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Original Article

EXTRACTION OF A WATER SOLUBLE BIOACTIVE HYPOXOSIDE AND ITS DEVELOPMENT INTO AN ETHOSOMAL SYSTEM FOR DEEP DERMAL DELIVERY

SEBENTA MENON#, DEVINA VERMA#, TAHIR KHUROO, SUSHAMA TALEGAONKAR, ZEENAT IQBAL*

Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, New Delhi 110062, India
Email: ziqbaljh@yahoo.co.in

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ABSTRACT

Objective: This study was aimed to extract Hypoxoside, a water soluble phytochemical, from the corms of *Hypoxis hemerocallidea*, and incorporate it into a suitable transdermal carrier system to increase its penetrability and deep dermal delivery for potential antioxidant and anticancer activity.

Methods: The extraction of *Hypoxis hemerocallidea* corms was carried out by continuous hot extraction method. This extract (20 mg/ml) was loaded into ethosomal vesicular system by cold method and optimized by varying proportions of lecithin and ethanol. The optimized system was then subjected to characterization in terms of particle size, polydispersity index (PDI), entrapment efficiency and invitro permeation and penetration studies.

Results: The optimized vesicle with size of 176.2±11 nm, PDI of 0.231 and entrapment efficiency of 74.2±2.3% was obtained which showed a sustained release pattern of the hypoxoside from the vesicular system. Confocal laser scanning microscopy (CLSM) demonstrated that the vesicles were able to efficiently traverse the skin to a depth of 117.29 µm whereas the mechanism of vesicle-skin interaction was confirmed by histopathological study.

Conclusion: The study indicated that with the development of an efficient delivery system a water soluble phytochemical with antioxidant and anticancer properties can be efficiently delivered to the skin.

Keywords: Hypoxoside, Rooperol, Transdermal, Skin Cancer, Antioxidant.

INTRODUCTION

Hypoxis hemerocallidea Fisch. (Hypoxidaceae), commonly known as hypoxis, African potato, is a plant native to the savanna regions of South Africa, Swaziland and Zimbabwe [1, 2]. This herb has been widely used in traditional African medicine for centuries for the alleviation of numerous diseases exhibiting activities such as anti-inflammatory, antioxidant, anti-epileptic, antidiabetic, antinociceptive, immunomodulatory and anticancer [3-5]. A major phyto constituent of this herb, hypoxoside, a norlignan glycoside, which though inactive undergoes degradation in the presence of an enzyme β-glucosidase, to form an aglycone, rooperol [6]. This enzyme, β-glucosidase, is produced 100 folds more in cancer cells than in normal cells, thus ensuring specific targeting of the cancerous tissue. Rooperol and has been patented in the US as an anticancer agent for skin, colon and lung cancer [7]. Cytotoxic activity of hypoxoside was found against B16-F10-BL-6 mouse melanoma cells in the presence of β-glucosidase, whereas the aglycone (rooperol), was found to be cytotoxic per se [8]. The modern day practice demands an assimilation of a drug (natural or synthetic) into a novel drug delivery system in order to make it suitable and successful conduit for targeting drug to desired site. Carriers like solid-lipid nanoparticles, nanostructured lipid carriers, nano emulsion gels have been suitably used for skin delivery [9-11]. More recently, ethanolic vesicular systems called ethosomes with an array of advantages have emerged as an acceptable dermal delivery tool specifically in skin conditions where the therapeutic requirement amounts to targeting drug delivery to deeper skin layers [12, 13]. These vesicles are composed of ethanol, phospholipid, and water. The presence of ethanol, a well-known permeation enhancer provides elasticity to the vesicles as well as increases fluidity of the intercellular lipids which in synergism with the ability of the vesicular lipids to fuse with skin lipids enhance the efficacy of the ethosomal carriers to release the drug in the deeper layers of the skin [14]. In addition, these vesicular systems have the capacity to load both lipophilic as well as hydrophilic drug. Unlike other vesicular systems, ethosomes can better encapsulate hydrophilic moieties both in the aqueous core of the ethosomal system as well as in the solution form in the ethanol molecules embedded in the lipid bilayer. The study, therefore focuses on developing and optimizing a model transdermal carrier system for improved loading and enhanced delivery of a hydrophilic phytochemical.

