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Original Article

Enhanced wound healing effects of herbal gel formulations in a rabbit model: a comparative study

Dunia A. Al-Farraj[1], Muhammad Kashif[2*], Fateh Ullah[2], Hafiz Muhammad Ali[3], Abdul Qayyum[3], Asma Yamin[4], Jawaria Aslam[5], Shabana Bibi[6], Sayed M. Eldin[7*], Iftikhar Ali[8], Abd El-Zaher M.A. Mustafa[1], Mohamed Soliman Elshikh[1]

[1]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia
[2]Department of Clinical Sciences, University of Veterinary and Animal Sciences, Lahore, Sub-campus Jhang, Pakistan
[3]Faculty of Veterinary and Animal Sciences, The Islamia University of Bahawalpur, Pakistan
[4]Department of Zoology, The Govt Sadiq College Women University, Bahawalpur, Pakistan
[5]Bahawalpur Medical and Dental College, Bahawalpur, Pakistan
[6]Department of Biosciences, Shifa Tameer-e-Millat University, Islamabad, Pakistan
[7]Center of Research, Future University in Egypt, New Cairo, Egypt
[8]Department of Genetics and Development, Columbia University Irving Medical Center, New York, USA

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**Corresponding author email:*
muhammad.kashif@uvas.edu.pk
sayed.eldin222@gmail.com

Abstract

The current study was conducted to evaluate the effects of herbal 2% topical gel formulations of either of *Allium sativum*, *Calotropis procera* and *Prosopis juliflora* or their combination compared to an antibiotic cream (Betaderm-N) on healing of full-thickness skin wounds in rabbit. The wound healing (contraction) rate of treated groups was found to be significantly ($p<0.05$) higher than the positive and negative control groups. The wound treated with *A. sativum* were healed on 12th day while those treated with *P. juliflora* or Betaderm-N cream healed on 15th day. The wounds treated with combination gel showed a significantly ($p<0.05$) higher healing rate and completely healed the wound by 9th day of the experiment and in the histo-pathological examination, there observed an increased number of collagen fibers in dermis of the skin compared to positive and negative controls. Catalase test was used to differentiate *S. aureus* from other staphylococcal species. *S. aureus* has golden or creamy colour colonies raised on mannitol salt agar with coagulase positive activity. While the pink colonies raised at Meckonky agar with Indol positive test were of *E. coli*. By disc diffusion method, the combination of three herbal extracts showed a significantly ($p<0.05$) higher antibacterial activity against *S. aureus* and *E. coli* than other groups and showed a significant increased level of superoxide dismutase (SOD) and reduced glutathione (GPx) at 7th ($p<0.05$), 14th ($p<0.05$) and 21st ($p<0.01$) days of treatments. It was thus concluded that the combined effects of three herbal extracts accelerated the healing process of surgical wound in rabbits due to presence of active metabolites.

Keywords: Medicinal plants, Phytochemical analysis, Wound healing, *S. aureus*, Rabbit

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Introduction

A wound is referred to as an injury in the living tissue caused by a cut or puncture into the skin (Hemmati and Mohammadian, 2000). It is a cellular, anatomical and functional dis-arrangement of living cells and tissues induced by physical, chemical, electrical or microbial agents (Sharma et al., 2021; Shehadul-Islam et al., 2017). The regeneration of the damaged tissue or wound healing is a complicated process involving angiogenesis with migration and proliferation of fibroblasts (Eming et al., 2014). One of several potential problems with wound healing is infection of the wound and hence, impairment in the healing indicates that a wound has not gone through all of its typical healing phases (Liu et al., 2021). Delay in the process of wound healing leads to additional costs and expenditures and in the complicated cases, causes huge economic loses (Sen, 2019; Coffey et al., 2019; Aslam et al., 2021).

