Original Article

A Biochemical and Molecular Insight to Wound Healing Properties of Traditional Indian Medicines in Normal and Diabetic Rats

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ABSTRACT

Background: Honey, ghee, *Glycyrrhiza glabra* L. (GG), and *Nerium indicum* Mill. (NI) have been effectively used in *Ayurveda* and Indian folk medicine for healing both normal and diabetic wounds. However, the exact biochemical and molecular mechanisms involved are less discussed. The present study explores the wound healing efficacy of these traditional medicines in normal and diabetic rats using biochemical and molecular parameters. **Materials and Methods:** Normal and Streptozotocin-induced diabetic Wistar rats were used for the study and inflicted with excision wounds. The test materials, i.e., honey, ghee, GG, and NI, were topically applied to the wounds singly and in combinations (H+G: Honey+Ghee and Tot-combination of all test materials). On the 8^{th} and 16^{th} days of healing, biochemical and molecular parameters were assessed using the tissues procured from the wound site. We quantified the levels of hydroxyproline and antioxidants (CAT-Catalase, GSH-Glutathione, SOD-Superoxide dismutase, MDA-Malondialdehyde). We also measured the mRNA expression of growth factors, i.e., Transforming Growth Factor-beta (TGFβ), fibroblast growth factor 2 (FGF2), Platelet-Derived Growth Factor (PDGF), and vascular endothelial growth factor (VEGF). **Results:** Biochemical analysis showed enhanced hydroxyproline levels in both normal and diabetic wounds treated with the test materials. The groups treated with GG in normal and NI and Tot in diabetic wounds showed statistically significant findings ($p<0.05$). Different study materials showed significant antioxidant properties at different intervals of wound healing. Similarly, different test materials enhanced the expression of different growth factors at the wound site. **Conclusion:** Result indicates that the wound healing properties of these natural traditional medicines are through their antioxidant and growth factor enhancing capability at the wound site.

Keywords: Wound healing, Honey, Ghee, *Glycyrrhiza glabra*, *Nerium indicum*, Diabetic wounds, Antioxidants, Gene expression.

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INTRODUCTION

In the process of wound healing, the inflammatory phase is of great importance. During this phase, the antioxidants, growth factors, and other inflammatory mediators are produced.[1] The Reactive Oxygen Species (ROS) are also secreted in this phase, which in low levels is vital to protect the wound from the invading bacteria and other micro-organisms.[2] However, overexposure to ROS causes oxidative stress resulting in delayed wound healing.[3]

Living cells comprise numerous vital antioxidants such as Catalase (CAT), Glutathione (GSH)-related Glutathione S-Transferase (GST), and Superoxide Dismutase (SOD) that avoid or mend the injury instigated by ROS and control redox-sensitive signaling pathways.[4]

Growth factors such as Platelet-Derived Growth Factor (PDGF), transforming growth Factor-ß (TGF-ß), Fibroblast Growth Factors (FGFs) Vascular Endothelial Growth Factor (VEGF) were also shown to play an important role in wound healing.[5-9]

The management of the wound is complicated, especially when the wound is of a non-healing type. The most common type of non-healing wounds is diabetic wounds. Diabetic wounds, unlike typical wounds, heal slowly, resulting in treatment with conventional topical medicines, a challenging process. The wound healing regulators become dysfunctional, which leads to a delay in the refurbishment.[10] Diabetic wounds present defective wound repair and a decrease in the formation of granulation tissue.

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Reduced amounts of collagen, protein, and Deoxyribonucleic Acid (DNA) is also observed in diabetic wounds.[11]

Although modern medicine has found advancement in the management of wounds, poor wound management is still commonly observed. This problem is attributed to unawareness, indecorous hygiene, the absence of essential health care needs, and health centers' remoteness. On the other hand, *Ayurveda* and folk medicinal systems are readily available and encompass many constituents that improve healing. Traditional medicines are therefore yearned for their high acceptability rate and real toleration.[12-15]

Ayurvedic scriptures have mentioned the effective use of honey, ghee, and *Glycyrrhiza glabra* (*Yasti madhu*) (GG) roots in treating wounds, i.e., *Vrana shodhana* and *ropana.*[16-18] *Nerium indicum* (NI) is also known for its wound-healing benefits in folk medicine.[19]

Honey and ghee have been regularly used in *Ayurveda* for wound healing.[16-18] The use of honey in treating both acute and chronic wound is also an approved practiced in the modern medicine.[20,21] Ghee based formulations have also been evaluated for wound healing activities.[22] GG and NI's healing benefits on both acute and chronic (diabetic) wounds, although mentioned,[23,24] have not been scientifically standardized and validated. Further, the healing benefits of combining test materials, i.e., H+G (Honey and Ghee) and Tot (all test materials combined), are also not explored.

Our previously published reports on animal models (normal-non-diabetic) studied the changes occurring in the wound site using these test materials. The biomechanical parameters such as rate of wound closure and re-epithelialization; histological findings such as the rate of wound healing, re-epithelization, and dermal rearrangement were significantly improved when treated with the test materials.[25,26] Further, the groups treated with the test materials showed favorable inflammatory changes (interleukin 1 beta, i.e., IL1 β) and an adequate myofibroblast activity at the wound site compared to control (untreated).[26] The traditional medicines also positively influenced the activity of fibroblasts and keratinocytes, as indicated by the *in vitro* studies.[27,28]

However, the exact biochemical and molecular mechanisms underlying this traditional medicinal mediated wound healing in normal and diabetic wounds are less explored. Further, effect of these test materials on the ROS, antioxidant levels and growth factors in the wounds is not investigated.

Therefore, this present study was designed to scientifically evaluate the biochemical and molecular markers of wound healing using these traditional medicinal preparations, i.e., honey, ghee, and extracts of two medicinal plants-GG and NI. This study also attempts to explain the actual healing process in normal and diabetic wounds, the mechanisms of which otherwise remains elusive.

MATERIALS AND METHODS

Test material-procurement and preparation

We obtained honey (unprocessed and raw), ghee (Cow's ghee), and roots of GG from Sri Dharmasthala Manjunatheshwara (SDM) Ayurvedic pharmacy, Udyavara, Udupi (Batch No. 2015). The leaves of NI were collected from its natural habitat. The plant was identified and authenticated by a botanist, and a voucher specimen is deposited in the herbarium of a local University (PP no. 603). The plant names of GG and NI have been checked with http://www.theplantlist.org (last accessed: 01/04/2020). The test materials were standardized by subjecting them to High-Performance Thin-Layer Chromatography (HPTLC) and phytochemical screening.