Thus, the objective of the study is to extract hypoxoside from the corms of *Hypoxis hemerocallidea* and formulate an optimized ethosomal system encapsulating the extracted bioactive to increase its availability to various skin layers serving as a potential tool in various skin afflictions.

MATERIALS AND METHODS

Chemicals and reagents

The African Potato corms were obtained from The Swaziland Agricultural Research Centre, Malkerns, Swaziland. The corms 4 kg were tested by the Phytosanitary Department of the Ministry of Agriculture in Swaziland and the identity of the species was ascertained as *Hypoxis hemerocallidea* (Hypoxidaceae). The African Potato corms were further authenticated by The National Bureau of Plant Genetic Resources (NBPGR), Pusa Campus, New Delhi. Soybean phosphatidylcholine (Phospholipon 90 G) was obtained as a gift sample from LIPOID, Germany. Ethanol absolute (99.99%) was purchased from Changshu Yangquan Chemical, China. Carbopol 934 was supplied by Ranbaxy laboratories ex-gratia. All other chemicals and reagents were from S. D. Fine Chemical, India.

Extraction of the Hypoxoside: the active phytochemical component

The extraction of *Hypoxis hemerocallidea* corms was carried out by continuous hot extraction method [15]. In the preparation of the African Potato extract, the following 3 main procedures were followed:

Preparation of dried coarse powder of the african potato corms

The fresh corms (3 kg) were washed properly using tap water. The adventitious roots were removed and the outer covering of the corm was peeled-off. The fresh corms (2 kg) was cut into small pieces and put in an oven at 45 °C for 12 h, with occasional removal from the oven every 3 h. Dried coarse powder (800 g) was successfully obtained and stored in an airtight container for future use.

Extraction of the dried coarse powder with methanol using a Soxhlet apparatus

The dried coarse powder (200 g) was exhaustively extracted with methanol (1000 ml) using the Soxhlet apparatus for 36 h to obtain the methanolic extract.

Concentration of the methanolic extract under reduced pressure

The thick methanolic extract was then further concentrated under reduced pressure using a rotary evaporator to obtain a viscous dark brown residue (170.5 g, 85.25 % yield). The viscous residue was stored in refrigerator at 2 °C for further use.

Enrichment of extract

The dried extract (100 g) was re dissolved in water (250 ml) and partitioned with chloroform (250 x 3). The aqueous layer and the chloroform layer were separated and concentrated to dry residue.

Preparation of ethosomes (Placebo and hypoxoside loaded vesicles)

Ethosomes were prepared by cold method [16]. Briefly, lecithin was weighed and dissolved in ethanol. Specific amount of distilled water with or without hypoxoside (20 mg/ml) was taken. To this, ethanolic mixture was transferred dropwise while stirring at 1000 rpm using a magnetic stirrer for 20 min in a closed assembly to prevent evaporation of ethanol. The whole assembly was maintained at 30 °C throughout the process. The ethosomes so formed were then transferred into glass vials and stored in a cool, dry place. In order to attain the optimized formulations, lecithin and ethanol concentration was taken as independent variables while the PDI, particle size and entrapment efficiency were taken as dependent variables. Soya lecithin (2, 2.5% and 3%) and ethanol (30, 40, 45%) concentration were taken at three different levels.

Vesicle size and size distribution

The mean size and polydispersity index of the vesicles were analyzed by dynamic light scattering technique also known as photon correlation spectroscopy using Zetasizer 1000 HS (Malvern instruments, UK) The sample was placed in a quartz cuvette diluted with distilled water and size measurements were carried out at a scattering angle of 90 °. All observations were recorded in triplicate for each formulation.

