

Full Proceeding Paper

GREEN TEA EXTRACT IN MICROEMULSION: STABILITY, DERMAL SENSITIZATION AND EFFICACY AGAINST UV INDUCED DAMAGES

ANOOPINDER KAUR¹, PURVA THATAI¹, SHIKHA SHARMA¹, BHARTI SAPRA^{1*}

**¹Pharmaceutics Division, Department of Pharmaceutical Sciences, Punjabi University, Patiala (India)
Email: bhartijatin2000@yahoo.co.in**

Received: 29 Oct 2015 Revised and Accepted: 12 Feb 2016

ABSTRACT

Objective: To determine the photoprotective effect of epigallocatechin (EGC) extracted from green tea using microemulsions as a carrier system.

Methods: In the present study, EGC was extracted from commercially available branded as well as loose green tea samples. Further, microemulsions (MEs) of these extracts were formulated and were evaluated for their antioxidant and photoprotective effects. MEs were formulated using Capmul MCM as oily phase, Tween 80 as a surfactant and Labrasol as co-surfactant after studying the phase behavior. MEs containing green tea extracts were applied to the rat dorsal skin after exposure to UV radiation (rate of exposure = 9.71 J/cm², dose = 0.9011 mJ/cm²/sec). The effect of the formulation was evaluated in terms of reduced glutathione level (GSH), radical scavenging activity (DPPH), trans-epidermal water loss (TEWL), irritation potential and histological changes. The EGC content of both types of green tea was estimated using HPTLC and photo-stability of ME formulation was evaluated using FTIR-ATR technique.

Results: The content of EGC in loose and branded tea leaf extracts was found to be 0.00322 % w/w and 0.00468 % w/w, respectively. FTIR studies revealed the instability of formulations prepared with water as aqueous phase after UV exposure. However, in the case of PB (pH 7.4) as the aqueous phase in MEs; no change in the spectra of formulations after UV exposure for different time intervals was observed. Results of TEWL studies indicated that the barrier perturbation was not severe which clearly states the safety aspect of the formulation. The extinction coefficient (EC₅₀) value for loose tea and branded tea leaf extract was found to be 170 µg/ml and 79 µg/ml, respectively. The yield of the extract was 0.00322±0.026 % w/w for loose tea and 0.00468±0.150 % w/w for branded tea.

Conclusion: The results of the present investigation indicated that pH of the aqueous phase used for preparing the formulation affected the photo-stability of the formulation. Further, MEs prepared using green tea extracts exhibited photoprotective effects. Hence, the green tea extract containing ME formulations have promising potential to be a cosmeceutical.

Keywords: Efficacy against uv induced damages, Microemulsion, Dermal sensitization

© 2016 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>)

INTRODUCTION

Numerous types of acute and chronic reactions in skin e. g. sun burns, photo immune suppression, and the photoaging has been coupled with sun exposure. In scrutiny of growing understanding of the potentially unfavorable long-term side effects of solar irradiation, there is a universal call for harmless and effective photoprotectants that may provide protection against ultraviolet (UV) radiations. Sunscreens are one of the best photoprotective measures that are in great demand these days. A sunscreen formulation contains components that provide protection against high energy photons of UV radiation, and these products work mainly by absorbing, reflecting or scattering UVA and UVB rays [1]. Hence, they attenuate the amount and nature of UV radiations reaching viable cells in the skin. No sunscreen prevents photo-damage, as it has been revealed that sub-erythral doses of UV radiations lead to a variety of molecular changes, for example, DNA damage in epidermal cells. However, the spectrum of UV radiations accessing viable epidermal cells can be altered by the use of topical sunscreens. Regular use of sunscreen has been shown to reduce actinic keratosis [2], solar elastosis [3], UV-induced immune suppression [4] and photosensitivity in humans and prevents the formation of squamous cell carcinoma in animals.

This is well established that UV radiations are one of the major ecological causes of skin cancers; however, skin cancers can be prevented by the use of physical and chemical sun-protective. Various synthetic and natural agents are available in the market for the purpose of photoprotection. However, many synthetic protectants have their own disadvantage e. g. contact dermatitis caused by cinnamates and oxybenzone and thus natural photo-protectants are preferred [5].

Tea polyphenols e. g. epigallocatechin-3-gallate (EGCG), catechin, epigallocatechin (EGC) have been found to possess antioxidant, anti-

inflammatory and anti-carcinogenic activity. Tea polyphenol e. g. catechins and EGCG have been explored for their antioxidant activity by many researchers, however; their sun-protective activity needs additional research in relation to UV irradiation. Hence, in the present study, EGC extracted from green tea has been investigated for its photoprotective effect.

In addition, the commonly used carrier system for sun photoprotectants is lotion, gel, etc. However, in the present investigation micro emulsions (MEs) have been formulated with the hypothesis that the penetration of micro particulate system based carriers will be rapid and deeper than conventional formulations. Recent advances in MEs show its promising role as a potential carrier due to their improved solubilization power for poorly soluble, poorly absorbed, labile herbal extracts and photo-chemicals. MEs have the ability to encapsulate nonpolar molecules such as lipids, flavorants, antimicrobials, antioxidants, and vitamins. Moreover, they are easy spreadable, non-sticky, waterproof and compatible with the skin [6]. Due to this fact, the efficiency of the product can be enhanced. In the previous investigations involving catechins, antioxidant activity has been demonstrated, but none of the researchers have used W/O MEs as the topical carrier system for their delivery. Moreover, the process of extraction used in the present study ensures extraction of EGC predominantly in the extract.

Moreover, catechins are not photostable [7] and in the present study, the formulation has been stabilized in order to improve the efficiency. This approach can be useful in making it a commercially viable product without the addition of other photoprotective excipients or stabilizer. Furthermore, this study considered histological aspect, i. e. microscopic studies and non-invasive studies, like TEWL in order to evaluate the efficacy of the formulation against UV radiation-induced dermal changes.

