Development of phytovesicles containing triterpenoids from *Samadera indica*

Vidya Viswanad, Subin Mary Zachariah, Aiswarya Sathi, N. A. Aleykutty[1]

Department of Pharmaceutics, Amrita School of Pharmacy, Amrita Vishwavidyapeetham University, AIMS, Kochi, [1]Department of Pharmaceutics, Caritas College of Pharmacy, Kottayam, Kerala, India

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ABSTRACT

Introduction: *Samadera indica* belonging to Simaroubaceae family is being used traditionally for many diseases including arthritis, edema, itching, skin diseases, constipation, and general debility. **Objective:** The effectiveness of any drug delivery system depends upon its ability to deliver the active components at therapeutic level. In this study, a novel phyto vesicular formulation for the enhanced topical delivery of methanol extract of *S. indica* in order to treat skin infections was developed. **Materials and Methods:** The methanol extract fraction of leaves of *S. indica* which showed more antifungal activity was purified to separate an antifungal compound. Phytovesicles were formulated using the more antifungal fraction in order to treat topical and deep seated fungal infections. Pytovesicles were prepared using 1:2 molar ratio of antifungal triterpenoid from *S. indica* (AFTSI)-phosphatidylcholine by film hydration method. **Results and Discussion:** Chloroform 100% fraction of methanol extract of *S. indica* showed more activity against the fungus *Candida albicans.* Further purification gave a fraction with minimum inhibitory concentration value of 15.6 µg/ml against *C. albicans* and showed positive test for triterpenoids. The fraction was named as AFTSI. A compound (20 mg) was isolated from this fraction at an R_F value. The phytovesicle gel formulated using AFTSI showed enhanced skin permeability and antifungal activity. **Conclusion:** The study demonstrated that the phytovesicular gel developed using methanol extract of *S. indica* would be beneficial for treating deep seated fungal infections.

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INTRODUCTION

Modern medicine has been using standardized extracts or isolates from the plant in formulation of drugs. Research in this area is increasing and opens a gateway for the development of new drugs.[1] Also present century is witnessing a high demand for herbal medicines as these are relatively safe with lesser side effects. This leads plant derived drugs a potential that can be exploited effectively through further research in the area.[2,3] Microbial skin infections especially bacterial and fungal are very common and threatening in many cases. Development of a newer antimicrobial drug from plant would be beneficial for treating these infections.[4] In this context drug delivery using a standardized plant extract will be beneficial and can be improved by integrating with modern technology. Considering this the plant *Samadera indica* (Simaroubaceae) has been selected for the study. The plant is being used traditionally for many diseases including arthritis, edema, itching, skin diseases, constipation, and general debility. *S. indica* belonging to Simaroubaceae family is rich in triterpenoids.[5,6] The previous study showed the methanolic extract of *S. indica* was found to be active against selected microorganisms that causes skin infections and anti-microbial activity was found to be more against the fungus *Candida albicans.* The antioxidant studies also proved that the methanolic extract of *S. indica* has the ability to scavenge free radicals produced in our body.[7,8] The effectiveness of any drug delivery system depends upon its ability to deliver the active components at therapeutic level. So the aim of present study was to develop a novel phyto vesicular drug delivery for the enhanced topical delivery of methanol extract of *S. indica.*

Address for correspondence: Dr. Vidya Viswanad, Department of pharmaceutics, Amrita School of Pharmacy, Amrita Vishwavidyapeetham University, AIMS Health Science Campus, AIMS, Ponekkara P.O, Kochi - 682 041, Kerala, India. E-mail: vidyaviswanad@aims.amrita.edu

MATERIALS AND METHODS

Fractionation of crude methanol extract[9,10]

The leaves of *S. indica* were collected from local areas of Ernakulam District and authenticated. Leaves after collection were shade dried and was subjected for extraction in methanol in a soxhlet assembly. The crude methanol extract was subjected for preliminary phytochemical screening and fractionation by gradient elution technique. The stationary phase was silica gel of mesh size 60–120 (SD fine chemicals Mumbai) and mobile phase was different combinations of petroleum ether, chloroform and methanol at variable proportions (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100). The fractions were collected in 50 ml portions. The thin layer chromatography (TLC) of all fractions were performed, based on TLC profile similar fractions were pooled together, concentrated and evaporated to dryness. All fractions were tested for antimicrobial activity by minimum inhibitory concentration (MIC) against selected organisms *Staphylococcus aureus, Psuedomonas aeruginosa and C. albicans* to know which fraction shows more activity.[10,11] Chloramphenicol for bacteria and cotrimazole for fungi was used as positive control at concentrations ranging from 100 to 3.12 g/ml. Dimethyl sulfoxide (DMSO) was used as the solvent control. The fractions were tested at different concentrations ranged from 1000 to 7.5 μg/ml.

