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ASSESSMENT OF THE ANTIOXIDANT ACTIVITY OF *QUERCUS INFECTORIA* GALL EXTRACT AND ITS EFFICACY IN WOUND HEALING USING OPTICAL COHERENCE TOMOGRAPHY IN SWISS ALBINO MICE

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ABSTRACT
Use of medicinal plants for wound healing is widely accepted as it is economical with almost no side effects. Galls of Quercus infectoria (QI) used in medicinal preparations are known for their anti-inflammatory properties. However, the effect of application of the QI plant extract on wound healing is poorly understood. The QI gall extract in methanol was prepared by Soxhlet method and the major phytochemicals such as total phenol, total flavonoid and total triterpenoid were analyzed for their antioxidant properties. The QI was also converted in a form of a gel for tropical application. The wound healing property of this gel was evaluated in tape stripped wounds using Optical Coherence Tomography (OCT), a non invasive optical imaging technique for histological assessment of edema formation, epithelialization, granulation tissue deposition and epidermis thickness. Wound contraction was calculated from the photographs taken on different days. QI gel treated mice on day 2 showed reduced edema, higher amount of granulation and faster epithelialization by OCT and histology as compared to control. The epidermis thickness of the treated groups as observed from OCT and histology was ~2 fold more than that of untreated wounds as observed from OCT on day 9. The increase in the collagen synthesis with absence of inflammatory cells was also seen in wounds treated with QI extract. Further, compared to untreated significant wound contraction was observed in treated wounds. The results from OCT, histology and wound contraction studies provide evidence for the ethno medicinal use of QI gall extract for wound healing, which occurs probably due to the presence of major phytochemicals and the antioxidant activity of the extract.

KEYWORDS: Polarization Sensitive Optical Coherence Tomography (PS-OCT), Optical Coherence Tomography (OCT) imaging, wound healing, Quercus infectoria.

1. INTRODUCTION
Wound healing involves three different stages of inflammation, proliferation and remodeling. However, in chronic wound models, healing is delayed due to various factors such as bacterial infection, oxidative stress-mediated diseases, inflammatory cytokines, delayed collagen synthesis and reduced angiogenesis etc.[1,2] Impaired wound healing requires timely treatment and regular monitoring of wounds during healing process. Assessment of wound healing is based on macroscopic observation, measurement of wound size, color, odour, drainage and scar formation.[3] The structural changes and the effect of particular drug over complete wound healing processes can be studied by a non invasive imaging technique. Optical Coherence Tomography (OCT) is a non invasive real time imaging technique *in vivo* and it allows evaluation of biological tissue up to a depth of 2-3 mm with an axial resolution of ~15 micrometers.[4] OCT measurement is based on light back scattered from inside the tissue by correlating it with light that has travelled a known reference path. The OCT enable evaluation of skin lesions, especially non melanoma skin cancers and inflammatory diseases, quantification of skin changes and accurately detects the presence or absence of the epidermal layer of skin, allowing noninvasive tracking of the wound re-epithelialization.[5] Hence, OCT provides greater information about a wound than visual monitoring during treatment and wound healing. Polarization –sensitive Optical Coherence Tomography (PS-OCT) measures the birefringence properties of skin[6] and has been used significantly for monitoring collagen content, loss of collagen structure and integrity, which is associated with the wound healing kinetics and for the quantification of various drug effect during wound healing.

Plant extracts due to the presence of bioactive components can be effective in various steps of wound

repair and tissue regeneration. However, a confined number of studies carried out so far, report the use of bioactive compounds to confirm the therapeutic efficacy, necessitating studies that are required to explore the bioactive components of medicinal plants as potential wound healing agents in the field of phytotherapy. An important group of biologically active compounds called proanthocyanidins belonging to polyphenolic bioflavonoids are produced by various medicinal plants and have received attention as possible wound healing and dressing agents.[7]

