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Research Article

Performance evaluation of *Bacopa monneri*-loaded ethosomes for topical delivery

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Abstract
Bacopa monnieri is a plant with a rich history of use in traditional Ayurvedic medicine, spanning several centuries. It is also referred to as Brahmi in some regions. This product serves as a treatment for various skin conditions, such as inflammation and wound healing. Its properties also aid in the production of collagen and improve circulation. The inclusion of antioxidants enhances your skin's overall health, resulting in a rejuvenated and vibrant appearance. The present study aimed to prepare a nano-lipoidal system loaded with *Bacopa monnieri* (BM) extract and its characterization. Twelve formulations (F1-12) were developed using the ether injection method using different ratios of BM extract, L-alpha phosphatidylcholine (SPC), ethanol, and water. Bacoside A was used as a marker compound for estimation purposes. BM extract-loaded ethosomes were characterized in which formulation F-5 showed the highest entrapment efficiency of 89 %, with vesicle size 188 nm, while the zeta potential was -29.19 mV, and the polydispersity index (PDI) was 0.221±1.45. *In vitro* extract release using a dialysis membrane was performed for 12 hours, and it was found to be 44 % and 61 % at the end of 8 and 12 hours. The formulation followed zero-order non-Fickian diffusion kinetics, which is best for transdermal formulations. The goat skin was used for Confocal laser scanning microscopy (CLSM) study which showed the fluorescence intensity of Rhodamine B entrapped in those was 16.783, while it was 8.580 in the blank hydroethanolic solution, which confirmed the penetration of the ethosomal systems up to 30-40 µm deeper into the skin which gives a possibility that ethosomes can contribute in collagen synthesis and decrease the degradation of elastin in the deeper layers.

Keywords: BM *(Bacopa mannieri)*, CLSM (L-alpha phosphatidylcholine), *In vitro*, Rhodamine B, Skin aging

INTRODUCTION

The human skin, the largest organ, is vital for shielding our internal organs from dangerous external stimuli and acting as a barrier against them (Rajeev *et al.* 2024). It comprises three layers: the subcutaneous layer, dermis, and epidermis. The epidermis is the outer layer of the skin and the main defense against damaging external stimuli (Baroni *et al.,* 2012).

It is crucial to comprehend and preserve the integrity of this layer because any interference with the keratinocyte cell cycle or differentiation in the epidermis might result in modifications to the thickness of the epidermis and impaired function. The outermost layer of the skin, known as the epidermis, acts as a waterproof barrier that shields the body from many environmental elements. The skin also acts as a sensory organ and aids in controlling body temperature. The skin's primary function is to shield the body from harmful substances and pathogenic organisms by acting as a physical barrier (Roostermanet *al.,* (2006). In addition, the skin possesses regenerative qualities that enable it to regenerate and repair damage. The complex layer that gives the skin strength and suppleness is called the

dermis, found underneath the epidermis.

The mechanism of skin aging is complex and involves numerous components. Internal aging, also known as chronological aging, is a normal process regulated by hereditary and hormonal variables (Mohiuddin, 2019). External aging, on the other hand, is induced by a variety of external causes, such as sun exposure, smoking, alcohol consumption, and exposure to environmental toxins. (Farage*et al.,* 2008). Furthermore, the locate and operational length of the muscles clipped to the orbicularis orris are important in maintaining a proper facial appearance and skin tone.

Brahmi, also known as extract, has been traditionally used in Ayurvedic medicine for its numerous health benefits. About skin aging, Brahmi plays a crucial role in maintaining youthful and healthy skin. Its antioxidant action assists in defending the skin from damage initiated by free radicals, which are one of the main contributors to premature aging. In addition, Brahmi contains compounds that boost collagen fabrication, upgrade skin elasticity, and decrease the arrival of wrinkles (Tirant*et al*, 2018). *BM* extract is a plant with a typical chemical composition consisting of dammarane-type triterpenoid saponins called bacosides. Twelve analogs from the Bacoside family have been identified, and bacosides I-XII are an important constituent. Other alkaloids include her saponin, apigenin, D-mannitol, monnierasides I-III, plantainoside B, cucurbitacin, and brahmine (Nandy, 2019). These compounds possess various pharmacological activities, including antioxidant, neuroprotective, and memory-enhancing effects. In addition, bacoside A has shown potential in reducing anxiety and stress levels in preclinical studies. (Valotto *et al*., 2024).

