Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i1.4271

DEVELOPMENT, CHARACTERIZATION, STABILITY AND TOXICITY EVALUATION OF TOPICAL ETHOSOMAL GEL LOADED WITH *BERBERIS VULGARIS L.* FRUIT EXTRACT AS A COSMECEUTICAL PRODUCT"

Nayla Javed[1*], Haji Muhammad Shoaib Khan[2], Naheed Akhtar[3], Umair Khurshid[4], Umer Shaukat[5], Marwah Rehman[6], Hafiz Arfat Idrees[7], Muhammad Younus[8], Muhammad Saleem Qureshi[9], Zafar Iqbal[10], Wajeeha Almas[11], Muhammad Naeem[12], Rihana Dilshad[13], Rizwana Dilshad[14], Muhammad Wajahat[15], Khadija Karim[16], Muhammad Shahzad Khan[17*]

[1*,2,3]Department of Pharmaceutics, The Islamia University of Bahawalpur, Bahawalpur 63100, Punjab - Pakistan
[4]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, The Islamia University of Bahawalpur, 63100, Punjab - Pakistan
[5]Rashid Latif College of Pharmacy, Rashid Latif Medical Complex, Lahore, Punjab - Pakistan
[6,17*]Abbas Institute of Medical Sciences, GC University Faisalabad, Layyah Campus, Layyah 31200, Punjab - Pakistan
[7]Faculty of Pharmacy, The University of Lahore, Punjab - Pakistan
[8]Department of Pharmacognosy, Faculty of Pharmacy, The Islamia University of Bahawalpur - Pakistan
[9]Akbar Institute of Medical Sciences, Bahawalpur, Punjab - Pakistan
[10]Bahawalpur College of Pharmacy, BMC Complex, Bahawalpur - Pakistan
[11]IBADAT International University, Islamabad - Pakistan
[12]Department of Pharmacy, Shah Abdul Latif University, Khairpur, Sindh - Pakistan
[13]Sheikh Zayed Medical College, Rahim Yar Khan, Punjab - Pakistan
[14]Swedish College of Pharmacy and Allied Health Sciences, Rahim Yar Khan – Pakistan
[15,16]Department of Pharmaceutics, Faculty of Pharmacy, Bahauddin Zakariya University, Multan 60800, Punjab - Pakistan

***Corresponding Author:** Muhammad Shahzad Khan[1], Nayla Javed[2]
[1]Abbas Institute of Medical Sciences, GC University Faisalabad, Layyah Campus, Layyah 31200, Punjab – Pakistan, Email: shahiub@yahoo.com, ORCID ID; https://orcid.org/0000-0002-4834-7287
[2]Department of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur 63100, Punjab – Pakistan, Email: naila.javed5@gmail.com

Abstract

Berberis vulgaris L. being copious with phenols, flavonoids, alkaloids, and vitamin C along with its value-added organic properties is thought to be a good contender of the cosmetic industry. Study was thus aimed to develop a *Berberis vulgaris L.* (BV) extract based optimized nano-ethosomal gel and its investigation for skin rejuvenation, acne and hydration effects aided by the increased solubility and trapping of various medicinal agents and plant extract by nanostructured vesicles.
Tyrosinase enzyme inhibition test, phenolic and flavonoid content, and in-vitro SPF (sun protection effect) evaluation of the BV extract were all studied. Zeta size, zeta potential, encapsulation efficiency

(%EE), and SEM (scanning electron microscopy) were used to analyse formulations of 09 ethosomal formulations based on BV extract. After being included into the gel system, the skin effects of the improved ethosomal formulation were investigated using minimally invasive in vivo methods. A research on the acute oral toxicity of Berberis vulgaris L. was also conducted to examine any potential harmful effects.
The optimized nano ethosomal formulation (F9) was spherical in shape, 116.5nm in size, −28.5 surface charge, and showed EE 97.38%. The BV loaded ethosomal topical gel was stable physicochemically throughout the study and rheological analysis revealed non-Newtonian behavior. *Ex vivo* permeation of ethosomal gel exhibited 69.88% release. Statistically ANOVA indicated that *in vivo* parameter skin effects were significant (P<.05).
A stable BV loaded nano ethosomal gel system is an inspiring approach for phytoextracts than conventional formulations as a cosmeceutical application that has substantial effects on skin.

Keywords: Cosmetic, *Berberis vulgaris L.*, Antioxidant, Nano Ethosomes, Stability testing, Non-invasive *in vivo* study

1. Introduction

The pharmaceutical and cosmetic industries are now doing a reflection search for bioactive compounds that have astounding human health advantages. The extraction of these bioactive components from medicinal plants or herbs is still favoured (Maia et al., 2019). This method was utilised to develop the new term "cosmeceutical," which was created by fusing the words "cosmetic" (which delivers the desired esthetical impact) and "pharmaceutical" (to cure dermatological conditions) (Espinosa-Leal & Garcia-Lara, 2019).

The ethosomal vesicular system, which are brand-new elastic phospholipid nano vesicles designed for better distribution of active ingredients in terms of quantity and depth, can increase medication administration via skin (Venus, Waterman, & McNab, 2010)’(Ainbinder, Godin, & Touitou, 2016). Ethosomes are topically non-invasive drug delivery systems that allow the highest drug concentration to reach the dermal layers deeply and minimise systemic adverse effects in comparison to traditional liposomes or hydroalcoholic solutions. (Verma & Pathak, 2010)’ (Touitou, Dayan, Bergelson, Godin, & Eliaz, 2000) (Khan et al., 2022). Ethosomes play a crucial function in controlling the pace of drug release over a lengthy period of time by protecting the medication from the body's immune system or clearance mechanisms (Aggarwal & Nautiyal, 2016; Rakesh & Anoop, 2012).
Human skin constitutes the epidermis, which is the top skin layer, the dermis, which lies underneath the epidermis, and the hypodermis make up the skin (deeper subcutaneous fat tissues) (Venus et al., 2010). As a result of a connection between one melanocyte and 30–40 connected keratinocytes, epidermal melanin units are created. Melanocytes, which are found in the basal layer of the epidermis, are responsible for absorbing certain potentially dangerous ultraviolet radiations (UV) from sunlight (Cichorek, Wachulska, Stasiewicz, & Tymińska, 2013). The buildup of elastin and inability of collagen during the photoaging process is the histological alteration. The dermal fibroblast is activated by the growth factor modification to produce collagen (building block of skin). Since the study showed that repeated UVR exposure might result in the creation of peroxyl free radicals, which then create malnodialdehyde, which collagen is constantly cross-linked by. The result is the appearance of pigmentation, melasma, erythema, early signs of ageing, wrinkles, DNA damage, and dry, rough skin (Mukherjee, Maity, Nema, & Sarkar, 2011). Skin barriers for powerful medication administration remain a concern now despite research into topical and transdermal drug delivery.
Plants have a plenty of primary and secondary metabolites that have been utilized in medicinal, food, and cosmetic goods and are typical in their broad variety of chemically produced structures. (Bansal, Reddy, & Kumar, 2017). The compounds that protect plants from harsh calamity conditions like UV, dearth, and drastic temperature scales can have same basic defensive mechanisms on human skin. (Apone et al., 2010)’ (Saewan, Vichit, & Prinyarux, 2018). Phenolic acids, flavonoids, glycosides,

tannins, lignins, alkaloids, hetrocyclic aromatics, terpenes (terpenoids, isoprenoids), vitamins, amino acids, peptides, essential oils, sugars, are the phytochemicals used in cosmetic products. (Antonopoulou et al., 2016).' These phytochemicals can thus be employed in the cosmetics sector as antioxidants, skin lighteners, anti-wrinkle, anti-aging, anti-inflammatory, antibacterial, sunscreen, emollients, in acne, and for skin cancer (Korkina, Mayer, & De Luca, 2017) (Khan & Akhtar, 2022). Secondary plant metabolites known as phenolic compounds act as hydrogen donors, reducing agents, radical scavengers, and metal chelators (Ghribia, Ghouilaa, Omrib, Besbesb, & Janneta, 2014). The integumentary structure of the body is the primary external layer of human skin. Thermal regulation, sensibility, vitamin D synthesis, vitamin B folates protection, bodily barrier against infections, and severe water loss are the energetic functions of skin (Brandner & Jensen, 2008).

Berberis vulgaris L. (BV), a member of the Berberidaceae family, naturally occurs in Europe, Asia, Africa, and North America (Rahimi-Madiseh, Lorigoini, Zamani-Gharaghoshi, & Rafieian-Kopaei, 2017). As an abundant source of phytochemicals known as isoquinoline alkaloids (such as berberine, berbamine, bargustanine, and palmatine) and secondary metabolites like phenolic compounds, anthocyanins, carotenes, aesculetin, ascorbic acid, caffeic acid, pectin, and tannins (Tabeshpour, Imenshahidi, & Hosseinzadeh, 2017). Fruits of *Berberis vulgaris* L. (BV) are used as an antioxidant, antibacterial, anti-inflammatory, analgesic, for liver disorders, bronchial and gastrointestinal discomforts, and as a circulatory system tonic. They are notably useful for renal damages (Končić, Kremer, Karlović, & Kosalec, 2010). By lowering sebum production and skin irritation, *Berberis vulgaris* L. effectively treats acne vulgaris (Fouladi, 2012).

In this study it was decided to develop an ethosomal preparation of BV extract, describe the formulation, include the optimum formulation into gel, and then estimate the optimal ethosomal formulation loaded with BV extract in vitro and in vivo.

Materials and Methods

Plant Material

Berberis vulgaris L. fruit were collected from local market, Carbopol® 940 (Lubrizol China) and Vit.C (ascorbic acid), DPPH, gallic acid (GA), quercetin, mushroom tyrosinase, kojic acid (KJA), ethanol (EtOH), propylene glycol (PG) and triethanolamine (TEA) (Sigma Aldrich Germany). Phospholipid® (Phospholipon 90 G) donated by Lipoid GmbH Germany.

Extract Preparation

Dried fruit was subjected to extraction by macerating in ethyl alcohol-water mixture (70:30; v/v) for 3 days at room temperature in a dark. Then in a rotary evaporator (Heidolph, GmbH & CO. Germany), solvent is evaporated to 1/3 of its initial volume and the hydro-alcoholic fraction of *Berberis vulgaris L.* (HABV) was further fractionated with n-hexane, chloroform and n butanol through solvent–solvent partitioning. The fractions obtained were titled as NHBV (n-hexane), CLBV (chloroform fraction), and NBBV (n-butanol fraction) of *Berberis vulgaris L.* fruit respectively. The fractional extracts obtained were kept in air tight containers at 4ºC and used freshly for phytochemical analysis as well as for other evaluation study (Chen, Wu, Shieh, Kuo, & Hsieh, 2006).

