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Flash chromatographic separation and diabetic wound healing study of *Hemigraphis alternata* leaves extract

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ABSTRACT:Diabetes, a multifaceted disorder associated with delayed wound healing due to persistent hyperglycaemia and related comorbidities, which increases morbidity, mortality and worsens quality of life. *Hemigraphis alternata* possess wound healing, antioxidant, anti-inflammatory and anti-diabetic properties. This study was conducted to assess the efficacy of *H. alternata* leaf extract in the treatment of diabetic wounds. Hydroalcoholic extract from *H. alternata* leaves was cold-extracted and fractionated via flash chromatography. Collected fractions underwent phytochemical screening for active constituents. An emulgel with the extract was developed and tested for wound healing in STZ + Nicotinamide induced diabetic rats.Flash chromatographic separation of extract yielded 12 fractions and its phytochemical screening showed presence of various phytoconstituents such as alkaloids, phenols, flavonoids, terpenoids, etc which might responsible for wound healing. Wound closure rate in rats treated with formulated emulgel was significantly faster than the untreated diabetic rats. The emulgel group showed low level pro-inflammatory cytokines (IL-6 and TNF-α) and high concentration of VEGF. This study has shown that *H. alternata* emulgel has a significant potential in diabetic wound management by enhancing the release of VEGF and reducing the production of pro-inflammatory cytokines which induces cell proliferation, migration and wound contraction.

KEYWORDS: Diabetic wound healing; Flash chromatography; Emulgel; *Hemigraphis alternata*; Pro-inflammatory cytokines; Diabetes.

1. INTRODUCTION

Diabetes, a chronic metabolic disorder [1, 2], has emerged as a significant public health concern in recent times. It is estimated that approximately 9.5% of the global population is affected by diabetes [3], and this prevalence continues to rise. Notably, 15% to 20% of diabetic patients contend with diabetic wounds [4], often necessitating extended hospitalization for effective management. As per the recent report of the International Diabetes Federation (IDF), in India more than 74 million people are diabetic. In 2021, despite the mortality risks posed by the Covid-19 pandemic, nearly 6.7 million deaths were attributed to diabetes and its associated complications. Diabetes delays the wound healing process by affecting each stage of wound healing, including haemostasis, inflammation, proliferation, and remodelling [4] which constitute a major health issue in diabetic individuals. Multiple complex factors such as hyperglycaemia, neuropathy [5], ischaemia [2], sustained cytokine production (IL-6, TNF-α), impaired keratinocyte and fibroblast proliferation and migration, apoptosis, and impaired vascularization affects each stage of wound healing [6], mainly inflammatory and proliferative phase with impaired or delayed diabetic wound healing.

In normal wound healing, multiple intercellular signals, including proinflammatory cytokines, chemokines, coagulating factors and healing growth factors are released by different cell types like keratinocytes, fibroblasts, endothelial cells, macrophages, etc [6]. However, in the case of diabetic wounds, the complex pathophysiology arises from persistent hyperglycaemia. This condition leads to several challenges, including reduced oxygen supply (hypoxia) to the wound, neuropathy in which patients are unable to realize the occurrence of wound, increased oxidative stress due to the overproduction of reactive oxygen species (ROS) triggered by advanced glycation end products (AGEs), and the continuous release of

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pro-inflammatory cytokines like TNF-α, IL-1, and IL-6. This chronic inflammation suppresses the production of anti-inflammatory cytokines, such as IL-10 [6], perpetuating the anti-inflammatory phase and delaying the healing process. High level of TNF-α causes tissue necrosis and low level of VEGF slows the revascularization or angiogenesis [7] leading to impaired wound healing [8].

Hemigraphis alternata, (Figure 1) belonging to the Acanthaceae family, is a plant indigenous to India [9]. This perennial herb possesses remarkable wound healing potency, hence in Malayalam it is called 'Murikootti' or 'Murianpacha' while in Sanskrit it is known as "Vranaropani" [10]. The plant also goes by various other names, including Red Ivy, Red flame Ivy, Waffle plant, Java Ivy, etc [11]. Prior research supports the presence of significant active phytoconstituents in this plant, such as phenols, flavonoids, terpenoids, saponins, coumarins, carboxylic acid, cinnamic acid, and tannins, which are believed to contribute to its wound-healing capabilities.

