Evaluation of wound healing effects of *Syzygium cumini* and laser treatment in diabetic rats

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**Abstract:** This study was aimed to investigate the antioxidant effects of *Syzygium cumini* (*S. cumini*) seeds extract along with wound healing score in correlation to glutathione and blood glucose levels in diabetic rats. Sprague-Dawley rats were divided into 8 groups (n=6) and diabetes was induced by a single intraperitoneal injection of streptozotocin (40 mg kg\(^{-1}\) body weight). An alcoholic extract of *S. cumini* was administered to punch plier induced wounded rats and changes in serum glutathione levels, wound healing score and blood glucose levels were examined. Laser treatment of 500 mW for 5 minutes was given once a day for 12 days at 810 and 630 nm respectively. Statistical analysis was performed using one way ANOVA. A p-value <0.05 was considered significant and p-value <0.001 was considered highly significant. There was significant increase in glutathione levels and wound healing score when *S. cumini* extract was administered oral and topical along with topical laser therapy. There was significant reduction in the blood glucose levels upon administration of *S. cumini* extract in diabetic rats. This study concludes that there was a positive relation between the glutathione levels and wound healing score, since there was increase in wound healing score with the increase in the glutathione levels.

**Keywords:** *Syzygium cumini*, glibenclamide, glutathione, wound healing score, diabetes mellitus

**INTRODUCTION**

Approximately 170 million people are affected by the Diabetes worldwide, including 20.8 million in the USA, and by 2030 these statistics are expected to double (Wild et al., 2004). The normal wound healing cascade consists of several steps, homeostasis, inflammation, granulation, tissue formation, and tissue remodeling. The delayed wound healing is the outcome of impaired regulation of the complex molecular and biological factors involved in normal healing process (Maruyama et al., 2007, Galiano et al., 2004, Lobmann et al., 2002). Free radicals scavenging activities are also known to play a very important role in healing of normal and delayed healing types of wounds. The magnitude of free radical generation and their disposal mechanisms are known to be changed in diabetic and aged animals (McDANIEL et al., 1998). It is also reported that defective wound healing is caused by increased production of reactive oxygen species (ROS) (Petreaca et al., 2012). Oxygen has a central role in the wound healing mechanism, such as aerobic bacterial killing, collagen production, angiogenesis, and epithelialization; hence the wound healing process is defected under hypoxia (Schreml et al., 2010, Sen, 2009). During inflammation, neutrophils and macrophages reach at injury site start to secrete large amounts of ROS along with pro-inflammatory cytokines (Goldman, 2004) and proteolytic enzymes such as matrix metalloproteinase (MMP). Laser therapy facilitates collagen production in hyperglycemic wound healing patients due to increased fibroblastic proliferation of cells, capillary proliferation, and epithelialization of wound by the release of growth factors from fibroblastic cells at some particular wavelength (632.8 nm). Photo-bio stimulation may be done by the absorption of energy through the respiratory chain in mitochondria that causes the oxidation of NADH in both the mitochondria and cytoplasm, thus activating the ETC (electron transport chain), resulting in an enhanced electrical potential across the mitochondrial membrane, an increased ATP production, and nucleic acid synthesis activation (Reddy et al., 2001). The degree and severity of wound can be assessed by quantitative evaluation based on six histological parameters to give healing score. The total healing score in either case was calculated by addition of scores of sole criteria, and the healing status is graded as good (16-19), fair (12-15) and poor (8-11)(Gupta and Kumar, 2015). Natural products are proved to have anti-diabetic effects as well as antioxidant affects that can help in wound healing process according to their available indigenous data (Oyenihi et al., 2014). *S. cumini* is slow growing plant known for its ornamental value. Seeds of *S. cumini* are used in diverse alternative healing systems like Ayurveda, Unani and Chinese medicine (Kumar et al., 2009). Its polyphenolic constituents are known for their antioxidant, anti-diabetic, anti-inflammatory, anti-asthmatic effect and for arthritis as well as heart diseases (Muruganandan et al., 2001). Plant extracts of *Syzygium* species are reported to have antibacterial effects against Salmonella typhimurium (Shaﬁ et al., 2002). The aim of the study was to evaluate antioxidant effects of the *S. cumini* extract.
cuminis and the correlation between glutathione levels and wound healing score by using S. cuminis extract along with laser therapy in streptozotocin induced diabetic rats.

