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Transdermal Delivery of Aceclofenac: Effect of *Gymnema Sylvestre* and *Caralluma Adscendens* With Its Mechanism of Action

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ABSTRACT

Saponins rich fraction of Caralluma adscendens, Gymnema sylvestre & their combination (C+G), was investigated for transdermal enhancer activity by using human cadaver skin in vitro with aceclofenac as the model drug. Moreover, FT-IR studies were conducted to understand to possible enhancement mechanism. Results shows significantly increased flux of the drug compared to control ($p<0.05$). Similarly permeability coefficient (Kp), cumulative amount release (Q_{24}) and enhancement ratio (ER) shown significant increase over control sample. FT-IR studies reveal that Gymnema & Caralluma combination (C+G) reduces peak area by 89.00 % and 75.76 % for symmetric and asymmetric stretching peaks. In addition significantly reduces percentage of secondary structures of keratin at amide I band. These results indicate that Caralluma & Gymnema enhances transdermal permeation of aceclofenac by biphasic mechanism involving partial extraction of stratum corneum (SC) lipid and interaction with SC keratin.

Keywords: Permeation enhancer; Caralluma adscendens; Gymnema sylvestre; FT-IR; Aceclofenac; Stratum corneum.

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INTRODUCTION

Even after wide spread acceptance of modern drug discovery tools such as combinatorial chemistry, High Throughput Screening (HTS), Computer Aided Drug Design the drugs with poor biopharmaceutical properties is yet not reduced. Many of these molecules are very successful and potential therapeutic agents, but suffer from serious drawback of very low bioavailability because of poor permeation across biological barriers [1, 2]. In an attempt to overcome the problems arising from skin impermeability and biological variability and to raise the drug candidate for TDDS, various approaches to reversibly remove the barrier resistance have been investigated. Among these approaches, co-administration of drug with chemical enhancer is widespread accepted and is explored for several drug molecules [3]. Among natural products, one of extensively studied class is essential oils and terpenes. Many mono and sesquiterpenoids have been reported as permeation enhancers. Being natural in origin, terpenes are regarded as relatively safe and clinically acceptable and have been explored as permeation accelerants for many lipophilic as well as hydrophilic drugs [4-9]. However, except terpenes, other phytochemicals are rarely investigated for their permeation enhancement/retardant properties. In attempt to investigate the phytoconstituents such as saponins for transdermal permeation enhancement of clinically used drugs, investigation on Caralluma & Gymnema fractions as a permeation enhancer for aceclofenac as model drug is reported here.

Aceclofenac {2-[[2-[2-[2, 6 dichlorophenyl] amino] phenyl] acetyl] oxy] acetic acid} is a NSAID of phenyl acetic acid type. It is frequently prescribed NSAID for minor traumas and soft-tissue inflammation and chronic inflammatory conditions such as rheumatoid arthritis [10].

MATERIALS AND METHODS

Materials

Aceclofenac was procured as gift sample from Leben Laboratories Pvt. Ltd., (India). All other chemicals used were of analytical grade.

Collection Of Plant Material

The whole plant of *Caralluma adscendens* Roxb. (Asclepiadaceae) was collected from Western Ghat region of Maharashtra in September and leaves of *Gymnema sylvestre* R.Br. (Asclepiadaceae) were collected from local forest in August. Herbarium specimens of both the plants were prepared and both plants were authenticated by taxonomist, Dr. D.A. Patil.

Extraction Procedure

***Caralluma adscendens* (Saponin rich fraction) [11, 12]**

Whole plant powder (500 gm) was extracted with methanol. The methanolic extract was concentrated on vaccum rotatory evaporator and subsequently dried in vaccum drier .The methanolic extract was further fractionated into ethyl acetate and n-butanol. The n-butanol fraction is rich in saponins and flavonoids. Saponins were further concentrated on vaccum rotatory evaporator and dried in vaccum drier. The saponin rich n-butanol extract were used as penetration enhancer.

***Gymnema sylvestre* (saponin rich fraction) [12]**

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Estimation of Total Saponins [13]

It was observed that, n-Butanol extract consist the saponins. So there was need to estimate the total saponins by using the method. In brief, 5 gm of drug powder, extracted with 90 % v/v methanol (25 ml) by refluxing for half an hour. The residue obtained was, again refluxed with 25 ml methanol. The soft extract, left after distillation of alcohol, was treated with petroleum ether (60-80^{0}C) by refluxing for half hour. At the end saponins get precipitated in it and were settled down in beaker.