Figure 1. *Hemigraphis alternata*

The pharmacological properties of *H. alternata* that support wound healing include its anti-inflammatory [9], antioxidant [12] and antimicrobial [13] properties. Despite these potential benefits, it is noteworthy that there has been limited research on this plant to date. Therefore, there is a need for a comprehensive investigation into its phytochemical composition and pharmacological properties to ascertain its potential for wound management. This study aims to identify the natural phytoconstituents present in the enriched fractions of HA (hydroalcoholic) extract from *H. alternata*, which were isolated using a flash chromatography system and further analysed using High Performance Thin Layer Chromatography (HPTLC) technique. Additionally, the study explores the wound healing potential of HA extract formulated as an emulgel in diabetic rats.

2. RESULTS

The phytochemical testing of the HA extract with percentage yield 10.35% (w/w) showed presence of major phytoconstituents including phenols, flavonoids, tannins, steroids and carbohydrates. Total phenolic and flavonoid content of HA extract, calculated from the standard calibration curve (R2 = 0.9924 and y = 0.0169x + 0.4772) and (R2 = 0.9959 and y = 0.0084x - 0.0161) was found to be 252.5 mg GA equivalents per g of extract and 456.6 mg rutin equivalents per g of extract respectively. DPPH assay of HA extract demonstrated maximum radical scavenging activity (95.13 %) with 6.88 µg/mL IC50 value when compared to ascorbic acid standard (IC50: 18.13 µg/mL).

2.1. HPTLC Fingerprinting analysis

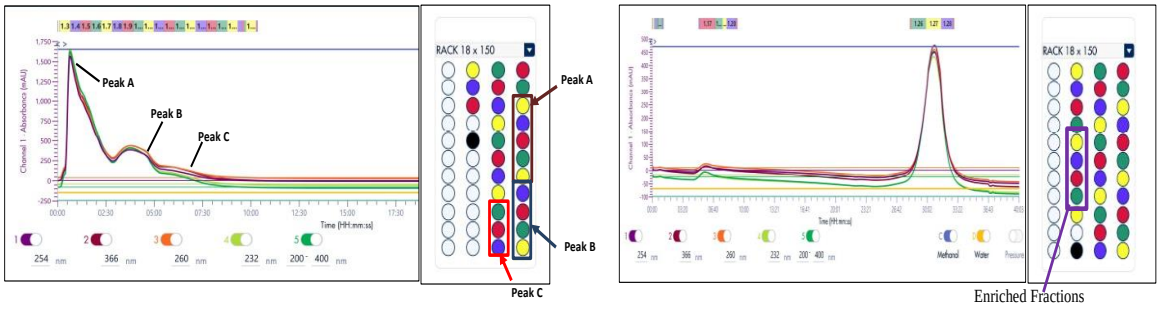
The HPTLC densitometric analysis of plates developed with methanol-water (95:5 v/v) at 366 nm showed fairly well resolved peaks with different Rf values. Post derivatization after heating the plate at 105^{o}C for 5 min, the plate with NP/PEG reagent produced intense fluorescence (orange-yellow and green bands) immediately after 5 min which indicated the presence of flavonols and flavones.

2.2. Flash chromatographic separation and HPTLC

Initially, total 8 enriched fractions i.e., fractions containing peaks (F1-F4 & F26-F29) were separated and collected from the HA extract. These collected fractions were further analysed by HPTLC on Reverse Phase (RP)-TLC plates to observe the band separation. Based on the HPTLC of enriched fractions i.e., F3 and combined F26-29 fractions were further loaded on to the RP flash column using same mobile phase for further separation of the enriched fractions.

Flash chromatographic separation of enriched Fraction

Flash chromatogram (Figure 2-a) of concentrated enriched fraction F3 showed 3 peaks i.e., peak A, B & C and fractions containing peak A, B and C were named as fractions F3-1, F3-2 and F3-3 respectively. Flash chromatogram (Figure 2-b) of combined and concentrated enriched fractions F26- F29 showed one tall peak which were named as fraction F26-1. Phytochemical evaluation of flash chromatographic enriched fractions showed the presence of natural phytoconstituents i.e., alkaloids, tannins, flavonoids and phenols [14].



(a) **(b)**

Figure 2. Flash *Chromatogram* of (a) Fraction F3 (b) combined fractions F26-F29.

