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Biocompatible Nanoparticles for Sustained Topical Delivery of Anticancer Phytoconstituent Quercetin

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Abstract: This study describes the release and retention of a herbal lipophilic drug in sustained and controlled manner in skin layers, given topically, intended for skin cancer. Quercetin -loaded nanoparticles were prepared by nanoprecipitation technique using ethylcellulose as polymer. Ethylcellulose was selected as it is biocompatible, but non-biodegradable and hence can act as a reservoir in skin furrows and ducts. It was observed that the Quercetin: Ethylcellulose: Tween 80 at different ratios affects particle sizes along with yield and entrapment efficiency. It was found that the size of nanoparticles could be varied by changing the speed of agitation and sonication. The nanoparticles were prepared in particle size range 228.77±2.0 nm and the zeta potential of the selected formulation were found to be -16.7 mV, which shows the stability of the preparation. The percent entrapment efficiency was found to be in the range from 51.96 to 53.93% and percent loading capacity in the range 34.19 to 5.12%. The amount of drug release from nanoparticles and of drug retained in skin was compared using *ex vivo* study which shows that the drug being lipophilic could be retained in the skin for longer duration thus reducing the dose and frequency of drug administration. Further the amount of drug reaching to other organs is also reduced since the systemic absorption of drug was low. Thus, Quercetin loaded nanoparticles were prepared for topical use.

Key words: Nanoprecipitation technique, ethylcellulose, skin retention, topical nanoparticles, antioxidant flavonoid

INTRODUCTION

In the skin cancer outer layer of skin become cancerous; which was treated traditionally by the use of herbal juices or extracts. The poultices of roasted onions and blood root were used by Americans against skin cancer. This erodes the skin to draw bout cancers and tumors. Polyphenolic phytoconstituents have various properties like anti-aging, antioxidant, hydrating, photo protection, astringent, anti-irritant and antimicrobial activity (Kaur and Saraf, 2011; Adesegun *et al*., 2008; Akond *et al*., 2011). The phenolic compounds obtained by varied herbal sources reduce the oxidative damage (Kaur and Saraf, 2012; Ashawat *et al*., 2007; Atrooz, 2009).

Quercetin (3,3', 4', 5,7-pentahydroxylflavone) is a flavonoid obtained from fruits and vegetables like apple, onion, tea, berries and brassica. Quercetin shows anti-proliferative effects and aids to the effectiveness of chemotherapeutic agents and is effective against ultra-violet radiation induced damage (Scambia *et al*., 1992).

Effects of herbs can be predetermined more accurately than newly approved synthetic drugs. Synthetic drugs might have some side effects but herbal drugs frequently face the problems related to dose. The herbs to be effective should be given in proper interval and proper dosage. Here, comes the use of a novel delivery system to make the drug release pattern predetermined (personal communication). The nanoparticles were found to be a good alternative owing to its controlled and sustained delivery and stability (Mohanraj and Chen, 2006).

Many *in-vitro* studies reveal that the quercetin obtained from onion, inhibits the growth of cancerous cells without affecting the normal cells growth. Further studies are required to establish the utility of quercetin. Hence, the comparison of drug release and drug retention of quercetin loaded nanoparticles was done. The study mainly focuses on the topical treatment of skin cancer with a drug of herbal origin and with reduced side effects to other organs of the body (personal communication).

Anticancer activity of quercetin has not been explored clinically because of low absorption when given orally. A study shows that to reach the therapeutic level 10 μM, 1500 mg of daily dose is required which is

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practically not beneficial (Hollman *et al*., 1997). The poor solubility and low stability of quercetin in aqueous alkaline medium also restricts its application in oral use (Van der Woude *et al*., 2003). The aim of the study was to deliver quercetin topically to show its beneficial effect on skin cancer coping with all above drawbacks. Though yet to be explored, topical chemotherapy is a well-known agenda in this era (Nogueira *et al*., 2011). Transdermal system is desired to maintain a constant and prolonged drug level with reduced frequency of dosing (Pattnaik *et al*., 2011).