MATERIALS AND METHODS

Materials

Oleic acid, polyoxyethylene sorbitan monooleate (Tween 80) and acetonitrile were purchased from s. d Fine Chemicals (Mumbai, India); isopropyl myristate and propylene glycol were obtained from Thomas Baker (Mumbai, India) and Qualigens Fine Chemicals (Mumbai, India), respectively. Glyceryl mono caprylate (Capmul MCM) was a gift from Abitec Corporation (Mumbai, India), glyceryl mono-oleatetype 40 (Peceol), caprylocaproyl macrogol-8-glycerides EP/USP NF (Labrasol) and oleoyl macrogol-6-glycerides EP/USP NF (Labrafil M 1944 CS) were obtained as gift samples from Gattefosse SAS, France. PEG-40 hydrogenated castor oil USP/NF (Cremophor RH 40) was also a gift from BASF chemicals (Burgbernheim, Germany). DPPH [(2,2-Diphenyl-1-2,4,6-trinitrophenyl) hydrazyl] was procured from Sigma Chemicals (USA). EGC was a kind gift from Institute of Himalayan Biosource Technology (Palampur, India). Branded green tea and commercially available loose green tea was purchased from local market. All the other reagents used in the present study were of analytical grades and procured from CDH Lab Reagent (New Delhi).

Methods

Extraction of green tea (*Camellia sinensis* L.)

Extraction was carried out using two samples of green tea; Branded green tea (TT) and Loose green tea (LT) according to modified extraction procedures [8]. Sulphuric acid was added during the extraction to break-down all the esters to obtain EGC.

Standardization of extract

Preparative thin layer chromatography (TLC)

Different solvent systems were tried for selection of solvent system using TLC plate consisting of chloroform, methanol, ethyl acetate, glacial acetic acid or acetonitrile in different ratios.

High-performance thin layer chromatography (HPTLC)

HPTLC was carried out in order to analyze green tea extract (GTE) for quantitative estimation of EGC in both the extracts (TT and LT) using the solvent system selected from TLC studies. Standard stock solution (50 µg/ml) of EGC was prepared in the concentration range of 100–1000 ng/ml. Further, stock solutions of LT and TT extracts (1000 µg/ml) were prepared in methanol and were injected and analyzed.

Sampling was done on the TLC plate with Linomat-5 autosampler, and the plates were scanned with CAMAG TLC scanner-4 detectors. Quantification of EGC in the extracts was performed from the peak area of the component and its corresponding calibration curve using Win CATS software.

Estimation of antioxidant activity

DPPH assay of GTE

Method

The DPPH radical scavenging assay was performed using a modified method of Brand-Williams *et al.* [9].

Antiradical activity was expressed as an inhibition percentage (% I) and was calculated using the following equation:

$$\text{Inhibition percentage (\% I)} = \frac{\text{Absorbance}(\text{control}) - \text{Absorbance}(\text{sample})}{\text{Absorbance}(\text{control})} \times 100$$

Different sample concentrations (50-1000 µg/ml) were used in order to obtain calibration curves and to calculate the EC₅₀ values (EC₅₀ is concentration required to obtain a 50 % radical scavenging activity). The sample concentration that could lower the initial absorbance of DPPH solution by 50% was chosen as the endpoint for measuring the antioxidant activity [10].

Formulation of MEs containing GTE

The MEs consisting of oil (Oleic acid/IPM/Capmul MCM/Peceol), surfactant (Tween 80), co-surfactant (propylene glycol 400/Cremophor RH40/labrasol/labrafil M 1944 CS), and the aqueous

phase (double-distilled water or phosphate buffer (PB, pH 7.4) were formulated. Oil to surfactant weight ratio was varied from 9:1 to 1:9 and the ratios of surfactant/co-surfactant were varied as 1:1, 1:2 and 2:1. To these mixtures, water/PB, pH 7.4 was added drop-wise and mixed using a magnetic stirrer (REMI, India) at 25±0.05 °C.

The phase behavior of all the systems was mapped, and ME area was calculated.

MEs were prepared using selected and optimized oil, surfactant, and co-surfactant and; GTE was dissolved in the aqueous phase of ME so as to achieve final concentration of extract in the formulation as 1%, 2% and 4% w/w concentrations.

Characterization of MEs

Following the phase behavior studies, MEs were prepared along the dilution line in phase diagrams.

All the formulations were diluted 10 times and 100 times with an aqueous phase at ambient temperature and then the contents were gently stirred by shaking. The equilibrated samples were assessed for clarity and transparency by visual inspection. % transmittance of these formulations was assessed at 650 nm using a UV spectrophotometer (Beckman DU 640B UV/VIS Spectrophotometer, USA). The pH measurements of the ME formulations were done using pH meter.

The electric conductivity (σ) was measured using microprocessor based pH-EC (ESICO, India) operating at 50 Hz. The conductivity of selected MEs was measured as a function of φ (% weight fraction of water). The error limit of conductivity measurements was ±5%. The cell constant of the conductivity meter was 1.099 µS/cm. Conductivity measurements were carried at 25±0.5 °C in triplicate (n = 3).

Viscosities of these formulations were determined using a Brookfield Viscometer (Spindle No. S18, LVDV-I-Prime Brookfield Engineering Laboratories, USA). Viscosity measurements were carried out at 25±0.5 °C in triplicate (n = 3).

Particle size measurements of finally selected ME formulations (100 times dilution) with and without extract were carried out using the Malvern Zetasizer (Malvern Instrument Ltd., USA).

To determine zeta potential, the MEs systems were diluted (100 times) with distilled water and measured with the Zetasizer using clear disposable zeta cell (Beckman Coulter, Delsa™ nano, USA).

Stability of MEs was tested by carrying out centrifugation at 4,000 rpm for 15 min immediately after their formation and by subjecting the MEs to a total of 3 complete cycles; each cycle consisting of 24 h at 60°C followed by 24 h at -4 °C immediately after the formation. After a specific period, particle size measurements of ME were done to observe the change if any.

Photo-stability study

ATR-FTIR studies were carried out using a Bruker Alpha instrument (Zn Se crystal detector) to analyze any changes during UV exposure in order to determine the photo-stability. ME formulations with GTE sealed in vials were exposed to UV source. The rate of exposure to UV radiation was 9.71 J/cm² with a dose of 0.9011 mJ/cm²/sec and observed for any type of change after regular periods (0 h, 2 h, 4 h, 6 h). Any kind of interaction that occurred in the formulation was determined by comparing spectral peaks.

Dose-dependent influence of GTE on the skin

Sensitization test (draize test)

The study employed Wistar rats (250 g) of either sex (n = 3), for testing the skin irritation. Hair of dorsal area of animals were removed in such a way that formulations could be accommodated in that area and divided into six groups, Group I: without any treatment (negative control), Group II: treatment with sodium lauryl sulphate (positive control), Group III: Treatment with blank formulation, Group IV: ME containing 1% GTE (TT), Group V: ME containing 2% GTE (TT), Group VI: ME containing 4% GTE (TT). Animals were observed for one month for any sign of erythema or edema and scored as reported by Muller-Decker *et al.*, 1997 [11].

