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Formulation and Evaluation of Allicin and Curcumin Gel Improves Normal and Diabetic Ulcers in Rabbits

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***SUMMARY*. Allicin is one of the pharmacologically active sulfur compound of *Allium sativum* and curcumin, the main curcuminoid of *Curcuma longa*. Both are used extensively as a household remedy for the wound care and diabetes mellitus in the sub-continent. The goal of this study was to have a preparation that is inexpensive to prepare so that individuals at risk for the development of non-healing skin ulcers can utilize it on a long-term basis as a wound preventative. A 20% w/v gel of each (allicin:curcumin with optimized ratio 1:2) was made using Carbopol-940 in the concentration of 5% and evaluated for normal and diabetic wounds. The potential of allicin and curcumin gel (ACG) treatment was studied against experimentally induced excision wound on the thigh of rabbits under ketamine anesthesia. The decrease in wound size was judged by using a scale. Rabbits were divided into six Groups (6 rabbits each). Group 1 (normal wound) and Group 2 (diabetic wound) treated with povidone-iodine (PI), Group 3 (normal wound) and Group 4 (diabetic wound) were treated with ACG and Group 5 (normal wound) and Group 6 (diabetic wound) treated with distilled water. After 9th post wounding day, ACG exhibited better and significant wound healing ($p < 0.05$) against normal and diabetic wounds. We concluded that the gel of allicin and curcumin possess excellent wound healing activity and can be used as an alternative medicine for wound care.**

RESUMEN. La alicina es uno de los compuestos de azufre farmacológicamente activos de *Allium sativum* y curcumina es el principal curcuminoide de *Curcuma longa*. Ambos se usan ampliamente como un remedio casero para el cuidado de heridas y diabetes mellitus en el subcontinente. El objetivo de este estudio fue tener una preparación que sea barata de preparar para que las personas en riesgo de desarrollar úlceras cutáneas no curativas puedan utilizarla a largo plazo como preventivo de heridas. Se preparó un gel al 20% p/v de cada uno (alicina:curcumina en relación optimizada 1:2) usando Carbopol-940 en una concentración del 5% y se evaluó para heridas normales y diabéticas. Se estudió el potencial del tratamiento con alicina y gel de curcumina (ACG) contra la escisión inducida experimentalmente en muslo de conejos bajo anestesia con ketamina. La disminución en el tamaño de la herida se juzgó usando una escala. Los conejos se dividieron en seis grupos (6 conejos cada uno). Grupo 1 (herida normal) y grupo 2 (herida diabética) tratados con povidona yodada (PI), grupo 3 (herida normal) y grupo 4 (herida diabética) fueron tratados con ACG y grupo 5 (herida normal) y grupo 6 (herida diabética) tratados con agua destilada. Después del noveno día posterior a la herida, ACG exhibió mejor y significativa curación de heridas ($p < 0.05$) frente a heridas normales y diabéticas. Concluimos que el gel de alicina y curcumina posee una excelente actividad de curación de heridas y puede usarse como medicina alternativa para el cuidado de heridas.

INTRODUCTION

Wound can be referred to as an injury which results in torn or punctured skin. Pathologically a wound means an injury which harms the dermis of the skin [1]. Wound healing is an active process consisting of four uninterrupted, overlapping, and exactly programmed phases [2]. The events of each phase happen in a fixed and synchronized manner. The first phase begins immediately after wounding, with vascular constraint and fibrin clot formation. The clot and neighboring wound tissue release pro-inflammatory cytokines and growth factors such as transforming growth factor (TGF) -β, platelet-derived

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growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF). On controlled bleeding, inflammatory cells travel into the wound and encourage the inflammatory phase, characterized by the chronological penetration of neutrophils, macrophages, and lymphocytes [3]. A vital function of neutrophils is the clearance of invading microbes and cellular debris in the wound area, these cells also generate substances such as proteases and reactive oxygen species (ROS), which cause some additional damage [4].

Herbal medicine has been used for many centuries with profound results. Healing capability of many natural plants has been observed since ancient times. Amongst those plants, Garlic and Curcumin share a vital importance [5,6].