Ethosomal characterization

Vesicular shape and surface morphology

Scanning Electron Microscopy (SEM) was used to characterize the surface morphology of the vesicles. One drop of ethosomal system was mounted on clear glass stub, air dried and coated with Polaron E 5100 Sputter and visualized under Scanning electron microscope (JEOL JSM 840, USA).

Transmission Electron Microscope (TEM) (Philips, CM-10 model) was used as a visualizing aid for vesicles. Samples were dried on carbon-coated grid and negatively stained with aqueous solution of phosphotungstic acid. After drying, the specimen was viewed under the microscope at 10–100 k-fold enlargements at an accelerating voltage of 100 KV.

Entrapment efficiency

The entrapment efficiency was determined with respect to drug concentration of the supernatant. The entrapment efficiency of hypoxoside loaded ethosomal formulation was obtained by the separation of free drug from that entrapped in the ethosomes by ultracentrifugation at 10000 rpm for 40 min at 4 °C. The supernatant containing free hypoxoside was collected and analyzed by UV spectroscopy at 258 nm. The encapsulation efficiency was calculated using equation (1)

$$\text{Encapsulation efficiency (\%)} = \frac{Total\ drug - Free\ drug}{total\ drug} \times 100 \quad (1)$$

***In vitro* skin permeation and skin deposition**

The rat was sacrificed by cervical dislocation and the abdominal skin was excised. The hair was carefully shaved with the help of a depilatory (Veet, Reckitt Benckiser). The fat adhered on the dermal side of the skin was removed using isopropanol. The Franz diffusion cell with a permeation area and receiver cell volume of 0.636 cm^2 and 12 mL, respectively, was set up by mounting the skin on the receptor compartment with stratum corneum side facing upwards.

The receptor compartment was filled with phosphate buffer solution and maintained at 32 °C±1 °C with constant stirring at 100 rpm. The upper chamber of the Franz diffusion cell was filled with the ethosomal formulation. Sampling was done for 24 h and samples were withdrawn through the sampling port at 30 min, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h and analyzed for drug content by UV spectroscopy at 258 nm. The receptor cell after each withdrawal was simultaneously replenished with an equal volume of fresh phosphate buffer. The study was also repeated with hydroethanolic solution of hypoxoside.

Drug kinetic modeling

The drug release profile from the ethosomal vesicles was determined by finding the best fit kinetic model amongst: zero order, first order, Higuchi and to better determine the release behavior Korsemeyer-Peppas model was further applied [17].

Depth of skin penetration

In order to determine the mechanism of penetration of the vesicles and their capacity to deliver drug along with its potential to traverse skin, confocal laser scanning microscopy, CLSM, was performed**.** Rats were sacrificed and the abdominal skin was removed. The skin was treated with Rhodamine B loaded ethosomal vesicles for 24 h and then observed under confocal microscope with Fluorescence Correlation Spectroscope-Olympus fluo view FV1000 (Olympus, Melville, New York) with an argon laser beam with excitation and emission wavelength of 488 nm and 590 nm respectively.

The skin sample was sliced in sections of 6–10 µm thickness through the z-axis by CLSM. The results were compared with skin sample treated with hydroethanolic solution of the dye.

Vesicle skin interaction

The vesicle skin interaction study was done to observe any ultra structural changes in the skin upon exposure to the vesicular system. The ethosomal formulation (placebo) was applied topically on the rat skin for 24hours. After sacrificing the animals by ether inhalation, skin was excised and fixed in buffered formalin (10% w/v). Sections of skin were stained with haematoxylin and eosin stain and observed for histological changes in stratum corneum, epidermis and dermis under an optical microscope. An untreated skin sample was used as control.

RESULTS AND DISCUSSION

Extraction of the hypoxoside

A viscous dark brown residue was obtained with a percentage yield of 85.25 %. The viscous residue was stored in refrigerator at 2 °C for further use. The aqueous layer gave a percent yield of 94.46 and dissolved majority of the compounds of H. hemerocallidea including hypoxoside. This hypoxoside rich fraction used for further studies. The chloroform extract gave a meager percent yield of 3.68% and consisted mainly of sterols (fig. 1).