These grades were averaged across all animals and grading time points, and then the averages were combined to derive a primary index (PI) [12]. All the experimental procedures were carried out in accordance with the standard guidelines approved by the Institute of Animal Ethical Committee, Punjabi University, Patiala. (Proposal no. 107/99/CPCSEA/2013-23).

Transepidermal water loss measurements (TEWL)

TEWL measurements were done on animals used for the draize test in parallel for a one month period using Tewa meter TM 210 (Courage and Khazaka Electronic GmbH, Koln, Germany). The laboratory temperature and relative humidity were maintained at 25 ± 2 °C and 55 ± 5 %, respectively. Animals were divided into 6 groups (n = 3) as mentioned in sensitization test.

Biochemical estimation of reduced glutathione

Reduced glutathione (GSH) assay

Levels of glutathione hormone were estimated in different treated and control rat skin. Animals were divided into nine groups; group I: untreated control group (without formulation and without UV exposure), group II: treatment with formulation but without UV exposure, group III: treatment with blank formulation and with UV exposure, group IV: treated with ME (1% w/w TT) but not UV exposed, group V: treated with ME (2 % w/w TT) but not UV exposed, group VI: treated with ME (4 % w/w TT) but not UV exposed, group VII: treated with ME (1% w/w TT) and UV exposed, group VIII: treated with ME (2 % w/w TT) and group IX: treated with ME (4 % w/w TT).

Irradiation and treatment with formulations

The UV source of irradiation consisted of UV tubes emitting continuous spectrum between 270-400 nm. Formulations (0.5 g/cm² of ME on the rat dorsal skin) were applied 15 min before irradiation and animals were irradiated for 3 h. The distance between source and rats was kept 30 cm. Animals were sacrificed by cervical dislocation, 6 h after the UV exposure [13].

Preparation and collection of skin samples

Dorsal skin was removed and homogenized in phosphate buffer, pH = 7.4 followed by centrifugation at 3000 rpm for 15 min. The supernatant thus obtained was used for GSH assay. Furthermore, remaining skin samples were preserved in formaldehyde for histological studies.

Estimation of reduced glutathione (GSH)

Estimation of reduced glutathione was carried out according to the procedure reported by Singh *et al.* [14]. The solutions were analyzed spectrophotometrically for GSH estimation at 412 nm.

$\text{Reduced glutathione} = \text{Absorbance}/\text{EC} \times \text{protein content} \times \text{volume of sample used} \times 100$

Where extinction coefficient (EC) is = $13600 \text{ M}^{-1} \text{ cm}^{-1}$.

Total protein content was estimated using the Biuret method (Biuret assay kit by Erba Mannheim) and calculated using the following formula;

$$\text{Total protein (g/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard} \times \text{concentration of standard (g/dl)}}$$

Histopathological studies

UV-induced histopathological changes were evaluated considering the parameters of infiltration of inflammatory cells, epidermal thickening and keratinocytes contents. Skin samples were stained with hematoxylin and eosin (H & E) and sections were examined using light microscopy. The images were captured at 10X magnifications [15].

Statistical analysis

All the data were analyzed for statistical significance by using Two-way Anova followed by post-test; Newman-Keuls Multiple Comparison Test.

RESULTS AND DISCUSSION

Standardization and quantitative estimation of epigallocatechin

TLC fingerprinting profiles of LT and TT extracts

Chemo-profiling of an extract of *Camellia Sinensis* leaves was performed by TLC using reference EGC. Comparative TLC fingerprint profiling confirmed the presence of epigallocatechin (EGC) in the ethyl acetate extract. EGC was visualized under ultraviolet light at 274 nm. The R_f value was observed at 0.85 which is in accordance with a literature R_f value of EGC (0.88). The yield of total crude extract was found to be 4 % w/w.

Estimation of epigallocatechin in camellia Sinensis leaves using HPTLC method

A standard plot was prepared at 274 nm between the concentration range of 100–1000 ng/ml of EGC and linearity of the calibration curve was observed to be in the range of 400-900 ng/ml. The content of EGC in LT and TT leaf extracts was calculated using the regression equation of standard plot and which was found to be 3.22 % w/w and 4.68 % w/w, respectively.

DPPH assay

EC_{50} is a useful parameter to evaluate and compare the antioxidant activity of various samples. Buenger *et al.* indicated that in order to obtain satisfactory results, the highest concentration tested should give a maximum value of radical scavenging activity [16]. In fact, it would be erroneous to employ the linear regression for the calculation of EC_{50} over this value.

The antioxidant potential of both the extracts was measured by DPPH free radical scavenging assay. In the present investigation, EC_{50} values were found to be 170 $\mu\text{g/ml}$ and 79 $\mu\text{g/ml}$ for LT and TT, respectively. The obtained results depict that extract from TT provides significantly ($p < 0.05$) greater antiradical activity as compared to LT extract (fig. 1). 1000 $\mu\text{g/ml}$ of TT and LT were used to quantify the % inhibition activity. In the case of TT % inhibition was observed to be 95.6 %, whereas, in case of LT it was 94.4 %.

In a similar study, Kim *et al.* (2007) compared the antioxidant activity of different types of green teas, fermented teas and other related common teas by examining radical scavenging activity using DPPH. The observations revealed that EC_{50} of epigallocatechin gallate (EGCG) for 0.1 mM DPPH radical was maximum (5.5 μM or 4.2 mg/l by weight) followed by catechin (14 μM or 2.5 mg/l) and vitamin C (22 μM or 3.9 mg/l). Therefore, it can be postulated after combining present investigation's data that scavenging activity of these antioxidants follows the order as EGCG > EGC > catechin > vitamin C [17].