Lipid nanocarriers adsorb to skin surface and allow lipid exchange between the outermost layers of the stratum corneum (Vyas *et al*., 2012). There is a need for delivery system with localized and controlled delivery of drugs for topical skin ailments (Singh *et al*., 2010).

Nanoparticles have versatile potential for efficient exploitation of different drug delivery formulations and routes because of the properties provided by their small size (Saraf *et al*., 2011a, b). There possible benefits include controlled release, protection of the active pharmaceutical ingredient and drug targeting. Nanoparticle can be here preferably used as a carrier for controlled release of quercetin in skin (Song *et al*., 2011). Thus nanoparticles have greater efficiency in enhancing the permeation of drugs into skin than many other vehicles (Chanchal and Swarnlata, 2008). Nanoparticles could be suitable for the encapsulation of bioactive compounds (such as flavonoids, vitamins, among others) (Pool *et al*., 2012). Ethylcellulose was considered a good option as it is biocompatible but non-biodegradable and hence can act as reservoir in skin furrows and ducts. The experiment was designed to formulate the nanosized particles that can reside in the outer layers of the stratum corneum and epidermis, with negligible penetration into the dermis (Tan *et al*., 2011). In this study the use of quercetin was explored as anti-cancer agent, with the aid of nanoparticle as delivery system.

MATERIALS AND METHODS

The study was carried out from Jan. 2010 to May 2010. Quercetin was purchased from Sigma Laboratories. Ethylcellulose and Potassium bromide were obtained from Hi-media laboratories Pvt. Ltd. Tween-80, IPA and Octanol were obtained from Loba Chemie. Pvt. Ltd. All the other chemicals and reagents used were of analytical grade.

Instrumentation: Absorbance was recorded using UV- Visible spectrophotometer (Shimadzu, Pharmaspec-1700) and interaction study was done using FTIR (Shimadzu 8400S FTIR spectrometer). The TEM analysis was performed from sophisticated analytical instrumentation facility, New Delhi. The particle size and zeta potential was measured using Zetasizer (Malvern DTS, UK) from CIF, BITS Ranchi.

Preformulation studies: Quercetin is a yellow colored, odorless and crystalline compound. Its melting point was found to be 316°C, which confirms its stability at room temperature under working conditions. The quercetin was found to be soluble in most of the organic solvents and was insoluble in distilled water showing its hydrophobic nature. The partition coefficient of quercetin was found 2.96, which confirm the lipophilicity of the drug and hence the nanoparticulate delivery of the drug offers a good alternative for the water insoluble drugs.

Interaction study: The drug polymer mixture was prepared at 1:2 ratio and was sealed in a Teflon-lined screw caped vial and stored at 50°C for 2 weeks. On periodically examining no unusual color change was found. After 2 weeks, dilutions of sample (in Isopropyl alcohol) were analyzed using UV-Visible spectrophotometer at 381 nm against blank. The FT-IR spectra of mixture in the frequency range between 4000 and 400 cm^{-1} were compared against pure drug spectra (Raju *et al*. 2009).

Preparation of nanoparticles systems: Drug-loaded ethylcellulose nanoparticles were prepared by desolvation- solvent evaporation method (Mukherji *et al*., 1990; Ravikumara *et al*., 2009). Different samples were prepared by varying the concentration of stabilizer and were visualized by transmission electron microscopy (TEM) (Morgagni 268-D). The formulation with 2% tween-80 was found non-aggregated, so further preparation were prepared using the same, varying drug-polymer ratio.

Characterization of nanoparticles (Hirsjarvi, 2008): The characterization of nanoparticles was performed by the determination of entrapment efficiency and the *in vitro* release study. Further particle size analysis, zeta potential determination and morphology study was performed.

