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Development and Standardisation of a Method for Inflicting Frostbite Injury in Rats and Formulation of Essential Oil in Treatment of Frostbite

Kumud Joshi[#], Danswrang Goyary[#,*], Bhaskar Mazumder[@], Sumit Kishor[#], Piyali Dey[#], Yangchen Doma Bhutia[#], Sanjeev Karmakar[#], Pronobesh Chattopadhyay[#], and Sanjai Kumar Dwivedi[#]

[#]*DRDO-Defence Research Laboratory, Tezpur - 784 001, India*
[@]*Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh - 786 004, India*
[*]*E-mail: goyary@gmail.com*

ABSTRACT

Frostbite is a cold induced injury which occurs due to exposure of a particular site of body to sub-zero temperature. One of the primary reasons for lack of proper studies about the underlying mechanism of frostbite injury is due to non-availability of any reliable animal model and method for inflicting frostbite. In our current research, a device was designed and standardised to inflict frostbite wound in wistar rat. A formulation comprising different combination of essential oils was also developed and its activity was assessed and found effective in the treatment of frostbite wound.

Keywords: Frostbite device; Formulation; Wound contraction; Vascular injury

1. INTRODUCTION

Frostbite is an ischemic injury; the main cause of frostbite is vascular damage post freezing injury. Frostbite generally occurs in extreme ends of body like finger tips and the distal end of the toes, where compromised blood perfusion may hamper blood flow[1,2].

As we can understand that the pathology of frostbite is far complicated than normal wounds, the frostbite wound seem to non-responsive to conventional wound healing agents e.g. silver sulphadiazine, iodine tincture and others. The primary reason for this is the vascular damage in frostbite, leading to thrombus formation and ensuing ischemic damage. In normal wounds the damage to epidermal layer of skin following trauma, opens the otherwise intact skin barrier. This allow for access of drug to the site of action. However in frostbite there is rarely breakage of epidermal barrier. Thus topically applied therapeutic agents cannot be applied directly. This is a major challenge for topical delivery of therapeutic agents in frostbite wound.

Further emulating the human pathology in animal is far difficult; given the structure of animal skin vary greatly, than that of human. Moreover there is no standard model for frostbite. This is one of the main reasons for lack of research in frostbite. Current work intends to standardise a simple and reliable method for induction of frostbite injury.

For inducing frostbite we need super cold agents. Generally, two main agents are used for inducing frostbite in animal model are liquid nitrogen and solid CO_2. Both of these agents are highly volatile and difficult to handle, having risk of injury, and are expensive. They require special setup at times. Many of these devices are hard to emulate and need a lot of controlled engineering work. Earlier, several methods have been developed for inducing frostbite injury. Auerbach[6], *et al*. developed a method using magnets frozen in dry ice to create wound of frostbite. Goertz[3], *et al*. designed a special device, using a Dewar vessel, with which applied air jet through a catheter orifice fixed at 3 mm to create frostbite wound. Ear injury model was also developed by Kulka[4]. Hamlet[5], *et al*. developed frostbite wound in rabbit foot using ethylene glycol-alcohol at -40 °C for producing fourth degree necrotising wound. Martin Heisig[6], *et al.* immersed rat tail in -22 °C liquid for 4 min for inducing frostbite. Similarly Hu[7], *et al*. studied pathophysiologic model of frostbite, stimulating high altitude frostbite using a specially designed equipment to deliver liquid nitrogen to the shaved back of the rat creating superficial, partial, and full-thickness frostbite wounds. They exposed rats directly to liquid nitrogen, using specially designed equipment. Rothenberger[8], *et al.* inflicted frostbite injury on the abdomen of Goettingen-Minipigs using an aluminium bar (300 g, circular with a radius of 1 cm) frozen with liquid nitrogen to -196 °C. The bar was applied for 1 s, 5 s, 10 s, 20 s, 30 s and biopsies were performed after four hours and 7 days post injury and evaluated the damaged tissue by Heamatoxyalin & Eosin (H & E) and Masson-trichome (MT) staining.