Analysis of Antioxidant Activity

DPPH Assay

Antioxidant potential of the *Berberis vulgaris L.* extract was analyzed by adding 2ml of DPPH (0.4 M in methanol), in 1ml of extract sample solutions (1mg/ml) and standard ascorbic acid (Vit. C). Then incubated for 25 minutes at room temperature (Shahwar et al., 2011).
Calculated the absorbance at 517 nm and percent inhibition was measured by the subsequent formula;
% inhibition = [(Absorbance of control−Absorbance of sample) ÷ Absorbance of control] × 100

Preliminary phytochemical analysis

Phytochemical analysis was performed for the detection of flavonoids, glycosides, tannins, alkaloids, saponins, carbohydrates and protein in the extract. Concisely, Mayer’s reagent, Hager’s reagent, Dragondroff’s reagent and Wagner’s reagent were used to identify the alkaloids. Ferric chloride test, Froth test, Lead acetate test, Ninhydrin test, Raymond's test, Biuret test, Molisch’s and Benedict’s test, Acrolein test, Salkowski’s and Libermann Burchard’s test were used to identify the phenols, tannins, saponins, amino acids, glycosides, flavonoids, protein, carbohydrates, reducing sugars, fixed oil and fats, sterols respectively.

Ferrous Reducing Antioxidant Power Assay (FRAP)

The FRAP assay of the *Berberis vulgaris L.* fruit was calculated by the method of Benzie & Strain with slight changes (Benzie & Strain, 1999)(Saini, Singhal, & Srivastava, 2011).

Tyrosinase Inhibition Assay

For this activity, oxidation of L-DOPA was measured by earlier described method with slight modifications (Eghbali-Feriz et al., 2018).

Approximation of Total Phenolic Content (TPC) and Total Flavanoild Content (TFC) TPC in plant sample were calculated by the Folin–Ciocalteu colorimetric assay with some modifications. TPC was indicated as mg of GAE/gram (Gallic acid equivalents) of dry weight of plant (Kamtekar, Keer, & Patil, 2014).

TFC (total flavonoid contents) was determined through the aluminum chloride colorimetric assay with slight modification (Zhishen, Mengcheng, & Jianming, 1999). The TPC were stated as mg quercetin (QE)/g of plant dry weight (Saini et al., 2011).

Measurement of Sun Protection Factor (SPF)

Each fraction sample of BV extracts, BVCG (BV control gel) and BVEG (BV ethosomal gel) was added to ethyl alcohol (1mg/ml) and ultra-sonicate it for 10 minutes. Then filtered the solution through Whatman No.1 filter paper and each sample absorption was taken at every 5nm in UV-B wavelength range (290–320 nm). Mansur’s equation was used to find the sun protection factor (Costa, Detoni, Branco, Botura, & Branco, 2015).

$$SPF = CF \times \sum_{290}^{320} \times EE(\lambda) \times I(\lambda) \times abs(\lambda)$$

Where CF is correction factor = 10, EE is erythemal efficiency spectrum,
I is solar intensity spectrum, Abs. is absorbance of the solution. The values of EE (λ) and I (λ) are constant according to Sayre method (Sayre, Agin, LeVee, & Marlowe, 1979).

Development of Ethosomes (BV-Eth)

Ethosomes were prepared by cold process defined by Touitou *et al* with minor modifications. The ethosomal system was composed of 1-3% phospholipid, 2% drug, 10-40% ethanol, 20% propylene glycol (PG) and water as quantity sufficient to 100% w/w as designated in **Table 2**. Firstly, phospholipid (SPC) was dissolved in ethyl alcohol and PG in a closed vial heated at water bath at 30°C. HABV extract solution (having highest antioxidant potential) in water (30°C) was added gently to the above solution in a fine stream by syringe and the entire system was stirred for 25 min. at 700 rpm on magnetic stirrer (VELP Scientifica, Italy). The temperature of the system was maintained at 30°C and the ethosomal formulation was positioned in a sonicator for 30 minutes at room temperature to get small vesicles and then refrigerated until further investigation was carried out. (Zhang et al., 2012) (Maurya, Prajapati, Gupta, Saxena, & Dhakar, 2010).

Table 2. Composition (% wt/wt) of (BV-Eth). Ingredients contain *Berberis vulgaris* L. (BV), ethanol (EtOH), propylene glycol (PG), phospholipid (SPC) and water (H2O).

Formulation codes	SPC	Drug	EtOH	PG	H2O
F1	1 %	2 %	20 %	20 %	q.s
F2	1 %	2 %	30 %	20 %	
F3	2 %	2 %	20 %	20 %	
F4	2 %	2 %	30 %	20 %	
F5	2 %	2 %	40 %	20 %	
F6	3 %	2 %	10 %	20 %	
F7	3 %	2 %	20 %	20 %	
F8	3 %	2 %	30 %	20 %	
F9	3 %	2 %	40 %	20 %	

SPC: Soyaphosphatidylcholine, q.s: quantity sufficient

Characterization of Nano Ethosomes

Vesicle Size, PDI and Zeta Potential Analysis

Zeta size, potential and PDI of BV-Eth were evaluated by Zetasizer Nano ZS 90 (Malvern Instruments, UK), equipped with software (version 6.34).

Entrapment Efficiency

The encapsulation efficiency of BV based ethosomes (BV-Eth) was calculated through indirect ultracentrifuge method. Ethosomal preparations were ultracentrifuge for 25 min at 12,000 rpm. The supernatant of all the nine formulations were collected and determined the drug amount in both vortexed and un vortexed tasters at 290 nm through UV-Visible spectrophotometer (Dave, Kumar, Lewis, & Paliwal, 2010). As berberine is the significant and main alkaloid present in the *Berberis vulgaris L.* and contributes in the pharmacological activity. It exhibited λmax on UV spectrum at 290 nm; thus, it was reflected as biomarker for approximation (Nasrollahzadeh, Maham, Rostami-Vartooni, Bagherzadeh, & Sajadi, 2015).

Encapsulation efficiency was measured by the formula:

Entrapment Efficiency = [(T initial drug amount − T free drug amount) ÷ T initial drug amount] × 100

Morphology

The surface morphology of the ethosomal vesicles were evaluated by scanning electronic microscopy (SEM) (JSM-7500F, Jeol, Japan). The ethosomal vesicular sample was taken in the grid and dried. The scanning was accomplished by the microscope at magnification (50,000 x) by possessing the grid (in range of 15-30kb).

Chemical Detection by FTIR

FTIR analysis of HABV extract and optimized nano ethosomal formulation was analyzed by infrared spectroscopy. The functional groups in each sample showed transmittance in percentage displayed in graph at specific wavelength and this FTIR analysis was recorded in the wave number region of 500-4000cm-1 (Uttra & Hasan, 2017).

Preparation of BV based Ethosomal Gel (BVEG) and Control gel (BVCG)

The BV loaded ethosomal gel (BVEG) was prepared by dispersing gelling agent carbopol 940 (1% w/w) to the distilled water. Carbapol 940 was compatible with ingredients of ethosomal formulation and was easily applicable. Then the mixture was kept to swell overnight and triethanolamine was used to adjust the pH of the gel to skin pH (6-6.8). To this gel, optimized ethosomal formulation was gradually added and mixed properly until a transparent gel formed. Control gel (BVCG) containing

BV extract was also prepared in the same way (S. Ijaz, Khan, Anwar, Talbot, & Walsh, 2019). Then BVEG and BVCG were kept for 24 hrs to remove the air.

Gel Stability Studies

All governing organizations approve only real time statistics for any drug or pharmaceutical for estimating the shelf life. ICH (International Council on Harmonisation) guidelines for accelerated stability studies may support the stability and screening of formulation related to transport or storage at room temperature. The ability of drug retention in the vesicles was evaluated by placing the ethosomal suspension at different temperatures. To evaluate the stability studies, control and optimized ethosomal gel formulation was stored at several temperatures in hot incubator (Sanyo MIR-162, Japan). During 12 weeks, organoleptic, homogeneity,spreadability,change in pH and rheology property were evaluated at different temperatures (S. Ijaz et al., 2019).

Organoleptic, pH and Spreading diameter Evaluation

Various organoleptic parameters like odor, color, appearance and texture were observed. The pH was recorded using the electrode of the pH meter (WTW pH-197i, Germany), immersed in to the formulation at room temperature and triplicate reading was taken (Ramadon, Goldie, & Anwar, 2017). The spread ability of control and ethosomal gel was evaluated by placing the weight on upper plate (125 gm) and calculated the spreading diameter of one gram of gel in millimeter (mm) after 1 minute between two parallel plates (20cmx 20cm) (Abdulhamid & Sani, 2016).

Rheological Evaluation

Rheological characteristics like shear stress, shear rate and viscosity of BV based control gels (BVCG) and ethosomal gel (BVEG) were determined. Viscosity at 25°C of BVEG and BVCG establish at a shear rate of 50 with a speed range from 20-100 rpm (M. Ijaz & Akhtar, 2020).

Ex-vivo Permeation Study

To evaluate the permeation study of vesicles by Franz diffusion cells, rat abdominal skin was used at 37°C for 24 hr. The skin sections were attached in the diffusion cell by soaking in phosphate buffer pH 7.4 through an open area of skin (1.76 cm^2) having a 15mL volume in the receiver compartment. To protect from light and evaporation, franz cell was shielded with parafilm on upper side. The skin side was submerged in phosphate buffer solution and 5ml of *Berberis vulgaris L.* based ethosomal gel and control gel samples were applied to the skin outer layer. The time intervals for samples were at 0 time, 0.25hr, 0.5hr, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr, 7hr, 8hr, 9hr, 10hr,11hr, 12hr, 18hr and 24 hr. UV Visible spectrophotometer analyzed the samples at 290nm (Lopez-Pinto, Gonzalez-Rodriguez, & Rabasco, 2005; Surini, Arnedy, & Iswandana, 2019). As main bioactive compound in *Berberis vulgaris L.* is alkaloid i.e. berberine and is responsible mainly for pharmacological activities of BV fruit (Hadaruga et al., 2010). Berberine exhibited λ-max on UV spectrum at 290 nm; so, it was reflected as biomarker for estimation (Nasrollahzadeh et al., 2015).

Non-invasive *In vivo* analysis

Study design

A single blind, randomized study for 12 weeks was planned for *in vivo* analysis. Present study was permitted by the Board of Advanced Study and Research (BASR) and the institutional ethics committee, Faculty of pharmacy and Alternative Medicines, The Islamia University, Bahawalpur against reference no. 111-2020-/PHEC. Moreover, this study was also in accordance with guidelines by Helsinki Declaration, International Ethical Guidelines for Biomedical Research Involving Human Subjects by The Council for International Organizations of Medical Sciences (CIOMS) (Upadhyay, Fatima, Sharma, Saravanakumar, & Sharma, 2017).

Ethosomal (BVEG) and control gel (BVCG) effects were observed on 13 healthy male volunteers (22-45 age). Before to start the study, a consent was signed by all volunteers and established protocols and conditions was instructed to them.
All volunteers were checked by the dermatologist for any dermal allergy or disorders. The volunteers were instructed about the diet routine and to evade the antioxidants, multivitamin intake throughout the study. BVEG and BVCG was given to each volunteer to use for 12 weeks two times a day on left and right cheeks. The readings were taken in triplicatey.

