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# *In vitro* permeation of aceclofenac through the shed skin of two different species

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**ABSTRACT**

*Saponins rich fraction of Caralluma adscendens, Gymnema sylvestre & Piperine, an amide alkaloid of black pepper, was investigated for transdermal enhancer activity using shed snake skin in vitro with aceclofenac as the model drug. The membranes used were the dorsal portions of shed skin of two different species of snake. Results shows excellent permeability in dorsal area with permeability order for different species is cobra > viper, this may be due to thickness of skin, 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. These results indicate that Caralluma, Gymnema & Piperine enhances transdermal permeation of aceclofenac significantly. The combination of two enhancer (C+G) showed most promising result.*

**Keywords:** Transdermal enhancer; Shed snake skin; *Caralluma adscendens; Gymnema sylvestre*; Piperine.

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## INTRODUCTION

Monitoring the release of number of drugs in vitro, for study the effects of a number of penetration enhance using the shed snake skin as a model membrane for diffusion studies is well known. The shed skin is not a mammalian integument, it has been reported that some compounds penetrate shed snake skin as human stratum corneum at similar rates [1]. In some countries it is difficult to obtain human skin for in vitro experiments so it is therefore important to have alternate biological or synthetic membranes which mimic human skin membranes for diffusion experiments. Since, snake moult periodically a single animal can provide repeated shed & skin is easily available from many snake parks. Skin can be obtained without injury to animal do not have to subject to chemical or heat stress prior to use. The epidermis is shed as a large intact sheet. Shed skin can be stored for long periods at room temperature and easily transported. Stored & fresh skin appear to show no difference [1]. The study designed to evaluate the diffusion characteristic of two different species of snake skin and to investigate the differences if any between dorsal and ventral sites.

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 [2,3]. 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 [4]. Among natural products, one of extensively studied class is essential oils and terpenes. 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 [5-10]. However, except terpenes, other phytochemicals are rarely investigated for their permeation enhancement/retardant properties. In attempt to investigate the phytoconstituents such as saponins & alkaloid for transdermal permeation enhancement of clinically used drugs, investigation on *Caralluma, Gymnema* fractions & Piperine, is a major alkaloid of *Piper nigrum* Linn. and *Piper longum* Linn., which are widespread consumed as a spice and medicinal compound since ages [11]. In addition, piperine is also known to improve the oral bioavailability of several drug and nutraceutical molecules[12,13] as a permeation enhancer for aceclofenac as model drug is reported here.

Aceclofenac is a NSAID of phenyl acetic acid type. It is frequently prescribed NSAID for minor traumas and soft-tissue inflammation and chronic conditions such as rheumatoid arthritis [14].

## MATERIALS AND METHODS

### 2.1.Materials

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

### Snake skin [1]

The shed skin of two different species forest cobra and viper of snake were used the shed snake skin sample were freshly moulted samples obtained from Aurangabad snake park. The skin were cut into size and hydrated by allowing them to soak overnight in water in a covered petri dish. The outer surface of skin was placed in contact for the study. The Study was duly approved by IAEC

### 2.2.Collection Of Plant Material

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

### 2.3.Extraction Procedure

***Caralluma adscendens* (Saponin rich fraction)[15, 16]**

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)[16]**

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.

### Isolation of piperine and its standardization

Piperine was extracted according to the method reported [17]. In brief, 500 gm powder was extracted with ethanol (95%) in Soxhlet extractor. The extract was concentrated using rotatory vacuum evaporator and to the concentrated extract, 70 ml of 10% KOH aqueous solution was added and left overnight. The yellow coloured solids were separated and recrystallized in acetone.

**2.4.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 shed snake skin 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* & Piperine the shed snake skin were immersed in 1 % w/v *Caralluma, Gymnema* & Piperine solution respectively and 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.