The majority of commercially available single synthetic medical formulations lack sufficient evidence to support their efficacy in treating the wounds. Additionally, the existing synthetic drugs despite having bacterio-static or bactericidal properties but have relatively restricted properties to accelerate the stages of wound healing (Hussain et al., 2020; Basit et al., 2021). Moreover, the therapeutic index of the majority of drugs is limited and if used continuously, has the risk to develop antimicrobial resistance (Chokshi et al., 2019). Natural substances are safe and alternate to synthetic drugs that could be used to accelerate the proliferation and remodeling phases of healing (Qasim, 2022; Alafnan et al., 2023). In Asia, Africa, Middle East and Latin America, traditional treatments by using natural products derived from indigenous plants like *Centella asiatica*, *Curcuma longa* (turmeric) and *Paeonia suffruticosa* (common garden peony) constitute the advanced therapeutic applications for wound healing (Shedoeva et al., 2019; El-Ashram et al., 2021). Throughout the history, herbal items and medications have played an essential role in the treatment of ailments in both humans and animals (Salman et al., 2020; Mehboob et al., 2022; Panezai et al., 2022; Paskudska et al., 2018). These herbal medicines can also be used to cure ulcers, skin infections, diabetes, jaundice, fever, inflammation and viral infections (Ashraf et al., 2021; Aslam et al., 2021; Aslam et al., 2023a). Herbal medicines also ensure wound healing by providing a suitable environment that promotes the healing process (Kaneez et al., 2022; Sharma et al., 2021). Moreover, the use of antibiotics for control of infectious agents and in wound healing leads to induction of stress (Alvi et al., 2021).

Aloe vera is one of the oldest known plants that promotes wound healing by improving keratinocyte growth factor and collagen type-1 and thus improves the wound healing (Attah et al., 2016). *Allium sativum* known as wild garlic, belongs to lily family, found in the region of south-west central Asia and has anti-fibrinolytic, antioxidant and anti-platelet properties (Farahpour et al., 2017). The active ingredients of *A. sativum* include alliin, allyl cysteine, allyl disulfide and allicin and have potent antioxidants that also stimulate the process of wound healing (Sobolewska et al., 2015). *Calotropis procera* (calotrope or milk weed) is found abundantly in South-West and South-East Asia, Africa and in the Caribbean Islands in Central and South America (Aderounmua et al., 2013; Gebremeskel et al., 2018). It is used to treat jaundice, eczema, asthma, fever, snake bite, malaria, dysmenorrhea, arthritis and leprosy and its latex is used for the prevention of dental and skin burn problems (Ali-Seyed and Ayesha, 2020). *Prosopis juliflora* (babul or mesquite) is found in arid and semiarid regions around the globe and contains flavonoids, tannins, alkaloids, phenolics and saponins that are used as anti-cancer, anti-fungal, anti-inflammatory, anti-bacterial and antioxidant activity (Yadav and Rana, 2020). According to WHO, 170 of the 194 member states (80% of the world population) reported the use of traditional herbal medication for their primary ailments because of their limited side effects and easy availability as compared to allopathic medicines. The current study was thus planned to observe the effects of topical application of alcoholic extracts of *A. sativum*, *C. procera and P. juliflora* on surgically generated full-thickness skin wounds in rabbits.

Material and Methods

Animal housing and management

Adult male rabbits (n=30) of an average weight of about 1 to 1.5 kg were kept at the animal house facility of College of Veterinary and Animal Sciences, sub-campus Jhang of University of Veterinary and Animal Sciences, Lahore at uniform feeding and under proper management conditions.

The animals were kept at the standard laboratory conditions at 25ºC temperature with equal hours of light and darkness. About 30 min before the surgical intervention, each experimental animal was administered with atropine sulfate @ 0.035 mg/kg b.wt. (body weight) intravenously. While a combination of ketamine (35 mg/kg b.wt.) and xylazine (5 mg/kg b.wt.) was also used intramuscularly to completely anesthetize the animals. The ethical committee of the College of Veterinary and Animal Sciences Jhang, sub-campus of University of Veterinary and Animal Sciences, Lahore granted the consent for animal experimentation for the current study (CVAS/ERC-CS-1960, Dated: 03-08-2021).

