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Research Article
Development and Evaluation of a Polyherbal Ointment for Enhanced Wound Healing and Antimicrobial Activity

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Abstract
Background and Objective: *Ziziphus nummularia* (ZN), *Nigella sativa* (NS) and *Thymus vulgaris* (TV) are traditional herbal remedies known for their wound-healing properties. This study aimed to develop and assess a polyherbal ointment containing ZN, NS and TV and to evaluate the synergistic potential of the polyherbal formulation for its wound healing and antimicrobial efficacy. **Materials and Methods:** Methanolic extracts of the leaves were utilized to prepare formulations using the fusion method. Four formulations were prepared: Three consisting of individual herbal extracts (I, II and III) and one as a polyherbal ointment (PHO). Physical parameters such as pH, stability and spreadability were evaluated. *In vivo* wound healing activity was assessed using an excision wound model, measuring wound contraction and epithelialization period. Antimicrobial potential was evaluated against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The results were analyzed using statistical methods, with significance defined as $p<0.05$ for all comparisons. **Results:** The prepared formulations were found to have satisfactory physicochemical properties, including spreadability, pH and loss on drying. Over time, all formulations showed a decrease in wound area compared to controls. The PHO showed the highest level of activity (11.66±0.37) when compared to the individual herbal formulations (*Z. nummularia*: 14.33±0.59 day, *N. sativa*: 16.33±0.37 days and *T. vulgaris*: 15.83±0.71 days). When tested against all organisms, PHO showed significant antibacterial activity at 200 µg/mL. **Conclusion:** The polyherbal ointment showed remarkable wound healing and antimicrobial properties, suggesting its potential for wound management. These effects likely stem from the synergistic interactions of active phytoconstituents within the herbs, including essential oils and phenolics.

Key words: Polyherbal ointment, *Z. nummularia*, *N. sativum*, *T. vulgaris*, wound healing, antimicrobial efficacy

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Various herbal medicines have been used to treat wounds as they are the richest source of bioactive components. Wounds are described as physical injuries that cause breaking or opening of the skin, accompanied by disruption of structure and function; this needs to be restored by appropriate methods. The healing process of wounds involves various steps, such as the inflammatory phase, the formation of granulation tissue, the repair of connective tissue, etc[1]. Natural medicines are popular due to the belief that they have fewer side effects, raw material availability and cost-effectiveness. The exploration of herbal wound products has emerged as a significant area of recent research, seeking to unravel the intricacies of the wound healing process. This field endeavors to expedite healing by regulating inflammatory mediators such as nitric oxide, cytokines, eicosanoids and various growth factors[2]. Throughout history, combinations of herbs have been employed for disease treatment, reflecting ancient therapeutic practices that recognized the synergistic benefits of herb-herb combinations.

Individual medicinal herbs contain active phytoconstituents, but these are present in small quantities and hence may be insufficient to produce the desired activity. Indeed, polyherbal formulations play a pivotal role in wound healing due to the diverse pharmacological targets addressed by the selected herbs. These targets include the suppression of inflammatory transduction cascades, reduction of oxidative factors and prevention of microbial infections at the wound site[1,3,4]. Through their multifaceted action, polyherbal formulations offer a comprehensive approach to wound management, addressing various aspects of the healing process simultaneously. Several reports of plants having wound-healing activity are available in the literature. However, there is still a bountiful reserve of unexplored plants. These agents of natural origin heal the wound by multiple mechanisms, as they consist of a wide array of phytoconstituents. The antioxidant, antimicrobial, anti-inflammatory and analgesic properties of the natural agents present in the plants influence the wound-healing activity and help expedite the wound-healing process through synergistic effects[5]. However, there is a need for standardization and scientific validation of the claims of their traditional use as wound healing agents before their recommendation for wound healing. A topical ointment containing antibacterial properties can be used in the treatment of bacterial infections of wounds. A polyherbal ointment can be more beneficial in healing wounds and preventing infections due to the presence of various phytoconstituents, which may possess synergistic effects. These ointments may serve several purposes by acting as astringent, emollient, protectant and antiseptic[6]. There has been an increasing trend of scientific research in herbal medicine.