Effect of lecithin and ethanol concentration

The optimization of the drug loaded ethosomal vesicles were carried out by varying the amount of lecithin (2%, 2.5%, and 3%) and ethanol (table 1). The result obtained showed that there is a correlation between the vesicle size and concentration of lecithin. When ethanol was kept as constant, the vesicle size increased with increase in lecithin concentration with the smallest vesicle size being 176.2±11 (2% lecithin) and the largest of 485.1±20 nm which was obtained with 3% lecithin. The particle size obtained for the drug loaded formulations ranged from 165.2 to 494.1 nm.

However, no significant difference in size was observed with an increase in ethanol concentration although the PDI decreased even though not in all the cases. Also, increasing the ethanol concentration beyond 40% significantly increased the size of the ethosomes (data not shown).

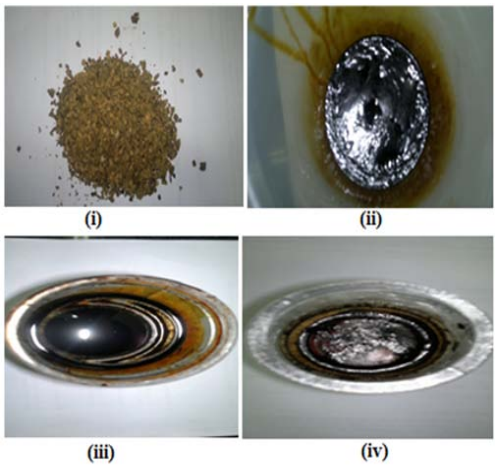

Fig. 1: It shows (i) Dried Coarse Powder of H. hemerocallidea; (ii) Concentrated extract of H. hemerocallidea; (iii) Aqueous Fraction; (iv) Chloroform Fraction

This may be possible due to thinning of the vesicle membrane resulting in aggregation of vesicles. Entrapment efficiency is a measure of suitability of the lipid bilayer of the vesicles to incorporate drug. The entrapment efficiency was affected adversely with an enhancement in ethanol concentration which was responsible for thinning of vesicular membrane allowing only a limited amount of drug incorporation.

The entrapment efficiency was shown to be highest for AP1 with an ethanol concentration of 30 %, which also yielded an acceptable size and PDI and were submitted for further characterization.

Surface shape and morphology

SEM is a dependable tool to ascertain the surface topology of vesicular system. The optimized vesicles showed a spherical shaped vesicle with a smooth surface as evident in fig. 2A and 2B. The TEM microscopy very well corroborated the size of 161.58 nm which was close to the one obtained by the zetasizer. (176.2±11 nm).

***In vitro* permeation**

The success of any dermal formulation strongly relies on the adequate permeation of drug/drug loaded system into the skin; hence the first step towards its evaluation remains the *in vitro* permeation studies. The optimized ethosomal preparation revealed an in vitro percentage release amounting to 77.61% in 24 h while the hydro ethanolic solution of the API showed a permeation of 70.27% in 12 h (fig. 3). The results indicated the sustained and prolonged release behavior of ethosomes which also confirmed the presence and retention of ethosomes within the skin tissue.

Table 1: It shows optimization of ethosomal vesicles showing the effect of independent variables (lecithin and ethanol concentration) on dependent variables (vesicle size, PDI and entrapment efficiency)

Formulation Code	Lecithin (%wt/wt)	Ethanol (%wt/wt)	Distilled water (%wt/wt)	Size (nm)	PDI	Entrapment efficiency
AP1	2	30	q. s	176.2±11	0.231	74.2±2.3
AP2	2	40	q. s	194.5±22	0.211	55.6±2.8
AP3	2.5	30	q. s	262.4±13	0.321	65.21±2.3
AP4	2.5	40	q. s	285.3±18	0.233	46.32±3.3
AP5	3.0	30	q. s	485.1±20	0.432	66.88±4.2
AP6	3.0	40	q. s	452.9±12	0.432	49.34±2.1