Preparation of plant extracts, their phytochemical analysis and gel preparation

The samples of *A. sativum*, *C. procera* and *P. juliflora* were air dried in the shade and then ground to form a powder. By applying cold extraction method, the powder was macerated in 95% methanol (1:4 w/v) and put on continuous shaking for 72 h. The alcohol was then evaporated by using a rotary evaporator set at 40ºC and the prepared extract was stored in an air dried container until further analysis.

To ascertain the existence of phyto-components, the methanol extracts of all the plants were initially subjected to phytochemical screening by Mayer's test (alkaloids), alkaline reagent test (flavonoids), Legals test (glycosides), Salkowski test (saponins, tannins, phytosterol), Libermann Burchard test (triterpenoids) and Ninhydrin test (proteins, amino acids) (Table 1) (Aslam et al., 2023a; Morsy et al., 2016; Srividhya et al., 2019).

Table-1: Qualitative analysis of the plant extracts to determine the phytochemicals

Phytochemicals	*A. sativum*	*C. procera*	*P. juliflora*
Alkaloids	+	-	+
Flavonoids	+	+	+
Glycosides	+	+	+
Tannins	+	-	+
Phenolic compounds	+	-	+
Terpenoids	-	+	-
Saponins	+	+	-
Amino acids	+	-	-

A. sativum was found to have maximum number of phytochemicals. + = present, ⁻ = absent

The gel was prepared by mixing a suitable volume of deionized water with carbomer powder using a magnetic stirrer at 1500 rpm. The 2% herbal extract was then added in the mixture to make 2% topical gel. Then 10% NaOH solution was then added to adjust the pH between 7 to 7.5 with continuous stirring (Budiman et al., 2019).

Animal experimentation and determination of rate of wound contraction

The animals (n=30) were randomly divided into six groups with 5 rabbits / group. The skin wound (3×3 cm) was experimentally created in the dorsal area of thoracic region of each animal by following the rules of ethics committee. The gel of each respective extract was applied at the site of wound of respective treatment group twice a day for a duration of three weeks i.e. the wounds of rabbits of group A were treated with *A. sativum*, those of group B with *C. procera* and of group C with *P. juliflora*. While the wounds of animals of group D were treated with gel formed by combination of the three extracts. The wounds of animals of group E were treated with an antibiotic preparation (betaderm-N cream having betamethasone valerate and Neomycin sulphate; control positive), while the wounds of group E animals were treated only with distilled water (control negative). At every days, the distance between the wound margins was measured until the incision was fully closed and scar tissue was formed (Masson-Meyers et al., 2020). The percent wound contraction was calculated by using the following formula:

$$\% \text{ Wound contraction} = \frac{Healed\ area}{Total\ wound\ area} \times 100$$

Histopathological examinations

At the end of the experiment (21 days), after collection of gross anatomical characteristics, the animals were sacrificed, tissue samples (1x1 cm) of wound scar at the skin were collected, washed with normal saline and fixed in 10% neutral buffered formalin. The tissues were processed by paraffin sectioning and 4-5 µm thick histological sections were stained by Hematoxylin and Eosin techniques, as previously described (Ali et al., 2017; Sikandar et al., 2020).

Serum biochemical examinations

The serum is separated from the collected blood (Qureshi and Ali, 2016; Malik et al., 2018; Iqbal et

al., 2021) at days-1st, 7th, 14th and 21st and enzymes of oxidative stress i.e. glutathione peroxidase (GPx) and superoxide dismutase (SOD) were determined by using the diagnostic kits; GPx: Cat No: SG-0120Rb, SOD: Cat No: SG-0061Rb (Ransod and Ransel, Randox, United Kingdom).

Bacterial isolation

The swabs were taken from the surgical wounds of animals for culture before the start of treatment, on 3rd day of wound induction (of non-treated group or from the wounds treated only with distilled water) and on 7th day of gel application. The swabs were put on the nutrient agar media for the growth of bacteria and the identification of various bacterial strains by different biochemical tests like coagulase and catalase tests, as described earlier (Hussain et al., 2012;Geletu et al., 2022). The colonies obtained at nutrient agar media were stained with gram staining and these bacteria were also grown on mannitol salt agar to differentiate staphylococci from other bacterial species. For the identification of *Escherichia coli* (*E. coli*), the bacteria were grown on Meckonky agar and were further tested by Indole test (Geletu et al., 2022).