The herbs chosen for the investigation-*Z. nummularia* (Burm. f.) Wight & Walk. Arn, *N. sativa* L. and *T. vulgaris* L. belong to the families Rhamnaceae, Ranunculaceae and Lamiaceae, respectively. Their selection was based on documented biological activities found in the literature. *Ziziphus nummularia* has been reported to possess a range of beneficial properties, including antioxidant, anti-inflammatory, antimicrobial, antiproliferative, hypoglycemic and hypolipidemic effects[7]. In traditional and indigenous medicine, various dosage forms of *N. sativa* have been used to manage a wide range of acute and chronic health issues. These conditions encompass respiratory issues such as asthma and bronchitis, as well as pain and inflammation, skin allergies, high blood pressure, diabetes, liver problems, rheumatism, mental health disorders, malaria, decreased appetite, insomnia, snake bites, digestive issues and menstrual pain[8]. *Thymus vulgaris* is reported for antibacterial and antifungal properties besides, it is traditionally claimed for antitussive, antibroncholitic, antispasmodic, anthelmintic, carminative and diuretic properties[9]. Overall, the three selected herbs are known for their anti-inflammatory, antibacterial and analgesic properties, which can influence various phases of the wound healing process. Additionally, traditional knowledge from the local population indicates that these plants were historically used to treat ulcers and acne and were valued for their astringent properties. This historical use and literature validation justifies their selection for our study. Also, polyherbal formulations, containing a combination of phytoconstituents, offer advantages over isolated chemicals[7,10-12]. Therefore, this study aimed to formulate, assess and explore herbal and polyherbal ointments' antimicrobial and wound-healing potential.

MATERIALS AND METHODS

Collection of plant material: This study was conducted from January to December, 2023. The plant material was obtained from the local market of Rafha, located in the Northern Border Province of Saudi Arabia and voucher specimens PC-2023-3, PC-2023-4 and PC-2023-5 were deposited at Northern Border University. A senior colleague at the Department of Pharmacognosy, College of Pharmacy, Northern Border University, Saudi Arabia, authenticated the samples. The leaves were pulverized to obtain coarse powders, which were stored in tightly sealed containers for future use.

Preparation of herbal extract: The dried powder (100 g) of *Z. nummularia*, *N. sativa* and *T. vulgaris* leaves was extracted separately with methanol by maceration for 24 hrs. The three extracts thus obtained were concentrated and stored in separate airtight containers until further use.

Preliminary phytochemical screening was performed for the methanolic extracts for the presence of phytoconstituents such as flavonoids, glycosides, alkaloids, etc., using standard methods[13].

An emulsifying ointment base was prepared by the fusion method[5]. The required quantity of emulsifying wax, soft white paraffin and liquid paraffin was weighed and melted together until congealed. Table 1 and 2 depict the contents of the formulations.

The herbal ointments were prepared by weighing and incorporating 1 g of each of the methanolic extracts separately into the emulsifying ointment base to obtain formulations I, II and III (1% concentration). The polyherbal ointment (formulation IV) was prepared by incorporating 1 g of extract each into the emulsifying base (3% concentration (1% of each extract)).

Physical evaluation of formulated ointments: The prepared herbal and polyherbal formulations underwent assessment for various physicochemical properties, including colour, odour, washability, homogeneity, pH and spreadability[14,15].

Organoleptic parameters: The evaluation of colour and odour for all formulations was conducted through physical examination.

Determination of pH: The pH of the ointments was determined using a digital pH meter (pH211-Hanna). The amount of the ointment sample was dissolved in distilled water and the pH was measured three times. The average of these three measurements was recorded as the pH of the ointment.

Loss on drying: This parameter is assessed by placing the formulations in a Petri dish in a water bath (Lab Zone Industries, Ambala, Haryana, India) and drying them until a constant weight is achieved.