Values represent mean±SD (n=3)

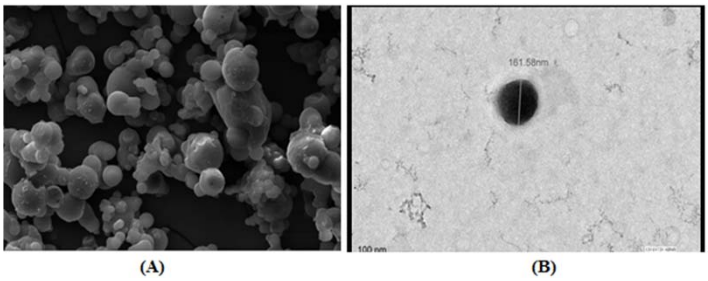

Fig. 2: It shows A) SEM micrographs of optimized formulation AP1; B) TEM micrographs of optimized formulation AP1

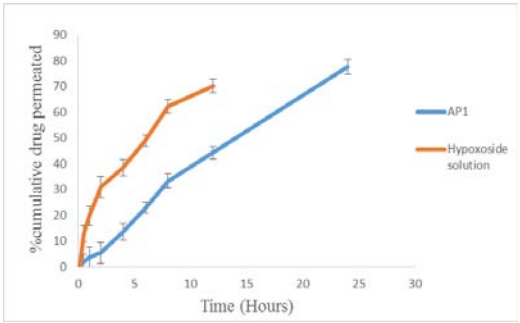

Fig. 3: It shows comparative release profile of optimized ethosomal formulation (AP1) and hydroethanolic solution of hypoxoside (mean±SD, n = 3)

Kinetic drug modelling

Each drug delivery system has a certain mechanism of drug release which is ascertained by fitting the drug release profile into standard drug kinetic models. The IVR profile of the ethosomal system, when subjected to kinetic modeling showed the best fit with zero order release model (concentration independent). Further the data was fitted into the Korsmeyer-Peppas model and the n value was used to determine the nature of drug release.

Since n value was greater than 0.89 it indicated drug release through non-Fickian super case-II transport [18] (table 2 and fig. 4).

Table 2: It shows release rate constants (K), regression coefficient (R^2) and release exponents (n) of optimized ethosomal formulation

Zero order		First order		Higuichi		Korsemeyer-Peppas	
K	R^2	K	R^2	K	R^2	Rel. expo.(n)	R^2
3.316	0.987	3.325	0.985	17.28	0.956	0.984	0.994