2.3. Formulation of Emulgel containing *H. alternata* extract (HA Emulgel)

Homogenous, consistent and non-greasy HA emulgel with pH 6.52 was prepared with good spreadability (35.01 g cm/sec), extrudability (249 g/cm^2) and minimum water loss. The prepared emulgel formulation showed shear thinning property with the viscosity returning to normal as soon as the shear was removed.

2.4. In-vivo diabetic wound healing study

In the skin irritancy test of HA emulgel and gel base, 0 score was observed, which indicated the safety of formulation for topical application.

Wound measurement

Wounds were serially photographed using a digital camera (CANON) and the wound size of each rat was measured on respective days (0,3,6,9,12,14,16,18,21) using digital vernier calliper (Baker, SDN10). Figure 3 depicts the percentage wound contraction of diabetic control and groups treated with standard and HA emulgel with photographs of wounds. The mean, SD, and percent wound contraction were then calculated and compared with diabetic control for that day. From day 9 wound healing in rats treated with HA emulgel was found to be much faster as compared to diabetic control. In groups treated with standard and HA emulgel re-epithelialization was observed from day 12 while in diabetic control group re-epithelialization was not observed. The mean body weight (248 g, 243 g, 238 g, 231 g) and blood glucose level (290 mg/dL, 269 mg/dL, 261 mg/dL, 259 mg/dL) of all diabetic rats were measured on 0, 7th, 14th and 21st day of the study respectively.

2.5. Histological Evaluation

Histological sections of active wound and surrounding tissue of the wound site was carried out, ensuring a comprehensive examination. Representative images were selected to depict key histological features accurately. These images were chosen based on their ability to illustrate important findings, such as tissue necrosis, epithelialization, collagen deposition, and neovascularization. Care was taken to include images that best exemplify the observed histological changes in both untreated diabetic rat wounds and those treated with standard and HA emulgel. The histological evaluation of wound tissue isolated from diabetic rats (untreated) showed severe multifocal tissue necrosis and no epithelialization, no neovascularization was observed. This finding indicates a significant impediment to the natural healing process in untreated diabetic rats, likely due to impaired vascular function and reduced tissue perfusion associated with diabetes. The absence of epithelialization in the untreated diabetic rat wounds suggests a failure of the epithelial cells to migrate and proliferate effectively to close the wound. This may be attributed to dysregulated inflammatory responses, altered growth factor expression, or other factors characteristic of diabetic wounds. Wound tissues isolated from diabetic rats treated with standard and HA emulgel displayed no tissue necrosis.

However epithelialization, collagen deposition and neovascularization were observed (Figure 4) which are extremely benefial in the wound healing process.The absence of tissue necrosis in wounds treated with standard and HA emulgel suggests that these treatments effectively restored tissue viability and controlled widespread cell death, indicating a positive effect of the treatments on improving tissue perfusion, reducing inflammation, or enhancing cellular survival mechanisms.The presence of epithelialization in standard and HA treated wounds indicates successful closure of the wound bed by a new layer of epithelial cells. This suggests that the treatments promoted epithelial cell migration, proliferation, and differentiation, facilitating wound closure and restoration of tissue integrity.