Testing of anti-bacterial activity

The zone of inhibition for different plant extracts was measured by using disc diffusion method (Aslam et al., 2023b). The five different preparations of the plant extracts (2% *A. sativum*, 2% *C. procera*, 2% *P. juliflora*, 2% combination (of three plant extracts) and betaderm-N cream were tested for their antibacterial activity. Sterile Whatman filter paper disc (0.5 cm) was used in the test; each containing one of the respective plant extracts (500 mg/ml of extract/disc). ADP-ribosylation factor (10µl) was added at each disc and then allowed to dry for an hour. After that, the disc was placed at the top of culture plates of *S. aureus* and left in the incubator for 24 h at 37°C. The anti-bacterial activity of the test sample was determined by measuring the diameter (mm) of the zone of inhibition (Qayyum et al., 2016)

Statistical analysis

The one-way analysis of variance (ANOVA) and Tukey's post hoc test were used to analyze the data by using IBM SPSS® Statistics version 22. All data are presented as mean ± SD (Standard deviation). $p<0.05$ was considered as statistically significant level.

Results

Rate of wound contraction:

There observed a daily decrease in the size of wound until the incision had completely closed and scar tissue was formed. The rate of wound contraction for the treated groups was found to be higher than the control negative group (Figure 1). The order of healing of wounds in different treatment groups is as under: *combination* gel > *A. sativum* > *P. juliflora* = betaderm-N > *C. procera*. The *A. sativum* had significantly ($p<0.05$) rapid rate of wound contraction as compared to *C. procera*, *P. juliflora*, betaderm-N cream and control negative group. Likewise, the combination of the three herbal plants had a significant ($p<0.05$) improved effects in the wound healing than all other treatment groups. Among those groups (group A to C) treated individually with herbal gels, there was a significant difference ($p<0.05$) between the groups treated with *P. juliflora* and antibiotics with *C. procera* i.e. the group treated with *C. procera* has delayed wound healing compared to the other two groups. On 3rd day of treatment, there was a significant contraction in wound size in the group treated with a combination of three herbal gels.

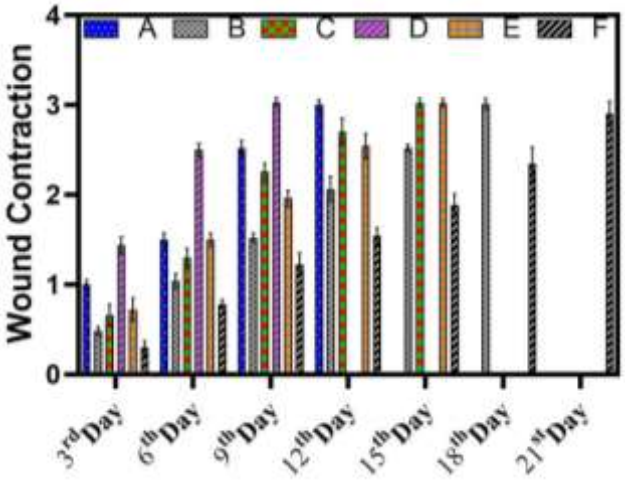

Figure-1: Contraction of wound at different time intervals on treatment with different plant extracts compared to control groups. The rate of wound contraction for the treated groups was found to be higher than the control negative group. The order of healing of wounds in different treatment groups is as under: *combination* gel > *A. Staivum* > *P. juliflora* = betaderm-N > *C. procera*.

A. sativum gel had a considerable wound reduction rate compared to other groups treated with individual herbal gels. On 6th day, the results were the same as noticed on the 3rd day. On 9th day, there was a complete reduction in the wound size i.e. wound closure in the group treated with the combination of herbal gels, while the groups treated with *A. sativum* also had a significant decrease in wound size compared to other groups. On 12th day, there was a complete healing of the wound treated with *A. sativum* compared to other groups treated with individual herbal gels. On 15th day, the groups treated with *P. juliflora* and Betaderm-N cream had a thorough reduction in the wound, while on 18th day, the *C. procera* treatment group was healed entirely and the control negative group was healed completely on 21st day.