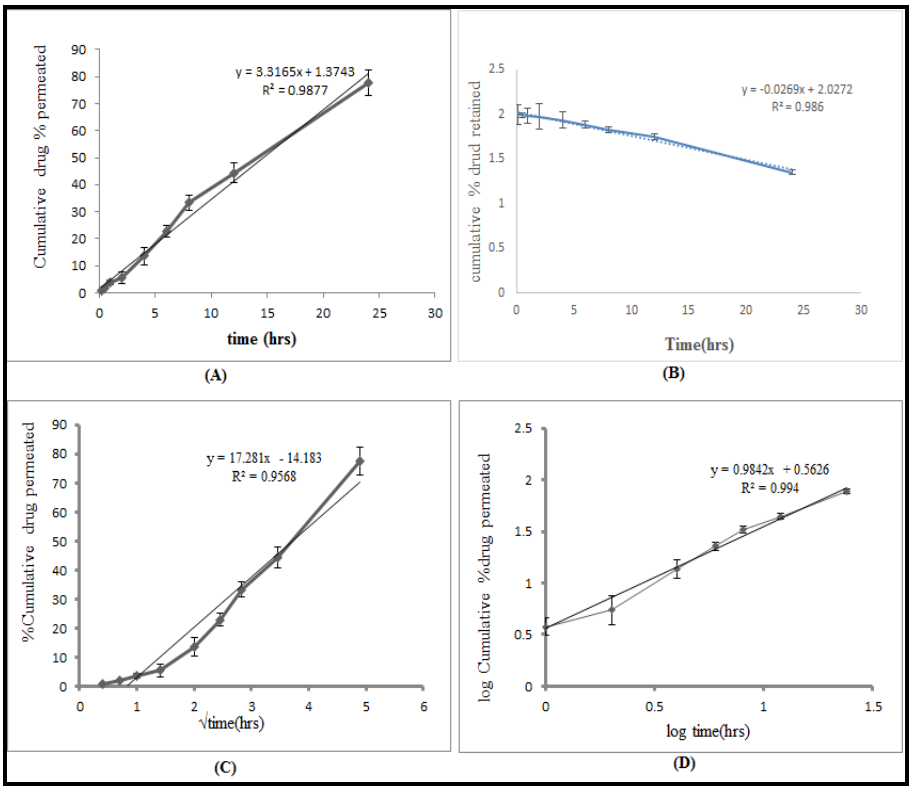

Fig. 4: It shows (A) Zero order (B) First Order (C) Higuchi (D) Korsmeyer-Peppas. Release rate constants (K), regression coefficient (R^2) and release exponents (n)

Confocal laser scanning microscopy

The permeation and retention studies had reflected onto the prolonged presence of the ethosomes in the deep skin layers.

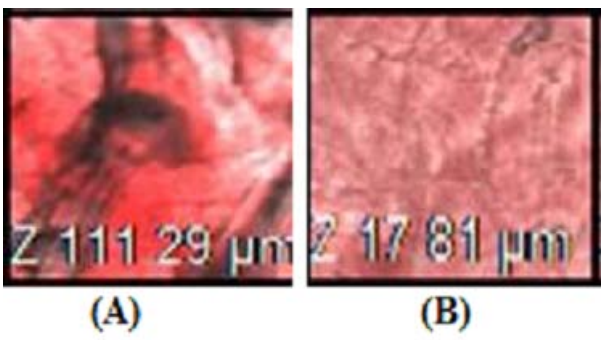

Fig. 5: It shows CLSM images of cross sections of abdominal rat skin after 24-h incubation in Franz diffusion cell A) with Rhodamine B loaded ethosomal vesicles; B) with Rhodamine B loaded hydroethanolic solution

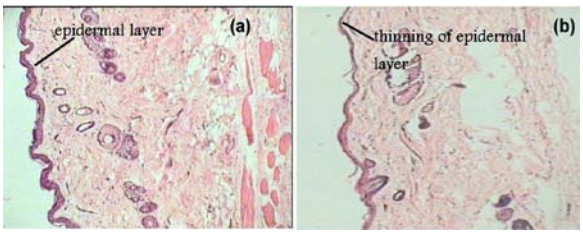

Fig. 6: It shows Histopathological sections of abdominal skin of rat (a) untreated; (b) skin treated with ethosomal vesicles

This entanglement and deposition ensures the suitability of the system for delivering the drug into deeper layers of the skin. Confocal microscopy successfully showed the penetration of drug loaded formulation up to 111.29 µm in z-axis as shown in fig. 5 which was much higher as compared to that of the hydroethanolic solution which reached up to only 17.81 µm.

Vesicle skin interaction

The interaction of the ethosomal vesicles with the different layers of the skin was demonstrated clearly when the ethosome treated and untreated skin samples were examined under the optical

microscope. The ethosome treated skin sample showed thinning of epidermal layer in contrast to the distinct epidermal and dermal layer seen the control skin sample. This indicated the mode of ethosomal entry into the skin. Ethanol leads to extraction of the skin lipids causing disruption in the stratum corneum layer through which the vesicles pass and release the drug in the deeper layers of the skin [19]. Also, safety of ethosomal vesicles was established as ethanol did not show any signs of edema or irritancy.