In the present study, extract prepared from the galls of Quercus infectoria (QI) were investigated for wound healing. QI is a small tree found mainly in Greece, Iran and Asian countries. The galls arise on young branches of the tree as a result of attack by the gall-wasp *Adleria gallae tinctoria*. QI is commonly known as majuphal and belongs to the family Fagaceae.[8] The plant is known for its various medicinal properties such as astringent, antidiabetic, antifungal and anti-inflammatory activities. It is very effective against inflamed tonsils and direct application of it on skin cures swelling or inflammation. The powdered galls in the form of ointments are also suggested to cure hemorrhoids caused by inflammation of the skin.[9] The main constituents of the galls have been reported to comprise of a large amount of tannins (50-70%) and some amount of gallic acids, ellagic acid and synergic acid.[10] Tannins promote the wound healing through several cellular mechanism, inhibiting reactive oxygen species, reducing the time of wound healing and increasing the formation of capillary vessels, fibroblast and keratinocyte formation.[11] In the present study, total phenol, flavonoid and triterpenoid contents of QI were estimated and the effect of the QI extract on wound healing was studied. The structural changes in wound healing process during the early days, post application of QI gel such as inflammation, edema, epidermal migration and late wound healing events such as collagen layer formation and hair follicle generation were monitored by OCT. The changes observed in OCT were further correlated with histology in control and extract treated wounds at regular intervals.

2. MATERIAL AND METHODS

2.1 Chemicals: 2,2,-diphenyl-1-picryl hydrazyl (DPPH), urosolic acid and quercitrin hydrate were procured from Sigma Chemical Co. St. Louis, Missouri, USA. Other chemicals and solvents of AR grade were purchased from Hi Media Co. Mumbai, India.

2.2 Plant material and extract preparation: The galls of QI were procured from the local market and the galls were authenticated by Dr A.B. Seerwani, Professor, Goverment Holkar College, Indore. The galls 10 g were grinded in to fine powder and extracted in methanol (300 ml) for 6-8 hr using Soxhlet apparatus**.** The extract was concentrated by evaporation at 35^0C in water bath. These extracts were stored at 4^0 C for analysis. The extraction and analysis were performed in replicates of four.

2.3 Total phenol content

Total phenol content of QI was determined using the Folin–Ciocalteu reagent.[12] The total phenol in the test samples were expressed as gallic acid equivalents using a standard curve of propyl gallate (1mg/ml).

2.4 Total flavonoid content The total flavonoids content was estimated according to the aluminium chloride method.[13] The total flavonoids in the test sample were determined using a Quercitrin hydrate as standard.

2.5 Total triterpenoids content

The total triterpenoids content was estimated by the method of Chang and Lin.[14] Urosolic acid was used as standard.

2.6 Free radical scavenging activity using DPPH

The free radical scavenging activity was measured using the method of Mellors and Tappel.[15] The model of scavenging, the stable 2,2 diphenyl-p-picryl hydrazine ((DPPH) radical is used to evaluate antioxidants activities. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. The assay is based on the reduction of DPPH at 517 nm.

2.7 Total antioxidant power using ferric reducing antioxidant power

The total antioxidant capacity (TAC) of QI was determined using the ferric reducing antioxidant power (FRAP) as described by Benzie and Strain.[16] TAC of the test samples in vitro was determined by reduction of a ferric tripyridyl triazine (Fe^{3+} -TPTZ) complex to the ferrous form, which has an intense blue colour and was monitored by measuring the change in absorption at 593 nm.

2.8 Reducing power The reducing power of the test sample was determined according to the method of Oyaizu.[17] The reductive ability was measured by the reduction of $FeCl_3$ in presence of plant extracts.

3.0 *In vivo* wound healing experiments

3.1 Tape stripping wound model

Twenty four Swiss albino mice (Male, 25-30 g) were used for the experiments on wound healing. The animals were housed individually in cages with free access to food, water and were maintained on a 12 h light/dark cycle at 22^0C ($\pm$ 2^0C). For creation of wounds, mice were anesthetized by an intraperitoneal injection of ketamine (80 mg/kg) along with xylazine (10 mg/kg). Tape stripping wounds were created according to the protocol described.[18] Briefly, hair was removed from a ~2 cm^2 area from the dorsal side of mice using a depilatory cream after anesthetizing the animals. The skin was cleansed with 70% alcohol and with betadine. The tape – stripping wound model was generated by applying an adhesive tape of 2 x 2 cm size for 15 times, as described.[18] Following this procedure, the skin became visibly damaged and appeared red and glistening.