Histo-pathological examinations

There observed an improved growth and proliferation of the epidermis (re-epithelialization of the wound area) and the components of the matrix. The thickness of the dermal layers was increased with organized collagen matrix i.e. wound remodeling in the wound of animals treated with a combination gel compared to other treatment groups of gels of individual plant extracts. The percentage of collagen fibers was found to be higher in the animals treated with a combination of medicinal gels (Figure 2a) compared to control positive (Figure 2b) and negative groups.

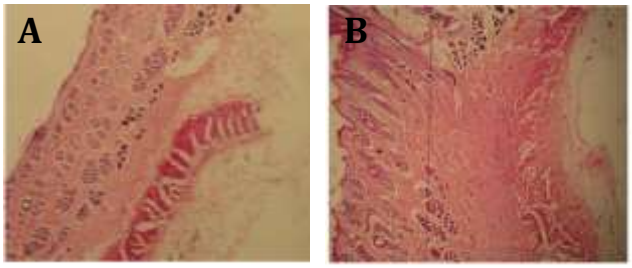

Figure-2: Wound treated with a combination of *A. sativum, C. procera and P. juliflora* (A) and betaderm-N cream (B). There observed an increased number of collagen fibers and blood vessels in the dermis treated with combination gel compared to control negative. The slide B is showing relatively less number of collagen fibers compared to the skin of the wound treated with combination gel of three plant extracts.

Quantification of glutathione peroxidase (GPx)

The level of GPx enzyme gradually increased with time and type of treatment (Figure 3). There was a statistically significant difference ($p<0.05$) between the time of healing and the type of treatments used in different groups that indicated that the herbal medicine used for healing the wounds also reduced the oxidative stress. On day 1st, there was no difference in the level of GPx but at the days-7th, 14th and 21st, there was a significant ($p<0.05$) difference in the enzyme level. The group treated with the combination gel of the herbal extracts revealed a higher level of GPx than the control groups (Figure 3).

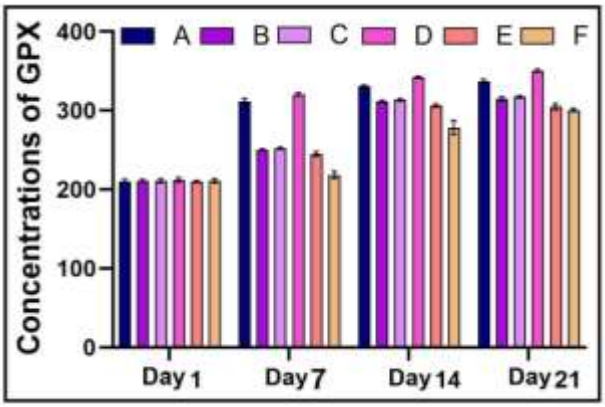

Figure-3: Quantification of glutathione peroxidase (GPx; U/L) at different time intervals after treatment with different plant extracts compared to control groups. On day 1st, there was no difference in the level of GPx but there observed a significant increased level of the enzyme at 7th ($p<0.05$), 14th ($p<0.05$) and 21st ($p<0.01$) days of treatment. The group treated with the combination gel revealed a higher level of GPx than the control groups. The wounds were treated with *A. sativum* (A), *C. procera* (B), *P. juliflora* (C) and combination gel (D) in comparison to wounds of control positive; betaderm-N cream (E) and control negative (F) groups.