MATERIALS AND METHODS

Chemicals
All chemicals used in the study were of analytical grade. Ethanol from Riedel-de-Haen Germany was used, while streptozotocin, ascorbic acid and DPPH from Sigma-Aldrich, Germany were used.

Preparation of the extract
S. cuminis was collected from Mirpur Azad Kashmir in the form of ripened fruits and pulp was removed from the seeds. It was identified by the In-charge Herbarium, Department of Pharmacy, Islamia University of Bahawalpur. Dried seeds were then crushed to fine powder and soaked in 99.8% ethanol for 15 days. Then extract was filtered and concentrated by Rotary evaporator. The extract so obtained was stored at 0°C until it was used.

Antioxidant activity
The antioxidant potential of crude extract Syzygium cuminis (SC), DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging method adopted by Vamanu and Nita, 2013 was used with minor modification. 0.8ml of 0.2mM methanol solution of DPPH was mixed with 0.2ml of 100, 500 and 1000µg/ml of each extract (SC and BA). Then the mixture was shaken and kept in dark for 30 minutes. Keeping ascorbic acid of similar concentrations as reference standard. Blank solution was prepared by adding 0.8 ml DPHH solution and 0.2 ml methanol. After 30 minutes, absorbance of all mixtures was measured at 517nm using UV-Spectrophotometer. % inhibition was calculated by using following formula:

\[
\text{% inhibition} = \left( \frac{A_c - A_s}{A_c} \right) \times 100
\]

Ac is the Absorbance of control whereas As is the absorbance of sample.

Animals
Pathogen free male Sprague Dawley rats obtained from NIH having weight 250-350 gram, age 12-16 weeks were used in the study. Rats were kept in caging with controlled environment at temperature 24 to 26 °C and were exposed to light and dark in a ratio of 13 and 11 hours respectively. They were provided with filtered sterilized water and initially housed in a group of 06 whereas, after wound induction housed separately. The dose of S. cuminis used in the study was selected on the basis of pilot study.

Induction of diabetes mellitus
Diabetes in experimental animals was induced by a single intraperitoneal (i.p) injection of STZ at a dose of 40mg/kg. (Al-Watban et al., 2007). The animals showed hyperglycemia 72 h. post STZ injection with blood sugar level (>250mg/dl) were included in the study. Experimental animals were divided into 8 groups of 6 animals each as mentioned below.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Drug/ Dose/Route</th>
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<tbody>
<tr>
<td>Pt</td>
<td>Normal saline 10 ml/kg oral and topical soft Paraffin</td>
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<tr>
<td>STZ-Pt</td>
<td>Diabetes induced Rats treated with topical soft Paraffin</td>
</tr>
<tr>
<td>STZ-GoPt</td>
<td>Diabetes induced Rats given 2mg/Kg oral glibenclamide and applied topical Paraffin</td>
</tr>
<tr>
<td>STZ-GoLt</td>
<td>Diabetes induced Rats given 2mg/Kg oral glibenclamide and treated with topical laser</td>
</tr>
<tr>
<td>STZ-GoSc</td>
<td>Diabetes induced Rats given 2mg/Kg oral glibenclamide and treated topical with S. cuminis extract (20%)</td>
</tr>
<tr>
<td>STZ-GoScLt</td>
<td>Diabetes induced Rats given 2mg/Kg oral glibenclamide and treated topical with S. cuminis extract (20%)</td>
</tr>
<tr>
<td>STZ-SCt</td>
<td>Diabetes induced Rats treated with topical S. cuminis extract (20%) and also given the extract 300mg/kg; orally</td>
</tr>
<tr>
<td>STZ-SCotLt</td>
<td>Diabetes induced Rats and treated with topical laser + S. cuminis extract (20%) and also given the extract 300mg/kg; orally</td>
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</table>

Wound induction
The excisions were placed on the rat after they were provided anesthesia with ketamine 110mg/kg and xylazine 7 mg/kg; i.p. Two wounds of same diameter, i.e. 7.5 mm were induced via belt punch plier on right and left side of dorsal scapular region by lifting it with the help of a forceps after removing dorsal surface hairs. A silicon sheet having a hole of same size as of wound was placed on the wound and fixed with the sutures on either side of the wound (Wang et al., 2013). The size of the wound was measured at day 1, 3, 5, 7, 9, 11 and 14 using Sony pixel 10. 1camera.