Nanocream containing Canola (*Brassica napus L.*) Oil was evaluated for skin aging activity, which indicated a greater percentage recovery by nano-creams than other conventional creams (Sumaiyah and Sumaiyah, 2021)

This study incorporated BM extract into nanolipoidal vesicles like ethosomes to improve its penetration inside the deeper layers of the goat skin, which might help enhance collagen synthesis and decrease elastin degradation in the dermal layer.

MATERIALS AND METHODS

The BM extract was received from Natural Remedies Bangalore as a gift sample. Bacoside A was purchased from Natural Remedies Bangalore. L-alpha Phosphatidylcholine (Egg yolk 60%) was purchased from Sigma. All other chemicals used are of analytical grade.

Preformulation studies

Extract Identification

HPLC (High-performance liquid chromatography)

A total of 10 mg of bacoside-A was dissolved in 10 ml of HPLC-grade methanol to prepare a stock solution. Suitable dilutions were made in the range from 10 µg/ml to 100 µg/ml. A Li Cryosphere RP-18 HPLC column with the isocratic flow was employed in the current experiment, and the HPLC system was outfitted with Shimadzu's LC 2010C HT software for lab solutions (Murthy *et al.,* 2006).

FTIR of BM extract

Extract Excipient interaction studies

Extract-excipient compatibility was determined by taking a physical mixture of BM extract and soya PC in a sealed vial using FTIR (Perkin Elmer Spectrum, version 10.03.06). (Adki*et al.,* 2019).

Organoleptic properties

BM extract was visually identified based on the organoleptic characteristics of color, odor, taste, and texture.

Solubility

The solubility of *Bacopa monneri*was determined by the Qualitative method in which a small amount of the extract (BM) was placed in a test tube and the desired solvent was added with slight shaking (Lala, 2019).

Acid-insoluble ash value

Total ash value

Two grams of precisely weighed extract were transferred into a crucible that had been previously tared (of silica or platinum). The material was evenly distributed and burned, progressively increasing the temperature to 600°C until it turned white, signifying the absence of carbon and the calculated total ash value (Pandey & Kumar (2022).

Acid-insoluble ash

25 ml of hydrochloric acid (~70 g/l) was added to the crucible, covering the complete ash with a watch glass, and boiled gently for 5 minutes. Pour hot water over the watch glass to rinse it, then pour the liquid into the crucible and acid insoluble as calculated using the formula (Mishra *et al.,* 2015).

$$\% \text{ Total ash} = \frac{\text{Ash Weight}}{Total\ Sample} x100 \quad \text{Eq.1}$$

Qualitative chemical testing of the BM extract

Preparation of the sample

Twenty millilitres of ethanol and methanol were mixed with five grams of the medication to create a test solution (Mishra *et al.,* 2015). All the analyzed tests are given in Table 1.

Preparation of endosomes

A weighed quantity of L-α phosphatidylcholine (Soya PC) and BM extract were dissolved in ethanol using a magnetic stirrer in different ratios (Table 2). The aque-

Table 1. Physiochemical tests of *Bacopa monneri* (BM) extract

S.No.	Test used for	Name of Phyto-chemical tests	Methanol	Ethanol	Reference
1.	Carbohydrates	Molisch's Test	+	+	(Jadhav et al., 2016)
2.	Proteins	Millon's Reagent Test	+	+	(Ravishankar et al., 2016
3.	Amino acids	Ninhydrin Test	-	-	Parveen et al., 2016
4.	Steroids	Liebermann Burchard Reaction	+	+	Indoliya et al., 2022
5.	Cardiac Glycosides	Legal's Test	+	+	Akhil et al., 2017
6.	Anthraquinone gly-cosides	Bontrager's Test	-	-	Pawar et al., 2015
7.	Saponin glycosides	Foam Test	+	+	Bhardwaj et al., 2019
8.	Flavonoids	Sodium hydroxide Test	+	+	Azad et al., 2012

ous phase was added drop by drop using a syringe while the solution was being agitated at 700–800 RPM on the magnetic stirrer. Stirring was continued for 15 minutes at room temperature, and the beaker was covered to prevent evaporation. The resulting dispersion was then sonicated and kept in the refrigerator (Das *et al.,* 2018).