CONCLUSION

It may be concluded that a popular folklore medicine of African origin, sourced from its native place, yielded a potential anticancer phytochemical hypoxoside which was successfully assimilated into a modern pharmaceutical tool and converted into a nanoethosomal preparation which has shown appreciable permeation through the skin.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

REFERENCES

1. Drewes SE, Khan F. The african potato (*Hypoxis hemerocallidea*): a chemical-historical perspective: review article. S Afr J Sci 2004;100:425-30.
2. Owira PM, Ojewole JA. African potato' (*Hypoxis hemerocallidea* corm): a plant-medicine for modern and 21st century diseases of mankind?–a review. Phytother Res 2009;23:147-52.
3. Ojewole JAO. Antinociceptive, anti-inflammatory and antidiabetic properties of *Hypoxis hemerocallidea* Fisch. and C. A. Mey. (Hypoxidaceae) corm ['African Potato'] aqueous extract in mice and rats. J Ethnopharmacol 2005;103:126-34.
4. Ojewole JA. Anticonvulsant activity of *Hypoxis hemerocallidea* Fisch. and CA Mey. (Hypoxidaceae) corm ('African potato') aqueous extract in mice. Phytother Res 2008;22:91-6.
5. Muwanga C. An assessment of extracts, and actives as natural antibiotic, and immune modulation phytotherapies. MSc. Dissertation. University of the Western Cape, Cape Town; 2006.
6. Bettolo GB, Patamia M, Nicoletti M, Galeffi C, Messana I. Research on African medicinal plants—II: Hypoxoside, a new glycoside of uncommon structure from hypoxis obtusa busch. Tetrahedron 1982;38:1683-7.
7. Drewes S, Liebenberg RW. Rooperol and its derivates; 1987.
8. Theron EJ, Albrecht CF, Kruger PB, Jerkins K, Van Der Merwe MJ. Glucosidase activity in fetal bovine serum renders the plant glucoside hypoxoside, Cytotoxic Toward B16-F10-B1-6 mouse melanoma cells. *In vitro* Cell Dev Biol: Anim 1994;30:115-9.
9. Jun H, Shi-wen Z. New research on development of solid lipid nanoparticles. J Med Coll PLA 2007;22:385-90.
10. Muller RH, Petersen RD, Hommoss A, Pardeike J. Nanostructured lipid carriers (NLC) in cosmetic dermal products. Adv Drug Delivery Rev 2007;59:522-30.
11. Kong M, Park HJ. Stability investigation of hyaluronic acid based nanoemulsion and its potential as transdermal carrier. Carbohydr Polym 2011;83:1303–10.
12. Akiladevi D, Basak S. Ethosomes: A non-Invasive approach for transdermal drug delivery. Int J Curr Pharm Res 2010;2:1-4.
13. Touitou E Godin B, Dayan N, Weiss C, Piliponsky A, Levi-Schaffer F. Intracellular delivery mediated by an ethosomal carrier. Biomaterials 2001;22:3053-9.
14. Ainbinder D, Paolino D, Fresta M, Touitou E. Drug delivery applications with ethosomes. J Biomed Nanotechnol 2010;6:558-68.
15. Heinrich M, Barnes J, Gibbons S. Williamson EM. Fundamentals of pharmacognosy and Phytotherapy, Elsevier Health Sciences; 2012.
16. Touitou Elka. "Compositions for applying active substances to or through the skin; 1996.
17. Kalia YN, Guy RH. Modeling transdermal drug release. Adv Drug Delivery Rev 2001;48:159-72.
18. Dash S, Murthy PN, Nath L, Chowdhury P. Kinetic modeling on drug release from controlled drug delivery systems. Acta Pol Pharm 2010;67:217-23.
19. Touitou E, Godin B, Weiss C. Enhanced delivery of drug into and across the skin by ethosomal carriers. Drug Dev Res 2002;50:406-15.