Curcumin is a yellow pigment substance and component of turmeric *(Curcuma longa),* which is well-known more than a century ago. Turmeric is a spice from the root of the *Curcuma longa* plant which belongs to the ginger family. It is being used in Southeast Asian cultures, especially in Ayurvedic medicine [4]. For centuries it has been known that turmeric shows anti-inflammatory activity, but extensive research completed within the past two decades has resulted in the evidence that this activity of turmeric is due to curcumin, diferuloylmethane [6].

This agent has been shown to control numerous transcription factors, cytokines, protein kinases, adhesion molecules, redox status and many enzymes linked to inflammation. The process of inflammation has played a major role in most autoimmune diseases, neoplastic diseases, chronic illnesses, including neurodegenerative, cardiovascular, pulmonary, metabolic disorders [7].

Curcumin (diferuloylmethane), is the main curcuminoid present in turmeric which gives its yellow color. Curcumin possesses significant anti-inflammatory, anti-oxidant, anti-carcinogenic, anti-mutagenic, anti-coagulant and anti-infective effects [4,6]. Curcumin has also revealed significant effects on wound healing properties. It acts on various stages of the natural wound healing process to hasten healing [7]. Researchers have shown the ability of curcumin to reduce the body's natural response to wounds such as inflammation and oxidation [8]. The recent literature on the wound healing properties of curcumin also provides evidence for its ability to improve granulation tissue formation, collagen deposition, tissue remodelling and wound contraction [9]. It is evident that curcumin has maximum therapeutic effects on skin wounds [6].

Allium sativum is a genus of some 500 species belonging to the family Liliaceae [10]. Its enzymes (for example, alliinase), sulfur-containing compounds such as alliin, and compounds produced enzymatically from alliin (for example, allicin) [11]. Other constituents such as arginine, oligosaccharides, flavonoids, and selenium are also available in garlic [11]. Allicin is a polar compound of phenolic and steroidal nature which shows interesting pharmacological properties [5]. Allicin decoction arouse immune functions such as proliferation of lymphocyte, release cytokine and phagocytosis and lessen cellular proliferation of virally infected cells [12].

Allicin induces antioxidant action by hunting reactive oxygen species (ROS), enhancing the cellular antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and increasing glutathione in the cells [13,14]. It protects DNA against free radicals and defends against UV-induced damage. It also protects against some forms of UV-induced immune-suppression [15]. Considering the medicinal importance of both the active phytoconstituents in wound repair, we planned to test in combination to produced synergistic effect in normal and diabetic ulcers.

MATERIAL AND METHODS

Drugs and chemicals

All the drugs used in this study were of pharmaceutical grade. Povidone-iodine (PI) solution (10% w/v) was purchased from Brooks Pharmaceuticals Pvt. (Ltd.) Pakistan. Lignocaine gel was purchased from Atco Pharmaceuticals Pvt. (Ltd.) Pakistan. Ketamine and streptozotocin (STZ) were purchased from Indus Pharmaceuticals Pvt. (Ltd.) Pakistan. The preparation of curcumin used here was obtained from LKT Laboratories, Inc. (St. Paul, MN), and allicin were purchased from All sure neutraceuticals, China.

Preparation of gel

Curcumin and allicin were dissolved in dimethyl sulfoxide (DMSO) at 10% for curcumin and 5% for the allicin. A 20% w/v gel of each (allicin and curcumin with optimized ratio 1:2) was made using Carbopol 940 in the concentration of 5%. A 20% w/v gel of each was used as in our previous study [2]. The concentrations chosen for the two active phytoconstituents were

based on the previous results of preliminary *in vitro* studies, and efficacy in phase-1 and phase-2 of wound healing. The gel formulated was stored at -4 °C in air tight jars in the laboratory refrigerator.