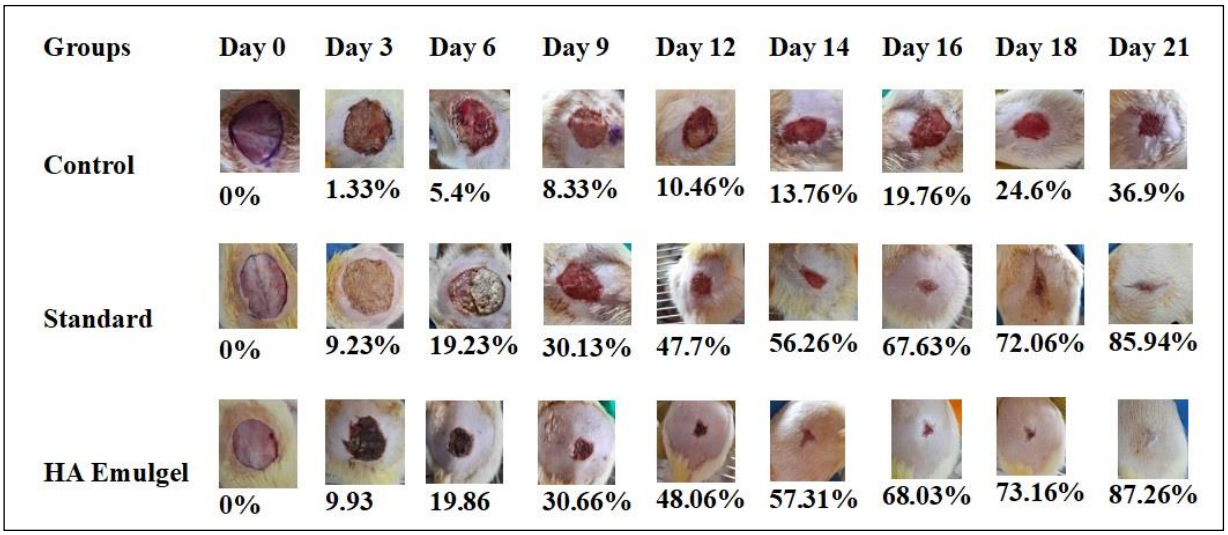


Figure 3. Respective images of wounds of diabetic control, standard and HA emulgel treated groups with % wound contraction on 0,3,6,9,12,14,16,18,21 days.

Figure 4. Histological examination of wound tissue of diabetic control and animals treated with standard and HA Emulgel

2.6. Statistical Analysis

Rats treated with HA emulgel formulation showed moderately significant (***p < 0.0001) wound contraction on day 9 and highly significant wound contraction (****p < 0.0001) from day 12 to day 21 as compared to the untreated diabetic control. Continuation of treatment till the 21 days, resulted in significant reduction in wound size of animals treated with HA emulgel formulation. All values are expressed as a Mean ± SEM, n = 6, vertical lines represent SEM. All data are subjected to one-way ANOVA following by Tukey's multiple comparison test. Disease control group was compared with all groups (Standard and HA emulgel) and standard group was compared with HA emulgel group. *p <0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: non-significant. Statistical data of wound contraction (Figure 5) showed significant reduction in wound size.

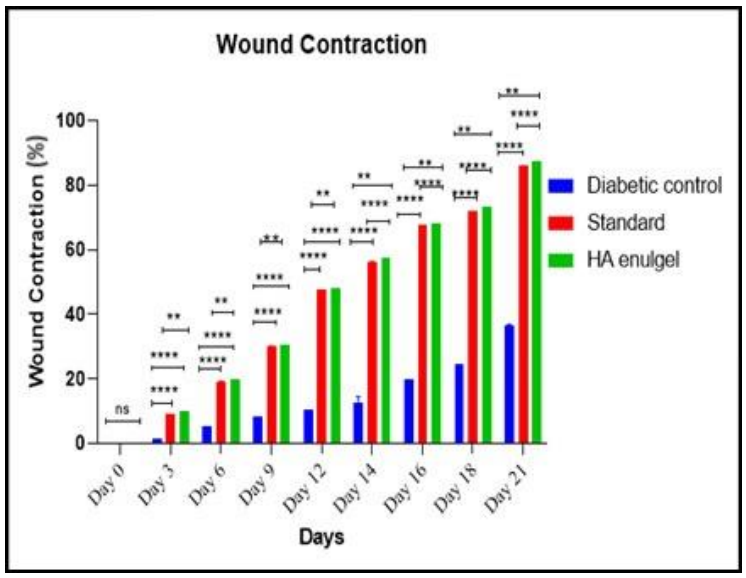


Figure 5. Statistical data of wound contraction (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: non-significant; Mean ± SEM, n = 6)

2.7. Effect of HA emulgel on the production of IL-6, TNF-α and VEGF

Figure 6 illustrated that in wound tissue of diabetic rats (untreated), the pro-inflammatory cytokine level was found to be significantly elevated with IL-6 (2740.333 pg/mL) and TNF-α (446.312 pg/mL) and level of healing growth factor (i.e., VEGF- 101.528 pg/mL) was found to be significantly low, while groups treated with standard and HA emulgel showed significant reduction of pro-inflammatory cytokines with IL-6 (653.5 pg/mL) and TNF-α (286.3125 pg/mL) in standard and IL-6 (438.5 pg/mL) and TNF-α (153.1875 pg/mL) in HA emulgel treated groups and level of healing growth factor (VEGF) was found to be significantly high in groups treated with standard (1180 pg/mL) and HA emulgel (910 pg/mL) as compared to diabetic control group which showed significantly high wound contraction in treatment groups.