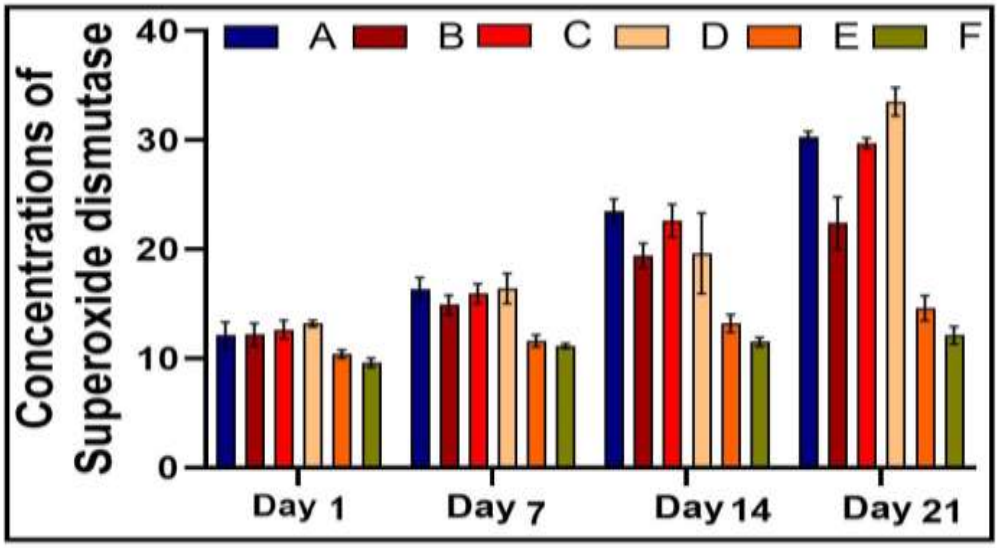

Figure-4: Quantification of superoxide dismutase (SOD; U/ml) at different time intervals after treatment with different plant extracts compared to control groups. There was no significant difference in the values of SOD at 1st first day of experiment, while the difference was significant at 7th (p<0.05), 14th (p<0.05) and 21st (p<0.01) days of treatments. The combination of the extracts was found to be more effective (p<0.01) in reducing the oxidative stress i.e. combined treatment significantly increased the antioxidant level. The wounds were treated with *A. sativum* (A), *C. procera* (B), *P. juliflora* (C) and combination gel (D) in comparison to wounds of control positive; betaderm-N cream (E) and control negative (F) groups

Table-2: Quantification of superoxide dismutase (SOD) after treatment with different medicinal plants

Treatment groups						
Days	***Alium Sativum***	***Calotropis Procera***	***Prosopis Juliflora***	**Combination**	**Betaderm-N**	**Control negative**
1	12.12±1.21	12.18±1.01	12.62±0.85	13.18±0.08	10.40±0.39	9.68±0.43
7	16.30±1.10*	14.90±0.89	15.96±0.95*	16.40±1.39*	11.60±0.55	11.16±0.23
14	**23.50±1.12****	19.40±1.14*	22.60±1.52*	19.66±4.73*	13.20±0.84	11.58±0.43
21	**30.30±0.45****	22.40±22.40*	29.70±1.48*	**33.50±1.32****	14.60±1.14	12.18±0.84

Quantification of Super Oxide Dismutase (SOD)

There was no significant difference in the values of SOD between different treatment groups at 1st day of experiment but the difference was significant at 7th (p<0.05), 14th (p<0.05) and 21st (p<0.01) days of treatments. The combination of the extracts was found to be more effective (p<0.01) in reducing the oxidative stress; combined treatment has increased the anti-oxidant level (Figure 4, Table 2).

There was no significant difference in the values of SOD at 1st day of the experiment, while the difference was significant at 7th (p<0.05), 14th (p<0.05) and 21st (p<0.01) days of treatments. The combination of the extracts was found to be more effective (p<0.01) in reducing the oxidative stress; combined treatment has increased the antioxidant level. Betaderm-N = Control positive.

Bacterial isolation

By catalase test, the staphylococci were found to be strongly catalase positive. On Gram staining, they appeared to be gram-positive bacteria which were round/cocci in shape and appeared as irregular chains or often grape-like clusters. A positive coagulase test differentiated the *Staphylococcus aureus* from other staphylococcal species. Then mannitol salt agar was used for specific growth of *S. aureus*. The colonies appear golden or creamy colour were considered to be of *S. aureus* (Figure 5). While the presence of *E. coli* was confirmed by pink colonies on Meckonky agar with Indole positive activity.