Microscopically, this procedure resulted in the partial removal of the epidermal layer. Wounds were left undressed to the open environment and the animals were kept individually in separate cages. The experimental procedures involving animals were approved by the Institutional Animal Ethics Committee (IAEC), vide project no CPCSEA/2016/01/ dt 20.2.2016. All the procedures involving wound creation, OCT imaging of wounds were carried out in anesthetized conditions. The animals were kept on warm cotton pads for recovery from anesthesia. Animals were euthanized by cervical dislocation. All research animals were treated humanely.

3.2 Application of herbal extract on wounds

The QI extract was transformed into herbal gel using petroleum jelly as base. The prepared gel was evaluated for physical appearance, homogeneity, spread ability, and pH. The gel was also examined for microbial and anti- microbial growth. The above gel was tested for skin irritation to observe toxicity or any other side effects.

Mice with tape stripped wound were divided into two groups, control and experimental group for evaluating healing potential of extract. Group 1 was control group in which wound of the animal was not treated with any gel or ointment. Group 2 was served as treated group in which wounded area of the animals were treated with tropical application of QI in petroleum jelly. Briefly, herbal extract powder (4% w/w) was mixed with petroleum jelly. Gel 250 mg was applied uniformly on the tape stripped wounds for 5 days, consecutively, at 24 h interval.

3.3 OCT imaging of wounds of mice

Figure 1 shows a schematic of the PS-OCT set up. Light from a 5 mW superluminescent diode (center wavelength ~840 nm, bandwidth ~40 nm) was collimated and passed through a polarizing beam splitter to get vertically polarized light beam. Details of the interferometer and the spectrometer are described earlier.[19] The fast fourier transform (FFT) of the re sampled interference spectrum generates the depth-resolved intensity images, which were then used to generate PSOCT images. The system features a signal-to-noise ratio of ~96 dB with axial and lateral resolutions of ~10 and 30 µm respectively.

3.4 Histology

For wound healing studies after recording the images of wound on day 14 post treatment, treated and untreated animals were scarified under anesthesia and wounds with surrounding tissues were collected. Specimens were fixed in 10% formaldehyde, embedded in paraffin, tissue sections of 5 µm were prepared using microtome and stained with hematoxylin and eosin and visualized under a light microscope at 50X magnification for the presence of crust, inflammatory cells, granulation layer, edema, epidermis, dermis and dermal papillae. For epidermal thickness measurement, 2 slides for each group and each time point were chosen and observed at 400X magnification. For each slide, 7 to 8 microscopic views were chosen and evaluated for thickness measurement of the epithelial layer by means of a calibrated ocular on a light microscope (Olympus, IX 70) at 400X magnification (40 X objective x 10 eye piece). Thus the data from all fields of view were averaged to calculate the mean and standard deviation.

3.5 Assessment of wound area reduction

Photographic images were taken on different post wounding time points to assess morphological status of wounds. From the images, wound area was calculated using Image J software. Further, to rule out, if the initial variation in wound size could have any effect on wound area reduction kinetics, relative percentage of wound area decrease for each wound was calculated from the initial (A0) and final (At) area on day, by using formula : $[A0 - At]/[A0] \times 100$.

3.6 Statistical analysis

All experiments were carried out at least four times. Data from independent experiments were used to calculate the mean and standard deviation. One way ANOVA followed by Fisher's post hoc test was carried out to determine the significance of the difference between treated and untreated groups of wounds. P value < 0.05 was considered significant.

4. RESULTS

4.1 Determination of phytoconstituents

The phytochemical composition of extract is represented in Table 1. The standards used were propyl gallate, quercitrin hydrate and urosolic acid for determination of phenol, flavonoid and triterpenoid respectively.

4.2 Total antioxidant power

The antioxidant properties of extract such as free radical scavenging activity (FRSA) using DPPH and total antioxidant power (TAP) using FRAP in 100 ul of test sample is represented in Table 2. Butylated hydroxy toluene (BHT) was used as a positive control. FRSA showed proportionate increase in % DPPH radical scavenging activity. $FeSO_4.7H_20$ was used as standard for calculating the FRAP value.

The ability of QI to reduce ferric ions was used as a measure of antioxidant activity. A concentration dependent increase in the reducing ability of the extract was observed as shown in the Fig. 2. The higher absorbance of the reaction mixture indicated greater reducing power.