Animals

Rabbits of either sex with an average weight of 1.5 kg were purchased from the pet market Hussain Agahi, Multan, Pakistan. Rabbits were kept under standard laboratory conditions at 27 °C room temperature with 12 h light and dark cycles. All animal experiments were performed according to the rulings of the "Animal Ethical Committee" of Muhammad Institute of Medical and Allied Science, Multan, Pakistan, reference number EC/12/2017 dated 7th December, 2017.

Induction of diabetes

Overnight fasting rabbits were injected intraperitoneally STZ (50 mg/kg) after titrating it with an ice cold citrate buffer (pH 4.5). After injection they had given 5% glucose solution to drink to overcome the hypoglycemic shock. After 48 h the development of diabetes was confirmed. Rabbits having fasting blood glucose levels more than 200 mg/dL were opted for the experiment [16].

Grouping of animals

Animals were divided into 6 Groups, each Group containing 6 rabbits as follows: *Group 1* treated to normal wounds with PI Solution (1 mL), *Group 2* treated to diabetic wounds with PI Solution (1 mL), *Group 3* treated to normal wounds with ACG (1 mL), *Group 4* treated to diabetic wounds with ACG (1 mL), *Group 5* treated to normal wounds with distilled water (1 mL), and *Group 6* treated to diabetic wounds with distilled water (1 mL).

Wound healing activity

Rabbits were anesthetized with ketamine before and during the creation of wounds as mentioned by Khan *et al.* [2]. Back surface hairs of rabbits were shaved and lignocaine gel (local anaesthetic) was applied. The scalpel was used to cut the skin of rabbits (full thickness) to make a circular wound of 80 mm^2 on the thigh. The whole wound was left open. The wound closure percentage was measured on day 0, 3rd, 6th and the 9th post wounding days. The recovery of the wound areas was calculated in cm with the help of scale. It was considered as the initial wound healing area. Topical treatment was done in all cases, once daily at 10 AM. On the 9th post wounding day, by using Eq. [**1**], the percentage wound closure was calculated (Fig.1).

$$Percent\ \ wound\ \ closure = \frac{Initial\ \ area\ \ of\ \ wound - n^{th}\ day\ of\ wound}{Initial\ \ area\ \ of\ \ wound} \times 100 \qquad [\mathbf{1}]$$

Estimation of total phenol content

Total phenol contents were estimated using standard protocol [17] with slight modification. For each replicate, 1 mL of plant extracts (0.5 g/20 mL), was prepared in sterile distilled water which was added to 4 mL of Folin-Ciocalteu's reagent (Sigma, USA). After 7 min, 5 mL of 20% sodium carbonate was added to each solution. The resultant solutions were incubated in darkness for 2 h at room temperature. The absorbance was measured at 740 nm with a spectrophotometer (UV 3000, ORI. Germany). Gallic acid (5, 10, 25, 50, 75, and 100 mg/L) was used as a standard chemical for calibration curve. Quantification of total phenolic content (TPC) was expressed in terms of gallic acid equivalent (GAE) mg/g of dried fraction. All samples were analyzed in triplicate.

Histopathological examination

Each Group were selected on days 3, 6 and 9. Excisional biopsies were taken from the wounded skin and were fixed in formaldehyde solution (10%) and then samples were prepared for tissue processing. After preparing the tissue slides, slides from each sample were randomly selected, and were stained with hematoxylin and eosin and also with the Trichrome Masson staining protocol. Pictures were taken from all slides using a microscope equipped with camera. Finally, two pathologists that were not aware of the slide classifications evaluated the slides and reported the results [10].

Acute dermal toxicity evaluation of ACG

The acute dermal toxicity of ACG was evaluated by fixed dose method in accordance with