Spreadability: The spreadability of the ointments was determined by measuring the time, in seconds, it took for two glass slides to separate when a specific load was applied. The spreadability (S) was calculated using the formula[15]:

$$S = \frac{M \times L}{T}$$

Where:
M = Weight attached to the upper slide
L = Length of the glass slides
T = Time taken for separation

Stability studies: The formulations underwent stability studies for 3 months under three different temperature conditions (4, 27 and 37°C). Phase separation and visual changes were monitored to assess stability.

Homogeneity: Visual inspection was employed to assess the homogeneity of each formulation.

Skin irritation study: The skin irritation study was evaluated using healthy rats. An area of approximately 4 cm^2 was depleted on either side. One side was utilized for the control

Table 1: Preparation of the emulsifying ointment base

Ingredients	Quantity required (g)	Quantity taken (g)
Emulsifying wax	300	150
White soft paraffin	500	250
Liquid paraffin	200	100
Total quantity	1000	500

Table 2: Preparation of the herbal and polyherbal formulations

	Formulation			
Treatment	I (ZNO)	II (NSO)	III (TVO)	IV (PHO)
Ziziphus nummularia	1 g	-	-	1 g
Nigella sativa	-	1 g	-	1 g
Thymus vulgaris	-	-	1 g	1 g
Emulsifying ointment base	up to 100 g	up to 100 g	up to 100 g	up to 100 g

ZNO: *Ziziphus nummularia* ointment, NSO: *Nigella sativa* ointment, TVO: *Thymus vulgaris* ointment and PHO: Poly herbal ointment and the other for the test ointment. The ointment containing *Z. nummularia* extract was applied once daily for seven days and was later observed for any sensitivity or adverse reactions like erythema and edema. The same procedure was performed with the other ointments.

Evaluation of the wound-healing activity of the formulations: The wound healing activity was evaluated using the excision wound model[5]. Male and female Sprague Dawley rats, weighing between 250 and 300 g, were obtained from a licensed local animal supplier for this study. Throughout the experiment, these animals were housed under standard conditions, provided with commercial pelleted rat food and had access to water *ad libitum*.

Ethical consideration: The study strictly adhered to ethical guidelines established by the Committee for Control and Supervision of Experiments on Animals and the experimental protocol obtained approval from the Institutional Animal Ethical Committee (SBRL/IAEC/09/23-24).

Study design: Each rat was individually housed in cages and anesthetized with ether. A mark was made on the dorsal thoracic region, specifically 1 cm away from the vertebral column and 5 cm away from the ear. Subsequently, a wound area of approximately 500 mm^2 was created by excising the full thickness of the skin.

Subsequently, the 42 animals were divided into seven groups, with each group containing six animals and treated as follows:

- **Group I:** Emulsifying base
- **Group II:** Standard nitrofurazone 0.2% (w/w) ointment
- **Group III:** Control
- **Group IV:** *Ziziphus nummularia* ointment (ZNO)
- **Group V:** *Nigella sativa* ointment (NSO)
- **Group VI:** *Thymus vulgaris* ointment (TVO)
- **Group VII:** Polyherbal ointment (PHO)

The formulated ointments were applied daily until complete wound healing was achieved. The ointment was gently applied evenly with the help of a swab in sufficient amounts to cover the entire wound area. The wound area was measured on alternate days using millimeter-scale graph paper and the percentage of wound healing was calculated based on these measurements. Complete epithelialization was determined by the absence of scar tissue and the duration required for this process was recorded as the period of epithelialization.

Antimicrobial activity: The cup-plate method was used to evaluate the formulations' antimicrobial activity. In a nutrient agar medium, the zone of inhibition was measured after 24 hrs of incubation at 37±2°C[16]. To assess the anti-microbial activity, Two-Gram Positive (*Bacillus subtilis*, NCTC8236) and Two-Gram Negative (*Escherichia coli*, AICC25922 and *Pseudomonas aeruginosa*, AICC27853) organisms were used. The prepared formulations were inserted into the plates and left overnight to be observed at 37°C in the incubator (Amaze Instruments, Noida, Uttar Pradesh, India). After 24 hrs, a zone of inhibition measured in millimeters was observed surrounding each well.