Characterization of extract-loaded endosomes

Particle size, polydispersity index (PDI), and zeta potential

Morphological studies of the prepared ethosomes were conducted using Phase contrast microscopy and Transmission Electron Microscopy and were determined using Zeta Sizer (Malvern Zeta Sizer 7.12) (Mohanty *et al.,* 2018)

Entrapment efficiency

Entrapment efficiency was determined using the ultracentrifugation technique. The aqueous dispersion was centrifuged for 30 minutes at 4°C and 10,000 RPM. The clear supernatant was carefully siphoned off. The sediment was then treated with 1 ml of 0.1% Triton X-100 to lyse the vesicles, and pH (6.8) buffer was added for dilution. The following formula was used to determine the entrapment efficiency.

$$\frac{\text{Amount of extract present in vesicles}}{\text{Total Extract incorporated}} \times 100 \qquad \text{Eq.2}$$

***In vitro* study**

Franz diffusion cell (made of glass) with a surface area of 2.54 cm^2 accessible for diffusion was used for an *in vitro* investigation. Before beginning the investigation, the dialysis membrane (50-LA, Himedia Lab. Pvt. Ltd.) was saturated for 4 h in PBS (6.8 pH). The autosomal dispersion was positioned in the donor compartment, and the dialysis membrane was placed between the donor and receptor compartments. The receptor compartment was filled with PBS (6.8 pH) and stirred continuously to maintain a temperature of 37±0.5°C. Parafilm was placed over the donor chamber to prevent evaporation (Pal*et al.,* 2024). Among 12 formulations (F-1 to F12), the Formulations F-2, F-5, F-6, and F-8 were selected based on zeta, vesicle size, PDI, and entrapment efficiency (Table 3) and further characterized for the *in vitro* diffusion study.

Permeation Data Analysis

Data obtained from the in-vitro extract release investigation were fitted using several kinetic equations to examine the release rate profile: Higuchi Release Kinetics represents the cumulative percentage of extract release versus SQRT Time, Zero Order represents the cumulative percentage of extract release versus time, and First Order Release represents the percentage age cumulative extract release versus time (Li*et al.,* 2019).

Confocal Laser Scanning Microscopy

The ability of dye-loaded ethosomes to access through the goat ear skin procured from the slaughterhouse was monitored using confocal laser scanning microscopy. Franz diffusion cells were used to execute the study in the same manner as the *in vitro* diffusion study. The dialysis membrane was replaced with goat ear skin with stratum corneum facing the donor com-

Table 2. Formulation table for those preparation

Formulation	% L-α Phosphatidyl-choline	Ethanol: Water
F1	1	10:90
F2	1	20:80
F3	1	30:70
F4	1	40:60
F5	2	10:90
F6	2	20:80
F7	2	30:70
F8	2	40:60
F9	3	10:90
F10	3	20:80
F11	3	30:70
F12	3	40:60

partment. The probe Rhodamine B (0.03%) was added to the solvent ethanol with SPC. Dye-loaded ethosomal dispersion was applied for 8 h to the dorsal skin of the animal in a Franz diffusion cell at 37°C. The skin sample was detached from the donor cell and carefully cleaned with distilled water at the end of the study. Using a microtone (LEICA CM1520), the treated region was separated from the skin. Skin specimens were observed under a confocal microscope (Laser Scanning Super-Resolution Microscope System, LEICA Microsystems. (Huangteerakul*et al.,* 2021).

Ethical approval

Goat ear skin was purchased from a slaughterhouse. So ethical approval is not required

Statistical Analysis

One-way ANOVA was used for the statistical analysis (Microsoft Excel). When $P < 0.05$, the difference was considered significant.

RESULTS AND DISCUSSION

The results of pre-formulation studies of the BM extract are tabulated in Table 4. BM extract was a yellowish powder with a characteristic odor and bitter taste; the log p-value was 1.82. The log value indicates more extract concentration in the lipid phase compared to the aqueous phase, i.e. the extract was more lipophilic.