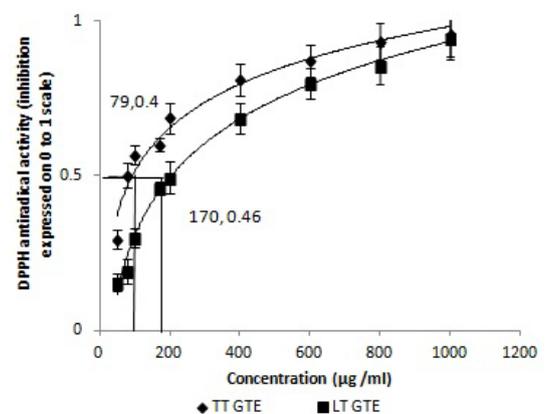


Fig. 1: Graph depicting EC_{50} values of branded green tea (TT GTE) and loose green tea (LT GTE)
*(TT = branded tea, LT = loose tea, GTE = green tea extract)

In vitro, antioxidant activities of water extract of 20 brands of different types of tea available in Bangladesh were assessed by

different techniques like by estimating total antioxidant capacity (TAC), DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP) and ferrous ion chelating (FIC) ability. The results revealed that green tea extract possessed the highest polyphenol content (103.0±0.3 mg gallic acid equivalent (GAE)/g), concurrently the highest DPPH-radical scavenging activity (IC50) (19.0±3.0 µg/ml). The scavenging activity in the aforesaid study is higher than the present study, which may be attributed to the presence of all phenolic compounds in the extract [18].

Preparation of phase diagram

Phase diagrams of MEs with and without co-surfactants were prepared to identify and calculate the ME area (A_t). The surfactant mixture having different weight ratio (1:1/1:2/2:1) were prepared by mixing Tween 80 with different co-surfactants.

A_t of all the systems is summarized in table 1. It is evident from the data that % A_t was significantly more when surfactant: co-surfactant ratio was 2:1, whereas few formulations were not stable also in this ratio. However, on the basis of ME area the ratio of 2:1 was selected for further studies. Amongst different components maximum ME area (38.44 %) was obtained using Capmul MCM as oil, Tween 80 as surfactant and Labrasol as co-surfactant and thus used further for the formation of the final formulations (table 1).

The selected batch of MEs was also prepared using PB, pH 7.4 as aqueous phase and there was no significant difference in their ME area with respect to MEs prepared using water as aqueous phase (p<0.005). Table 1 depicts the composition and ME area of the selected surfactant to the co-surfactant ratio (2:1).

Table 1: Composition of different MEs Systems along with their A_t/unstable formulation

Oil	Surfactant					Surfactant: Co-surfactant	Formulation code	%A _t /Unstable formulation (water as aqueous phase)	%A _t /Unstable formulation (PB (7.4) as aqueous phase)
	Tween 80	Cremonophor RH 40	Labrasol	Labrafil	Propylene Glycol				
Oleic acid	+	+	-	-	-	2:1	OTC3	14.11	14.02
IPM	+	+	-	-	-	2:1	ITC3	Unstable	Unstable
Peceol	+	+	-	-	-	2:1	PTC3	19.64	19.82
Capmul MCM	+	+	-	-	-	2:1	CTC3	35.12	35.22
Oleic acid	+	-	+	-	-	2:1	OTL3	33.22	33.64
IPM	+	-	+	-	-	2:1	ITL3	Unstable	Unstable
Peceol	+	-	+	-	-	2:1	PTL3	31.88	31.73
Capmul MCM	+	-	+	-	-	2:1	CTL3	38.44	38.63
Oleic acid	+	-	-	+	-	2:1	OTLb3	11.85	11.72
IPM	+	-	-	+	-	2:1	ITLb3	Unstable	Unstable
Peceol	+	-	-	+	-	2:1	PTLb3	19.05	19.20
Capmul MCM	+	-	-	+	-	2:1	CTLb3	34.63	34.79
Oleic acid	+	-	-	-	+	2:1	OTPGS3	20.77	20.67
IPM	+	-	-	-	+	2:1	ITPG3	Unstable	Unstable
Peceol	+	-	-	-	+	2:1	PTPG3	Unstable	Unstable
Capmul MCM	+	-	-	-	+	2:1	CTPG3	Unstable	Unstable

*(n=3)

Characterization of MEs

All the formulations along the dilution line were clear. % Transmittance of blank MEs with water as well as with phosphate buffer as aqueous phase was measured after 10 and 100 times dilutions and it ranged between 30–70 %. The transmittance of MEs formulated with water and PB, pH 7.4 as aqueous phases were found to be statistically similar (p<0.05). The pH values of MEs containing GTE were in the range from 2.75 to 4.29 and 6.15 to 7.48 with water and PB, pH 7.4 as the aqueous phase, respectively.

Conductivity measurement is an important tool to access the structure of MEs and to evaluate their percolation behavior. The plot of electrical conductivity (σ) as the function of % aqueous phase (w/w) is drawn. Variation in conductivity with respect to aqueous phase is shown in the fig. 2. The conductivity was found to increase with an increase in water fraction. The smooth increase in conductivity indicated the stability of MEs [19].

The conductivity was initially low in oil/surfactant-co-surfactant mixture and was found to increase with an increase in the % weight fraction of aqueous phase (φ) (fig. 2). As the value of φ increased conductivity (σ) of the system also increased, but to a lesser extent until a specific φ. After this point a drastic increase in conductivity

was observed, this phenomenon is called percolation and φ at which change is observed is known as the percolation threshold (φ_p).

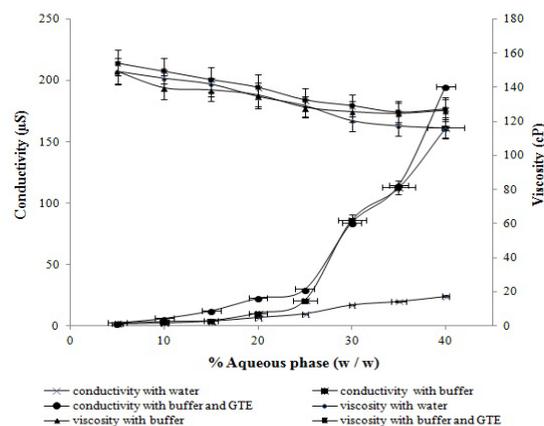


Fig. 2: Conductivity and viscosity profiles of MEs with water and buffer

Percolation threshold (ϕ_p) for the systems containing water, buffer or buffer+1% GTE (TT) is at 30 % aqueous phase. Drastic increase in conductivity at 30 % w/w buffer is most likely caused due to the transition from an oil continuous ME system to water continuous ME system [20]. A similar trend was observed with GTE MEs. On this basis, the final w/o formulation was selected in which ϕ was 25 % w/w.

