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Dual Therapeutic Action of Wheatgrass Extract Loaded Lipidic Vesicles for Simultaneous Dermal and Systemic Delivery

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Abstract

The present research work aims to prepare a unique formulation (Gel) containing a lipidic vesicle of extract having the dual therapeutic purpose of achieving great skin simultaneously with improved haemoglobin content. Ethosomes were prepared using soya phosphatidylcholine (1-4%) and ethanol (10-40%) by a modified cold method and were characterized for various pharmaceutical parameters. The prepared ethosomes were unilamellar in nature and were in the size range of 110.98-357.34 nm and had PDI values in the range of 0.167 to 0.528 while Zeta Potential (ZP) was between -54.0 and -79.4 mV. The Entrapment Efficiency (EE) of ethosomes was found to be in the range of 62.13±1.05 to 89.82±1.18%. Ethosomes were further added to Carbopol 934P for gel formation, and subsequently, evaluated for their physicochemical properties. *In vitro* permeation studies and ex-vivo permeation studies were conducted for drug solution, ethosomes incorporated in viscous gel, and conventional gel using Franz diffusion cells for the compound. The results revealed that the ethosomal gel formulation was having 4 times better permeation ability as compared to plain solution and plain gel formulation revealing the fact that ethosomal gel serves the dual purpose of achieving sustained delivery of wheatgrass extract over some time into the systemic circulation as well as treating skin manifestations simultaneously.

Introduction

Anemia affects one-third population of the world and is more prevalent in low- and middle-income countries [1]. Anemia is characterized by a low level of Hemoglobin (Hb) in the blood that may occur due to loss of blood or a decrease in the production of blood cells in the body [2]. RBC count, mean corpuscular volume, blood reticulocyte count, blood film analysis, or Hb electrophoresis are some of the methods used to diagnose anemia [3]. The WHO-defined Hemoglobin (Hb) cut-offs, specific to age, sex, and pregnancy status, are most widely used to diagnose anemia, with the threshold being <120 g/L for non-pregnant and <110 g/L for pregnant women of 15-49 years of age [4]. This concentration may vary as per age, sex, pregnancy

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status, and genetic and environmental factors [5]. The consequences are a decrease in productivity of work, impaired neurological development [6], low birth weight [7], delayed child development [8], and increased morbidity and mortality [9,10].

Anemia prevalence also varies by geographical region. Sub-Saharan Africa and South Asia have the highest anemia prevalence, and at the country level, anemia among Women of Reproductive Age (WRA) remains a moderate-to-severe public health problem (prevalence of 20% or greater) in most WHO member states including India [11]. Globally, anemia prevalence among both non-pregnant and pregnant WRA decreased by less than 1% per year (non-pregnant WRA: from 33% to 29%; pregnant WRA: from 43% to 38%) in these years irrespective of excursive campaigns and awareness programs [12]. The pathophysiology involves is a decrease in oxygen supply to various tissues that may affect multiple organ systems and the various other effects like a decrease in iron content can affect the brain function and its development before the development of anemia which can further cause a negative effect in health and development [13]. Schistosomiasis, Malaria, HIV, Tuberculosis, Thalassemia, and Sickle cell disorders are some of the diseases that are primarily associated with anemia [14].

Wheat (*Triticum* species) a cereal grass of the *Gramineae* (Poaceae) family is the world's largest edible grain cereal-grass crop and is reported to improve hemoglobin content drastically and thus can benefit anemic patients [15]. Further, several other reports highlight the antioxidant/anti-inflammatory properties of this herb [16] and thus can be used to treat various skin manifestations and reduce the problem of aging and wrinkles [17].

It has been also observed that females especially working women don't have enough time to focus on this problem very attentively and regularly. So, the researchers have aimed to develop a suitable formulation of wheatgrass extract for achieving the dual benefit of improved Hb content simultaneously with enhanced skin texture by minimizing skin problems.