Preparation of test materials

Aqueous GG and NI: GG roots and NI leaves were dried in shade for a week and then powdered. Their aqueous extracts were prepared using the hot maceration method.[25]

Combinations of test materials: Two combinations of test materials were used: i.e., Honey+Ghee (H+G) and Tot (H+G+GG+NI) (all test materials combined). The formulations were prepared using equal parts of the test materials.

Experimental animals

Healthy adult albino Wistar rats (*N*=168) of either sex (84 males and 84 females) weighing 150-200 g were used in the study. They were placed in separate cages under standard conditions. The temperature, humidity (25±0.5ºC) and 12 hr' light/dark cycle was appropriately maintained. The animals were provided a standard diet and water *ad libitum*. The experiments were carried out in the central animal house facility and were approved by the Institutional Animal Ethics Committee (IAEC/KMC/49/2013). The animals were randomly allocated into two main sets: normal (*N*=84) and diabetic (*N*=84). We further grouped each set into two, i.e., 8 (*N*=42) and 16 (*N*=42) days treatment plan. Supplemental image 1 provides a schematic representation of the experimental groups.

Induction of diabetes

The animals (*N*=84) ordained to belong to the diabetic group were induced with diabetes using Streptozotocin (STZ).[29]

After seven days of acclimatization, the rats were induced with diabetes. We injected the rats with a single intraperitoneal dose of STZ (35 mg/kg) after overnight fasting. STZ induced hyperglycemia was overcome by adding glucose (5%) to the drinking water. Accu-Chek glucometer with glucose oxidase-peroxide reactive strips (Roche Diagnostic India Pvt.

Ltd., Mumbai) was used to measure fasting blood glucose levels. The animals with Fasting Blood Sugar (FBS) levels >250 mg/dL were considered for diabetic models. The animals with FBS levels <250 mg/dL were excluded from the study.

Wound model and treatment groups

We classified the animals into two sets (normal and diabetic) of seven groups each (6 animals in each group): Control (untreated) and honey, ghee, GG, NI, H+G, and Tot treated groups. The animals were followed to study the nature of wound healing for 8 and 16 days, respectively (Supplemental 1).

Creation of wound

The rats (*N*=84) were anesthetized with ketamine, and excision wounds were inflicted using a standard protocol.[25] We shaved the dorsal fur, and the area to be wounded was outlined on the animals' back using a circular stainless-steel stencil and a marker. A full thickness of the excision wound of 2.5 cm width (circular area=4.90 cm^2) and 0.2 cm depth was made along the markings using toothed forceps, a scalpel, and pointed scissors. The wound was left open.

Treatment plan

The open wounds were topically applied with the reference dose of test materials every day, i.e., 500 mg once a day. The dosage was calculated based on the amount of test material required to cover the entire wound and the adjacent area uniformly. The two subclasses of animals (normal and diabetic) with seven groups each (control and six treated groups, i.e., honey, ghee, GG, NI, H+G, and Tot) were followed to study the nature of wound healing for 8 and 16 days, respectively. The rats of the control group (both normal and diabetic) were left untreated.

At two different time points of the treatment (8^{th} and 16^{th} day), the animals' corresponding group was sacrificed (using an excess of thiopentone sodium). The skin tissue with the excision was collected separately for biochemical and molecular studies.

Biochemical Analysis

Hydroxyproline estimation

Skin tissues of uniform weight, i.e., 250 mg, were collected from the wound site. The tissues were homogenized and processed to analyze hydroxyproline content using the colorimetry method.[30]

Estimation of antioxidants

The level of antioxidants, such as CAT,[31] GSH,[32] SOD,[33] was estimated using standard protocols in wet granulation tissue homogenates. An increase in the level of these antioxidants indicates the reduced activity of oxidative stress.

The free Radicals-Lipid Peroxidation (LPO)/Malondialdehyde (MDA) levels, was estimated using the MDA assay. A decrease in the MDA level indicates reduced oxidative stress.[34]

The tissues were homogenized in phosphate-buffered saline (PBS, pH 7) using a glass-Teflon homogenizer (10% w/v) at four degrees Celsius temperature. The homogenate was then used for the estimation of antioxidants and free radicals.

Molecular Analysis

Ribonucleic acid (RNA) isolation and reverse transcription

Total RNA was obtained from wound tissues (250 mg) on day 16 using the PureLink RNA isolation kit (Invitrogen, USA). Complementary DNA (cDNA) was then prepared using the Superscript III First-strand synthesis kit (Invitrogen, USA) according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

Semi-quantitative PCR was run using primers designed explicitly for messenger RNA (mRNA) templates of the growth factors TGFβ, FGF2, PDGF, and VEGF. To normalize the gene expression level, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as the internal control.

The primer sequences are as follows

Gene	Name	Primers
GAPDH	Forward	CTAGAGACAGCCGCATC
GAPDH	Reverse	GGGTAGAGTCATACTGGAAC
TGF-b1	Forward	CTGGAAAGGGCTCAACACCT
TGF-b1	Reverse	GGCCCCCAGATGGGCTT
FGF-2	Forward	GCCGAACGGGACAGATTCTT
FGF-2	Reverse	TTCGCACACACTCCCTTGAT
PDGF	Forward	GTGTGAGACAGTAGTGACCCC
PDGF	Reverse	ACGGACGAGGGGAACAACATT
VEGF	Forward	CGTCCAACTTCTGGGCTCTT
VEGF	Reverse	GCTTTCTGCTCCCCTTCTGT

The intensity of the bands obtained was then measured using ImageJ software (National Institute of Health-NIH and Laboratory for Optical and Computational Instrumentation-LOCI, University of Wisconsin, Madison, Wisconsin, USA). The control group's intensity was considered 100%, and that of the remaining groups was calculated and documented.

Statistical Analysis

The findings were expressed as mean±Standard Errors of the Mean (SEM). Graph Pad Prism software (Microsoft, San Diego, California, USA) was used for data analysis. One-way ANOVA, followed by Dunnett's posthoc test, was used to compare control and treated groups within days 8 and 16. We separately analyzed the normal and diabetic groups. *P*-value ≤0.05 was considered statistically significant.