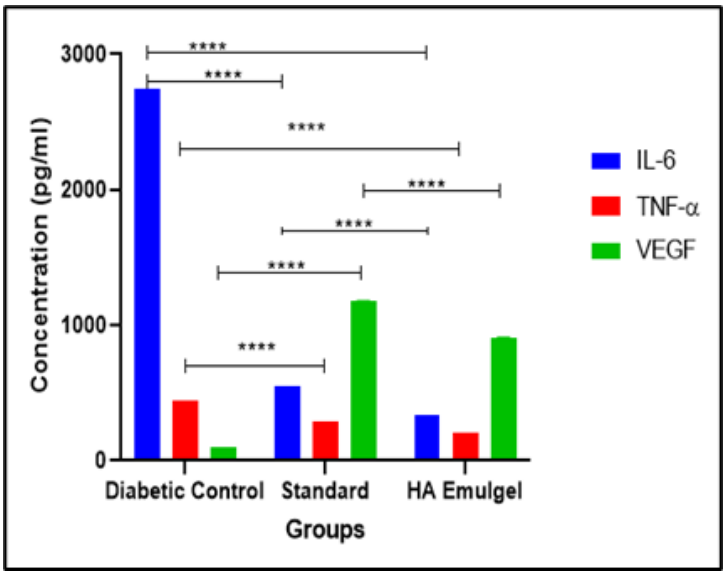


Figure 6. Effect of HA emulgel on production of IL-6, TNF-α and VEGF in wound tissue

3. DISCUSSION

The present study explored the phytochemical constituents and wound healing properties of *H. alternata* (HA) extract, with a focus on its potential application in diabetic wound management. The high levels of total phenolic and flavonoid content of HA extract are indicative of the potential antioxidant capacity of HA extract. Antioxidants play a crucial role in wound healing by neutralizing harmful free radicals and reducing oxidative stress, which is known to delay the wound healing process. The DPPH assay

further confirmed the antioxidant potential of HA extract, with the superior antioxidant activity than ascorbic acid, a well-known antioxidant standard. This result highlights the efficacy of HA extract in combating oxidative stress, which is a common impediment in diabetic wound healing.

The HPTLC fingerprinting analysis of HA extract provided insight into its phytochemical composition, with a particular emphasis on flavonols and flavones. The flash chromatographic separation of HA extract yielded enriched fractions containing various bioactive compounds, including alkaloids, tannins, flavonoids, and phenolics. These compounds have the potential to exhibit antimicrobial, anti-inflammatory, and wound healing properties. By controlling infection, reducing the production of reactive oxygen species, and promoting collagen deposition, these compounds can aid in the acceleration of wound healing in diabetic individuals.

For topical application, an emulgel containing HA extract is prepared. In the in-vivo diabetic wound healing study, HA emulgel was found to be both safe and effective for topical application. The results demonstrated a significant improvement in wound contraction from day 9, with re-epithelialization observed in groups treated with HA emulgel. This suggests that the HA emulgel promotes wound closure and accelerates the healing process, a vital outcome for diabetic individuals who often experience delayed wound healing. Histological evaluation further supported the positive effects of HA emulgel, showing that tissue necrosis was absent in treated groups, while collagen deposition and neovascularization were observed. These histological changes are indicative of successful wound healing and tissue regeneration. Finally, the evaluation of pro-inflammatory cytokines (IL-6 and TNF-α) and the healing growth factor VEGF in wound tissues revealed a significant reduction in inflammation and an increase in tissue regeneration in HA emulgel-treated groups. This further substantiates the potential of HA extract in promoting a favourable wound healing environment in diabetic individuals.

4. CONCLUSION

In conclusion, the results of this study provide strong evidence for the wound healing properties of *H. alternata* extract, especially when formulated into an emulgel. The presence of beneficial phytoconstituents, high levels of antioxidants, and the observed effects on wound contraction, tissue regeneration, and inflammatory cytokines all support the potential use of HA extract in diabetic wound management. Further research is essential to explore the detailed mechanisms underlying these effects and to assess its safety and efficacy in clinical settings.