The reduction in the viscosity with the increase in water content was observed in all the systems. When the water concentration was

increased from 5 % w/w to 40 % w/w viscosity decreased slowly from 149.1±1.82 cP to 115.8±0.3 cP. In the case of buffer MEs viscosity decreased from 149.5±0.55 cP to 126.6±0.35 cP and in the case of GTE MEs (prepared using a buffer), viscosity decreased from 154.4±0.40 cP to 126.8±1.36 cP (fig. 2).

The results of particle size distribution depicted that as the water content increased from 5 % to 25 % w/w, particle size decreased, whereas, when the water content was increased from 30 % to 40 % w/w particle size increased (table 2).

Table 2: Particle size of ME formulations and ME formulations with 1% green tea extract

S. No.	% Water	Particle size of ME formulation (nm)	Polydispersity index (P. I.) of ME formulation	Particle size of ME formulation with 1% GTE (nm)	Polydispersity index (P. I.) of ME formulation with 1% extract
1	5	248.233±7.606	0.282±0.172	ND	ND
2	10	242.667±4.041	0.311±0.179	ND	ND
3	15	232.212±6.244	0.198±0.120	ND	ND
4	20	221.666±7.637	0.319±0.150	ND	ND
5	25	211.665±12.583	0.233±0.140	185.966±5.559	0.286±0.145
6	30	243.333±11.372	0.215±0.140	228.966±8.538	0.176±0.106
7	35	255.533±11.038	0.153±0.151	ND	ND
8	40	284.512±7.0533	0.404±0.091	ND	ND

*(All values are represented as mean±SD, n=3)

For the selected ME (ME with 25 % aqueous phase) particle size and zeta potential were determined for extract loaded ME also. There was a decrease in particle size of loaded ME.

Zeta potential of selected ME without drug and with the drug was found to be -10.59 and -16.82, respectively. However, there was no significant change in zeta potential in potential zeta ME with and without the extract.

In most of the investigations, no change/or a slight change in particle size of drug loaded MEs has been observed as compared to unloaded/blank MEs [21]. However, particle size reduction after loading was observed in a recent investigation carried out by Patel *et al.*, (2013) while designing lumefantrine-oleic acid self-nano emulsifying ionic complex [22].

Decrease in particle size was attributed to two possibilities, first; a certain portion of the drug could act as an emulsifying agent by depositing itself at ME interfaces [23]. The second due to deposition of the drug at ME interface, the reduced mobility of surfactant is thought to decrease the particle size of loaded MEs [24]. Further, EGCG has been reported to exhibit surfactant-like properties to a lesser extent [25]. Hence, the presence of EGCG in the extract may be the reason of such type of behavior in case of GTE.

Stability testing showed that all the formulations on dilution water line were stable as no phase separation was observed and after a definite period no change in particle size of selected ME was observed.

Photostability studies

It is evident from the fig. 3 and 4, that the spectra of formulations with GTEs at 0 h, 1 h were same whereas, after 2 h peaks were obtained at a similar frequency, but, their stretching altered. Thereafter, at 3 h, 4 h and 6 h changes in spectra were also observed in the relation of intensity as well as stretching of peaks which indicated the instability of formulations prepared with water as aqueous phase after UV exposure. But in the case of PB, pH 7.4 as aqueous phase no prominent change in the spectra of formulations with extract after UV exposure for different time intervals was observed (fig. 4). It has been reported by Mochizuki *et al.* that catechins are stable at physiological pH [26] whereas, another group revealed that the effect of pH on the stability of catechins is lesser as compared to theaflavins and furthermore, amongst all stability of catechin is maximum at pH 5-6 [27]. In the present study PB, pH 7.4 has been used, whereas the final pH values of formulation range from 6.15-7.48.

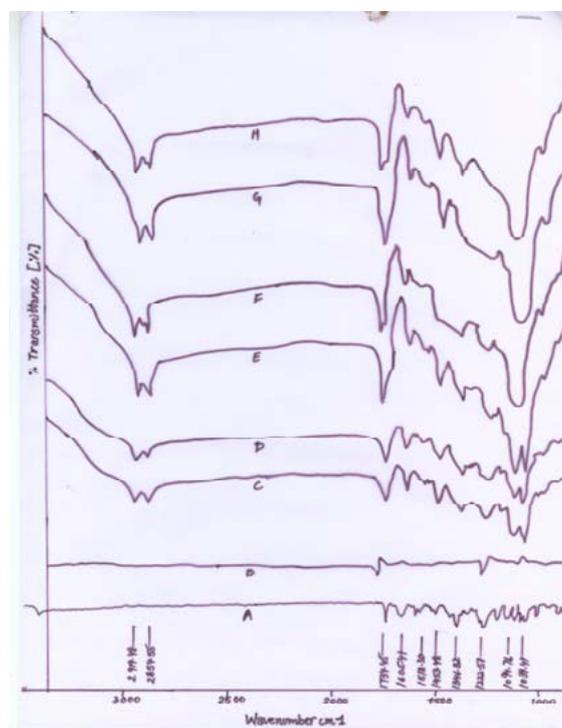


Fig. 3: FTIR spectra of ME with water as aqueous phase A) Standard, B) GTE (TT), C) ME+1 % GTE (TT) at 0 h, D) ME+1 % GTE (TT) at 1 h UV exposure, E) ME+1 % GTE (TT) at 2 h UV exposure, F) ME+1 % GTE (TT) at 3 h UV exposure, G) ME+1 % GTE (TT) at 5 h and H) ME+1 % GTE (TT) at 6 h

*(GTE = Green tea extract, TT = Branded green tea)

Dose-dependent influence of GTE on the skin

Draize test

The draize test was conducted for one month with daily application of formulations (0.5 g/cm²) on the dorsal surface of animals. The results of the draize test didn't suggest any irritation potential of the formulation on rat skin (table 3).