RESULTS

Rate of wound closure/healing (Figure 1)

Normal group

Ghee, H+G, and Tot treated wounds showed significant wound closure on day eight compared to control (normal-untreated). On day 16, only H+G and Tot treated wounds showed significant findings.

Diabetic group

All the treated groups (except ghee) showed significant wound closure on day eight compared to control (diabetic-untreated). Honey continued to show consistently better-wound closure till day 16. Ghee, on the contrary, showed reduced wound closure and healing both on 8th and 16th day of observation.

Biochemical Analysis

Estimation of Hydroxyproline (Table 1)

Normal group

Although higher hydroxyproline levels were noted in all the treatment groups unlike control (normal-untreated) on both days 8 and 16, the findings were not significant statistically. Only GG treated animals showed statistically higher hydroxyproline values on day 8 of treatment ($p<0.05$).

Diabetic group

In general, total hydroxyproline content was lesser in diabetic wounds compared to normal wounds. In comparison to diabetic-untreated, only the groups treated with NI ($p<0.05$) and Tot ($p<0.01$) on day 8 showed a significant increase. Rest of the groups (except Ghee on day 8) although showed an increase, the findings were however not significant statistically.

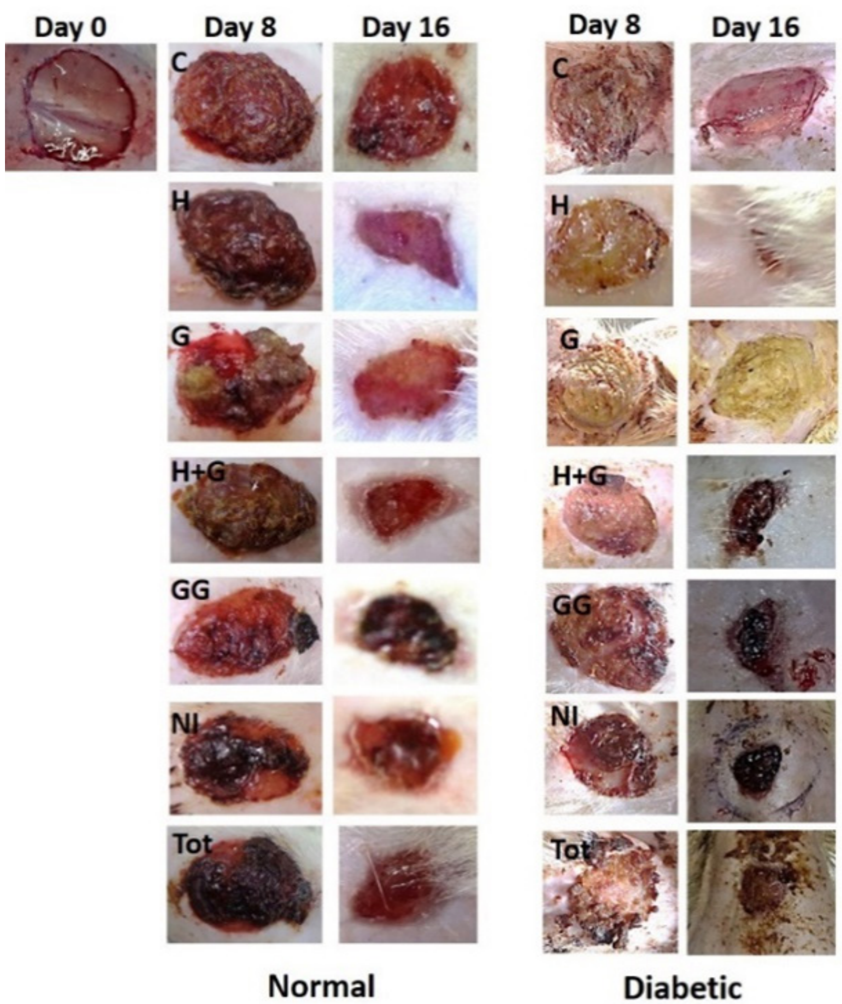

Figure 1: Representative images of the wounds in the experimental groups (normal and diabetic) captured on days 0, 8, and 16.

Estimation of Antioxidants

CAT Levels (Figure 2.1)

Normal group

The CAT levels were higher in the treatment groups (normal-untreated) on both days 8 and 16. The CAT levels showed a significant increase in ghee ($p<0.001$), H+G ($p<0.05$), GG ($p<0.001$) and Tot ($p<0.05$) treated groups on day 8th and ghee ($p<0.01$) and Tot ($p<0.05$) treated groups on day 16th were observed (Figure 2.1a).

Diabetic group

The CAT levels were found to be variable in the treatment groups. All the treated groups showed higher CAT values (diabetic-untreated) on both days 8 and 16. But the statistically significant finding was found only in the groups treated with NI ($p<0.05$) on day 8. By day 16, the CAT levels were relatively lowered in all the groups compared to day 8, indicating the decrease in the ROS activity as healing progressed (Figure 2.1b).

GSH levels (Figure 2.2)

Normal group

The GSH levels were higher in all the treatment groups (normal-untreated). Groups treated with ghee showed a statistically significant increase on day 16 ($p<0.01$). However, the levels in the rest of the groups were statistically insignificant compared to the control (normal-untreated) (Figure 2.2a).

Diabetic group

On day 8, the GSH levels were similar in all the experimental groups, while on day 16, it was higher in all the treated groups (diabetic-untreated). However, the findings were statistically significant only in the groups treated with Honey and H+G on day 16 ($p<0.05$). However, the levels in the rest of the groups were statistically insignificant compared to control (diabetic-untreated) (Figure 2.2b).

