damage of skin is the key happening dermatological problems worldwide [25].

A bulk of the UVB (290 to 320 nm) is absorbed through the epidermis of skin, however UVA (320-400 nm) reaches into the dermal layer as well as in systemic circulation, tissues and generates reactive oxygen species (ROS).[26] The ROS influence lipid peroxidation in biological membranes leading to dermatitis, melanoma, inflammations, ageing and other serious skin complications [26-27].

The distance between the UV lamp and dorsal rat skin is crucial factor and it was 40 cm. A specified area of about 2×3 cm2 on the dorsal skin of the rat was shaved using a soft hair-removing lotion. The rats were observed for 48 h to exclude rats showing abnormal hair growth or any adverse reaction to the depilatory preparation. A soft hair-removing lotion was favored over a shaving blade to minimize free radical production due to trauma from the blade.

For each subject, the minimally erythemogenic dose (MED) [138 mJ/cm2, obtained by exposure for approximately 4.30 min from a distance of approximately 40 cm] was determined preliminarily and a UVA irradiation dose (610 mJ/cm2) for 20 min corresponding to the 4.42 times of the MED was used throughout the study.

Animal grouping and application of gel formulations

The rats were divided into 5 groups, each containing six animals (n = 6). A thin and uniform layer of gel formulation (500 mg ≈ 5 mg of FA) was applied to the demarcated shaved area on the dorsal skin of the rats. Group I (control, untreated UV radiation) and Group II (UV irradiated) were treated with placebo formulation. Group III was applied for Q10 conventional gel (Q10-CG1 equivalent to 0.1% Q10). Group IV was applied to statistically optimized formulation F5 containing Q10-ME loaded NLC gel (Q10 ME-NG5 equivalent to 0.1% Gallic acid). Group V was applied to GA conventional gel (GA-CG2 equivalent to 0.1% GA).

Then the exposure of UVA radiation was done to UVA irradiated, Q10-CG1, and Q10 ME-NG5 immediately after topical application while to GA-CG2 group after 4 h of topical application for seven days.

On the eighth day, all the animals were anesthetized using diethyl ether and sacrificed by the cervical dislocation. The UVA treated portion of cutaneous (epidermis and dermis) tissues was quickly dissected, washed with ice-cold saline and the homogenate were prepared in 0.1 M PBS (pH 7.4) and centrifuged at 13500 rpm for 5 min (Spinwin MC-02, Tarson, India). Then the supernatant was collected and stored at -20 °C for further use.

RESULTS

Stability Studies of gel formulation:

As per ICH guidelined Stability of the selected NLCs gel was determined by storing the selected NLCs at 4°C ± 0.5°C. Vesicle size, zeta potential and entrapment efficiency of the nanoparticles were measured after 90 days by methods described in earlier sections. The changes in the parameters were analyzed statistically by Student t test. The significance was set at a level of $p< 0.05$.

***In vitro* sun protection factor determination by UV spectrophotometer**

The ratio of UV doses of protected to unprotected gives the sun protection factor (SPF). This *in vitro* method measures the reduction of the irradiation by measuring the transmittance after passing through a film of product.

The method employ 1.0 g of optimized formulation which is transferred to a 100 mL volumetric flask, diluted with ethanol, followed by ultrasonication for 5 min followed by filtering through cotton. Furthermore 5.0 mL aliquot was transferred to 50 mL volumetric flask and diluted to volume with ethanol. Then a 5.0 mL aliquot was transferred to a 25 mL volumetric flask and the volume completed with ethanol [28].
The absorption data were obtained in the range of 290 to 320, every 5 nm, and 3
determinations were made at each point to get the best average, followed by the application of Mansur equation [29].

$$\text{SPF (spectrophotometric)} = \text{CF} \times \sum_{290}^{320} \text{EE}(\lambda) \times \text{I}(\lambda) \times \text{Abs}(\lambda)$$

Where CF = correction factor

EE (λ) = erythmogenic effect of radiation with wavelength λ,
Abs (λ) = spectrophotometric absorbance values at wavelength λ.
The values of EE, I are constants.
Correction factor=10

On the experimental basis the SPF value of the optimized formulation was found out to be 15.24 ±0.05 as depicted in table 2.