The fraction that showed greater antifungal activity was purified to separate an antifungal compound.

Formulation of phytovesicle gel[11,12]

Phytovesicles were formulated using more active antifungal fraction in order to treat topical and deep seated fungal infections. Pytovesicles were prepared using 1:2 molar ratio of antifungal triterpenoid from *S. indica*-phosphatidylcholine (AFTSI-PC) by film hydration method with an aim to increase its permeability through skin. The complex formation was studied by determining the Fourier transform infrared (FTIR), solubility and melting point. It was then characterized for shape, size distribution. Loading efficiency and zeta potential.

The phytovesicles were later incorporated into carbopol gel to get a final 1% concentration. The gel was evaluated for parameters.

Characterization of complex

Shape and size distribution

The shape of vesicles was observed under scanning electron microscope. Vesicular size distribution studies were evaluated by dynamic light scattering method. The instrumental setting was fixed at temperature 20°C, viscosity 0.01 poise and refractive index − 1.333.

Loading efficiency

Phytovesicles containing AFTSI were separated from untrapped drug by centrifugation at 9000 rpm for 45 min. The supernatant was recovered and assayed spectrophotometrically using Shimadzu ultraviolet spectrophotometer.

$$\text{Loading efficiency} = \frac{Ct - Cr}{Ct} \times 100$$

Where, Ct - concentration of total AFTSI; Cr - concentration of free AFTSI.

Zeta potential determination

The zeta potential of preparation was determined by zetasizer.

Evaluation of phytovesicle gel

Permeation studies[13]

The permeation studies were carried out for the phytovesicle gel. It was compared with conventional carbopol gel, marketed formulation and control formulation without drug.

Hundred milligram of the formulation was applied to the donor compartment of the prepared Franz diffusion cell. At a predetermined time of 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 20 and 24 h 0.5 ml of the sample was withdrawn and the cell was refilled with same amount of the receptor solution. Withdrawn samples were analyzed spectrophotometrically at 230 nm. Each permeation experiment was replicated three times. The amount of drug permeated to the receptor compartment was calculated. The drug solution was used as the control. The cumulative amount of drug permeated per cm^2 was plotted against time. The steady state flux was calculated from the slope of the linear portion.

$$\text{Steady state flu x } \left(J_{ss}\right) = \frac{dM}{Dt}$$

Where, M = cumulative amount of drug permeated per unit area.

The apparent permeability coefficient (Kp) can be calculated using equation:

$$\text{Apparent coefficient, Kp} = \frac{JSS}{C\,donor}$$

The lag time (r) was calculated using the X-intercept of the linear portion of the curve. With known thickness of the skin (δ) and (r), diffusion coefficient was calculated.

In-vitro drug release study[14]

Release study was carried for the phytovesicle gel, marketed formulation and conventional carbopol gel 100 mg of the formulation was placed in a cellophane membrane immersed into 30 ml of dissolution medium

of phosphate buffer of pH 7.4-methanol mixture medium. To simulate the human skin condition, during the experiment, temperature was maintained 37°C ± 0.5°C. The 5 ml samples were withdrawn at predetermined rate 1, 2, 3, 4, 5, 6, 7, 8 and 24 h, and replaced with equivalent amount of the samples. The withdrawn samples were analyzed spectrophotometricaly at 230 nm. The amount of drug released was calculated and the percentage drug released was plotted against time. The data obtained for phytovesicular gel from *in vitro* drug release studies were fitted in:

Various kinetic models

In-vitro antifungal activity by cup plate method

The antifungal activity was determined by cup plate method. The activity was compared with conventional carbopol gel and marketed formulation of cotrimazole. The concentrations of samples were taken at 1000 µg/ml and zone of inhibition was measured in mm.