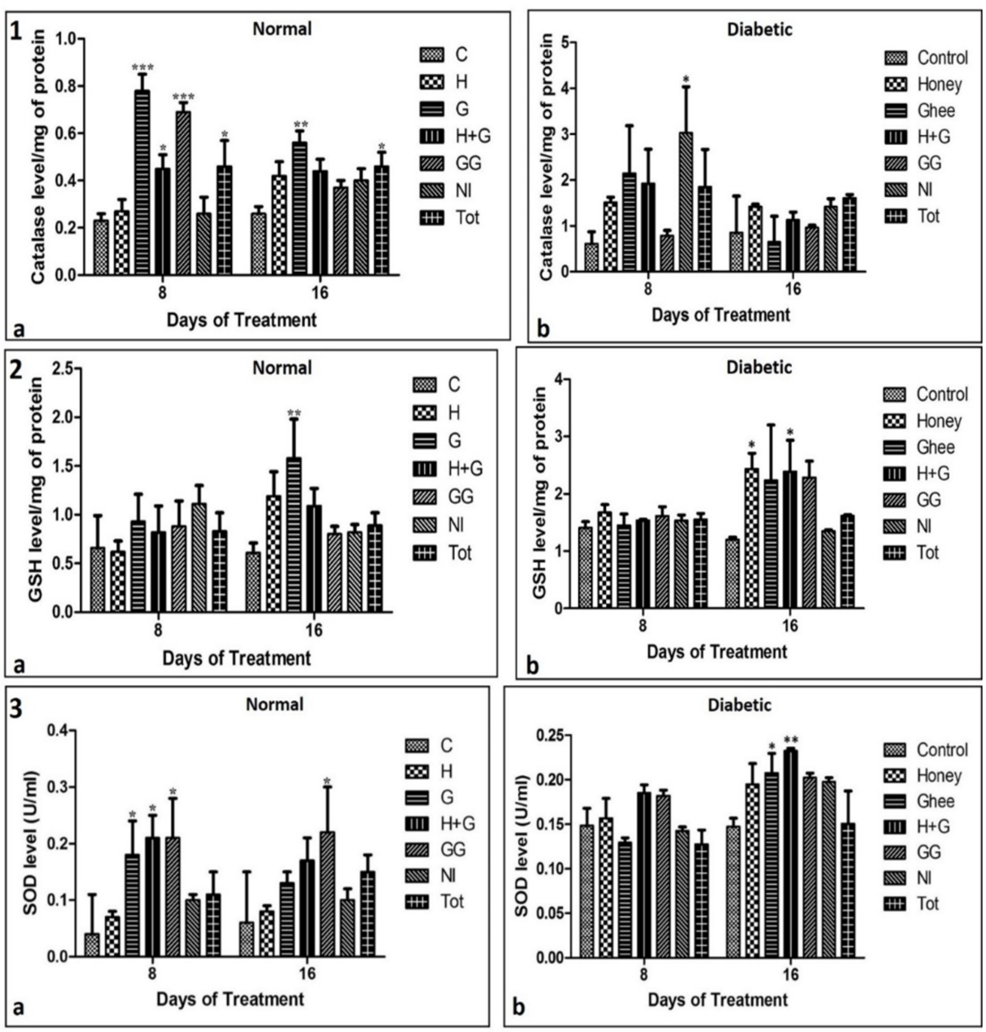

Figure 2: Graphical representation of the catalase (Figure 2.1), GSH (Figure 2.2), and SOD (Figure 2.3) levels at the wound site in normal and diabetic animals on days 8 and 16. *$p<0.05$, **$p<0.01$ and ***$p<0.001$in comparison with control (untreated group) within days 8 and 16.

Table 1: Hydroxyproline Estimation in Normal and Diabetic Excision Wounds.

Experimental Group	Normal: Hydroxyproline/mg of tissue (Mean±SEM)		Diabetic: Hydroxyproline/mg of tissue (Mean±SEM)	
	8 Days	16 Days	8 Days	16 Days
C	2.21±0.46	4.25±0.20	0.79±0.09	0.92±0.31
H	2.65±0.77	5.27±0.86	0.87±0.06	1.46±0.33
G	3.70±0.96	6.02±0.29	0.73±0.14	1.04±0.07
H+G	3.25±0.54	4.56±0.29	1.41±0.24	1.36±0.26
GG	4.52±0.73*	5.44±0.43	0.98±0.06	0.68±0.10
NI	2.48±0.45	3.83±0.38	1.76±0.43*	0.77±0.13
Tot	3.14±0.46	4.71±0.54	1.99±0.42**	1.29±0.26

*$p<0.05$ and **$p<0.01$ compared to control (untreated group) within days 8 and 16.

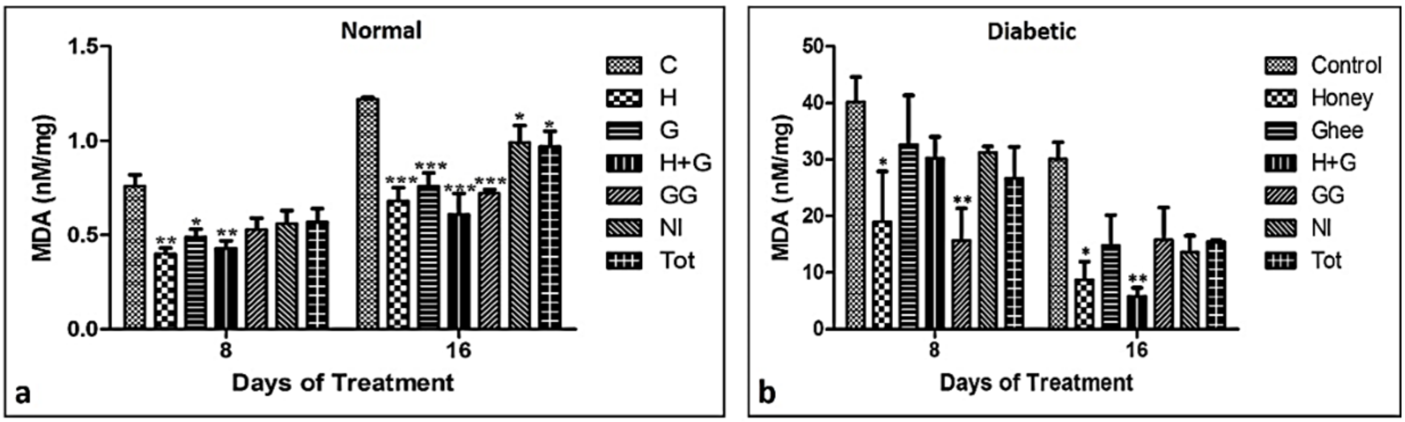

Figure 3: Graphical representation of the MDA levels at the wound site in normal (Figure a) and diabetic (Figure b) animals on days 8 and 16. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ in comparison with control (untreated group) within days 8 and 16.