4.3 Effect of the gall extract on wound healing

4.3.1 OCT and PSOCT imaging studies

In vivo monitoring of wound healing kinetics was investigated using OCT and retardation image using PSOCT. The untreated group on day 2, the crust layer was visible as single scattering layer, below which there was scattering poor zones, suggesting edematic region. These changes are expected on day 2 post wounding, due to the ensuing inflammation in the wound at this time

point (Fig. 3). The OCT images of treated group showed formation of early crust (yellow arrow) along with another scattering layer (not fully formed, marked by pink arrow) below the topmost scattering crust layer. Early formation of new epithelium, epidermal - dermal junction, scar and granulation tissue was also seen in treated groups as compared to the control groups. Also, the overall scattering from the dermis region is much higher in the treated group.

On day 9, the decrease in the tissue imaging depth and increase in dermal Scattering in the treated group(red double arrow) as compared to the control group indicates improved tissue remodeling. The changes in the thickness of epidermis were measured from OCT images. The epidermis thickness was 30-40 µm and was not uniform in the untreated group. On the contrary, in the treated group, the epidermis thickness (black double arrow) was observed to be 60-80 µm and was uniform. The dermis-epidermis junction (red arrow) was visible in both the group, but more conspicuous in untreated group. Only in the treated groups, hair follicles were visible.

4.3.2 Histological assessment

In order to correlate the scattering changes observed in OCT with structural alterations of wound bed histology of tissue sections excised on day 2 and 9 post wounding was performed. The histology images of untreated and treated wounds are shown in Fig. 4. On day 2, the untreated wounds, higher amount of inflammatory cells, complete lack of epidermis and edematic regions were observed (Fig. 4 A). The extract treated wounds on the other hand showed more granulation tissue and lesser inflammatory cells (Fig. 4 B). By day 9, the untreated wounds showed some amount of inflammatory cells, ~100-150 µm thick crust and thinner collagen layer in the dermal region, indicating incomplete wound closure (Fig. 4 C). In contrast, the treated wounds showed a complete and uniform epidermis, lack of crust and showed thicker collagen layers, presence of dermal papillae in dermal region, suggesting the recovery of wounds (Fig. 4 D). The histology images also showed similar trend for epidermis thickness as that of OCT data (Fig. 4 E).

4.3.3 Wound area reduction

In order to establish whether the OCT and histology changes actually correlate with wound closure, wound area from the photographic images was measured. The photographic images of untreated vis-a-vis treated wounds (at day 2 and 9) and the wound area reduction data for the corresponding days are presented in Fig. 5A and B, respectively. It can be seen that as compared to untreated wounds, the decrease in wound area in treated wounds was significantly higher.

Caption for Tables

Table1. Total phenolic, flavonoid and triterpenoid content of QI. Values are Mean ± S.E. of four replicates.
a GAE mg/g of the test sample.
b QE mg/g of the test sample.
c UE mg/g of the test sample.

Table 2. Evaluation of the antioxidant power of QI. Values are Mean ± S.E. of four replicates.
a % of DPPH radical scavenging activity.
b The test sample 100 µl was used for the estimation of FRAP $FeSO_4 7H_20$ was used for the standard.

Caption for Figures

Figure1. Scheme of the PS-OCT set up showing superluminescent diode (SLD), polarizing beam splitter (PBS), nonpolarizing beam splitter (NPBS), quarter waveplate (QWP), glass plate (GP), lens (L), mirror (M), line scan camera (LSC), transmission (TG) grating and wave form function generator (WFFG).

Figure 2. Reducing power of QI.

Figure 3. Effect of QI extract on wound inflammation, epithelial layer formation, as studied by OCT. Back scattered intensity image and retardation image of untreated control and treated skin on day 2 and day 9 was shown in yellow arrow: crust. pink arrow: new epithelium layer. circle: scattering free/poorly scattering region, suggesting fluid filled region, possibly an edematic region. Black double arrow: epidermis was observed that in the treated group, on day 9, the epidermis thickness (black double arrow) was more formed and uniform. Increase in the dermal scattering (red double arrow) in the treated group. Dermis - epidermis junction (red arrow) was visible in both the groups. In the treated groups, hair follicles (dashed black arrow) were visible. Scale bar: 500 µm. The data represent 3 independent experiments for untreated and treated wounds (n = 6 per group per each time point).