Ethosomes can be used for transdermal as well as topical delivery and are soft, malleable vesicles composed mainly of phospholipids (phosphatidylcholine, phosphatidylserine, and phosphatidic acid), ethanol (relatively high concentration) and water [18,19]. Ethanol is a major ingredient and acts as a penetration enhancer. The mechanism of its penetration-enhancing effect is well known. Ethanol interacts with lipid molecules in the polar head group region, resulting in a reducing the rigidity of the stratum corneum lipids, and increasing their fluidity [20]. The interaction of ethanol into the polar head group environment can result in an increase in membrane permeability [21,22]. *In vitro* and *In vivo*, in animal and clinical studies have shown that ethosomes are efficient at improving dermal/stransdermal delivery of both hydrophilic and lipophilic moieties [19].

So, in this current research work, ethosomal gel was prepared to comprise wheatgrass extract to ameliorate hemoglobin levels and refine skin texture at the same time.

Materials and Method

Plant material and chemicals

Seeds of Triticum *aestivum* (Wheat grass Seed) were obtained from National Seeds Corporation Limited, UAS Campus, Hebbal, Bangalore, India and were authenticated by the Department of Ayurveda, Banaras Hindu University, Varanasi. Soya phosphatidylcholine was purchased from Sigma-Aldrich New-Delhi. Ethanol, chloroform, propylene glycol, and cholesterol were procured from Degussa India (P) Limited, Mumbai; India. All other chemical used during the study was of analytical grade.

Cultivation, collection, and extraction of Wheatgrass

An adequate quantity of seeds of wheatgrass was soaked overnight in water. The soaked seeds were spread on the surface of soil filled in plastic trays. A thin layer of soil was sprinkled on seeds of wheatgrass. The trays were covered to provide darkness which helps in sprouting [23]. The plantlets after 9 days, which grew- up to a height of about 15-16 cm were selected for the extraction procedure. The grasses were harvested and dried in well-ventilated dark rooms for 4 days. For extraction dried wheat Grasses were powdered in the mill. The crushed wheatgrass was completely exhausted by adding small quantities of methanol and filtering off every time in a successive manner for 24 hours. This extract was evaporated to dryness at 35^{O} C to remove methanol. Similarly, successive extraction was also carried out. In successive extraction, crushed wheatgrass was exhausted by adding small quantities of petroleum ether a few times and filtering off every time in a successive manner. The filtrate was evaporated to remove petroleum ether and residues were again exhausted by adding acetone a few times. Here also filtrate was evaporated, and residues were exhausted with methanol and then with water, and each time filtrate was evaporated. Finally, the powder was obtained in successive extraction from petroleum ether, acetone, methanol, and water was combined and kept at 4^{o} C for further use.

Calibration curve

The calibration curve of wheatgrass extract was prepared in skin surface fluid (pH 7.4). A stock solution of 1000 µg/ml was prepared followed by the preparation of a calibration curve in the range of 2-12 µg/ml in skin surface fluid (pH 7.4). The calibration curve was prepared by measuring the absorbance of these test solutions under U.V Spectrophotometer at 263 nm [24].

Experimental design

A total of 9 formulations were prepared varying two independent variables at different concentration and their effect on the dependent variable were evaluated. The independent variables are ethanol (10-30%) and Soya phosphatidylcholine SPC (2-4 %). The detailed composition of different formulations is provided in (Table 1)

Preparation of Ethosomes

Ethosomes were prepared by the cold method followed by ultrasonication. Phospholipids and Wheat Grass Extract (WGE) were dissolved in ethanol and propylene glycol mixture (Table 1). This mixture was heated at 30º C in the water bath. To this mixture distilled water was added slowly in a fine stream with a constant mixing (Mechanical stirrer, Remi equipment, Mumbai) at 700 rpm. The temperature was maintained at 30º C throughout the experiment. The mixing was continued for 5 minutes. The preparation was stored at 4^{O} C. The colloidal dispersion prepared by the above procedure was subjected to sonication at 4ºC using a probe sonicator (Ultrasonic Homogenizer, Model No. UAI-PS 750-20LED) in 3 cycles of 5 minutes with 5 minutes rest between the cycles. The formulation was then homogenized at 15,000 psi pressure, in three cycles, using a high-pressure homogenizer to get nano-sized Ethosomes. The prepared ethosomes were stored under refrigeration and evaluated for the following parameters.

Characterization of wheatgrass extract loaded vesicles

Physical appearance

Colloidal appearance, color, and phase separation of all ethosomal formulations were observed visually for the evaluation of their physical appearance.