5. MATERIALS AND METHODS

5.1. Plant material

The experimental plant *Hemigraphis alternata* was collected from "Chaitanya Vilas Nursery", Bhiwandi, Thane, Maharashtra, India in December 2021 and authenticated at Regional Ayurveda Institute for Fundamental Research, Pune, Maharashtra, India with authentication number 14666. The leaves of the plant were used for the study.

5.2. Chemicals used

All analytical grade solvents (ethanol and methanol), excipients (carbopol 934, liquid paraffin, propylene glycol, span 20, tween 20, methyl and propyl paraben) were purchased from Loba Chemie Pvt Ltd, Mumbai, India. Standard drugs (Gallic acid, Rutin, Ascorbic acid Streptozotocin and Nicotinamide) and reagents (Aluminium chloride, DPPH, 2-aminoethyldiphenylborate and Folin-Ciocalteu) used were supplied by Sigma Aldrich, Darmstadt, Germany and Sisco Research Laboratories Pvt. Ltd, Andheri, Mumbai, India.

5.3. Preparation of Extract

The hydroalcoholic (HA) extract of *H. alternata* leaves was prepared by cold extraction method by soaking the fine powder of dried leaves (56 g) in mixture of ethanol and distilled water (in 50:50 ratio) with periodic stirring & shaking for 4-5 days followed by filtration using Whatman filter paper. The filtrate was then evaporated to dryness using vacuum oven (Being; DZF6032) under reduced pressure at 70°C. The prepared extract was then stored in the refrigerator (LG) until further use.

5.4. Total Phenolic Content (TPC)

The TPC of HA extract was determined by the Folin–Ciocalteu method with some modifications using Gallic acid (GA) as the standard [15]. 1 mL sample solution of HA extract (1000 µg/mL) was mixed with 1 mL of 10% Folin-Ciocalteu (FC) reagent solution& kept for 3 min, then 10% Na_2CO_3 solution (2 mL) was

added. and final volume was made with distilled water up to 10 mL. The mixture was then allowed to stand for 30 mins in dark room and the absorbance was measured spectrophotometrically at 650 nm. Using the standard (GA) calibration curve, the TPC was calculated and expressed as mg of GA equivalent per g of dry HA extract.

5.5. Total Flavonoid Content (TFC)

The TFC of HA extract was estimated by the aluminium chloride colorimetric method with some modifications using Rutin as the standard [16]. 1 mL sample solution of HA extract (1000 µg/mL) was mixed with 0.3 mL 10% $AlCl_3$, 5% $NaCO_2$ solution and 1 M NaOH solution (2 mL) and final volume was made with distilled water up to 5 mL. The mixture was then left at room temperature for 15 min and the absorbance of the mixture was measured spectrophotometrically at 415 nm. From the standard (Rutin) calibration curve, the TFC was calculated and expressed as mg of rutin equivalent per g of dry HA extract.

5.6. Antioxidant activity (Radical scavenging activity) using DPPH assay

The HA extract of *H. alternata* was screened to evaluate its free radical scavenging activity by DPPH assay with slight modifications using ascorbic acid as the standard [17]. 0.135 mM DPPH solution prepared in methanol was mixed with 1 mL sample solution of HA extract at different concentration (5, 10, 20, 40, and 60 µg/mL) and then allowed to stand in the dark room at RT for 30 min. The final absorbance was then measured at 517 nm. Percent radical scavenging activity of HA extract was calculated using following equation:

$$\% \text{ RSA} = \frac{(\text{A control}) - (\text{A sample}) \times 100}{(\text{A control})}$$

where,

A control = absorbance of blank DPPH solution

A sample = absorbance of sample solution

% RSA (Radical scavenging activity) was plotted against concentration in µg/mL and IC50 value of HA extract was calculated.

5.7. HPTLC Fingerprinting analysis

The HPTLC fingerprinting analysis was carried out using CAMAG HPTLC system (Switzerland), controlled by winCATS software (Version 1.4.6) and consisting of an automatic sample applicator (Linomat 5) with a 100 μL syringe, UV chamber (CAMAG) and the CAMAG TLC scanner 3. For the separation of phytoconstituents, reverse phase precoated silica gel 60 RP-18 F254s aluminium plates (5 x 7.5 cm; Merck, Germany) was used as the stationary phase and methanol-water (95:5 v/v) was employed as the mobile phase. Saturation of development chamber was done according to standard procedure of USP-203 [18]. After development, the TLC plate was dried in air and visualized at 254 nm/ 366 nm under CAMAG UV chamber to observe the separation of bands. HPTLC densitometric analysis was performed at slit dimension 6 × 0.45 mm by scanning the TLC plate at 366 nm with 20 mm/s of scanning speed using tungsten lamp as the radiation source in absorbance mode before derivatization with NP/PEG and Dragendroff's reagents.