Drug entrapment efficiency and loading capacity: For determination of drug entrapment, the suspension was centrifuged at 15,000 rpm for 30 min in a micro ultra-centrifuge. The supernatant was analyzed spectrophotometrically for quercetin content at 257 nm. The percent entrapment was calculated from the formula:

$$\frac{W_{sys} - w_{sup}}{W_{sys}} \times 100$$

The percent loading capacity was calculated from the formula:

$$\frac{W_{sys} - w_{sup}}{W_{sys} + P} \times 100$$

Where:

W_{sys} = The amount of drug added to the system
W^{sup} = The amount of drug in the supernatant
P = The amount of polymer added to the system

***In vitro* release studies:** *In vitro* release studies were performed using modified Franz diffusion cell. Dialysis membrane was prepared by treating the cellophane membrane (Jain *et al*., 2011; Waghmare *et al*., 2011). The prepared membrane was stored in the saline phosphate buffer (pH 4.5). Phosphate buffer pH 5.6 containing 0.5% w/v of polysorbate 80 was used as release media. Nanoparticle dispersion (2 mL) was placed in the donor compartment and the receptor compartment was filled with 0.5% polysorbate 80 in phosphate buffer, pH 5.6 (40 mL). During the experiments, the solution in receptor side was maintained at 37±0.5°C and stirred at 800 rpm with Teflon-coated magnetic stirring bars. At fixed time intervals, 2 mL of the sample was withdrawn from receiver compartment and analyzed by UV spectroscopy. The kinetic models used were zero order equation, first order equation, Higuchi release and Korsemeyer-Peppas (Bourne, 2002; Higuchi, 1963).

Optimization of formulation: It was done by response surface method using stat-ease 7.1.6 software (Li *et al*., 2011). Optimum formula was developed which designates the level of independent variable that results in maximum percent drug entrapment efficiency and percent drug loading capacity with best release kinetics.

***Ex-vivo* skin penetration study:** A system employing modified Franz diffusion cells with a diffusional area of 2.50 cm^2 was used for penetration studies. The prepared goat skin was set in place with the stratum corneum facing the donor compartment and the dermis facing the receptor compartment (Pillai *et al*., 2010; Kumar and Verma, 2010; Mamatha *et al*., 2009; Saraf *et al*., 2011c). The donor compartment was filled with 2 mL of nanoparticle preparation and the receptor compartment of the cell was filled with 40 mL of phosphate buffer (pH 7.4) with 0.5% polysorbate 80 wherein was maintained at 37±0.5°C and stirred at 800 rpm with Teflon-coated magnetic stirring bars. From the receptor side 2 mL aliquots were collected at designated time intervals and an equivalent volume of receptor fluid was supplied to the receiver compartment immediately after each sample collection. The samples were analyzed using UV-VIS spectrophotometer at 257 nm and at each sampling time points the cumulative amount diffused Q (mg cm^{-2}) was calculated. At the end of 24 h, the skin was cut, into and small pieces and extracted, with isopropyl alcohol and analyzed spectrophotometrically at 257 nm.

Surface/shape morphology of nanoparticles: Samples were studied by placing a drop of nanoparticle formulation on to a copper grid and air dried, followed by negative staining with a drop of aqueous solution of sodium phosphotungstate for contrast enhancement. The air-dried sample was examined under the transmission electron microscope. The study was performed from AIIMS Sophisticated Analytical Instrumentation facility, New Delhi.

Particle size and zeta potential determination: The particle size and zeta potential of nanoparticles were determined by Dynamic Light Scattering (DLS) method using a Malvern Zetasizer 4700 (Malvern Ltd., Malvern, UK) with a 25 mW He-Ne laser and the Automeasure (version 3.2) software.

Statistical analysis: Data were expressed as mean±standard deviation (SD). Statistical analysis was carried out by one-way Analysis of Variance (ANOVA) followed by Dunnett's test using Graph Pad Prism 3 (Graph Pad Software, Inc., La Jolla, CA, USA) and the differences were considered as statistically significant at ***$p<0.001$.

RESULTS AND DISCUSSION

Quercetin is polyphenolic drug having anti oxidant and anticancerous activity (Di Carlo *et al*., 1999). But, the poor solubility and low stability of quercetin in aqueous alkaline medium (Van der Woude *et al*., 2003) restricts its application in oral use. The aim of this study was to explore quercetin as anticancer agent via sustained topical route. The overall objectives were reduction of dose, sustained release, convenient route and avoidance of side effect to other organs. The drug was characterized for its melting point, partition coefficient, solubility and IR spectra as shown in Table 1 which reveals the primary characteristics of the drug. From the Infra-Red Spectroscopy the presence of aromatic ring, hydroxyl group and carbonyl group presence was revealed as shown in Fig. 1. The absorbance maxima were found to be