Laser therapy
The animals were radiated for 14 days after wound induction via MDL 500 enhanced multifunction semiconductor laser device by Shanghai Sunny Optoelectronic Technology Co., Ltd, China (red; 650nm and IR; 808nm). The energy density and the power density was calculated as Diode laser and LED laser of near infrared wavelength i.e. 810 nm and 630 nm respectively at a power of 500 mW for 5 minutes, once a day. The beam of LED was used as pulse mode, each day (Huang et al., 2009).

Experimental protocols
The animals were divided into eight different groups, each consisting of six animals as mentioned in the section.
of induction of diabetes mellitus. Animals were injected with STZ to induce diabetes (at the dose of 40mg/kg; i.p) and treated for 14 days after diabetes induction. At the end of the experimental period; i.e. 14th day, animals were anesthetized by ketamine/xylazine, blood was collected by cardiac puncture and sera were separated by centrifugation at 4500 rpm for 15 minutes. (Takahashi et al., 2014). The skin at the site of wound was separated by belt punch plier and stored in 10% formalin and sent for histopathology for the estimation of various histopathological parameters (Fiette and Slaoui, 2011). All the serum samples were stored for future use at -20°C (Ruehl-Fehlert et al., 2003).

**Healing score (HS)**

Wound healing was assessed by calculating healing score, a quantitative method used by Gupta and Kumar. It was calculated on the bases of numbers assigned to six histological parameters including, amount of granulation tissues, inflammatory infiltrate, collagen fiber orientation, pattern of collagen, amount of early collagen and amount of mature collagen (Gupta and Kumar, 2015).

**Biochemical parameters**

The sera were used for the estimation of different biochemical parameters; e.g. glutathione levels and blood glucose levels as described by Look et al (Look et al., 1997).

**Histopathological parameter**

The skin sample was taken from healed wound on 14th day of study and fixed in 10% neutral buffered formalin. The slides were stained with H & E (Hematoxylin- Eosin) and trichrome mason stain. All specimens were evaluated separately by a histopathologist and several histomorphological parameters were analyzed.

**STATISTICAL ANALYSIS**

Statistical analysis was done by Graph pad prism version 6.1 using one way ANOVA and Bonferroni’s post hoc test. *p<0.05 to *p<0.01 was considered as statistically significant and **p<0.001 was considered as highly significant.

**RESULTS**

**Antioxidant assay of crude extract**

Free radical scavenging activity of ascorbic acid and the crude extracts of *S. cumini* was performed. The percentage inhibition of *S. cumini* at 100, 500 and 1000 (µg/ml) was found 57.03, 87.01 and 88.85%. The percentage inhibition of standard (ascorbic acid) at 1000 µg/ml was 90.07%. The results showed that *S. cumini* has almost equal antioxidant potential to ascorbic acid.

**Wound healing Score**

Wound healing score of 8 animal groups (n= 6 animals) are presented in fig. 1. Diabetic-induced group (STZ-Pt) has wound healing score (mean ±SEM) value of 7.3±0.33 while disease control group (STZ-Go-Pt) has 8.5±0.42. Normal group (Pt) treated with paraffin has shown wound healing score of 9.8±0.47. STZ-Go-Lt group showed significant increase (11.8±0.30) in wound healing score when compared with disease control group (STZ-Go-Pt). This effect may be attributed to the addition of laser therapy along with glibencamide. When *S. cumini* extract was administered topically (STZ-Go-Sc) showed decreased wound healing (11.1±0.70) as compared to topical *S. cumini* along with laser treatment group (STZ-Go-Sc-Lt) 13±0.51. Best wound healing results were achieved by *S. cumini* topical and oral treatment along with laser therapy (18.3±0.33).

