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# Efficacy of Quercetin Treatment and Low Level Laser Therapy on Wound Healing in Normal and Diabetic Rats

**Ebtesam Aboud[1], Osama M. Ahmed[2], Tarek Mohamed[3], Hany Hamdy[3], Hala Moustafa[5]**

[1, 5]Biomedical Equipments Department, Faculty of Applied Medical Science, October Six University, Egypt

[2]Physiology Division, Zoology Faculty of Science, Beni-Suef University, Beni-Suef, Egypt

[3, 4]Physics Department, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt

**Abstract:** ***Background: Low level laser therapy (LLLT), commonly known as photo-stimulation or phototherapy has emerged. Methods: One hundred rats were used and streptozotocin (45mg /kg body weight) was applied for diabetic induction. An 2cm$^2$ full thickness skin wound was created a specifically with a scalped in 10 groups of non-diabetic rats and diabetic rats on the shaved back of the animal. The study was performed using 630.8 nm laser incident dose of 6.36 J/cm$^2$ and treatment schedule of 3 times/week with quercetin were used in the experiment. The area of wound in all rats was measured. LTB4, PGE2, TNF-α, and IL-4 in the serum level in rats were determined using ELISA technique. Results: Serum LTB4, PGE2, TNF-α, and IL-4 levels were significantly improved as a result of treatment of non-diabetic and diabetic wounded rats with quercetin and LLLT. The concomitant treatment with quercetin and LLLT produced the most potent effects on altered cytokines as a result of quercetin treatment or LLLT. Reduction in the wound size was also observed as a result of treatment with quercetin and LLLT. The pro-healing actions seem to be due to increased collagen deposition as well as better alignment & maturation in second week. The weak linear response of the stress strain characteristic curve for the control group is due to the fact that tissue is mainly composed of two components, collagen and hydroxyapatite crystals. The stress/strain exhibited significant changes between the treated and non-treated groups. Conclusion: The biomechanical and biochemical analysis and observation suggested that 6.36 J/cm$^2$ laser photo-stimulation and quercetin treatment facilitates the tissue repair process by accelerating collagen production in diabetic wound healing.***

**Keywords:** Low level laser, quercetin, cytokines, stress strain characteristic curve, LTB4, PGE2, TNF- α, and IL-4

## 1. Introduction

Wound healing is an interaction of complex cascade of cellular and biochemical actions healing to the restoration of structural and functional integrity with regain of strength of injured tissues **(Kumarasamyraja *et al*., 2012).** It Involves continuous cell – cell interaction and cell matrix interactions that allow the process to proceed in different overlapping phases and process including inflammation, wound contraction, re- epithelialization tissue, remodeling, and formation of granulation tissue with angiogenesis **(Vetriselvan 2013).** In tissue homeostasis remodeling and repair, mast cells store and release various potent mediators in particular histamine, proteases, lipid mediators and cytokines through which they can influence different stages of cutaneous wound healing **(Kumarasamyraja *et al*., 2012).**

The medicinal value of the medicinal plants lies in the bioactive phytochemical compounds that produce normal physiological action on the human body **(Akinmoladun *et al*., 2007).** Some of the most valuable bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, phenolic compounds and many more compounds **(Edeoga *et al*., 2005)**. Quercetin, a bioflavonoid has anti-proliferative effects on normal and malignant cells and it has antihistamine release effects; these properties could theoretically prove its beneficial effects in reversing the proliferative and inflammatory responses in hypertrophic scars **( Saulis *et al*., 2002)**.

The application of phototherapy or low-level laser therapy (LLLT) has generated considerable interest within surgery, dentistry, dermatology, somatology, pain management and wound healing **(Moustafa *et al*., 2012)**. LLLT is a therapeutic method that involves the application of laser light, at a particular wavelength and at low intensities, to the tissue to stimulate biological processes **(Nteleki and Houreld 2012; Abass *et al*., 2012).** The use of laser as a non-surgical medical treatment modality for assisting the normal processes of healing has increased over the last few years **(Hawkins and Abrahamse, 2010)**. However, the efficacy of laser in reducing pain or promoting tissue repair still remains controversial **(Enwemeka *et al*., 2004)**. Laser therapy aims to restore the normal biological function of injured or stressed cells so normalization which is the keystone of laser therapy. The stimulatory effect of laser therapy can be seen in wounded cells or in cells that are growing sub-optimally whereas cells that are normal or fully functional remain unaffected and no therapeutic effect can be observed **(Hawkins and Abrahamse, 2010).** Therefore, this study is designed to assess the efficacy of quercetin treatment and LLLT on wound healing in normal and streptozotocin (STZ)-induced diabetes mellitus.