5.8. Flash chromatographic separation

For separation of enriched fractions (i.e., fractions containing peaks) from HA extract of *H. alternata*, an automated flash chromatography system (puriFlash 5.020, Interchim), controlled by the Intersoft x "Genius" software and consisting of quaternary gradient pump, RJ45 control unit, UV detector and fraction collector was utilized. To separate the enriched fractions, pre-packed reversed phase flash column IR-50C18-F0012 (50 µm; PURIFLASH C18-STD F0012) as a stationary phase and methanol-water (95:5 v/v) as the mobile phase was utilized. Flash chromatographic separation was done by loading the sample onto the pre-packed 12 g IR-50C18-F0012 RP flash column via direct injection mode in liquid form followed by 10 min equilibration of the column with 100% methanol at 15 mL/min flow rate, peak threshold 0.1 mA units, peak collections into 8 mL test tubes. The individual enriched fractions were collected and further analysed by HPTLC method using previous procedure to visualize the separation of bands. Enriched fractions collected from flash chromatography further concentrated and screened phytochemically for the presence of phytoconstituents (Tannins, Alkaloids, Flavonoid and Phenol).

5.9. Formulation of Emulgel containing *H. alternata* extract (HA Emulgel)

The HA emulgel was prepared by incorporating the Carbopol 934 based gel phase and emulsion (w/o) in 1:1 ratio with gentle stirring. The aqueous phase containing of HA extract, tween 20, propylene glycol, methyl and propyl paraben in purified water was dispersed into the oil phase (containing span 20 in light liquid paraffin), with constant mechanical stirring with heating of the two phases to 50-60°C prior to the addition to obtain the emulsion [19]. The pH of emulgel formulation was adjusted to 6-6.5 using triethanolamine (TEA) [19,20]. Table 1 represents the composition of HA emulgel.

Table 1. Composition of HA emulgel formulation in % w/w.

Sr. No.	Excipients	Quantity (%w/w)	Indication
1	HA extract	4%	Active constituent
2	Carbopol 934	2%	Gelling agent
3	Light liq. paraffin	5%	Thickening agent
4	Propyl paraben	0.02%	Preservative
5	Methyl paraben	0.18%	Preservative
6	Propylene glycol (PG)	1%	Thickening agent
7	Tween 20	1.5%	Emulsifier
8	Span 20	1%	Emulsifier
9	Purified Water	q.s.	Solvent

5.10. Physicochemical evaluation of HA Emulgel

The prepared emulgel formulation was evaluated for various physicochemical parameters such as visual inspection for its colour, homogeneity, consistency, phase separation and grittiness; pH measurement using a digital pH meter (Elico pH meter, LI-120); viscosity measurement to investigate its flow property using Brookfield viscometer (Model-RVDV-II + pro), spindle 96 TFat 37 ± 0.5ºC] [21]; spreadability [22] using the spreadability apparatus fabricated in laboratory. The spreadability factor (S) was calculated by recording the time in seconds required to separate the two slides using formula, S = M.L / T, where M = weight tied to pulley; L = length of glass slide and T = time required to separate the slide. The rate of evaporation of water from the emulgel was measured to assess its potential to lose/ adsorb water which is also a critical factor for wound healing. The %water loss was calculated as, Percent water loss (%) = $(W_0\text{-}W_t)/W_0 \times 100$ Where, W_0: Initial weight; W_t: Weight after time 't'. The extrudability of emulgel was measured by recording the force in g/cm² required to extrude the emulgel from collapsible tubes.

5.11. In-vivo Diabetic Wound healing study

5.11.1. Animals

After due approval (DYPIPSR/IAEC/Oct/21-22 P-07") from Institutional Animal Ethic Committee (IAEC), the diabetic wound healing study was carried out on animal model (male wistar rats weighing 200–300 g). Seven days before the commencement of study, experimental animals were housed separately in polypropylene cages at a regulated ambient temperature (25-2°C) and 60-70% relative humidity. The animals received a conventional pellet feed, water, and was kept in a typical light/dark cycle for care. The experimental protocols were carried out in accordance with the standards defined by the Indian National Science Academy.

5.11.2. Skin irritation Test

According to the OECD 404 guideline [23], the skin irritancy test of the prepared HA emulgel was done by progressively applying the emulgel and gel base to the experimental animals (up to three test patches), and monitoring the animals for a period 14 days.

5.11.3. Induction of diabetes

In experimental rats, diabetes was induced by intraperitoneal injection of Streptozotocin (STZ) (45 mg/kg body weight) and Nicotinamide (110 mg/kg) after 12 hours of fasting. Fasting blood glucose level was measured after 72 hours of STZ and nicotinamide injection by collecting blood from the retro-orbital plexus (ROP) route using glucometer (Accu-chek active) [24]. The animals with blood glucose level greater than 250 mg/dL were considered as diabetic and utilised for the further study [25].

5.11.4. Excision wound model

The diabetic rats were divided in 3 groups (n=6/group) and after anaesthetizing the rats using diethyl ether, the dorsal region of each animal was shaved and then cleaned with sterile water [26]. The full-thickness 30 x30 mm excision on the shaved skin was made [27,28]. Group 1 was kept as the diabetic control without any treatment. Group 2 and Group 3 were treated with standard formulation (Himalaya antiseptic cream, each 1g contains 200 mg aloe vera, 10 mg nirgundi and vatadha, 5 mg manjishta extracts) and test formulation (HA emulgel) respectively. The formulations, both standard and test were applied evenly in sufficient quantity to cover the entire wound. Wounded animals were treated twice a day for a period of 21 days and the re-epithelisation was recorded. Rats were housed in separate cages.

5.11.5. Measurement of wound size

The wound closure rate was estimated by measuring the wound size using digital Vernier calliper on respective days (0, 3, 6, 9, 14, 16, 18 and 21) after wound creation. The percent wound contraction was calculated for each animal as below [29].

% Wound Contraction:

$$\frac{\text{0 Day wound size} - \text{n Day wound size X 100}}{\text{0 Day wound size}}$$

n= No of days (3,6,9,12,14,16,18,21)

5.12. Histological examination

To verify the maturity of the repaired tissues, cellular responses and wound tissue vascularization was tracked and histological examination was performed at the termination of study by dissecting the skin tissue samples from the wound region, including the dermis, epidermis, and subcutaneous layers and fixing it into a 10% formalin buffer solution. A pathologist prepared the paraffin-embedded slices (3-5 m thick) of tissue, stained them with haematoxylin and eosin, and then examined under a 40x microscope to determine the degree of tissue development, collagen deposition, neovascularization, re-epithelization and scar formation. The concentration of proinflammatory cytokines, including IL-6and TNF-α as well as healing growth factor (VEGF) in wounded tissue, were measured according to standard procedure i.e., Enzyme Linked Immunosorbant Assay (ELISA).

5.13. Statistical analysis

We used Graph Pad InStat (version 8) software to analyse the results of relative wound size and percent wound contraction, and compared the data using One-Way Analysis of Variance (ANOVA) followed by Dunnett's tests and Tukey's multiple comparison test, with multiple comparisons such as Control vs Standard; Control vs HA emulgel; Standard vs HA emulgel. A *p <0.05 or **p <0.01 was considered to be statistically significant.

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Author contributions: Concept – A.T., S.C; Design – A.T., L.K., N.S.; Supervision – A.T.; Resources – N.S., A. T., S.C.; Materials – A.T., V.B.; Data Collection and/or Processing – N.S., V.B., P. J.; Analysis and/or Interpretation – A.T., L.K.; Literature Search –N.S., V.B.; Writing – P.J., A.T.; Critical Reviews – A.T., L.K., S.C.

Conflict of interest statement: The authors declared no conflict of interest.

Ethics Approval and Consent to Participate: After due approval from Institutional Animal Ethic Committee (IAEC) with protocol number (DYPIPSR/IAEC/Oct/21-22 P-07"), the diabetic wound healing study was carried out in accordance with the standards defined by the Indian National Science Academy. Skin irritation test was performed according to the OECD 404 guideline.

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