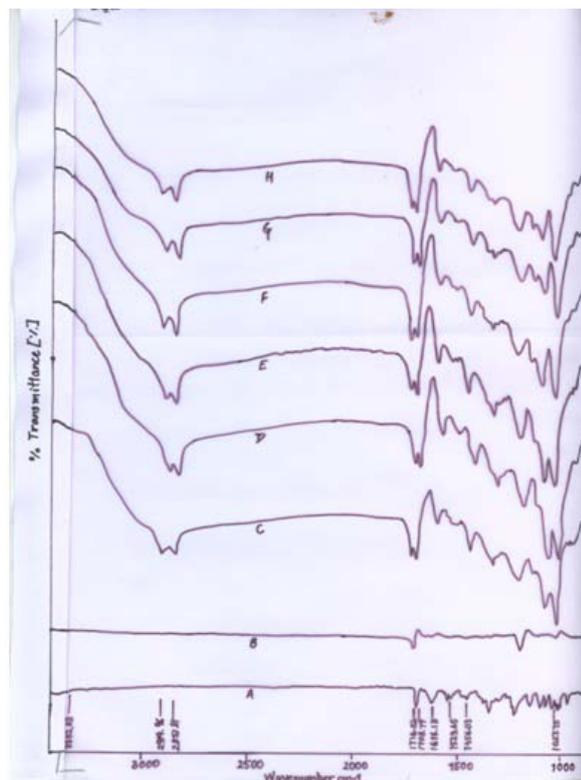


Fig. 4: FTIR spectra of ME with phosphate buffer pH 7.4 as aqueous phase A) Standard, B) GTE (TT), C) ME+1 % GTE (TT) at 0 h, D) ME+1 % GTE (TT) at 1 h UV exposure, E) ME+1 % GTE (TT) at 2 h UV exposure, F) ME+1 % GTE (TT) at 3 h UV exposure, G) ME+1 % GTE (TT) at 5 h and H) ME+1 % GTE (TT) at 6 h

*(GTE = Green tea extract, TT = Branded green tea)

Table 3: Irritation potential of the formulations along with primary index

Group	Primary index (PI)	Level of irritation
Group I	1.0	Mild
Group II	5.5	Severe
Group III	0.9	Mild
Group IV	1.0	Mild
Group V	0.9	Mild
Group VI	1.5	Mild

*(n=3)

TEWL

A high TEWL indicates defects in the barrier function of the skin. As the skin barrier function is believed to be primarily located in the intercellular domains [28], the lipid phase acts as a barrier against water loss.

TEWL of animals was measured using tewameter before application of sample formulations and at a time different intervals as shown in fig. 5. In the case of Group I, TEWL was more for initial 2 h and after that, it started coming close to control value, which indicates a reversal of barrier perturbation. The complete reversal was observed at 6 h. In group II, TEWL values were found to be high, and no reversal was observed. In Group III, TEWL was more for initial 4 h and after 4 h it got normalized. Similarly, in different groups, a complete reversal of TEWL was observed after 6–8 h. In addition, it is worth mentioning that the barrier perturbation was not severe as the difference in minimum and maximum TEWL values were 0.6, 1.4 and 1.3 in animals treated with the formulation containing 1 %, 2 % and 4% GTE, respectively. However, the barrier perturbation is considered to be severe when the reversal of TEWL values takes

longer duration, e. g. in the case of sodium lauryl sulphate reversal to control values was not observed even after 48 h [29].

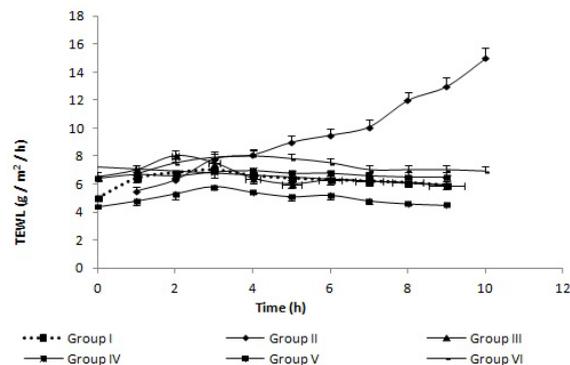


Fig. 5: Transepidermal water loss (TEWL) studies after application of formulations in different groups

Reduced glutathione (GSH) assay

GSH is considered to be a major exogenous antioxidant produced by cells which participate directly in the neutralization of free radicals and reactive oxygen compounds as well as maintained exogenous antioxidants such as vitamin C and vitamin E in the reduced (active) form [30]. The decreased glutathione level indicates an impaired antioxidant enzyme system of the skin cell [31].

For GSH activity animals were divided into 9 groups as mentioned before. The GTEs from both the samples (LT and TT) were formulated in different concentrations. The amount of GSH in the control group (Group I) without any treatment or exposure was $0.62 \pm 0.02 \mu\text{M}/100 \text{ mg}$ of skin protein. After UV exposure (Group II) it decreased to $0.26 \pm 0.015 \mu\text{M}/100 \text{ mg}$ of skin protein. However, treatment with MEs containing different extracts, amount of GSH increased in the range of 0.51 ± 0.017 to $0.55 \pm 0.03 \mu\text{M}/100 \text{ mg}$ of skin protein which is significantly similar (table 4). In addition, in normalizing the GSH, TT extracts were more effective than LT extracts whereas, the dose-dependent effect was not observed. On this basis, ME containing 1% w/w extract were considered to be equivalent to those of 4 % w/w.

Groups treated with different concentrations of GTE (1%, 2%, 4%) showed no significant difference, both in the case of TT as well as LT extract containing ME. However, a decrease in GSH level was significantly lesser in the case of TT as compared to LT ($P < 0.05$). These results further illustrate the better bioactivity of TT, these results are in consonance with those obtained for DPPH activity.

A similar study was conducted by Katiyar and coworkers (2001) in which they investigated the effects of topical application of EGCG to human skin before UV irradiation on UV-induced markers of oxidative stress and antioxidant enzyme. The results of immunohistochemistry revealed the application of EGCG ($1 \text{ mg}/\text{cm}^2$ skin) before a single UV exposure of $4 \times$ minimal erythema dose (MED) decreased the production of reactive oxygen species which was measured using markers such as H_2O_2 and NO levels. Analytical enzymes assays unveiled that single UV exposure of $4 \times$ MED to human skin increased catalase activity (109-145%) and decreased glutathione peroxidase activity (36-54%) and total GSH level (13-36%) at different time points studied. However, pretreatment with EGCG was found to be restored the UV-induced decrease in GSH level and afforded protection to the antioxidant enzyme, glutathione peroxidase [32].