## 2. Materials and Methods

### Experimental animals

A total of one hundred male albino rats weighting 100-120 g and aging 8-9 weeks obtained from National Research Centre (Dokki, Giza, Egypt) were used in the present investigation. The rats were kept at a constant temperature of

22-25°C with a 12-hours light/dark cycle and free access to water and standard diet *ad libitum*. All animal procedures are in accordance with the guidelines of Experimental Animal Ethics Committee, Faculty of Science, Beni-Suef University, Egypt. All efforts were done to reduce the number and suffering of animals.

**Induction of diabetes mellitus**

Streptozotocin (STZ) was dissolved in cold citrate buffer, pH 4.5 and was prepared freshly for immediate use within 5 min. STZ injection was intraperitoneally injected as a single dose of 45 mg/kg to rats deprived of food and water for 16 hours. The dose of STZ was administered at a volume 1 ml/kg b. wt of rats. Ten days after STZ injection, rats were fasted overnight (10-12 hours) and then orally given glucose at dose level of 3 g/kg b.wt after 2 hours of glucose intake, blood was collected from lateral tail vein of each rat and blood glucose level was measured by Glucometer (Model Bionime Rightest GM 100). The rats that have blood glucose level ranged from 180-300 mg/dl were considered as mild diabetic rats and were included in the experiment.

**Surgical procedure**

Animals were anaesthetized by intraperitoneal administration of ketamine at dose level of 60 mg/kg b. wt. The dorsal fur of the animals was shaved with an electric clipper, and the anticipated area of the wound to be created was outlined on the back of the animals with a marker pen. An excision wound of size 2 $cm^2$ was made by cutting out a 2×2 cm piece of skin from the shaved area. The wounds were of full thickness type extending up to the subcutaneous tissue **(Maiya *et al.*, 2009).**

**Dose prepration of quercetein:-**

Quercetin was administered to rats at dose level of 25 mg /kg b. wt **(Skaper *et al.*, 1997)** every other day for two weeks. For dose Preparation 25mg of quercetein was dissolved in 5 ml, 1% carboxy methyl cellulose, then was given to kg of rats

**Low level laser therapy (LLLT):-**

Laser used was He-Ne laser (NEC, Japan). Laser has a given wavelength of 632.8 nm. Its energy density is the most important factor in determining the tissue reaction **(Plaetzer *et al.*, 2002)**. Power density was 3 $mW/cm^2$, wavelengths was 632.8 nm, spot size of the laser beam was 1 $cm^2$ with incident doses of 6.36 $Joules/cm^2$ beam cross-section was 0.3 $cm^2$, and application time was 10 min. Power density measured the potential thermal effect of those photons at the treatment area **(Bruce *et al.*, 1992)**. Despite the high output power, this laser also has biostmulating effects **(Turner and Hode, 2002; Ahmed *et al.*, 2012).** The energy deposited was calculated through the relation

$$\text{Energy density (Joule/cm}^2\text{)} = \frac{\text{Laser output power (watts) x Time (secs)}}{\text{Beam area (cm}^2\text{)}}$$

**Animal grouping:-**

After induction of diabetes mellitus and wounding, the rats were allocated in the following groups each of ten rats.

Group 1: Rats included in this group are non-diabetic non-wounded rats.

Group 2: Rats of this group are non-diabetic wounded rats.

Group 3: Rats in this group are non-diabetic wounded rats administered quercetin at dose level of 25 mg/kg b. wt by oral gavage every other day for 15 days.

Group 4: Rats included in this group are non-diabetic wounded rats subjected to LLLT every other day for 15 days.

Group 5: Rats within this group are non-diabetic wounded rats treated with quercetin by oral gavage and subjected to LLLT every other day for 15 days.

Group 6: Rats included in this group are diabetic non-wounded control rats.

Group 7: Rats of this group are diabetic wounded rats.

Group 8: Rats within this group are diabetic wounded rats that are administered quercetin at dose level of 25mg/kg by oral gavage every other day for 15 days.

Group 9: Rats included in this group are diabetic wounded rats subjected to LLLT every other day for 15 days.

Group 10: Rats within this group are diabetic wounded rats treated with quercetin by oral gavage and subjected to LLLT every other day for 15 days.

**Collection of the blood samples**

After 7 and 15 days of stabilization period, blood samples were collected from jugular vein of fasting animals. The blood was left to coagulate at room temperature and then centrifuged at 3000 rpm for about 15 minutes. The supernatant sera were aspirated and fractioned into three vials.

**Measurement of wound diameter and wound closure (healing factor):-**

The wound diameter was measured on days 1, 5, 10 and 15 after incision and percentage of wound closure (healing factor) was calculated using the following formula:

Wound closure rate on day X (%) = [(wound diameter on day 0 – wound diameter on day X) / (wound diameter on day 0)] X 100 **(Maiya *et al.*, 2009).**

**Estimation of serum cytokine levels:-**

PGE2, TNF-α, LTB4 and IL-4 level were determined by ELISA kit obtained from Scientific Group. S. A. according to the manufacturer's instructions.