Table 1: Preformulation studies of quercetin

Property	Description
Solubility	Soluble in ethanol, methanol, isopropyl alcohol and chloroform
Functional groups present in IR spectra	Aromatic CH stretching (3310 1 cm^{-1}), O-H stretching (3450 1 cm^{-1}),-CO stretching (1685-1660 1 cm^{-1}), Aromatic ring (1000 1 cm^{-1})
Partition coefficient	2.96±0.1

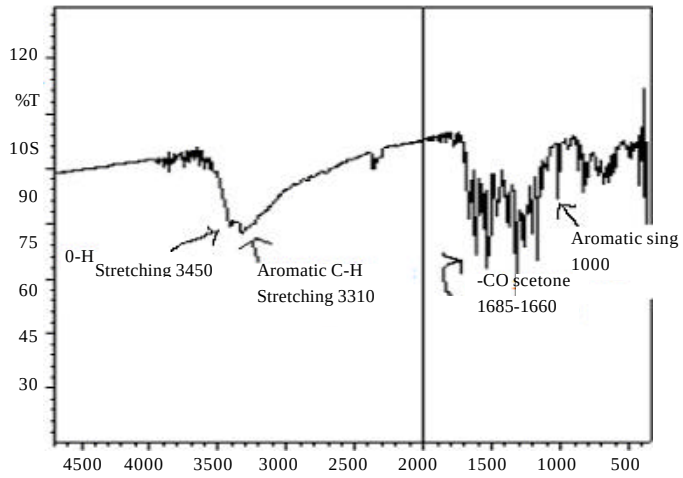

Fig. 1: IR Spectra of quercetin

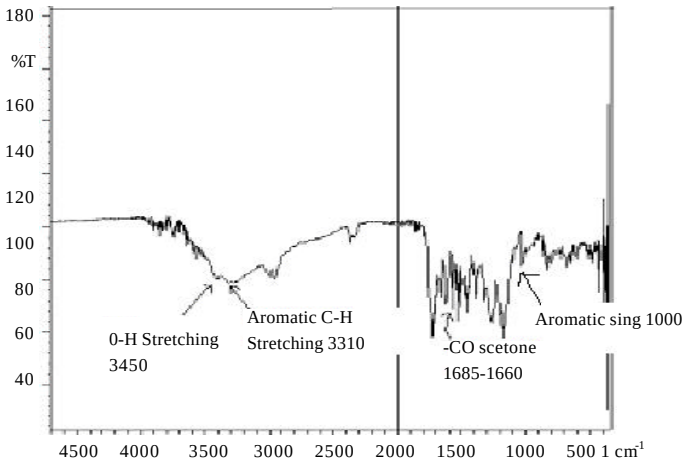

Fig. 2: IR spectra of ethylcellulose and quercetin

257 nm by the UV Spectroscopy. The other most important study performed was the drug-interaction study of the drug and polymers. The presence of the peaks of the pure drug belonging to different functional group of the drug in the drug polymer mixtures (Quercetin and Ethylcellulose) as shown in Fig. 2 confirms the stable nature of the drug in the drug-polymer mixture.

The quercetin-loaded nanoparticles were prepared by nanoprecipitation technique using solvent evaporation method. Quercetin act as anti-cancer agents due to its action like interaction with type-II estrogen binding sites tyrosin kinase inhibition (Lamson and Brignall, 2000). But it has been reported by Hollman *et al.* (1997), that a very low quantity is observed when given orally. A single dose of 100 mg only creates 0.8 μM quercetin concentration in serum but for anti-cancer activity serum quercetin concentration of 10 μM was required. Ultimately the required dose can be extrapolated to 1500 mg. Low bioavailability, higher dose and less stable nature of quercetin have attracted researchers to work on topical delivery of quercetin. In this study an effort was made to develop a delivery

system that can explore the use of quercetin as a weapon against cancer. A research by Alvarez-Roman *et al.* (2004) reports that of fluorescent material containing nanoparticles, fluorescence was perceptible at greater depths (up to 60 micron) within the skin (Alvarez-Roman *et al*., 2004).