Figure 4. Histological images on day 2 and day 9 for untreated (A and C) and treated wounds (B and D), respectively. Black arrow: absence of epidermis on day 2. E: epidermis. Blue arrow: Inflammatory regions. White arrow: Dermal papillae. It was observed that in the treated group, on day 9, the epidermis thickness was more formed and it was uniform. Scale bar of each image: 200 µm. The figures represent 2 wounds for untreated and treated wounds per each time point, (n = 2 per group per each time point). Figure 4 E. Effect of QI extract on ET as measured from the histology images (15 microscopic views per group). Values are Mean ± S.E.

Figure 5. Effect of QI on wound closure.

Figure 5A. Photomicorgraphs of wounds on day 0, 2 and 9 for untreated (left) and treated wounds (right). Scale bar of each image: 10 mm.

Figure 5 B. Percent wound area reduction on day 9 with respect to the initial tape stripped wound area. The data represent 3 independent experiments for untreated and treated wounds (n = 6 per group per each time point).

Table.1 Total Phenolic, Flavonoid and Triterpenoid content of QI

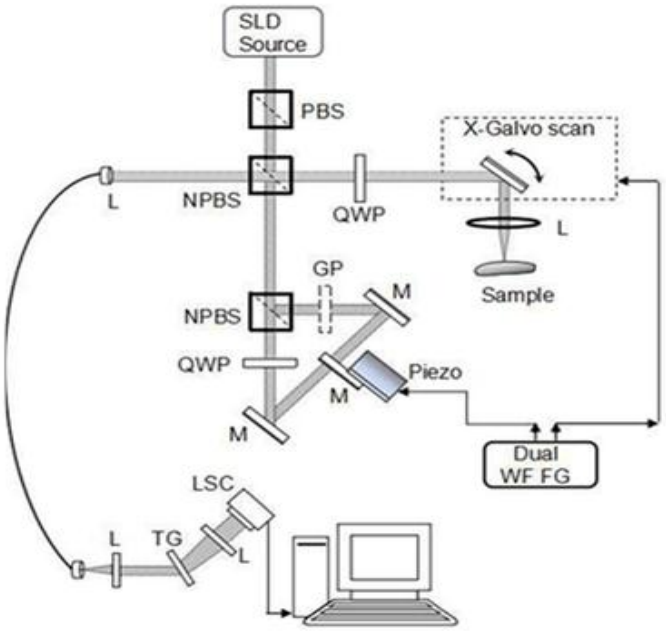
Parameters	Results
Total phenol content[a]	104.11±1.79
Total flavonoid content[b]	16.10±0.88
Total triterpenoid content[c]	4.17±0.56

Values are Mean ± S.E. (n=4) P < 0.05

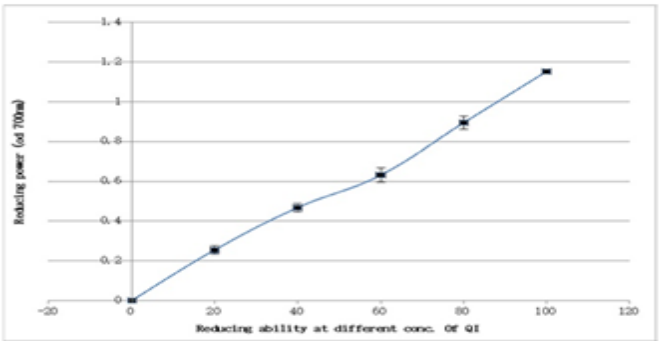
Table.2 Evaluation of the antioxidant power of QI