**2.7.Data Analysis [20,21]**

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

$Jss = dQr / Adt,$

Where Jss is steady-state flux in µg/$cm^2$ 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

**Table No.1: Effect of Piperine on transdermal permeation of aceclofenac in vitro on dorsal shed snake skin of Cobra and Viper**

**Cobra**

| Enhancer | (% w/v) | Flux ( ) | Lag time (h) | $Q_{24}$ ( ) | Kp ( ) | ER | SC[a] ( ) |
|---|---|---|---|---|---|---|---|
| **Control** | --- | 19.50±1.90 | 0.40±0.6 | 410.16±55.22 | 1.06± 0.05 | 1 | 179.7±13.4 |
| **Piperine** | 1.0 | 32.77± 3.40** | 0.34±0.8 | 689.33±67.40** | 1.76±0.12** | 1.68 | 133±9.64** |
| **Piperine** | 2.0 | 35.88± 3.90** | 0.30±0.9 | 755.50±75.50 | 1.96±0.15 ** | 1.84 | 124.3±15.2** |

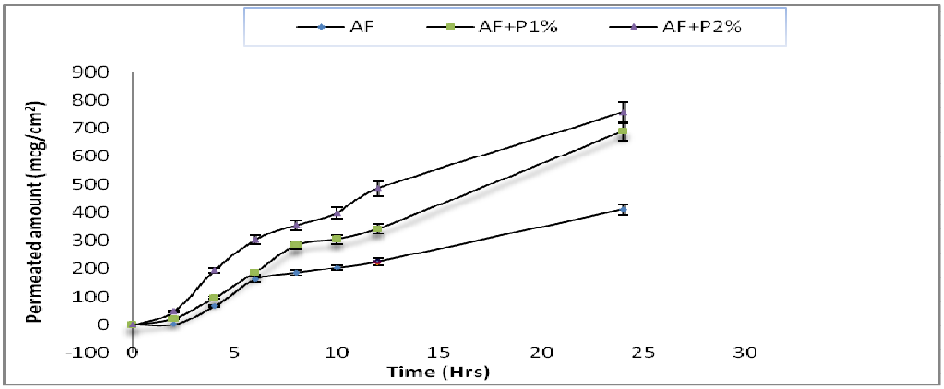
**Viper**

| Enhancer | (% w/v) | Flux (µg/cm² per hr) | Lag time (hr) | $Q_{24}$ (µg/cm²) | Kp ($10^4$)(cm $h^{-1}$) | ER | SC[a] (µg/g) |
|---|---|---|---|---|---|---|---|
| **Control** | --- | 17.80±1.30 | 0.45±0.7 | 405.4 ±48.80 | 1.04± 0.02 | 1 | 185.5±12.54 |
| **Piperine** | 1.0 | 24.38± 1.40** | 0.38±0.8 | 553.50±50.20** | 1.46±0.09** | 1.37 | 148±9.50** |
| **Piperine** | 2.0 | 27.94± 1.75** | 0.33±0.10 | 635.83±80.80** | 1.69±0.12** | 1.57 | 130.3±19.2** |

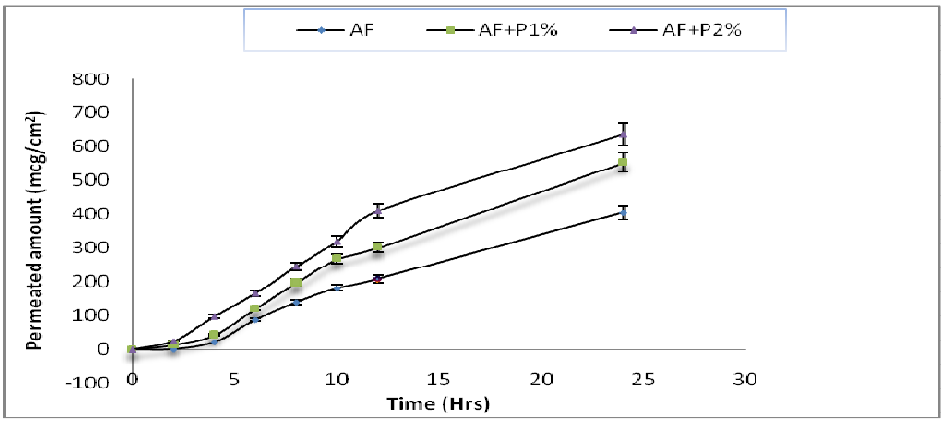
*All above values expressed as the mean ±S.D of four readings (n=6).*
**P < 0.05 (one way ANOVA followed by Dunnet test).*
*** P< 0.01 (one way ANOVA followed by Dunnet test)*

**Fig. No.1: In-vitro transport of Aceclofenac through dorsal shed snake skin of Cobra & Viper.**
**Each data point is the Mean ± S.D of four readings (n = 6).**
*Key: (◆) Control , (■) Piperine 1% (▲) Piperine 2%.*

**Cobra**




**Viper**




**Permeation Studies**

Aceclofenac flux, lag-time, enhancement ratio, permeation coefficient and skin content of drug of untreated and Piperine treated shed snake skin were summarized in (Table 1). It is evident from results that *in vitro* permeation of aceclofenac through treated shed snake skin of Cobra & Viper gives significant increase in permeability coefficient (Kp) of drug at (1 % w/v & 2 % w/v) concentrations compared to control ($p<0.01$). It enhanced Kp by 1.76,1.96 & 1.46,1.69 respectively with increase in enhancement ratio (ER) 1.68,1.84 & 1.37,1.57 folds at 1 % w/v & 2 % w/v respectively at Cobra & Viper shed snake skins.. The flux of aceclofenac was 32.77,35.88 & 24.38,27.94

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 higher concentration the reduction of skin content of drug and it is proportionate decrease in lag-time. Thus, increased flux, Kp and reduced lag-time and skin content of drug are better correlated with Piperine treated shed skin at different concentrations. However, Viper species shed skin shows lower permeation as compared to Cobra shed skin (Fig.1).

Furthermore, significant results for Caralluma & Gymnema but more promising results for their combination (C+G) shown the excellent synergistic effect as compare to individual effect which were summarized in (Table 4). It is evident from results that *in vitro* permeation of aceclofenac through treated shed snake skin of Cobra & Viper species epidermis (C+G) gives significant increase in permeability coefficient (Kp) of drug at 1 % w/v & 2 % w/v concentrations compared to control ($p<0.01$). It enhanced Kp by 2.48, 2.90 & 2.36, 2.76 respectively with increase in enhancement ratio (ER) 2.37, 2.78 & 2.26, 2.68 folds at 1 % w/v & 2 % w/v respectively at Cobra & Viper shed snake skins. The flux of aceclofenac was 46.21, 54.21 & 40.23, 47.70 shown significant increase in flux. The skin content of drug was significantly low ($p<0.01$) compared to control. Thus, increased flux, Kp and reduced lag-time and skin content of drug are better correlated with Combination of *Caralluma & Gymnema* (C+G) treated shed skin (Fig.4).

**Table No.4: Effect of combination of *Caralluma & Gymnema* (C+G) on transdermal permeation of aceclofenac in vitro on dorsal shed snake skin of Cobra and Viper**

**Cobra**

| Enhancer | (% w/v) | Flux (µg/cm²per | Lag time (hr) | $Q_{24}$ (µg/cm²) | Kp ($10^4$) (cm $h^{-1}$) | ER | SC[a] (µg/g) |
|---|---|---|---|---|---|---|---|
| **Control** | --- | 19.50±1.90 | 0.40±0.8 | 410.16±55.22 | 1.06± 0.05 | 1 | 179.7±13.4 |
| **C+G** | 1.0 | 46.21± 6.20** | 0.23±0.10 | 970.16±58.25** | 2.48±0.10** | 2.37 | 126±10.74** |
| **C+G** | 2.0 | 54.21± 6.95** | 0.16±0.12 | 1139.66±60.8** | 2.90±0.18 ** | 2.78 | 115.3±21.2** |

**Viper**

| Enhancer | (% w/v) | Flux (µg/cm² per hr) | Lag time (hr) | $Q_{24}$ (µg/cm²) | Kp ($10^4$)(cm $h^{-1}$) | ER | SC[a] (µg/g) |
|---|---|---|---|---|---|---|---|
| **Control** | --- | 17.80±1.30 | 0.45±0.7 | 405.4 ±48.80 | 1.07± 0.03 | 1 | 185.5±12.54 |
| **C+G** | 1.0 | 40.23± 2.60** | 0.28±0.8 | 918.50±80.20** | 2.36±0.11** | 2.26 | 135±11.64** |
| **C+G** | 2.0 | 47.70± 3.20** | 0.20±0.10 | 1087.50±60.45** | 2.76±0.10 ** | 2.68 | 125.3±22.42** |

*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)*

**Fig. No.4: In-vitro transport of Aceclofenac through dorsal shed snake skin of Cobra & Viper. Each data point is the Mean ± S.D of four readings (n = 6). Key: (◆) Control , (■) Caralluma + Gymnema (C+G) 1% (▲) Caralluma + Gymnema (C+G) 2%.**

**Cobra**

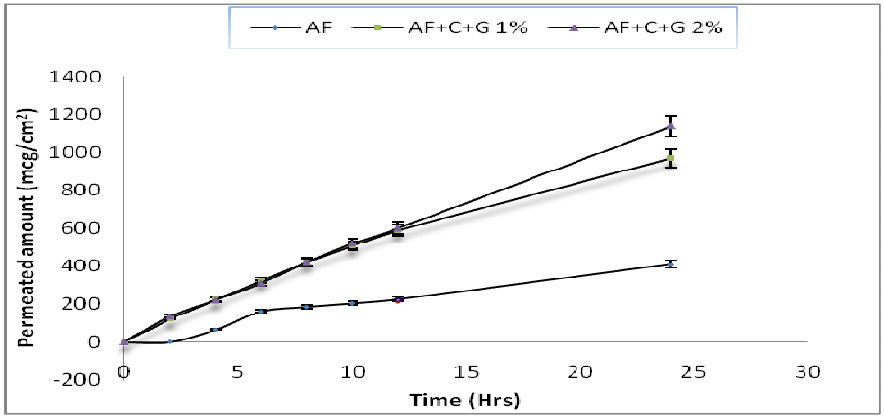



**Fig. No.4: In-vitro transport of Aceclofenac through dorsal shed snake skin of Cobra. Each data point is the Mean ± S.D of four readings (n = 6). Key: (◆) Control , (■) Caralluma + Gymnema (C+G) 1% (▲) Caralluma + Gymnema (C+G) 2%.**

**Viper**

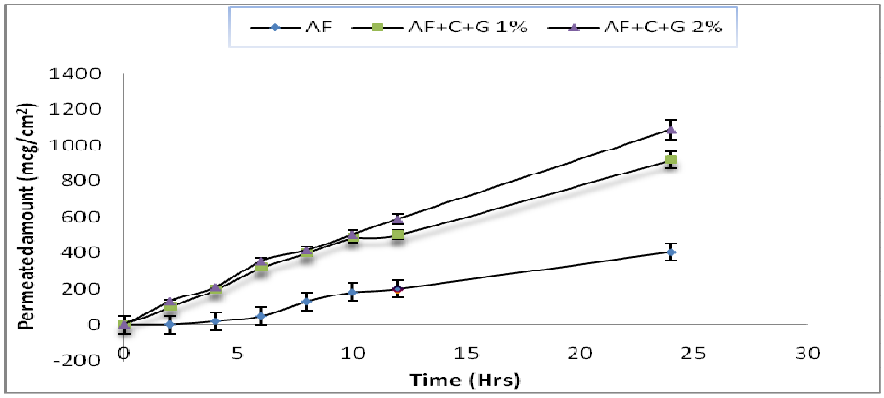


**Table No.2: Effect of *Gymnema* (GYM) on transdermal permeation of aceclofenac in vitro on dorsal shed snake skin of Cobra and Viper**

**Cobra**

| Enhancer | (% w/v) | Flux (µg/cm²per hr) | Lag time (hr) | $Q_{24}$ (µg/cm²) | Kp ($10^4$) (cm $h^{-1}$) | ER | SC[a] (µg/g) |
|---|---|---|---|---|---|---|---|
| Control | --- | 19.50±1.90 | 0.40±0.8 | 410.16±55.2 | 1.06± 0.05 | 1 | 179.7±13.4 |
| **GYM** | 1.0 | 40.36± 4.10** | 0.36±0.10 | 837.50±65.3 | 2.15±0.10** | 2.07 | 136±10.44** |
| **GYM** | 2.0 | 44.85±4.80** | 0.30±0.12 | 945±70.80** | 2.41±0.14 ** | 2.30 | 128.3±16.32** |
| **Viper** | | | | | | | |
| Control | --- | 17.80±1.30 | 0.45±0.7 | 405.4 ±48.8 | 1.07± 0.03 | 1 | 185.5±12.54 |
| **GYM** | 1.0 | 30.62± 1.90** | 0.37±0.8 | 697.16±58.4 | 1.79±0.9** | 1.72 | 151.5±13.4 |
| **GYM** | 2.0 | 38.27± 2.15** | 0.32±0.9 | 872.83±60.8 | 2.26±0.12 ** | 2.15 | 159±14.52** |