By various reviews the utility of this solvent evaporation method for hydrophilic as well as hydrophobic drugs have been studied (Mohanraj and Chen, 2006). The formation of nanoparticles using Ethylcellulose and tween-80 had been confirmed by the formation of dummy nanoparticles. First of all the stability of the nanoparticles were studied with respect to the effect of concentration of stabilizer (tween-80) by keeping the drug: polymer ratio and other parameters constant, as shown in Table 2. It was observed that when 0.1% and 0.5% of tween-80 were used for the preparation of nanoparticles, they were either not prepared or the one prepared were unstable after 1 week. The nanoparticle suspension prepared using 1, 1.5 and 2% tween-80 was found to be stable. Further TEM studies were carried out to reveal the aggregation status. It was found that only formulation prepared using 2% tween-80 (F5) was non-aggregated as depicted in Fig. 3. Hence further preparations were prepared using 2% tween-80 by varying drug: polymer ratio and were named as F5a-F5f. The prior optimization of the surfactant concentration was for the sake of time and materials. These were characterized by various parameters like percent entrapment efficiency (% EE), percent loading capacity (% LC) and *in vitro* release study which was later on fed in to the optimization software as responses. Results of the following parameters are shown in Table 3. The % EE was found to range from 51.96 to 53.93% and % LC was found to range from 34.19 to 5.12%.

Release profile of quercetin from all the formulations (F5a-F5f) was fit into various kinetic models to find out the mechanism of drug release. Among various models highest correlation coefficient was shown in Higuchi plot as depicted in Table 4. The data obtained was also fit in to the Krosemeyer–Peppas in order to find out the 'n' value, to describe the drug release mechanism. The 'n' value ranged between 0.297 and 0.483 and was found to be less than 0.5 indicating that the mechanism of drug release is diffusion controlled (Fickian diffusion) and Korsmeyer-Peppas model showed high correlation between each other. The results were in congruence with the release kinetics produced by nimesulide-loaded ethylcellulose nanoparticles (Ravikumara *et al*., 2009).

The results as depicted in the Table 3 show that with the increase in polymer quantity the percent entrapment efficiency was increased while percent loading capacity and percent *in vitro* release was decreased. Further optimization of best formulation ratio was done by response surface method using stat-ease 7.1.6 software taking the percent drug entrapment efficiency, percent drug loading capacity and release kinetics as responses in factorial design. Condition for the selection of optimum

Table 2: Optimization of percentage of tween-80

Formulation code	Drug : polymer(ethyl cellulose) (D:P) ratio	Percent of stabilizer (tween-80) used (%)	Initial Status of nanoparticles	Status of nanoparticles after 1 week
F1	1:1	0.1	Not prepared	--
F2	1:1	0.5	prepared	unstable
F3	1:1	1.0	Prepared	Stable
F4	1:1	1.5	Prepared	Stable
F5*	1:1	2.0	Prepared	Stable

*Optimized preparation

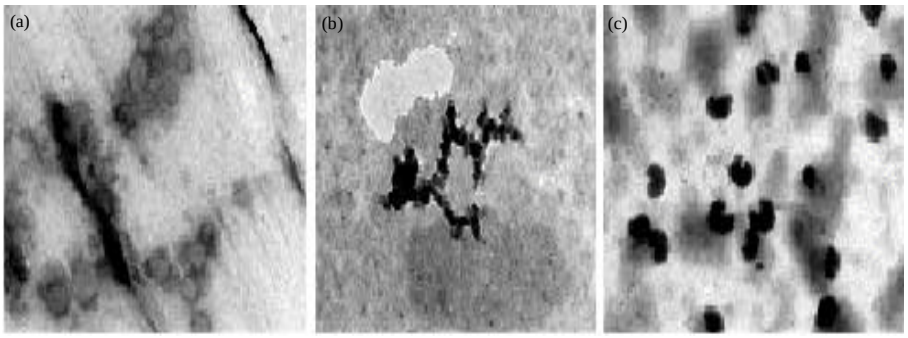

Fig. 3: TEM image of (a) Formulation F3: Aggregated, (b) Formulation F4: Aggregated and (c) Formulation F5: Not aggregated