Entrapment efficiency

The Entrapment Efficiency (EE) of ethosomes was determined by using an ultracentrifugation apparatus (Remi Instrument Limited, Mumbai). The first 5 mL of each formulation were centrifuged at 60,000 rpm, at 4 °C for 2 h. The supernatant was collected and analyzed at 263 nm using a UV spectrophotometer (UV-visible Spectrophotometer 1700 Pharmaspec Shimadzu, Kyoto, Japan) to determine the unentrapped drug. In addition, the ethosomes were lysed with the same volume of 20% v/ v Triton® X-100, diluted with ethanol, and analyzed for the total drug amount in the formulation [25,26]. The EE was calculated from the following equation.

$$Entrapment\ efficiency(EE)\% = \frac{T - F}{T} \times 100$$

Where T: Total amount of drug in the formulation.

And F: Fraction of drug unentrapped.

Vesicle size and size distribution

Dynamic Light Scattering (DLS) was employed to determine the vesicle size and the size distribution of the developed Ethosomes. Vesicular suspension, 1 mL, was diluted to 10 mL with normal saline and the size of the vesicles was determined using the Malvern Zeta master (ZEM 500962, Malvern, UK). The mean value of three repeated measurements for each sample was reported as the final observation [27-29].

Zeta potential

The Zeta potential of undiluted Ethosomal formulation was carried out using Zetasizer (Nano-ZS, Malvern U.K.) The samples were injected into a zeta potential measurement cell. The measurements were done at 25 °C at an electric field strength of 23.2 V/cm [30].

Surface morphology

The surface morphology of optimized Wheat grass-loaded ethosomes formulation (F6) was examined by Transmission electron microscopy (Model LEO 435 VF, Leo electron microscopy Ltd., Cambridge, England). Prior to analysis, the ethosomes were mounted onto double-sided tape that has previously been secured on copper stubs and coated with platinum. Then the SEM images were recorded at a different magnification at the acceleration voltage of 80Kv [31].

Incorporation of vesicular formulation into gel

For incorporation of Wheat Grass loaded Ethosomes into a gel, Carbopol 934P 1% w/v was soaked in a minimum amount of water for an hour and then optimized Ethosomal dispersion. Optimized ethosomal formulation was added to the swollen polymer under continuous stirring (700rpm) in a closed vessel and the temperature was maintained at 30º C. Stirring was continued to form homogeneous ethosomal gels and finally, triethanolamine was added to neutralize the formulation.

Evaluation of vesicular gel

Viscosity and pH determination

Viscosity of the ethosomal gel formulations were determined using Brookfield viscometer (Model No DV-III ULTRA) at 100rpm using spindle no. 06. Digital pH meter (RI-152-R) was used to determine the pH of the prepared ethosomal gel. The glass electrode was dipped completely into the gel formulation [32].

Spreading diameter

The spreadability of gel formulation was determined by placing the 1gm of gel within a pre-marked circle over a glass plate (20 cm × 20 cm) of smooth surface. Over which a second glass plate was placed.The standard weight of 125gm was applied on the upper plate and allowed to rest for 5 min. The increase in diameter due to spreading was noted [33,34].

Drug content of the formed gels

About 500 mg of gel was taken and dissolved in 10 ml of ethanol followed by 40 ml of pH 6.8 PBS. The solution was then passed through the filter paper, and 50 µl of the filtrate was withdrawn. The filtrate was diluted by adding distilled water, and the drug content was measured spectrophotometrically at 263 nm against the corresponding gel concentration [33,34].

In-vitro drug release study

In vitro release study was carried out using Franz Diffusion Cell with a diffusion area of 9.42 cm^2 and a receiver volume of 14 ml. An egg membrane of suitable size was mounted between the donor cell and acceptor cell of the Franz diffusion cell. The donor compartment was filled with the known quantity of optimized ethosomal Formulation (F), ethosomal gel formulation, and plain gel formulation of wheat grass oil, and the cumulative amount of drug released in each case was compared. Phosphate buffer (pH 6.8) was used in the acceptor compartment. Studies were carried out at 37 ± 1°C at a speed of 400rpm/min for 24h. Samples (1ml) were withdrawn through a sampling port at predetermined time intervals over 24 h and analyzed using UV spectrophotometer at 243 nm. Samples were immediately replenished with an equal volume of fresh phosphate buffer. All experiments were conducted in triplicate. Sink condition was maintained throughout the experiment [35,36].