Evaluation of skin irritation by patch test
Patch test was accomplished on forearm of selected volunteers to evaluate the skin irritation by any formulation constituent, A 4×4 cm marked area on forearm was applied by BVEG and BVCG on right and left forearm. After 24 hrs., this zone was tested by Mexameter® to create the base line for erythma and melanin. Then again about 1g of BVEG and BVCG was applied on right and left forearm by covering with sterilized surgical gauze. After 48 hrs., measurements for skin melanin and erythma was again noted by Mexameter® and evaluated for any skin irritation or hypersensitivity.
Before to conduct the study, panel test was executed in expression of irritation, moisturizing, ease of application, spreadability, shine on skin and sense after application by 5 qualified judges. They requested to smear the formulations on hand and give scores 0-5 (weak- powerful).

Assessment of skin erythema and melanin
Melanin level was determined using Mexameter® from the strength of light absorbed and reflected at wavelength of 660 and 880 nm while ski erythema level was measured at 568 and 660 nm (Wan et al., 2017).

Assessment of skin sebum level
Sebumeter® was used to quantify the skin sebum index on the mechanism of grease spot photometric technique through a sebumeter tape. This tape becomes transparent on contacting with skin sebum and after implanting into device opening; its transparency was determined by the light emission (Hameed, Akhtar, Khan, & Asrar, 2019). Three successive readings and their mean were taken for study.

Assessment of skin hydration index
Skin hydration index was measured by corneometer® CM825 joined with Multiprobe Adapter MPA5. The mechanism involved is the electrical field enters into epidermis and skin hydration is determined as dielectric constant-dependent capacitance changes of water (Manosroi, Jantrawut, Akihisa, Manosroi, & Manosroi, 2011). Three successive readings and their mean were recorded to lessen the error.

Assessment of skin elasticity
Skin elastometer® EM 25 has the rapid and non-invasive elasticity measuring pressure probe, with 2 mm diameter and determines skin elasticity on 0-99 scale. The working mechanism is the skin forcing and stretching and was employed to appraise the aging of skin with elasticity value. The result of elasticity is shown on LED of device in terms of percentage (S. Ijaz et al., 2019). Three successive readings and their mean were recorded for the study.

Acute toxicity study
This study was conducted after the approval of the ethical committee and the voucher no was given IUB/2021/032 for future perspective. Acute oral toxicity study was performed on the extract of *Berberis vulgaris L.* according to OECD guidelines 425. A single oral dose 2000mg/Kg was given firstly to only one animal. If the animal was survived for up to 24 hrs., then next dose was given to

the remaining four animals. Mortality and morbidity were observed for 48hrs and then till the 14 days (Anwar et al., 2021).

Mathematical and statistical valuation

The percentage changes in various factors were calculated by following formula;

Percentage change = [(X−Y) ÷ Y] ×100

Where X = value at specific time break, Y = ‘0’ hr value.

Each interpretation was calculated for three times and results were signified as mean ± SD. For statistical data estimation, GraphPad prism 7.00 was employed. Two-way ANOVA (analysis of variance) was executed to evaluate the changes between various time breaks, while paired sample *t* test was valuable to compare the properties of BV ethosomal and control preparations. P < .05 was recognized as significant level.

Results

DPPH Free Radical-Scavenging Assay

BV fruit extract fractions have tremendous antioxidant potential as compared to Vit.C (control). The free radical scavenging assay of HABV, NHBV, CLBV, and NBBV was 84.36% ± 0.021, 67.68% ± 0.05, 72.18% ± 0.041, and 78.56% ± 0.037 respectively while that of ascorbic acid has 97.89% ± 0.021 representing in (**Fig.1(a)**. The crude hydro-alcoholic fraction of BV has the maximum phenolic and flavanoid contents than other fractions and this could be due to the ethanol has greatest polarity than other solvents used.

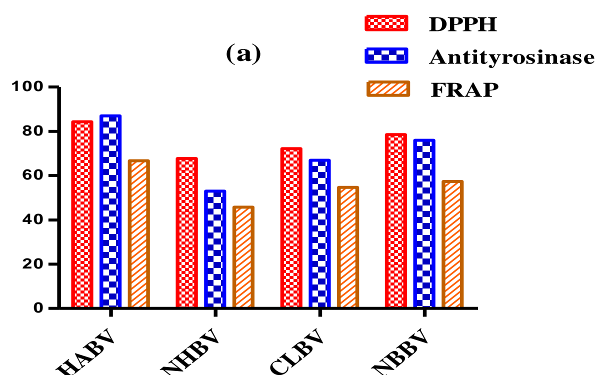

Figure 1. (a): Antioxidant, anti-tyrosinase activities, FRAP

Phytochemical analysis

Phytochemical screening of the crude extract of *Berberis vulgaris L.* were executed to identify the presence of secondary metabolites as depicted in (**Table 1).** The formation of yellow, red, brown colored precepitates with the particular readgent confirms the existence of alkaloid in BV sample extract. Other metabolites in the BV extract sample were also identified.

Table 1. Phytochemical screening of BV extract

Sr No.	Secondary metabolites	BV extract
1	Alkaloids	+
2	Phobatannins	-
3	Phenols	+
4	Flavonoids	+
5	Terpenoids	+
6	Glycosides	+
7	Saponins	+
8	Steroids	-

‘+’ designates the presence of secondary metabolites, ‘–’ designates the absence of secondary metabolites

Ferric Reducing Antioxidant Power (FRAP)
Fraction samples with grater absorbance are good reducing agents. FRAP values in HABV, NHBV, CLBV, and NBBV were 66.76 ± 0.03 µmol, 45.75 ± 0.042 µmol, 54.64 ± 0.051 µmol and 57.36 ± 0.073 µmol TE/ g of dry samples respectively as given in (**Fig.1(a).**

Mushroom Tyrosinase Inhibition Analysis
% tyrosinase inhibition of crude HABV fruit extract exhibited maximum activity as specified in (**Fig.1(a).** Mushroom tyrosinase inhibition of HABV, NHBV, CLBV, and NBBV was 87 ± 0.032 %, 53 ± 0.047%, 67 ± 0.031% and 76 ± 0.027 % respectively as relative to Kojic acid showing activity 99 ± 0.016 %. These outcomes indicated that HABV fraction again had maximum tyrosinase inhibitory assay than other fractions.

Total Phenolics Contents (TPC) and Total Flavanoid Contents (TFC)
Total phenolic contents in HABV crude fraction of BV fruit exhibited highest number of phenolic compounds as compared to other fractions as displayed in (**Fig.1(b).** The total phenols in HABV, NHBV, CLBV, and NBBV were about 286.38 ± 0.024 mg, 182.16 ± 0.062 mg, 198.54 ± 0.045 mg and 218.16 ± 0.032 mg GAE/ g respectively.

Total flavanoid contents in HABV fraction of BV fruit indicated the maximum number of flavonoid compounds when related to other fractions as shown in (**Fig.1(b**). The total flavonols in HABV, NHBV, CLBV, and NBBV were approximately 186.47 ± 0.023 mg, 136.54 ± 0.041 mg, 153.67 ± 0.076 mg and 168.37 ± 0.054 mg QE/g respectively.

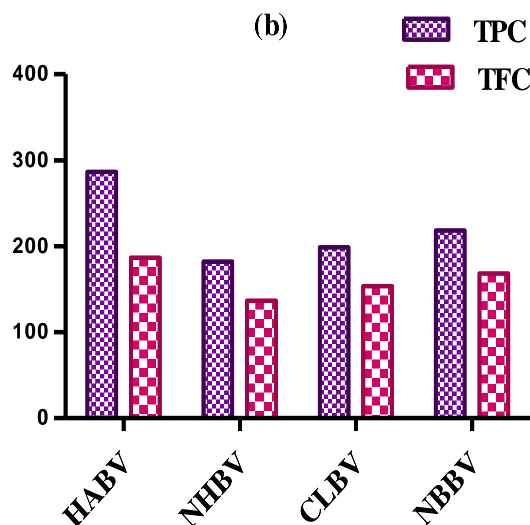

Figure 1. (b): Total phenolic and total flavonoid contents (TPC & TFC)

Sun protection factor (SPF)
The SPF is a computable assessment of the efficacy of a sunscreen preparation. In averting sunburn and other skin damages, a sunscreen agent should have a wide-ranging between 290 to 400 nm. SPF values of HABV, NHBV, CLBV, NBBV, BVCG and BVEG were 14.29 ± 0.013 %, 11.64 ± 0.035 %, 13.13 ± 0.041 %, 10.63 ± 0.064, 15.32 ± 0.033 and 17.74 ± 0.025% respectively as shown in (**Fig.1(c).**

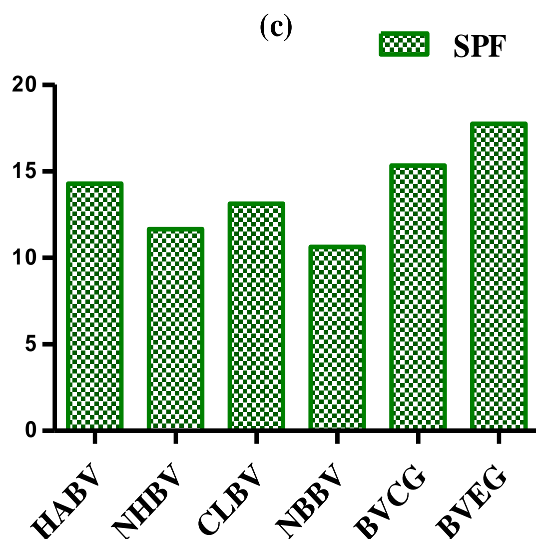

Figure 1. (c): SPF of fraction

Particle size, Poly dispersity index, Zeta potential and Entrapment efficiency

The concentration of ethanol and SPC was optimized by formulating different ethosomal formulations. An optimized formulation was chosen on basis of vesicle size, encapsulation efficiency, Zeta potential and PDI as given in (**Table 3**). The size of the formulations is dependent on the concentration of SPC and ethyl alcohol and it was found to be in range of 116.5 to 318.31 nm in table 3.

Table 3. Entrapment efficiency, Vesicle size, PDI and Zeta potential of (BV-Eth)

Formulation Code	EE (%±SD)	Vesicle size(nm)	PDI	Zeta potential (mV)
F1	66.18 ± 0.05	285.57	0.993	− 13.9
F2	69.74 ±0.072	270.98	1.36	−15.2
F3	75.79 ± 0.058	242.52	0.564	−18.5
F4	83.74 ± 0.047	310.32	0.531	−20.4
F5	89.54 ± 0.036	259.06	0.720	−22.5
F6	84.19 ± 0.045	318.31	0.561	−25.7
F7	86.39 ± 0.0216	289.09	0.475	−21.9
F8	92.70 ± 0.031	224.34	0.651	−26.3
F9	97.38 ± 0.02	116.5	0.500	−28.5

EE: Entrapment efficiency, PDI: polydispersity index, The results are mean ± SD (n=3),
SD: Standard deviation

The PDI value lies between 0.475 to 1.36 and the range of ZP value is from −30mV to 30mV, as found in all the formulations from −13.9 to −28.5 produced by the lipid net charge of the lipid in the formulation. The range of entrapement efficiency of ethosomes were from 66.18% to 97.38%.

On the basis of smaller vesicle size, uniform size distribution **(Figure 2. (a),** stability and higher encapsulation efficiency, formulation F9 was selected as optimized formulation for *in vitro* studies and further incorporated into gel formulation for *in vivo* studies.

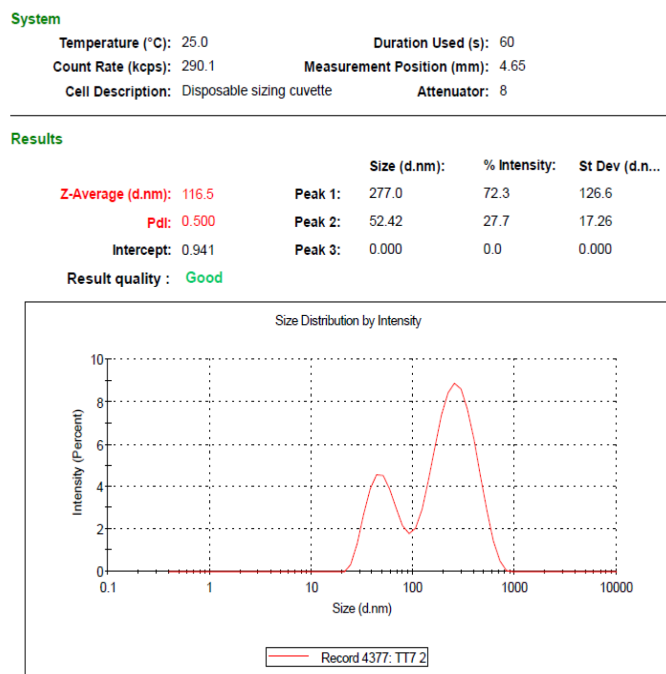

Figure 2: (a): Zeta size and PDI of optimized F9 formulation

Morphology

Shape and size of the transdermal drug delivery systems vesicle is a fundamental factor in the therapeutic efficacy. Surface morphology of ethosomes were further confirmed by Scanning electronic microscopy, mitigating the vesicular features carrying by this novel ethosomal system as represented in (**Fig.2 (b)**.

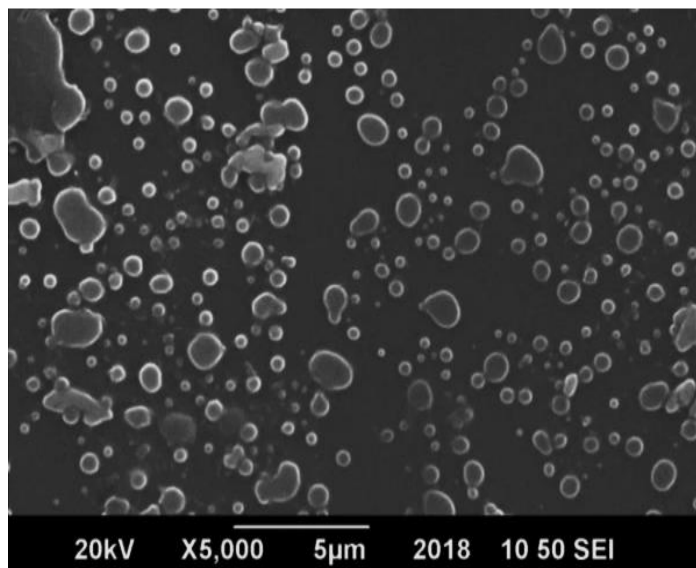

Figure 2. (b) SEM microphotograph of optimized formulation(F9) at 5µm

Fourier Transform Infrared Spectroscopy Analysis

The possible interaction among the drug and the excipients of ethosomal preparation were analyzed by IR spectroscopy. IR spectra of pure *Berberis vulgaris L.* extract and the prepared ethosomal formulation are shown in (**Fig.3 (a, b)** respectively. The FTIR spectrum of the Crude *Berberis vulgaris L.* depicted major peaks at 3335.14cm^{-1}, 2978.67cm^{-1}, 1639.51cm^{-1}, 1412.61cm^{-1}, 1083.98cm^{-1}, and 1043.68cm^{-1}.

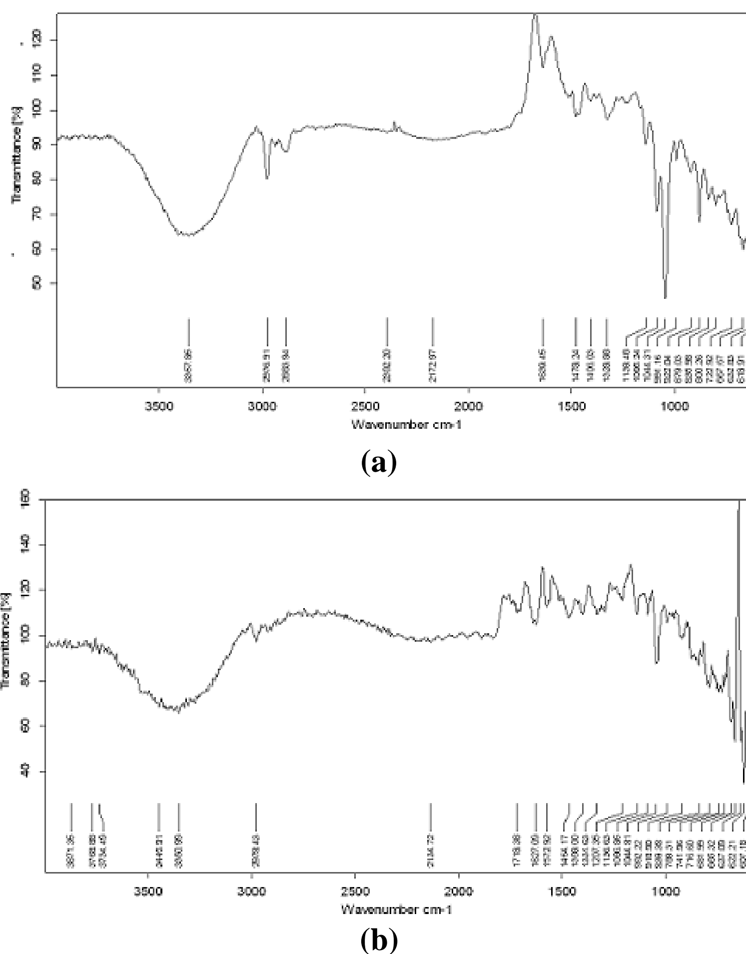

Figure 3: **(a)** Infrared spectrum of *Berberis vulgaris L.* extract **(b):** Infrared spectrum of ethosomes (F9)

Physicochemical Properties of Gel

Freshly formulated BVEG and BVCG were light pink in color, with no smell and having smooth,soft texture. Color of BVEG and BVCG is due to the pure *Berberis vulgaris L.* extract color. Physicochemical properties of both the gels were almost remain the same through the 90 days of study.The results of the evaluation parameters for gels are presented in (**Table 4).**

Table 4. Evaluation of physicochemical properties of BVEG and BVCG

After 90 days										
Parameters			**8°C**		**25°C**		**40°C**		**40°C±RH75%**	
	E	**C**	**E**	**C**	**E**	**C**	**E**	**C**	**E**	**C**
Color change	-	-	-	-	-	-	-	-	-	-
Odor change	-	-	-	-	-	-	-	-	-	-
Physical change	-	-	-	-	-	-	-	-	-	+

BVEG = E, BVCG = C

pH determination

BVCG and BVEG were kept at 8°C, 25°C, 40°C and 40°C ± RH 75% for a duration of 90 days and these formulations are almost neutral in nature represented in (**Fig.4 (a , b)** respectively. The changes in pH with time in control and ethosomal topical gel formulation is in the range to be used on the human skin.

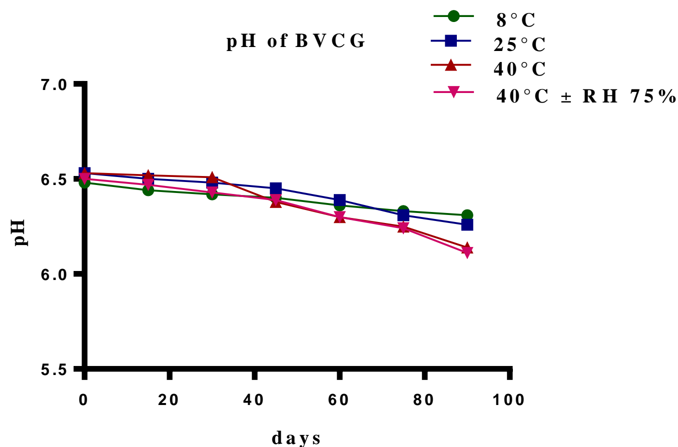

Figure 4 (a): pH changes in BVCG

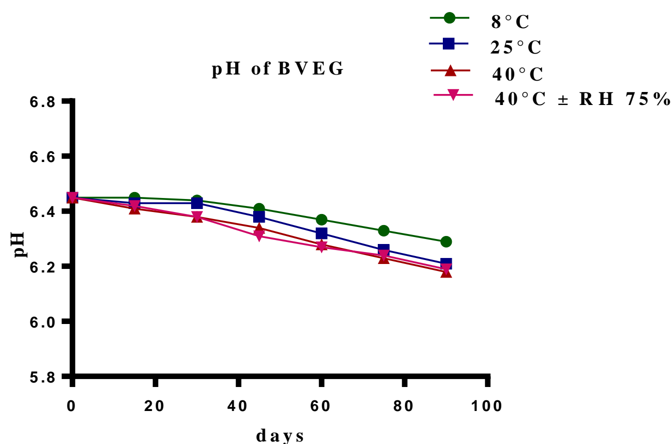

Figure 4 (b): pH changes in BVEG

Spreadability Determination

Spreadability of BVCG and BVEG at different temperature i.e. 8°C, 25°C, 40°C and 40°C ± RH 75% against time is represented in (**Fig.5 (a, b)** respectively. Spreadability of freshly prepared BVCG and BVEG were 3.3 ± 0.21 and 3.5 ± 0.32 and after 90 days at 8°C there was minor change in spreadability of BVCG and BVEG i.e. 3.1 ± 0.41 and 3.3 ± 0.14 indicating for both the gels were physically stable.