**Biomechanical Measuring System:-**

To measure the thickness of the skin strips, an electronic micrometer (Micro 2000, Moore & Wright and Sheffield, UK) was used. Tensile testing machine (Force meter BG500, USA) was used to stretch the strips, using 10 mm of their length at both ends to clamp them to the machine. A sample was spaced 40 mm between the jaws they were located and stretched to failure by the tensile testing machine. Load-deformation curve, tensile strength, strain and Young's modulus of elasticity were recorded by a computer program (Mark-10).

The elastic modulus of an object is defined as the slope of its stress-strain curve in the elastic deformation region:

$\lambda$ = Stress / Strain

Where: λ (lambda) is the elastic modulus
Stress is the force per unit area
Stress (σ) = Force / Area ($N/m^2$)
Force = m × g
Area = $\pi r^2$
M: mass
G: gravity of acceleration (9.8 cm /$s^2$)
$\pi r^2$= 3.14 ×radius

## 3. Statistical Analysis

The data were analyzed using a one-way analysis of variance (ANOVA) (PC-STAT, University of Georgia, 1985) followed by the LSD test to compare various groups with each other. The results were expressed as the mean ± SE, and values of P>0.05 were not considered statistically significantly different, whereas values of P<0.05 were considered significant.

## 4. Results

**Effects of quercetin treatment and LLLT on stress/strain and wound healing in rats:-**

Figures 1, 2, 3 and 4 showed the stress-strain characteristic curves of skin of 10 animals of each group. The results indicated two portions in the characteristic curve where in the first part an approximately linear dependence of the strain upon the applied stress, re-followed by a nonlinear dependence of the strain on the stress, after which the skin is cutting. By comparing the treated non-diabetic groups with non-treated non-diabetic control groups after 1 and 2 weeks, the stress/strain was significant as a result of quercetin treatment and/or LLLT (Figures 1 and 2) similarly for diabetic rats the stress /strain was also significantly affected in the treated diabetic groups in comparison with the non-treated diabetic ones (Figures 3 and 4).