***Ex-vivo* skin permeation studies**

The 6-8 weeks rats of Sprague-Dawley strain having weight in the range of 130-160gm were sacrificed using chloroform. Then the hair was trimmed carefully (<2mm) and the skin from the abdomen was removed using a scalpel and placed in the aluminum foil. Any adhering fat or subcutaneous tissue from the dermal side of the skin was pulled gently.

The ex-vivo skin permeation of wheatgrass from optimized selected Formulation (F8), ethosomal gel formulation, and plain Gel of drug was studied using locally fabricated diffusion cell. The excised skin was soaked in a buffer for 6-8 hours then it was clamped in between the donor and receptor compartment of the cell (area 2.011 cm^2). 20 ml of Phosphate buffer (pH 6.8) was taken in the receptor compartment. The formulation of the known quantity was spread uniformly on the epidermal side of the excised skin. The donor compartment was kept in contact with the receptor compartment. The studies were carried out at 37 ± 0.1^{O} C and the receptor medium was stirred by externally driven Teflon-coated magnetic bars (400 rpm/min). At predetermined time intervals,an aliquot of the sample was withdrawn and replaced by Phosphate buffer (pH 6.8). The samples were analyzed at 263 nm using a UV spectrophotometer (UV-visible Spectrophotometer 1700 Pharmaspec Shimadzu, Kyoto, Japan).

This study was carried out in triplicate and the average value was reported. The flux was also determined from the linear portion of the graph by plotting between cumulative amounts of drug permeated per unit area per unit time (CDP/A) across the membrane. This flux was compared with the target flux [37,38].

Stability Study

Optimized ethosomal gel formulation was selected for stability studies of vesicles. The vesicular suspension was kept in sealed vials at 4 ± 2^{o} C and at room temperature for 60 days. Percent entrapment, zeta potential, and vesicle size were determined at different time intervals using the methods described above in the manuscript [39,40].

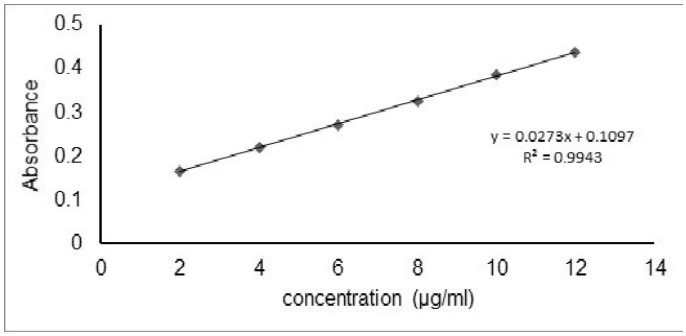

Figure 1: Calibration curve of wheat grass extrac.

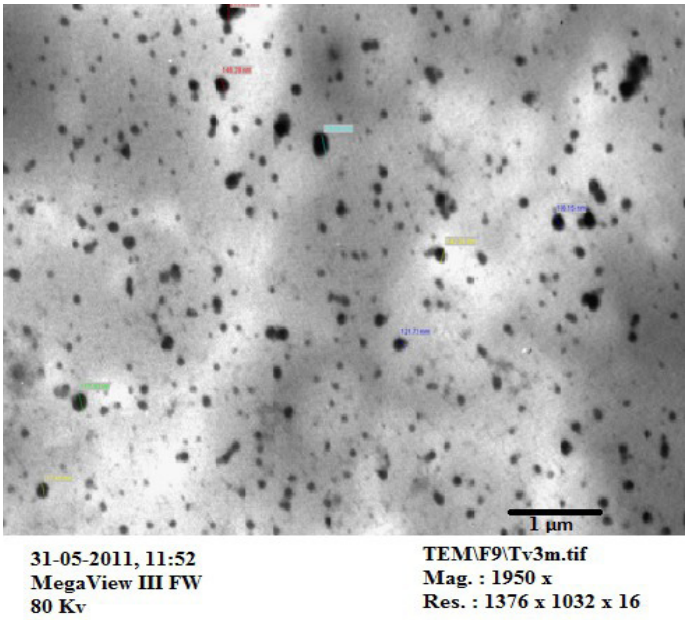

Figure 2: Transmission electron microscopic image of F6 ethosomal formulation.