Table 3: Characterization of ethylcellulose nanoparticles

Formulation code	Drug: polymer (ethyl cellulose) ratio	Amount of drug (mg)	Amount of polymer (ethyl cellulose) (mg)	Percent v/v of stabilizer (tween-80) used	Percent Entrapment Efficiency (EE) (Response 1)	Percent Loading Capacity (LC) (Response 2)	Percent *In-vitro* release after 24 h (Response 3)
F5a	1:1	25	25	2	51.96±0.35***	34.19±0.42***	50***
F5b	1:2	25	50	2	52.26±0.08***	20.71±0.03***	39.9***
F5c	1:4	25	100	2	52.76±0.12***	11.65±0.02***	28.6***
F5d	1:6	25	150	2	52.72±0.04***	8.076±0.01***	27.6***
F5e	1:8	25	200	2	52.73±0.05***	6.16±0.01***	26.8***
F5f	1:10	25	250	2	53.93±0.11***	5.12±0.02***	12.6***

*The statistical analysis of the experimental data by the one-way ANOVA was performed and the differences were considered as statistically significant at ***p<0.001

Table 4: *In-vitro* release profile of quercetin

				Kroseneyer-peppas	
Formulation code	Higuchi model r	Zero-order r	First-order r	k	n
F5a	0.9900	0.915	0.9586	0.9887	0.4839
F5b	0.9529	0.8638	0.8973	0.9565	0.4627
F5c	0.994	0.9557	0.9714	0.9947	0.4156
F5d	0.9534	0.9758	0.9792	0.9419	0.2973
F5e	0.9964	0.9922	0.9968	0.9749	0.4348
F5f	0.9329	0.9859	0.9854	0.948	0.4405

Table 5: Tables generated from software by Design summary (ECN) and solutions for 6 combinations of categoric factor levels (ECN)

Design summary: ECN

Study type:	Factorial
Initial design:	Full factorial
Design model:	Main effects
Runs:	6

Response	Name	Units	Minimum	Maximum	Mean	Std.dev	Ratio
Y1	EE	%	51.96	53.93	52.72	0.67	1.03
Y2	LC	%	5.12	34.19	14.31	11.25	6.67
Y3	DR	%	12.6	50	30.91	12.75	3.96

Solutions for 6 combinations of categoric factor levels: ECN

Number	Drug: polymer	EE	LC	DR	Desirability
1	1:2	52.26	20.71	39.9	0.391
2	1:4	52.76	11.65	28.6	0.339
3	1:6	52.72	8.076	27.6	0.251
4	1:8	52.73	6.16	26.8	0.174

EE: Entrapment efficiency, LC: Loading capacity, DR: Drug release

formulation was, maximum magnitudes for all the 3 responses. The data generated from the software are shown in Table 5 according to which four solution ratios were found as for 6 combinations of categoric factor levels. The best formulation F5b from among the above solutions was selected on the basis of highest desirability score.

A further *ex vivo* study of this selected formulation (F5b) was carried out in order to demonstrate the retention of drug in the skin to extend the duration of action and the applicability of nanoparticles for topical delivery. The goat skin was taken for the purpose (Saraf *et al*., 2011c). The *ex vivo* drug release was compared with the skin retained in the skin, a major portion was retained in the skin hence reducing approach of drug to other organs through systemic route and availability of drug to the desired skin tissue for a comparatively longer duration of time. From the percent cumulative drug permeated versus time plot, the slope values were determined as the skin permeation rate. The cumulative amount of drug permeated at the end of 24 hwas found to be 78.4 μg cm^{-2} with skin permeation rate constant of 0.4559 percent/cm^2/h. The release of drug from these formulations followed a Fickian pattern with n value 0.423-0.426. A greater amount of drug was present in the skin (25.56%) subjected to the extraction and compared with the drug released in the receptor compartment 19.6%, shown in Fig. 4. The slower release of drug from nanoparticle dispersions maintained the drug in skin for longer period of time. The retention of greater percentage of drug in skin shows that the high drug concentration was maintained in the skin as compared to the amount released in the blood stream hence skin targeting was achieved. Further the zeta potential and particle size of these selected formulation were found to be -16.7 and 228.77±2.0 nm, respectively which confirms the stable