Qualitative tests performed on the BM extract are shown in Table 1 and it is clearly shown in the table physiochemical test was found to be positive in reference to BM extract. The HPLC chromatogram of Bacoside A showed a Retention time of 17.323 as shown in Fig 1. The presence of a specific peak for Bacoside A at a retention time of around 17.045 was verified and thought a positive result for the BM extract. FTIR spectra of BM extract, soya PC, and physical mixture of BM extract and soya PC are shown in Fig 2. In the FTIR study, the extract showed the characteristic peak at 3367.53 corresponding to O-H stretching, 1065.43 corresponding to C-O-C stretching, soya PC showed a peak at 3780.10 corresponding to O-H stretching, and 1234.26 showed C-O-C stretching. The same was present in the physical mixture of extract and soya PC as well. All peaks of the pure extract and pure polymer were present in the FTIR spectrum of the physical mixture of extract and polymer. There was no new peak generation, confirming no incompatibility issue between the extract and polymer. Fig 1 shows the HPLC chromatogram of BM extract and Bacoside A which confirms that Bacoside gives a peak with retention time17.045 which confirms that Bacoside A is found in BM extract. Twelve batches of ethosomes were prepared by varying the amount of SPC from 1% - 3%, whereas alcohol concentration varied from 10% - 40%. Ethosomes are nano vesicular lipid carriers that are extensively used for transdermal delivery. The ethanol content in ethosomes alters the arrangement of intercellular lipids in the stratum corneum, increasing its fluidity. Ethosomes possess flexibility and deformability, enabling them to penetrate the disordered stratum corneum and reach deeper skin layers. (Zhang *et al.*, (2022) They can easily pass through the skin and in-

Table 3. Prefomulation study results of BM extract

Standard plot of BM extract	Methanol using UV Spectrophotometer
Absorption maxima	271 nm
Beer's Law limit	1-10 µg/ml
Regression equation	0.2956x + 0.0036
Intercept	0.0036
Slope	0.0818
R^2	0.9991
Log P	1.82
pH	6.09
Solubility	Soluble in methanol and etha-
Color, odor, and taste	Yellowish color powder with a characteristic odor and bitter
Melting Point	312°C-316°C

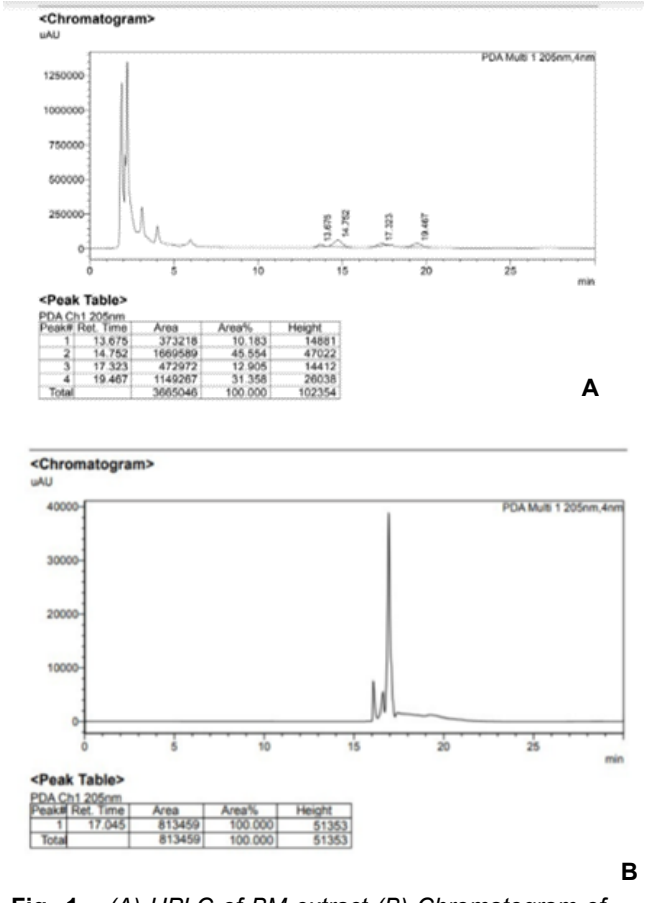

Fig. 1. *(A) HPLC of BM extract (B) Chromatogram of Bacoside -A*

Table 4. Showing characterization studies of entrapment efficiency, vesicle size, polydispersity Index, zeta potential