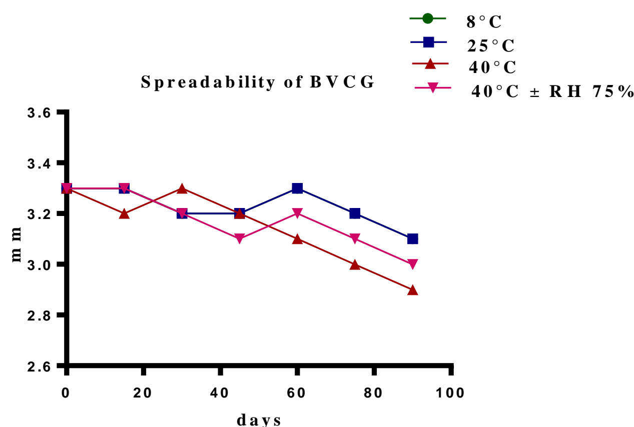

Figure 5. (a): Spreadability analysis of BVCG **(b):** Spreadability analysis of BVEG

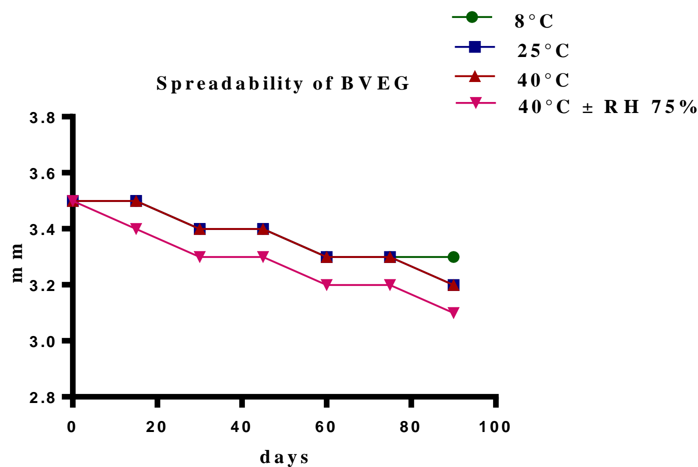

Figure 5. (b): Spreadability analysis of BVEG

Rheological Evaluation

The correlation between shear rate and shear stress of freshly formulated BVEG and BVCG at 0 hr. and after 12 weeks can be taken by Rheograms are represented in (**Fig. 6 (a, b).**

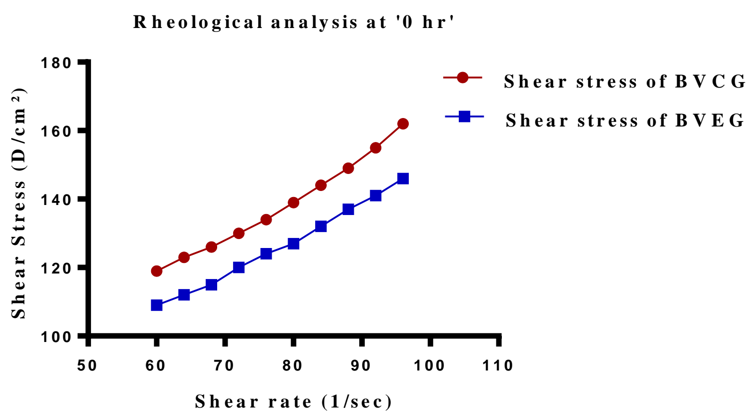

Figure 6. (a): Rheological analysis of the BVCG and BVEG at "0"hr

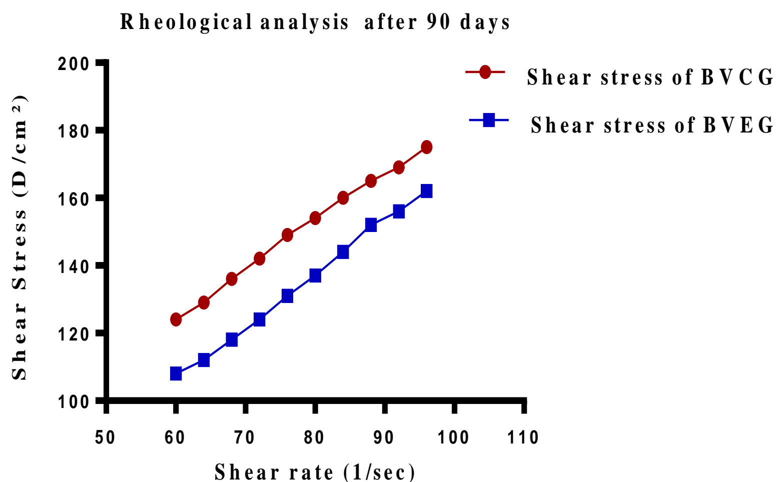

Figure 6. (b): Rheological analysis of the BVCG and BVEG at "12"weeks

Ex-Vivo Permeation Studies

The cumulative release of the drug from the control gel was 45.12 ± 0.072 % and from the ethosomal topical gel was 69.88 ± 0.016 % in 24 hrs. as displayed in (**Fig. 7**).

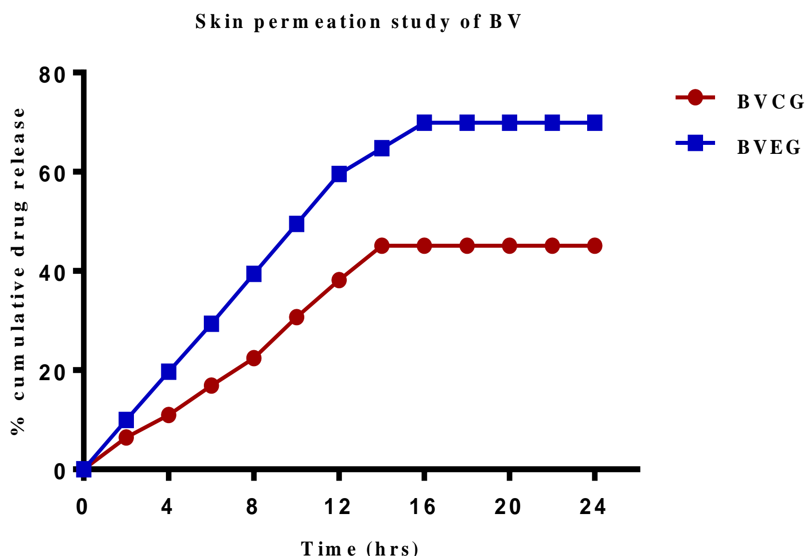

Figure 7: Comparative permeation analysis of BVCG and BVEG

Non-invasive *In vivo* studies

Evaluation of primary skin irritation by Patch test

Before to start the *in vivo* study, Patch test was performed on designated human volunteers to check any allergy, hypersensitivity or irritation to skin from any ingredient of BVCG and BVEG. From the results, it was confirmed that there was no allergic or skin irritation in any volunteer after 48 hrs. as shown in (**Fig. 8). Hence** patch test approves that these formulations were appropriate, safe and compatible for further 12-week *in vivo* study.

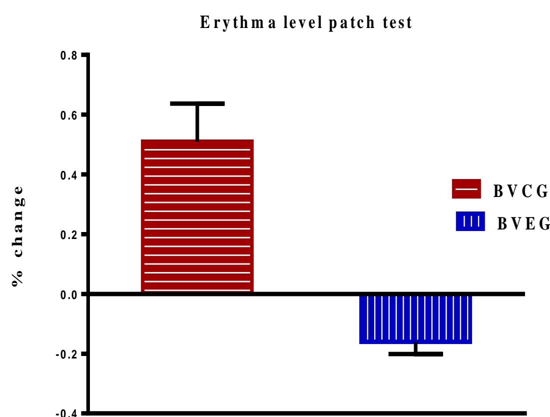

Figure 8: Erythma index for Patch test

Panel test

The different sensory factors assessed by judges were significant as shown in (**Fig. 9).** The results of the sensory parameters were positive and non-irritant for both BVCG and BVEG.

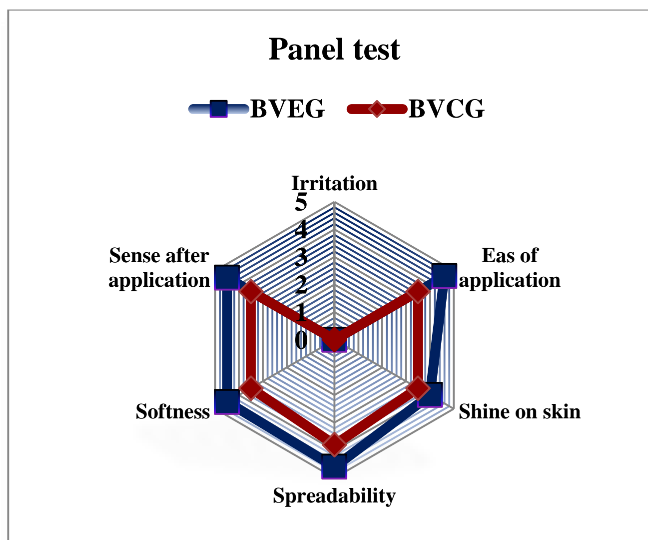

Figure 9: Panel test (n=5), average scores (±S.D) of BVCG and BVEG

Skin melanin and erythma evaluation

The melanin value was estimated during the course of the study at 0, 2, 4, 6, 8, 10, 12 weeks. From the results, it was observed that minor increase in melanin value by the application of BVCG whereas significant decrease in melanin after BVEG application and percentage change in melanin was 12.02% ±0.052 from the baseline zero time as represented in (**Fig. 10**).

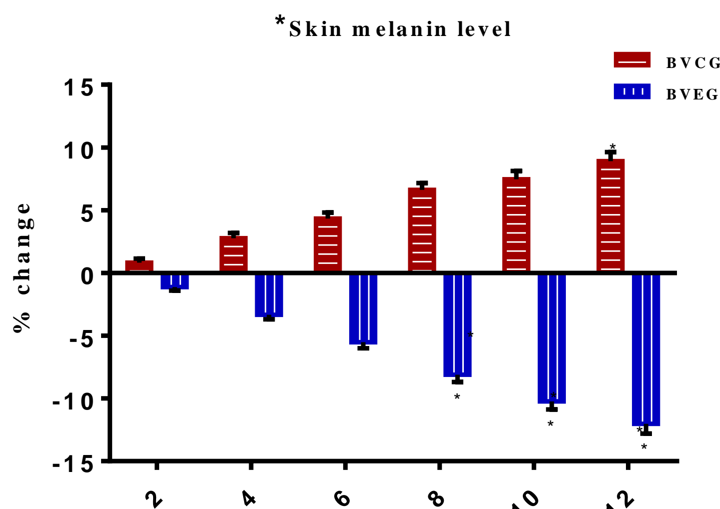

Figure 10: Mean % change in melanin by BVCG and BVEG

Two-way ANOVA specifies the significant change in melanin (P< .01) for BVEG with respect to time. Paired sample *t* test revealed that variation in melanin value of BVEG was significant as relevant to BVCG. LSD test also designates a significant change for the BVEG during the whole study. BVEG falls the erythma value by 10.90% ±0.036 and BVCG increased the erythma by 4.30% ±0.064 from the baseline zero time after 12 week of study **(Fig. 11).** Statistically ANOVA shows that BVEG effects are significant as compared to control formulation and paired *t* test indicated that change in erythma value between BVEG and BVCG was also significant. LSD test for the BVEG specified the significant change during the 12 week of study period.

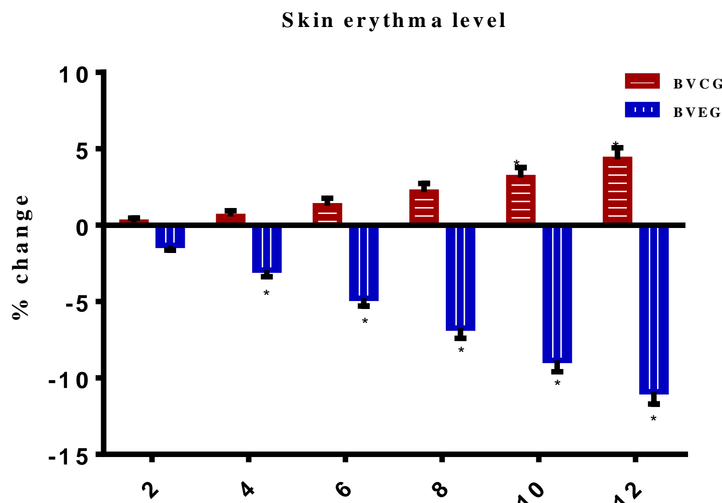

Figure 11: Mean % change in erythma by BVCG and BVEG

Skin sebum index evaluation

The active formulation of BV presented a remarkable reduction in the sebum value of skin by 19.8% ± 0.10 while BVCG increases sebum value of skin by 11.5 ± 0.27 as shown in (**Fig. 12).** Paired sample *t* test exhibited a substantial variation between the BVCG and BVEG effects during the whole study. LSD directs those effects of BV active formulation was significant for study period of 12 weeks. ANOVA analysis demonstrated that reduction in sebum value by BVEG was significant while increased in sebum content by BVCG was insignificant.

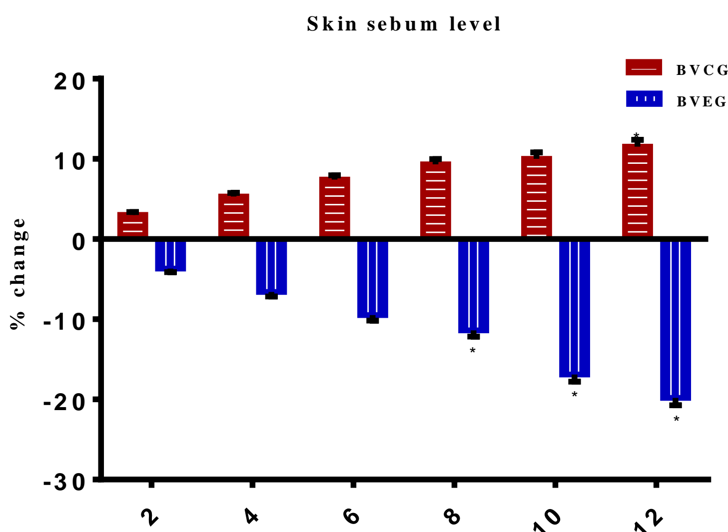

Figure 12: Mean % change in sebum by BVCG and BVEG

Skin hydration value assessment

BVEG increased the skin moisture content by 26.7% ±0.17 and BVCG also increased the skin moisture by 10.1%±0.121 at 2,4,6,8,10,12 weeks from the 0 hr. values **(Fig. 13).** LSD test indicated a significant variation for whole study period of 12 weeks and statistically ANOVA for BVEG also displayed significant change ($P < .05$). Paired *t* test between the BVCG and BVEG stated a significant difference throughout the study of 12 weeks.

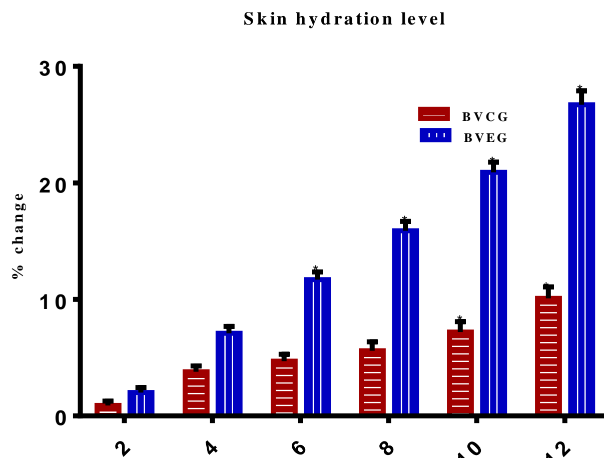

Figure 13: Mean % change in skin hydration by BVCG and BVEG

Skin elasticity evaluation

In the current study, active formulation (BVEG) revealed a remarkable increase in skin elasticity i.e. 14.0% ±0.18. Paired sample *t* test represented a significant skin elasticity difference between BVCG and BVEG effects **(Fig. 14).** LSD indicated the significant elasticity effects on skin by the active formulation during the whole study period. Two-way ANOVA demonstrated that BVEG exhibited a significant effect as compared to time from zero time to 12 weeks.

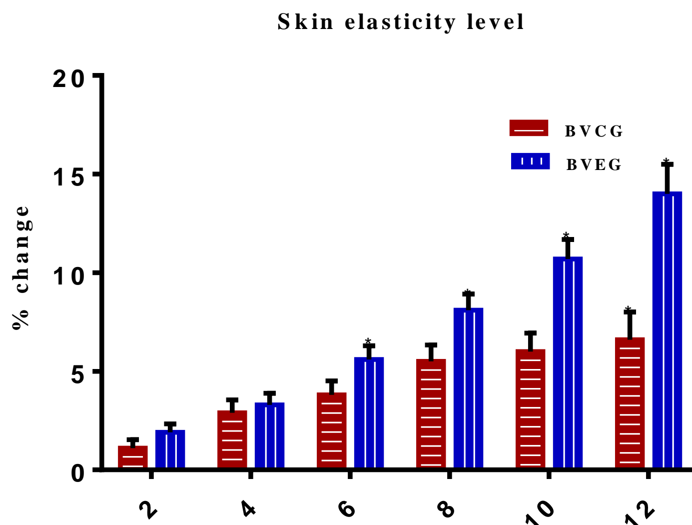

Figure 14: Mean % change in skin elasticity by BVCG and BVEG

Acute toxicity study

No mortality and morbidity were observed throughout the study period. LD_{50} of the *Berberis vulgaris L.* was greater than 2000mg/Kg.

Discussion

In this study, antioxidant, phenolic, flavanoids, anti-tyrosinase, and SPF values of an ethanolic fruit extract from Berberis vulgaris L. were investigated. In comparison to other BV fruit extract fractions, the hydro-alcoholic crude fraction of Berberis vulgaris L. displayed the highest antioxidant capacity, as measured by FRAP and mushroom tyrosinase inhibition experiment. This is because of presence of polyphenols, alkaloids, and flavonoids. (Asensio et al., 2020). In this study, HABV extract reduced pigmentation and premature ageing by scavenging free radicals, preventing oxidative stress in cells,

improving skin suppleness, and curing acne vulgaris (Özgen, Saraçoğlu, & Geçer, 2012). The SPF value of BV extracts and its formulations might be attributed to the presence of flavanoid and phenolic contents that could be employed as natural sunscreens in cosmeceuticals to prevent photo-induced skin damage (Gajardo et al., 2016).

The deeper skin stratums are said to be permeable to vesicles with a size of 300 nm or less. The results show that vesicle size increased when SPC concentration was raised from 1% to 3%, however vesicle size was negatively impacted by ethanol concentration (i.e. higher ethanol concentration, lower will be the vesicle size) (Elsayed, Abdallah, Naggar, & Khalafallah, 2007). The result confirms the fact that the size of vesicles also reduced as ethanol concentration increased because it provided the vesicular systems with a surface (-ve) net charge by modifying specific surface properties (Chourasia, Kang, & Chan, 2011). Polydispersity index (PDI), or the level of particle heterogeneity, is another physical characteristic. The PDI value of 0.7, which indicates stable and repeatable vesicles, indicates homogenous dispersion (Ramadon et al., 2017). The improved ethosmal formulation F9 in the current investigation has a PDI value of 0.5, indicating a monodisperse narrow size distribution. This PDI value can be a result of the phospholipid's lipophilicity having a specific location in the membrane of the vesicles (Ascenso et al., 2015). The lipid structure net charge in the vesicular formulation generates the zeta potential, a critical parameter that controls the stability of the vesicular system. Electrostatic repulsion and resistance force generated by the (-ve) charge on the ethanol in the solution can stop vesicles from aggregating (Samnani, Shahwal, Bhowmick, Joshi, & Dubey, 2012).

Entrapment efficiency (EE) is the ethosomal system's delivery potential, which is directly related to its ability to transport drugs. The quantity of ethyl alcohol and soy phosphatidyl choline has a good impact on the trapping of plant extract inside lipid molecules. The ethanol content should not exceed 40%, since greater levels might result in drug seepage from the lipid bilayer, which would result in EE reductions (Touitou et al., 2000)' (Srivastava, Singh, & Amrit, 2015).

The free OH in the molecule and the OH group creating hydrogen bonds, the carbonyl group C=O, stretching the C=C aromatic ring, and the C-OH and C-H stretching vibrations, respectively, are all shown by the FTIR spectrum of the BV extract. These peaks may have indicated the presence of some compounds in the Berberis vulgaris L. extract, including polyhydroxyl phenols and flavonoids (Fu, Zhang, Guo, & Chen, 2014). In the extract excipients combination, the same peaks were similarly visible albeit with little shifting. When comparing the Berberis vulgaris L. extract in the physical mixture to pure Berberis vulgaris L. extract, the results showed no appreciable change, suggesting that no interaction had occurred (Dave et al., 2010).

The spreadability of the control gel showed a modest shift in the early weeks, and then it declined as the water content evaporated. Because to the fluidizing effects of ethanol and the gelling ingredient carbopol 940, the spreadability of the ethosomal topical gel somewhat enhanced. Spreadability may be analysed more quickly when considering phospholipid concentration (Iizhar, Syed, Satar, & Ansari, 2016).

The physical stability estimation during safe application and formulation shelf life into skin is significantly influenced by the rheological parameter in topical formulation (Gilbert, Picard, Savary, & Grisel, 2013). Drug dispersion is caused by the microstructural environment of the rheological measures (Di Mambro & Fonseca, 2005). By applying shear tension, the material's primary structure experiences advanced fragmentation. Then, due to Brownian movement, the core structure is once again restored once the shear force is removed (Gaspar & Campos, 2003). Additionally, the consistency of the formulation during storage and the simplicity of application would be supported by the shear thinning and viscoelastic characteristics of the semisolid system (Rathapon, Sirivat, & Vayumhasuwan, 2005).

Because ethanol gives vesicles delicate, flexible characteristics, they can enter the skin's dermis well. Additionally, propylene glycol serves as a permeation enhancer, increasing the permeability of vesicles through biological membranes thanks to the synergistic effects of ethanol (Touitou et al., 2001). Ethanol primarily alters the stratum corneum's lipid bilayer and increases the lipid's fluidity. Ethosomal vesicles can then infiltrate the disturbed stratum corneum as a result. The transdermal

absorption of the medicine into the deeper layers of the skin may result from the drug being released at various points along the penetration pathway as ethosomes and skin lipid merge (Jain, Umamaheshwari, Bhadra, & Jain, 2004).
Melanin pigment is created in humans by melanocytes in melanosomes through a combined process known as melanogenesis (Videira, Moura, & Magina, 2013). Tyrosinase, phenylalanine hydroxylase (PAH), tyrosine hydroxylase isoform I (TH-1), tyrosinase related protein-1 (TRP-1), and tyrosinase related protein-2 are the main enzymes involved in melanogenesis (TRP-2) (D'Mello, Finlay, Baguley, & Askarian-Amiri, 2016). Melanocytes control extrinsic factors like UV light and chemicals (Yamaguchi & Hearing, 2009). The melanin pigment is crucial for protecting against damaging UV rays, skin hyperpigmentation, melasma, and scavenging toxic medicines and chemicals. Elevated melanin synthesis results in an increase in skin melanin and erythma levels (Lee, Baek, & Nam, 2016). The tyrosinase enzyme is inhibited by phenols and flavanoids in the BV fruit extract, which lowers the generation of melanin and lowers skin erythema levels in the current study (Nawaz et al., 2019).
Sebum is a natural bodily oil that is generated by sebaceous glands and is an oily, waxy substance that lubricates the stratum corneum. Skin sebum content was significantly decreased in the current investigation. Increased sebum production on the skin leads to the development of acne vulgaris. Due to its antioxidant activity, presence of poly phenols, and specifically berberine alkaloid, BV fruit extract significantly decreased the amount of sebum in the skin and may inhibit the development of acne (Imanshahidi & Hosseinzadeh, 2008).
The level of water in the skin affects how it ages, works, and desquamates. When trans epidermal water loss (TEWL) is higher, insufficient enzymatic activity can lead to early ageing, dryness, and wrinkles in the skin. Due to the suppression of skin erythema and inflammation in the current study, (TEWL) levels are decreased and skin moisture content is increased (Ovaere, Lippens, Vandenabeele, & Declercq, 2009).

CONCLUSION

The Berberis vulgaris L. fruit extract used in the current study contains large amounts of polyphenols and flavonoids, and it also exhibits outstanding antioxidant activity, as well as significant mushroom tyrosinase inhibition and higher SPF value. Vesicular nanostructured ethosomes derived from Berberis vulgaris L. were favorably generated and then added to carbopol-940 gel. It demonstrated effective drug release, making it appropriate for topical administration. For 90 days, BV ethosomal gel exhibited stability at room temperature, and its pH remained in the skin's normal range. pH demonstrates that no skin irritation was discovered. Additionally, BVEG affects skin elasticity, hydration, and melanin statistically significantly (P.05). The BVEG decreased readings for skin erythma and sebum. Therefore, it is proposed that this topical BV ethosomal gel be investigated further in order to assess its potential for the treatment of clinical conditions such psoriasis, dermatitis, and acne.

Conflict of interest

There is no conflict of interest in this project.

References

1. Abdulhamid, A, & Sani, I. (2016). Preliminary phytochemical screening and antimicrobial activity of aqueous and methanolic leave extracts of Balanites aegyptiaca (L.). *International Research Journal of Pharmaceutical and Biosciences, 3*(1), 1-7.
2. Aggarwal, Divya, & Nautiyal, Ujjwal. (2016). Ethosomes: A review. *Int J pharm med res, 4*(4), 354-363.
3. Ainbinder, Denize, Godin, Biana, & Touitou, Elka. (2016). Ethosomes: Enhanced delivery of drugs to and across the skin *Percutaneous Penetration Enhancers Chemical Methods in Penetration Enhancement* (pp. 61-75): Springer.

4. Antonopoulou, Io, Varriale, Simona, Topakas, Evangelos, Rova, Ulrika, Christakopoulos, Paul, & Faraco, Vincenza. (2016). Enzymatic synthesis of bioactive compounds with high potential for cosmeceutical application. *Applied microbiology and biotechnology, 100*(15), 6519-6543.
5. Anwar, Fareeha, Saleem, Uzma, Rehman, Atta-Ur, Ahmad, Bashir, Froeyen, Matheus, Mirza, Muhammad Usman, . . . Ahmad, Sarfraz. (2021). Toxicity evaluation of the naphthalen-2-yl 3, 5-dinitrobenzoate: a drug candidate for Alzheimer disease. *Frontiers in Pharmacology, 12*, 607026.
6. Apone, Fabio, Tito, Annalisa, Carola, Antonietta, Arciello, Stefania, Tortora, Assunta, Filippini, Lucio, . . . Chrispeels, Maarten J. (2010). A mixture of peptides and sugars derived from plant cell walls increases plant defense responses to stress and attenuates ageing-associated molecular changes in cultured skin cells. *Journal of biotechnology, 145*(4), 367-376.
7. Ascenso, Andreia, Raposo, Sara, Batista, Cátia, Cardoso, Pedro, Mendes, Tiago, Praça, Fabíola Garcia, . . . Simões, Sandra. (2015). Development, characterization, and skin delivery studies of related ultradeformable vesicles: transfersomes, ethosomes, and transethosomes. *International journal of nanomedicine, 10*, 5837.
8. Asensio, Esther, Vitales, Daniel, Pérez, Iván, Peralba, Laia, Viruel, Juan, Montaner, Celia, . . . Sales, Ester. (2020). Phenolic Compounds Content and Genetic Diversity at Population Level across the Natural Distribution Range of Bearberry (Arctostaphylos uva-ursi, Ericaceae) in the Iberian Peninsula. *Plants, 9*(9), 1250.
9. Bansal, Mahima, Reddy, M Sudhakara, & Kumar, Anil. (2017). Optimization of cell growth and bacoside-A production in suspension cultures of Bacopa monnieri (L.) Wettst. using response surface methodology. *In Vitro Cellular & Developmental Biology-Plant, 53*(6), 527-537.
10. Benzie, Iris FF, & Strain, JJ. (1999). [2] Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration *Methods in enzymology* (Vol. 299, pp. 15-27): Elsevier.
11. Brandner, JM, & Jensen, JM. (2008). The skin: an indispensable barrier. *Exp Dermatol, 17*, 1063-1072.
12. Chen, Fu-An, Wu, An-Bang, Shieh, Pochuen, Kuo, Daih-Huang, & Hsieh, Chi-Ying. (2006). Evaluation of the antioxidant activity of Ruellia tuberosa. *Food chemistry, 94*(1), 14-18.
13. Chourasia, Manish K, Kang, Lifeng, & Chan, Sui Yung. (2011). Nanosized ethosomes bearing ketoprofen for improved transdermal delivery. *Results in pharma sciences, 1*(1), 60-67.
14. Cichorek, Mirosława, Wachulska, Małgorzata, Stasiewicz, Aneta, & Tymińska, Agata. (2013). Skin melanocytes: biology and development. *Advances in Dermatology and Allergology/Postępy Dermatologii I Alergologii, 30*(1), 30.
15. Costa, Sônia CC, Detoni, Cassia B, Branco, Carla RC, Botura, Mariana B, & Branco, Alexsandro. (2015). In vitro photoprotective effects of Marcetia taxifolia ethanolic extract and its potential for sunscreen formulations. *Revista Brasileira de Farmacognosia, 25*(4), 413-418.
16. D'Mello, Stacey AN, Finlay, Graeme J, Baguley, Bruce C, & Askarian-Amiri, Marjan E. (2016). Signaling pathways in melanogenesis. *International journal of molecular sciences, 17*(7), 1144.
17. Dave, Vivek, Kumar, Dhirendra, Lewis, Shaila, & Paliwal, Sarvesh. (2010). Ethosome for enhanced transdermal drug delivery of aceclofenac. *International journal of drug delivery, 2*(1).
18. Di Mambro, Valéria M, & Fonseca, Maria JV. (2005). Assays of physical stability and antioxidant activity of a topical formulation added with different plant extracts. *Journal of pharmaceutical and biomedical analysis, 37*(2), 287-295.
19. Eghbali-Feriz, Samira, Taleghani, Akram, Al-Najjar, Hadi, Emami, Seyed Ahmad, Rahimi, Homa, Asili, Javad, . . . Tayarani-Najaran, Zahra. (2018). Anti-melanogenesis and anti-tyrosinase properties of Pistacia atlantica subsp. mutica extracts on B16F10 murine melanoma cells. *Research in pharmaceutical sciences, 13*(6), 533.

20. Elsayed, Mustafa MA, Abdallah, Ossama Y, Naggar, Viviane F, & Khalafallah, Nawal M. (2007). Lipid vesicles for skin delivery of drugs: reviewing three decades of research. *International journal of pharmaceutics, 332*(1-2), 1-16.
21. Espinosa-Leal, Claudia A, & Garcia-Lara, Silverio. (2019). Current methods for the discovery of new active ingredients from natural products for cosmeceutical applications. *Planta médica, 85*(07), 535-551.
22. Fouladi, Rohollah F. (2012). Aqueous extract of dried fruit of Berberis vulgaris L. in acne vulgaris, a clinical trial. *Journal of dietary supplements, 9*(4), 253-261.
23. Fu, Rao, Zhang, Yuting, Guo, Yiran, & Chen, Fang. (2014). Antioxidant and tyrosinase inhibition activities of the ethanol-insoluble fraction of water extract of Sapium sebiferum (L.) Roxb. leaves. *South African Journal of Botany, 93*, 98-104.
24. Gajardo, Sandra, Aguilar, Marilú, Stowhas, Tamara, Salas, Felipe, Lopez, José, Quispe, Cristina, . . . Benites, Julio. (2016). Determination of sun protection factor and antioxidant properties of six Chilean Altiplano plants. *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas, 15*(5), 352-363.
25. Gaspar, LR, & Campos, PMBG Maia. (2003). Rheological behavior and the SPF of sunscreens. *International journal of pharmaceutics, 250*(1), 35-44.
26. Ghribia, Lotfi, Ghouilaa, Hatem, Omrib, Amel, Besbesb, Malek, & Janneta, Hichem Ben. (2014). Antioxidant and anti–acetylcholinesterase activities of extracts and secondary metabolites from Acacia cyanophylla. *Asian Pacific journal of tropical biomedicine, 4*, S417-S423.
27. Gilbert, Laura, Picard, Céline, Savary, Géraldine, & Grisel, Michel. (2013). Rheological and textural characterization of cosmetic emulsions containing natural and synthetic polymers: relationships between both data. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 421*, 150-163.
28. Hadaruga, Daniel I, Hadaruga, Nicoleta G, Bandur, Geza N, Rivis, Adrian, Costescu, Corina, Ordodi, Valentin L, & Ardelean, Aurel. (2010). Berberis vulgaris extract/β cyclodextrin nanoparticles synthesis and characterization. *Rev Chim (Bucharest), 61*, 669-675.
29. Hameed, Abdul, Akhtar, Naveed, Khan, Haji Muhammad Shoaib, & Asrar, Muhammad. (2019). Skin sebum and skin elasticity: Major influencing factors for facial pores. *Journal of cosmetic dermatology, 18*(6), 1968-1974.
30. Iizhar, Syed Ahmed, Syed, Ismail Ahmed, Satar, Rukhsana, & Ansari, Shakeel Ahmed. (2016). In vitro assessment of pharmaceutical potential of ethosomes entrapped with terbinafine hydrochloride. *Journal of advanced research, 7*(3), 453-461.
31. Ijaz, Musarrat, & Akhtar, Naveed. (2020). Fatty acids based α-Tocopherol loaded nanostructured lipid carrier gel: In vitro and in vivo evaluation for moisturizing and anti-aging effects. *Journal of cosmetic dermatology, 19*(11), 3067-3076.
32. Ijaz, Shakeel, Khan, Haji Muhammad Shoaib, Anwar, Zeeshan, Talbot, Brian, & Walsh, John J. (2019). HPLC profiling of Mimosa pudica polyphenols and their non-invasive biophysical investigations for anti-dermatoheliotic and skin reinstating potential. *Biomedicine & Pharmacotherapy, 109*, 865-875.
33. Imanshahidi, Mohsen, & Hosseinzadeh, Hossein. (2008). Pharmacological and therapeutic effects of Berberis vulgaris and its active constituent, berberine. *Phytotherapy research, 22*(8), 999-1012.
34. Jain, S, Umamaheshwari, RB, Bhadra, D, & Jain, NK. (2004). Ethosomes: a novel vesicular carrier for enhanced transdermal delivery of an antiHIV agent. *Indian journal of pharmaceutical sciences, 66*(1), 72.
35. Kamtekar, Samidha, Keer, Vrushali, & Patil, Vijaya. (2014). Estimation of phenolic content, flavonoid content, antioxidant and alpha amylase inhibitory activity of marketed polyherbal formulation. *Journal of applied pharmaceutical Science, 4*(9), 61.

36. Khan, Palwasha, & Akhtar, Naveed. (2022). Phytochemical investigations and development of ethosomal gel with Brassica oleraceae L.(Brassicaceae) extract: An innovative nano approach towards cosmetic and pharmaceutical industry. *Industrial Crops and Products, 183*, 114905.
37. Khan, Palwasha, Akhtar, Naveed, Khan, Haji Muhammad Shoaib, Tasneem, Rabia, Zaka, Hafiz Saqib, Akhtar, Naheed, & Sharif, Arfa. (2022). Assessment of Brassica oleraceae L.(Brassicaceae) Extract loaded Ethosomal gel as a versatile Vesicular carrier system for Dermocosmetic application: A noninvasive split-faced study. *Journal of Cosmetic Dermatology*.
38. Končić, M Zovko, Kremer, Dario, Karlović, Ksenija, & Kosalec, Ivan. (2010). Evaluation of antioxidant activities and phenolic content of Berberis vulgaris L. and Berberis croatica Horvat. *Food and chemical toxicology, 48*(8-9), 2176-2180.
39. Korkina, Liudmila G, Mayer, Wolfgang, & De Luca, Chiara. (2017). Meristem plant cells as a sustainable source of redox actives for skin rejuvenation. *Biomolecules, 7*(2), 40.
40. Lee, Sang Yeul, Baek, Namhuk, & Nam, Tae-gyu. (2016). Natural, semisynthetic and synthetic tyrosinase inhibitors. *Journal of Enzyme Inhibition and Medicinal Chemistry, 31*(1), 1-13.
41. Lopez-Pinto, JM, Gonzalez-Rodriguez, ML, & Rabasco, AM. (2005). Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. *International journal of pharmaceutics, 298*(1), 1-12.
42. Maia, Juliana Leão, Dantas, Thayse Naianne Pires, Neto, Bento Pereira da Costa, Borges, Kátia Cristina, Lima, Erica Cortez, da Mata, Ana Lúcia de Medeiros Lula, . . . Pereira, Camila Gambini. (2019). Extract of spray-dried Malay apple (Syzygium malaccense L.) skin. *Journal of Food Process Engineering, 42*(8), e13275.
43. Manosroi, Aranya, Jantrawut, Pensak, Akihisa, Toshihiro, Manosroi, Worapaka, & Manosroi, Jiradej. (2011). In vitro and in vivo skin anti-aging evaluation of gel containing niosomes loaded with a semi-purified fraction containing gallic acid from Terminalia chebula galls. *Pharmaceutical biology, 49*(11), 1190-1203.
44. Maurya, Sheo Datta, Prajapati, S, Gupta, A, Saxena, G, & Dhakar, Ram Chand. (2010). Formulation development and evaluation of ethosome of stavudine. *Int J Pharm Edu Res, 13*, 16.
45. Mukherjee, Pulok K, Maity, Niladri, Nema, Neelesh K, & Sarkar, Birendra K. (2011). Bioactive compounds from natural resources against skin aging. *Phytomedicine, 19*(1), 64-73.
46. Nasrollahzadeh, Mahmoud, Maham, Mehdi, Rostami-Vartooni, Akbar, Bagherzadeh, Mojtaba, & Sajadi, S Mohammad. (2015). Barberry fruit extract assisted in situ green synthesis of Cu nanoparticles supported on a reduced graphene oxide–Fe 3 O 4 nanocomposite as a magnetically separable and reusable catalyst for the O-arylation of phenols with aryl halides under ligand-free conditions. *RSC advances, 5*(79), 64769-64780.
47. Nawaz, Mehwish, Khan, Haji Muhammad Shoaib, Akhtar, Naveed, Jamshed, Talha, Qaiser, Rubina, Shoukat, Hina, & Farooq, Mehrin. (2019). Photodamage and Photoprotection: An In vivo Approach Using Noninvasive Probes. *Photochemistry and photobiology, 95*(5), 1243-1248.
48. Ovaere, Petra, Lippens, Saskia, Vandenabeele, Peter, & Declercq, Wim. (2009). The emerging roles of serine protease cascades in the epidermis. *Trends in biochemical sciences, 34*(9), 453-463.
49. Özgen, Mustafa, Saraçoğlu, Onur, & Geçer, Esma Nur. (2012). Antioxidant capacity and chemical properties of selected barberry (Berberis vulgaris L.) fruits. *Horticulture, Environment, and Biotechnology, 53*(6), 447-451.
50. Rahimi-Madiseh, Mohammad, Lorigoini, Zahra, Zamani-Gharaghoshi, Hajar, & Rafieian-Kopaei, Mahmoud. (2017). Berberis vulgaris: specifications and traditional uses. *Iranian Journal of Basic Medical Sciences, 20*(5), 569.
51. Rakesh, R, & Anoop, KR. (2012). Ethosomes for transdermal and topical drug delivery. *Int J Pharm Pharm Sci, 4*(3), 17-24.
52. Ramadon, Delly, Goldie, Aisha Wirarti, & Anwar, Effionora. (2017). Novel transdermal ethosomal gel containing green tea (Camellia sinensis L. Kuntze) leaves extract: formulation and in vitro penetration study. *Journal of Young Pharmacists, 9*(3), 336.

53. Rathapon, A, Sirivat, Anuvat, & Vayumhasuwan, Panida. (2005). Viscoelastic properties of Carbopol 940 gels and their relationships to piroxicam diffusion coefficients in gel bases. *Pharmaceutical Research, 22*(12), 2134-2140.
54. Saewan, Nisakorn, Vichit, Wannisa, & Prinyarux, Thanon. (2018). Anti-aging efficacy of Thai red rice callus cosmetic product. *The Journal of Applied Science, 17*(Special), 63-72.
55. Saini, NK, Singhal, M, & Srivastava, B. (2011). Evaluation of antioxidant activity of Tecomaria capensis leaves extract. *Inventi Rapid Ethnopharmacol, 2011*(2), 117.
56. Samnani, A, Shahwal, V, Bhowmick, M, Joshi, A, & Dubey, BK. (2012). Design and evaluation of ultradeformable soft elastic nano vesicle ethosomes for dermal delivery. *Int J Biomed Adv Res, 3*(02), 111-117.
57. Sayre, Robert M, Agin, Patricia Poh, LeVee, Gordon J, & Marlowe, Edward. (1979). A comparison of in vivo and in vitro testing of sunscreening formulas. *Photochemistry and Photobiology, 29*(3), 559-566.
58. Shahwar, Durre, Ullah, Saif, Ahmad, Mobasher, Ullah, Sami, Ahmad, Naeem, & AKMAL, KHAN MUHAMMAD. (2011). Hypoglycemic activity of Ruellia tuberosa linn (Acanthaceae) in normal and alloxan-induced diabetic rabbits.
59. Srivastava, N, Singh, K, & Amrit, K. (2015). Formulation and evaluation of seabuckthorn leaf extract loaded ethosomal gel. *Asian J Pharm Clin Res, 8*, 316-320.
60. Surini, Silvia, Arnedy, Antik R, & Iswandana, Raditya. (2019). Development of Ethosome Containing Bitter Melon (Momordica charantia Linn.) Fruit Fraction and In Vitro Skin Penetration. *Pharmacognosy Journal, 11*(6).
61. Tabeshpour, Jamshid, Imenshahidi, Mohsen, & Hosseinzadeh, Hossein. (2017). A review of the effects of Berberis vulgaris and its major component, berberine, in metabolic syndrome. *Iranian journal of basic medical sciences, 20*(5), 557.
62. Touitou, E, Dayan, N, Bergelson, L, Godin, Biana, & Eliaz, M. (2000). Ethosomes—novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. *Journal of controlled release, 65*(3), 403-418.
63. Touitou, E, Godin, Biana, Dayan, N, Weiss, C, Piliponsky, A, & Levi-Schaffer, F. (2001). Intracellular delivery mediated by an ethosomal carrier. *Biomaterials, 22*(22), 3053-3059.
64. Upadhyay, Tarun K, Fatima, Nida, Sharma, Deepak, Saravanakumar, V, & Sharma, Rolee. (2017). Preparation and characterization of beta-glucan particles containing a payload of nanoembedded rifabutin for enhanced targeted delivery to macrophages. *EXCLI journal, 16*, 210.
65. Uttra, Ambreen Malik, & Hasan, Umme Habiba. (2017). Anti-arthritic activity of aqueous-methanolic extract and various fractions of Berberis orthobotrys Bien ex Aitch. *BMC complementary and alternative medicine, 17*(1), 1-16.
66. Venus, Matt, Waterman, Jacqueline, & McNab, Ian. (2010). Basic physiology of the skin. *Surgery (Oxford), 28*(10), 469-472.
67. Verma, Poonam, & Pathak, K. (2010). Therapeutic and cosmeceutical potential of ethosomes: An overview. *Journal of advanced pharmaceutical technology & research, 1*(3), 274.
68. Videira, Inês Ferreira dos Santos, Moura, Daniel Filipe Lima, & Magina, Sofia. (2013). Mechanisms regulating melanogenesis. *Anais brasileiros de dermatologia, 88*, 76-83.
69. Wan, Miaojian, Hu, Rong, Xie, Xiaoyuan, Gong, Zijian, Yi, Jinling, Chen, Haiyan, . . . Lai, Wei. (2017). Skin erythema, pigmentation and hydration kinetics after ultraviolet radiation-induced photodamage in southern chinese women. *Photochemistry and photobiology, 93*(5), 1276-1281.
70. Yamaguchi, Yuji, & Hearing, Vincent J. (2009). Physiological factors that regulate skin pigmentation. *Biofactors, 35*(2), 193-199.
71. Zhang, Zhen, Wo, Yan, Zhang, Yixin, Wang, Danru, He, Rong, Chen, Huijin, & Cui, Daxiang. (2012). In vitro study of ethosome penetration in human skin and hypertrophic scar tissue. *Nanomedicine: Nanotechnology, Biology and Medicine, 8*(6), 1026-1033.
72. Zhishen, J, Mengcheng, T, & Jianming, W. (1999). Research on antioxidant activity of flavonoids from natural materials. *Food Chem, 64*, 555-559.