Parameters	Results
FRSA[a]	83.86±0.42
TAP[b]	118.86±0.36

Values are Mean ± S.E. (n=4) P<0.05

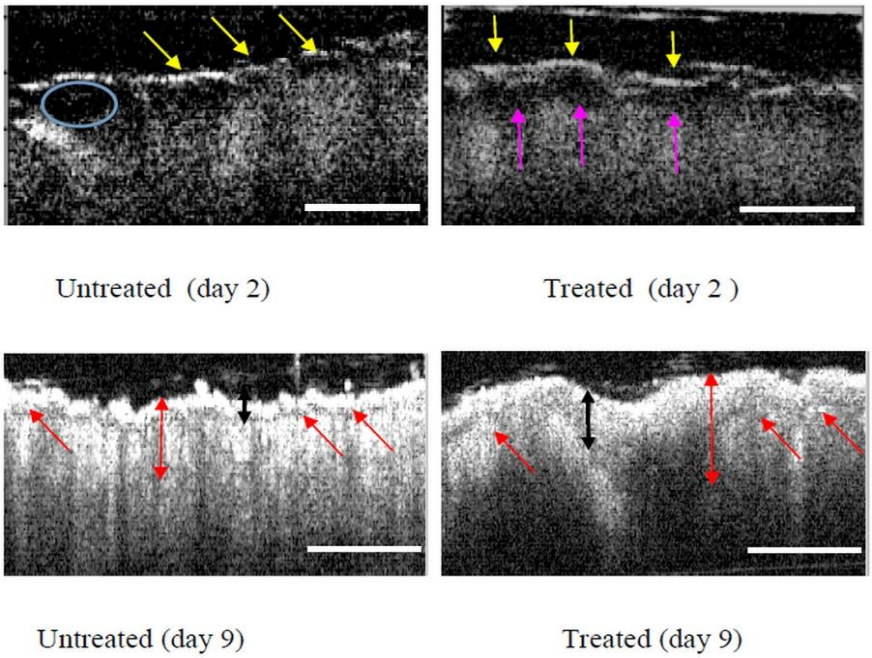


"Fig.1" PS-OCT setup

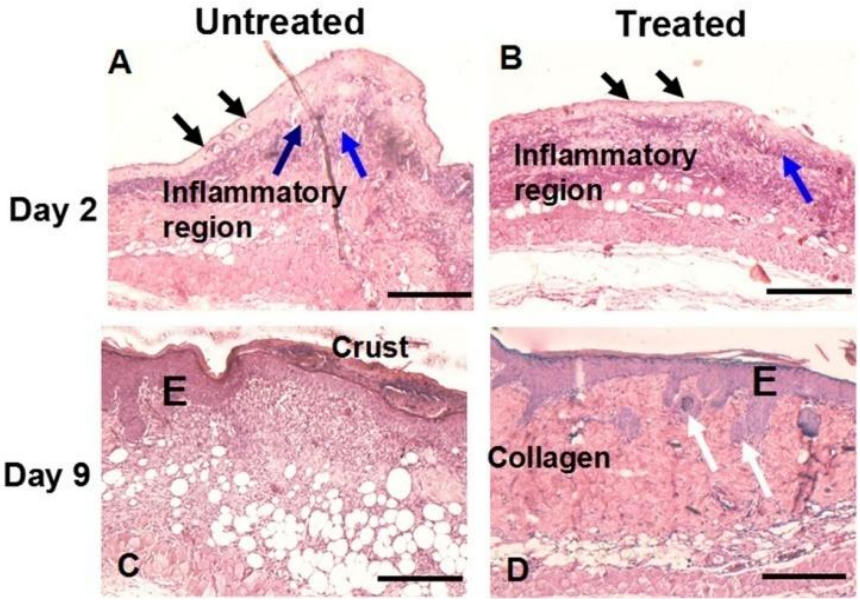


"Fig.2" Reducing power of QI.

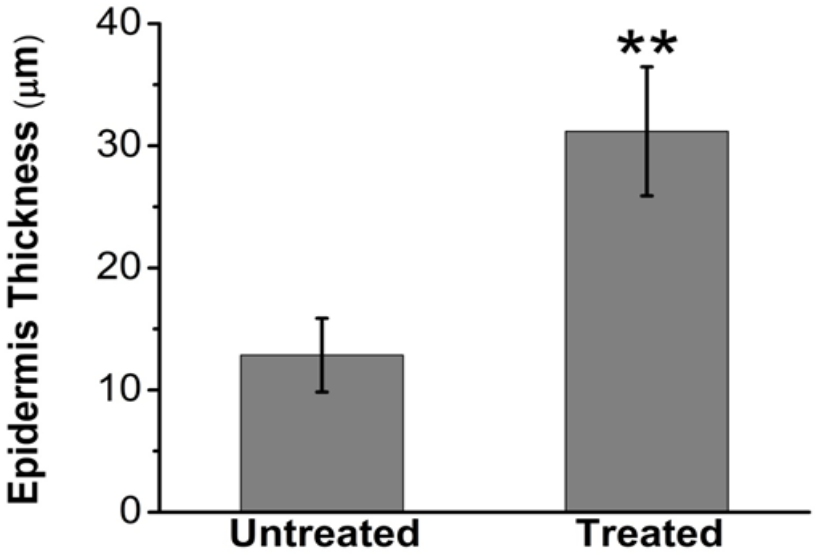
Values are Mean ± S.E. (n=4)