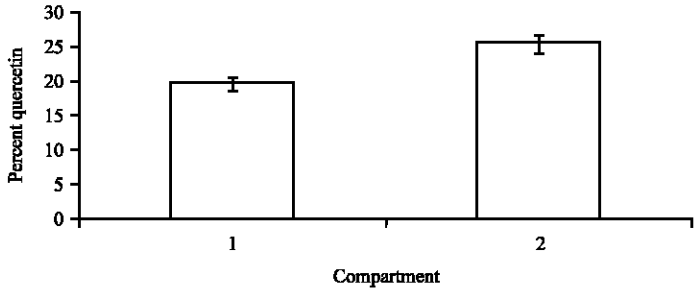

Fig. 4: Comparison of drug levels in 1: recipient compartment and 2: skin

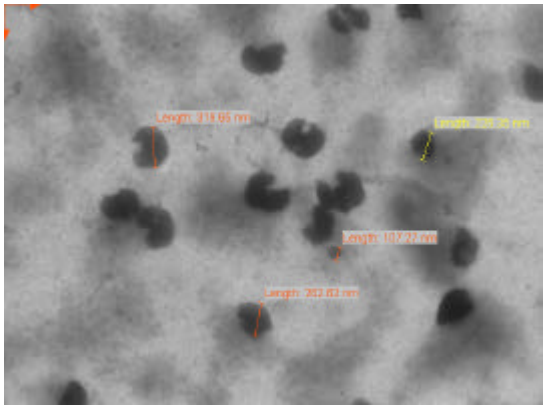

Fig. 5: TEM of formulation F5b

nature and appropriate particle size of the preparation. TEM image of the optimized formulation is shown in Fig. 5. Thus the quercetin loaded nanoparticles were successfully prepared for the topical sustained effect.

Nanometric systems have a great surface area, which renders them highly satisfactory for the application of lipophilic substances promoting a homogeneous drug release (Bouchemal *et al*., 2004). As quercetin is lipophilic in nature the high surface area of nanoparticulated systems could have played an important role in penetration in the different layers of skin and might have facilitated the contact with the stratum corneum. Similarly (Alvarez-Roman *et al*., 2001, 2004) had also prepared nanometric systems of a sunscreen, the Octyl Methoxycinnamate (OMC) and found that it remained in the skin layers but did not reach systemic circulation. As discussed by Guterres *et al*. (2007) the polymeric nanoparticles intended for cutaneous delivery could be prepared with biocompatible polymers generally presenting particle diameters around 200 to 300 nm. (Guterres *et al*. 2007). Pople and Singh had prepared solid lipid noparticles of Vitamin A in the size range 350 nm and reported their localized action in the skin (Pople and Singh, 2006). In the present study the size range of the prepared nanoparticle formulations was 228.77±2.0 nm which could also be one of the reasons for the retention of drug in the skin. While Wu *et al*. (2008), prepared quercetin-loaded nanoparticles having particle size less than 85 nm and correlated with the improvements in physicochemical characterization and dissolution property and enhanced antioxidant effect (Wu *et al*., 2008). Thus, nanoparticles increase the drug adhesivity or its time of permanence in the skin and can be used as reservoirs of lipophilic drugs to deliver them in the stratum corneum or could be reduced in size and by the alteration of physicochemical properties could also be given systemically.

CONCLUSION

The quercetin loaded nanoparticles were prepared by selecting the proper excipients and optimized process variables. The change in polymer concentration affected the nanoparticle size and release performance. The colloidal carrier, being sub micron in size, enhances the drug penetration into the skin layers, but because of lipoidal nature and size more than 200 nm, the penetrated drug concentrates in the skin and remains localized there for a longer period of time, thus enabling drug targeting to the skin. The sustained release and retention study of drug suggest that the frequency of administration and dose could be reduced. Moreover adverse effect to other organs could also be minimized, as compared to when the same was given through other routes. In future a greater interest should be focused on nanoparticle of quercetin, as it's a potential anti-cancer agent.

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