Statistical analysis: The statistical analysis conducted in this study aimed to assess the significance of differences between the control group and the treated samples. A $p<0.05$ was considered significant for all comparisons. This was achieved using the Student's t-test, with all results presented as mean values accompanied by their respective standard deviations.

RESULTS

Phytochemical analysis: The methanolic extracts of all three plants underwent preliminary phytochemical investigation using standard methods to determine the presence or absence of various groups of phytochemical constituents. This analysis identified alkaloids, glycosides, flavonoids, tannins, carbohydrates, proteins and saponins. Methanol, being a polar solvent, can extract a wide range of phytochemicals.

Evaluation of physical properties of the formulated ointments: The formulations of *Z. nummularia* (ZN), *N. sativum* (NS) *and T. vulgaris* (TV) were observed to exhibit distinct colorations: Dark green for ZN, light green for NS and brownish for TV, respectively. In contrast, the polyherbal formulation appeared pale green. The odor and texture of each formulation were documented and summarized in Table 3. Additional parameters, including pH, spreadability, stability, skin irritation and phase separation, were also assessed and recorded.

Wound healing activity by excision method: The polyherbal formulation exhibited the most rapid epithelialization, with a period of 11.66±0.37 days, in contrast to ointments containing individual extracts (*Z. nummularia:* 14.33±0.59 days, *N. sativum:* 16.33±0.37 days *T. vulgaris:* 15.83±0.71 days), while the control group required 22.66±0.37 days for complete epithelialization. The wound-healing activity followed the order: PHO>ZNO>TVO>NSO.

Table 3: Physiochemical properties of the herbal and polyherbal formulations

Physicochemical parameter	ZNO	NSO	TVO	PHO
Colour	Dark green	Light green	Brownish	Pale green
Odour	Characteristic	Characteristic	Characteristic	Characteristic
Texture	No grittiness	No grittiness	No grittiness	No grittiness
pH	7.33±0.19	6.63±0.05	6.66±0.05	6.9±0.16
Spreadability (g/cm/sec)	14.36±0.42	13.36±0.32	13.93±0.23	15.43±0.19
Phase separation	Nil	Nil	Nil	Nil
Skin irritation study	No skin irritation was observed	No skin irritation was observed	No skin irritation was observed	No skin irritation was observed
Stability (4, 24 and 37°C)	Stable	Stable	Stable	Stable

Values are expressed by Mean±SEM of six readings, ZNO: *Z. nummularia* ointment, NSO: *N. sativum* ointment, TVO: *T. vulgaris* ointment and PHO: Poly herbal ointment

Table 4: Effect of different formulations on contraction of wound and epithelization

Treatment	Contraction[1]	Epithelization[2]
Emulsifying base	10.33±0.37	19.16±0.29
Control	11.5±0.4	22.66±0.37
Standard nitrofurazone	7.66±0.59	14.83±0.29
Ziziphus nummularia ointment (ZNO)	7.16±0.55**	14.33±0.59**
Nigella sativum ointment (NSO)	8.33±0.37*	16.33±0.37*
Thymus vulgaris ointment (TVO)	8.16±0.29*	15.83±0.71**
Polyherbal ointment	6.5±0.4**	11.66±0.37***

Values are expressed by Mean±SEM of six readings, *p <0.01, **p<0.001, ***p<0.0001 compared to control, [1] 50% contraction of wound expressed in days and [2] Period of epithelization expressed in days

Table 5: Comparative antimicrobial efficacy of herbal and polyherbal ointment formulations