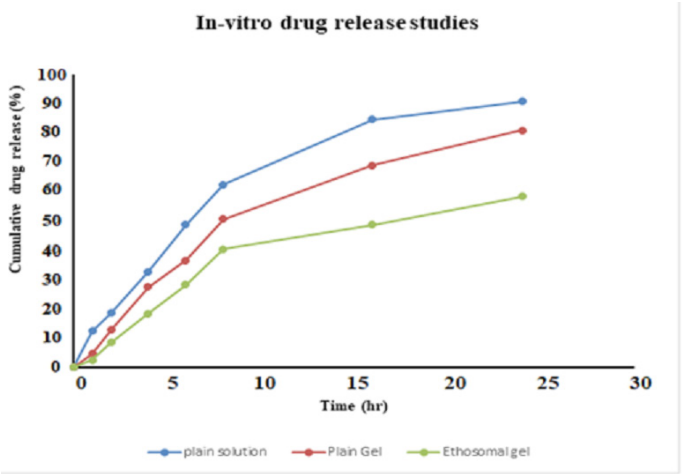

Figure 3: In-vitro release data comparison of different formulation.

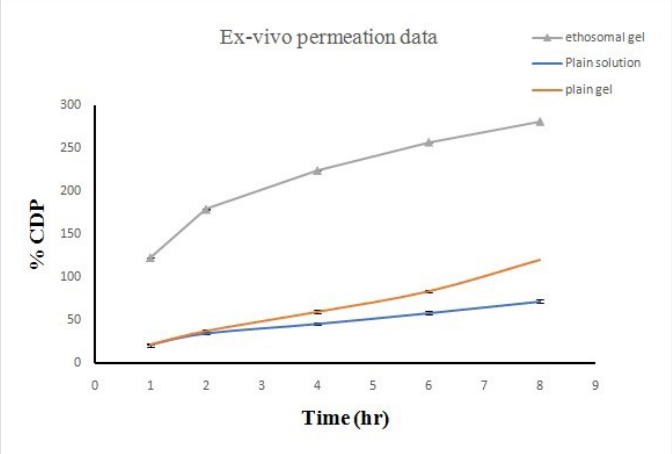

Figure 4: Ex-vivo permeation data comparison of different formulation.

Table 1: Composition of various ethosomal formulations.

Composition in % w/w	F1	F 2	F 3	F 4	F 5	F 6	F 7	F 8	F 9
Drug (wheatgrass)	1	1	1	1	1	1	1	1	1
Soya phosphatidylcholine	2	2	2	2	3	3	3	3	4
Ethanol	10	20	30	40	10	20	30	40	40
Propylene glycol	1	1	1	1	1	1	1	1	1
Cholestrol	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Water	q.s	q,s	q.s	q.s	q.s	q.s	q.s	q.s	q.s

Table 2: Characterization report of ethosomal formulation.

Formulation code	Vesicle Size	PI	Zeta Potential	% Entrapment	pH
F1	254.00±0.11	0.310±0.076	-54.17±0.23	62.13±1.05	6.6
F2	238.61±5.23	0.260±0.36	-59.67±0.45	65.43±0.98	6.5
F3	220.00±2.92	0.215±0.23	-60.12±0.03	69.25±0.56	7.8
F4	223.85±4.41	0.167±0.45	-61.23±0.54	70.11±1.04	5.7
F5	208.93±2.32	0.459±0.53	-63.76±0.49	70.15±1.25	5.3
F6	187.71±6.13	0.331±0.28	-67.89±0.34	79.04±0.56	6.8
F7	202.17±4.84	0.271±0.65	-69.11±0.39	80.63±1.92	7.4
F8	194.34±2.56	0.202±0.35	-70.11±0.53	80.24±1.07	7.0
F9	183.73±5.96	0.528±0.29	-79.87±0.67	89.82±1.18	6.4

Table 3: Evaluation Parameters of physiochemical properties of ethosomal Gel.