SOD level (Figure 2.3)

Normal group

SOD levels showed a statistically significant rise in groups treated with ghee, H+G, and GG on day 8th and GG on day 16th, respectively ($p<0.05$). The rest of the treatment groups showed higher SOD values than control (normal-untreated). These findings were not significant statistically (Figure 2.3a).

Diabetic group

The activity of SOD in the treated groups on day 8 was variable. But the activity was higher and statistically significant in ghee ($p<0.05$) and H+G ($p<0.01$) treated groups on day 16. The rest of the treated groups, although showed biologically higher values of SOD day 16, these findings were, however, not statistically significant (Figure 2.3b).

LPO using MDA assay (Figure 3)

Normal group

The wound tissues subjected to LPO showed markedly lower MDA values in all the treatment groups (normal-untreated). The levels were significantly lower in honey ($p<0.01$), ghee ($p<0.05$), and H+G ($p<0.01$) treated groups on day 8th and in all the groups on day 16th (Honey, Ghee, H+G, and GG: $p<0.001$; NI and Tot: $p<0.05$) (Figure 3a).

Diabetic group

Statistically significant reduction in the MDA levels was observed in the groups treated with honey ($p<0.05$) and GG ($p<0.01$) on day 8th; honey ($p<0.05$) and H+G ($p<0.01$) on day 16th. The rest of the groups, although they showed lower values of MDA on both days 8 and 16, were, however, insignificant statistically (Figure 3b).

Molecular Analysis

Expression of growth factors

On day 8, extraction of the RNA from the granulation tissue was not successful and, therefore, was not considered for the assessment of expression of the growth factors. On day 16, we could extract the RNA from the wound tissue, and it was further processed for evaluating the expression of growth factors (Figures 4.1 and 4.2 a).

Normal group

TGFβ expression was not detected in any of the experimental groups (This particular segment was repeated thrice, but we were unsuccessful in detecting the expression). FGF2 expression was detected and was significantly greater in honey, ghee, and H+G treated groups in comparison to control (normal-untreated) ($p<0.05$). Significantly increased PDGF expression levels were found in groups treated with GG, H+G, and Tot ($p<0.05$). Ghee, H+G, and Tot treated groups showed a significant increase in the levels of VEGF compared to control (normal-untreated) ($p<0.05$) (Figure 4.1).

Diabetic group

TGFβ showed a significant rise in the expression levels in groups treated with Honey, GG, and NI ($p<0.001$). Further, FGF2 and PDGF expression was found to be increased in the groups treated with GG and NI ($p<0.001$). Expression of VEGF was statistically higher in the groups treated with honey ($p<0.05$), GG ($p<0.001$), NI ($p<0.001$), and Tot ($p<0.001$). The better expression of the growth factors was observed in the treated diabetic wounds permitting better healing, unlike the untreated diabetic group (Figure 4.2).

DISCUSSION

The test materials showed significant influence on better healing in normal and diabetic groups than control (normal and diabetic-untreated). It may be attributed to various indigenous factors or biomarkers present in them.

Honey

Honey is effectively involved in the activation of human keratinocytes by up-regulating the expression of growth factors such as IL-1β, TGF-β, TNF-α (Tumor Necrosis Factor-α), and MMP-9 (Matrix Metalloproteinase 9). It also encourages type IV collagen degradation in the skin through the activation of MMP-9 which is essential in remodeling the skin during healing.[35]

Ghee

The Polyunsaturated Fatty Acids (PUFA) present in ghee controls the cell to cell interaction and intracellular signal transduction.[36] It also shown to enable the vitro proliferation of epithelial cells and aids in wound healing.[37]

GG

Glycyrrhizic Acid (an active component of GG) inhibits the cyclooxygenase activity and formation of prostaglandins (i.e., prostaglandin E2). It also indirectly inhibits the platelets' aggregation, thereby reducing the rate of inflammation and edema formation, thereby promoting wound healing.[38,39] Some of the constituents of GG possess substantial antioxidant properties, i.e., glycyrrhizin and glabridin. These components inhibit the production of ROS at the inflammation site.[40,41]

NI

NI is considered useful in healing chronic wounds, both diabetic and non-diabetic type.[19] Although the leaves and latex of NI is a known skin irritant and a systemic toxic agent, it is still in use traditionally by herbalists as a folk remedy for a wide variety of conditions, including dermatitis, wound healing, abscesses, psoriasis, sores, warts, corns, ringworm, scabies, herpes, skin cancer, and tumors.[19]

The wound healing potential of NI may be attributed to the phytoconstituents present in it such as tannins, flavonoids, phenols which are known to carry good antioxidant properties. They promote wound contraction by causing the chelation of the free radicals and reactive species of oxygen species. They further aid in increasing the capillary formation and assists in fibroblasts and keratinocyte proliferation and collagen rearrangement. Potent anti-inflammatory capacity is also reported in them which also can be attributed to its wound healing properties.[42,43]