	Zone of inhibition							
	Staphylococcus aureus		*Bacillus subtilis*		*Escherichia coli*		*Pseudomonas aeruginosa*	
Sample	100 (µg/mL)	200 (µg/mL)	100 (µg/mL)	200 (µg/mL)	100 (µg/mL)	200 (µg/mL)	100 (µg/mL)	200 (µg/mL)
PHO	13.1±0.19	18.43±0.14	14.26±0.14	21.5±0.29	9.7±0.24	12.7±0.24	6.5±0.32	12.0±0.05
ZNO	8.06±0.10	12.7±0.16	7.53±0.32	10.4±0.09	NI	5.6±0.32	NI	9.8±0.05
NSO	9.6±0.40	14.23±0.05	11.3±0.18	14.8±0.05	NI	4.5±0.27	NI	NI
TVO	11.2±0.19	15.3±0.18	10.6±0.14	17.3±0.14	NI	7.73±0.19	NI	8.9±0.053
Standard ampicillin	16.73±0.19	28±0.09	11.7±0.19	24.8±0.23	10.7±0.24	22.53±0.37	9.5±0.37	20.2±0.14

Values are expressed by Mean±SEM of six readings. NI: No inhibition, Zone of Inhibition was absent in the control group, *S. aureus: Staphylococcus aureus*, *B. subtilis*: *Bacillus subtilis*, *E. coli*: *Escherichia coli*, *P. aeruginosa*: *Pseudomonas aeruginosa*, ZNO: *Z. nummularia* ointment, NSO: *N. sativum* ointment, TVO: *T. vulgaris* ointment and PHO: Poly herbal ointment

These results underscored the synergistic effect of the polyherbal ointment compared to individual methanolic extracts on wound healing (Table 4).

Antimicrobial properties of formulations: The antibacterial potential of the herbal and polyherbal ointments was evaluated at two concentrations, 100 and 200 µg/mL. The results are shown in Table 5. At the high concentration of 200 µg/mL, all formulations demonstrated antimicrobial activity, indicating their efficacy. The zones of inhibition ranged from 4.5±0.27-21.53±0.29 mm. The findings suggested that herbal and polyherbal formulations were more effective against Gram-positive bacteria, specifically *S. aureus* and *B. subtilis* than Gram-negative bacteria.

DISCUSSION

The polyherbal formulation (PHO) developed in this study, comprising *Ziziphus nummularia, Nigella sativa* and *Thymus vulgaris*, exhibited outstanding wound healing and antimicrobial properties. The synergistic effect of these medicinal plants in a single formulation enhances its therapeutic efficacy, positioning it as a promising option for wound care, infection prevention and the acceleration of the healing process. The findings of this study aligned with numerous investigations in the literature that underscore the role of various herbs and their formulations in promoting wound healing[17]. These natural remedies exhibit a range of beneficial properties, including antimicrobial, analgesic, anti-inflammatory and antioxidant effects, which are all essential for the effective wound healing process[17]. The investigation revealed a significant acceleration in wound healing among groups treated with both herbal and polyherbal extracts compared to the control group, which showed delayed wound contraction. Consistently, a reduction in wound area was noted until complete healing was achieved in all groups within 22 days. Both herbal ointments and the polyherbal ointment expedited epithelialization, requiring significantly fewer days for complete epithelialization compared to both the control and base groups. The shorter epithelialization period observed with both polyherbal ointment and herbal extracts, as opposed to the emulsifying base and control, might be attributed to the enhanced viability of newly formed epithelial cells. Wound healing duration relies on the rate of wound contraction, enabling rapid closure through re-epithelialization and the reduction of the extracellular matrix needed for damaged epithelium recovery[18]. This could be attributed to the synergistic effect of the three herbs, which contain phytoconstituents such as flavonoids, phenolic acids and saponins, known to expedite various stages involved in wound healing. Thus, the present study provides evidence that polyherbal ointments are more efficient than individual herbal ointments, corroborating findings in existing literature[14]. Bacterial infections can impede wound healing progress. The use of herbs for treating wound infections is common, owing to the variety of phytoconstituents they contain that are capable of inhibiting microbial growth, depending on the plant and specific organisms. Hence, traditionally used plants are being explored for the development of safe and effective antimicrobial formulations[19]. The study found it worthwhile to assess antibacterial activity. Both herbal ointments and the polyherbal ointment expedited epithelialization, requiring significantly fewer days for complete epithelialization compared to both the control and base groups.