Determination Of Foaming Index. [13]

From chemical it was observed that, n-butanol extract contains the saponins. When an aqueous decoction was shaken the persistent foam was observed. In brief, 1 gm of powder was taken and passed through sieve no. 1250, transfer it to 500 ml conical flask containing 100 ml of boiling water, boiled for 30 min, then cooled, filtered and transferred to 100 ml volumetric flask, sufficient water was added to make up the volume. Poured the decoction in to the 10 Stoppard test-tubes (height 16 cm, diameter 16 mm). Measure the height of foam after 15 min.

Haemolytic Activity [14]

Sheep Blood was taken for the procedure, activity performed with aqueous, methanolic extracts. Extract Conc. 5, 10, 25, 50, 100, 250, 500 & 1000 mg/lit. Normal Saline and Distilled Water was taken as 0% and 100% activity respectively.

Preparation of Epidermis And Stratum Corneum [15-17]

Full thickness human abdominal skin samples (female age 27 years) were obtained post-mortem from Rural Hospital, Amalner (Maharashtra) India and stored at -20^0C in doubled-sealed evacuated polyethylene bags. On removal of subcutaneous fat from skin, it was immersed in water at 60^0C for 2 min followed by removal of epidermis.

The stratum corneum (SC) sheet was obtained by floating freshly prepared epidermal membranes (SC side up) on an aqueous solution of trypsin (0.001% w/v) and sodium bicarbonate (0.5% w/v), at room temperature for 3 hr. The SC was removed, thoroughly washed and dried in a vacuum desiccators. The Study was duly approved by IAEC.

***In-vitro* Permeation Study [18, 19]**

The diffusion cells, similar to vertical Franz diffusion cells, with 10 ml and 4 ml capacity of receptor and donor compartments respectively with 2.5 cm^2 diameter (2.2 cm^2 effective diffusion area) were used for permeation studies. The epidermal skin layer was mounted carefully on the lower half of the cell with the epidermis facing upwards. The receptor compartments were filled with 0.1M phosphate buffer (pH 6.8). The prepared diffusion cells, containing the buffer, were equilibrated for 1 hr in a water bath at 37°C, prior to the addition of saturated aceclofenac solution to the donor compartment. The receptor compartment was kept at 37°C and stirred with a magnetic stirrer at 400 rpm. After an hour, 3 ml of freshly prepared saturated solution of the aceclofenac in phosphate buffer (pH 6.8) was added to each donor compartment, which was immediately covered with parafilm, to avoid the loss due to evaporation. To determine the effect of the Caralluma & Gymnema, the epidermal membranes were immersed in 1 % w/v Caralluma, Gymnema solution prepared in phosphate buffer (pH 6.8) for 24 hr, rinsed and mounted in the diffusion cells. Aliquots of 1 ml were withdrawn periodically and replaced with the same volume of receptor fluid for 24 hr. and analyzed on Shimadzu-1610 UV-spectrophotometer for aceclofenac content according to the method reported. After 24 hr, the skins were removed and analyzed for drug content using a modified method.

FTIR Study [20]

The circular disc of SC of approximate 1.5 cm diameter was prepared and hydrated in sodium chloride (0.9 % w/v) solution containing antimicrobial agents for 3 days. Before

hydration of the SC discs for 3 days, FT-IR (Shimadzu-8400S, Japan) were recorded in the frequency range 400 to 4000 cm^{-1} with 2 cm^{-1} resolution. Each spectrum was an average 10 scans. After 3 days of hydration, these discs were thoroughly blotted over filter paper and IR spectra were recorded. Then SC discs were kept in 5 ml of Caralluma, Gymnema & Combination of both (C+G) (1 % w/v) prepared in phosphate buffer solution (pH 6.8) at room temperature for 24 hr. Then after 24 hr the SC discs were thoroughly washed, blotted dry and FT-IR spectra were taken. Each sample served as its own control.

Data Analysis [21, 8]

The skin flux was determined from Fick's law of diffusion.

$$Jss = dQr / Adt$$

Where Jss is steady-state flux in $\mu g/cm^2$ per hr, dQr is the change in quantity of material passing through the membrane into receptor compartment in µg, A is the active diffusion area in cm^2 and dt is the change in time.