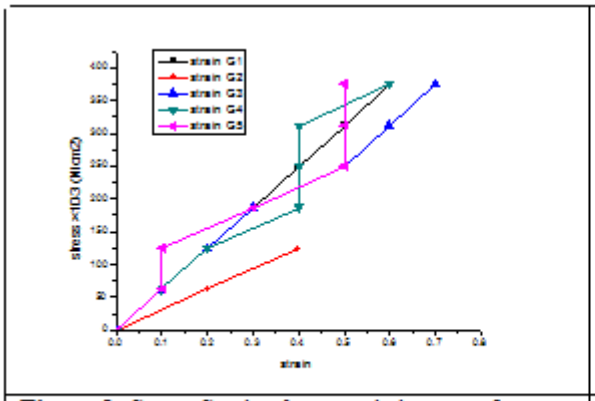

**Figure 1:** Stress-Strain characteristic curve for non-diabetic rats from groups 1, 2, 3, 4 and 5 after one week.

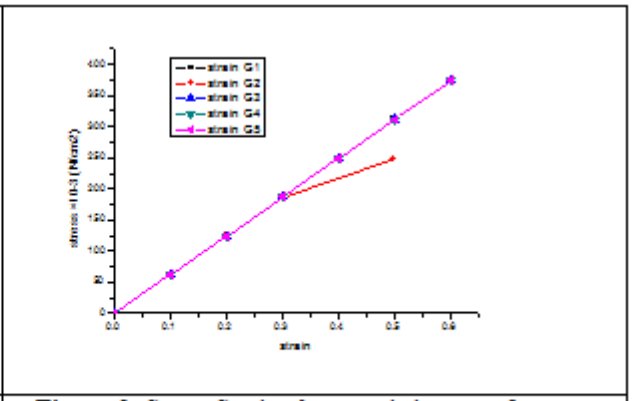

**Figure 2:** Stress-Strain characteristic curve for non-diabetic rats from groups 6, 7, 8, 9 and 10 after two weeks.

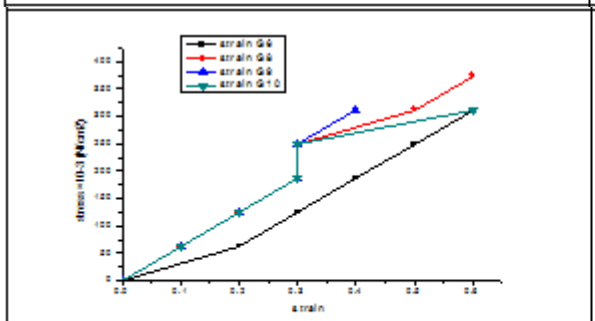

**Figure 3:** Stress-Strain characteristic curve for diabetic rats from groups 1, 2, 3, 4 and 5 after one week.

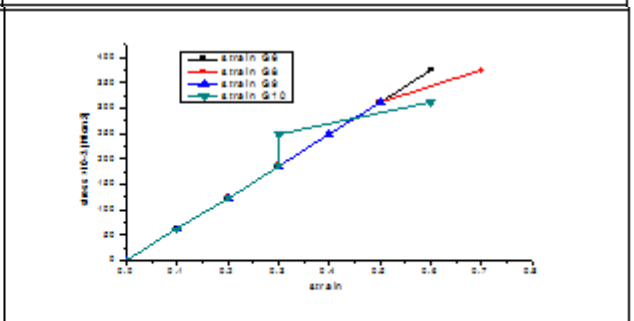

**Figure 4:** Stress-Strain characteristic curve for diabetic rats from groups 6, 7, 8, 9 and10 after two weeks.

There were significant decreases (P<0.001) in the wound closure rate (healing factor) of wounded non-diabetic treated with quercetin and LLLT as compared with wounded non-diabetic control (group 2; G2) (Figure 5). Similarly, there were significant increases (P<0.001) in the wound healing acceleration in wounded diabetic treated rats as compared with wounded diabetic control group (group 7; G7) (Figure 6). The experimental wounds were, on average, nearly completely healed by the 15$^{th}$ day of quercetin treatment and LLLT, whereas those of control groups were not. The treatments are arranged according to their potency to enhance wound closure rate in the following order: quercetin in combination with LLLT, LLLT and quercetin,

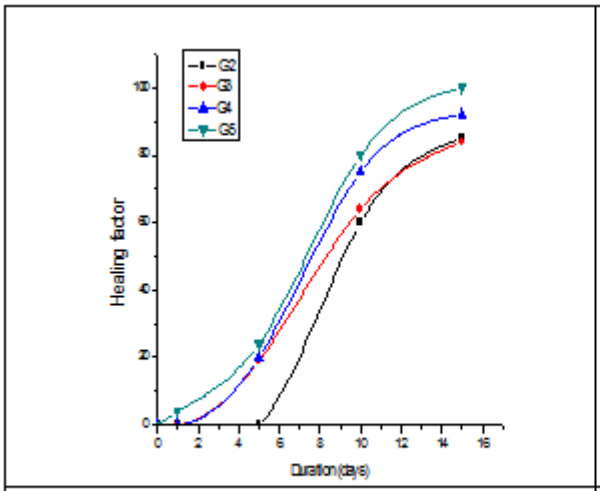


**Figure 5:** Effect of quercetin treatment and LLLT on wound closure rate (healing factor) in wounded no-diabetic rats.

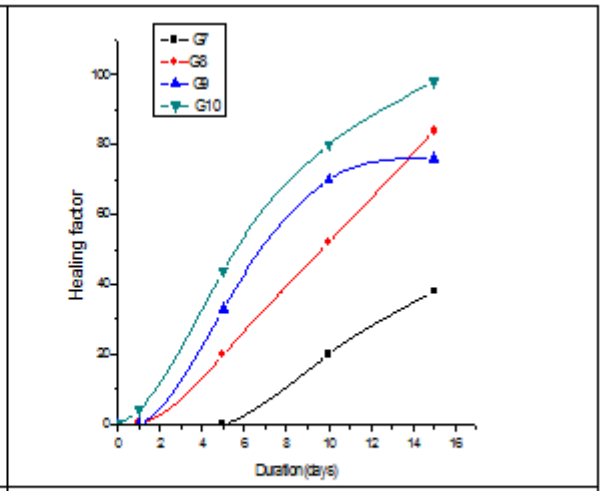


**Figure 6:** Effect of quercetin treatment LLLT and on wound closure rate (healing factor) in wounded diabetic rats.

**Effect of quercetin treatment and LLLT on various serum cytokine levels:-**

Data showing the effects of quercetin administration and/or LLLT for one week on serum LTB4, PGE2, TNF-α and IL-4 levels were respectively illustrated in figures 7, 8, 9 and 10.

Serum LTB4 level was vigorously elevated in wounded diabetic group at the 7$^{th}$ day after wounding as compared to non-wounded diabetic and non-wounded non-diabetic groups although it was non-significantly affected in that group at the 15$^{th}$ day. The treatment of wounded diabetic rats with quercetin or with quercetin in combination with LLLT induced a significant decrease of the elevated LTB4 the 7$^{th}$ and 15$^{th}$ days. While serum LTB4 level was significantly decreased as a result of LLLT of wounded diabetic rats the 7$^{th}$ day, it was significantly increased the 15$^{th}$ day as compared with the corresponding with the wounded diabetic controls (Figure 7).

Serum PGE2 was significantly elevated in wounded non-diabetic and wounded diabetic rats as compared with the corresponding non-wounded controls. The treatment of these wounded rats with quercetin and/or LLLT successfully improved the elevated PGE2 at the 7$^{th}$ and 15$^{th}$ days as compared with corresponding non-treated controls (Figure 8).