**Table No.3: Effect of *Caralluma* (CAR) on transdermal permeation of aceclofenac in vitro on dorsal shed snake skin of Cobra and Viper**

**Cobra**

| Enhancer | (% w/v) | Flux (µg/cm² per hr) | Lag time (hr) | $Q_{24}$ (µg/cm²) | Kp ($10^4$)(cm $h^{-1}$) | ER | SC[a] (µg/g) |
|---|---|---|---|---|---|---|---|
| Control | --- | 19.50±1.90 | 0.40±0.8 | 410.16±55.2 | 1.06± 0.05 | 1 | 179.7±13.4 |
| **CAR** | 1.0 | 41.15± 5.20** | 0.30±0.10 | 861.66±66.6 | 2.20±0.11** | 2.11 | 130±9.24** |
| **CAR** | 2.0 | 49.34± 5.60** | 0.19±0.12 | 1038.33±72. | 2.65±0.16 ** | 2.53 | 120.3±19.15** |
| **Viper** | | | | | | | |
| Control | --- | 17.80±1.30 | 0.45±0.7 | 405.4 ±48.8 | 1.07± 0.03 | 1 | 185.5±12.54 |
| **CAR** | 1.0 | 35.60± 2.50** | 0.33±0.9 | 809.83±74.4 | 2.11±0.12** | 2.0 | 144±10.84** |
| **CAR** | 2.0 | 40.41± 2.60** | 0.25±0.11 | 921±60.48** | 2.39±0.14 ** | 2.27 | 129.3±20.32** |

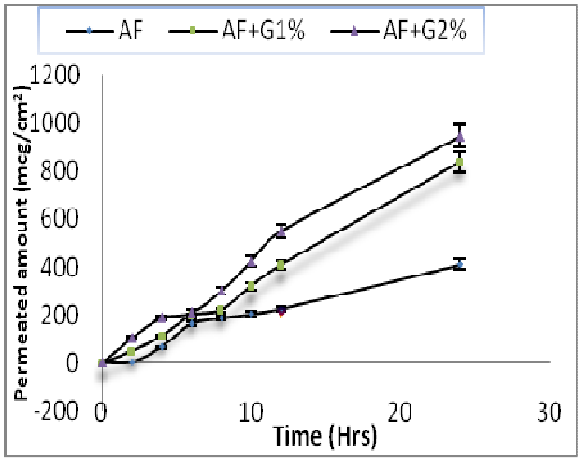
*All above values expressed as the mean ± S.D of four readings (n=6).*
** P < 0.05 (one way ANOVA follwed by Dunnet test).*
*** P< 0.01 (one way ANOVA follwed by Dunnet test)*

Similarly, Gymnema shows significant increase in permeability coefficient (Kp) of drug at 1 % w/v & 2 % w/v concentrations compared to control ($p<0.01$) shown in (Table 2). It enhanced Kp by 2.15, 2.41 & 1.79, 2.26 respectively with increase in enhancement ratio (ER) 2.07,2.30 & 1.72,2.15 folds at 1 % w/v & 2 % w/v respectively at Cobra & Viper shed snake skins. The flux of aceclofenac was 40.36, 44.85 & 30.62, 38.27 shown significant increase in flux. The skin content of drug was significantly low ($p<0.01$) compared to control. The results of Caralluma shows more prominent increase in permeability coefficient (Kp) of drug at 1 % w/v & 2 % w/v concentrations compared to control ($p<0.01$) shown in (Table 3).

It enhanced Kp by 2.20,2.65 & 2.11,2.39 corresponding increase in enhancement ratio (ER) 2.11, 2.53 & 2.0, 2.27 folds at 1 % w/v & 2 % w/v respectively for Cobra & Viper shed snake skins. The flux of aceclofenac was 41.15,49.34 & 35.60,40.41 shown significant increase in flux. The skin content of drug was significantly low ($p<0.01$) compared to control. Cobra species shed snake skin shows more prominent permeation as compared to Viper shed skin (Fig.2 & Fig 3).

**Fig. No.2: In-vitro transport of Aceclofenac through dorsal shed snake skin of Cobra & Viper.**
**Each data point is the Mean ± S.D of four readings (n = 6).**
**Key: (◆) Control , (■) Gymnema 1% (▲) Gymnema 2%.**

**Cobra**




**Viper**

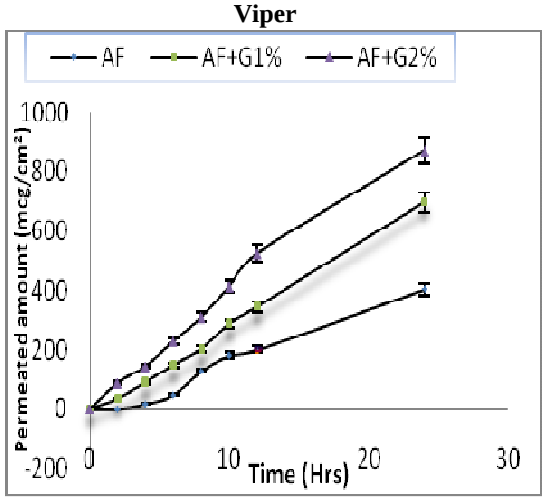
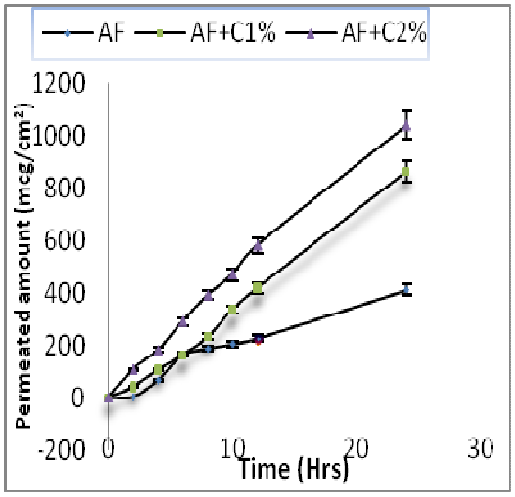
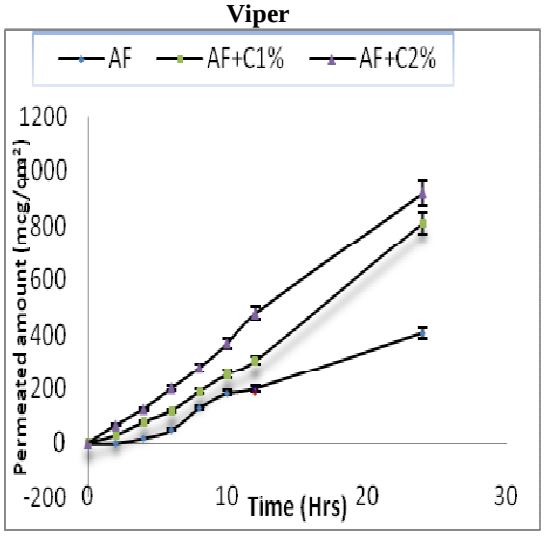



**Fig. No.3: In-vitro transport of Aceclofenac through dorsal shed snake skin of Cobra & Viper.**
**Each data point is the Mean ± S.D of four readings (n = 6).**
**Key: (◆) Control , (■) Caralluma 1% (▲) Caralluma 2%.**

**Cobra**




**Viper**




## CONCLUSION

The results indicates an excellent permeability in dorsal area with permeability order for the two different snake species is cobra > viper, this may be due to thickness of skin. Thus, economically chief, relatively safe, effective at lower concentration shown by *Caralluma*, *Gymnema* (saponin rich extracts) & Piperine an amide alkaloid which makes it an 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. Earlier study proves their mechanism of action in Human stratum corneum with biphasic mode of permeation enhancement [21].

The shed snake proves to be an alternate biological or synthetic membranes which mimic human skin membranes in the diffusion experiments. The result of previous studies suggested 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 [21] .

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