The cumulative amount of aceclofenac permeated per unit skin surface area was plotted against time and the slope of linear portion of plot was estimated as steady state flux (Jss). The lag time was determined by extrapolating the linear portion of the abscissa.

The permeability coefficient (Kp) was calculated as

$$Kp = Jss / Cv$$

Where Cv is total donor concentration of aceclofenac.

Enhancement ratio (ER) was calculated by dividing permeability coefficient of aceclofenac through epidermis treated with Caralluma & Gymnema by permeability coefficient of aceclofenac through the untreated epidermis.

Statistical Analysis

Results are expressed as mean ± SD of at least 6 experiments. The permeation study data and FT-IR data were analyzed by analysis of variance (ANOVA) followed by Dunnet test and paired t-test respectively using GraphPad Prism software (version 5.0). The level of significance was selected as ($p< 0.05$).

RESULTS AND DISCUSSION

Permeation Studies

Aceclofenac flux, lag-time, enhancement ratio, permeation coefficient and skin content of drug of untreated and Caralluma & Gymnema treated epidermis were summarized in (Fig.1, Table 1). It is evident from results that in vitro permeation of aceclofenac through treated epidermis gives significant increase in permeability coefficient (Kp) of drug at (1 % w/v) concentrations compared to control (p<0.01). It enhanced Kp by 2.14 & 1.91 corresponding increase in enhancement ratio (ER) 1.91 & 1.78 folds at 1 % w/v respectively. The flux of aceclofenac was 43.07 & 40.23 respectively shown significant increase in flux. The skin content of drug was significantly low (p<0.01) compared to control. Thus, lag-time data and data of skin content suggest that, at concentration at 1 % lipid extraction occurs, which is also reflected in reduction of skin content of drug and also proportionate decrease in lag-time. Thus, increased flux, Kp and reduced lag-time and skin content of drug are better correlated with Caralluma & Gymnema treated induced lipid extraction and due to interaction with keratin (Fig.2, Table 2). However, their combination (C+G) shown the promising synergistic effect as compare to individual effect which were summarized in Table 1. It is evident from results that in vitro permeation of aceclofenac through treated epidermis (C+G) gives significant increase in permeability coefficient (Kp) of drug at (1 % w/v) concentrations compared to control (p<0.01). It enhanced Kp by 2.31 corresponding increase in enhancement ratio (ER) 2.17 folds at 1 % w/v. The flux of aceclofenac was 48.75 shown significant increase in flux. The skin content of drug was significantly low (p<0.01) compared to control. Thus, lag-time data and data of skin content suggest that, at concentration at 1 % lipid extraction occurs, which is also reflected in reduction of skin content of drug and also proportionate decrease in lag-time. Thus, increased flux, Kp and reduced lag-time and skin content of drug are better correlated with Combination of Caralluma & Gymnema (C+G) treated induced lipid extraction and due to interaction with keratin (Fig.2, Table 2).

Fig. No.1 *In-vitro* transport of Aceclofenac through human skin .Each data point is the Mean ± S.D of four readings (n = 6). Key: (◆) Control , (▲) Gymnema 1%, (■) Caralluma 1%,(●) Combination (car+gym) 1%.

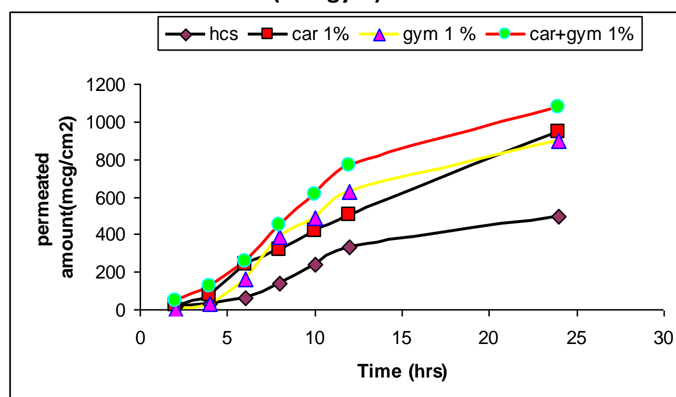

Table No.1: Effect of Caralluma & Gymnema along with their combination (C+G) on transdermal permeation of aceclofenac in vitro human cadaver skin model