Hong *et al.* observed the photoprotective effect of a formulation containing tannase-converted green tea extract on UV-B irradiated mice skin ($5.2075 \times 10^{-4} \text{ W cm}^{-2}$ at a distance of 40 cm). Tannase-catalyzed hydrolysis of catechin gallates (EGCG and EGC) in green tea increased the scavenging of radicals, superoxide anions, and hydrogen peroxide. It has also been reported that the antioxidant activities and chelation of metal ions in green tea catechins are enhanced with

tannase treatment [33]. The observations revealed that there was a significant prevention of the reduced form of GSH depletion in mice

treated with tannase-treated green tea extract [34]. These results further support the use of EGC as the photo-protective agent.

Table 4: Average glutathione level in different animal groups

Group	Glutathione Level	Decrease in level
Group I: Without any treatment and exposure	0.62±0.021	-
Group II: treatment with formulation, but without UV exposure	0.25±0.013	2.38±0.12
Group III: treatment with blank formulation and with UV exposure	0.22±0.023	2.81±0.22
Group IV: treated with ME (1% w/w) but not UV exposed	0.51±0.024	1.21±0.14
Group V: treated with ME (2 % w/w) but not UV exposed	0.52±0.011	1.19±0.16
Group VI: treated with ME (4 % w/w) but not UV exposed	0.55±0.014	1.12±0.18
Group VII: treated with ME (1% w/w) and UV exposed	0.32±0.017	1.93±0.15
Group VIII: treated with ME (2 % w/w)	0.35±0.003	1.77±0.21
Group IX: treated with ME (4 % w/w)	0.38±0.007	1.63±0.27

*(All values are represented as mean±SD, n=3)

Histology

UVB-induced histopathological changes were evaluated considering the parameters of infiltration of inflammatory cells, epidermis thickening, and apoptotic keratinocytes. The microscopic results revealed an ulcerative epidermis with a band like inflammatory infiltrates in dermal connective tissue as apoptotic keratinocytes in UV-irradiated skin. E and K represent epidermis and keratinocytes in fig. 6-8.

The histology of control skin sample (i.e. without UV exposure and treatment) revealed a normal epidermis without infiltration of inflammatory cells. The topical treatment with blank ME showed similar epidermal changes depicting no effect of blank ME. In some places keratinocytes were swollen, showing the effect of surfactant or co-surfactant present in the formulation. Furthermore, topical application of all MEs containing GTEs decreased the severity of UVB-induced histological effects, and the effect was qualitative and not quantitative as illustrated in fig. 6-8.

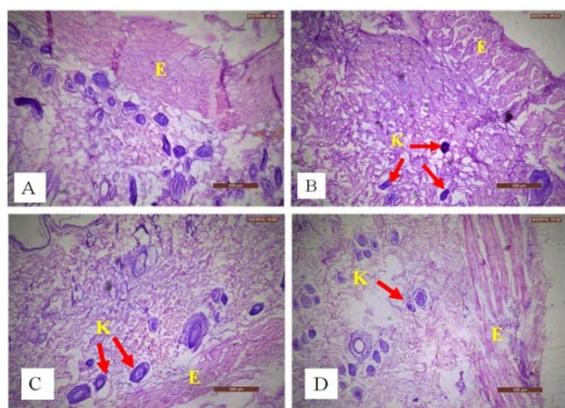


Fig. 6: Histological slide of H and E staining of normal skin A) normal skin at 10 X and B) UV treated skin at 10 X, C) treated with UV and blank formulation at 10 X and D) UV and 1% GTE (TT) formulation at 10 X

*(GTE = Green tea extract, TT = Branded green tea)

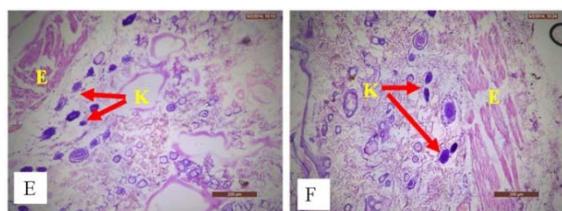


Fig. 7: Microscopy slides of H and E staining E) UV and 2% GTE (TT) formulation at 10 X, F) UV and 4% GTE (TT) formulation at 10 X

*(GTE = Green tea extract, TT = Branded green tea)

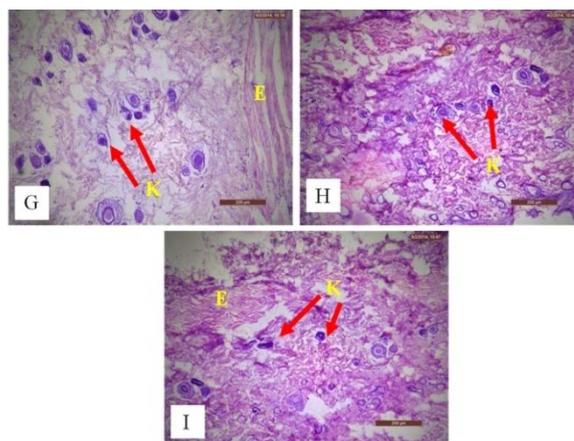


Fig. 8: Histological slides of H and E staining of skin treated with, G) UV and 1% GTE (LT) at 10 X and H) UV and 2% GTE (LT) formulation at 10 X, I) UV and 4% GTE (LT) formulation at 10 X

*(GTE = Green tea extract, LT = Loose green tea)

CONCLUSION

On the basis of DPPH activity, GSH assay, biophysical and microscopic studies, it is suggested that MEs formulated using EGC may prove beneficial for preventing UV-induced photodamage. Hence, supplementation of skin protectants with GTE may possess a potential to be a cosmeceutical.

CONFLICT OF INTERESTS

Declared none

REFERENCES

- Kaur A, Thatai P, Sapra B. Need of UV protection and evaluation of the efficacy of sunscreens. *J Cosmet Sci* 2014;65:315-45.
- Berman B, Cockerell CJ. Pathobiology of actinic keratosis: Ultraviolet-dependent keratinocyte proliferation. *J Am Acad Dermatol* 2012;68: S10-2.
- Laethem AV, Claerhout S, Garmyn M, Agostinis P. The sunburn cell: Regulation of death and survival of the keratinocyte. *Int J Biochem Cell Biol* 2005;37:1547-53.
- Ullrich SE. Mechanisms underlying UV-induced immune suppression. *Mutat Res* 2005;571:185-205.
- Chatelain E, Gabard B. Photostabilization of butyl methoxydibenzoylmethane (Avobenzone) and ethyl hexyl methoxycinnamate by bis-ethyl hexyl oxyphenol methoxyphenyl triazine (Tinosorb S), a new UV broadband filter. *Photochem Photobiol* 2001;74:401-6.
- Sapra B, Thatai P, Bhandari S, Sood J, Jindal M, Tiwary AK. A critical appraisal of microemulsions for drug delivery. *Ther Delivery* 2013;5:1649-60.