![Fig. 1: The effects of orally and topically applied crude extract of *S. cumini* with and without laser on healing score number in punch plier-induced wound in diabetic rats at 14th day of study. The values are expressed as mean ±SEM of six animals in each group and analyzed using one way ANOVA. The STZ-induced diabetic group is compared with normal control group (**p<0.001: Highly significant) and treatment groups (STZ-Scot-L, STZ-Scot, STZ-Go-Sc-Lt, Go-Lt) and disease control (Go-Pt) is compared with STZ-induced diabetic group (**p<0.001: Highly significant). The treatment groups (STZ-Go-Sc-Lt, STZ-Go-Sc and STZ-Go-Lt) were also compared with disease control group (##p<0.001: highly significant), (###p<0.001: highly significant). STZ: Streptozotocin, SC: *S. cumini*, G: Glibenclamide, L: Laser, P: Paraffin.](image)

**Table 1: Effects of *S. cumini* and laser on serum glutathione levels**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum glutathione levels (mean ±SEM)</th>
</tr>
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<tbody>
<tr>
<td>STZ-Sc-ot-Lt</td>
<td>11.63±0.19**</td>
</tr>
<tr>
<td>STZ-Scot</td>
<td>10.23±0.13**</td>
</tr>
<tr>
<td>STZ-Go-Sc-Lt</td>
<td>10.18±0.07**</td>
</tr>
<tr>
<td>STZ-Go-Lt</td>
<td>9.72±0.08**</td>
</tr>
<tr>
<td>STZ-Go-Sc</td>
<td>9.88±0.04**</td>
</tr>
<tr>
<td>Pt</td>
<td>9.65±0.10**</td>
</tr>
<tr>
<td>STZ-Go-Pt</td>
<td>9.35±0.06**</td>
</tr>
<tr>
<td>STZ-Pt</td>
<td>6.62±0.09</td>
</tr>
</tbody>
</table>

The values are expressed as mean± SEM The mean of each group is compared with the mean of diabetic-induced control.
group (STZ-Pt) indicating ** as statistically highly significant (P< 0.001).

**Fig. 2**: Blood glucose levels at day 0. The values are expressed as mean ± SEM of six animals in each group and analyzed using one way Anova. R: Rat, STZ: Streptozotocin, SC: S. cumini, G: Glibenclamide, L: Laser, P: Paraffin.

**Glutathione levels**

According to our study, the diabetic-induced group (STZ-Pt) showed decreased serum glutathione levels represented by mean ±SEM as shown in table 1 when compared with the treatment groups. Treatment group given S. cumini topical extract (20%) indicated significant increase in glutathione levels (9.88±0.04**) as compared to diabetes induced group (6.62±0.09). Topical and oral administration of S. cumini extract showed significant increase in GSH levels (10.23±0.13**) when compared with diabetes induced and disease control group. Laser therapy alone is less effective in increasing serum GSH levels (9.72±0.08**) when compared with combination of topical S. cumini extract and laser treatment. The group treated with oral and topical S. cumini along with laser treatment showed highly significant antioxidant effect (11.63±0.19**) when compared to the diabetes induced and disease control groups.

**Fig. 3**: The effects of oral and topical crude extract of (S. cumini) with and without laser on blood glucose levels in diabetic rats at 5th day of study. The values are expressed as mean ± SEM of six animals in each group and analyzed using one way Anova. The diabetic-induced group is compared with normal control group Pt (**p<0.0001: highly significant) and treatment groups (STZ-SCot-Lt, STZ-SCot, STZ-SCo-Lt, STZ-Go-Lt) and disease control (STZ-Go-Pt) is compared with diabetes-induced group (**p<0.0001: highly significant), (*p<0.05: significant). R: Rat, STZ: Streptozotocin, SC: S. cumini, G: Glibenclamide, L: Laser, P: Paraffin.