Enhancer	(% W/V)	Flux (µg/cm².h)	Lag time (h)	Q_{24} (µg/cm²)	Kp (10^4)(cm h^{-1})	ER	SC (µg/g)
Control	__	22.56±1.44	0.50±0.11	492.4±57.22	1.07±0.03	1.00	202.75±12.40
Caralluma	1 %	43.07±3.14**	1.55±0.32**	944±73.92**	2.14±0.18**	1.91	178.20±10.12
Control	__	22.56±1.44	0.50±0.11	492.4±57.22	1.07±0.03	1.00	202.75±12.40
Gymnema	1 %	40.23±2.30**	2.51±0.48**	913.3±45.18**	1.91±0.11**	1.78	136±9.54**
Control	__	22.56±1.44	0.50±0.11	492.4±57.22	1.07±0.03	1.00	202.75±12.40
Combination(G+C)	1 %	48.75±4.01**	0.35±0.15	1079±129.9**	2.31±0.16**	2.17	108.30±20.2**

All above values expressed as the mean ± S.D of four readings (n=6).

*P < 0.05 (one way ANOVA followed by Dunnet test).

** P< 0.01 (one way ANOVA followed by Dunnet test)

Fig 2: FT-IR spectra of SC showing reduction in asymmetric and symmetric CH_2 stretching absorbance after Caralluma, Gymnema & their combination (C+G) treatment.

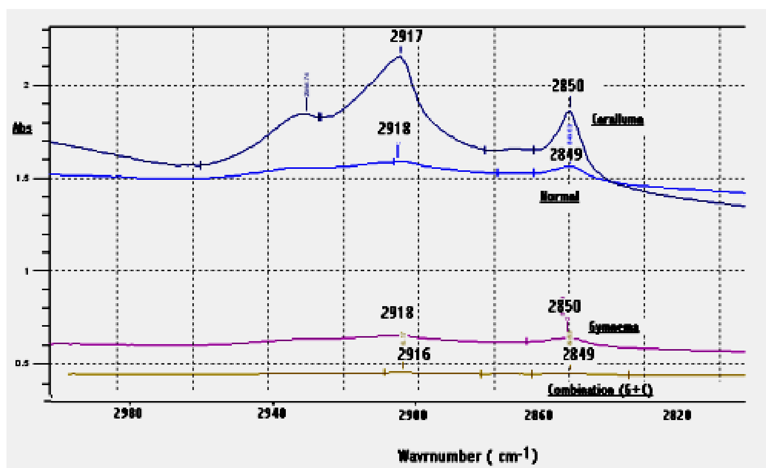

Table 2: The peak height and area of symmetric and asymmetric CH_2 before and after treatment of SC with Caralluma & Gymnema along with their combination (C+G) 24 h and their percentage decrease

	Symmetric C-H stretching				Asymmetric C-H stretching			
	Peak Height	% decrease	Peak Area	% decrease	Peak Height	% decrease	Peak Area	% decrease
Control	1.38±0.15	---	156.6±18.7	---	1.39±0.16	---	72.82±10.26	---
Gymnema	0.66±0.10	52.17*	86.09±10.88	45.02**	0.67±0.12	51.80*	46.88±11.75	35.62*
Caralluma	0.75±0.14	45.65*	47.5±7.44	69.98**	0.50±0.11	64.03*	38.22±10.44	47.51*
Combination (G+C)	0.45±0.10	67.39**	17.22±1.28	89**	0.47±0.10	66.19*	17.65±1.53	75.76**

% decrease or increase in peak height or area = (peak area or height of enhancer treated SC – peak height or area of untreated SC) / (peak height or area of untreated SC) × 100.
All above values expressed as the mean±S.D of four readings (n=3).
*P < 0.05 (one way ANOVA followed by Dunnet test).
** P< 0.01 (one way ANOVA followed by Dunnet test).