S. No.	Formulation Code	Entrapment efficiency (%)	Vesicle size (nm)	Polydispersity Index (PDI)	Zeta Potential (mV)
1	F1	66 ± 0.32	241 ± 1.67	0.387 ±1.32	-45.44
2	F2	83 ± 1.07	109 ±1.57	0.266±1.43	-24.22
3	F3	69 ± 1.21	342 ± 1.43	0.324 ± 1.43	-31.44
4	F4	56 ± 0.23	243 ± 2.43	0.297 ±1.04	-43.23
5	F5	78 ± 0.17	176 ±1.43	0.107±1.29	-27.12
6	F6	89 ±1.33	188 ±1.22	0.221±1.06	-29.19
7	F7	63 ± 1.56	254.32 ± 1.45	0.322 ± 1.32	-51.48
8	F8	72 ±1.32	199 ±1.31	0.267±1.45	-22.11
9	F9	63 ± 0.43	365 ± 1.43	0.284 ± 1.78	-36.34
10	F10	69.57 ± 1.65	298 ± 1.34	0.314 ± 1.43	-47.87
11	F11	58.76 ± 0.22	264 ± 1.32	0.298 ± 1.74	-43.67
12	F12	63.87 ± 1.89	297 ± 1.87	0.289 ± 1.87	-38.54

For Standard deviation (□=3)

crease the accumulation of extracts in the skin. Sailaja and Meghana (2024) prepared an ethosomal gel of clotrimazole for fungal infection and found that the result was very satisfactory; hence, ethosomes are an efficient carrier for the topical administration of clotrimazole. Phase contrast microscopy of all batches revealed the surface morphology of the ethosomes showed a unilamellar shape with a near-spherical structure as shown in the TEM photomicrograph (Fig.3.) Vesicles are 128 nm in size which is best to penetrate inside the skin easily (Fig. 4). Unlike ethosomal vesicles, which have a spherical shape, this could be due to the lack of cholesterol in the vesicle and the presence of ethanol, which gives the bilayer membrane some fluidity (Munir *et al.(*2024).

The results of EE, Zeta Potential, Poly Dispersity index, and Vesicle Size are tabulated in Table 5, in which formulation F-5 showed the highest entrapment efficiency of 89 ±1.33 %, with vesicle size 188 ±1.22 nm, while the zeta potential was -29.19 mV, and the polydispersity index (PDI) was found to be 0.221±1.45.

Entrapment efficiency can be defined as the delivery potential of the ethosomal system and is directly affected by its extract-carrying capacity. The entrapment efficiency of the ethosomes was determined for all the formulations. The amount of soya PC and ethanol was found to be directly proportional to the entrapment efficiency of the BM-loaded ethosomes. The entrapment efficiency ranged from 56% to 89%.

The polydispersity index was used to measure the homogeneity of the prepared ethosomes based on their particle size distribution. The ethosomal preparation with a lower PDI value was selected because it represents more homogeneity in size distribution.

Ethosomal vesicles ranged in size from 109-254 nm. The literature suggests that vesicles below 300 nm can carry their extract into the deep layer of the skin to a