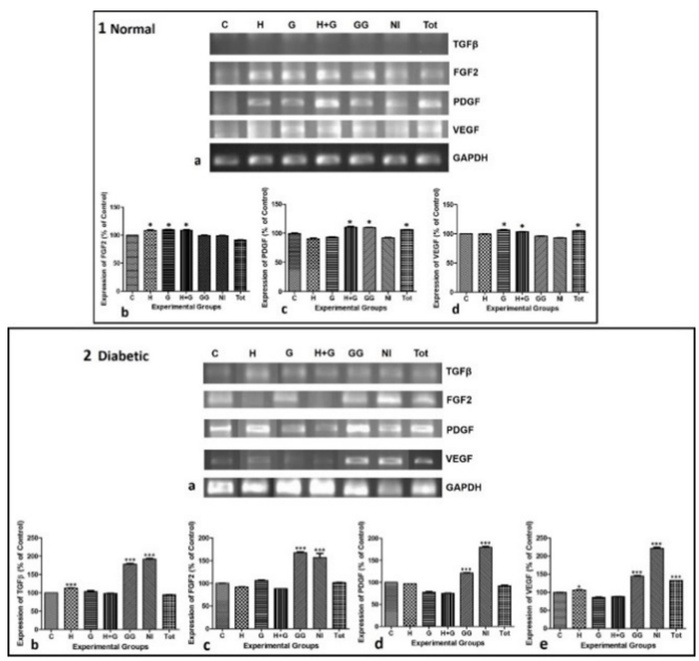

Figure 4: Showing the alteration of growth factors at the wound site in normal and diabetic groups. Figure a. Representative images of gel analysis of TGFβ, FGF2, PDGF, and VEGF expression in the wounds of all the experimental groups of normal (Figure 4.1) and diabetic (Figure 4.2) animals on day 16. TGFβ was not expressed in any of the treatment groups in normal animals. Figure 4.1, b-d: Graphical representation of FGF2, PDGF, and VEGF levels, respectively. *$p<0.05$ in comparison with control (untreated group). Figure 4.2, b-e: Graphical representation of TGFβ, FGF2, PDGF, and VEGF levels, respectively. *$p<0.05$ and ***$p<0.001$ in comparison with control (untreated group).

Biochemical Analysis

Hydroxyproline estimation

Increased levels of hydroxyproline in the wound tissue suggest an increased fibroblastic activity. It thereby indicates increased synthesis of collagen. Collagen provides integrity and strength to the tissue matrix and plays a vital role in epithelization and homeostasis.[44]

Studies have revealed a lower quantity of hydroxyproline in diabetic patients with foot ulcers that delay the repair process. They further suggested that the usage of approved available drugs and applications would increase the tissue hydroxyproline content.[45] The present study advocates the same and has found promising results in improving the tissue hydroxyproline content in diabetic wounds treated by the test materials.

In the normal excision wounds, all the treated groups' tissues showed higher values of hydroxyproline compared to control (normal-untreated) on days 8 and 16. On the contrary, NI showed lower hydroxyproline levels compared to control (normal-untreated) on day 16. The findings, however, were not significant statistically. Only GG treated skin showed statistically significant value on day 8 of healing.

Among the diabetic models, NI and Tot treated groups showed significant results on day 8. The rest of the groups, although showed an increase, the findings were, however, not significant statistically (Table 1).

Nisbet *et al*. carried out the hydroxyproline estimation. They demonstrated that the collagen and hydroxyproline levels in the groups treated with honey were significantly more than the untreated control on days 7 and 14.[20] Although a marginal increase was found in the hydroxyproline levels in the present study, the findings were, however, not significant statistically in both normal and diabetic wounds.

Earlier studies using anti-aging *Ayurvedic* formulations have shown that the combination of fruit extract of *Phyllanthus emblica (Amalaki)* and ghee exhibited a slight enhancement in the hydroxyproline levels, but was not statistically significant.[18] In the current study, too, the ghee and H+G treated wounds, although showed an increase in the hydroxyproline levels; the findings were, however, statistically insignificant in normal wounds. The diabetic wounds treated with ghee and H+G also showed similar results.

The healing ability of the aqueous GG extract on dermal ulcers showed a significant rise in the number of fibroblasts, capillary buds and improved the tensile strength of the ulcers. Increased collagen content, as indicated by the higher hydroxyproline levels, was also observed.[23] Similarly the current study also found a significant upsurge in hydroxyproline levels in the normal wounds treated with GG on day 8. Increased hydroxyproline levels were also observed in diabetic wounds treated with GG but were not statistically significant.

Treatment of the wounds with NI showed better healing, as indicated by increased hydroxyproline content in a previous study.[24] In the present study in normal excision wounds, NI showed slightly higher hydroxyproline values on day eight, which was not significant statistically.

However, the diabetic wounds treated with NI exhibited a significant rise in the hydroxyproline levels on day 8.

The combination of test materials (Tot) showed a marginal increase in the hydroxyproline levels on both days 8 and 16. The findings were, however, not statistically significant. The diabetic wounds treated with Tot showed statistically increased hydroxyproline levels on day 8. This observation further glorifies the benefits of the combination of the test materials in treating diabetic wounds.

Antioxidants: CAT, GSH, SOD, MDA

ROS is useful during healing as they offer defense against invading pathogens and mediate cellular signaling.[46] The intracellular concentration of ROS is determined by its production and elimination by the antioxidants. Many antioxidants are present in a cell that repairs the damage caused by ROS and regulates the redox-sensitive signaling pathways.

Earlier studies have also shown that overexposure to ROS, i.e., oxidative stress, results in poor wound healing.[3,47] An increase in the antioxidant level indicates ROS's decreased activity, thereby indicating better healing.[48]

Animal experiments conducted in the past have proposed that the application of powerful antioxidants at the wound site helps bring the ROS to normal levels. In turn, it aids in reversing the chronicity of the wounds such as diabetic wounds and favors healing.[49]

A previous study on the gastroprotective influence of manuka honey in rats showed that it considerably improved the levels of Nitric Oxide (NO), GSH, Glutathione Peroxidase (GPx), and SOD and decreased the levels of MDA. The CAT levels were, however, unaffected.[50]

In the present study on normal wounds, honey showed a biological increase in the CAT activity, GSH, and SOD compared to control on days 8 and 16. But the GSH levels on day 8 showed a slight decline. These findings were, however, not significant statistically. On the contrary, we found a substantial reduction in the levels of MDA in the normal groups indicating decreased activity of ROS in the honey-treated wounds on both days 8 and 16.

The diabetic wounds applied with honey exhibited a significant rise in the level of GSH on day 16. The levels of CAT and SOD, although saw a biological increase, the findings were, however, statistically insignificant. The levels of MDA were significantly decreased in the honey treated diabetic wounds on both days 8 and 16, thereby indicating potent antioxidant activity (Figures 2 and 3). Interestingly it was even better compared to normal wounds (Figure 1).

However, the antioxidative ability of ghee (solely), although mentioned in *Ayurvedic* scriptures, is not documented experimentally.[11] The wound healing ability of two polyherbal formulations in ghee showed an increase in SOD and GSH. Simultaneously, the free radicals (lipid peroxidation) were reduced significantly.[51]

In the present study on normal wounds, ghee showed increased levels of the antioxidants. The catalase levels in the wound tissue were significantly higher on both days 8 and 16. Ghee treated groups showed significantly higher levels of GSH on day 16. SOD also showed increased activity on day 8. Although the activities of GSH on day 8th and SOD on day 16th were higher, however, was statistically insignificant. MDA levels were significantly reduced on both days 8 and 16 in the ghee treated wounds.