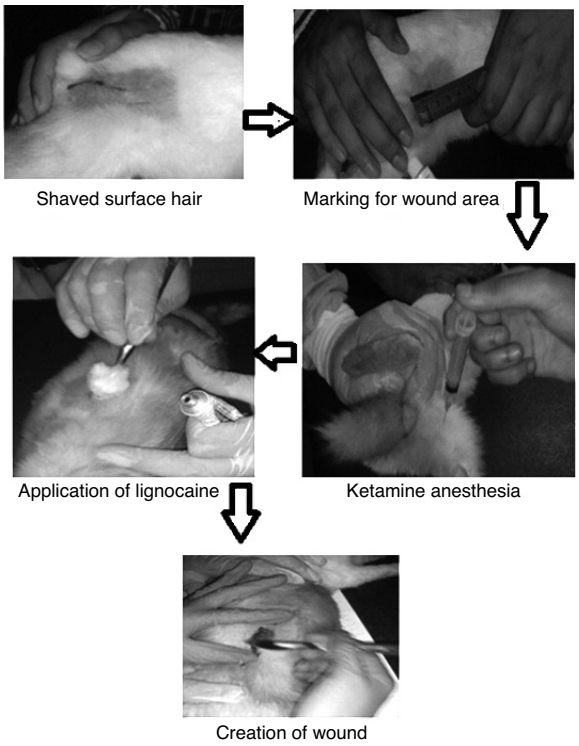

Figure 1. Photographic representation of the methodology.

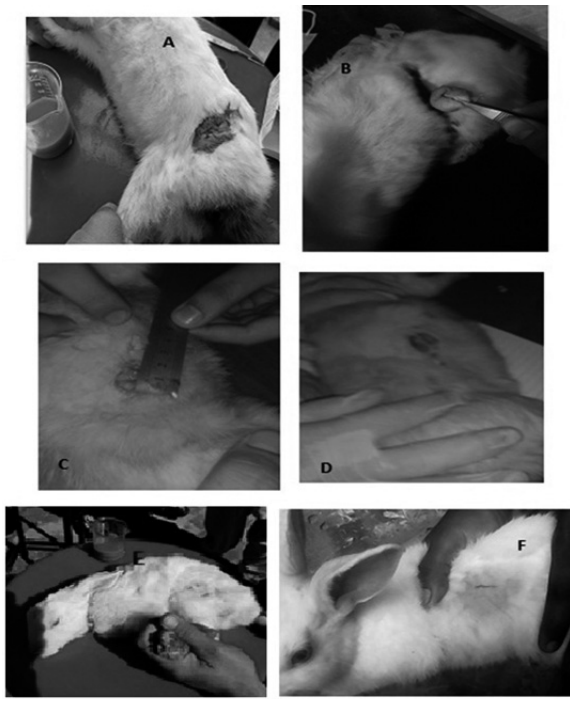

Figure 2. Photographic representation showing the % wound contraction on different post wounding days from A-F.

the guidelines of OECD (Organization for Economic Co-operation and Development). Briefly, dorsal area of the rabbits were shaved and the gel was applied topically on rats at a dose of 2000-3000 mg and observed for 48 h for irritation, redness and inflammation [15].

Statistical analysis

Data was analyzed using SPSS software (Spss Inc., Schicago, USA) and results were expressed as mean ± SD. Whereby variables first being tested for normality through the Shapiro Wilk test, while the difference between experimental Groups was analyzed using one-way ANOVA followed by Bonferroni test.

RESULTS

This study of the excision wound model expressed that Groups 1-4 showed decreased wound area day by day (Fig. 2). However, 89.34% wound healing was observed on 9th post wounding day in Group 1 animals, where as a Group 2 treated animals expressed 69.50% healing. While Group 3 showed 92.34% healing and 86.55% percentage of healing in Group 4 (Table 1). Both the Groups treated with ACG were found to be statistically significant (p value = 0.019 and 0.026), while Groups5 and 6 proved insignificant. Table 2 shows phenol contents (GAE mg/g).

DISCUSSION

Our goal, ultimately, is to have a preparation that is inexpensive to prepare so that individuals at risk for the development of non-healing wounds can utilize it on a long-term basis as a wound healing agent. In the present study, we show that topical treatment with a combination of two natural products, i.e. curcumin and allicin extract, improves healing of experimentally induced excision wounds in normal and diabetic rabbits (Fig. 3).