**Fig. 4**: The effects of oral and topical crude extract of (SC) with and without laser on blood glucose levels in diabetic rats at 10th day of study. The values are expressed as mean ± SEM of six animals in each group and analyzed using one way ANOVA. The diabetes-induced group is compared with normal control group Pt (**p<0.0001: highly significant) and treatment groups STZ-SCot-Lt, STZ-SCot, STZ-SCo-Lt, STZ-Go-Lt and disease control (STZ-Go-Pt) is compared with diabetes-induced group (**p<0.0001: highly significant), (*p<0.05: significant). R: Rat, STZ: Streptozotocin, SC: S. cumini, G: Glibenclamide, L: Laser, P: Paraffin.

**Fig. 5**: The effects of oral and topical crude extract of (SC) with and without laser on blood glucose levels in
diabetic rats at 14th day of study. The values are expressed as mean ± SEM of six animals in each group and analyzed using one way Anova. The diabetes-induced group is compared with normal control group Pt (*p<0.0001: highly significant) and treatment groups (STZ-SCot-Lt, STZ-SCot, STZ-SCo-Lt, STZ-Go-Lt) and disease control (STZ-Go-Pt) is compared with diabetes-induced group (**p<0.0001: highly significant). R: Rat, STZ: Streptozotocin, SC: *S. cumini*, G: Glibenclamide, L: Laser, P: Paraffin.

**Blood glucose levels**

Blood sugar levels of all 8 groups (day 0, day 5, day 10, and day 14) are graphically represented in figs. 2-5. The blood glucose levels were measured after the 72 hours of administration of STZ (40mg/kg). The rats with glucose level higher than 250 mg/dL was considered diabetic. After the administration of STZ, glucose levels increased highly significantly (*p<0.0001) in all groups as compared to normal control group R-P as shown in fig. 2.
The results at day 5 showed that groups receiving glibenclamide orally have more significantly decreased levels of glucose as compared to group treated with crude extract of *S. cumini* as shown in fig. It was observed that group treated with oral glibenclamide and topical laser (STZ-Go-Lt) have decreased glucose levels (227.8±29.37) highly significantly as compared to diabetes induced group (378.0±23.85). The glucose levels of R-STZ-G-P was 234.2±35.89 which was significantly (**p<0.01) less than the diabetic-induced group. On investigation, the glucose levels in the groups treated with glibenclamide oral and crude extract of SC topical (STZ-Go-SCt and STZ-Go-SCo-Lt) was found significantly (*p<0.05) less 273.3±15.42 and 239.3±12.70 respectively. The groups administered with *S. cumini* blood glucose levels. On day 14, it was observed that glibenclamide orally have more significantly decreased levels of glucose as compared to treat with crude extract of *S. cumini* as shown in fig. It was observed that group treated with glibenclamide orally and laser topically (STZ-Go-Lt) have highly significant (**p<0.001) reduction in glucose levels (115.7±12.54) as compared to diabetic-induced group (378.0±23.85). The glucose levels of STZ-Go-Pt was 123.0±17.15 which was significantly (**p<0.01) less than the diabetic-induced group. On investigation, the glucose levels in the groups treated with glibenclamide orally and crude extract of *S. cumini* topically (STZ-Go-SCt) were increased (147.7±15.44) as compared to (STZ-Go-SC-Lt) group 133.0±6.51. When SC extract was administered orally and topically with and laser therapy, there was non-significant (ns) increase in blood glucose levels 186.0±9.42 and significant increase (*p<0.05) 189.2 ±10.11 respectively as compared to STZ-Go-SCt and STZ-Go-SCt-Lt groups.

**Histopathology of Skin**

The H and E staining (fig. 6) of skin carried out at 14th day of study. The granulation tissue stained dark pink and amount of granulation tissue was found absent in STZ-SCt-Lt. Only few inflammatory infiltrate was present and orientation of collagen fiber (stained blue in Masson’s trichrome staining) was horizontal. The pattern of collagen was reticulate. The Masson’s trichrome staining (fig. 7) showed that there was large amount of mature collagen (dark blue) as compare to early collagen (light blue). These all parameter showed that the skin has been healed. The diabetic-induced group have moderate amount of granulation tissue and inflammatory infiltrate while orientation of collagen was mixed. The amount of mature collagen was found less as compared to treatment groups. These results depict poor healing as shown in figs. 6 and 7.