The ghee treated diabetic wounds displayed significantly increased SOD levels on day 16. Although CAT and GSH levels showed a rise, the findings were, however, not significant statistically. The MDA levels also failed to show a substantial decrease (Figures 2 and 3). Ghee treated diabetic wounds also showed poor healing and wound closure than normal wounds as indicated by the macroscopic observations (Figure 1). Fat embolism at the wound site might have been responsible for poor healing. The factor underlying this observation, however, needs further exploration.

H+G treated wounds of normal animals presented a significant upsurge in catalase and SOD levels on day 8. Their activity on day 16, although higher, was statistically insignificant. GSH also showed increased activity on both days 8 and 16. The finding was, however, not statistically significant. MDA levels found a considerable reduction on both days 8 and 16. The diabetic wounds treated with H+G also showed better antioxidant activity. GSH, SOD, and MDA levels showed significant findings on day 16 (Figures 2 and 3). This finding is unique and is not documented previously.

GG's antioxidant properties have been discussed previously, aiding its wound healing and rejuvenating effects.[52] In a rat model of metabolic syndrome X, glycyrrhizin was found to reduce oxidative stress, as indicated by increased antioxidants (CAT and SOD) and reduced MDA levels.[53] In the current study on normal animals, GG treated wounds showed significantly higher levels of CAT on day 8 and SOD on both days 8 and 16. The levels of catalase on day 16 and GSH levels on both days 8 and 16, although higher but were not significant statistically. Significantly reduced MDA levels were also observed on day 16. Day 8, although showed a decrease, the finding, however, was statistically insignificant.

In diabetic wounds, although the levels of catalase, GSH, and SOD were biologically higher in the GG treated groups, the findings were, however, not significant statistically. The MDA levels, on the contrary, were significantly reduced on day 8 (Figures 2 and 3). This finding affirms the antioxidant ability of GG.

In a non-wound model, NI increased the activity of endogenous antioxidants such as SOD, CAT, GSH and decreased levels of MDA in blood and brain tissues.[54] In the present study on normal animals, biologically higher antioxidant activity was observed in the NI treated wounds. But these findings were not significant statistically. Although lowered on both the 8th and 16th day, the MDA levels were significantly reduced only on the 16th day.

The diabetic wounds treated with NI showed a significant level of catalase activity on day 8. The rest of the antioxidants' activity was although better; the findings were, however, statistically insignificant (Figures 2 and 3).

The combination of test materials (Tot) also increased antioxidant activity at the wound site in normal wounds. A significant value was found in the CAT activity on day 16. MDA levels also showed a substantial reduction on day 16. The diabetic wounds treated with Tot, although showed the antioxidants' better activity, the findings were, however, not significant statistically (Figures 2 and 3).

Therefore, it could be stated that these natural medicines may aid in enhancing the antioxidant activity and thus lowers the activity of the ROS. The reduced activity of ROS, in turn, prevents tissue damage and improves wound healing in both normal and diabetic wounds.

Molecular Analysis

Assessment of alteration of the growth factors at wound site using RNA isolation and reverse transcription

The pathophysiology of chronic wounds has been attributed to a delayed healing process, probably due to the wound's impaired ability to harness the growth factors responsible for healing. The chemotaxis of growth factors towards chronic wounds is complicated, the details of which are still being unraveled. However, it is now known that wound healing is promoted by some growth factors such as TGF, FGF, PDGF, VEGF, Keratinocyte Growth Factor (KGF), Epidermal Growth Factor (EGF), and Insulin-like Growth Factor (IGF).[55]

TGF-β plays a significant role in enhancing the inflammatory response and tissue debridement. TGF-β, along with PDGF, also helps in converting fibroblasts into myofibroblasts, thereby bringing about wound closure.[56]

In earlier studies, increased activity of TGF β was found in the wound tissues treated with curcumin[57] and aloe vera gel.[58]

However, in the present study, normal wound tissue did not show the expression of TGF β. Inability to detect the TGF β expression in normal wound may be due to very low level of TGF which could not be detected using the current method of semi quantitative PCR. Further, RT-PCR with SYBR green assay may be more sensitive, but beyond the scope of the present study.

In diabetic wounds, the expression of TGF β was observed. It was significantly higher in the groups treated with Honey, GG, and NI (Figure 4).

FGF2 is believed to be helpful in tissue regeneration and wound healing.[59] Studies have also indicated that the topical application of FGF2 on skin wounds quickens both epidermal and dermal recovery.[60,61] Our previously published *in vitro* studies on dermal fibroblasts showed that all the treated groups had significantly greater FGF2 expression levels in comparison to control (normal-untreated).[27] In the current study, FGF2 displayed a significantly higher expression in the wounds treated with honey, ghee, and H+G in normal animals. Among the diabetic groups, GG and NI treated animals showed better expression of FGF2 (Figure 4).

Further, PDGF is a potent stimulator of the cells of mesenchymal origin. It instigates chemotaxis, proliferation, and expression of new genes in the monocytes, macrophages, fibroblasts, and the other types of cells crucial in wound healing.[62] PDGF-BB (a PDGF homodimer, similar to the one present in humans) is used to treat injuries. Herein the proliferation of fibroblasts and smooth muscle cells are recruited in wound healing.[63] Earlier Greenhalgh *et al*. revealed that recombinant PDGF-BB treated genetically diabetic mice showed better healing of full-thickness skin wounds.[64] Recombinant PDGF-BB was also found to increase the healing skin's tensile strength in diabetic rats, thereby promoting wound healing.[65,66] In the current study, PDGF expression was statistically higher in the groups treated with GG, H+G, and Tot in normal animals. Among the diabetic groups, GG and NI showed a significant increase in the PDGF levels (Figure 4).

VEGF is a potent proangiogenic growth factor, and its amount in the wound can considerably influence healing. VEGF affects various healing process mechanisms, including angiogenesis, epithelization, and collagen deposition.[67] Since angiogenesis upholds a principal role in wound healing, in the future, VEGF (alone or in combination) may be employed on non-healing wounds.[68]

In the current study, ghee, H+G, and Tot treated groups displayed increased VEGF levels in normal animals. The rest of the groups showed an increase; the findings were not significant statistically. On the contrary, in diabetic animals, the groups treated with honey, GG, NI, and Tot showed statistically significant results (Figure 4).