As we noticed that some of these devices developed for inflicting frostbite are very complicated and difficult to design for emulation. Moreover the exposure time for frostbite in some of the methods is very long, up to 20 min. Thus we tried to develop a simple device which can quickly give uniform, reproducible frostbite wound to animals, while providing easy and safe handling to the user.

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One of the important feature of frostbite is a local injury and there is no specific treatment for it. Recently several researches in field of frostbite topical therapy have been reported. Shen[9], *et al.* developed nanogel using *Ganoderma lucidum* (GLT) nanogels for treatment of frostbite, They also developed lipid based nano-formulation for treatment of frostbite[10]. Similarly Auerbach[11], *et al.* developed poly L arginine cream for treatment of frosbite. More recently heparin based nanogel has been developed for healing frostbite, suggesting that anticoagulant drugs have high efficacy in treatment of frostbite[12]. As the essential oils have significant antiplatelet effect, our study involves development of an essential oil based formulation for topical therapy for frostbite injury and evaluation of healing potential using wound contraction and histopathological analysis.

2. MATERIALS AND METHODS

2.1 Materials

Essential oils (Industrial Grade) of wintergreen, rosemary and curcuma were obtained from Surajbala Exports Pvt. Ltd, New Delhi. Eucalyptus, olive oil, bees wax and lanolin of laboratory grade were obtained from HiMedia, Pvt Ltd, Mumbai. All other chemicals were of analytical grade.

2.2 Methods

2.2.1 Design of Device and its Standardisation

The device for induction of frostbite wound was fabricated using solid circular rod of aluminium (height 15 cm and a diameter of 3 cm) and incorporating a long stainless steel rod (45 cm) with a support shaft. The device can be completely immersed in liquid nitrogen cylinder for cooling, provides safety to the user, while handling cooled device. The weight of the device is approximately 300 g. The weight of the device provides a uniform pressure, when the cold device is applied on the back of the rat, producing uniform wound in different group of animals, ensuring reproducibility.

2.2.2 Standardisation of Frostbite Injuries

A total of 32 Wistar rats (180-250 g) were taken in the study and the animals were divided into four groups with different exposure time to evaluate the extent of frostbite injury. All animals used for experiments were obtained from the animal housing facility of Defence Research Laboratory (Tezpur, Assam, India) and all the experiments were performed in accordance with the Guide for the care and use of laboratory animals. All animals were acclimatised for 21 days prior to the beginning of the experiments (IAEC No. 20/8/16).

For standardisation of the animal model, the animals were exposed to the cooled surface of device under its own weight without external pressure. The duration of exposure was 5 s, 10 s, 20 s, and 30 s, respectively. The body temperature was measured by a rectal thermometer. The wound healing was recorded visually for next 30 days; the area of wound was determined using wound contraction studies. Histopathological evaluation of injured tissue was also performed, at 0 and 7 days, to record the degree of necrosis and damage induced by exposure for different time intervals.

2.2.3 Histopathological Evaluation

The histopathological evaluation of the healing tissues was done at different time intervals during the treatment period by using Heamatoxyalin & Eosin staining and Masson trichome staining (day 3, day 7, day 14 and day 21 post injury) as compared to control group. The histopathological analysis helped in determining the extend of ischemic damage and depth of injury.

2.2.4 Development and Evaluation of Essential Oil Combination Formulation for Treatment of Frostbite

As frostbite is a vascular ischemic injury, wherein vascular damage leads to severe platelet aggregation leading to thrombus formation which further results in stunting of blood supply to the affected tissue and ischemic cell death. Therefore antiplatelet and antioxidant agents such as essential oils can be beneficial. Essential oils are known to possess anti-platelet aggregation effect; moreover they possess excellent wound healing action and excellent vasodialatory action as well. Essential oils are also known to have good permeation across the skin barrier. These physiochemical and biological activities of essential oils make them a suitable candidate for possible use in frostbite. In this study, a combination of essential oils was prepared as a topical formulation and evaluated their frostbite healing activity.