**DISCUSSION**

Healing score analysis of 8 groups (n= 6) showed significant differences in the inflammatory phase of the healing process between diabetes-induced and treatment groups. Our results shows highest healing score with oral and topical extract of *S. cumini* along with topical laser treatment. This may be attributed to the free radical scavenging activity of the *S. cumini* extract as reported previously that fruit extract of *S. cumini* has outstanding antioxidant properties (Katayir et al., 2016). Early antimicrobial properties of *S. cumini* by using its extract against multi resistant strains of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. The results concluded in this study propose a potential use of *S. cumini* for treatment of skin wounds (Oliveira et al., 2007, Dweck, 2002). The antimicrobial action of the *S. cumini* leaves hydro alcoholic extract may be due to tannins and other phenolic components. *S. cumini* contain high concentration of gallic and ellagic acid polyphenol derivatives (Mahmoud et al., 2001, Timbola et al., 2002). Laser treated diabetic rat group showed improved wound healing as compared to groups treated with *S. cumini* topical extract. The possible mechanism due to which laser technology improves wound healing is due to cell proliferation, increase the ATP synthesis, and increase tensile strength of scar tissue and promoting the reduction of pain and trauma. The effects of low-level laser can be observed in the behavior of lymphocytes, increasing their proliferation and activation; on macrophages, increasing phagocytosis; and on fibroblasts, increasing the secretion of growth factors and enhancing the uptake of both fibrin and collagen. Its action can be observed on the reduction of the area of skin wounds in humans and animals (Andrade et al., 2014). When laser treatment was combined with *S. cumini* topical extract, results were significant as compared to laser and plant extract alone. This may be due to additive antioxidant effects of the plant extract along with wound healing effect of laser therapy.

According to our results, the diabetic-induce group (STZ-Pt) showed decreased serum glutathione levels as shown in (table-1) when compared with the treatment groups. The groups treated with *S. cumini* oral and topical along with diabetes induced and laser treatment showed highly significant increase in glutathione levels of the extract when compared to the diabetes-induced group. These results are supported by the findings of the wound healing score analysis (fig. 1).The underlying mechanisms behind this increase involve the synthesis of glutathione which is important macro molecules of body and protects against reactive oxygen species (Mukherjee et al., 1994, Jagetia, 2017). Our results are supported by the study conducted on the antioxidant effects of *S. cumini*. They used streptozotocin (STZ) induced diabetic Wister female rats to analyze the antioxidant effects of *S. cumini* extract. *S. cumini* caused an increase in the levels of superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and reduced glutathione (Rijt et al., 2015) and subsequent reduction in free radical formation in liver tissues of the diabetic rats (Rakha et al., 2008).
Another study conducted to analyze the antioxidant effects of \textit{S. cumini} seed extract on streptozotocin induced diabetic wistar rats. \textit{S. cumini} caused significant reduction in the levels of peroxidation in diabetic rats in treatment group. \textit{S. cumini} extract also caused significant increase in the glutathione levels. These results suggest the antioxidant effects of \textit{S. cumini} that are helpful in treating the complications of diabetes mellitus (Farswan et al., 2009). Topical laser therapy is helpful in wound healing along with topical and oral extract of \textit{S. cumini}. Ahmed et al (2016) suggested that low level laser therapy and quercetin (an anti-inflammatory and antioxidant) treatment facilitates the tissue repair process by accelerating collagen production in diabetic wound healing. Our results indicated that the treatment with \textit{S. cumini} along with topical laser therapy was superior in improving wound healing in diabetic rats than the use of either of each. Moreover the hypoglycemic effects of selected doses of \textit{S. cumini} were almost comparable to that of glibenclamide.

**CONCLUSION**

Present study concludes that \textit{S. cumini} and Laser have wound healing effects. It was observed that the groups treated with \textit{S. cumini} oral and topical along with laser have greater healing effects. It was also observed that the group treated with \textit{S. cumini} oral and topical along with laser has high levels of glutathione which also correspond with better healing. It was also observed that glucose lowering effects of glibenclamide and oral \textit{S. cumini} were almost comparable. However further studies on large number of animals as well as humans are required to support and implement the use of oral and topical \textit{S. cumini} along with laser in wound healing.

**REFERENCES**


