Formulation development and evaluation of anti-acne activity of ethosomal gel prepared using \textit{Plumbago zeylanica} root extract

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\textbf{ABSTRACT}

The objective of present research work is, to develop an ethosome, as a carrier system for Plumbago root extract, its incorporation into gel formulations and to characterize the developed gel formulations by estimation of plumbagin content and study of anti-acne activity. Roots of \textit{Plumbago zeylanica} L (Plumbaginaceae) contains Plumbagin and is used for skin infections and intestinal worms. Topical therapy for acne includes comedolytic agents, antibiotics and anti-inflammatory drugs. The excessive use of antibiotics has led to the increased resistance of acne-causing bacteria. In this research work, plumbago roots have been screened for their potential use for the treatment of acne and hydroalcoholic extract of the roots was found to be effective. Seven batches (EF1-EF7) of ethosomes were prepared using soya lecithin (1-3%) and ethanol (10-45%) and the hydroalcoholic extract. The range of entrapment efficiency varied from 17.12 to 80.82%. The ethosomes EF6 having highest entrapment efficiency was incorporated into gel formulation. Carbopol 934P (0.5-2%) was used to prepare ethosomal gel and evaluated for physicochemical properties, drug content and diffusion characteristics. The pH of the gel was in the range of 6.87 to 7.03. Viscosity was between 5600 - 9800 centipoises. The % drug content was in the range of 95.91% to 100.7%. The ethosome, in their gel formulation, showed good physicochemical properties, drug content and diffusion pattern. The anti-acne activity of F3 showed good zones of inhibition comparable with standard Clindamycin. The present study suggested ethosomal gel as an efficient carrier for plumbago root extract for anti-acne activity.
reduced bioavailability. Hence, novel drug delivery system like phytosome, ethosome, dendrimer, nanosome can help in increasing the absorption of phytoconstituents and subsequently their bioavailability (Williams, 2006; Atmakuri and Dathi, 2010; Grace et al., 2014). One of the vesicular carriers that is being explored in recent years is ethosome. The remarkable feature of this carrier is its high deformability because of which it can penetrate intact through the human skin. Ethosomes are soft, flexible vesicles that can transfer active substances efficiently through the stratum corneum into the skin better than liposomes (Bhowmik et al., 2012; Touitou et al., 2000).

Plumbago zeylanica L is a multipurpose medicinal herb of the family Plumbaginaceae. It contains important chemical constituents like naphthoquinones, alkaloids, glycosides and phenolic compounds (Mandavkar, 2011) Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is the principal active compound primarily present in roots in higher amounts. The roots of the plant is used as a cure for skin infections and intestinal worms. Paste prepared from the roots of the plant is applied to the skin to treat ulcers, sores, acne, wound healing and also a filarial leg (Jain et al., 2014; Tyagi and Menghani, 2014).

Acne vulgaris, is a common skin condition, causing changes in pilosebaceous units (PSU) and skin structure consisting of a hair follicle and its associated sebaceous gland, via androgen stimulation. It is characterized by non-inflammatory follicular papules or comedones (blackheads) and by inflammatory papules, pustules, cysts and nodules in its more severe forms. The anaerobic bacteria Propionibacterium acne (P.acne) residing within the pilosebaceous follicle cause inflammation when exposed to the dermis with the ruptured follicle. Staphylococcus epidermidis, an aerobic organism and part of natural skin, is usually involved in superficial skin infection within the sebaceous unit (Thappa et al., 2009). Traditional treatments include anti-inflammatory and antimicrobial agents. Topical therapy includes comedolytic agents, antibiotics and various anti-inflammatory drugs. Systemic therapy includes antibiotics and zinc hormones. The excessive use of antibiotics has led to the increased resistance of acne-causing bacteria. They exhibit several side effects like dryness of skin, dermatitis, darkening of skin and recurrence after withdrawal. Down the ages, extracts of plants have evoked interest and have been screened for their potential uses as alternative remedies for the treatment of various infectious diseases (Sharma and Singh, 2015).

With this background, it was decided to develop an efficient ethosome as a carrier system for a hydroalcoholic extract of Plumbago, its incorporation into gel formulations and to characterize the developed gel formulations using various parameters including estimation of plumbagin content and antiacne activity.