7. Bianchi A, Trotta V, Scalia S. Photostability of epigallocatechin-3-gallate in topical formulations-effect of sunscreens and antioxidant agents. *H&PC Today* 2013;8:18-22.
8. Madaan R, Bansal G, Kumar S, Sharma A. Estimation of total phenols and flavonoids in extracts of *Actaea acuminata* roots and antioxidant activity studies. *Indian J Pharm Sci* 2011;73:666-9.
9. Brand-Williams W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. *LWT-Food Sci Technol* 1995;28:25-30.
10. Molyneux P. The use of the stable free radical diphenyl picrylhydrazyl (DPPH) for estimating antioxidant activity. *J Sci Technol* 2004;26:211-9.
11. Muller-Decker K, Heinzekmann T, Furstenberger G, Kecskes A, Lehmann WD, Marks F. Arachidonic acid metabolism in primary irritant dermatitis produced by patch testing of human skin with surfactants. *Toxicol Appl Pharmacol* 1998;153:59-67.
12. <http://www.fda.gov/ohrms/dockets/dailys/04/oct04/101904/04n-0050-rpt0002-Tab-19-vol9.pdf>. [Last accessed on 10 Feb 2015].
13. Casagrande R, Georgetti SR, Verri WA, Dorta DJ, dos Santos AC, Fonseca MJ. Protective effect of topical formulations containing quercetin against UVB-induced oxidative stress in hairless mice. *J Photochem Photobiol B* 2006;84:21-7.
14. Singh B, Sharma B, Jaggi AS, Singh N. Attenuating effect of lisinopril and telmisartan in intracerebroventricular streptozotocin induced experimental dementia of Alzheimer's disease type: possible involvement of PPAR- γ agonistic property. *J Renin Angiotensin Aldosterone Syst* 2013;2:124-36.
15. Vicentini FMC, Fonseca YM, Pitol DL, Iyomasa MM, Bentley MVLB, Fonseca MJV. Evaluation of protective effect of a water-in-oil microemulsion incorporating quercetin against UVB-induced damage in hairless mice skin. *J Pharm Pharm Sci* 2010;13:274-85.
16. Buenger J, Ackermann H, Jentzsch A, Mehling A, Pfitzner I, Reiffen KA, Schroeder KR, et al. An interlaboratory comparison of methods used to assess antioxidant potentials. *Int J Cosmet Sci* 2006;28:135-46.
17. Oh JH, Kim EH, Kim JL, Kang JS. Study on the antioxidant potency of green tea by DPPH method. *FASEB J* 2007;21:700-10.
18. Dutta AK, Siddiquee MA, Hossain S, Kabir Y. Finlay green tea possesses the highest *in vitro* antioxidant activity among the 20 commercially available tea brands of Bangladesh. *Malays J Pharm Sci* 2013;11:11-20.
19. Al-Malah K, Mousa H, Al-Remawi. Microemulsions of potassium oleate, tween-20, propylene carbonate, and ethylene glycol as drug vehicles for mefenamic acid. *J Dispersion Sci Technol* 2011;32:1509-15.
20. El-Laithy HM. Preparation and physicochemical characterization of dioctyl sodium sulfosuccinate (aerosol OT) microemulsion for oral drug delivery. *AAPS PharmSciTech* 2003;4:80-9.
21. Thakar H, Nangesh J, Parmar M, Patel D. Formulation and characterization of lipid-based drug delivery system of raloxifene microemulsion and self micro emulsifying drug delivery system. *J Pharm Bioallied Sci* 2011;3:442-8.
22. Patel PP, Shah SK, Patel KN, Patel MS. A detailed review on sustained release drug delivery system. *Int J Med Pharm Res* 2013;1:1145-53.
23. Martin AN. Coarse dispersions. In: Martin A, Bustamante P, Chen AHC. Eds. *Physical Pharmacy: Physical Chemical Principles in the Pharmaceutical Sciences*. Philadelphia, Lea & Febiger; 1993. p. 477-511.
24. Park KM, Kim CK. Preparation and evaluation of flurbiprofen-loaded microemulsion for parenteral delivery. *Int J Pharm* 1999;181:173-9.
25. Sun Y, Hung WC, Chen FY, Lee CC, Huang HW. Interaction of tea catechin (-)-epigallocatechin gallate with lipid bilayers. *Biophysical J* 2009;96:1026-35.
26. Mochizuki M, Yamazaki S, Kano K, Ikeda T. Kinetic analysis and mechanistic aspects of autooxidation of catechins. *Biochim Biophys Acta* 2002;1569:35-44.
27. Su LY, Leung LK, Huang Y, Chen ZY. Stability of theaflavins and catechins. *Food Chem* 2003;83:189-95.
28. Williams AC, Barry BW. Penetration enhancers. *Adv Drug Delivery Rev* 2004;56:603-18.
29. Sapra B, Jain S, Tiwary AK. Transdermal delivery of carvediol in rats: probing the percutaneous permeation enhancement mechanism of soybean extract-chitosan mixture. *Drug Dev Ind Pharm* 2009;35:1230-41.
30. Niwa Y, Lizawa O, Ishimoto K, Akamatsu H, Kanoh T. Age-dependent basal level and induction capacity of copper-zinc and manganese superoxide dismutase and other scavenging enzyme activities in leukocytes from young and elderly adults. *Am J Pathol* 1993;143:312-20.
31. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun* 1976;71:952-8.
32. Katiyar SK, Afaq F, Perez A, Mukhtar H. Green tea polyphenol (-)-epigallocatechin-3-gallate treatment of human skin inhibits ultraviolet radiation-induced oxidative stress. *Carcinogenesis* 2001;22:287-94.
33. Lua MJ, Chenb C. Enzymatic tannase treatment of green tea increases *in vitro* inhibitory activity against N-nitrosation of dimethylamine. *Process Biochem* 2007;42:1285-90.
34. Hong YH, Jung EY, Shin KS, Kim TY, Yu KW, Chang UJ, et al. Photoprotective effects of a formulation containing tannase-converted green tea extract against uvb-induced oxidative stress in hairless mice. *Appl Biochem Biotechnol* 2012;166:165-75.