Table 2: Normalized product function for the SPF determination

Wavelength λ (cm)	**EE×I**
290	0.014
295	0.082
300	0.287
305	0.329
310	0.186
315	0.087
320	0.015
Total=1	

Table 3: Pharmacotechnical evaluation of NLC loaded hydrogel

Formulation code	**pH ± SD**	**Viscosity (cp) T- spindle S- 95 50 rpm ± SD**	**Spreadiability (gm.cm/min)**	**Percent drug content ± SD**
Control	6.27 ± 0.03	5879 ± 2.1	247/2.5	87.46 ± 0.19
F1 (G1)	6.78 ± 0.04	7231 ± 1.2	225/2.5	87.33± 0.45
F2 (G2)	6.87 ± 0.03	7461 ± 3.2	212/2.5	88.60 ± 0.67
F5 (G3)	6.90 ± 0.12	7426 ± 1.4	217/2.5	92.12 ± 0.29
F6 (G4)	6.82 ± 0.06	7319 ± 2.0	223/2.5	87.94 ± 0.58

Table 4: Skin permeation parameters of Q_{10}-ME calculated from *in vitro* permeation studies using Franz diffusion cell through rat's skin (after 12 hr)

Formulation code (Gel code)	**Percent cumulative drug permeated ± SD**	**Flux ± SD (µg/cm²/min)**	**Release rate ± SD (µg/cm²/min½)**	**Enhancement ratio**	**Lag time (hr)**
G0	56.14 ± 1.31	1.191 ± 0.14	19.28 ± 1.24	----	0.4
F1 (G1)	82.37 ± 1.02	2.76 ± 0.28	43.19 ± 1.89	2.28	0.6
F2 (G2)	89.63 ± 2.19	3.06 ± 0.68	47.22± 1.54	2.59	0.6
F5 (G3)	90.18 ± 0.76	3.26 ± 0.12	50.54 ± 1.10	2.84	0.5
F6 (G4)	87.52 ± 1.51	2.89 ± 0.31	46.18 ± 1.43	2.50	0.7

Skin irritation study

The skin irritation study was performed test as per OECD Test Guidelines 404 and in European chemical bureau Annex. V part B-4 using dorsal side of hairless rats (n = 6). The gel formulations (~500 mg) of F5 and placebo-(without drug) were applied. The animals were observed and evaluated for any sign of erythema, oedema and erosion for a period of 7 days.

Physicochemical characterization of NLC gel

The prepared NLC loaded hydrogel were translucent in appearance with the pH between 6.78 ± 0.04 to 6.90 ± 0.12 (Table 4) that lies in the normal range of skin pH (4.5 to 7), indicating the gels to be potentially free from irritation. The drug content closely ranged between 87.33 %± 0.45 to 92.12% ± 0.29 suggesting uniformity of the drug dispersion in the gels. The viscosity of the gels also closely ranged between 7231 ± 1.2 to 7461 ± 3.2 cps. The viscosity is predominantly dependent on the gelling agent used. Carbopol 934 upon hydration forms a physically bonded structure that provides sufficient mechanical strength to the gel. The pH, viscosity and drug content of the control gel (G0) were 6.27 ± 0.03, 5879± 2.1 cps and 87.46 ± 0.19 respectively. There was a noticeable difference in viscosity values of control gel (pure drug based) and NLC based gels. This clearly defines the contribution of the nanocarrier on the viscosity of the topical formulation. This clearly defines the contribution of the nanocarrier on the viscosity of the topical formulation as conferred in table 3.

In-Vitro Drug Release Studies: The *ex-vivo* permeation profiles of Q_{10}-ME hydrogels

The in-vitro drug release of the prepared gel formulation was evaluated with the help of Franz Diffusion cell. In this 2.5 g of each gel was placed in a donor compartment. The receptor compartment was filled with 10 ml physiological buffer solution (pH 7.4), at 37°C and magnetically stirred at 400 rpm with a semi permeable membrane area of 1.8 cm^2. The 2 ml of receptor fluid was withdrawn at an interval of 0.5, 1, 2, 3, 4, 8 and 10 h. An equal volume of PBS was consecutively added to the receptor compartment after each sampling to sustain the sink conditions. Each sample was filtered through a 0.45 µm polyamide membrane filter and then determined for Gallic acid content by HPLC analysis.[31] .

The concentrations of all the formulations were calculated and then the % drug content release was determined, as shown in Table 4.

The outcomes of UVA exposure on the skin antioxidant biochemical marker enzymes

The antioxidant enzyme levels in the UVA irradiated rat skin was reduced significantly in comparison to the control group (without UVA exposure) ($P < 0.01$). The levels of skin antioxidant enzymes (GPX, SOD and CAT) were significantly higher in case of the groups pre-treated with Q10-CG1and Q10-ME NG5, than UVA irradiated group ($P < 0.01$) [Figure 1 (A-C)].

The group pre-treated with NG did not produce any effect on the skin antioxidant enzymes as compared to the UVA irradiated group, which may be due to the placebo NG [Figure 1 (A-C)].

Significant increase in the level of SOD, GPX and CAT was observed with GA-NG2 group ($P < 0.01$) comparing to GA-CG2 group ($P < 0.05$), after 4 h of UVA irradiation and treatment with Q10-ME loaded nano-gel and GA conventional gel respectively as shown in Figure 1(A-C).

The raised level of TBARS was significantly reduced with Q10-CG1 and GA-NG2 treated groups compared to the UVA irradiated group ($P < 0.01$). GA-CG2 group reduced the TBARS to the minimum extent compared to Q10-ME NG5 ($P < 0.05$), after 4 h of UVA irradiation [Figure 1 (D)]. This may be due to less permeability of Q10 conventional gel.

GA-NG2 showed the better therapeutic effect because of its enhanced permeability than the conventional gel. Thus, the optimized formulation Q10-ME NLC gel containing GA has the potential to enhance UV protective activity of the skin for longer periods rather than conventional topical formulations.

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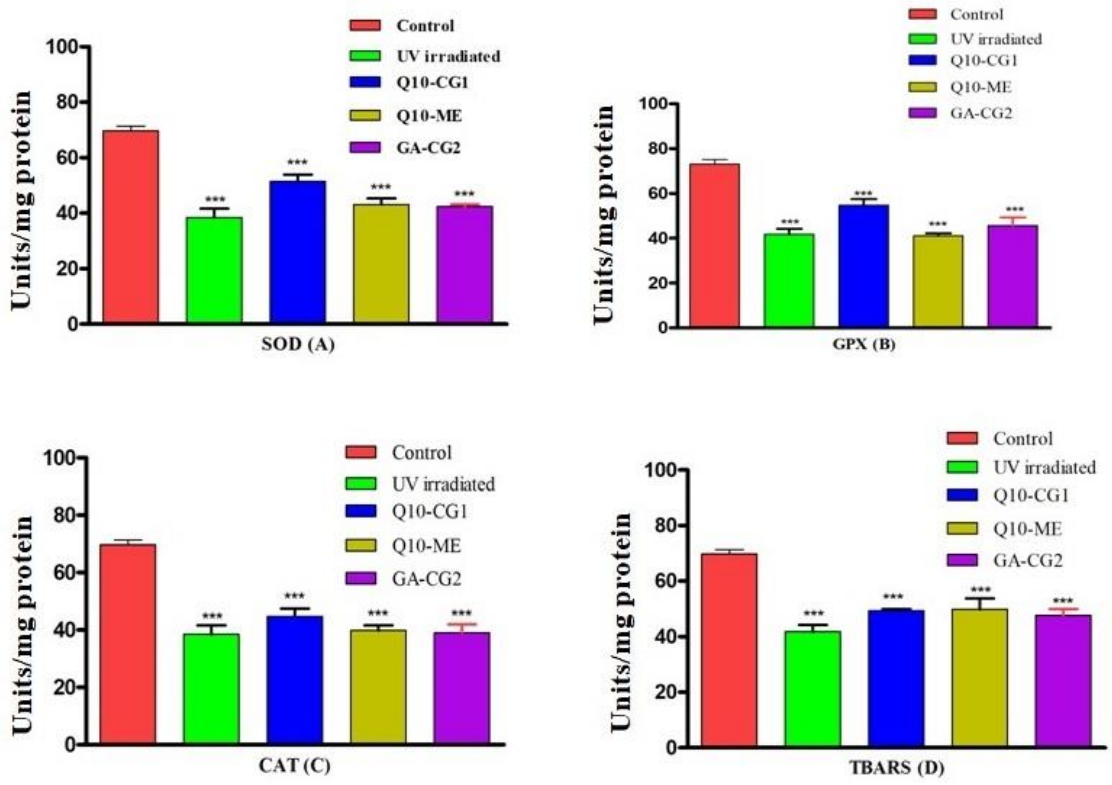

Fig 1: Effect of Q10 loaded different gel formulations on (A) SOD, (B) GPX, (C) CAT and (D) TBARS levels in rat skin [Values were mean ± SEM (n = 6), $^{**}P < 0.05$, $^{***}P < 0.01$]

The topical or transdermal drug delivery route can avoid hepatic first-pass metabolism resulting in the possible prolongation of the $t_{1/2}$ and a sufficient concentration in the systemic circulation [32].

Q10-ME NLC gel containing GA exhibited better UVA protection activity even after 4 h of application to the skin surface due to its improved permeability and sustained-release profile compared to conventional gel.

CONCLUSION

Nanostructured lipid carrier of Coenzyme Q_{10} & *Myrica esculenta* leaves extract was successfully developed and statistically optimized by using 2^3 factorial design. The obtained NLCs were nanosized particles with high entrapment efficiency. The optimized formulations were selected for the gel preparation. The NLC loaded gel represented higher permeability than the control gel and marketed product of Coenzyme Q_{10} indicating its higher antiaging potentials. Flux rate was highly dependent on the amount of gel applied per unit area. The enhanced permeation of the drug through NLC carriers might help in enhancing the pharmacological efficacy of Coenzyme Q_{10}, subsequently enhancing antiaging potential of Coenzyme Q_{10} with polyphenolic herbal extract of *myrica esculenta* leaves. The statistically optimized Q10-ME NLC gel containing GA has proved the prominent photo protective ability and hence enhanced the antiaging potential of CO Q10. The optimized formulation has no any sign of skin irritation.

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