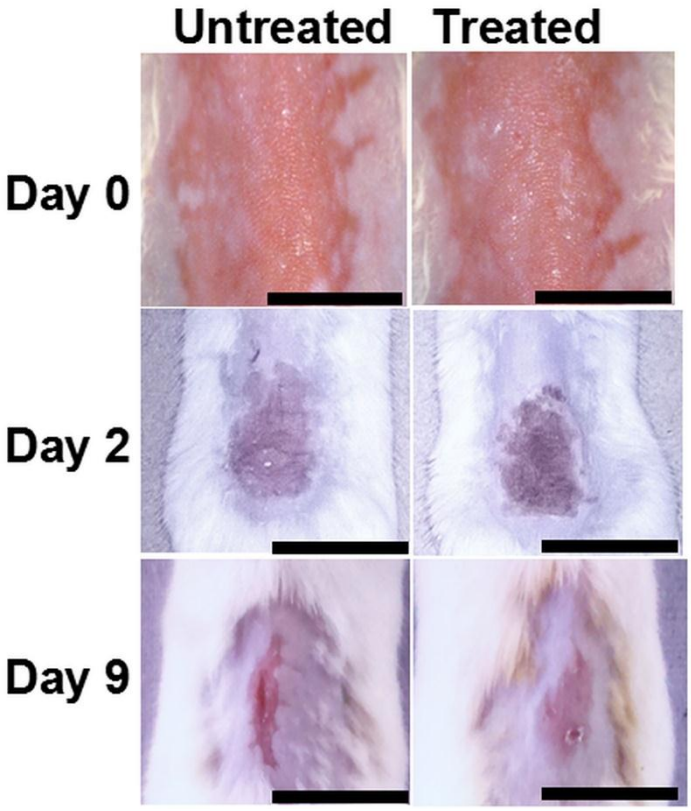
"Fig.3" OCT and PS-OCT images of the skin of untreated control and treated group on day 2 and day 9.



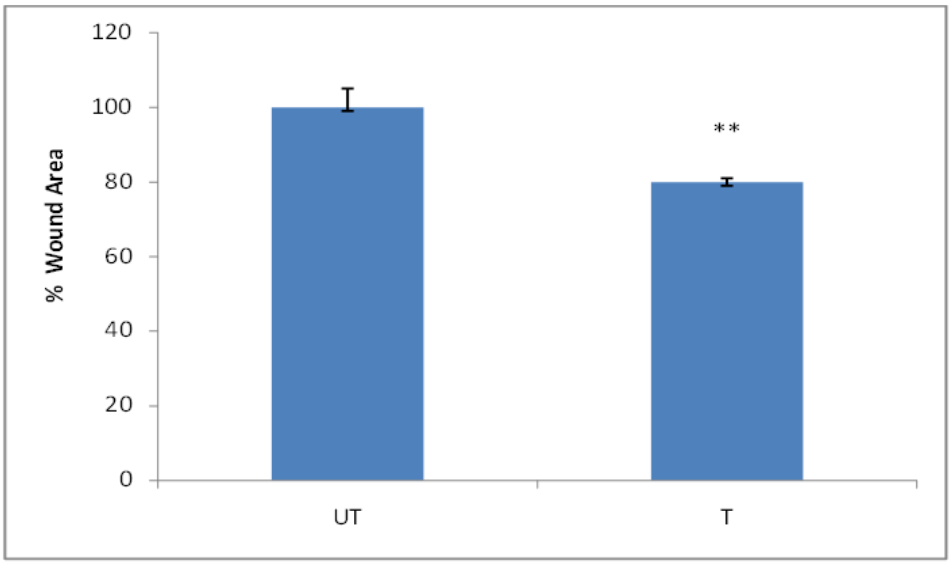
"Fig. 4" Histological images of skin of untreated control and treated group on day 2 and day 9.



" Fig . 4 E" Effect of QI on Epidermal thickness



"Fig. 5 A" Photomicrographs of wounds.



"Fig. 5 B" