Design and optimization of a novel herbosomal-loaded PEG–poloxamer topical formulation for the treatment of cold injuries: a quality-by-design approach

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Abstract
The spectrum of cold injuries ranges from frostnip, chilblains to severe frostbite. Cold injuries occur upon prolonged exposure to freezing temperature and are pathologically a combination of ice crystal formation in the tissue resulting in inflammation, thrombosis and ischemia in the extremities, often necessitating limb amputation in extreme cases due to tissue necrosis. Severe forms of frostbite are a cause of major concern to patients as well as the treating physician. Due to the lack of effective treatment modalities and paucity of research on prophylaxis and therapeutics of cold injuries, we developed a novel herbosomal-loaded PEG–poloxamer topical formulation (n-HPTF) employing quality-by-design (QBD) approach. Natural compounds exhibiting potent therapeutic potential for the management of cold injuries were incorporated in novel lipid vesicles (herbosomes) loaded in PEG–poloxamer polymers. The herbosomal formulation effectively creates an occlusion barrier that promotes epithelial regeneration, desmosome scale-up and angiogenesis and thus promotes rapid healing, indicating controlled release of herbosomes. Optimized novel herbosomes showed entrapment efficiency > 90% and < 300 nm mean particle size and in vitro drug permeation of about 2 µg/cm² followed Higuchi’s release kinetics. Skin irritancy study on female Sprague–Dawley rats showed no edema or erythema. In vivo bio-efficacy study revealed significant efficacy (p < 0.05) when compared to the standard treatment groups.

Keywords Cold injury · Novel herbosomal-loaded PEG–poloxamer topical formulation (n-HPTF) · Quality by design (QBD) · Frostbite healing study · Histopathology

Introduction
Cold injury is defined as an injury in which prolonged exposure to cold wind causes severe tissue damage, even lead to amputation due to exposure to freezing temperature. Cold injury is basically characterized by four interconnected, often consecutive, pathophysiological phases: (i) desensitization of sensory neuronal receptor or TRPV1 channels, (ii) prolonged exposure leads to local vasoconstriction and ischemia due to pre-freezing response of local tissue, (iii) imbalanced salt concentration of intracellular fluid that leads to influx of extracellular fluid and leads to ice-crystal formation due to altered osmotic pressure as well as initiation of cytokines release and (iv) thrombosis and its progression, which can even lead to amputation, if left untreated [1]. The human body initially responds to the initiation of tissue freezing by the act of “hunting reaction” which involves the alteration of vasodilation and vasoconstriction cycle up to a limit; however, due to loss of sensory receptors, microvascular changes cannot be restricted [2]. Thus, it becomes imperative to understand the thermodynamics of the skin and subcutaneous tissue in order to unravel the mechanism of cold injury. The spectrum of cold injuries clinically presents itself between the range of freezing type (frostbite) and non-freezing types, e.g., frostnip (a milder form of cold injury and completely reversible), pernio, i.e., chilblains (this represents a more severe form of injury than the frostnip and occurs due to cold moisture exposure for 3–6 h), and trench foot (a form of non-freezing type cold injury that
includes purple phalanges, tingling and itching and can progress to the numbness of the foot, if not diagnosed).

Frostbite—the severe form of cold thermal injuries, results due to damage to the skin and often occurs in tissues primarily due to freezing [2]. Cold injuries ranges from minimal tissue loss to severe necrosis often leading to amputation of limbs. Frostbite is important from both civil and military perspective as it poses a great risk to the severe cold-exposed persons [1]. During high-risk military operations, frostbite severely affects the operational efficiency of soldiers and can present immense medical and logistic challenges in terms of treatment, evacuation and rehabilitation [3, 62].

Depending on the severity, the commonly available prophylactic and therapeutic modalities for the management of cold injury/ies include topical application of Aloe vera cream, followed by a combination therapy of pentoxifylline, aspirin and vitamin C for non-freezing-type cold injuries, whereas rapid rewarming should be avoided. Other treatments, based on severity of cold injury, include thrombolitics (tissue plasminogen activator; tPA) [2], vaso-dilators (prostaglandins E1, prostacyclin analogue, nitroglycerin, reserpine) [4], hypobaric oxygen therapy [5], anti-coagulants (heparin), hydrogel formulation (recombinant human granulocyte–macrophage colony stimulating factor; rhGM-CSF) [6], etc.

Prevention is considered better than cure, and effective topical formulations can play a key role by preventing the induction of cold injuries even before they occur. The novel topical formulation designed by us is a next generation formulation and is based on herbosomes incorporated in a polymer matrix and contains natural ingredients for holistic healing for precisely targeting the multiple sites affected by frostbite.

The rationale for inclusion of the phytoconstituents in the novel formulation was as follows: Aloe barbadensis is a well-known plant reported in Ayurveda to promote wound healing. One of the pharmacologically active constituents found in the leaves of Aloe vera, viz. acemannan, is a very potent compound that acts as a TXA2 (thromboxane A2) inhibitor [7], which is involved in thrombosis formation in case of frostbite. Aloe also exhibits excellent anti-inflammatory and vasodilatory activity mediated via COX (cyclooxygenase) inhibition [7]. Azadirachta indica is mentioned in Ayurveda for its antimicrobial and wound healing efficacy. The active ingredients like nimbolide and azadirachtin present in A. indica modulate signaling pathways like transcription factor (NFκB) and apoptotic (Bcl2). A. indica also plays a role in regulation of pro-inflammatory enzyme activities through COX and LOX (lipooxygenase), and these factors are required to support the treatment of initial response that occurs in first- and second-degree frostbite [8]. In this study, Camellia sinensis was chosen as one of the key ingredients due to its richness in antioxidant compounds, which functionally minimizes the effect of reactive oxygen species (ROS). The active constituents, viz. epigallocatechin gallate (EGCG), gallicatein gallate (GCG), catechin (C) and epicatechin (EC), inhibit COX-1 (inflammatory markers) and thromboxane synthase (TXAS commonly associated with platelet aggregation) [9, 10], which is very important progeny of the diseased condition like frostbite. On the other hand, Glycyrrhiza glabra is a rich source of tannins, phytosterols, coumarins, vitamins (B1, B2, B3, B5, E and C), glycosides, pantothenic acid, biotin, etc. Glycyrrhiza glabra has the ability to down-regulate the expression of pro-inflammatory cytokines, e.g., tumor necrotic factor alpha (TNF-α) and associated interleukins like IL-1 and IL-6 [11], and thereby plays an important role to control the progression of the diseased condition at initial stage itself. Calendula officinalis flower also possesses active ingredients that are responsible for inhibiting the pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IFN-γ, besides possessing COX-2 inhibitory activity [12]. Arnica montana was selected for incorporation into the herbosomal preparation, along with Curcuma longa, due to their agonist activity of TRPV1 (transient receptor vanilloid subfamily, member 1), which is responsible for the sensation of heat/cold and is an important regulator of sensory response for the nerve endings found in subcutaneous tissue [13]. Curcuma longa was incorporated in the herbosomes along with Arnica montana because TRPV1 channel is de-sensitized in the presence of inflammatory conditions and Curcuma longa is a well-known anti-inflammatory agent [14]. Pro-vitamin B5 or dexamethasone, a pharmacologically active compound known for re-generation of epithelial lining of skin when applied topically as well as helps in remodeling and acts as a potent compound for cold injury condition to prevent amputation of extremities from ice-crystal formation and subsequent injuries [15]. Tocopherol acetate is a well-known antioxidant used in the treatment of various skin ailments has an inhibitory effect on ROS and superoxide generation and was incorporated in the designed formulation due to its potent antioxidant activity [16].

Development of an optimal drug delivery system requires control of several critical parameters to ensure that the formulation of different batches consistently remains uniform. Quality control parameters in formulations containing herbal extracts are quite critical. Quality-by-design (QbD) approach in pharmaceutical product development has been introduced by pharma regulatory authorities to build the basis for robust and effective product development with a statistically designed risk assessment and risk management parameters within a design space. A QbD emphasizes uniform built parameters for the analytical quality control as well as finished product. Design of experiments (DoE) for process design and development and process optimization are widely used in
pharmaceutical product development [17, 18]. In this paper, we report the design and development of a novel herbosomal-loaded PEG–poloxamer topical formulation (n-HPTF) by QbD approach for formulation optimization by controlling the critical processing variables, entrapment efficiency, particle size, in vitro drug permeation through skin and rheology. Vesicles prepared from natural polymer and lipid possess less shelf life and are stable mostly in refrigerated condition. To increase the shelf-life of herbosomes in the herbal pharmaceutical preparations, we incorporated a polymeric base containing a potent carrier for the model disease. A wide range of variability between two similar crude materials is often observed due to natural conditions of growth, germplasm type and origin, time of cultivation and harvesting, etc. Thus, it becomes imperative to control CQAs of the herbal pharmaceuticals [19, 20].

A standard pharmaceutical concept with high rate of approvals of pharmaceutical products envisages quality by design (QbD) for designing, developing and optimizing the processing variables for robust product. Guidelines dedicated to QbD are enumerated by International Conference of Harmonization (ICH) of ICH Q8 (R2) [21–23]. Pharmaceutical manufacturing industries widely accept the concept of QbD to achieve an invariable product batch-to-batch by implementing the QbD relationship links of critical material attributes (CMAs) and critical quality attributes (CQAs) within a design space [22].

The aim of this study was to optimize a novel herbosomal-loaded PEG–poloxamer topical formulation (n-HPTF) for the treatment of cold injuries (both freezing and non-freezing), primarily occurring at high altitude by employing QbD approach at each CQAs. The product efficiency and product critical attributes were measured at each stage to minimize the quality risk factors, leading to cost-effective quality optimized finished product. Process control steps were monitored at each point of designing of n-HPTF, e.g., percentage entrapment efficiency for herbosomes, particle size and zeta potential, with their independent variables like drug ratio and lipid ratio. Optimization of final formulation, i.e., n-HPTF, was critically controlled in terms of selection of polymeric ratio, penetration enhancer, etc., which had a significant role on the rheological parameters and in vitro drug permeation. Stability testing was carried out as per ICH guidelines and the efficacy of n-HPTF in prevention of cold injury was evaluated in Sprague–Dawley rat model.

Materials and methods

Materials

Ascorbic acid; 2,2’-azio-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS); 2,2’-diphenyl-1-picrylhydrazyl (DPPH); ferric chloride hexahydrate (FeCl3.6H2O); Folin-Ciocalteu reagent; potassium persulfate; sodium nitroprusside; sodium hydroxide; sodium carbonate; ferrous sulfate; sodium phosphate; aluminum chloride; ammonium molybdate; tocopherol acetate; gallic acid; rutin trihydrate; L-α-phosphatidylcholine; cholesterol; stearic acid; sorbitol; pro vitamin B5; PEG 3350; poloxamer-188; Carbopol® 940; cetyl alcohol; xanthan gum; triethanolamine; Garcinia indica; Transcutol®; glycerol; tween-60; and PEG-200 were procured from Sigma-Aldrich (St Louis USA). Solvents such as methanol (HPLC grade) and chloroform (HPLC grade) were also procured from Sigma-Aldrich (St Louis, USA). Ultra-pure water with 18.2 MΩ resistivity obtained by Milli-Q unit (Merck ELIX®) was used throughout the study whenever required.

The plant material was collected from different regions of India during February–October, 2020, or procured from reputed herbal suppliers. The plant material was duly authenticated by a reputed botanist and tested for the absence of heavy metals. Quality control methods for herbal materials as stipulated by WHO (2011) were strictly followed. Authentic specimens of the material have been maintained in the Department of Phytochemistry, Defence Institute of Physiology and Allied Sciences, Delhi, India. The plant material (Aloe barberdensis Mill. (Family: Asphodelaceae) (leaf) (Aq.); Azadirachta indica A. Juss. (Family: Meliaceae) (leaf) (Aq.); Curcuma longa L. (Family: Zingiberaceae) (rhizome) (Aq.: Ethanol, 50:50 v/v); Camellia sinensis (Family: Theaceae) (leaf) (Aq.); Glycyrrhiza glabra L. (Family: Fabaceae) (stem) (Aq.: Ethanol, 30:70 v/v); Arnica montana L. (Family: Asteraceae) (Aq.: Ethanol, 50:50 v/v); and Calendula officinalis L. (Family: Asteraceae) Aq.: Ethanol, 50:50 v/v) was extracted by homogenizer-assisted extraction method.

Homogenizer-assisted extraction of herbas

A homogenizer-assisted herbal extraction method was opted over conventional maceration method to obtain high antioxidant capacity content extracts [24]. Other advantages of this method over conventional methods of extraction includes: high yield extraction, less time-consuming, less energy expenditure and reduced chances of bacterial or fungal contamination. The extraction of herbal materials, viz. Aloe barberdensis (leaf) (Aq.); Azadirachta indica (leaf) (Aq.); Curcuma longa (rhizome) (Aq.: Ethanol, 50:50 v/v); Camellia sinensis (leaf) (Aq.); Glycyrrhiza glabra (stem) (Aq.: Ethanol, 30:70 v/v); Arnica montana (flower) (Aq.: Ethanol, 50:50 v/v); and Calendula officinalis (flower) Aq.: Ethanol, 50:50 v/v), was carried out using GRAS solvents (water and ethanol) separately. Each extract was collected separately and filtered using muslin
cloth and then further purified by using ultracentrifugation at 5000 rpm for 10 min at 4°C (ROTA 4R-V/Fm Plasto Craft, India) and lyophilized by using (Allied Frost FD-5 Lyophilizer, India). Lyophilized herbal extracts were collected and packed in an air-tight container; percent yield of each extract was calculated and extracts stored for further analysis. Percent yield of the lyophilized extract was calculated by the following formula:

\[
\text{Percent Yield} = \frac{\text{Actual Yield}}{\text{Theoretical yield}} \times 100
\]

**Attenuated total reflection-fourier transform infrared spectroscopy (ATR-FTIR)**

Fourier transform infrared spectroscopy analyzes the infrared light from the light source passing through a Michelson Interferometer along with the optical light. The parallel polarized light affects the absorption by the sample which can be enhanced by using polarizer. FTIR is dedicated to study the functional groups present in the sample perpendicular to its dipole moment vis-a-vis to internal reflection metal. To study the functional groups present in the all-herbal extracts viz; Aloe barbadensis (leaf) (Aq.); Azadirachta indica (leaf) (Aq.); Curcuma longa (rhizome) (Aq.: Ethanol, 50:50 v/v); Camellia sinensis (leaf) (Aq.); Glycyrrhiza glabra (stem) (Aq.: Ethanol, 30:70 v/v); Arnica montana (Aq.: Ethanol, 50:50 v/v); and Calendula officinalis (Aq.: Ethanol, 50:50 v/v), ATR-FTIR spectrophotometer (Bruker EQUINOX 55 FTIR, Germany) equipped with a liquid nitrogen-cooled mercury cadmium telluride (MCT) was used at room temperature (25 ± 1 °C) with an equipped detector of nominal resolution of 2 cm for each spectra. For ATR reflection, diamond is embedded with FTIR as an internal reflection element (IRE), placed at an angle of 45°, and this scans each functional group 32 times to give one reflection, which is equivalent to resolutions. After successful spectral reading, an advance ATR correction was applied to all spectra in the region lying between 4000 cm\(^{-1}\) to 400 cm\(^{-1}\); subsequently peak fitting was performed by using Opus software integrated with Bruker ATR-FTIR instrument [25].

**Determination of total polyphenolic content and total flavonoid content**

Total polyphenolic content in the herbal extracts was quantified by using Folin-Ciocalteu reagent (FCR) adopted from the method of Do et al. [26] with some modifications. Briefly, a standard of gallic acid was prepared between concentration ranges of 5 µg/mL to 45 µg/mL to obtain a standard curve. Simultaneously, accurately weighed 1 mg of all the test compounds [extracted herals, viz. Aloe barbadensis (leaf) (Aq.); Azadirachta indica (leaf) (Aq.); Curcuma longa (rhizome) (Aq.: Ethanol, 50:50 v/v); Camellia sinensis (leaf) (Aq.); Glycyrrhiza glabra (stem) (Aq.: Ethanol, 30:70 v/v); Arnica montana (Aq.: Ethanol, 50:50 v/v); and Calendula officinalis (Aq.: Ethanol, 50:50 v/v)] was dissolved in 10 mL of Milli-Q water. After that, each dilution of standard and test compounds was mixed with 1 mL of FCR and kept aside for 5 min. of incubation in dark. Then 2 mL of sodium carbonate solution (20%) was added to the above incubated mixtures and kept aside for further 60 min. more. After complete incubation time period, absorbance of each standard and test compounds was recorded at 750 nm wavelength using Jenway (6505) UV/Vis spectrophotometer. Experiments were carried out in triplicate, and results were expressed in mg of gallic acid equivalents of per gram (GAE/g) of dry weight of extract (DWE) [26–28]. Total flavonoid content in the herbal extract was quantified by using aluminum chloride method described by Zhishen et al. [28] with some modification. Briefly, a standard solution of rutin trihydrate (RT) in a concentration range of 10 µg/mL to 300 µg/mL in Milli-Q water to obtain a standard curve. Simultaneously, accurately weighed 1 mg of test compounds [extracted herals, viz. Aloe barbadensis (leaf) (Aq.); Azadirachta indica (leaf) (Aq.); Curcuma longa (rhizome) (Aq.: Ethanol, 50:50 v/v); Camellia sinensis (leaf) (Aq.); Glycyrrhiza glabra (stem) (Aq.: Ethanol, 30:70 v/v); Arnica montana (Aq.: Ethanol, 50:50 v/v); and Calendula officinalis (Aq.: Ethanol, 50:50 v/v)] was prepared in 1 mL of Milli-Q water. Then, in a 10 mL test tube, 0.5 mL of herbal extract solution, 0.5 mL of 30% methanol, 0.3 mL of NaNO₂ (0.5 M) and 0.3 mL of AlCl₃·6H₂O (0.3 M) were mixed together and kept aside for 5 min. of incubation in dark and same procedure was followed for the standard solution. After 5 min. of incubation, 1 mL of NaOH (1 M) was added and then total volume was made up to 10 mL with Milli-Q water. Readings of each sample (in triplicate) were recorded at 715 nm wavelength using Jenway (6705) UV/Vis Spectrophotometer. The total flavonoid content in each extract was expressed as mg of rutin trihydrate equivalents per gram (RT/E)/g of dry weight of extract (DWE) [27–30, 36].

**In vitro antioxidant potential study**

Antioxidant capacity is the overall capacity of extracts, food or antioxidative enzymes such as SOD (superoxide dismutase), catalase, GPx (glutathione peroxidase) and glutathione-S-transferase to scavenge free radicals, which prevents the harmful effects generated by these free radicals. Antioxidant potential study of herbal extracts is necessary to establish the protective efficacy against the harmful free radicals especially superoxide ions, nitrogen and hydroxyl ion. Antioxidant...
potential of extracted herbas was assessed by using in vitro assays, viz. DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric reducing antioxidant power), ABTS (2,2'-azino-bis (3-ethylbenzothiazolin-6-sulfonic acid)), NO (nitric oxide) scavenging potential and TAC (total antioxidant capacity).

**Determination DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity**

DPPH free radical scavenging activity of the herbal extracts was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) chemical method described by Blois [30] and Uddin [31]. The hydrogen donating capacity of the extracts leads to change in color of the DPPH solution from violet to yellow showing antioxidant activity in the given extracts. Briefly, in a 10 mL of test tube 0.1 mM methanolic DPPH (3 mL) and 0.2 mL of extracts (ranged from 0.5 mg/mL to 10 mg/mL) was added, followed by vortexing and kept aside in dark for 30 min. of incubation. Similarly, standard solution of ascorbic acid in the range of 10 µg/mL to 100 µg/mL was taken as a reference for estimating the potential inhibition capacity. After a 30 min of incubation at room temperature (25 °C), absorbance of each solution was recorded spectrophotometrically at 517 nm wavelength.

The percentage inhibition of DPPH free radical was calculated by the following equation:

\[
\%\text{ inhibition of DPPH} = \left[\frac{Ac - As}{Ac}\right] \times 100
\]

where Ac is absorption of control; As is absorption of extracts/standard.

The % inhibition of standard solution was plotted against concentration, and an IC₅₀ value was calculated from the graph. Results obtained from extracts were correlated with standard. Each value obtained from the reading was calculated from sample of each extract in triplicate.

**Determination of FRAP (ferric ion reducing antioxidant power) activity**

FRAP activity of the given extracts was determined by the method reported by Benzie et al. [32]. Briefly, 1 mg/mL of each extract in Milli-Q water was taken in a test tube and 2.8 mL of FRAP solution [(300 mM acetate buffer (25 mL); 10 mM 2,4,6-trpyridyl-s-trazine (TPTZ) in 40 mM HCL (2.5 mL) and 20 mM FeCl₃.6H₂O (2.5 mL))] was added to it. To determine FRAP activity in the given extract, a standard solution of ferric sulfate was prepared in Milli-Q water and a concentration range of 50 µg/mL to 500 µg/mL was taken to plot a standard curve and incubated with FRAP solution. Each sample in triplicate was incubated in dark at room temperature (25 °C) for 30 min, after which a standard plot was drawn and results were expressed in mg of ferrous (II) equivalent per gram dry weight of extracts (Fe (II)E/g of DWE).

**Determination of ABTS (2,2'-amino-bis (3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging activity**

ABTS free radical activity was determined by ABTS⁺ radical cation decolorization (from blue/green stable cationic radical chromophore to colorless) by addition of the antioxidant agents described by Re et al. [33]. ABTS⁺ cation radical was prepared by reaction mixture (1:1) of 7 mM ABTS in water and 2.45 mM of potassium persulfate in an amber color reaction mixture bottle and stored in dark for 12–16 h before use. Before carrying out the experiments, ABTS⁺ solution was diluted with methanol to achieve an absorbance of 0.700±0.02 at 734 nm [34].

Briefly, 50 µL of plant extracts (1 mg/mL) was added to 3.995 mL of diluted ABTS⁺ solution and set aside for 30 min. of incubation at room temperature (25 °C) in dark. Absorbance was recorded at 734 nm in triplicate samples of each extract. Trolox was used as a standard in the range of 10 µg/mL to 200 µg/mL and Milli-Q water as blank.

Calculation of ABTS⁺ value was done by using the formula mentioned below [35]:

\[
\text{ABTS⁺ free medical scavenging capacity (µgTEAC/gDWE)} = c \times V \times t/m
\]

where, ABTS⁺ free radical scavenging capacity of the extracts was expressed in TEAC/g DWE (Trolox equivalent antioxidant capacity per gram of dry weight of extract). In which, c is the Trolox concentration (µg/mL), V is the sample volume taken (ml), t is the dilution factor (if any), and m is the mass of the sample, i.e., dry weight of extract in gram.

**Determination of NO (nitric oxide) scavenging potential activity**

Nitric oxide scavenging potential of the extracts was measured by the method reported by Hazra et al. [35] with slight modification. Briefly, 1 mL of extracts (1 mg/mL) was mixed with 0.8 mL of 10 mM sodium nitroprusside solution prepared in phosphate buffer saline (pH 7.4) followed by incubation at 40 °C in dark for 150 min. Then 1 mL of 0.33% sulfanilamide prepared in 20% glacial acetic acid was added and incubated again for 5 min. in dark at room temperature (25 °C). Afterward, 1 mL of 0.1% of naphthylethendiamine dihydrochloride solution was added and further more incubated for 30 min. in dark at room temperature (25 °C). After successive incubation of extracts with reagent, a light pink color chromophore developed, which was analyzed by using
UV/Vis spectroscopy at 540 nm. Ascorbic acid (10 µg/mL to 100 µg/mL) was used as standard against test sample. Each sample was analyzed in triplicate.

The percentage inhibition of the nitric ion, i.e., nitric oxide free radical, was calculated by using the following formula described below:

\[
\text{% Inhibition} = \left( \frac{A_c - A_s}{A_c} \right) \times 100
\]

where Ac is the absorbance of control and As is the absorbance of test samples/standards.

**Determination of total antioxidant capacity (TAC)**

Total antioxidant capacity of the extracts was quantified by phosphomolybdenum complex formation method as described by Prieto et al. [37]. Phosphomolybdenum assay is based on reduction of Mo (VI) to Mo (V) by the given test samples (analytes), which subsequently forms green phosphate/Mo (V) complex when it reached to acidic pH. This test was performed by taking 0.3 mL of given sample (1 mg/mL) in a test tube mixed with 3 mL of reducing reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Subsequently, samples were incubated at 95°C for 90 min. and kept aside to cool down at room temperature (25°C). All samples were analyzed in triplicate. Ascorbic acid (in the concentration range of 10 µg/mL to 600 µg/mL) was taken as a standard and a calibration curve was plotted against absorbance. The absorbance was recorded at 695 nm spectrophotometrically, and results were expressed in mg of ascorbic acid equivalent per gram of dry weight of the extract ((mg AAE)/g of DWE).

**Preparation of novel herbosomes (n-herbosomes)**

Herbosomes are basically herbal-liposomes specifically designed to enhance the bioavailability of the herbals so that they possess targeted mode of action. In this study, we designed herbosomes by using conventional method of preparation, i.e., thin film hydration (TFH) method of liposomes described by Bangham [38] and Dave et al. (2017) [39] with few modifications. The ratio of natural plant products (NPPs) viz; *Curcuma longa* (CL); *Arnica montana* (AM); lipid (L-α-phosphatidylcholine); cholesterol; solvents (chloroform: methanol); surfactant (stearic acid); cryopreservant (sorbitol) and vehicle (phosphate buffer saline, pH 7.4) is depicted in Table 1. Method of optimization of n-herbosomes was done by employing response surface method analysis. Briefly, lipid, cholesterol, stearic acid and sorbitol were weighed accurately and dissolved in the solvent taken in a round-bottom flask (RBF), mixed thoroughly to obtain a clear lipid solution. Subsequently, *Curcuma longa* was added to lipid solution (oil/organic phase) and *Arnica montana* to PBS (aqueous phase). The organic phase was continuously rotated by using Rota evaporator (BUCHI Rotavapor® R-300, Switzerland) at 75 rpm and temperature 45°C to ensure organic solvent removal and successive thin layer formation. RBF was taken out and after complete vacuum drying, flask was kept over dry ice bath and taken out before any cracks were evident over the thin film. After the successful step of thin film formation, aqueous layer containing aqueous soluble ingredients was added drop-wise under continuous stirring at 300 rpm (too high or too low stirring rate can lead to generation of MLV (multilamellar vesicle)), for 24 h to obtain n-herbosomes. n-herbosomes were then subjected to Probe sonication (PCI Analytics) using ice bath for each 5 min of sonication cycle and 3 min. of thawing to avoid any excess heat generation and lipid disruption. n-herbosomes were then successfully collected and used for further drug development.

**Incorporation of n-herbosomes into polymeric base to form novel herbosomal-loaded PEG–poloxamer topical formulation (n-HPTF)**

Novel herbosomal-loaded PEG–poloxamer topical formulation (n-HPTF) was prepared by fusion method. Briefly, oil phase containing PEG-3350, poloxamer-188, *Garcinia indica* (Kokum butter, Pharmaceutical grade), cetyl alcohol, stearic acid and xanthan gum were fused vis-a-vis their melting point.

| Batch no | n-H-01 | n-H-02 | n-H-03 | n-H-04 | n-H-05 | n-H-06 | n-H-07 | n-H-08 | n-H-09 |
|----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Drug* (mg) | 75:50 | 100:50 | 125:50 | 75:50 | 100:50 | 125:50 | 75:50 | 100:50 | 125:50 |
| Lipid** (mg) | 100:15 | 100:15 | 100:15 | 125:15 | 125:15 | 125:15 | 175:15 | 175:15 | 175:15 |
| Solvents*** (ml) | 1:1 | 1:1 | 1:1 | 1:1 | 1:1 | 1:1 | 1:1 | 1:1 | 1:1 |
| Stearic acid (mg) | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Sorbitol (mg) | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| PBS pH 7.4 (ml) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

Drug*: (*Curcuma longa*: *Arnica montana*), Lipid**: (L-α-Phosphatidylcholine (95%), Cholesterol), Solvents***: (Chloroform: Methanol), PBS: (Phosphate Buffer Saline, pH 7.4)
temperature in a decreasing order on a water bath at 60 °C and water phase containing Aloe barbadensis, Azadirachta indica, Camellia sinensis, Glycyrrhiza glabra, Calendula officinalis, pro-vitamin B5, acryl 10/30, PEG-200, Tween-60, Transcutol®, glycerol, triethanolamine. The mixture was dissolved and warmed at 60°C on a water bath before mixing. After complete fusion of oil phase, water phase was added to it slowly with continuous stirring on water bath and tocoferol acetate was added when mixture was slightly cooled down to avoid oxidation of tocoferol acetate. After a homogenous mixing of the dual phase, mixture was taken out from the water bath and transferred to mortar. Mixture was vigorously mixed (continuously) using mortar-pestle. When the temperature of the formulation reached 40–45°C, 10 ml of previously prepared n-herbosomes was added slowly and stirred properly until a smooth homogenous cream was obtained. A number of formulations were prepared by controlling processing variables, and the ingredients were weighed and mixed together as listed in Table 2, and further a final optimization was done by using QbD approach.

**Design of experiment (DoE)**

Design of experiment (DoE) is an empirical tool for quality risk management (QRM) in pharmaceutical ICH guideline of ICH (Q9) including risk identification, risk analysis, risk evaluation and risk acceptance [40] which works within a defined design space ICH (Q8) [41]. Response surface methodology (RSM) is a statistical and mathematical tool employed to access such critical attributes by justifying input of critical processing variables during product development [41–46].

In the present study, Box-Behnken full-factorial design (3^2) was used to study the critical variable (independent variable) affecting product development and their risk management by employing Statistica V.10 software (StatSoft, Inc. USA) [38, 47, 95]. In RSM study, independent variables parameter affects one or more dependent variable (s); thus, we had applied RSM for the optimization of n-herbosomes as well as optimization of the final formulation (n-HPTF). In herbosomes the effect of independent variables, i.e., X1: drug ratio (Curcuma longa: Arnica montana) and X2: lipid ratio (L-α-phosphatidylcholine: cholesterol) on Y1 (CL): percentage entrapment efficiency (%EE) of Curcuma longa; Y1 (AM): percentage entrapment efficiency (%EE) of Arnica montana and Y2: particle size (nm), was studied. After optimization of herbosomes, final formulation was prepared and their processing variables were also studied by using full factorial design in which independent variables were taken as: X1: polymer ratio (PEG-3350: poloxamer-188) and X2: penetration enhancer ratio (Transcutol®:PEG-200) and their effect

**Table 2 Formulation designing for optimization of novel herbosomal-loaded PEG–poloxamer topical formulation (n-HPTF)**

| Batch no | n-HPTF-01 | n-HPTF-02 | n-HPTF-03 | n-HPTF-04 | n-HPTF-05 | n-HPTF-06 | n-HPTF-07 | n-HPTF-08 | n-HPTF-09 |
|----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| n-herbosomes (ml) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Aloe barbadensis (gm) | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 |
| Azadirachta indica (gm) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Camellia sinensis (gm) | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Glycyrrhiza glabra (gm) | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| Calendula officinalis (gm) | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Pro-vitamin B5 (mg) | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Tocopherol acetate (mg) | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 |
| PEG-3350 (gm) | 3.0 | 5.0 | 7.0 | 3.0 | 5.0 | 7.0 | 3.0 | 5.0 | 7.0 |
| Poloxamer-188 (gm) | 2.5 | 2.5 | 2.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 |
| Garcinia indica (gm) | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Carbopol® 940 (gm) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Stearic acid (gm) | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Cetyl alcohol (gm) | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Xanthan gum (gm) | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 |
| Tween-60 (gm) | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Triethanolamine (gm) | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 |
| Transcutol® (ml) | 2.5 | 2.5 | 2.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 |
| PEG-200 (ml) | 1.5 | 1.5 | 1.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Glycerol (ml) | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Aqua (q.s.) (w/w) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
was studied on the most critical attributes (for herbosomes) in finished product (topical formulation), i.e., Y1 (CL): In vitro drug permeation Q (µg/cm²) of *Curcuma longa*; Y1 (AM): In vitro drug permeation Q (µg/cm²) of *Arnica montana* Y2: Spreadability (mm). Tables 3, 4 and 5 summarize the experimental run, their coded value and corresponding actual value and their subsequent responses. The responses were calculated by using quadratic equation given below:

\[
y = \beta_0 + \beta_1 X + \beta_2 X^2 + \beta_3 X^3 + \beta_4 X_1 X_2 + \beta_5 X_1 X_3 + \beta_6 X_2 X_3 + \beta_7 X^1 Y + \beta_8 Y^2
\]

\[Y(\text{responses}) = b_0 + b_1 X + b_2 X^2 + b_3 X^3 + b_4 X Y + b_5 Y^2\]

**Characterization of novel herbosomes**

**Percentage entrapment efficiency (%EE) of novel herbosomes**

Percentage entrapment efficiency (%EE) of the designed herbosomes was quantified by analyzing the unentrapped drug remains in the supernatant, when exposed to a very high speed of centrifugation. Briefly, the prepared herbosomes were subjected to ultracentrifugation at 14,000 rpm at 4 °C for 15 min, using Thermo Scientific (SORVALL™ LEG- END™ MICRO 21R) centrifuge. The supernatant obtained was collected, and unentrapped amount of drug was quantified by using UV/Vis spectroscopy at 425 nm (*Curcuma longa*) and 345 nm (*Arnica montana*). Each formulation was analyzed in triplicate, and %EE was calculated by the formula given below:

\[
%\text{EE} = \frac{\text{Actual loaded drug} - \text{Free Drug (unentrapped drug)}}{\text{Actual loaded drug}} \times 100
\]

**Particle size, polydispersity and zeta potential of novel herbosomes**

A controlled and uniform particle size of the novel herbosomes is indispensable due to larger mass and complex structure of the NPs in comparison to synthetic drugs with precise molecular mass. Particle size (nm), polydispersity index (PDI) and zeta potential (mV) of the prepared novel herbosomes were characterized by using the principle of dynamic light scattering (DLS) with Malvern Zetasizer Nano ZS (Malvern Instrument, UK) at fixed angle of

**Table 3** Coded levels for experimental design

| Independent variables | Coded levels |
|-----------------------|--------------|
| Amount of drug (*Curcuma longa*: *Arnica montana*) mg | 75:50 -1 125:50 100:50 175:15 5.5:5 17:5 10.5:5 |
| Amount of lipid (*L*-α- Phosphatidylcholine: Cholesterol) mg | 100:15 125:15 175:15 3.0 5.0 7.0 |
| Amount of polymer (PEG-3350) gm | 3.0 5.0 7.0 |
| Amount of polymer (poloxamer-188) gm | 2.5 5.5 10.5 |
| Volume of penetration enhancer (Transcutol®:PEG-200) ml | 2.5:5 5.5:5 10.5:5 |

**Table 4** Optimization of n-herbosomes by RSM

| Batch Code | X1 | X2 | Y1 (CL) | Y1 (AM) | Y2 (nm) |
|------------|----|----|--------|--------|--------|
| n-H-01     | 0.00 | 0.00 | 59.84±1.39 | 60.86±1.02 | 240.1 |
| n-H-02     | -1.00 | 0.00 | 56.31±1.63 | 62.11±1.64 | 244.1 |
| n-H-03     | +1.00 | 0.00 | 74.14±0.26 | 66.17±1.02 | 278.0 |
| n-H-04     | 0.00 | -1.00 | 82.68±1.46 | 74.12±1.56 | 240.4 |
| n-H-05     | -1.00 | -1.00 | 89.68±1.44 | 79.11±1.11 | 260.2 |
| n-H-06     | +1.00 | -1.00 | 97.62±1.76 | 90.11±1.18 | 214.8 |
| n-H-07     | 0.00 | +1.00 | 87.14±1.44 | 77.14±1.74 | 285.5 |
| n-H-08     | -1.00 | +1.00 | 84.46±1.82 | 79.02±1.46 | 274.5 |
| n-H-09     | +1.00 | +1.00 | 82.80±1.14 | 77.79±1.14 | 280.1 |

Independent variables: X1 (Drug ratio), X2 (Polymer ratio)
Dependent variables: Y1 (CL) (%EE of *Curcuma longa*); Y1 (AM) (%EE of *Arnica montana*), Y2 (Particle size of n-herbosomes)

**Table 5** Optimization of n-HPTF by RSM

| Batch Code | X1 | X2 | Y1 (CL) | Y1 (AM) | Y2 (mm) |
|------------|----|----|--------|--------|--------|
| n-HPTF-01  | 0.00 | 0.00 | 0.14±0.07 | 0.29±0.08 | 7.0±0.07 |
| n-HPTF-02  | -1.00 | 0.00 | 0.12±0.12 | 0.29±0.17 | 7.7±0.07 |
| n-HPTF-03  | +1.00 | 0.00 | 0.69±0.18 | 0.33±0.09 | 6.1±0.01 |
| n-HPTF-04  | 0.00 | -1.00 | 0.22±1.12 | 0.22±0.14 | 5.0±0.14 |
| n-HPTF-05  | -1.00 | -1.00 | 0.94±0.08 | 0.39±0.12 | 11.1±0.12 |
| n-HPTF-06  | +1.00 | -1.00 | 1.14±1.10 | 0.42±0.14 | 10.1±0.01 |
| n-HPTF-07  | 0.00 | +1.00 | 0.29±0.09 | 0.26±0.14 | 5.2±0.01 |
| n-HPTF-08  | -1.00 | +1.00 | 0.45±0.14 | 0.28±0.02 | 4.1±0.1 |
| n-HPTF-09  | +1.00 | +1.00 | 0.86±0.01 | 0.39±0.17 | 4.5±0.01 |

Independent variables: X1 (polymer ratio), X2 (penetration enhancer ratio)
Dependent variables: Y1 (CL) (in vitro drug permeation Q of *Curcuma longa* in mg/cm²), Y1 (AM) (in vitro drug permeation Q of *Arnica montana* in mg/cm²), Y2 (Spreadability (mm))
90° at room temperature (25 °C) and standard experimental condition. Sample was diluted with de-ionized water before carrying out the measurement and repeated trice for each sample at 15 cycle run. PDI is another characteristic of the herbsomes, a narrow range of polydispersity index (PDI) reveals about its homogeneity. Zeta potential reveals a charge (+ive/-ive) present over the surface of the particles, which signifies the stability of the particles upon long-term storage (4 °C). Theoretically, best range of zeta potential for vesicle (s) is defined above the value of +30 mV or -30 mV [48].

Characterization of optimized n-HPTF

Physical evaluation

Optimized n-HPTF-06 was physically evaluated for their color, homogeneity and apparent phase separation upon long-term storage (30 °C ± 2 °C) vis-a-vis the initial formulation phase.

pH of optimized n-HPTF

pH of optimized n-HPTF-06 was measured by using digital pH meter Thermo Scientific (EUTECH Cyberscan PC 300, pH/conductivity/TDS/°C/°F meter). Briefly, 5 gm of optimized n-HPTF was placed in a clean beaker and allowed to equilibrate for 15 min. after keeping the reference electrode in the beaker. The average pH value represents the value three different measurements.

Rheological behavior

To assess the rheological property of designed nine formulations (n-HPTF-01 to n-HPTF-09), a digital viscometer (make ATAGO, VISCO™-895 Package B) at room temperature (25 °C) was used. Briefly, 25 gm of the n-HPTF was placed in a narrow head beaker and allowed to equilibrate for 15 min before recording the dial reading. Shear rate (sec⁻¹) for the optimized formulation was recorded in triplicate, and average value was plotted against viscosity (η) [50].

Spreadability

Spreadability is another critical rheological parameter based on “slip” and “drag” characteristics of the optimized formulation. Spreadability of n-HPTF-06 was measured by using a modified spreadability apparatus [49]. Briefly, 1 gm of the optimized n-HPTF-06 was placed (in a sandwich form) on glass plate (upper) having measurement (L × B) 10 × 4 cm and was fixed with another glass plate (below) with same measurement and a graph paper embedded below it. Upper slide was fixed with thin string hooked with weighing pan along with a pulley. After that, a constant weight (increasing weight) was placed over the pan and sliding of upper glass plate with respect to weight was measured and repeated trice. This gradual increase in slide of glass plate with respect to weight was calculated as per the formula described below to estimate the spreadability [50] of n-HPTF.

\[ S = M \times L/t \]

where, M is the mass applied; L is the distance travelled by the glass plate with respect mass applied; and t is time in seconds (60 s) taken by glass plate to move L (cm) distance.

Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The ATR-FTIR spectra of optimized n-H-06 and its individual components, viz. (Curcuma longa, Arnica montana, L-α-phosphatidylcholine, cholesterol, stearic acid as well as optimized n-HPTF-06, and its composition like (PEG-3350, poloxamer-188, Garcinia indica, cetyl alcohol, stearic acid and xanthan gum, Aloe barbadensis, Azardichca indica, Camellia sinensis, Glycyrrhiza glabra, Calendula officinalis, pro-vitamin B5, acryl 10/30, PEG-200, Tween-60, Transcutol®, glycerol and triethanolamine were analyzed by using ATR-FTIR spectroscopy (PerkinElmer FTIR Spectrum Two, UATR Two) equipped with a Dynascan™ interferometer with a set of LiTaO₃ (lithium tantalate) MIR detector that gives value in the range of 4000 cm⁻¹ to 450 cm⁻¹ at best resolution of 0.5 cm⁻¹ at room temperature (25 ± 1 °C). Peak fitting and analysis were done by in-built software “Spectrum” (Perkin Elmer IR Version 10.6.2),

In vitro drug permeation (Q)

In vitro drug permeation of the optimized n-HPTF-06 was calculated by using Franz’s diffusion cell (Orchid scientific six station diffusion cell, EMFDC 06, India) having 2.5 cm² orifice diameter (total drug diffusion area). Briefly, pre-treated cellophane membrane (12,000–14,000 kDa) in 0.1 M HCl was used as a semi-permeable skin membrane. Weighed accurately, 0.5 gm of the formulation (n = 6) was applied over membrane and mounted on the Franz’s diffusion cell’s mouth (between donor and receptor compartment). Further receptor compartment was filled with phosphate buffer saline pH 6.8 (12 mL) and 10% ethanol to maintain sink condition at a stirring rate of 270 rpm at a set temperature of 37 ± 1 °C maintained by continuous flow of water inside water jacket in the equipment. Samples were withdrawn by using injection equipped with instrument at 0, 0.5, 1, 2, 4, 6, 8 and 24 h.
and replaced by buffer to maintain a constant volume for release of drug throughout the process. The samples were filtered by using 0.45 µm syringe filter and reading observed using UV/Vis spectroscopy at 286.0 nm (Aloe barbadensis), 289.0 nm (Azadirachta indica), 425.0 nm (Curcuma longa), 302 nm (Camellia sinensis), 333.0 nm (Glycyrrhiza glabra), 345.0 nm (Arnica montana) and 287.0 nm (Calendula officinalis). In vitro drug permeation rate was calculated by estimating the cumulative amount of drug released through a semi-permeable membrane from a specified surface area with vis-a-vis given time and represented graphically as $Q$ (µg/cm²) versus time (h) [51–55].

### Kinetic modeling

To study the kinetics of drug release from optimized n-HPTF-06, a set of mathematical release kinetic model, viz. zero-order kinetics (Eq. 1); first-order kinetics (Eq. 2); Higuchi model (Eq. 3); and Korsmeyer–Peppas model (Eq. 4), was employed to assess the release of molecule from polymeric core/ matrix to the physiological buffer media [56–61].

$$Q_t = Q_0 + k_0 t$$  \hspace{1cm} (1)

$$\log Q_t = \log Q_0 - (k_1/2.303) \times t$$  \hspace{1cm} (2)

$$Q_t = k_H t^{1/2}$$  \hspace{1cm} (3)

$$M_t/M_\infty = k_f t^n$$  \hspace{1cm} (4)

where, in Eq. 1, $Q_t$ is the amount of drug present at absorption site at time $t$; $Q_0$ is the initial amount of drug present in the buffer solution or physiological media and in most of the cases this remain zero; $k_0$ is the constant for zero order kinetics.

In Eq. 2, $Q_t$ is the amount of drug dissolved at time $t$; $Q_0$ is the initial amount of drug present in the buffer solution or physiological media; and $k_1$ is constant for first order kinetics.

In Eq. 3, $Q_t$ is the amount of drug present in the media released from core/matrix of the polymer at given time $t$; and $k_H$ is the constant for Higuchi model [59].

In Eq. 4, $M_t/M_\infty$ represents the fraction of drug released at time $t$, $k_f$ is the release constant which is characteristics to the polymer–drug interactions, and power $n$ is the diffusion exponent characteristic to the release mechanism [60].

When (i) $n = 0.5$, equation becomes equal to Higuchi model indicating that release mechanism is of Fickian type of release, (ii) $n = 0.5$ to 1.0, suggested release corresponds to an anomalous, i.e., non-Fickian type of drug transport to the release media, (iii) $n = 1.0$, indicates release kinetics of drug is of zero-order kinetics, (iv) $n > 1.0$ suggested that release of drug is process dependent which rely on the number of polymeric chains in the given drug matrix [56].

### Stability study

Stability study of the optimized n-HPTF was performed by using ICH guidelines [ICH Q1 A (R2)] for long-term stability study of the topical pharmaceuticals for a given period of six months at temperature (5 °C ± 3 °C and 30 °C ± 2 °C) and percentage relative humidity (% RH) (65% ± 5%) using stability chamber (M.K. Scientific instruments, M.K SI-142-B, India) [63]. The formulation was observed subsequently at 0, 30, 60, 90, 120 and 180 days for color, texture, pH, spreadability and phase separation (if any) and furthermore evaluated by using ATR-FTIR spectroscopy for any physicochemical incompatibility after long-term stability study.

### In vivo study

#### Experimental animals

Throughout the study protocol, animal handling was performed as per the prescribed guidelines given by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) and the experiments were approved by the IAEC (Institutional Animal Ethical Committee) of Defence Institute of Physiology and Allied Sciences (DIPAS), DRDO, India, with approval number DIPAS/IAEC/2019/Dec-03. Animals were acclimatized prior to study for 7 days before carrying out the experiments under standard laboratory conditions (25 ± 1 °C); 12-h light/dark cycle; the animals received water and food ad libitum.

#### Skin irritancy study of optimized n-HPTF

Skin irritancy study of the optimized n-HPTF-06 was performed by selecting healthy young adult female Sprague–Dawley rats (nulliparous and non-pregnant), weighing 174–180 gm. In this study, female rats were selected in view of their high sensitivity to any chemical/other treatment [64]. The rats were acclimatized after grouping for at least 7 days before performing the experiments. Approximately 24 h before carrying out experiments, animals were anesthetized by administering a cocktail of Ketamine (90 mg/kg B.W.) and Xylazine (9.1 mg/kg B.W.) and dorsal hair of the rats was shaved by using electrical trimmer. Acute dermal toxicity of n-HPTF was performed as per prescribed OECD guidelines for acute dermal toxicity (402) [65]. Briefly, 1 gm of the optimized n-HPTF was applied over the dorsal skin of the rats (4 × 2.5 cm²) and observed for any visible changes such as erythema at 1 h, 6 h, 24 h and 72 h after application. The mean erythema scores were
recorded by using range between 0–4 at arbitrary scale depending upon the degree of erythema after application categories as: (i) no erythema = 0; (ii) slight erythema (barley, light pink skin) = 1; (iii) moderate erythema (dark pink) = 2; (iv) moderate to severe erythema (dark pink) = 3; and (v) severe erythema (extreme redness) = 4 scores.

**Preparation of skin before induction of frostbite**

Before induction of frostbite injury on the skin of the rats, skin preparation was carried out to suitably perform the experiments. Twenty-four hours before the experiment, rats were individually anaesthetized by using a cocktail of Ketamine (90 mg/kg B.W., i.m.) and Xylazine (9.1 mg/kg B.W., i.m.). Thereafter, the dorsal skin of the rats, covering from basal region of neck to the rear haunches, was shaved using trimmer (WAHL® Battery Trimmer), whereas small hair left behind on the skin were not further removed by application of any hair remover to mimic the actual skin condition (as hair appendages can act as a skin barrier to topical drug delivery system) [66]. After trimming, the skin was gently wiped with isopropyl alcohol and the animals were housed again in cages.

**Induction and validation of frostbite model using Sprague–Dawley rats**

Induction of frostbite on the skin of rats was carried out by the method previously described by Auerbach et al. [66] with slight modification. Briefly, after twenty-four hours of skin preparations, rats were anesthetized by using a cocktail of Ketamine (90 mg/kg B.W., i.m.) and Xylazine (9.1 mg/kg B.W., i.m.) and frostbite was created by using Ferrite magnet having diameter 1.29 cm, thickness 0.56 cm and weight 2.9 gm. Two magnets of the same diameter were placed in crushed dry ice (-80 ° ± 2 °C) for fifteen minutes and then placed on the skin of the rats by slightly sliding skin in upward direction for a duration of 1 min. The magnet was then removed and the skin was allowed to thaw for 5 min and the freeze-thawed cycle was repeated thrice with a freezing cycle interval of 5 min. Within two minutes of frostbite induction, skin of the animal appears bluish in color, which is an indication of the first sign of frostbite, i.e., cold injury. The model was further validated by continuous observation for change in skin color, which was followed up to 3 days, and it was observed that the exposed skin was completely darkened.

**Study design and dosing**

The experimental design constituted of grouping of the animals in subcategories for a comparative analysis of therapeutic efficacy of n-HPTF with a standard formulation. The animals were grouped into three groups (n = 12 each), viz. (i) Group I: Frostbitten (untreated); (ii) Group II: Frostbitten + Standard formulation (ALOCAL); (iii) Group III: Frostbitten + treatment (n-HPTF). About 0.5 gm of the standard drug and formulation were applied to the animals daily based on the groups assigned. The animal tissue was harvested (n = 2) at 0, 3, 7, 14 and 21 days for histopathological examination, as well as for the biochemical assays and marker-based studies.

**Morphometric analysis of frostbitten wound area**

Following induction of cold injury exposure achieved by ferrite magnet pre-frozen in dry ice, morphometric analysis aimed at studying the diameter of the darkened skin with and without treatment. In skin wound condition, a precise wound appears after exposure to validated wound model, but in case of cold injury the diameter of affected area is directly proportional to the cytokine response and body’s pathophysiological response. Thus, it becomes necessary to carry out morphometric analysis on a routine basis to assess the changes in the different treatment groups and control groups. This analysis was carried out by the method described by Vaghasiya et al. [67], where routine measurement of the frostbitten area was done with the help of Vernier caliper until complete healing of the cold-injured area was accomplished. Percentage area (contracted wound) was calculated by the formula given below:

\[
\text{Frostbitten Wound Contraction (\%) = \left( \frac{\text{Initial frostbitten area} - \text{Frostbitten area on a specific day}}{\text{Initial wound area}} \right) \times 100}
\]

**Histopathological study**

The histopathological observation was made by harvesting the animals (n = 2) 0, 3, 7, 14 and 21 days under euthanasia condition, by using high dose of anesthesia (Ketamine HCL/ Xylazin, 100 mg/mL). The cold injured skin tissue was wiped with sterile swabs and then excised and the affected area was washed with phosphate saline buffer (pH 7.4) and further fixed in 10% buffer formalin solution. For histological studies, the excised formalin-fixed tissue was embedded in paraffin blocks by opting the routine tissue embedding procedure. Briefly, the fixed tissue sample was dehydrated first by using graded concentration of ethanol and then in xylene and further embedded to the pre-fixed paraffin blocks. The section was cut to a thickness of 5 μm by using standard microtome. After obtaining the tissue sections, it was rehydrated before following the hematoxylin and eosin (H&E)
staining protocol. The stained tissue was further observed under optical microscope (LMi EA #LM061, Fisher Scientific, USA).

**Results and discussion**

Pharmaceuticals based on natural plant products (NPPs) are quite complex as compared to the synthetic ones because finished product quality is dependent on the source and nature of the material. Thus, during selection and processing procedure of the herbal ingredients, validation of each step is required for obtaining maximum yield and quality product [68].

**Homogenizer-assisted extraction of herbals**

Homogenizer-assisted extraction technique was used to extract the herbals used in the development of the n-HPTF-06, and the percentage yield of the extracted herbals was as follows: *Aloe barbadensis* (leaf) (Aq.) whole extract: 200x; *Azadirachta indica* (leaf) (Aq.): 16.37%; *Curcuma longa* (rhizome) (Aq.; Ethanol, 50:50 v/v): 11.37%; *Camellia sinensis* (leaf) (Aq.): 11.33%; *Glycyrrhiza glabra* (stem) (Aq.; Ethanol, 30:70 v/v): 7.01%; *Arnica montana* (Aq.; Ethanol, 50:50 v/v): 9.26%; and *Calendula officinalis* (Aq.; Ethanol, 50:50 v/v): 15.22%. Selection of suitable extraction method is important to achieve maximum yield of active biomass possessing rich proportion of antioxidant properties [93]. Optimized condition of homogenizer-assisted extraction of herbals gives high yield of biologically active constituents in a very less time of extraction in comparison to other methods of extraction of herbals [24]; therefore we selected this method of extraction to extract maximum active biomass from the herbs in order to achieve a maximum range of total antioxidant capacities, elucidated by using different method assays, viz. DPPH, FRAP, ABTS, NO, TAC, etc.

**Attenuated total reflection-fourier transform infrared spectroscopy (ATR-FTIR)**

ATR-FTIR spectroscopy was used to characterize the prominent functional groups present in the extracted herbals, which can be further used as a quality control parameter for the final form of formulation. The spectra of all the NPPs are depicted in Fig. 1. Characteristic ATR-FTIR spectra of *Aloe barbadensis* were at 3932.14 cm⁻¹ showing sharp O–H free alcohol (stretching), 3851.00 cm⁻¹ wide O–H (stretching), 2360.69 cm⁻¹ due to O = C = O group (stretching), 1782.60 cm⁻¹ due to C = O anhydrous (stretching) and 1733.14 cm⁻¹ due to N–O nitro compound (stretching), and similar finding was elucidated by Bajer et al. [69]. In case of extract of *Azadirachta indica*, characteristic peaks were at 3744.51 cm⁻¹ showing medium O–H (stretching), 3113.13 cm⁻¹ weak O–H carboxylic group (stretching), 2368.58 cm⁻¹ strong C = O primary (free, stretching), 1557.07 cm⁻¹ due to N–O (stretching) and 1409.72 cm⁻¹ medium S = O (stretching). Similar spectra were depicted by the authors Sriplatha et al. [70]. In *Curcuma longa*, characteristic peaks were found at 3740.75 cm⁻¹ strong O–H, alcohol (stretching), 2425.99 & 2331.65 cm⁻¹ strong C = O–H (stretching), 1688.8 cm⁻¹ strong C-H aromatic alkyl (bending) and 1549.75 cm⁻¹ medium C = C cyclic alkenes (stretching), and similar spectral findings were reported by Rohman et al. [71]. In *Camellia sinensis*, the characteristic peaks were identified as 3226.44 cm⁻¹ low O–H (stretching), 2967.94 cm⁻¹ C-H (stretching), 1605.53 cm⁻¹ α-β-unsaturated ketone (stretching), 1236.66 cm⁻¹ C-O alkyl aryl ether (stretching), 1144.55 cm⁻¹ C-O alcohol (stretching) and 1035.89 cm⁻¹ CO–O-CO (stretching), and similar spectral analysis results were shown by Senthilkumar et al. [72]. In *Glycyrrhiza glabra* characteristic peaks lie at 3743.63 cm⁻¹ medium O–H free alcohol (stretching), 3546.22 cm⁻¹ strong O–H intermolecular bonded alcohol (stretching), 1520.55 strong N-O nitro compound (stretching), 1416.60 cm⁻¹ O–H (bending), 1228.99 cm⁻¹ strong C-O aromatic (stretching) and 1040.50 cm⁻¹ strong CO–O-CO anhydride (stretching), and similar FTIR spectra of *G. glabra* were depicted by Kumari et al. [73]. In *Arnica montana*, the characteristic peaks were found at 3326.71 cm⁻¹ medium O–H (stretching), 2977.36 cm⁻¹ strong C-H aldehyde (stretching), 1647.69 cm⁻¹ strong C = C monosubstituted alkene (stretching), 1387.42 cm⁻¹ medium C-H Gem dimethyl (bending), 1325.94 cm⁻¹ O–H phenol (bending) and 1085.17 cm⁻¹ strong C-H (stretching); similar findings were reported by Purkait et al. [14]. In *Calendula officinalis* prominent peaks were identified at 3276.90 cm⁻¹ strong O–H (stretching), 2980.22 cm⁻¹ weak O–H (stretching), 2133.41 cm⁻¹ strong C = C = O ketone (stretching), 1645.58 cm⁻¹ C = O δ-lactone (stretching), 1416.82 cm⁻¹ medium O–H (bending), 1326.60 cm⁻¹ O–H phenol (bending), 1084.77 cm⁻¹ strong C-O (stretching), 1043.04 cm⁻¹ strong CO–O–CO anhydride (stretching) and 871.77 cm⁻¹ strong C-H 1,3-disubstituted (bending). Similar spectral analysis was delineated by Al-Mussawi et al. [12].

**Determination of total polyphenolic content and total flavonoid content**

Total polyphenolic content and total flavonoid content of the extracted herbals were quantified by using Folin-Ciocalteu reagent (FCR) and aluminum chloride method, respectively, analyzed by UV/Vis spectrophotometrically. Total polyphenolic compounds at 0.1 mg/mL of *Aloe
barbadensis (leaf) (Aq.); Azadirachta indica (leaf) (Aq.); Curcuma longa (rhizome) (Aq.: Ethanol, 50:50 v/v); Camellia sinensis (leaf) (Aq.); Glycyrrhiza glabra (stem) (Aq.: Ethanol, 30:70 v/v); Arnica montana (Aq.: Ethanol, 50:50 v/v); and Calendula officinalis Aq.: Ethanol, 50:50 v/v) were found to be 2.43 ± 0.04, 2.26 ± 0.21, 2.85 ± 0.22, 27.05 ± 0.06, 15.44 ± 0.15, 2.78 ± 0.11 and 11.18 ± 0.11 GAE of g/DWE, respectively. Total flavonoid content of Aloe barbadensis (leaf) (Aq.); Azadirachta indica (leaf) (Aq.); Curcuma longa (rhizome) (Aq.: Ethanol, 50:50 v/v); Camellia sinensis (leaf) (Aq.); Glycyrrhiza glabra (stem) (Aq.: Ethanol, 30:70 v/v); Arnica montana (Aq.: Ethanol, 50:50 v/v); and Calendula officinalis Aq.: Ethanol, 50:50 v/v) was 6.66 ± 0.03, 7.30 ± 0.02, 2.83 ± 0.01, 52.50 ± 0.04, 46.41 ± 0.03, 7.12 ± 0.11 and 14.72 ± 0.07 RTE of g/DWE, respectively. The maximum content of both polyphenolic and flavonoid groups was present in Camellia sinensis and Glycyrrhiza glabra extracts. Results were statistically analyzed by employing one-way ANOVA using Tukey’s multiple comparison test (Table 6).
In vitro antioxidant potential study

Pathophysiology of cold injury includes the sequelae of inflammatory pathways (cytokines mediators), oxidative stress (reactive oxygen species, superoxide and nitric oxide radicals), tissue necrosis, thrombosis and progression of which leads to amputation [92]. Active phytoconstituents of (i) Aloe barbadensis (leaf) (polysaccharides and flavonoid) [74], (ii) Azadirachta indica (leaf) (nimboide, azadirachtin, asorbate) [65], (iii) Curcuma longa (rhizome) (curcumin) [75], (iv) Camellia sinensis (leaf) ((-)epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC) epicatechin-3-gallate (ECG) and epicatechin (EC) [76], (v) Glycyrrhiza glabra (stem) (hispaglabridin B, isoliquiritigenin, isoflavone, glabridin, etc.) [77] (vi) Arnica montana (flower) (helanalin, flavonoids, chlorogenic acid, caffeic acid, apigenin, etc.) [78] and (vii) Calendula officinalis (flower) (flavonoids and phenolic acids) [79] are known to possess potent antioxidant properties, and were selected to minimize the effect of oxidative stress mediators species, viz. reactive oxygen species, superoxide and nitric oxide free radicals. Numerous studies conducted over the years have shown the immense potential of medicinal plants playing a role in acceleration of wound healing by their potent antioxidant compounds such as flavonoids and phenolic acid. In this study individual antioxidant potential of the herbals was determined via different assays methods, viz. DPPH, FRAP, ABTS, NO and TAC. The results of each components vis-a-vis antioxidant assays method are explained in subsequent paragraphs.

Determination DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

DPPH free radical scavenging activity of the extracted herbs was performed by using DPPH assay. Percentage inhibition of each extract was calculated and plotted against the tested concentrations (0.5, 1.0, 2, 4, 6, 8 and 10 mg/mL) and then IC_{50} value of each extract was calculated from graph as represented in Table 7. IC_{50} value of extracts, viz. Aloe barbadensis (leaf) (Aq.); Azadirachta indica (leaf) (Aq.); Curcuma longa (rhizome) (Aq.); Ethanol, 50:50 v/v); Camellia sinensis (leaf) (Aq.); Glycyrrhiza glabra (stem) (Aq.: Ethanol, 30:70 v/v); Arnica montana (Aq.: Ethanol, 50:50 v/v); and Calendula officinalis (Aq.: Ethanol, 50:50 v/v) was reported to be 1.11 ± 0.16, 2.14 ± 0.11, 1.72 ± 0.17, 7.81 ± 0.14, 2.56 ± 0.11, 2.23 ± 0.17 and 0.93 ± 0.02, respectively. Results were statistically analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. The order of free radical DPPH scavenging activity of the extract was: Camellia sinensis > Glycyrrhiza glabra > Arnica montana > Azadirachta indica > Curcuma longa > Aloe barbadensis > Calendula officinalis.

Determination of FRAP (ferric ion reducing antioxidant power) activity

The FRAP reducing activity of the extracted NPPs was performed and expressed in Fe (II) E/g of DWE as depicted in Table 7. FRAP reducing activity of Aloe barbadensis (leaf) (Aq.); Azadirachta indica (leaf) (Aq.); Curcuma longa (rhizome) (Aq.); Ethanol, 50:50 v/v); Camellia sinensis (leaf) (Aq.); Glycyrrhiza glabra (stem) (Aq.: Ethanol, 30:70 v/v); Arnica montana (Aq.: Ethanol, 50:50 v/v); and Calendula officinalis (Aq.: Ethanol, 50:50 v/v) was found to be 15.11 ± 0.28, 8.31 ± 0.01, 17.92 ± 0.05, 168.03 ± 0.33, 19.47 ± 0.16, 14.44 ± 0.12 and 11.12 ± 0.22 Fe (II)E/g of DWE, respectively. Results were statistically analyzed by one-way ANOVA, followed by Tukey’s multiple comparison test. The order of free radical FRAP activity reported

Table 6 Total Polyphenolic content and Total Flavonoid content in the natural plant products (NPPs)

| Parameters                      | Aloe bar badensis (Aq.) | Azadirachta indica (Aq.) | Curcuma longa (Aq.: Ethanol, 50:50 v/v) | Camellia sinensis (Aq.) | Glycyrrhiza glabra (Aq.: Ethanol, 30:70 v/v) | Arnica montana (Aq.: Ethanol, 50:50 v/v) | Calendula officinalis (Aq.: Ethanol, 50:50 v/v) |
|--------------------------------|------------------------|--------------------------|-----------------------------------------|-------------------------|-----------------------------------------------|------------------------------------------|-----------------------------------------------|
| Total Polyphenolic content (mg GAE/g of DWE) | 2.43 ± 0.04***          | 2.26 ± 0.21***           | 2.85 ± 0.22***                          | 27.05 ± 0.06***         | 15.44 ± 0.15***                               | 2.78 ± 0.11***                           | 11.18 ± 0.11***                               |
| Total Flavonoid content (mg RTE/g of DWE)     | 6.66 ± 0.03***          | 7.30 ± 0.02***           | 2.83 ± 0.01***                          | 52.50 ± 0.04***         | 46.41 ± 0.03***                               | 7.12 ± 0.11***                           | 14.72 ± 0.07***                               |

Total polyphenolic content and total flavonoid content of the extracted herbals are expressed as the mean ± SEM (n = 3) by standards as GAE, i.e., gallic acid equivalent, and RTE, i.e., Rutin trihydrate equivalent capacity of DWE, i.e., Dried weight of the Extract.

One-way ANOVA using Tukey’s multiple comparison test statistic was employed p value as: p < 0.001(*); p < 0.01(**); p < 0.05 (***)

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In vitro antioxidant potential of the homogenizer-assisted extracted natural plant products (NPPs)

| Parameters | Aloe barbadensis (Aq.) | Azadirachta indica (Aq.) | Curcuma longa (Aq.: Ethanol, 50:50 v/v) | Camellia sinensis (Aq.) | Glycyrrhiza glabra (Aq.: Ethanol, 30:70 v/v) | Arnica montana (Aq.: Ethanol, 50:50 v/v) | Calendula officinalis Aq.: Ethanol, 50:50 v/v |
|------------|------------------------|--------------------------|----------------------------------------|------------------------|-------------------------------------------|----------------------------------------|------------------------------------------|
| DPPH (IC₅₀) | 1.11 ± 0.16**          | 2.14 ± 0.11***          | 1.72 ± 0.17***                        | 7.81 ± 0.14***         | 2.56 ± 0.11***                            | 2.33 ± 0.17**                          | 0.93 ± 0.02**                             |
| FRAP (mg Fe (II) E/g of DWE) | 15.11 ± 0.28**        | 8.31 ± 0.01**           | 17.92 ± 0.05**                       | 168.03 ± 0.33**       | 19.47 ± 0.16**                            | 14.44 ± 0.12**                         | 11.12 ± 0.22**                            |
| ABTS (µg TEAC/g of DWE) | 14.23 ± 0.11***       | 1.26 ± 0.17*           | 0.89 ± 0.14*                         | 2.75 ± 0.16*         | 3.14 ± 2.14*                             | 2.14 ± 0.07*                           | 1.18 ± 0.11*                             |
| NO (%I) | 38.43**               | 78.00%*                 | 58.80%**                              | 62.14%*               | 66.14%*                                   | 54.12%**                               | 49.46%**                                 |
| TAC (mg AAE/g of DWE) | 4.23 ± 0.01*          | 8.12 ± 0.17*           | 4.15 ± 0.17*                         | 7.14 ± 0.16*         | 4.71 ± 0.01*                             | 1.12 ± 0.14*                           | 0.77 ± 0.01**                            |

In vitro antioxidant capacities of the extracted herals are expressed as the mean ±SEM (n = 3) evaluated by DPPH (2, 2-diphenyl-1-picyrhydrazyl), FRAP (Ferric Reducing Antioxidant Power), ABTS (2, 2'-azio-bis(3-ethylbenzothiazoline-6-sulfonic acid), NO (Nitric oxide) and TAC (Total Antioxidant Capacity), where TEAC is Trolox equivalent antioxidant capacity, AAE is Ascorbic acid equivalent antioxidant capacity and DWE is Dried weight of the Extract

One-way ANOVA using Tukey’s multiple comparison test statistic was employed

$p$ value: $p < 0.001(*)$; $p < 0.01(**)$; $p < 0.05 (***);$ ns (non-significant)

was: *Camellia sinensis > Glycyrrhiza glabra > Curcuma longa > Aloe barbadensis > Arnica montana > Calendula officinalis > Azadirachta indica.*

**Determination of ABTS (2, 2’-amino-bis (3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging activity**

Trolox equivalent activity of the extracted herals was determined by using ABTS assays method. ABTS free radical scavenging activity of the NPPs extracts, viz. *Aloe barbadensis* (leaf) (Aq.); Azadirachta indica (leaf) (Aq.); Curcuma longa (rhizome) (Aq.: Ethanol, 50:50 v/v); Camellia sinensis (leaf) (Aq.); Glycyrrhiza glabra (stem) (Aq.: Ethanol, 30:70 v/v); Arnica montana (Aq.: Ethanol, 50:50 v/v); and Calendula officinalis (Aq.: Ethanol, 50:50 v/v), was found to be 14.23 ± 0.11, 1.26 ± 0.17, 0.89 ± 0.14, 2.75 ± 0.016, 3.14 ± 0.14, 2.14 ± 0.07 and 1.18 ± 0.11 µg TEAC/g of DWE, respectively. Results were statistically analyzed by employing one-way ANOVA, using Tukey’s multiple comparison test. The order of ABTS scavenging activity was: *Aloe barbadensis > Glycyrrhiza glabra > Camellia sinensis > Arnica montana > Azadirachta indica > Calendula officinalis > Curcuma longa.*

**Determination of NO (Nitric oxide) scavenging potential activity**

Nitric oxide scavenging potential activity of the extracted NPPs was performed by using NO method and percentage inhibition of nitric oxide free radical by given extract was expressed as capacity of inhibition as depicted in Table 7 The percentage inhibition of the NO by extracts was

**Determination of total antioxidant capacity (TAC)**

Total antioxidant capacity of the extracted NPPs was performed by phosphomolybdenum complex formation method. Herbal extracts, viz. *Aloe barbadensis* (leaf) (Aq.); Azadirachta indica (leaf) (Aq.); Curcuma longa (rhizome) (Aq.: Ethanol, 50:50 v/v); Camellia sinensis (leaf) (Aq.); Glycyrrhiza glabra (stem) (Aq.: Ethanol, 30:70 v/v); Arnica montana (Aq.: Ethanol, 50:50 v/v); and Calendula officinalis (Aq.: Ethanol, 50:50 v/v), showed TAC as 4.23 ± 0.01, 8.12 ± 0.17, 4.15 ± 0.17, 7.14 ± 0.16, 4.71 ± 0.01, 1.12 ± 0.14 and 0.77 ± 0.01 mg AAE/g of DWE, respectively. Results were statistically analyzed by one-way ANOVA using Tukey’s multiple comparison test and are depicted in Table 7. The order of
TAC activity was as follows: *Azardchata indica* > *Camellia sinensis* > *Glycyrrhiza glabra* > *Aloe barbdensis* > *Curcuma longa* > *Arnica montana* > *Calendula officinalis*.

In case of cold injury, potent antioxidant activity of the herbal ingredients is necessarily required to inhibit the various pro-inflammatory cytokines mediators, superoxide and reactive oxygen species (ROS); hence, it becomes important to consider and ensure that the critical ingredients in the topical formulation for skin frostbite prophylaxis and therapeutics are enriched in antioxidative, anti-inflammatory and regenerative properties [10, 11].

**Preparation of novel herbosomes (n-herbosomes)**

Preparation of n-herbosomes was carried out by method by thin film hydration method in different batches with ingredients described in Table 1 and later on optimized by using response surface methodology using Design of experiment (DoE) subsequently optimized by statistical models. Based on this, n–H-06 was optimized as n-herbosomes incorporated in topical formulation (n‑HPTF) and n-HPTF-06 as the final formulation.

**Characterization of novel herbosomes**

Percentage entrapment efficiency (% EE) of n-herbosomes

Percentage entrapment efficiency of both entrapped drug *Curcuma longa* and *Arnica montana* was estimated and is depicted in Table 4. The order of entrapped drug *Curcuma longa* was: n–H-06 > n–H-05 > n–H-07 > n–H-08 > n–H-09 > n–H-04 > n–H-03 > n–H-01 > n–H-02, respectively; order of entrapped drug *Arnica montana* was: n–H-06 > n–H-05 > n–H-08 > n–H-09 > n–H-07 > n–H-04 > n–H-03 > n–H-02 > n–H-01, respectively. Similar entrapment efficiency of one of the components of *Curcuma longa* that is curcumin showed > 80% entrapment efficiency when incorporated into liposomes Ng et al. [80].

**Particle size, polydispersity and zeta potential of n-herbosomes**

The mean vesicle size (nm), size distribution (PDI) and zeta potential (mV) are essential parameters to characterize for vesiculosomes like herbosomes, liposomes, cubosomes,
Fig. 2 Response surface methodology for optimization of n-herbosmes and n-HPTF: (A) % EE (CL), (B) %EE (AM) (C) PS (nm), (D) in vitro drug permeation (CL), (E) in vitro drug permeation (AM) and (F) spreadability.
Fig. 2 (continued)
etc. In this study, the n-herbosomes were characterized for their particle size (nm) and found to be in the range between 214.8 nm to 280.1 nm as depicted in Table 4 and Fig. 3, whereas polydispersity lies in between the range of 0.299 to 0.387 and zeta potential of all formulations was found to be +26 mV revealing formation of stable herbosomes. The positive charge on zeta potential was due to stearic acid, which is a surfactant that maintains the stability of the herbosomes and was taken as constant in all the formulations, hence measured for the optimized one, i.e., n–H-06 as shown in Fig. 3. To deliver the drug to the deeper dermis layer, the optimum particle size should be ≤300 nm [81, 82] and our optimized particle size was within this range; hence, the herbosomes could reach the target site with more receptor binding affinity.

**Characterization of optimized n-HPTF**

**Physical evaluation**

Physical evaluation of the optimized n-HPTF-06 was done by observing color, homogeneity, apparent phase separation upon long-term storage at room temperature (25 °C ± 1 °C) and cold storage condition (4 °C ± 1 °C). No color change was observed at tested temperature, which was further evaluated in stability chamber.

**pH of optimized n-HPTF**

Physiological pH is a very important parameter to understand the release of drug in a unionized form. Hence, it is very important to design the formulation within the range of target site of absorption of drug. pH of the optimized formulation n-HPTF-06 was evaluated by using a digital pH meter and it was found to be 6.5 ± 0.11 (n = 3). pH of the topicals can widely affect the permeation and absorption of the drug. The study suggested that an optimal pH of 6–7 was appropriate to increase the permeability through dermal layer [83, 84].

**Rheological behavior**

All the formulations (n-HPTF-01 to n-HPTF-09) were to assessed their for rheological behavior by using ATGO
digital viscometer. The rheological behavior of all the formulations were found satisfactory and consistent. It was observed that formulation having high amount of polymeric ratio, i.e., from n-HPTF-07 to n-HPTF-09, possessed slightly more shear stress with respect to spinning of spindle at a given time. The rheological behavior of the all formulations tested is depicted in Fig. 4 showing viscosity (η) with respect to the shear rate (sec⁻¹) applied.

### Spreadability

Spreadability of the n-HPTF was evaluated by the method described earlier and is depicted in Table 5. It was observed that upon increasing the concentration of PEG-3350 (from 3.0 gm to 7.0 gm) and poloxamer-188 (from 2.5 gm to 10.5 gm), the spreadability of the formulation decreased and remained more consistent. Spreadability is a critical sensory parameter that is highly dependent upon the vehicles and humectant used in the formulation [88] and middle range of combination of PEG-3350: poloxamer-188 (5.0: 5.5 gm) was found to be the suitable range for ease-of-application (spreadability) i.e., 11.1 ± 0.12 mm in 60 s.

### Attenuated total reflection-fourier transform infrared spectroscopy (ATR-FTIR)

The ATR-FTIR spectra of the optimized n–H-06 and n-HPTF-06 were characterized vis-a-vis the individual ingredients and their characteristic peaks and overlay spectra of the optimized formulation obtained with ingredients are depicted in Fig. 5. The characteristic spectra of L-α-phosphatidylcholine showed prominent peaks at 3281.81 cm⁻¹ broad O–H alcohol (stretching), 3010.32 cm⁻¹ medium C-H (stretching), 2922.12 cm⁻¹ medium C-H (stretching), 1737.28 cm⁻¹ strong C + O (stretching), 1618.99 cm⁻¹ strong C + C (stretching), 1464.98 cm⁻¹ medium C-H (bending), 1053.54 cm⁻¹ strong C-O (stretching) and 822.29 cm⁻¹ medium C-H (bending). Cholesterol showed their characteristic peaks at 3431.97 cm⁻¹ broad O–H (stretching), 2929.85 cm⁻¹ medium O–H (stretching), 2866.39 cm⁻¹ doublet C-H (stretching), 1670.7 cm⁻¹ weak C = C (bending) and 1463.98 cm⁻¹ weak O–H (bending), and similar finding was observed by Dave et al. [39]. The optimized n-herbosomes (n–H-06) showed prominent peaks at 3350.91 cm⁻¹ broad O–H (stretching), 2929.86 cm⁻¹ medium O–H (stretching), 2978.15 cm⁻¹ intermolecular O–H (stretching), 1643.33 cm⁻¹ strong C = C (stretching), 1044.31 cm⁻¹ strong CO–O–CO (stretching) and 879.13 cm⁻¹ strong C-H (bending). Formulation base like PEG-3350 showed characteristics peaks at 3425.32 cm⁻¹ broad O–H (stretching), 2860.61 cm⁻¹ medium C-H (stretching), 2694.78 cm⁻¹ medium C-H (stretching), 1340.99 cm⁻¹ medium C-H (bending) and 1097.67 cm⁻¹ strong C-O aliphatic alcohol (stretching) Shameli et al. [85]. Poloxamer (P-188) showed characteristics peaks at 2969.93 cm⁻¹ medium C-H (stretching), 2882.50 strong C-H (stretching), 2953.46 cm⁻¹ medium C-H (bending), 1100.81 cm⁻¹ strong C-O (stretching) and 946.98 cm⁻¹ monosubstituted C = C (bending), and similar finding was observed by Manikandan et al. [86]. Garcinia indica showed prominent peak at 2953.46 cm⁻¹ medium C-H (stretching), 2914.57 cm⁻¹ alkene C-H (stretching), 2849.33 cm⁻¹ alkane C-H (stretching), 1730.08 cm⁻¹ ester C = O (stretching) and 1174.39 cm⁻¹ medium C-O (stretching). Cetyl alcohol showed characteristic peaks at 3271.03 cm⁻¹ broad O–H (stretching), 2916.33 cm⁻¹ strong C-H (stretching), 2848.94 cm⁻¹ medium C-H (stretching) and 1464.81 cm⁻¹ strong C = C (stretching). Stearic acid showed characteristics peaks at 2962.62 cm⁻¹ medium C-H (stretching), 2914.94 cm⁻¹ medium C-H (stretching), 1698.93 cm⁻¹ conjugated ketone C = O (stretching), 1429.01 cm⁻¹ medium O–H (bending) and 940.62 cm⁻¹ weak C = C (bending). Xanthan gum showed characteristics peaks at 3265.27 cm⁻¹ broad O–H (stretching), 2882.20 cm⁻¹ C-H (stretching), 1599.48 cm⁻¹ medium C = C (bending) and 1368.86 cm⁻¹ medium C-H (bending). Tocopherol acetate exhibited characteristics peaks at 2925.39 cm⁻¹ strong C-H (stretching), 2867.63 cm⁻¹ alkene C-H (stretching), 1758.31 cm⁻¹ strong C = O (stretching), 1460.16 cm⁻¹ medium C-H (bending) and 1204.43 cm⁻¹ C-O (stretching). Carbapolo 940 showed characteristic peaks at 3048.70 cm⁻¹ broad O–H (stretching), 2946.83 cm⁻¹ strong C-H (stretching), 2659.46 cm⁻¹ medium C-H (stretching), 1703.76 cm⁻¹ C = O (stretching), 1414.68 cm⁻¹ alcohol O–H (bending) and 1452.30 cm⁻¹ medium C-H (bending). PEG-200 showed characteristic peaks at 3410.49 cm⁻¹ broad O–H (stretching), 2868.24 cm⁻¹ strong C-H (stretching), 1453.92 cm⁻¹ medium C-H (bending), 1406.35 cm⁻¹ alcohol O–H (stretching).

![Fig. 4 Rheological property of the novel herbosomal-loaded PEG-poloxamer topical formulation (n-HPTF)](image-url)
Fig. 5  ATR-FTIR overlay of optimized n-HPTF and their ingredients

(stretching) and 1099.26 cm\(^{-1}\) medium C-O (stretching). Tween-60 showed characteristics peak at 3489.83 cm\(^{-1}\) broad O–H (stretching), 2922.26 cm\(^{-1}\) strong C-H (stretching), 2854.34 cm\(^{-1}\) medium C-H (stretching), 1735.87 cm\(^{-1}\) strong C=O (stretching), 1638.90 cm\(^{-1}\) conjugated C=O (stretching) and 1097.09 cm\(^{-1}\) medium C-O (stretching), and similar findings were observed by Gaikwad et al. [87]. Transcutol\(^\text{®}\) showed characteristic peaks at 3430.56 cm\(^{-1}\) broad O–H (stretching), 2975 cm\(^{-1}\) strong C-H (stretching), 2867.54 cm\(^{-1}\) strong C-H (stretching), 1455.38 cm\(^{-1}\) medium C-H (bending), 1287.47 cm\(^{-1}\) medium C-O (stretching) and 1068.86 cm\(^{-1}\) secondary alcohol C-O (stretching). Glycerol showed their characteristic peaks at 3293.89 cm\(^{-1}\) broad O–H (stretching), 2879.34 cm\(^{-1}\) strong C-H (stretching), 2933.20 cm\(^{-1}\) strong C-H (stretching), 1412.14 cm\(^{-1}\) medium C-H (bending) and 1029.64 cm\(^{-1}\) medium C-O (stretching). Triethanolamine showed characteristic peaks at 3307.47 cm\(^{-1}\) strong O–H (stretching), 2948.61 cm\(^{-1}\), 2478.52 cm\(^{-1}\) and 2822.85 cm\(^{-1}\) ternary amine, 1655.19 cm\(^{-1}\) medium C=O (stretching) and 1067.15 cm\(^{-1}\) medium C-O (stretching). Physical mixture of the designed formulation showed characteristics peak at 3350.60 cm\(^{-1}\) broad O–H (stretching), 2980.01 cm\(^{-1}\) strong C-H (stretching), 2816.75 cm\(^{-1}\) C-H (stretching), 1639.07 cm\(^{-1}\) weak C=C (stretching), 1457.85 cm\(^{-1}\) C-H (bending) and 1044.43 cm\(^{-1}\) medium C-O (stretching). The optimized formulation n-HPTF-06 showed their characteristics peaks at 3359.71 cm\(^{-1}\) broad O–H (stretching), 2883.55 cm\(^{-1}\) strong C-H (stretching), 2816.75 cm\(^{-1}\) C-H (stretching), 1638 cm\(^{-1}\) weak C=C (stretching), 1457.85 cm\(^{-1}\) C-H (bending) and 1094.38 cm\(^{-1}\) medium C-O (stretching). The identical peaks in the physicochemical mixture and optimized formulation revealed that there was no physicochemical incompatibility in this ideal combination of drug and polymers.
In vitro drug permeation (Q)

In vitro drug permeation from n-HPTF-06, containing herbesomes and extracted NPPs, is depicted in Fig. 6. The aim of preparing herbosomes was to increase the bioavailability of *Curcuma longa* at the target site due to its ability to inhibit TNF-alpha factor in deep tissue injury, besides it also exhibits anti-inflammatory, antimicrobial properties. Secondly, *Arnica montana*, which is hydrophilic in nature, was selected to incorporate into herbosomes due to their potent analgesic effect which required an immediate action at the targeted site. Other ingredients like *Aloe barbadensis*, *Azadirachata indica*, *Camellia sinensis*, *Glycyrrhiza glabra*, *Calendula officinalis* and Pro-Vitamin B5 showed controlled release from the polymeric matrix. Initially, a burst release pattern was seen vis-a-vis all ingredients due to their hydrophilic charged surface. Diverse release pattern for all the tested NPPs suggested that it may be due to nature of solubility and affinity of entrapment in by the polymer and lipid inside the core matrix as well as to the polymeric end chain; secondly this may be due to their intricate molecular size molecular [89] and partition efficiency to the semi permeable membrane at the diffusion site. Similar release parameter has been cited by Vuddanda et al. [88].

Kinetic modeling

The release kinetic model’s R² value, estimated for all the ingredients in the optimized formulation n-HPTF-06, is shown in Table 8. Overall, the best fit model for optimized formulation was first-order model in which amount of drug dissolved directly in the buffer from the formulation, followed by Higuchi’s model in which drug dissolution takes place from the matrix of the polymeric core. The different behavior of the NPPs may be due to their complex molecular chain and larger molecular size. In this study, PEG–poloxamer was loaded with herbosomal formulation in which herbosomes were packed in core vesicular matrix. It is reported in the literature that the following two types of release kinetic may be exhibited by the formulation due to variable solubility in the physiological buffer and matrix effect for the release of ingredients such as *Curcuma longa* from the polymeric matrix. Similar effect of vesicles on release kinetic was described by Muthappa et al. [90].

Stability study

Stability study of the final optimized formulation was carried by placing the optimized formulation n-HPTF-06 at two different temperatures as per ICH guideline for long-term stability study of the topical formulation, i.e., 5 °C ± 3 °C and 30 °C ± 2 °C; 65% ± 5% RH. n-HPTF-06 was assessed for color, texture, pH, spreadability and phase separation with respect to time (in days) for sampling, i.e., 0, 30, 60, 90, 120 and 180 days of storage. No significant change in any parameter was observed, and observed data are tabulated in Table 9. Further any physicochemical incompatibility upon long-term storage

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**Fig. 6** In vitro drug permeation (Q) of the optimized n-HPTF-06
was assessed by using ATR-FTIR (Fig. 7), which showed characteristic peaks at 3359.71 cm⁻¹ broad O–H (starching), 2918.63 cm⁻¹ strong C–H (stretching), 2131.75 cm⁻¹ and 2132.75 cm⁻¹ C–H (stretching), 1641 cm⁻¹ weak C=C (stretching), 1459.85 cm⁻¹ C–H (bending) and 1094.38 cm⁻¹ medium C–O (stretching), which corroborated to the results obtained for the final n-HPTF-06 formulation, and no physicochemical incompatibility [94] was observed in the spectra and hence the optimized formulation is stable even in exposed condition.

### Skin irritancy study on optimized n-HPTF

The optimized n-HPTF-06 was used for the skin irritancy study on female Sprague–Dawley rats (n = 6) as per standard prescribed protocol (OECD 402). The formulation was applied over the dorsal area of the rat’s skin by dividing the skin surface into two sections on the same rat to see any immediate contact irritation on the leftover skin (if any). Subsequently, rat skin was observed initially for 1 h and 6 h and observations were recorded and further the skin was restrained by using medical adhesive so that the formulation remained in contact with the skin for a long time. On the next day, the adhesive was removed and wiped out and the skin was gently sanitized by using 70% ethanol and observation was recorded and further observations were made till 72 h and primary dermal irritation index (PDII) was scored. No erythema or edema was observed on skin of the rats at any point of study (1, 6, 24, 48 and 72 h, respectively). Hence, the optimized formulation was considered as a safe formulation possessing no-irritancy upon application to rat’s skin.

### Table 8 Release kinetic model for the optimized formulation n-HPTF-06

| Kinetic model | Aloe barbadensis (Aq.) (R²) | Azadirachta indica (Aq.) (R²) | Curcuma longa (Aq.: Ethanol, 50:50 v/v) (R²) | Camellia sinensis (Aq.) (R²) | Glycyrrhiza glabra (Aq.: Ethanol, 30:70 v/v) (R²) | Arnica montana (Aq.: Ethanol, 50:50 v/v) (R²) | Calendula officinalis (Aq.: Ethanol, 50:50 v/v) (R²) | Pro vitamin B5 (R²) |
|--------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Zero order   | 0.874          | 0.874          | 0.875          | 0.969          | 0.901          | 0.974          | 0.969          | 0.971          |
| First order  | 0.814          | 0.914          | 0.941          | 0.984          | 0.914          | 0.923          | 0.914          | 0.978          |
| Higuchi model| 0.819          | 0.921          | 0.874          | 0.914          | 0.901          | 0.907          | 0.907          | 0.905          |
| Korsmeyer-Peppas | 0.748       | 0.748          | 0.784          | 0.873          | 0.822          | 0.904          | 0.987          | 0.808          |

### Table 9 Stability study of the optimized n-HPTF by using ICH guidelines Q1 A (R2) (Long-term study)

| Characteristics of Optimized formulation (n-HPTF-06) | Temperature (°C) and %Relative Humidity (%RH) | Time (in days) | Initial observations | 30 days | 60 days | 90 days | 120 days | 180 days |
|-----------------------------------------------------|-----------------------------------------------|---------------|----------------------|---------|---------|---------|----------|---------|
| **Color**                                           |                                               |               |                      |         |         |         |          |         |
| 5 °C ± 3 °C                                         | Pale yellow                                   | Pale yellow   | Pale yellow          | Pale yellow | Pale yellow | Pale yellow | Pale yellow | Pale yellow |
| 30 °C ± 2 °C; 65% ± 5% RH                           | Pale yellow                                   | Light brown   | Light brown          | Light brown | Light brown | Light brown | Light brown | Light brown |
| **Texture**                                         |                                               |               |                      |         |         |         |          |         |
| 5 °C ± 3 °C                                         | Smooth & no grittiness                        | Smooth & no grittiness | Smooth & no grittiness | Smooth & no grittiness | Smooth & no grittiness | Smooth & no grittiness | Smooth & no grittiness |
| 30 °C ± 2 °C; 65% ± 5% RH                           | Smooth & no grittiness                        | Smooth & no grittiness | Smooth & no grittiness | Smooth & no grittiness | Smooth & no grittiness | Smooth & no grittiness | Smooth & no grittiness |
| **pH**                                              |                                               |               |                      |         |         |         |          |         |
| 5 °C ± 3 °C                                         | 6.5 ± 0.11                                    | 6.5 ± 0.10    | 6.5 ± 0.12           | 6.5 ± 0.10 | 6.4 ± 0.14 | 6.4 ± 0.12 | 6.4 ± 0.11 |
| 30 °C ± 2 °C; 65% ± 5% RH                           | 6.5 ± 0.17                                    | 6.5 ± 0.11    | 6.5 ± 0.11           | 6.4 ± 0.13 | 6.4 ± 0.12 | 6.4 ± 0.12 | 6.4 ± 0.12 |
| **Spreadability** (Spreading in 1 min at a force of 25 gm) |                                               |               |                      |         |         |         |          |         |
| 5 °C ± 3 °C                                         | 11.02 mm²                                     | 11.00 mm²    | 11.01 mm²            | 11.10 mm² | 11.17 mm² | 15.14 mm² | 15.19 mm² |
| 30 °C ± 2 °C; 65% ± 5% RH                           | 11.11 mm²                                     | 11.02 mm²    | 11.13 mm²            | 11.15 mm² | 11.14 mm² | 15.14 mm² | 15.19 mm² |
| **Phase separation**                                |                                               |               |                      |         |         |         |          |         |
| 5 °C ± 3 °C                                         | No phase separation                           | No phase separation | No phase separation | No phase separation | No phase separation | No phase separation | No phase separation |
| 30 °C ± 2 °C; 65% ± 5% RH                           | No phase separation                           | No phase separation | No phase separation | No phase separation | No phase separation | No phase separation | No phase separation |
Fig. 7 ATR-FTIR spectra of n-HPTF-06: (A) 5°C ± 3°C/ 65 ± 5% RH and (B) 30°C ± 2°C/ 65 ± 5% RH
Experimental design and dose optimization for bio-efficacy study of n-HPTF

Sprague-Dawley rats, used in this study, were grouped into three groups: Group (I, II and III) as mentioned above and frostbite was induced and comparative study of the frostbite condition was monitored in formulation-treated groups as well as in untreated condition. Visual observation was done and changes recorded and further the condition was analyzed by histological study. No treatment complications or death of animals were observed during the course of this study.

n-HPTF ameliorates cold injury, promotes frostbitten wound healing and maintains skin integrity in vivo

A sequential analysis was required before reaching the final conclusion vis-a-vis the optimized n-HPTF formulation. In cold injury, tissue undergoes multifarious pathological alterations that may even lead to severe consequences: vis-a-vis amputation of exposed extremities if the sequences of changes/alteration(s) are not timely controlled. When the skin comes in contact with chilled wind or sub-zero temperatures, it impairs the normal physiological condition and causes desensitization of the non-selective cationic channel, vasoconstriction, tissue hypoxia, alteration in flow of electrolyte concentration inside the cells, ice-crystal formation, generation of reactive oxygen species and tissue necrosis. Cumulative and prolonged exposure accentuates thrombus formation, thereby leading to amputation/loss of extremities. In this study, animals were grouped in three major groups, viz. Group I (untreated), Group II (standard formulation twice a day; 0.5 gm application) and Group III (treatment was followed by applying n-HPTF twice a day; 0.5 gm/each application), and observation was recorded on 3, 7, 14, 21 and 28 days till the complete healing of frostbite wound.

At day 0, purplish skin was observed after 2 h. of induction of frostbite in all the animals and the area was marked for further morphometric analysis. At day 3, the major changes in the skin were noticed in all the three groups: [Group I: the skin became more injured and appeared more wounded in comparison to Groups II and Groups III animals, which received topical formulations]. The severity of cold injury among the groups was evident from visual inspection of the dead epidermis and more inflamed and flared skin. At day 7, Group I animals of the skin became oozier and developed hard crust over skin as well as pus formation was also observed. In Group II and Group III the hard crust was also observed, but in case of Group III the skin around the crust was normal and no sign of inflammation was observed in comparison to Group I and II. At day 14, the hard crust from the animal skin was crust out in almost all groups, but in case of untreated groups the condition of skin worsened and was a significant sign of ice-crystal formation, which upon nucleation damaged the deep skin layers in both Groups I and II with very slight observable differences, whereas the skin of Group III animal which were treated with n-HPTF was remain in contact which reveals that n-HPTF increases the ionic interaction and thus maintains integrity of the skin. At day 21, Group III showed accelerated healing rate followed by contraction and narrowing the wound area, suggesting an earlier re-epithelialization than the others groups of animals. At day 28, Group III showed complete healing and no scars over skin was witnessed by necked eyes over the skin of animals.

In conclusion, based on the ingredients incorporated in n-HPTF which was a priori selected based on their innate beneficial characteristics and targeted mode of action over the entire sequelae helped in alleviation of the frostbite symptoms and promoted rapid tissue healing as compared to untreated group and standard drug treated group. The comparative representation of all the three groups with respect to days 0, 3, 7, 14, 21 and 28 is depicted in Fig. 8.

Morphometric analysis of frostbitten wound area

Animal groups, followed by their prescribed dosage form were observed for their healing rate and frostbitten wound was measured by using Vernier caliper at day 3, 7, 14, 21 and 28, respectively. The percentage frostbitten wound contraction was calculated by formula mentioned earlier. The results were analyzed by using Student t-test and found to be significant (p < 0.05). n-HPTF showed ≈98.96% recovery than the standard treatment (65.65%) at day 28 suggesting an improved healing rate; the supporting morphometric measurement in graphical form is depicted in Fig. 9.

Channelized recovery of frostbitten skin by n-HPTF evaluated histopathologically

The histological study was designed to observe the detailed changes occurring during the course of induction of frostbite in cold injury induced model. During the study protocol, detailed histology of skin layers, demarcation and thickness of skin layers, morphology of underlying papillary layer as well as dermis layer with abundant blood capillaries and connective tissue cells was studied.

Day 3: Animals of all three groups represented similar changes symptomatically to frostbite condition. Few differences were noticed in the animal Group I and II, in which the skin layers were less inflamed (Fig. 10 (B1 and C1) as
compared to untreated groups as marked by red arrows in Group I (Fig. 10(A1) Auerbach et al. [66]).

Day 7: In Group I and II (Frostbitten and frostbitten+standard drug treated) underlying papillary layer was completely delocalized due to progression of frostbitten wound (Fig. 10(A2 and B2), but in Group III the papillary layer just below the epidermis was found intact, which is attributed to the antioxidant properties possessed by the extracted NPPs incorporated in n-HPTF as they are able to maintain papillary (which is widely known for the regulation of vascular temperature), thereby inhibiting the ice-crystal formation as depicted in Fig. 10(C2). Similar observation was reported by Auerbach et al. [66].

Day 14: Group I (untreated) exhibited complete disruption of all layers of the skin and loss of skin integrity, disruption of blood vessels and connective tissue and is histopathologically depicted in Fig. 10(A3), whereas, in Group II, the demarcation was observed and skin gaps between epithelial junctions could be clearly observed. However, in case of Group III, a clear morphology with defined skin integrity was observed at day 14 suggesting that n-HPTF is effective in controlling the subsequent ischemic condition inside the cells that results due to cold-induced stress. The herbal ingredients in n-HPTF possess significant amount of ROS scavenging capacity as revealed in the in vitro antioxidant capacity of the homogenizer assisted extracted NPPs, which exhibited promising therapeutic action against the cold injury condition.

Day 21: Group I showed formation of larger vacuoles during the phase of natural healing attained by dermis and epidermis layer of mammalian skin (Fig. 10(A4)). Similar results were seen in Group II with a smaller number of larger gap filling vacuoles Fig. 10(B4)). However, in case of Group III, the skin layer exhibited a well-defined structure and a clear delineation between epidermis and dermis layer of skin suggesting that the herbal ingredients of n-HPTF are able to maintain ionic interaction between cells and thus accelerate the production of collagen as well as connective tissue which is clearly evident in Fig. 10(C4) as well 40X resolution image (Fig. 10(E)_ showing dense collagen fiber, suggesting recovered healing.

![Fig. 8 Digital depiction of images of pre-frozen ferrite magnet induced cold injury on Sprague-Dawely rat skin and in vivo study for the bioefficacy of the optimized n-HPTF. The study was subgrouped in three groups (n=10) vis-a-vis; Group I: Frostbitten (Untreated); Group II: Frostbitten+Standard Formulation; Group III: Frostbitten+n-HPTF](image)

![Fig. 9 Morphometric observation of the frostbitten wound contraction (%) at day 3, 7, 14, 21 and 28, respectively. The results were analyzed statistically using Student’s t test and values were compared as mean±SEM and *p<0.05 (standard treatment groups versus n-HPTF groups)](image)
**Conclusion**

Quality-by-design (QbD) model is a preferable model used in the development of targeted pharmaceuticals to control their critical processing variables and save the time and cost of the finished product. These models are frequently used in pharmaceutical development so that in-process quality control (IPQC) and finished product quality control (FPQC) can be given design space. In this paper we have formulated a novel herbosomal topical formulation for the management of cold injury encountered in high altitude at sub-zero temperatures. The developed n-HPTF was optimized under critically controlled conditions in terms of their entrapment efficiency, particle size, zeta potential, spreadability, viscosity, in vitro drug permeation, etc. The NPPs were characterized by using ATR-FTIR to obtain characteristic peaks so that any physicochemical changes in the product can be identified. The developed formulation was studied for optimizing its shelf-life by using long-term stability study as per ICH guideline Q1 A (R2) and results were found satisfactory. Furthermore, the optimized formulation was used to assess the skin irritancy (if any) using female Sprague–Dawley rats and no dermal erythema or edema was observed. In vivo results of n-HPTF with respect to standard treatment was found significantly effective ($p < 0.05$), suggesting that the n-HPF can be used as an effective novel herbal prophylactic as well as therapeutic regimen for the management of severe cold injuries like frostbite. In future, the optimized n-HPTF will be further used for molecular pathway elucidation as well as characterizing the canonical biochemical pathways modulated during cold injuries.

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**Fig. 10** Histopathological studies depicted induction of frostbite and its recovery by designed topical formulation. Frostbite untreated rats (A1-A4), Standard formulation treated rats (B1-B4) and n-HPTF treated rat skin (C1-C4). The study was carried out by harvesting (n=2) animal in each group at days 3, 7, 14 and 21, respectively. At day 3, in A1, B1 and C1 some morphological changes in the dermis layer with presence of vacuoles as well as loss in integrity of basal membrane were observed. At day 7 in A2, a complete disruption of stratum corneum, stratum lucidum and stratum granulosum was observed, which is due to intracellular swelling and endothelial line separation and generation of ‘Gaps’ in between cells. A similar histopathological change was observed in B2 with the presence of basal membrane with desmosomes and in C2 morphological changes in the tissue were minimal which may be due to inhibition of O2 uptake by interstitial cells which prevents production of free radicals due to antioxidants rich ingredients incorporated in n-HPTF. At day 14, in A3, a complete loss of strata at the cold-exposed surface was observed (marked by red arrows) and in B3 the presence of desmohyalin granules with disruption in irregular lamellar body was seen, but loss in epidermal cells was found to be under control and in sample C3 the presence of large vacuoles was evident but uniform structure was observed with undamaged basal membrane, suggesting that healing is in upward direction from the basal vasculatures. At day 21, in sample A4 some reversible changes in the skin tissue were observed but a large number of granulocytes were also observed in the dense collagenous tissue indicating that loss in smooth muscle contraction takes place which is required for the thermoregulation and natural healing through contraction; in sample B4 similar histopathological changes were observed as in A4 with change in basal membrane integrity, but in sample C4 at day 21 a dense collagen fiber with aliened morphology was observed, and proliferation of desquamed cells at the upper edge of epidermis was also observed.
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Author's contributions All authors discussed experimental protocols, performed experimentations, discussed the results and contributed to the final manuscript.

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Data availability This study has not been published anywhere, and data provided in this study were generated to prove the theoretical aspect in topical formulation development.

 Declarations

Ethical approval and consent of participate The research work done is in compliance with all relevant ethical standards. For conducting animal studies, ethical approval was given by IAEC (Institutional Animal Ethical Committee) of Defence Institute of Physiology and Allied Sciences (DIPAS), DRDO, India, having approval number DIPAS/IAEC/2019/Dec-03.

Consent for publication This paper reflects the view of authors and should not be construed to reflect the views of the Govt. of India or the Ministry of Defence/DRDO or any other institution(s)/agency(ies) whatsoever. This article includes only unclassified information and is purely scientific in nature. The research work done is in compliance with all relevant ethical standards.

Competing interests The authors declare no competing interests.

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