Testing of anti-bacterial activity

Plant extracts of *A. sativum, C. procera, P.*

juliflora, their combination and Betaderm-N cream have high inhibition ability against *S. aureus* and *E. coli* (Table 3). The maximum zone of inhibition (mm) was observed by the combination gel ($p<0.05$). The zone of inhibition for *S. aureus* was observed in the following order: combination of extracts > *A. sativum* > *P. juliflora* > *C. procera* = Betaderm-N cream. For *E. coli*, the zone of inhibition was as follows: combination of extracts > *A. sativum* > *C. procera* = Betaderm-N cream > *P. juliflora*.

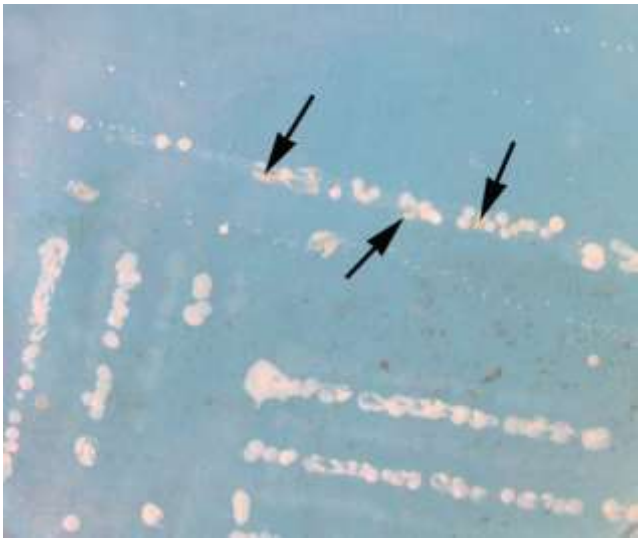

Figure-5: Golden color bacterial colonies of *S. aureus* at mannitol salt agar. The colonies having golden or creamy white colour with coagulase positive activity were considered to be of *S. aureus*.

Table-3: Disc diffusion test to determine the antibacterial activity of medicinal plants against *S. aureus* and *E. coli*

Groups	Concentration of the extracts (mg /ml)	Zone of inhibition against *S. aureus* (mm)	Zone of inhibition against *E. coli* (mm)
A. sativum (2%)	500	17	16
C. procera (2%)	500	14	15
P. juliflora (2%)	500	15	14
Combination gel	500	**25***	**26***
Betaderm-N	500	14	15

The maximum zone of inhibition was observed by the combination gel ($p<0.05$). The zone of inhibition for *S. aureus* was observed as: combination of extracts > *A. sativum* > *P. juliflora* > *C. procera* = Betaderm-N cream. For E. coli, the zone of inhibition was as: combination of extracts > *A. sativum* > *C. procera* = Betaderm-N cream > *P. juliflora*.

Discussion

Skin wounds are the disruption of the epithelium and the underlying connective tissue layers, developed by physical trauma that breaks and opens the skin (Lima and Passos, 2021). Wound healing involves multiple inter-related stages depending upon the type and severity of the injury; an early inflammatory phase which is within the first two days of an injury, a late inflammatory phase that starts at 2-3 days of injury, a proliferative phase that takes place between 4-21 days following the damage and a tissue remodeling phase that takes place from 21 days to one year (Birdane et al., 2014). In the current experiment, the rate of wound contraction of the treated groups was found to be relatively higher than the control negative group, as previously observed (Zaenal et al., 2023); while in the current study the authors found that by applying the gel of herbal extracts, the time of healing is reduced in the treated groups compared to the control groups. The effects of garlic on the wound healing process are due to active constituents of garlic including allicin, flavonoids and triacremonone; as anti-inflammatory agents (Abazari et al., 2022). Similarly, protocatechuic acid, chlorogenic acid, caffeic acid and ferulic acid are claimed to have anti-inflammatory and anti-oxidant characteristics in *Prosopis cineraria* that work synergistically to hasten the healing of cutaneous injury (Yadav and Rana, 2020). Moreover, it was also previously observed that traditionally the herbal extracts of *Prosopis juliflora* and *Prosopis africana* have been used for curing and healing of surgical wounds (Yadav and Rana, 2020).