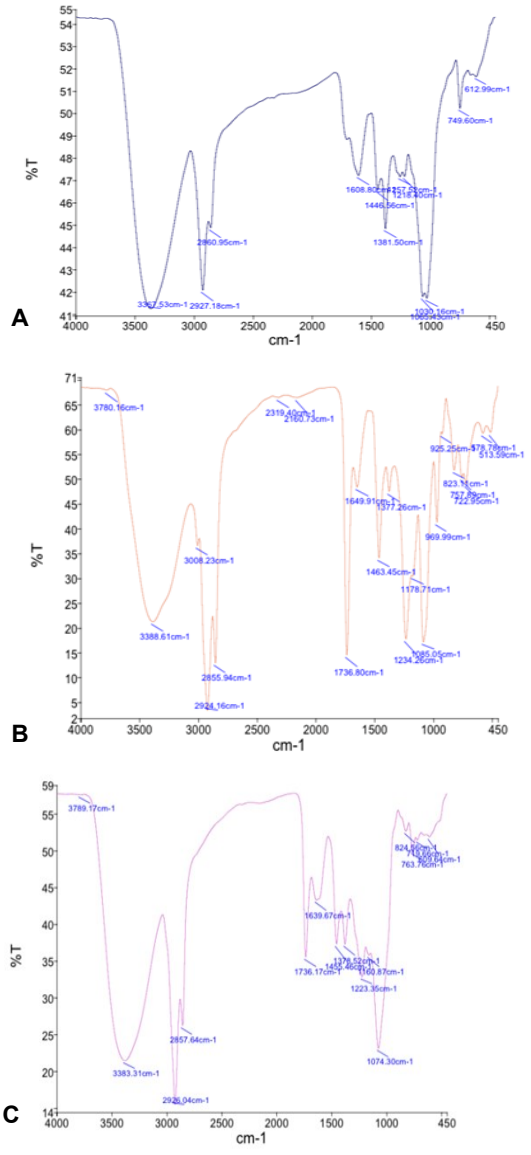

Fig 2. A) *FTIR spectrum of BM extract B) FTIR spectrum of the L- alpha C) FTIR spectrum of physical mixture of BM extract and phosphatidylcholine*

certain extent. The vesicle sizes increased with improving SPC concentrations from 1-3%, whereas the alcohol concentration had the opposite effect. Higher concentrations of alcohol resulted in lower vesicles. It has been observed that high concentrations of ethanol create a surface-depleting net charge by manipulating certain surface characteristics, resulting in smaller vesicles. This shows that the size of the vesicles increased with the concentration of SPC.

Zeta potential is the difference between the surfaces of a solid particle immersed in a conducting liquid. The stability of the ethosomal formulation can be determined from the zeta potential value. All ethosomal for-