Serum TNF-α level was significantly increased in wounded non-diabetic and wounded diabetic rats as compared with the corresponding non-wounded controls; the effect was more deteriorated as the time extended from 7 to 15 days. The treatment of wounded groups with quercetin and/or LLLT significantly decreased the elevated TNF-α level as compared with the corresponding controls. Moreover, quercetin in combination with LLLT seemed to me the most effective in improving the elevated TNF-α level at 15$^{th}$ day after wounding (Figure 9).

Serum IL-4 level was significantly increased in wounded non-diabetic rats at the 7$^{th}$ day while it was non-significantly altered in these animals at 15$^{th}$ day. The diabetic non-wounded rats exhibited a significant increase at 7$^{th}$ day while they showed a significant decrease at 15$^{th}$ day as compared with the corresponding non-diabetic groups. However the level of this cytokine was significantly depleted in wounded diabetic rats at the 7$^{th}$ week, it was significantly increased at the 15$^{th}$ week (Figure 10).

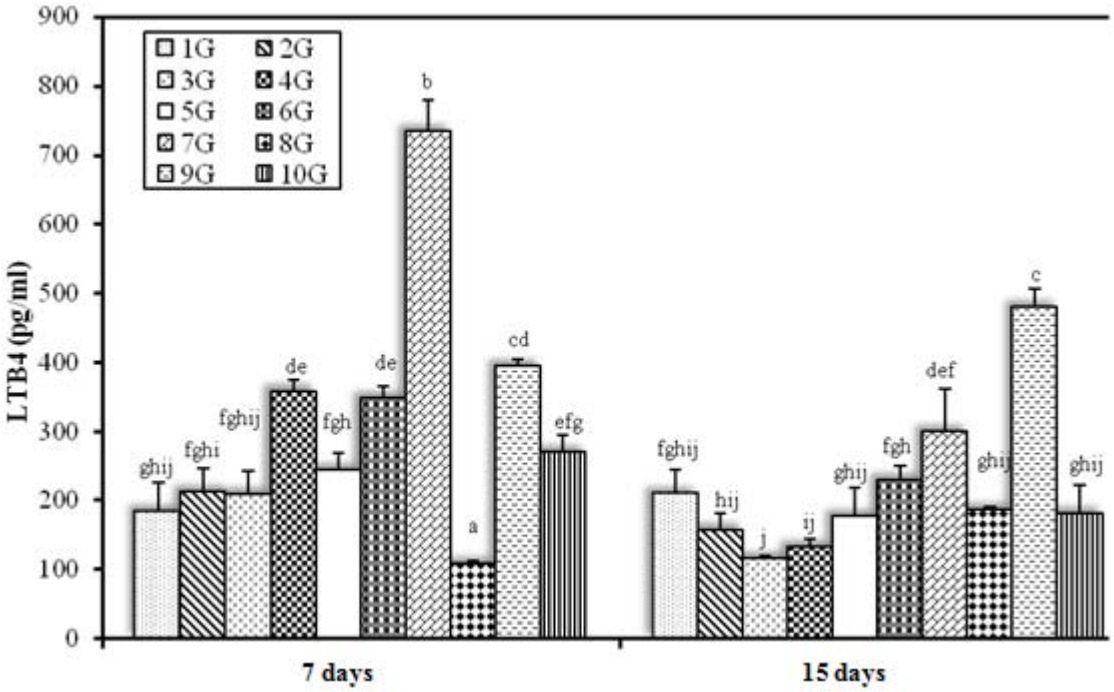


**Figure 7:** Effect of quercetin treatment and LLLT on serum LTB4 level in non-diabetic and diabetic wounded rats. Means, which have the same superscript symbol(s), are not significantly different