Formulation	Homogeneity	Viscosity	pH	Texture	Spreading diameter	Drug content
Ethosomal gel	Homogenous	4500	6.7	Smooth	52	75.73

Table 4: Ex-vivo permeation data of different formulation.

Time	Plain Solution	Plain Gel	Ethosomal Gel
0	0	0	0
1	22 ±0.186	21.3±0.056	122.40± 0.221
2	35.1± 0. 836	37.43 ± 0.38	178.62±0.127
4	46.2±0.527	59.5± 0.831	223.54±0.214
6	58.5± 0.382	83.45±0.081	256.50±0.117
8	71.8± 0.932	120.34±0.037	280.42±0.245

Result & Discussion

Calibration curve

Standard wheatgrass extract was estimated spectrophotometrically at λmax 263nm. It was found to be linear in the concentration range between 2.5-12 µg/ml. The standard equation for the linearity curve was found to be y = 0.0273x + 0.1097 with a coefficient of correlation r^2= 0.9943.

A total of nine formulations were prepared using cold sonication followed by ultrasonication. The developed formulations were then evaluated for the following parameters.

Characterization of ethosomal formulation

Entrapment efficiency

The entrapment efficiency of all ethosomal formulations were determined using ultracentrifugation. The effect of the two independent variables ethanol concentration and soya phosphatidyl concentration was observed on the dependent variable (entrapment efficiency) of ethosomes. The entrapment efficiency ranged from 62.13 percent for F1 formulation to 89.82 for F9 formulation. The results clearly revealed that there is a direct relationship between the concentration of ethanol and entrapment efficiency as can be seen in table 2. The entrapment was found to increase with the increase in ethanol concentration to a certain limit above which the higher concentration of solvent causes somewhat leakage of drugs from the bilayers of vesicles resulting in decreased entrapment efficiency. The entrapment efficiency increased with an increase in the concentration of SPC as can be seen from the results in table 2. There was a direct relationship between the polymer concentration and capturing ability of the vesicle [41].

Vesicle Size and shape and zeta potential

The vesicle size of the formulated Ethosomes was found to be in the range of 183.73 nm to 254 nm. It was clearly observed that vesicle size is inversely proportional to the concentration of ethanol as can be seen from (Table 2). The data in Table 2 also indicates that as the concentration of phospholipid is increased, the size of vesicles increases. As literature reports are available that vesicles up to 300 nm are able to penetrate to dipper layers of skin easily and release the medicament to the target site [41]. The Polydispersity Index (PDI) of various formulations is shown in (Table 2). PDI essentially gives a representation of the particle size distribution of a given sample. The numerical value of PDI ranges from 0.0 (as in the case of the perfectly uniform sample) to 1.0 (for a highly polydisperse sample. It has been demonstrated by various researchers that a PDI value below 0.3 is acceptable and is indicative of a homogeneous population of particles in the formulation of lipid-based carriers, such as liposome and nano-liposome formulations [42,43].

The value of the zeta potential plays a very important role in the potential stability of the colloidal system. The large magnitude of zeta potential whether positive (>+20 mV) or negative (< -20 mV) represents that the colloidal particle will have the tendency to repel each other and coalescence or aggregation would be avoided resulting in greater stability of the system. However, if the particles have low zeta potential values, then there will be no force to prevent the particles to come together and flocculate. The zeta potential was in the range of

-54 to -79 confirming the overall uniform dispersion of the ethosomes and a stable formulation. The charge of vesicles is an important parameter that can influence both stability and skin vesicle interaction. The pH of the ethosomal formulation was found to be in the range of 5.3 ± 0.58 to 7.8 ± 0.11 [44, 45].

Selection of the optimized formulation

Optimization was done based on entrapment efficiency, vesicle size, zeta potential, and polydispersity index. Thus, F8 formulation was employed for further studies to formulate ethosomal gel as it was having a size below 200 nm and also an optimum value of zeta potential above ± 25, and a PDI value of below 0.3. All these factors are considered very important for better permeation ability through the skin.

Surface morphology

TEM images depicted the presence of almost uniform, spherical-shaped vesicles and the absence of aggregates. It can be seen in fig. 2 that smaller size vesicles with an almost uniform nature were prepared.