This expedited healing could be attributed to the presence of phenolic constituents and essential oils. Oxygenated monoterpenes in plant essential oils have been reported as one of the major constituents of volatile oils and contribute to wound healing[20]. Constituents like thujone, camphor, borneol, 1,8-cineole, carvacrol, pinene, etc., have been reported to possess antioxidant and anti-inflammatory activity which could be another contributing factor[21]. Volatile oils exhibit several mechanisms for their antimicrobial activity. They affect the cell wall and cytoplasmic components by their hydrophobic nature. They act by degradation of the cell wall, cytoplasmic degradation, deterioration and coagulation and increase in permeability leading to loss of cellular contents[22]. Reviewing the literature suggests that essential oils are more effective against Gram-positive bacteria than Gram-negative bacteria[23,24]. The three herbs used in the formulations are rich in the components of essential oils like thymol, carvacrol, pinene, cymene, etc., as reported in various studies[9,25-27]. Similarly, the position, number and length of the chain of the substituents in the phenolic compound influence the antimicrobial and wound-healing potential of the phenolic compounds. Various mechanisms by which polyphenolic compounds exert their antimicrobial activity have been documented in the literature. They may act by any of the following mechanisms: By inhibiting the synthesis of nucleic acid, by perturbation of cytoplasmic membrane and by damaging the cell wall integrity and the physiological state of the bacterial cell[28]. Plant metabolites like phenolic acids, flavonoids[29] and volatile oil components have been reported to intervene the anti-inflammatory activity by their capacity to intervene in various mechanisms such as the production of inflammatory mediators, inhibition of phospholipase, cyclooxygenase and lipoxygenase enzymes[30]. Most of these phytoconstituents possess antimicrobial activity and anti-inflammatory activity and their combinations can be more potent than individual compounds. Flavonoids and polyphenolic compounds have been reported to possess antimicrobial activity against a wide range of microorganisms[31-33]. Thus, the activities exhibited by the polyherbal ointment are likely due to the synergistic effect of flavonoids, polyphenolics and essential oils present in these herbal extracts. In summary, the present study validated the antimicrobial activity of the herbs incorporated into the polyherbal ointment, suggesting a substantial role in warding off wound infections and advancing wound recovery. This link between the constituents of plants and the wound healing mechanisms emphasizes the therapeutic promise of polyherbal formulations. By engaging with microbial cell membranes, these preparations hold the potential to halt microbial growth, prevent infections and positively impact the wound healing process.

CONCLUSION

The study unequivocally showcases the exceptional wound-healing capabilities of polyherbal formulations containing *Z. nummularia*, *N. sativum* and *T. vulgaris*. The observed antimicrobial activity within the polyherbal formulation played a significant role in expediting wound healing. Notably, this formulation exhibited effectiveness against both Gram-positive and Gram-negative bacteria, highlighting its broad spectrum of action. Given the multifaceted benefits of polyherbal formulations, further investigation into their biological efficacy is warranted. The presence of phenolic compounds and essential oils in this polyherbal formulation is believed to underpin its remarkable healing properties. Future studies should aim to explore the wound-healing potential of this formulation using diverse animal models to elucidate its broader applicability.

SIGNIFICANCE STATEMENT

This study highlights the enhanced wound-healing and antimicrobial potential of a polyherbal ointment formulated from *Ziziphus nummularia*, *Nigella sativa* and *Thymus vulgaris*. By combining these traditional herbs, the formulation exhibited superior efficacy compared to individual herbal extracts, emphasizing the benefits of synergistic interactions among phytoconstituents. The findings suggest that the polyherbal ointment holds promising therapeutic value for wound management, offering an effective natural alternative with broad-spectrum antimicrobial properties. This research contributes to the growing interest in polyherbal formulations as viable options for improving wound care and infection control.

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