2.2.4.1 Profiling of Essential Oils using GCMS

All the essential oils were of industrial grade and procured from market, and they were chemically profiled using GC-MS analysis. Gas Chromatograph (Model: GC 7890B, Make: Agilent Technologies, CA) equipped with a fused silica capillary column (Model: HP-5MS, Make: Agilent Technologies, CA), and a mass spectrometry detector (Model: MSD 5977 A, Make: Agilent Technologies, CA) Inlet and detector source temperatures were held at 280 °C and 230 °C, respectively. The initial oven temperature was held at 50 °C and then ramped at rate of 20 °C/min to the temperature was raised 250 °C at the rate of 6.0 °C/min for 0 min. The essential oil was in a volume of 1 mL and helium was used as the carrier gas at 1 ml/min with a splitless mode. MSD voltage was 70 eV in scan mode. Eventually, essential oil profiling was done using MassHunter Quantitative Analysis software, v B.07.01 SP1/Build 7.1.524.1 (Agilent Technologies, CA).

2.2.4.2 Formulation of Ointment

The essential oils with good anti-platelet aggragatory activity along with good wound healing action have been selected for frostbite healing formulation. The formulation of frostbite ointment comprises as shown in Table 1.

The bees wax and lanolin were melted at 70 °C. All the essential oils were mixed with olive oil and then added to the mixture of bees wax and lanolin, stirred continuously for 5 min then allowed to cool down to room temperature. The ointment thus formed was used for evaluation of frostbite healing activity.

2.3.2 Evaluation of Frostbite Healing Activity of Essential Oil Combination

The frostbite injury was induced by the methods discussed in previous section 2.2.2, with an exposure of 10 s. The tissue was allowed to thaw at room temperature and the treatment was started 2 h post injury. The ointment was applied twice daily to the injured tissue.

2.3.3 Wound Contraction Rate

The wound contraction rate of the injured tissue was determined by measuring the area of wound at various intervals post injury as compared to control group.

2.3.4 Histopathological Evaluation

The histopathological evaluation of the healing tissues was done at different time intervals during the treatment period by using H&E staining (on day 3-, 7-, 14- and 21- post injury) as compared to control group.

3. RESULTS

3.1 Development and Standardisation of Frostbite Injury Model

All the group of animals showed wound post cold device exposure. An exposure of 5 s created a wound of asymmetric shape with patches of damaged areas. Damage is higher at centre, while less in outer areas (Fig. 1). Histological observation suggest that the thickness of damage in 5 s and 10 s wounds was also less in comparison to 20s and 30 s wounds, where the complete necrosis and tissue mummification was observed, suggesting that longer duration exposure lead to much deeper damage. The histological study (Fig. 2) revealed that 5 s exposure wound on 7- day did not show a complete necrosis demarcation, and patches of preserved tissue was observable in the area where wound was induced. On the other hand, in 10 s exposure wound group, the tissue damage was uniform and well marked. The epidermal and dermal damage was visible. In 20 s and 30 s exposure wounds, deeper irreversible necrosis is observed within first week. In 30s, the mummified tissue has necrotised and sloughed off completely from underlying muscles. The absence of epidermal structure and any vasculature is also evident in 7-day histology of 30 s exposure group. These suggest a complete necrosis similar to 4th degree frostbite. In 20 s exposure wound also, the demarcation of dead tissue and deeper tissue damage is observable with high degree suggesting severe irreversible damage to both dermal layer and underlying muscles and tissues. We found that 10 s exposure wound showed the presence of extensive blood clotting with tissue damage due to tissue shrinkage post ischemia and uniform wound in the H&E staining. However, the irreversible demarcation necrotic tissue is not observed before 10 day. The neutrophil infiltration, oedema and formation of blood clots in some regions were present suggesting a limited vasculature survival and slower ischemia. Injury of limited depth is observed with intact deeper tissues. Such wounds are suitable for evaluation of frostbite healing agents.