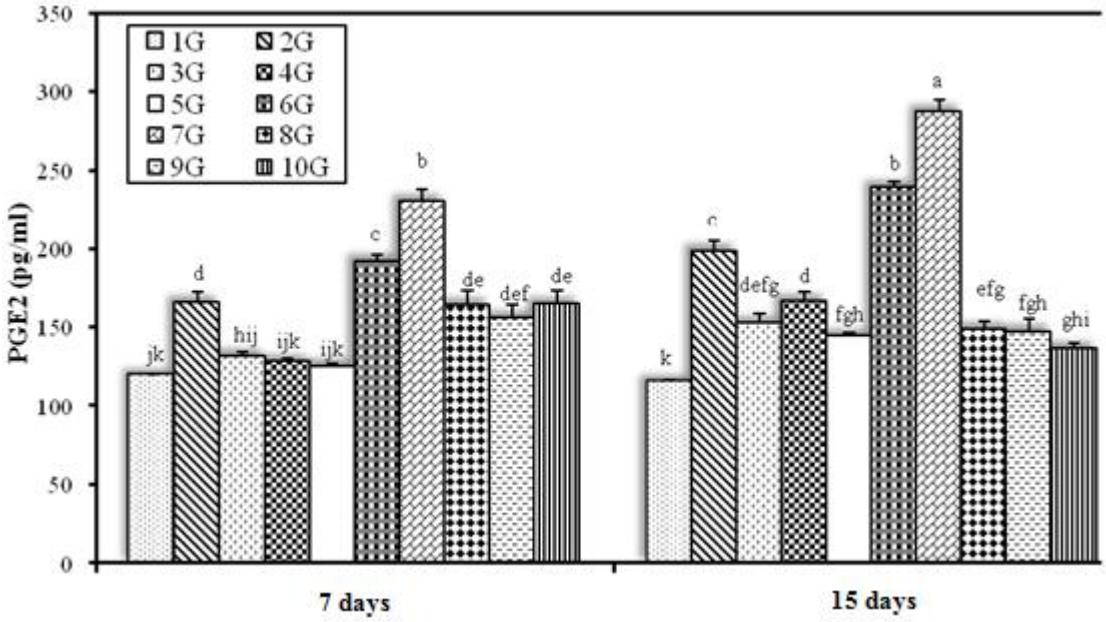


**Figure 8:** Effect of quercetin treatment and LLLT on serum PGE2 level in non-diabetic and diabetic wounded rats. Means, which have the same superscript symbol(s), are not significantly different.

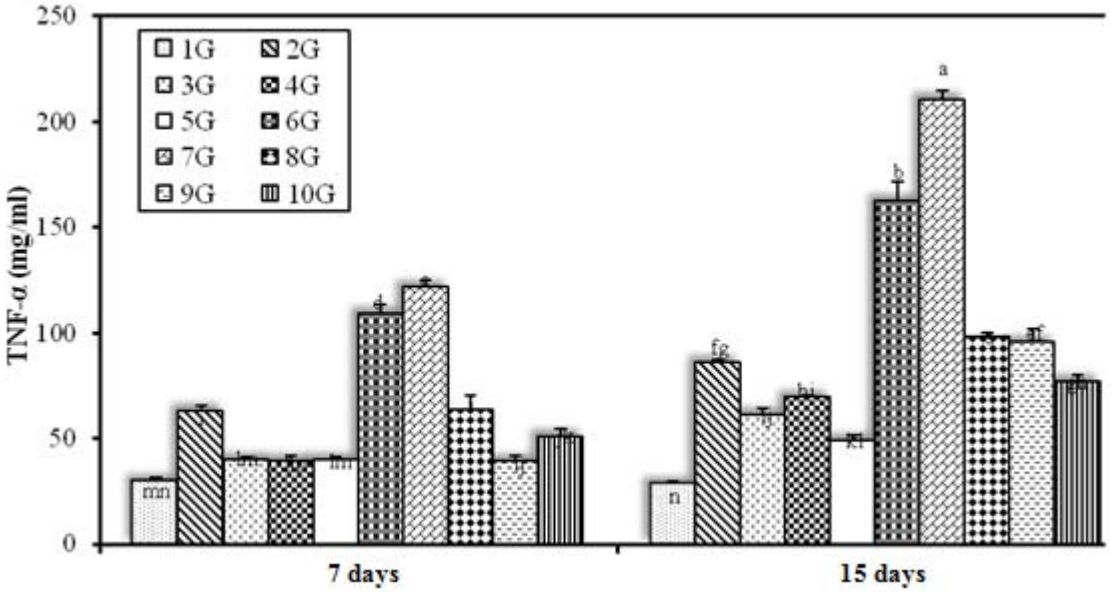


**Figure 9:** Effect of quercetin treatment and LLLT on serum TNF-α level in non-diabetic and diabetic wounded rats. Means, which have the same superscript symbol(s), are not significantly different.

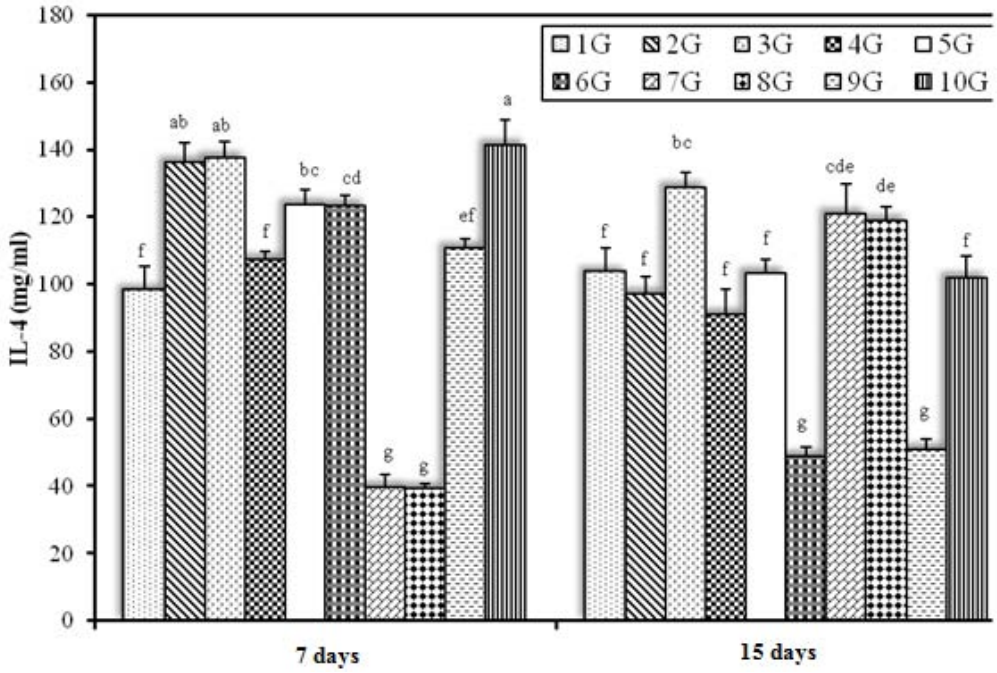


**Figure 10:** Effect of quercetin treatment and LLLT on serum IL-4 level in non-diabetic and diabetic wounded rats. Means, which have the same superscript symbol(s), are not significantly different

## 5. Discussion

In humans and in all mammalian species, the wound healing process can be subdivided into distinct consecutive and overlapping stages: hemostasis, inflammation, debridement, new tissue formation and remodeling **(Contran *et al*., 2001; Ebaid *et al*., 2013)**. The hemostatic phase occurs immediately after the appearance of the lesion and depends on platelet activity and on blood coagulation process, which includes a complex release of vasoactive substances, adhesive proteins, and growth factors for the development of other stages **(Contran *et al*., 2001; Mandelbaum, 2003)**. Quercetin is a bioflavonoid known to inhibit free radical processes in cells **(Kastsarou *et al*., 2000)**. It is able to protect cutaneous tissue type cell populations, fibroblasts, keratinocytes and endothelial cells of human origin from cytotoxic oxidative stress induced by protracted depletion of cellular glutathione **(Skaper *et al*., 1997).** In addition, quercetin has been shown to have an anti-inflammatory effect by stabilizing mast cell membranes and inhibit histamine release from basophils and mast cells as well as an antiproliferative effect in both normal and malignant cells of various types **(Saulis *et al*., 2002; Alexandrakis *et al*., 1999)**. Limiting inflammation is paramount in the control of scar growth and scar associated symptoms **(Denielson and Walter, 2004)**. The anti-inflamatory effect of quercetin, that was previously reported, may be reasonable explanation of its antifibrotic effect **(Lee *et al*., 1999)**. In the present study, after two weeks of quercetin treatment, the wounds were of most completely healed and scar formed. In the present study, the effect of the LLLT accompanied with quercetin was more effective in the treatment of animals at a dose of 6.36 Joules/cm$^2$. The enhanced wound healing may be

attributed to the improvement in collagen formation which can lead to enhancement in wound strength and healing in diabetic or non-diabetic rats treated with quercetin and LLLT. Marked improvement in wound strength and healing may also be due to the activity of the epithelial covering in wounded rats treated with quercetin and laser phototherapy. These explanations were supported by the results of the present study which indicated improvement in stress/strain and wound closure rate or healing factor as result of treatment with quercetin and LLLT. Stress/strain detected at days 1, 5, 10 and 15 post-wounding showed a positive relation between healing factor and treatment time. The treatment with quercetin concomitant with LLLT was found to be the most effective on improving the wound closure and wound healing rate. Moreover, the treatments are arranged according to their potency to enhance wound closure rate in the following order: quercetin in combination with LLLT, LLLT and quercetin. These findings are consistent with the findings of **Pinheiro and Frame (1992)** who reported that laser therapy is used in Biomedicine because it improves tissue regeneration and healing, reduces postoperative pain and reduces inflammation. The reason for the effective acceleration of wound healing in diabetic rats using low-power lasers was that perhaps the absorption of laser light with specific wavelength by target tissue resulted in the enhancement of fibroblast proliferation and the promotion of collagen metabolism and granulation tissue formation in the diabetic wound. The healing process of diabetic wound is a complicate done and is initiated by a complex series of events that include chemotaxis, growth factor pathways, complement generation, and the energy-poor environments created by low oxygen tensions, low pH and high lactate concentrations **(Knighton *et al.*, 1990).** The quercetin treatment or laser exposure at low energy and wavelength of 632.8 nm may modulate the serum and the release of growth factors and cytokines expression. In this way, the quercetin treatment alone or associated with LLLT marked decreased the elevated levels of inflammatory cytokines LTB4, PGE2 and TNF-α in non-diabetic and diabetic wounded rats. The lowered level of anti-inflammatory cytokine IL-4 was significantly increased in wounded diabetic rats as a result of LLLT or LLLT associated with quercetin treatment at the 7$^{th}$ day of treatment period. These results are in agreement with **Dyson and Young (1986)** who showed that positive effect of laser photostimulation on wound healing may involve the enhancement of growth factor release, which in turn promotes extracellular matrix production and degradation.