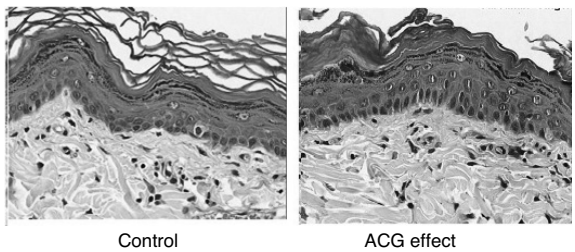

Figure 3. Photographic representation of histopathological changes in ACG treated Group as compare to control after 9th post wounding day.

Post wounding days	0	3	6	9	*p* value
Group 1	80.57 ± 12.5 (0%)	38.50 ± 7.3 (51.05%)	23.76 ± 9.3 (69.79%)	9.20 ± 2.7 (89.34%)	0.021
Group 2	79.32 ± 12.8 (0%)	46.19 ± 8.4 (41.77%)	28.97 ± 10.5 (64.45%)	24.19 ± 3.1 (69.50%)	0.043
Group 3	78.57 ± 12.5 (0%)	47.19 ± 6.8 (39.11%)	26.28 ± 6.6 (66.81%)	5.97 ± 1.5 (92.34%)	0.019
Group 4	79.32 ± 12.8 (0%)	46.19 ± 8.4 (36.32%)	28.97 ± 10.5 (76.87%)	15.19 ± 3.1 (86.55%)	0.026
Group 5	80.49 ± 10.8 (0%)	77.57 ± 10.8 (3.62%)	68.44 ± 10.8 (14.97%)	65.39 ± 10.8 (18.76%)	0.073
Group 6	80.22 ± 10.8 (0%)	80.00 ± 10.8 (0%)	76.88 ± 10.8 (4.16%)	73.25 ± 10.8 (8.68%)	0.081

Table 1. Effect of ACG on the normal and diabetic excision wound model. Value is mean ± SEM of Six animals (n = 6) in each Group. The number in parenthesis indicates percentage of wound contraction. All are significant at $p < 0.05$ as compared to Group 1 (control) and indicate not significant.

Sample (GAE mg/g)	Phenol Contents
Allicin	61.21 ± 0.15
Curcumin	29.85 ± 0.48

Table 2. Polyphenol strength of allicin and curcumin extracts. Values are expressed as means ± standard deviation (n = 3).

No irritation was observed in the treated animals at any time during wounding/wound-healing. The combination of curcumin and allicin gel functions to improve wound-healing in a synergistic manner by uplifting various contributory factors of wound healing. Taking past observations into account, along with the findings presented here, we hypothesize that the two agents act through complementary mechanisms one is more effective in phase-1 and second is more effective in phase-2 of wound healing. A major contribution of the allicin extract, we suggest, is to increase blood flow in the repairing tissue as it has been reported vasodilator in previous studies, [19] established antiseptic and antibiotic potential equivalent to penicillin18. while the major effect of curcumin is on matrix remodeling, this may be responsible for its better wound healing in diabetic wounds as compared to PI which is not effective in 2nd phase of wound healing [2]. Allicin is reported for re-epithelization while, curcumin has a strong positive effect on collagen synthesis [20]. It should be noted that these are not the only potential mechanisms by which the two agents may work. A number of past studies have shown that curcumin and allicin extract have anti-oxidant properties and broad anti-inflammatory antimicrobial activities [19-25]. These data provide a basis for our suggestion that the two agents have complementary effects. Obviously, additional studies will be needed to substantiate this overall hypothesis. While our vision is a topical product containing a combination of curcumin and allicin, both agents have been used orally. Thus, it is conceivable that beneficial effects could be more promising systemically with these agents provided as a nutritional supplement.

CONCLUSION

Combinational effect of both curcumin and allicin extract was found to have better and faster wound healing effect than standard drug Povidone Iodine ointment on excision wound model. Both the phytoconstitutents used together in a ratio of 1:2 considering their potential in both the phases of wound healing Thus, it can be concluded that when both were applied in combination externally on rabbits, it showed faster as well as better wound closure and wound contraction as compared to other treatment. Therefore, we may interpret that the observation of such response may be due to the synergistic effect of the two plant materials

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