FTIR

To know the underlying mechanism of enhancement, the biophysical study using FT-IR was undertaken. The molecular vibration of lipids and proteins are related to various peaks of FT-IR spectrum of SC. The band at 2918 cm^{-1} and 2850 cm^{-1} are due to the asymmetric and symmetric CH_2 vibrations of long-chain hydrocarbons of lipids [22]. Since, the height and area of these two bands are proportional to the amount of the lipids present, any extraction of lipids from SC results in decrease of peak height and area [23]. Further, fluidization of SC lipids also enhances the permeation of drug. The shift of CH_2 stretching peaks to higher wave number (trans to gauche conformation) and increase in their peak widths indicate fluidization of the SC lipids [24, 25]. The CH_2 stretching peaks in the spectra of untreated and treated SC was analyzed for change in peak heights and areas and the shift in of peak frequency after making baseline correction. It was evident from results (Fig.2, Table 2) that treatment of Caralluma & Gymnema (1 % w/v solution) reduces both peak height and peak area by 45.65 %, 52.17% & 69.98 %, 45.02% respectively for symmetric C-H stretching (at 2850 cm^{-1}). Similar, reduction in peak height and area for asymmetric C-H stretching (at 2917 cm^{-1}) by and 64.03 %, 51.80 % & 47.51, 35.62 % respectively recorded. Thus, it is clearly indicated that lipid extraction do occur along with the shift to lower wave number in CH_2 stretching peak was observed (at 2917 cm^{-1}).

The bands at 1650 cm^{-1} and 1550 cm^{-1} are due to the amide I and amide II stretching vibration of SC proteins. The amide I band arises from C=O stretching vibrations and the amide II bands from C-N stretching and N-H bending vibrations. The frequencies of these two bands, especially amide I band are sensitive and shift to higher or lower frequencies according to the change in protein conformation [26]. In Caralluma & Gymnema treated SC, shifts were observed only in Caralluma since amide I band consisting of component bands that represents various secondary structures of keratin and determination of percentage of these secondary structures will be useful parameter to know the interaction with keratin. The percentage of secondary structure was determined by deconvolution of amide I band and curve-fitting analysis of deconvoluted spectra (refer Table 3 and Fig.3). It is evident from results that percentage of secondary structure of β-sheets, anti-parallel β-sheets and β-turns were shown significant reduction (paired t-test, p< 0.0001). Similar reduction of percentage of secondary structures of α-helix and random coils were also recorded (p< 0.001). Thus, it is obvious that reduction of the percentage of secondary structure compared with the untreated SC was due to interaction of Caralluma & Gymnema with keratin. The result are more promising in their combination (C+G) reduces both peak height and peak area by 67.39 % & 89.00% respectively

for symmetric C-H stretching (at 2850 cm^{-1}). Similar, reduction in peak height and area for asymmetric C-H stretching (at 2917 cm^{-1}) by 66.19 % & 75.76 % respectively recorded. Thus, it is clearly indicated that lipid extraction do occur along with the shift to lower wave number in CH_2 stretching peak was observed (at 2916 cm^{-1}).

Taken together, results presented here suggest that an increase in thermodynamic activity and or decrease in diffusion path length Caralluma, Gymnema & their combination (C+G) can be attributed by extraction of SC lipids and substantial interaction with SC keratin. The study, therefore, confirms that Caralluma, Gymnema & their combination (C+G) induces alteration in membrane dynamics and permeation characteristic of SC and thereby increased permeation of aceclofenac across human epidermal membrane by lipid extraction and interaction of keratin.

Fig.3. Protein deconvolution and curve-fitting spectra of amide I and amide II bands of untreated SC (A) and SC treated with Caralluma (B), SC treated with Gymnema (C), SC treated with combination (C+G) (D).

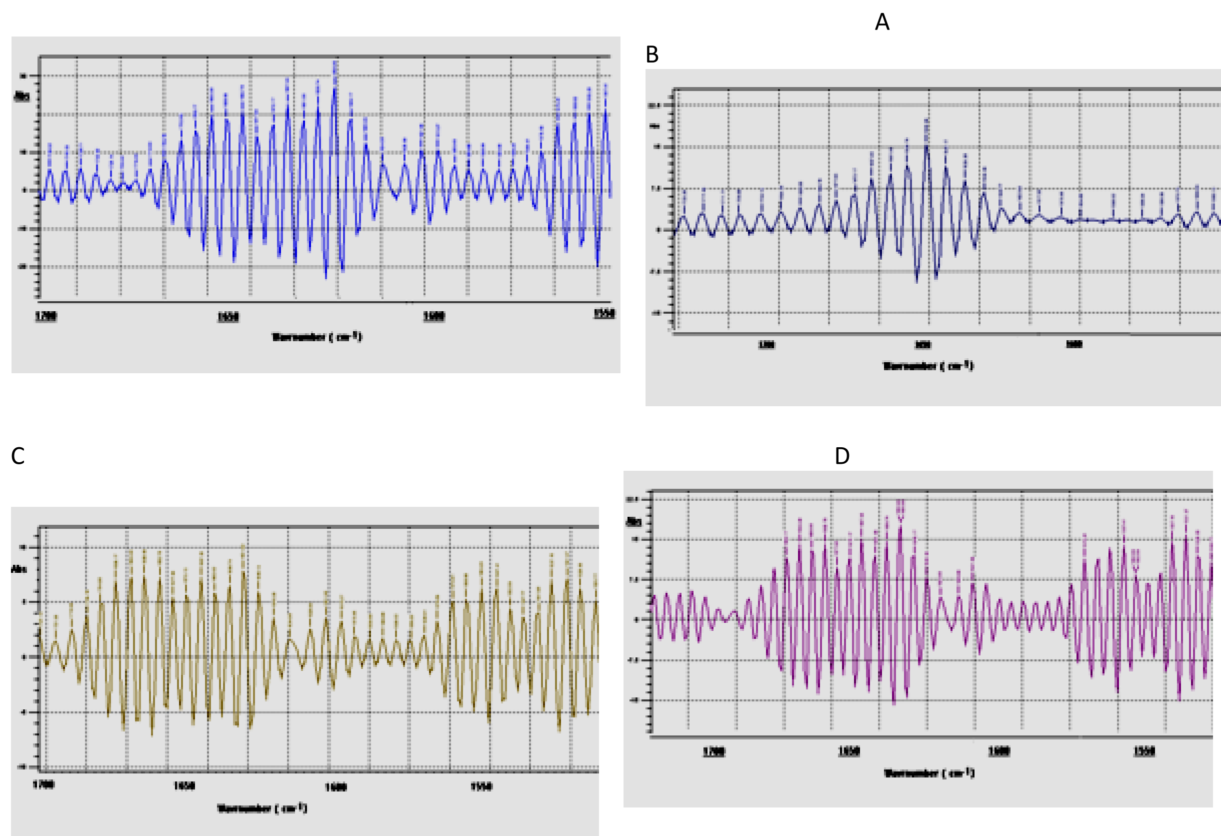

Table No. 3 The percentage secondary structures of keratin before and after treatment of SC with control or enhancer Caralluma & Gymnema along with their combination (C+G) solution for 24 h.

	% α-Helix (1650-1660)		% Anti-parallel β-sheet and β-turns (1660-1695)		% Random coil (1640-1650)		% β-sheet (1620-1640)	
	Control	Enhancer	Control	Enhancer	Control	Enhancer	Control	Enhancer
Caralluma	12.69±1.55	41.11±1.93[c]	60.58±2.46	90.62±2.72[c]	25.40±1.08	20.41±1.47[a]	31.96±1.70	58.57±1.6[c]
Gymnema	12.95±1.99	35.68±1.11[c]	61.29±3.62	93.06±1.98[b]	26.37±2.58	15.57±1.35[b]	32.56±2.56	12.62±1.51[b]
Combination(G+C)	13.30±1.67	7.66±0.93[b]	61.81±3.06	18.34±1.86[b]	25.62±3.14	3.05±0.42[b]	31.66±3.08	8.05±0.82[b]

All above values expressed as the mean ± S.D of four readings (n=6).

[a]*p<0.05

[b]**p<0.005

[c]***p<0.0001

Estimation Of Total Saponins, Foaming Index & Haemolytic Activity

The result suggested in Table No. 4 indicates the total saponin % is reasonable in extracts, also Gymnema shows lesser Haemolytic activity as compared to Caralluma so in Gymnema is relatively safe and for Caralluma one has to look after other the safety parameters as both showing promising enhancer effects & the foaming index is normal.

Observation	Caralluma adscendens	Gymnema Sylvester
Total saponin(%)	24.34%±1.22	29.33 % ± 1.55
Haemolytic (%) (500 µg/ml)	83.22%±2.56	21.77% ± 0.77
Foaming index	321 ± 1.88	433.2 ± 1.22

Thus, economically chief, relatively safe, effective at lower concentration and biphasic mode of permeation enhancement of Caralluma, Gymnema (saponin rich extracts) make it attractive natural extracts for further investigation for various polar and non-polar drugs. Alternatively, it can be exploited as template or scaffold for development of various analogues and semi-synthetic derivatives with improved efficacy and safety as transdermal permeation enhancer. Using addition as biophysical techniques for elucidating further mechanism of enhancement.

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