MATERIALS AND METHODS

Plant Material and Chemicals
Plant samples roots of Plumbago zeylanica L were procured from the local market at Kalbadevi Mumbai. It was authenticated in Agarkar Research Institute, Pune and was assigned the voucher no. (AHMA-24141). All chemicals and reagents used were of analytical grade. The standard plumbagin was procured from Sigma Aldrich. All culture Medias were purchased from Himedia Laboratories.

Preparation of the Extract
The powdered roots were extracted with 70% ethanol for a period of 16-17 hours in a Soxhlet extractor. The extract was filtered, concentrated under reduced pressure below 40 °C to obtain the dry extract. The dried extract was stored at 2-8 °C.

Characterization of the Extract
A stock solution of plumbagin (1000 µg/ml) was prepared. It was diluted to obtain a working standard of 100 µg/ml. The calibration curve of plumbagin was studied in the concentration (2.5 -12.5 µg/ml) at λmax 265nm using ethanol as blank. A
Table 1: composition of various ethosomal vesicles and its entrapment efficiency

| Ingredient | EF1  | EF2  | EF3  | EF4  | EF5  | EF6  | EF7  |
|------------|------|------|------|------|------|------|------|
| HA extract (%) | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 |
| Phospholipid (%) | 2    | 2.5  | 3    | 2    | 2.5  | 3    | 4    |
| CHCl3: MeOH | 2:1  | 2:1  | 2:1  | 3:1  | 3:1  | 3:1  | 3:1  |
| Cholesterol (%) | 0.2  | 0.2  | 0.2  | 0.2  | 0.2  | 0.2  | 0.2  |
| Ethanol (%) | 10   | 20   | 40   | 45   | 40   | 45   | 45   |
| Water (%) qs | 100  | 100  | 100  | 100  | 100  | 100  | 100  |
| Entrapment Efficiency | 17.12 | 25.36 | 55.33 | 35.35 | 58.17 | 80.82 | 41.46 |

Table 1 Finally, all the formulations were stored in the refrigerator.

Table 2: Preparation of ethosomal gel formulation

| Ingredients (gm) | F1 | F2 | F3 | F4 |
|------------------|----|----|----|----|
| Carbopol 974P    | 0.5| 1  | 1.5| 2  |
| Ethosomes (%)    | 2.5| 2.5| 2.5| 2.5|
| Propylene glycol(ml) | 1  | 1  | 1  | 1  |
| Glycerine (ml)   | 2  | 2  | 2  | 2  |
| Water (ml)       | q.s. 20 | q.s. 20 | q.s. 20 | q.s. 20 |

Table 3: Evaluation of gel formulation

| Parameters                  | F1                                    | F2                                    | F3                                    | F4                                    |
|-----------------------------|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|
| Appearance                  | Homogeneous                           | Homogeneous                           | Homogeneous                           | Homogeneous                           |
| Colour                      | Brownish                              | Brownish                              | Brownish                              | Brownish                              |
| pH                          | 6.87±0.12                             | 6.97±0.06                             | 7.03±0.06                             | 6.9±0.1                               |
| Spreadability (gm.sec/cm)   | gel liquid in nature                  | 39                                    | 35.5                                  | 30.57                                 |
| Viscosity (centipoise)      | gel liquid in nature                  | 5600                                  | 9400                                  | 9800                                  |
| Drug content(%w/w)          | 95.91                                 | 99.03                                 | 100.7                                 | 97.22                                 |

Table 4: Anti acne activity

| Microorganisms/ Zone of inhibition (mm ± S.D.) |
|----------------------------------------------|----------------------------------------------|
| Formulation                                  | S. epidermidis                               | P. acnes                                  |
| Gel formulation F2                           | 8.5±0.5                                      | 8.67±0.57                                 |
| Gel formulation F 3                          | 9.5±0.78                                     | 9.4±0.67                                  |
| Gel formulation F 4                          | 8.4±0.63                                     | 8±0.89                                    |
| 2.5% ethanolic (70%) extract                 | 14.26±0.051                                  | 13.45±0.39                                |
| 1.25% ethanolic (70%) extract                | 14.89±0.19                                  | 12.77±0.34                                |
| 1% ethanolic (70%) extract                   | 13.33±0.34                                  | 11.6±0.51                                 |
| Clindamycin                                  | 14.54 ± 0.39                                 | 11.67 ± 0.571                             |
Figure 2: Antiacne activity of formulations against *Staphylococcus epidermidis*

graph of concentration v/s absorbance was plotted to study the standard equation and coefficient of correlation. A test sample containing 100 µg of the extract was prepared and the concentration of plumbagin in the extract was estimated using the standard equation of the calibration curve (Bothiraja et al., 2011).

**Preparation of Ethosome**

Ethosome were prepared as reported by (Mogal and Puranik, 2013). In brief, lecithin was dissolved in chloroform: methanol mixture in a clean, dry round bottom flask followed by removal of the organic solvents using a rotary vacuum evaporator. The solvents were evaporated at 55°C and the thin film formed was allowed to stay for 12 hrs for complete evaporation. This was followed by hydration with different concentration of drug extract by rotation at the corresponding temperature and sonication. The dispersion was made up to 100ml with further addition of ethanol and water (qs 100 ml) and mixed well using a magnetic stirrer. 7 batches of ethosome were prepared using varying concentrations of lecithin(1-3%), chloroform: methanol mixture, and ethanol (10-45%).

**Evaluation of the Prepared Ethosomes**

The evaluation was done on the basis of vesicular size and entrapment efficiency

**Vesicular Size Determination**

The drop of ethosomal suspension was taken on a glass slide with a drop of glycerine. For visualization, Rhodamine Red was added and the ethosomes were observed under Digital Motic Optical Microscope.

**Entrapment Efficiency**

Aliquots of ethosomal suspension (10 ml) were subjected to centrifugation using a cooling centrifuge at 10,000 rpm for 90 minutes. The clear supernatant was separated from ethosome pellet. The pellet was dried in a vacuum oven. Accurately weighed 25 mg of the pellet was dissolved in 10ml of methanol and sonicated for 20-30 min to lyse the ethosomes. The solution was suitably diluted and absorbance measured at 265nm. Amount of plumbagin in supernatant and sediment gave a total amount of drug in the dispersion. The per cent entrapment was calculated using the formula, % entrapment= amount of plumbagin in sediment/amount of plumbagin added ×100.

**Preparation of Topical Gels**

For the preparation of topical gels, carbopol 976P NF (0.5-2%) was soaked in water for a period of 2 hours. The mixture was stirred for 1 hour with the help of magnetic stirrer. To this, propylene glycol and glycerine (1%) was added. The formulation was neutralized by drop-wise addition of 50% (w/w) triethanolamine until transparent gel appeared. Ethosomal suspensions were mixed into a vehicle (gel) to produce the final concentration of ethosome in the gel as 2.5% (w/w). The formulation was allowed to equilibrate for 24 hours. The ethosome EF6 was selected for incorporation into the gel. Four formulations as shown in Table 2 (F1, F2, F3 and F4) were prepared using a varying concentration of carbopol 976P (Dave et al., 2010; Helal et al., 2012).

**Anti-acne Activity**

The study was carried out by agar well plate method (Chaudhary and Sandhya, 2013). The two microbial cultures used for the study were obtained from IMTECH Chandigarh: *Propionibacterium acnes* (MTCC NO: 1951, Muller Hinton agar supplemented with 5% defibrinated sheep blood) and *Staphylococcus epidermidis* (MTCC NO: 435, Brain Heart Infusion agar). *P. acnes* is an anaerobic bacteria which is incubated for 72 hrs in an anaerobic jar containing gas pack and indicator vial. *S. Epidermidis* is an aerobic bacteria which is incubated for 48 hrs. The anti-acne activity was measured as a function of the diameter of the zone of inhibition against standard clindamycin (1 ppm) at 37±0.5°C. The anti-acne activity was studied for the hydroalcoholic extract in the concentrations 1%,1.25%, 2.5%,w/v and the gel formulations (F1 to F4).
RESULTS AND DISCUSSION

The roots of the plant were authenticated in Agarkar Research Institute at Pune and a voucher no. (AHMA-24141) was assigned. The 70% hydro alcoholic extract of plumbago roots gave a yield of 3.5%w/w. Standard Plumbagin was estimated spectrophotometrically at λmax 265nm. It was found to be linear in the concentration range between 2.5 – 12.5 µg/ml. The standard equation for the linearity curve was found to be y = 0.057x + 0.0504 with a coefficient of correlation r² = 0.9941. The plumbagin content in the hydro alcoholic extract was found to be 9.6%w/w.

Colloidal suspensions of ethosomes of 1.25% hydro alcoholic extract was prepared by film rehydration method. Variation in the different formulation were brought about by changing the concentration of phospholipid from 1.5% to 4% and varying the solvent proportion. Seven different ethosomal vesicles were prepared and they were visualized using a digital motic microscope and their entrapment efficiency was calculated. It was observed that ethosomes were round-shaped with particle size ranging from 400 nm- 1µm.

In order to determine the drug holding capacity of the prepared ethosomes, the entrapment efficiency was evaluated. Both, the amount of lecithin and ethanol, influenced the entrapment of the herbal extract inside lipid vesicles in a positive way. The entrapment efficiency of EF6 was found to be highest (80.82%) while EF1 showed the least entrapment efficiency. Increase in per cent drug entrapment was observed with an increase in ethanol concentration. This may be due to the nature of the drug. As the drug is a hydro alcoholic extract, more drug gets encapsulated when ethanol concentration increases. Increase in phospholipid concentration to 4%, decreases the entrapment efficiency. 3% phospholipid concentration showed maximum entrapment efficiency. The ratio of organic solvents also played an important role in drug entrapment. Maximum entrapment efficiency was observed in chloroform: methanol (3:1). Thus formulation EF6 had maximum entrapment efficiency. It contained 3% phospholipid, chloroform: methanol (3:1) and 45% ethanol.

The ethosomal vesicle EF6 was then taken up for further studies. A topical gel of 2.5 % w/w concentration was prepared using carbopol 974P. The concentration of carbopol 974P was varied in the range of 0.5 – 1.5%. The prepared formulations was evaluated for physical characteristics like homogeneity, spreadability, pH and drug content. It was observed that increasing the concentration of poly-mer increased the viscosity. Higher viscosity leads to low spreadability values. The result showed that the developed herbal gel was brownish in colour, translucent in appearance and showed good homogeneity with an absence of lumps and skin irritation. Formulation F3 containing 1.5% carbopol 974P showed good spreadability, pH, drug content and viscosity. The % drug content was observed in the range of 95.91% to 100.7% with formulation F3 showing a drug content of 100.7%. (Table 3) The cumulative release of ethosomal gel was maximum in F3 formulation (100.85%). The in vitro release efficiency of ethosomal gel shows that a significant amount of extract transported across the membrane when entrapped in ethosome.

The anti-acne activity of gel was performed on Propionibacterium acnes (MTCC NO: 1951, Muller Hinton agar supplemented with 5% defibrinated sheep blood) and Staphylococcus epidermidis (MTCC NO: 435, Brain Heart Infusion agar). The antiacne activity of the hydroalcoholic extract and the formulation F1 to F4 was studied. (Figures 1 and 2) It was observed that the formulation F3 had a good zone of inhibition as compared with other formulations. (Table 4).

CONCLUSIONS

Use of advanced lipid vesicles in transdermal drug delivery systems is a need of time since it is believed that conventional liposomal systems have limited applicability as carriers due to their confinement to the Stratum corneum of the skin, instead of its deep penetration. Permeation enhancers, such as Ethanol penetrates into intercellular lipids and increases the fluidity of cell membrane lipids and decrease the density of lipid multilayer of the cell membrane. Herbal ethosomes offers a good opportunity for non-invasive delivery of all sized drug molecules. On the basis of observations of the present study, it can be concluded that a combination of 45% ethanol, 3% lecithin and 70% hydroalcoholic plumbago extract (2.5%w/w) can be used for the preparation of ethosomes with good entrapment efficiency. The in vitro release efficiency of ethosomal gel was found to 100.85%, which support the potential of these carriers in penetrating the lipid-rich biological membrane. The present study revealed ethosomal gel as an efficient carrier for herbal extract.

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Conflict of Interest
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