Fig. 3. *TEM image of formulation F5 spherical shapes of vesicles*

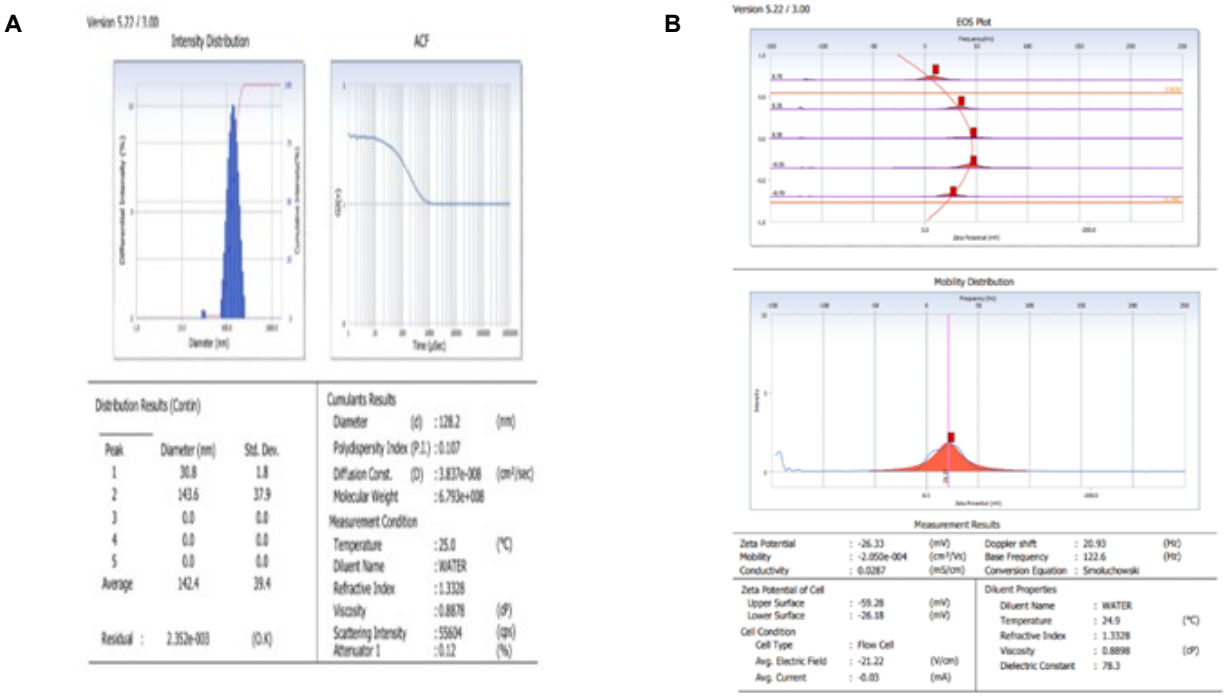

Fig 4. A) *Particle size,* B) *Zeta Potential, PDI of optimized formulation F-5*

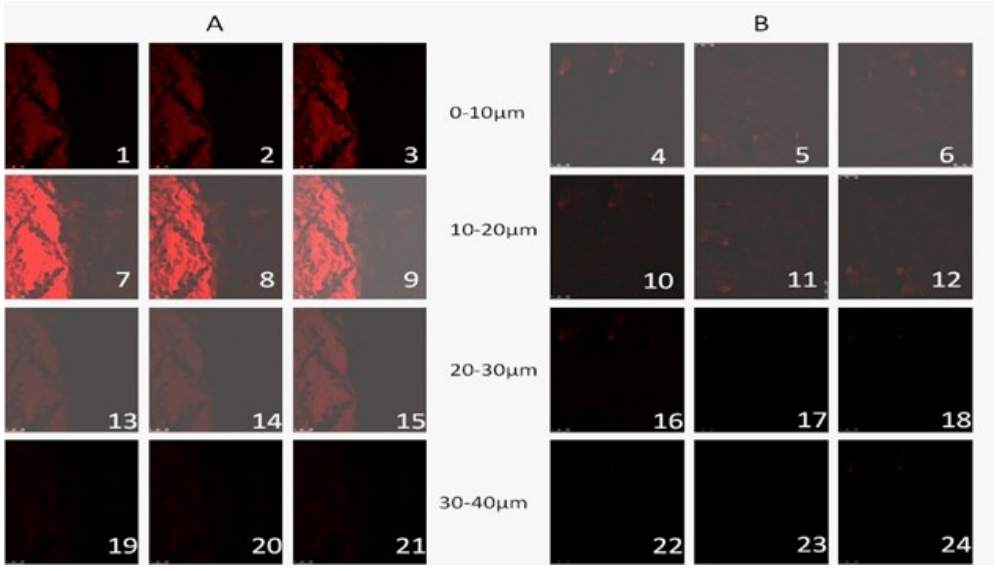

Fig. 5. *Confocal microscopy showing penetration of fluorescent probe Rhodamine B into the skin: (A) Penetration of Rhodamine B entrapped in ethosomes and (B) showing penetration of fluorescent probe from hydroalcoholic solution*

Table 5.Kinetic assessment of the release profile of Extract loaded ethosomal aqueous dispersion by using of dialysis membrane (50-LA, HI media Lab. Pvt. Ltd)

Formulation code	Zero-order Model R^2	First order model R^2	Higuchi order model R^2
F 2	0.9857	0.9827	0.9234
F 5	0.9841	0.9766	0.9143
F 6	0.9842	0.9781	0.9156
F 8	0.9819	0.9716	0.9118

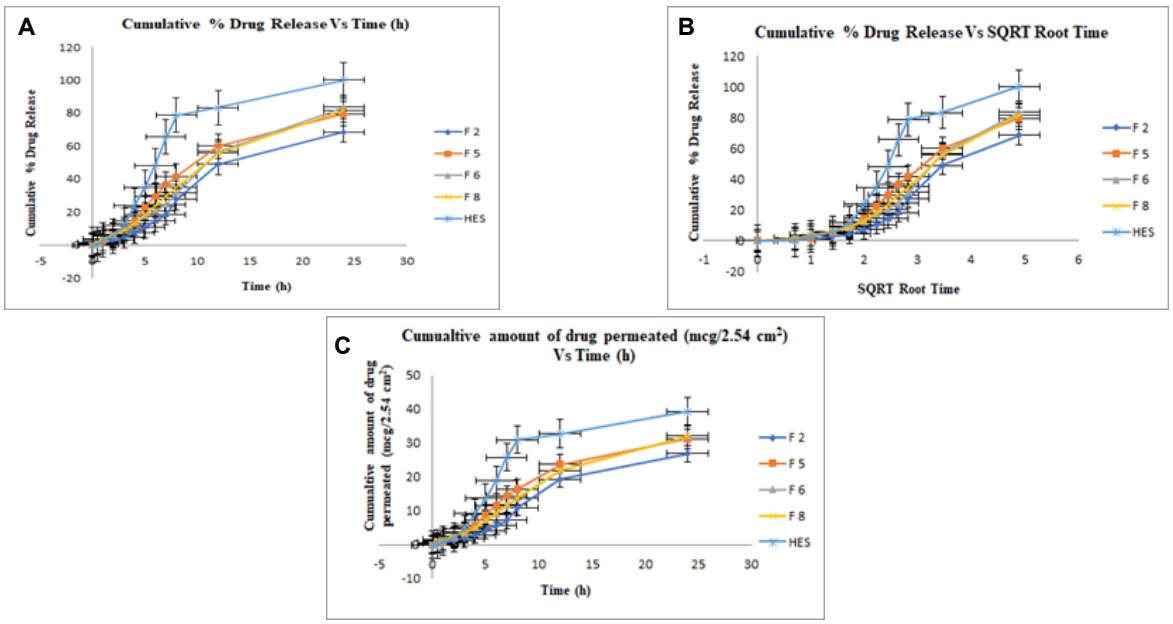

Fig. 6. *In vitro release profile of B M extract-loaded ethosomal formulations through the dialysis membrane (50-LA, Himedia Lab. Pvt. Ltd) (50-LA, Himedia Lab. Pvt. Ltd) Where Image A) shows the relationship between the % drug release and time B showing the % drug release and SQRT Time, C) shows the relationship between the cumulative amount of drug permeated (mcg/2.54 cm^2 and time*

mulations were found to have negative zeta potentials ranging from 22.11 to 45.44 mV.

Laser Scanning Super-Resolution Microscope images are shown in Fig.5, where penetration of probe-loaded ethosomes is shown by images i.e. at which depth of skin the ethosomes were able to penetrate. The fluorescence intensity of the loaded fluorescent probe in the present case Rhodamine B, shows the penetration depth into the skin. Thumbnails 1–6, 7–12, 13–18, and 19–24 show skin penetration at depths of 0–10, 10–20, 20–30, and 30–40 µm, respectively. Fig 5. shows a comparison of dye-loaded ethosomes with the hydroalcoholic solution. The fluorescence intensity of the dye-encapsulated ethosomes was higher.

At 30–40 µm within the skin, the fluorescence intensity of Rhodamine B entrapped in lipid vesicles was determined to be 16.783, whereas that of the hydroethanolic solution was 8.580. CLSM data demonstrated that Rhodamine-B-loaded ethosomes permeate deeper layers (Alshetaili, 2024) and the percent release was decreased with increasing amounts of lipid inethosomes, though increasing the concentration of alcohol somewhat increases the release. The observed increase in percent release with higher amount of alcohol could be due to increased fluidity of the bilayer membrane with increasing concentration of alcohol (Raghav*et al.*, 2024)

In vitro, release data were subjected to kinetics study according to Zero order, first order, and Higuchi Kinetics equation to determine the release mechanism. The R2 values of different formulations were higher in zero-order and first-order (Table 5). Therefore, the formulations that followed first-order kinetics imply controlled and slow release by diffusion as proposed by the first order, which is non-Fickian diffusion and best for transdermal formulations. Fig. 6 shows the release graph pattern of the four best-fitted formulations (F-2, F-5, F-6, F-8) and hydroethanolic solution, which makes it evident that F-5 shows the best release pattern. Permeation Data Analysis suggested that systems that obey zero-order release are ideal for transdermal extract delivery because they constantly release extracts over an extended period and reflect an improved therapeutic index.

Conclusion

Herbal extracts have always been an important part of the human healthcare system. They have a lot of potential and their incorporation in the novel nano vesicu-

lar delivery systems enhances their permeation in deeper layers of the skin, resulting in improved therapeutic benefits. The present study showed that the ethosomes loaded with *B.monnieri* (BM) extract are a useful delivery method for releasing entrapped extract into deeper layers of the skin, thus improving the therapeutic benefits of BM extract. The BM extract was rich in antioxidants and polyphenols, which have proven anti-photoaging effects. Incorporating the extract into ethosomes can now reach deeper skin layers up to 30-40 µm of goat skin. The BM extract-loaded ethosomes could be an anti-aging product that might help manage skin aging. Clinical studies in this direction can further support the usefulness of herbal extracts.

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Conflict of interest

The authors declare that they have no conflict of interest.

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