Histo-pathological evaluation of the treated wounds in our study showed a better regeneration of the epidermis in the animals treated with a blend of three herbal plants. This wound healing with a mixture of herbal extracts were far better than the results of the wounds treated with individual extracts and even better than those treated with Betaderm-N cream. Hence, it could be concluded that the combination of three plant extracts has a rapid wound healing and antioxidant effects compared to the other treatments. Histo-pathologically, the collagen fibers were also found to be higher in the animals treated with medicinal plants compared to those treated with antibiotics. It was found that the anti-inflammatory properties of garlic speed up the proliferative phase of wound healing, characterized by angiogenesis, growth of new fibers and re-epithelialization (Zaenal

et al., 2023). Moreover, in another study, *C. procera* showed a two-fold impact on wound healing and due to its anti-bacterial, anti-inflammatory and anti-oxidant capabilities, phenolic compounds significantly accelerated the collagenation, wound closure and epithelialization during wound recovery phases (Aderounmua et al., 2013). It was also observed that *P. cineraria* had significant effects on re-epithelization, promoting the formation of collagen, angiogenesis and mainly the remodeling of cutaneous appendages (Yadav and Rana, 2020).

Serum biochemical profile showed a significant increase in SOD and GPx in the groups treated with herbal extracts compared to the control (Yadav and Rana, 2020). Similarly, it was found that methanolic extract of *C. procera Lin* elevated the level of anti-oxidants (Yesmin et al., 2008). One of the potential cause of effectiveness and mechanism of action of these extracts is their capacity to scavenge the free radicals. Anti-oxidant qualities of many plants are due to majority of phenolic chemicals like tannins and flavonoids. Moreover, it has been demonstrated that oxidative stress is critical for regulating the blood flow to the area of wound healing and a rise in the total antioxidant status is crucial for the process of wound healing (Comino-Sanz et al., 2021). In our study, all the plants displayed antioxidant potential and their free radical scavenging actions seen are most likely the result of polyphenols present in the plants.

At performing the coagulase test and checking for differential gram staining for staphylococcal species, golden or creamy color colonies and coagulase positive results were indicative of *S. aureus*.

It was observed that *S. aureus* and *E. coli* were sensitive to the ethanolic extract of garlic (Maharjan et al., 2019). Hence, a large zone of inhibition was noticed when ginger and garlic were used in combination that confirms that the blend of the herbal extracts has synergistic effects in antibacterial activity (Noman et al., 2023). Many traditional plant remedies are present in the folk medicine that are used for the treatment of several ailments and now some have been validated by scientific studies to exert their biological action against infectious agents (Imran et al., 20222; Ahsan et al., 2022) and wound healing. Hence, it could be concluded that the use of these herbal remedies are both affordable and effective in wound healing, mainly when are also used for anti-bacterial activity and to reduce the oxidative stress.

Conclusion

The effects of topical gels of *A. sativum*, *C. procera* and *P. juliflora* were quite promising in healing of skin wounds in rabbit. Moreover, the combined gel has also superior results than the effects of an individual plant extract. The histo-pathology further confirmed these results be depicting an increased number of collagen fibers in the dermis of treated skin wound. The combination of these three herbal extracts also exhibited a higher antibacterial activity and significant increased levels of SOD and GPx. Hence, it was concluded that our selected herbal plants have efficient potential to accelerate and their combination has much better results in the process of wound healing in rabbits due to presence of active metabolites. Hence, these plants could be effectively used in topical pharmaceutical preparations and ointments for the therapy of surgical and other accidental wounds.

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Contribution of Authors

Al-Farraj DA, Bibi S, Eldin SM, Ali I: Performed literature review, reviewed and edited the manuscript
Kashif M & Ullah F: Conducted experiments, collected data, wrote first draft, edited and approved the final manuscript
Ali HM, Qayyum A, Yamin A, Aslam J, Mustafa AEMA & Elshikh MS: Performed the data analysis and interpretation and contributed in write up

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