Previous studies on cancer cells have found GG to be anti-angiogenic, and it was proposed as a possible supplementary source for the treatment of cancer.[69] In the current study, the groups treated with GG did not show any significant alteration in VEGF expression in normal wounds. However, the diabetic wounds treated with GG showed a considerable rise in the VEGF expression (Figure 4). VEGF is known to be specific to the endothelial lining of blood vessels. It is also found that a variety of other cell types involved in wound repair, e.g., keratinocytes and macrophages, can also express VEGF Receptors that can respond directly to VEGF.[70] Although GG is anti-angiogenic, its progressive effect on the proliferation of keratinocytes and macrophages would have been responsible for the increased VEGF production, thereby healing diabetic wounds, as observed in the present study. We also propose that VEGF activity differs in normal and cancerous tissues.

Therefore, traditional medicines have a positive influence on the growth factors involved in wound healing, as specified by the current study. The study also indicates that the test materials showed better results in the wounds of diabetic animals compared to normal. It could be due to the differential healing properties of these traditional medicines that are influenced by the differences in the wound healing mechanisms and microenvironment in normal and diabetic wounds.

Limitations of the study

Further research is required to identify the specific exogenous and endogenous factors influencing wound healing in different wound types. A thorough evaluation is also essential to explain the test materials' differential behavior singly and in combination and also in different wound types, i.e., normal and diabetic. The nature of the healed skin post treatment should also be assessed to identify the rejuvenating benefits of these traditional medicines.

Further, a comparison between oral and topical application of the test materials may provide a better idea about the drug's systemic effect. There is also a need to compare the mechanisms and healing benefits of Honey, Ghee, GG, and NI (singly and in combination) with the standard mode of treatment (allopathic) for wound healing. Alternative (*in vitro*) experimental models such as co-cultures of keratinocyte–fibroblast, 3D skin cultures, etc., may also be considered for exploring the molecular mechanisms of wound healing. It, in turn, would reduce the usage of animals in future research.

CONCLUSION

The present study provides scientific explanations to the biochemical and molecular modes of action of these traditional medicines, i.e., Honey, Ghee, GG, and NI singly and in combination. The study, therefore, concludes that these traditional medicines can play a vital role in treating and managing medically challenging wounds. The evidence presented by the current research provides increased value to traditional Indian medicines' efficacy in the national and international scenario.

Additionally, the findings derived from the present study may serve as stepping stones for further evaluation of various other intricate mechanisms that may aid in developing new approaches to the treatment of medically challenging wounds.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ROS: Reactive oxygen species; **CAT:** Catalase; **SOD:** Superoxide dismutase; **GSH:** Glutathione; **GST:** Glutathione s-transferase; **TGF-β:** Transforming growth factor-beta; **FGF2:** Fibroblast growth factor 2; **PDGF:** Platelet-derived growth factor; **VEGF:** Vascular endothelial growth factor; **IL1 β:** Interleukin 1 beta; **GG:** *Glycyrrhiza glabra* L.; **NI:** *Nerium indicum* Mill.; **H+G:** Honey+Ghee; **HPTLC:** High-performance thin-layer chromatography; **STZ:** Streptozotocin; **LPO:** Lipid peroxidation; **MDA:** Malondialdehyde; **PBS:** Phosphate buffered saline; **DNA:** Deoxyribonucleic acid; **RNA:** Ribonucleic acid; **cDNA:** Complementary DNA; **mRNA:** messenger RNA; **PCR:** Polymerase chain reaction; **GAPDH:** Glyceraldehyde-3-phosphate dehydrogenase; **PUFA:** Polyunsaturated fatty acids; **NO:** Nitric oxide; **GPx:** Glutathione peroxidase; **KGF:** Keratinocyte growth factor; **IGF:** Insulin-like growth factor; **EGF:** Epidermal growth factor.

SUMMARY

Honey, ghee, *Glycyrrhiza glabra* L. (GG), and *Nerium indicum* Mill. (NI) have been effectively used in *Ayurveda* and Indian folk medicine for healing both normal and diabetic. However, the exact biochemical and molecular mechanisms involved is less discussed. The present study explores the wound healing efficacy of these traditional medicines in normal and diabetic rats using biochemical and molecular parameters. Normal and Streptozotocin-induced diabetic Wistar rats were used for the study and inflicted with excision wounds. The test materials, i.e., honey, ghee, GG, and NI, was topically applied to the wounds singly and combined (H+G: honey+ghee and Tot-combination of all test materials). On the 8th and 16th days of healing, biochemical and molecular parameters were assessed using the tissues procured from the wound site. We quantified the levels of hydroxyproline and antioxidants (CAT-Catalase, GSH-Glutathione, SOD-Superoxide dismutase, MDA-Malondialdehyde). We also measured the expression of growth factors, i.e., Transforming Growth Factor-beta (TGFβ), Fibroblast Growth Factor 2 (FGF2), Platelet-Derived Growth Factor (PDGF), and Vascular Endothelial Growth Factor (VEGF). Biochemical analysis showed enhanced hydroxyproline levels in both normal and diabetic wounds treated with the test materials. Better antioxidant properties and increased expression of growth factors were also observed. The study attempts to provide a biochemical and molecular insight to wound healing by traditional medicines and glorifying their rejuvenating properties.

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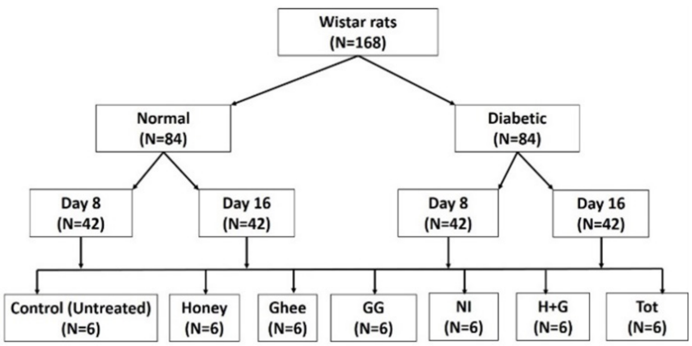

Supplemental 1: Schematic representation of the experimental groups involved in the study.