Characterization of ethosomal gel formulation

Ethosomal gels were evaluated for their physicochemical properties, drug content, and drug release profiles. The results of all the evaluation parameters for gels are presented in (Table 3).

The physicochemical properties of the prepared ethosomal gel were very much in the range of what is needed for efficient application and spreading of the formulation on the skin of the patient with good texture and effective drug concentration.

In Vitro Drug Release Studies

On comparing the basic parameters of different formulations F-8 was selected as the best formulation having optimum parameters. Three formulations i.e., plain drug solution, plain gel formulation, and ethosomal gel formulation were utilized and compared. Drug solution in the release medium was used as a control to make sure that the dialysis membrane was not a barrier to drug diffusion. After 24 h, this solution showed a cumulative percent drug release of 90.671 ± 5.07%, which was significantly higher than that released from any of the tested formulations after the same time (p < 0.05). This confirms that

the dialysis membrane did not impede the diffusion of the released therapeutic moiety.

The results as detailed in Fig.3 revealed that the ethosomal gel formulation was having great potential in sustaining the release rate of the drug from the preparation thereby being able to produce a sustained therapeutic effect at last. The release of drug from ethosomal gel was 58.32 ± 3.07% as compared to the plain gel formulation where 80 percent of drug was released in the defined period as the gelling polymer Carbopol is hydrophilic. The results indicated the potential of ethosomal Gel formulation as a transdermal drug delivery system for sustained therapeutic action [46,47].

Various research reports previously published have reported that drug release from vesicles suspended in gel bases occurs through two steps; drug release from the vesicles, which act as a drug reservoir followed by diffusion through the gel network[48].The presence of the gel network results in an increased diffusion path and subsequent reduction in drug release rate. Gel viscosity was reported to have an important influence on the drug release rate where increased viscosity was associated with a diminished drug release rate [49].

Ex Vivo Permeability Studies

Rat abdominal skin was used to compare the potential of selected ethosomal gel formulation with plain drug solution and plain gel of the drug. Figure 4 shows the cumulative amount of the therapeutic moiety permeated through the skin from various preparations as a function of time. Plain drug solution in receptor medium, used as controls had slow permeation and the amount permeated after 8 hours was found to be 71.86 ± 5.56 (Table 4). The drug permeation from the plain gel formulation was higher compared with this control, but the difference was non-significant ($p > 0.05$).

In contrast, the drug permeation from the ethosomal gel was significantly higher (289.6 ± 13.82) than that from any of the other tested preparations ($p < 0.05$). Data in Table 4 show that the cumulative drug amount permeated from the ethosomal gel was around 3.5 to 4-fold higher than from drug in plain gel and drug solution in receptor medium respectively (significant difference, $p < 0.05$). The greater potential of ethosomes to permeate through the skin has been previously observed in several other studies and was attributed to the presence of high ethanol concentration in the ethosomes [48,49]. Ethanol can increase the flexibility and fluidity of the vesicles allowing them to deform and penetrate through skin pores that are smaller than their size. However, ethanol is not the only factor operating since anthralin ethosomal gel had significantly higher drug permeation compared with the drug solution. This is in agreement with previous reports which showed that ethosomes had higher drug permeation through the skin compared to the drug hydroalcoholic solution [50].

Based on these observations, it was suggested that the observed enhanced drug permeation for ethosomes is dependent on several factors including the lipid-softening effect of ethanol, as well as the interaction of ethanol and those lipids with the stratum corneum lipids. This interaction might influence the bilayer structure of the stratum corneum [51].Together, these effects might act in a synergy resulting in enhanced penetration of the drug molecules across the stratum corneum and eventually better drug permeation.

Conclusion

The prepared novel ethosomal gel incorporating wheatgrass extract has shown an enhanced permeation profile as compared to the conventional formulation of the extract. The results were indicative of the potential of ethosomal gel for effective topical and transdermal delivery of the compound. We conclude that the ethosomal system may be a better approach for simultaneously dealing with dermatological disorders and at the same time delivering the compound to the deeper layers of skin from where better absorption in systemic circulation can take place. However, further biological and clinical research have to be carried out to explore the therapeutic potential of this extract for treating the problems of lowered haemoglobin content.

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