Skin irritation studies[15]

The skin irritation tests were performed using draize primary skin irritation test on albino rabbits. New Zealand white rabbits were climitazied for 7 days before the study. The fur from the dorsal surface was removed without damaging the skin 4 h prior to the study. The animals were divided into two groups. Group I received sodium chloride (0.9%) were used as the control. Group II received phytovesicular gel. The formulations were applied to approximately one square in the skin. The animals were observed for a period of 24, 48 and 72 h and noted for erythema and edema. The mean erythemal scores were determined depending on the degree of edema; no erythema/edema = 0, slight edema/edema = 1, moderate erythema/edema = 2, severe erythema/edema = 3.

Skin penetration studies

The penetration study was carried out by studying the morphology of the pig skin after application of gel.

Stability studies

The formulations were kept for stability studies for 6 months at room temperature (30°C ± 2°C) and at refrigerated temperature (4°C ± 2°C) to determine physical and chemical stabilities.[16] The amount of drug degraded at different time intervals was analyzed.

Statistical analysis

All experimental measurements were carried out in triplicate and were expressed as average of three analyses ± standard deviation. Statistical analyzes was performed by the *t*-test using the Statistical Package for the Social Sciences 17 (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc.) for windows package.

RESULTS AND DISCUSSION

The preliminary phytochemical screening showed the presence of triterpenoids as the main active constituent in all fractions of crude methanol extract. Antimicrobial activity study indicated that all fractions exhibited antimicrobial activity. Chloroform (100%), Pet ether: Chloroform (50:50) has shown greatest antimicrobial activity. The activity was found to be more for the fungus *C. albicans* indicating that they would be a potent antifungal drug.

Chloroform 100% fraction having comparatively high yield was subjected for preparative TLC purification, the separated fractions were subjected to antimicrobial assay by MIC. Chloroform: Methanol (1:0.125) fraction has shown MIC value of 15.6 µg/ml against *C. albicans* and showed positive test for triterpenoids. The fraction was named as AFTSI. A compound (20 mg) was isolated from this fraction at an R_F value. It was isolated as a light yellow colored compound soluble in methanol/DMSO and showed positive test for triterpenoids.

Infrared, mass and nuclear magnetic resonance (NMR) data suggested that compound has a molecular weight of 426 along with an oxygen function as OH group in the molecule. It can be inferred that isolated compound is a hydroxy derivative of amyrin[17,18] with molecular formula: $C_{30}H_{50}O_2$. It showed an MIC value of 15.6 µg/ml the compound was isolated for first time from *S. indica* and the possible structure of compound is shown in Figure 1. The spectral datas for the compound is given below.

Fourier transform infrared

Infrared (KBr) 3427, (OH stretch), 2955.04 (C-H stretch), 2926 (C-H stretch), 2856.67 (C-H stretch), 1734 (C = O stretch), 1573.97, 1423.91 (Benzene ring stretching) 1101.39 (C-O stretch), 802.41 (olefinic C-H bend aliphatic), 651.96 (C-H bend), 619.17 (C-H bend)/cm.

Nuclear magnetic resonance spectrum

Hydroen NMR (^{1}HNMR) spectra of the isolated compound showed characteristic signals for the number and types of carbon and hydrogen atoms present in the molecule. The NMR signals ^{1}HNMR (400 MHz, DMSO) δ 0.79, 0.87, 0.89, 0.96, 1.02, 1.08, 1.15 ppm and at 1.27 ppm indicated the presence of eight CH_3 groups attached to the tertiary carbon atoms. Two secondary methyls resonated at 0.96 and 0.98 were indicative of ursane skeleton. The multiplet signal at δ 4.05 ppm is due to the CH_2 protons under oxygen function. The signal at δ 5.1 ppm for one proton is due to the presence of an unsaturated proton.

Mass spectrum

Atmospheric pressure chemical ionization mass spectrometry ((-) APCI MS) m/z (% relative abundance):

441 $(M-H)^-$, 90 for $C_{30}H_{50}O_2$, and other diagnostic peaks at 427,105,191. The APCI MS spectrum indicated a peak at m/z 441 for $(M-H)^-$ ion suggesting a molecular weight of 442.

Formulation of phytovesicle gel

The λ_{max} of AFTSI was found to be 230 nm in phosphate buffer-methanol mixture. A Calibration curve for AFTSI in phosphate buffer-methanol was plotted the slope was found to be 0.012 with regression coefficient 0.999.
n = 3 (mean ± standard deviation of 3 determinations)
The compatibility studies of the physical mixture of components were studied and there were results were no physical incompatibilities observed.

Evaluation of antifungal triterpenoid from *Samadera indica*-phosphatidyl choline vesicles

The phytovesicles resulted due to the bonding of PC with AFTSI were analyzed for complex formation by solubility, FTIR and melting point studies, entrapment efficiency and vesicular size evaluation Figures 1 and 2.

Solubility

The solubility of AFTSI, and AFTSI-PC complex was checked in different solvents and was given in Table 1. The solubility studies concluded that AFTSI, PC and AFTSI-PC complex were soluble in methanol, DMSO and chloroform. The change in solubility of the complex in hexane as compared to AFTSI and PC may be due to interaction of AFTSI with the PC.

Fourier transform infrared

The FTIR spectras has shown following peaks for AFTSI and Phytovesicular gel confirming bonding of AFTSI with PC.

Antifungal triterpenoid from *Samadera indica*

Infrared (KBr/cm) 3414/cm, (OH stretch), 2937 (C = O stretch), 1727 (C-H stretch), 1378, 1247 (C-O stretch), 1456 (C = C bend) 817, 457, 608 (C-H bending) Figure 1.

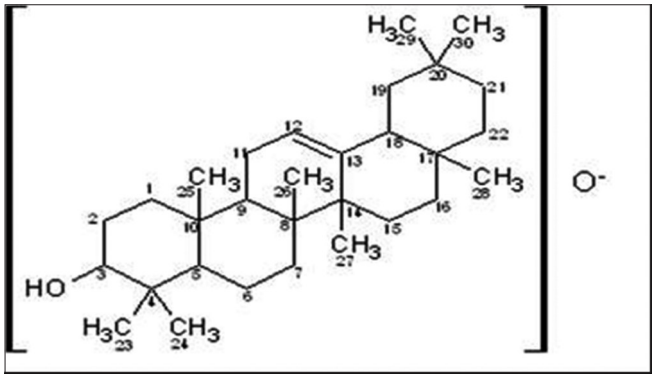

Figure 1: Structure of isolated compound

Phytovesicle gel

Infrared (KBr/cm) 3445/cm, (OH stretch), 2772 (C = O stretch), 1631 (C-H stretch), 1209 (C-O stretch), 1631 (C = C bend) 769, 732, 677 (C-H bending).

It has shown that the spectra of AFTSI were different from that of AFTSI-PC complex and characteristic peaks were noted. All the major peaks are maintained. A broad peak at 3445 can be seen and from the peaks it was clear that the drug has entrapped inside the phytovesicle complex.

Melting point

Melting point of AFTSI, PC and AFTSI-PC complex were found to be 194–196°C, 112–114°C and 82–86°C respectively. The change in melting point further revealed the formation of complex.

The result confirms that there is change in solubility, FTIR and melting point. This infers the complex formation is present.

Size and size distribution

The average size of particles was between 216.44 nm ± 31.45 nm. The polydispersity index was found to be 0.206, this shows that particles were of uniform size. The size distribution was given in Figure 3. The scanning electron microscopy revealed the shape and formation of phytovesicle. It was shown in the Figure 4.

Table 1: Solubility study

Solvent	AFTSI	PC	AFTSI-PC complex
Distilled water	Partially soluble	Micellar solution	Micellar solution
Methanol	Soluble	Soluble	Soluble
DMSO	Soluble	Soluble	Soluble
Chloroform	Soluble	Soluble	Soluble
Hexane	Soluble	Soluble	Partially soluble

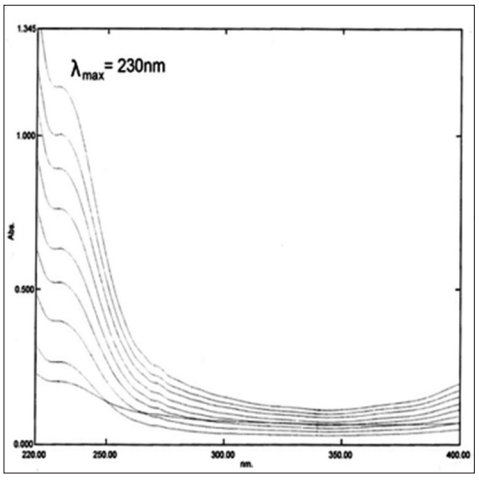

Figure 2: Overlain ultraviolet spectra of antifungal triterpenoid from *Samadera indica* in phosphate buffer-methanol mixture compatibility

Entrapment efficiency

The loading efficiency of formulation was found to be 82.46%. This indicated the amount of drug entrapped by phytovesicles.

Zeta potential determination

The zeta potential of the sample was found to be − 52.65. This shows that the preparation was stable.

Evaluation of phytovesicle gel

Physical parameters of phytovesicle gel

Physical parameters: The phytovesicle gel was shown in Figure 5. The physical parameters like pH, viscosity and extrudability shows that it has satisfactory values. The values were tabulated in Table 2.

Ex-vivo permeation studies

The *ex-vivo* permeation studies of phytovesicle gel, conventional carbopol gel, control and marketed formulation were carried out at 37°C for 24 h [Figure 6]. The permeability coefficient of the phytovesicular gel, carbopol gel, control and marketed formulation was found to be 5.6×10^{-3}, 2.31×10^{-3}, 1.11×10^{-3}, 1.77×10^{-3} respectively The flux data has shown that there is statistically significant difference ($P < 0.05$) in the permeation of drug through the skin. The penetration was enhanced five times when compared to the control.

Drug release kinetics

The *in-vitro* release data after 24 h of phytovesicle gel formulation was compared with marketed gel and conventional carbopol gel. The release datas of all the three formulations were represented graphically Figure 7. The cumulative percentage amount of drug release was found to be 68% ±2.476%, 40% ±7.243% and 59% ±1.689% respectively for phytovesicle gel, carbopol gel and marketed gel at the end of 24 h.

The *in-vitro* release data has shown that the drug release from the phytovesicle formulation is in controlled manner

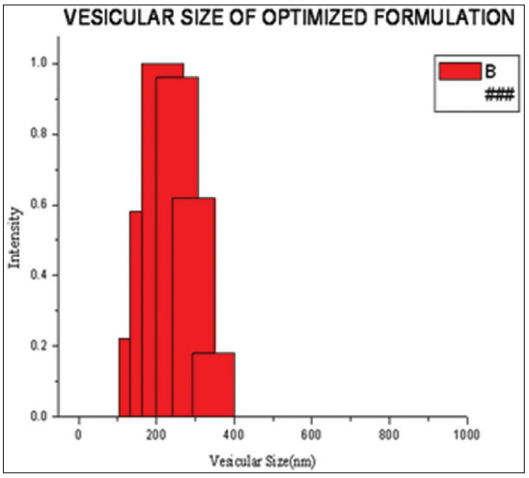

Figure 3: Size distribution of phytovesicles

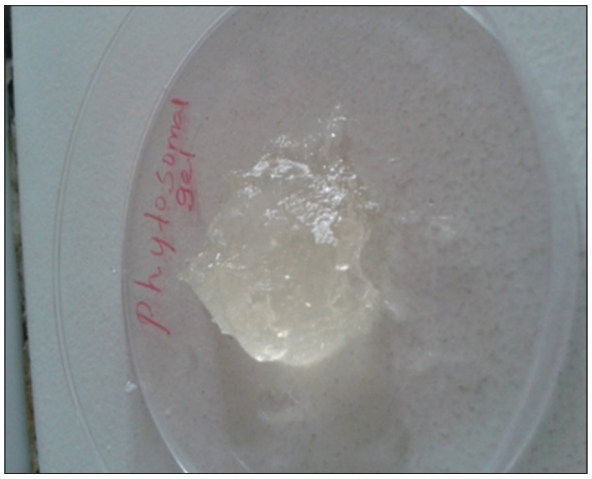

Figure 5: Phytovesicle gel

Table 2: Physical parameters of the phytovesicle gel

Formulation	pH	Viscosity	Spreadablity	Extrudability
Phytoveside gel	6.91±0.003	95.29±0.36	39.37±0.0861	Extrudable

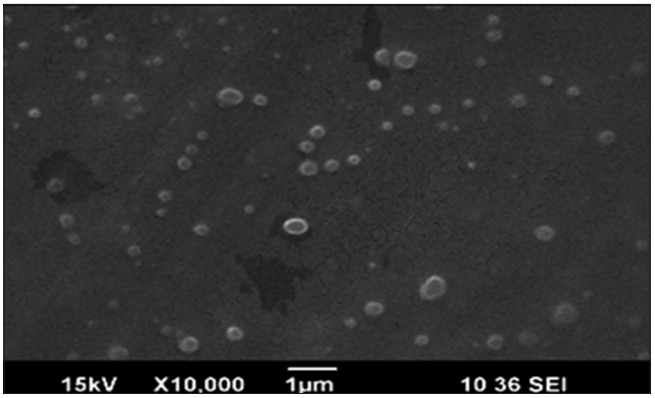

Figure 4: Scanning electron microscopy image of phytovesicles

Figure 6: *Ex-vivo* permeation studies

and with no lag time. The release profiles suggest that the release of drug has occurred slowly and then immediate release. The percentage amount of drug release was greater for phytovesicle gel formulation followed by marketed and conventional gel.

Formulation of the drug

The datas were fitted into different models to find out the release pattern. The release pattern was found out by noting the best fit model. It was given in Table 3.

Kinetic modeling of *in-vitro* drug release

The kinetic study revealed that phytovesicle gel follows zero order kinetics as the regression coefficients approaches unity, indicating the drug release is independent of drug concentration. The *n* values from Korsmeyer Peppas model show that the drug release pattern follows mainly nonfickian diffusion mechanism, the summation of both diffusion and dissolution controlled drug release.

Microbiological evaluation

Microbial contamination

The microbial contamination of the phytovesicle gel after 24 h was found to be 1.0 colony-forming unit (CFU) for bacteria and 1.51 CFU for fungi at temperature of 37°C and 2.19 and 1.76 CFU for bacteria and fungi at the end of 5 days. The values were within the limits.

In-vitro antifungal activity by cup plate method

The antifungal activity of the phytovesicular gel, conventional carbopol gel and marketed formulation was carried out against *C. albicans*. The zone of inhibition was found to be 30 ± 0.57, 24 ± 0.04 and 27 ± 0.60 respectively and shown in Figure 8. The antifungal activity of phytovesicle gel was higher than the marketed formulation ($P < 0.05$). This enhanced antifungal activity may be due to increased penetration of vesicle containing plant constituent through the fungal cell wall.

Zone of inhibition by cup plate method

Skin irritation studies

The phytovesicle gel did not show any skin irritation as the irritation score was zero.

Skin morphological studies

Histology of pig ear skin treated with phytovesicle gel, conventional carbopol gel, water and marketed formulation was given in Figures 9-12. In case of carbopol gel (control) and skin treated with water it was clear from figure that stratum corneum, epidermis and dermis were closely packed. Whereas, in case of phytovesicle gel, the stratum corneum is highly disrupted and hence rapid permeation of the drug through the skin, hence easily by passing the skin barrier thereby enhanced skin permeation. Thus, the mechanism of penetration of phytovesicles can be confirmed from the histological study that phytovesicle gel undergoes enhanced permeation as it alters the lipid fluidity of the stratum corneum.

Stability studies

Stability studies of phytovesicular gel at room temperature (30°C ± 2°C) and at refrigerator temperature (4°C ± 2°C) were carried out for 6 months. The physical appearance does not show any significant changes compared to the freshly prepared formulation. *In-vitro* drug release from formulation at two different temperatures is represented in the Figure 13 and shows that there are no significant changes in the cumulative

Table 3: Kinetic modeling of *in vitro* drug release

Formulation code	Kinetic Model								Best fit model
	Zero Order		First Order		HIGUCHI		PEPPAS		
	R	K_0	R	K_1	R	K_H	R	*n*	
Phytoveside gel	0.9776	3.5283	0.8378	0.0551	0.9647	18.887	0.7234	0.9342	Zero order

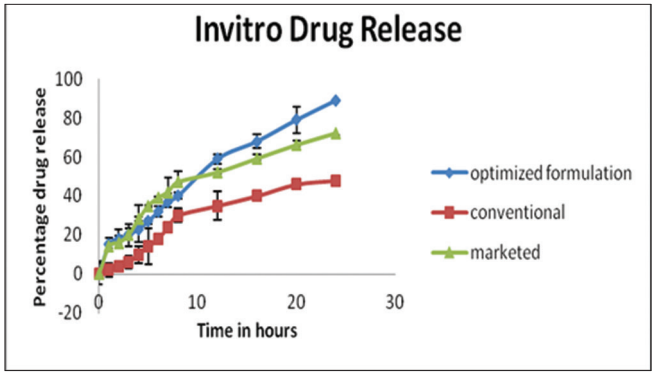

Figure 7: Kinetic modeling of *in-vitro* drug release of phytovesicle gel

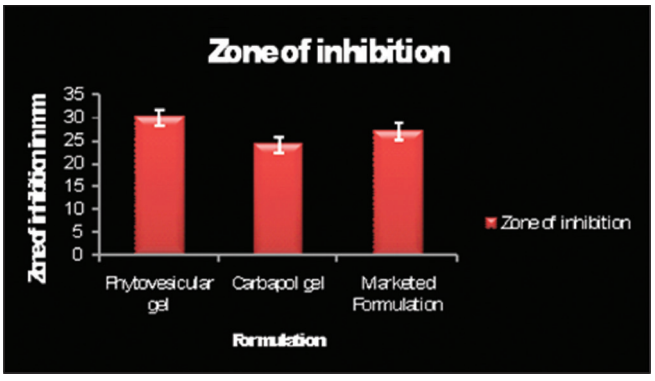

Figure 8: Zone of inhibition in mm

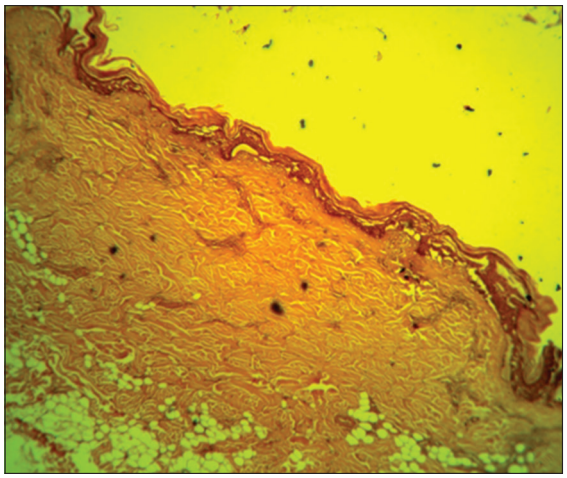

Figure 9: Skin treated with phytovesicle gel

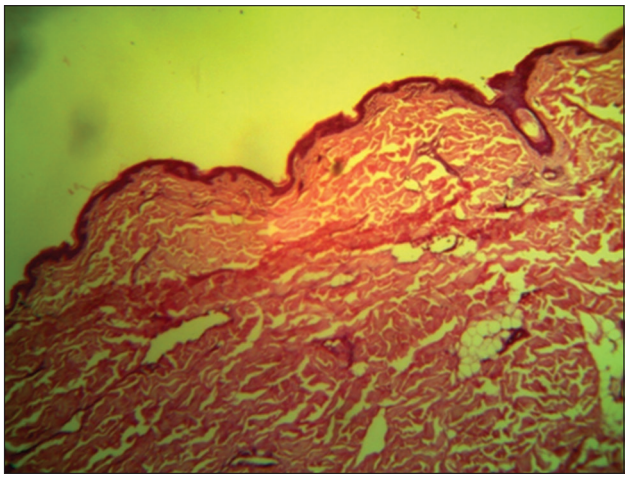

Figure 10: Skin treated with conventional carbopol gel

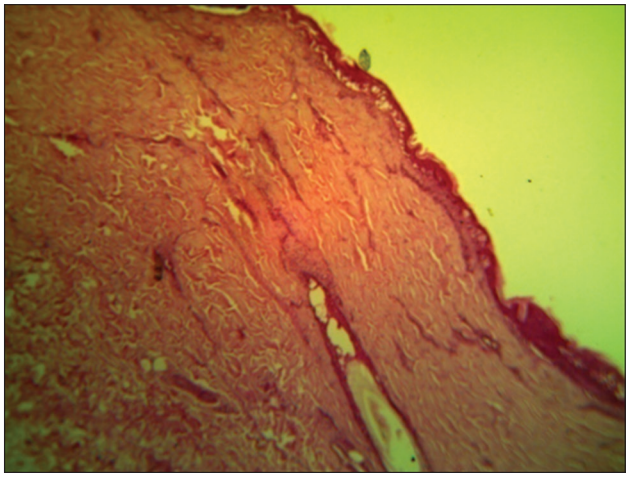

Figure 11: Skin treated with water

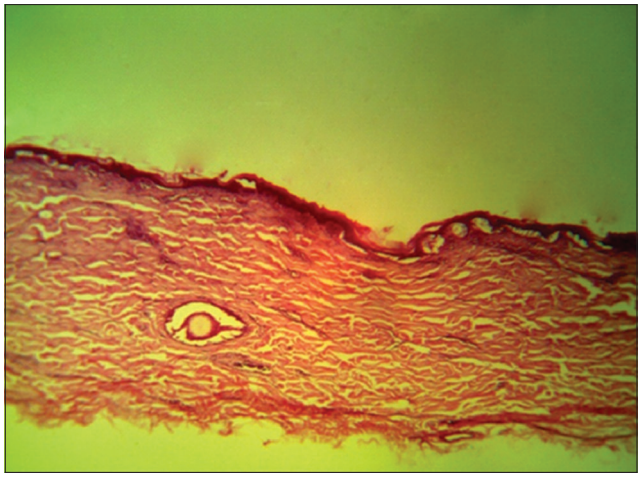

Figure 12: Skin treated with marketed formulation

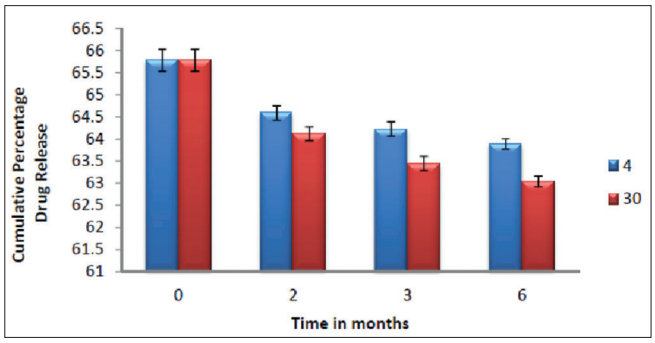

Figure 13: Stability studies of phytovesicle gel

amount of drug release during storage for 6 months in both conditions. However, the formulation is more stable at low temperature compared to the room temperature.

CONCLUSION

Samadera indica belonging to Simaroubaceae family is rich in triterpenoids. Triterpenoids are one of the major classes of phytoconstituents used as antimicrobials.[15,16] Methanolic extract of *S. indica* was found to be active against selected microorganisms and anti-microbial activity was found to be more against the fungus *C. albicans*. The antioxidant studies also proved that the methanolic extract of *S. indica* has the ability to scavenge free radicals produced in our body. This may be due to the presence of polyphenolic compounds present in the extract. The crude methanolic extract in topical formulations, ointment and gel exhibited antimicrobial activity. Gel was found to be comparatively stable than ointment preparation. The antimicrobial activity guided fractionation and purification of crude methanolic extract led to the isolation of hydroxy derivative of amyrin from chloroform fraction. This compound is isolated for first time from this plant. The compound exhibited activity against tested organisms *C. albicans, P. aeruginosa* and *S. aureus*. The activity was high for fungus *C. albicans*. Using the chloroform fraction of crude methanolic extract from the compound was isolated was named as AFTSI. The fraction showed equally potent activity towards the fungus *C. albicans*. In order to increase the permeability of drug

through skin and to treat topical and deep seated fungal infections a novel drug delivery system was developed using AFTSI. The complextionn of AFTSI with phoshatidyl choline and the resultant formation of phytovesicles were incorporated into carbopol gel. The phyto-vesicular gel formulation of the AFTSI showed excellent antifungal activity, *in-vitro* drug release and skin permeability when compared with a marketed and conventional formulation. The stability studies for a period of 6 months at room temperature and at refrigerator temperature was studied and inferred that the formulation was stable during the study period. It was found to be more stable at refrigerated temperature than room temperature. The present study provided the scientific evidence for the antibacterial and antifungal activity of the leaves of *S. indica*. The gel formulated with the triterpenoid fraction showed very good activity against *C. albicans* which is very promising. Further research is required to study the formulation aspects using the isolated compound and its advanced studies.

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