In the current study, it is worth mentioning that quercetin like LLLT induced marked amelioration of wound healing in comparison with non-treated wounded non-diabetic and diabetic rats. Quercetin is a bioflavonoid known to inhibit free radical process in cells and it is able to protect cutaneous tissue type cell population fibroblasts, keratinocytes and endothelial cell of human origin from cytotoxic oxidative stress induced by protected depletion of cellular glutathione **(Skaper *et al.*, 1997)**. In addition quercetin has been shown to have an anti-inflammatory effect by stabilizing mast cell membrane and in habiting histamine released from basophils (Alexandrakis et al., 1999). Quercetin treatment also lead to cells cycle arrest and poptosis **(Yoshida *et al.*, 1992; Wei *et al.*, 1994)**. LTB4, PGE2, TNF-α, and IL-4 are apleiotropic cytokine that acts on wide range of tissue and cells exerting growth including, growth, and differentiation induction effects depend on the nature of the target cells **(Kishimoto *et al.*, 2003)**. IL-4, TNF-α, PGE2 and LTB4 regulates immune activity in response to injury and infection, inflammation, oncogenic and hematopoiesis **(Castell *et al* 1990; Kishimoto *et al.*, 2003)**. Serum LTB4, PGE2, TNF-α, and IL-4 altered levels in non-diabetic and diabetic wounded rats were potentially alleviated and returned towards the non-wounded cytokine levels as a result of quercetin treatment and/or LLLT. These results are in agreement with previous studies which indicated that the anti-inflammatory effect of quercetin has a strong effect on wound healing, altering tissue regeneration, inflammatory reaction and immunologic function (**Pinheiro and Frame, 1992; Murugan and Pari, 2006)**. In the present study, the wound closure rate was remarkably decreased concomitant with the modulatory effects on inflammation as a result of treatment of wounded rats with quercetin and/or LLLT. These results are in agreement with many publications **(Wu *et al.*, 1999; Baumagartner-Parzer *et al.*, 1995; Ho *et al.*, 2000; Vincent *et al.*, 2005; Susztak *et al.*, 2006)**. Based on the results of the present study and previous publications, it can be suggested that quercetin treatment or laser irradiation for 10 minutes every other day for two weeks possibly may be due to an inhibition of pro-inflammatory and inflammatory cytokines expression due to an accumulated effect.

The characteristic mechanical properties of the tissue for animals from all groups are differences to those previous reported for healthy rats. Irradiation 10 minutes every other day with 6.36 Joules/cm$^2$ for one week showed a significant change in LTB4, PGE2, TNF-α, and IL-4 compared to unirradiated animals. Moreover irradiation 10 minutes every other day with laser 6.36 Joules/cm$^2$ for two weeks showed more pronounced amelioration in LTB4, PGE2, TNF-α, and IL-4 levels compared to irradiated animals. These improvement in altered detected cytokines were associated with marked decrease in wound closure rate and amelioration in strain-stress ratio. The concomitant treatment with quercetien and LLLT seemed to be more potent in improving wound healing and cytokines levels. In the present study, there were significant differences as a result of quercetien treatment and LLLT in the wound healing acceleration in diabetic. The experimental wounds were, on average, fully healed by the 15th day, whereas the wounded control groups were not. Regarding stress-strain ratio, the results indicate two portions in the characteristic curve where in the first part an approximately linear dependence of the strain upon the applied stress, re-followed by a nonlinear dependence of the strain on the stress, after which the skin is cutting. The data of the present study revolved significant changes in wounded non-diabetic or diabetic rats due to treatment with quercetien and LLLT. The weak linear response of the stress strain characteristic curve for the control group is due to the fact that tissue is mainly composed of two components, collagen and hydroxyapatite crystals. Each component of the tissue has its linear stress-strain response and what we measure is the net resultant of the two components. One more point worthy to be mentioned here is that the measuring method for the mechanical properties of tissue applied tensile forces, not compressive. Since collagen is mainly responsible for tensile

strain and hydroxyapatite has smaller responsibility, one may find that the characteristic curves for tensile strain demonstrate the role of collagen. Therefore, it can be concluded that quercetin and low level laser energy (632.8 nm) (He-Ne) with spot size 1 $cm^2$ and exposure duration of 10 minutes every other day at dose of 6.36 Joules/$cm^2$ have a significant beneficial effect on wound healing. The present study highlights the possible utility of 632.8 nm (He-Ne) laser with appropriate energy density as an adjunctive modality for diabetic wound healing in clinical practice. Our study suggests that low-level laser therapy 632.8 nm as well as quercetin can accelerate and promote wound healing in rats. The use of quercetin treatment concomitant with LLLT is more recommended than the use of each alone.

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