From the above findings, it is indicated that a frostbite injury model of different degree may be created by the device uniformly by varying the duration of exposure. It may also be concluded that 8 s - 10 s exposures are suitable

Table 1. Major Constituents in different oils as determined by GC-MS profiling

	Compound	RT	Mass
Rosemary oil	D-Limonene	5.352	$C_{10}H_{16}$
	Eucalyptol	5.402	$C_{10}H_{18}O$
	Trans Verbenol	6.053	$C_{10}H_{16}O$
	Thymol	6.622	$C_{10}H_{14}O$
	Cyclohexane, 1-methylene-4-(1-methylethenyl)-	4.939	$C_{10}H_{16}$
	Camphene	4.689	$C_{10}H_{16}$
	Benzaldehyde	4.783	C_7H_6O
	Isobornyl acetate	7.391	$C_{12}H_{20}O_2$
	Benzyl Benzoate	10.444	$C_{14}H_{12}O_2$
Curcuma Oil	Dibutyl phthalate	11.363	$C_{16}H_{22}O_4$
	Citronellol	6.866	$C_{10}H_{20}O$
	Citral	7.185	$C_{10}H_{16}O$
	Geraniol	7.047	$C_{10}H_{18}O$
	o-Cymene	5.308	$C_{10}H_{14}$
	Camphene	4.689	$C_{10}H_{16}$
	Benzaldehyde	4.783	C_7H_6O
	Linalool	5.89	$C_{10}H_{18}O$
	cis-Verbenol	6.509	$C_{10}H_{16}O$
	Methyl salicylate	6.691	$C_8H_8O_3$
	beta.-Bisabolene	8.849	$C_{15}H_{24}$
	aR-Turmerone	9.8	$C_{15}H_{20}O$
Oil of wintergreen	Methyl Salicilate	6.708	$C_8H_8O_3$
Eualyptus oil	3-Carene	4.533	$C_{10}H_{16}$
	(1S,3S,4S,5R)-1-Isopropyl-4-methylbicyclo[3.1.0]hexan-3-ol	4.952	$C_{10}H_{18}O$
	p-Cymene	5.033	$C_{10}H_{14}$
	Eucalyptol	5.39	$C_{10}H_{18}O$
	Trans-Verbenol	6.059	$C_{10}H_{16}O$
	.alpha.-Campholenal	6.166	$C_{10}H_{16}O$
	Trans-Verbenol	6.372	$C_{10}H_{16}O$
	p-Cymen-7-ol	6.635	$C_{10}H_{14}O$
	(-)-Myrtenol	6.741	$C_{10}H_{16}O$
	D-Carvone	7.073	$C_{10}H_{14}O$
	Limonen-6-ol, pivalate	8.08	$C_{15}H_{24}O_2$
	Aromandendrene	8.505	$C_{15}H_{24}$
	(-)-Globulol	9.45	$C_{15}H_{26}O$

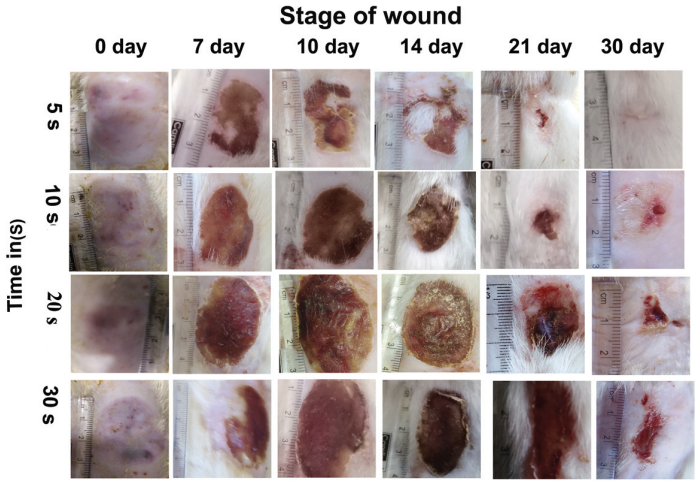

Figure 1. Wound contraction in different stages of healing after cold exposure for various time intervals.

for 1st and 2nd degree frostbite, whereas 20 s and 30 s exposure are suitable for deeper third and fourth degrees of frostbite.

3.2 Development and Evaluation of Frostbite Healing Formulation

3.2.1 Essential Oil Combination Preparation for Frostbite Healing

The results of GCMS profiling of the essential oils i.e. rosemary oil, curcuma oil, wintergreen oil, and eucalyptus oil are presented (Fig. 3, Table 2). The major constituents of these essential oils have specific vasodilatory and anti platelet activity. The essential oils possess significant anti-inflammatory, antioxidant and wound healing activity; such activity helps in treatment of frostbite.

3.2.2 Evaluation of Frostbite Healing Activity of Essential Oil Formulation

The treatment with Essential oil combination (EOC) produced significant ($p \leq 0.05$) wound healing in terms of wound contraction rate as compared to untreated group as shown in Fig. 4. The wound healing was achieved 80 % on 21-day in the treatment group, while the healing was only 35 % in non-treatment group. The histopathology also suggested a progressive healing in the treatment group as shown in Fig. 5. The oedema in treatment group was significantly lesser than control group. Moreover lower inflammation and lesser neutrophil infiltration were

Table 2. Formulation of EOC ointment

Constituent	Percentage of constituent (%)
Rosemary oil	3-5
Eucalyptus oil	5-7
Oil of curcuma	4-6
Oil of wintergreen	1-3
Olive oil (Base)	33
Bees wax	30
Lanolin	30

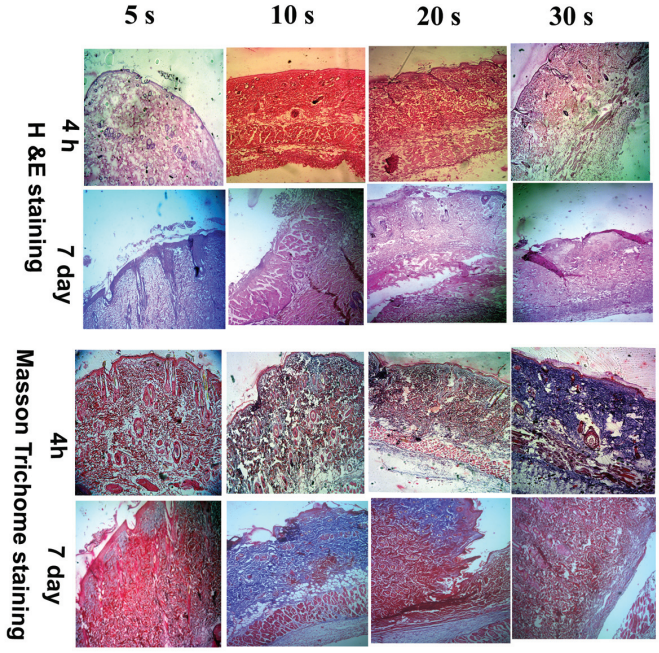

Figure 2. Histopathological evaluation using Haematoxylin-eosin stain and Masson-trichome stain at different stages of wound healing for different cold exposure groups.

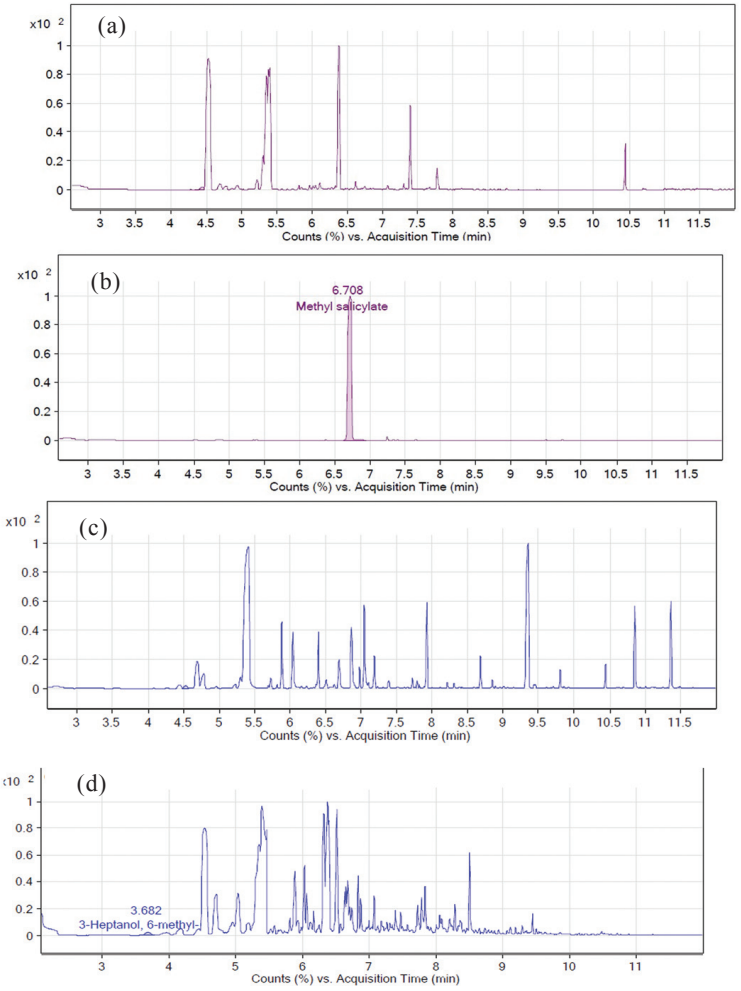

Figure 3. GC-MS Profile for essential oils (a) Rosemary oil, (b) Curcuma oil, (c) Oil of wintergreen, (d) Eucalyptus oil.

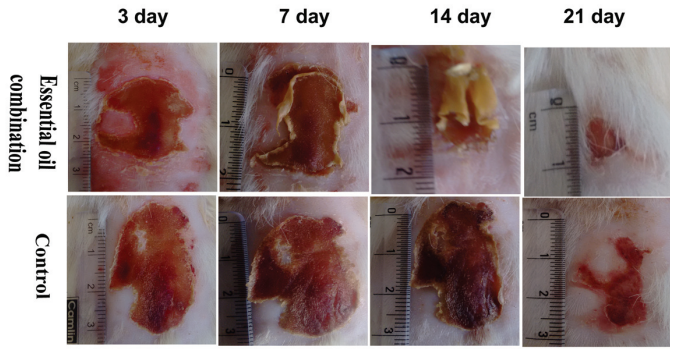

Figure 4. Wound contraction in different stages of frostbite healing by essential oil combination group *vs* control group.

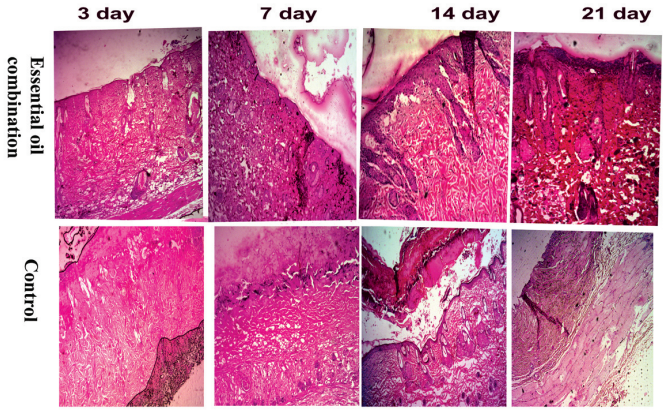

Figure 5. Histopathology of frostbite healing by essential oil combination group *vs* control group.

observed in treatment group in comparison to control group. A clear setting of necrosis post injury can be seen in control group on 7-day, while by 14-day, a complete separation of necrotic tissue is evident. No such severe ischemia is observed in EOC treated group, thus suggesting that the EOC formulation was able to prevent ischemia of tissue. Moreover, due to the healing activity and the vasodialatory activities of EOC, there is an evident restoration of vasculature. This result suggested that the EOC possess a good frostbite healing activity.

4. DISCUSSION

The above results suggest that with the designed device, 5 s exposure could induce partial thickness frostbite and 10 s exposures could create a full thickness wound and induce dermal tissue damage, and upper muscular layers was clearly visible in histology. Such injuries are suitable for evaluation of topical frostbite healing formulation. However 20 s and 30 s exposure lead to irreversible necrosis in epidermal and dermal layer, while severe tissue damage to underlying muscle, fat and inner tissues was also observed. Such wounds are mostly irreversibly damaged and therefore demand tissue sloughing and amputation, thus do not serve the purpose of evaluation of frostbite healing.

Moreover thickness of epidermal fat layer may also cause variation in degree of injury on exposure. However, to our best understanding major variation will only be seen, if there is a major difference of more than 25 % in the body weight and body fat in animals. Validation of such parameters need separate studies. Thus, our study indicates that an exposure of 7-10 s may be recommended suitable for inducing full thickness frostbite wound in wistar rat.

The study of EOC formulation against the frostbite injury showed high efficacy in healing as was evident from the wound contraction rate (80 % reduction) in treatment group on 21-day, while it was only 37 % for the control group. The histopathological evaluations also suggested reduction in oedema, neutrophile infiltration and ischemic damage and increased angiogenesis. These results suggest that essential oil combination possess a good frostbite wound healing property.

5. CONCLUSION

In summary, the device which was designed for inflicting frostbite wound was found to be precise and reproducible. The formulation comprising combination of essential oils was also found to be effective in the treatment of frostbite wound.

Conflict of Interest: None to declare.

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CONTRIBUTORS

Mr Kumud Joshi is a Senior Research Fellow in DRDO-Defence Research Laboratory, Tezpur, India and pursuing PhD from Dibrugarh University. His area of interest includes wound healing, Hemostat development, frostbite and novel drug delivery System.
In the current study, he is involved in conducting the research, and writing of the manuscript.

Dr Danswrang Goyary has completed his MSc and PhD in Biochemistry from NEHU, Shillong. He is currently working as a Scientist 'E' in DRDO-Defence Research Laboratoy, Tezpur, India. His current research interest includes wound healing, Hemostat development, and molecular research of medicinal agents.
In the current study, he is involved in designing, and guiding the research work. He is also involved in writing and reviewing of the manuscript.

Dr Bhaskar Mazumdar, is currently working as Professor at Dibrugarh University, Assam. His research area includes novel drug delivery systems against different diseases including tuberculosis, vector borne disease control and wound healing.
In the current study, he is involved in designing and guiding the research project.

Mr Sumit Kishor, is currently working as a Scientific Technical Assistant, DRDO-Defence Research Laboratory, Tezpur, India. He did his M.Sc from BHU and his area of interest includes microbiology, chromatographical techniques like GCMS, LCMS and high altitude medicine.
He contributed in GCMS experiments and analysis.

Ms Piyali Dey, MPharm is a Senior Research Fellow in Defence Research Laboratory, Tezpur, India and pursuing PhD from IFTM University. She is currently working on vector borne disease control, formulation and development of novel drug delivery system.
She is involved in conducting the research work and editing the paper.

Dr Yangchen Doma Bhutia, is a Scientist 'D' in Defence Research Laboratoy, Tezpur Assam. She completed Her MVSc (Pharmacology) from Indian Veterinary Research Institute, Barelliy. Her research interest includes exploration of pharmacological actions of plants including Haemostatic action and wound healing activities, and toxicity studies in animals.
In the current study, he contributed in conducting research and compiling the data.

Dr Sanjeev Karmakar, MSc, PhD, is currently working as Technical Officer 'B' in DRDO-Defence Research Laboratoy, Tezpur Assam, He has earned his PhD from Gauahati University, His research interest includes immunomodulatory drugs, microbiological research, and cell line based evaluations.
In the current study, he contributed in experiment and editing the paper.

Dr Pronobesh Chattopadhyay, (MPharm, ME, PhD, FNASc) is currently working as a Scientist 'F' in DRDO-Defence Research Laboratoy, Tezpur, India. His major research areas include, vector borne disease control, formulation and development of therapeutic agents like capsaicin riot control agents, snake repellent, sunscreen cream and antidote for toxins.
In the current study, he is involved in designing and guiding the research project.

Dr Sanjai Kumar Dwivedi, PhD, Scientist 'G' and Director, DRDO-Defence Research Laboratoy, Tezpur, has extensively contributed in protected cultivation, characterisation, propagation and conservation of plant genetic resources of Ladakh.
He is involved in guiding the research work and providing all logistic help to accomplish the research work.