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Evaluation of synergistic effect of *Chlorophytum borivilianum* extract on transdermal delivery of Pramipexole with its mechanism of action.

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ABSTRACT

Saponin rich fraction of Chlorophytum borivilianum was investigated for transdermal enhancer activity by using human cadaver skin in vitro with pramipexol 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 Chlorophytum borivilianum reduces peak area by 89.00 % for symmetric and asymmetric stretching peaks. In addition significantly reduces percentage of secondary structures of keratin at amide I band. These results indicate that C. borivilianum enhances transdermal permeation of pramipexol by biphasic mechanism involving partial extraction of stratum corneum (SC) lipid and interaction with SC keratin.

Keywords: Permeation enhancer; Stratum corneum; *Chlorophytum borivilianum*; Pramipexol.

INTRODUCTION

Pramipexole is prescribed for parkinsons but it is also indicated for aphrodisiac value. The general issues observed with this drug are nausea, vomiting and burning stomach. Attempt has made to prepare an alternate route of delivery but due to its bioavailability issue all went in vain.[1]

In Indian system of ayurveda tubers of *Chlorophytum borivilianum* family liliaceae are very famous for its adaptogenic and aphrodisiac properties. About 256 species of this plant are found in world and all are comes under the name of safed musli. Among this *Chlorophytum borivilianum* has very great market demand. Tuberous roots of *C. borivilianum* also possess immunomodulatory properties and are used to cure impotency, sterility and enhance male potency. The main active principles of roots, saponins are stimulants and metabolic enhancers and have been shown to possess anti-tumour activity [2, 3]. The extract of dried root tubers of *C. borivilianum* acts as psychostimulant and has a beneficial effect on the brain and human body by increasing alertness, mental ability, intelligence and sexual characters. Due to its therapeutic activity and diversified uses, demand for C. borivilianum is increasing in Indian and the international market.[4]

The plant is also used for dysuria [5]. Leaves are eaten by the tribal people of Western Ghats of India as an expectorant [6]. Root is used as aphrodisiac, diuretic, astringent useful in dysentery, as an antidiabetic and as appetizing agent [7]. Tubers are used as lactogogue [8]. The plant is rich in many pharmaceutical active constituents, mainly glycoside which has been isolated from leaves [9] and roots [10].

Pramipexole being a good aphrodisiac, its usage is limited due to its poor permeability across biological membranes. In an attempt to overcome the problems arising from skin impermeability and biological variability so as to raise the drug candidate for trasderma drug delivery system (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 [11]. 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 [12-16]. 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 *C. borivilianum* fraction as a permeation enhancer for pramipexole as model drug is reported here. We expect synergistic aphrodisiac effects of dual drug approach wherein we deal with the Ayurvedic and allopathic medicine simultaneously.

MATERIALS AND METHODS

2.1. Materials

Pramipexole was procured as from Rovathin (ID no S15847), China. All other chemicals used were purchase from Sigma, Mumbai and were of analytical grade.

2.2. Collection of Plant Material

The leaves and roots of *C. borivilianum* family liliaceae was collected from Western Ghat region of Maharashtra in May. Herbarium specimens of both the plants were prepared and both plants were authenticated by taxonomist, Dr. Kapil Shah, Department of Pharmacognosy, Gangamai College of Pharmacy, Dhule, Maharashtra.

2.3. Preparation of Extracts

The plant parts (Leaves and Roots) were washed, shade dried and extracted with methanol for 36 h. by soxhlet extraction. The MeOH solution was concentrated under reduced pressure, below 45 °C, to dryness. The resulting crude MeOH extracts (ME) was further fractionated to give purified saponin fraction (PSF) and labelled as GPSF (leaves) and CPSF (roots). The extracts were subjected to preliminary phytochemical screening [17] which indicated the presence of mainly saponins.

Estimation of Total Saponins [18]

In brief, 5 gm of drug powder (leaves & roots), 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 (60-800C) by refluxing for half hour. At the end saponins get precipitated in it and were settled down in beaker.

Determination of Foaming Index.[18]

The aqueous decoction was shaken to observe persistent foam. In brief, 1 gm of drug 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. The decoction was then poured in to the 10 Stoppard test-tubes (height 16 cm, diameter 16 mm). The height of foam was measured after 15 min.

Haemolytic Activity [19]

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

2.4. Preparation of Epidermis and Stratum Corneum [20-22]

Full thickness human abdominal skin samples (female age 27 years) were obtained post-mortem from Rural Hospital, Amalner (Maharashtra) India and stored at -20 °C in doubled-sealed evacuated polyethylene bags. On removal of subcutaneous fat from skin, it was immersed in water at 60 °C 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 vacuum desiccators. The Study was duly approved by IAEC.

2.5. In Vitro Permeation Study [23,24]

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 cm2 diameter (2.2 cm2 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 pramipexole 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 pramipexole 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 *C. borivilianum*, the epidermal membranes were immersed in 1 % w/v *C. borivilianum* 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 pramipexole content according to the method reported. After 24 hr, the skins were removed and analyzed for drug content using a modified method.

2.6. FTIR Study [25]
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 *C. borivilianum* both (CPSF & GPSF) (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.

2.7. Data Analysis [26]
The skin flux was determined from Fick's law of diffusion.

$$Jss = dQr / Adt$$

Where Jss is steady-state flux in µg/cm2 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 d_t is the change in time.

The cumulative amount of pramipexole 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 pramipexole.

Enhancement ratio (ER) was calculated by dividing permeability coefficient of pramipexole through epidermis treated with *C. borivilianum* by permeability coefficient of pramipexole through the untreated epidermis.

2.8. 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 ($p < 0.05$).

Table No.1: Effect of CPSF & GPSF along with their combination (CPSF + GPSF) on transdermal permeation of pramipexole in vitro human cadaver skin model

Enhancer	(% W/V)	Flux (µg/cm².h)	Lag time (h)	Q24 (µg/cm²)	Kp (10^4)(cm h^{-1})	ER	SC (µg/g)
Control	NIL	22.56±1.44	0.50±0.11	492.4±57.22	1.07±0.03	1.00	202.75±12.40
CPSF	1 %	43.07±3.14**	1.55±0.32**	944±73.92**	2.14±0.18**	1.91	178.20±10.12
Control	NIL	22.56±1.44	0.50±0.11	492.4±57.22	1.07±0.03	1.00	202.75±12.40
GPSF	1 %	40.23±2.30**	2.51±0.48**	913.3±45.18**	1.91±0.11**	1.78	136±9.54**
Control	NIL	22.56±1.44	0.50±0.11	492.4±57.22	1.07±0.03	1.00	202.75±12.40
CPSF + GPSF	1 %	48.75±4.01**	0.35±0.15	1079±129.9**	2.31±0.16**	2.17	108.30±20.2**

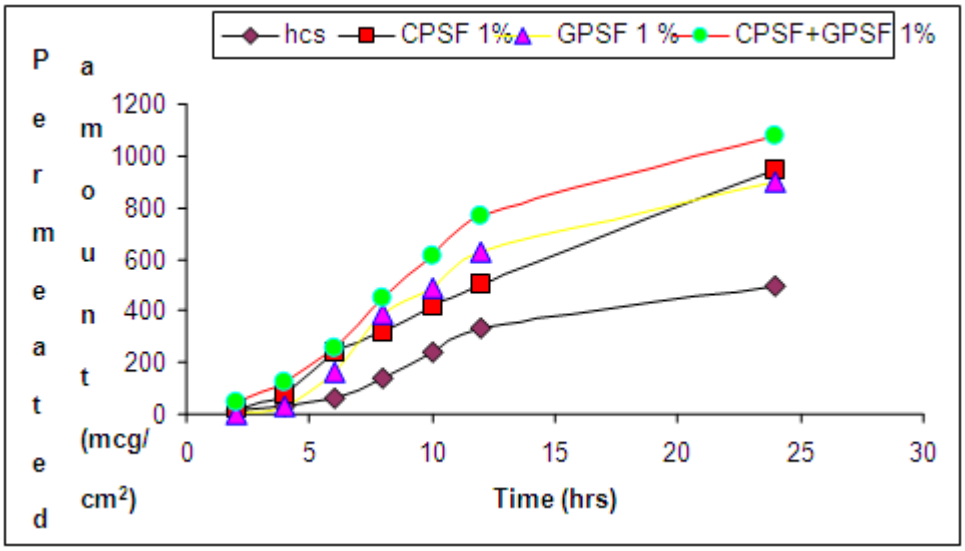
All above values expressed as the mean ±S.D of four readings (n=6).
**P < 0.05 (one way ANOVA follwed by Dunnet test).*
*** P< 0.01 (one way ANOVA follwed by Dunnet test)*

RESULTS AND DISCUSSION

3.1. Permeation Studies

Pramipexole flux, lag-time, enhancement ratio, permeation coefficient and skin content of drug of untreated and *C. borivilianum* treated epidermis were summarized in (Fig.1, Table 1).

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



It is evident from results that in vitro permeation of pramipexole 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 pramipexole was 43.07 & 40.23 respectively shown significant increases 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 *C. borivilianum* treated induced lipid extraction and due to interaction with keratin (Fig.2, Table 2). However, their combination (CPSF + GPSF) 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 pramipexole through treated epidermis (CPSF + GPSF) 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 pramipexole was 48.75 shown significant increases 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 *C. borivilianum* (CPSF + GPSF) treated induced lipid extraction and due to interaction with keratin (Fig.2, Table 2).

Table 2: The peak height and area of symmetric and asymmetric CH2 before and after treatment of SC with CPSF & GPSF along with their combination (CPSF + GPSF) 24 h and their % 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	---
GPSF	0.66±0.10	52.17*	86.09±10.8	45.02**	0.67±0.12	51.80*	46.88±11.75	35.62*
CPSF	0.75±0.14	45.65*	47.5±7.44	69.98**	0.50±0.11	64.03*	38.22±10.44	47.51*
GPSF + CPSF	0.45±0.10	67.39**	17.22±1.28	89**	0.47±0.10	66.19*	17.65±1.53	75.76**

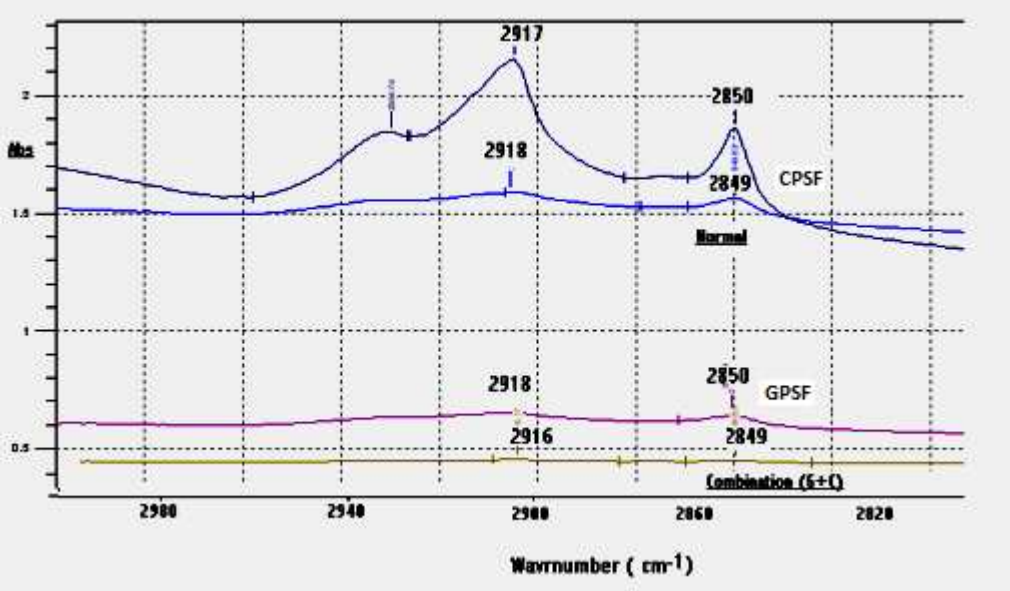
% decrease or increase in peak hight or area = (peak area or height of enhancer treated SC – peak height or area of untreated SC) / (peak height or area of untreated SC) × 100.

All above values expressed as the mean±S.D of four readings (n=3).

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

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

Fig 2: FT-IR spectra of SC showing reduction in asymmetric and symmetric CH_2 stretching absorbance after CPSF, GPSF & their combination (CPSF + GPSF) treatment



3.2. FT- IR

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 [27]. 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 [28]. Further, fluidization of SC lipids also enhances the permeation of drug. The shifts of CH_2 stretching peaks to higher wave number (trans to gauche conformation) and increase in their peak widths indicate fludization of the SC lipids [29, 30]. The CH2 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 *C. borivilianum* (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}).

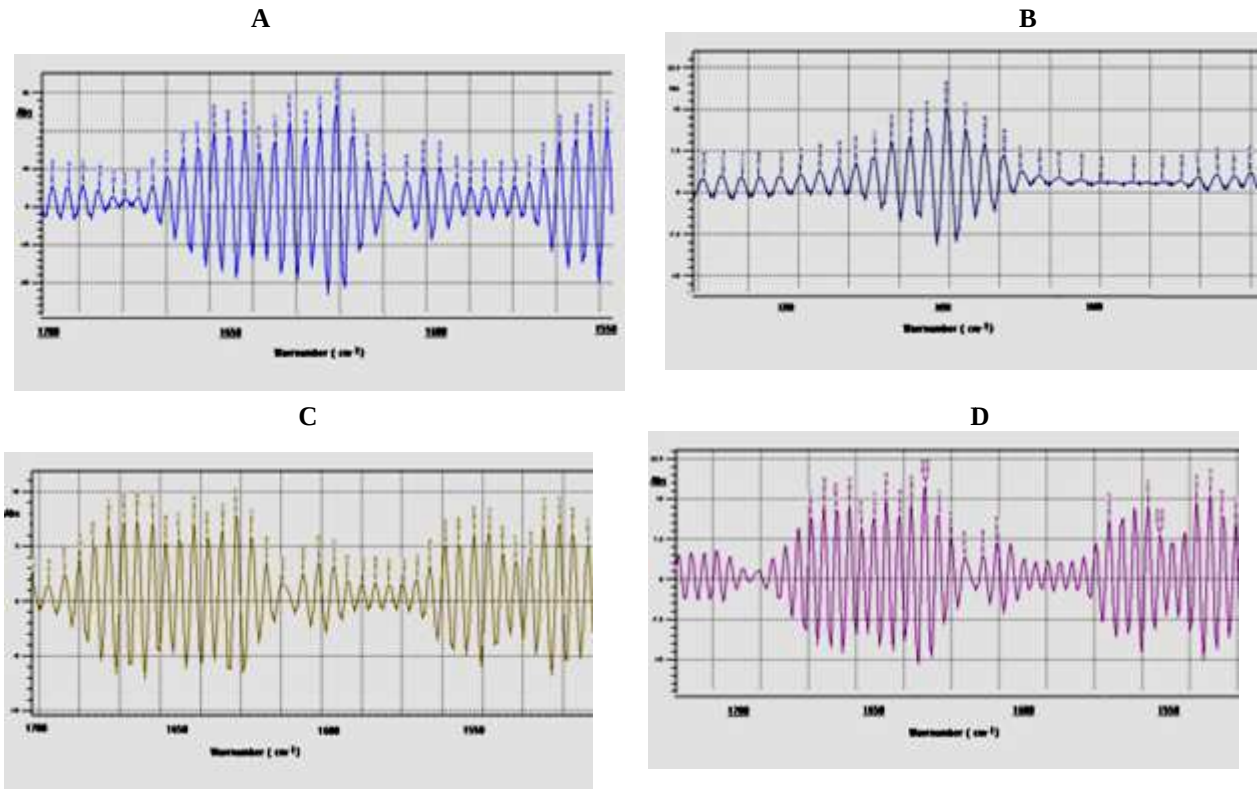
The bands at 1650 cm^{-1} and 1550 cm^{-1} are due to the amide I and amide II stretching vibration of SC proteins. The amide I band arises from C=O stretching vibrations and the amide II bands from C-N stretching and N-H bending vibrations. The frequencies of these two bands, especially amide I band are sensitive and shift to higher or lower frequencies according to the change in protein conformation [31]. In *C. borivilianum* treated SC, shifts were observed only in CSPF 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).

Table No. 3: The percentage secondary structures of keratin before and after treatment of SC with control or enhancer CPSF & GPSF along with their combination (CPSF + GPSF) 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
CPSF	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}
GPSF	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}
CPSF + GPSF	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*

Fig.3. Protein de-convolution and curve-fitting spectra of amide I and amide II bands of untreated SC (A) and SC treated with GPSF (B), SC treated with GPSF (C), SC treated with combination (CPSF + PSFG) (D).



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 *C. borivilianum* with keratin. The results are more promising in their combination (CPSF + GPSF) 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 CH2 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 *C. borivilianum* (CPSF + GPSF) can be attributed by extraction of SC lipids and substantial interaction with SC keratin. The study, therefore, confirms that *C. borivilianum* (CPSF + GPSF) induces alteration in membrane dynamics and permeation characteristic of SC and thereby increased permeation of pramipexole across human epidermal membrane by lipid extraction and interaction of keratin.

3.3. Estimation of Total Saponins, Foaming Index and Haemolytic Activity

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

Table No: 4: Total saponin percentage

Observation	CSPF (%)	GSPF (%)
Total Saponins (%)	24.34 ± 1.22	29.33 ± 1.55
Haemolytic (%) (500µg/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 CSPF & GSPF (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.

CONCLUSION

We herewith report successful synergistic combination of two drugs from different medical background. It's called synergistic because, the aphrodisiac effect of pramipexole was enhanced with the Ayurvedic "safed musali" which is a strong aphrodisiac as per the Ayurveda. We even are successful in overcoming the permeability issue of pramipexole by combining with the safed musali. The pharmacological effect on rabbits and mice is still on and will be communicated as we approach the final output.

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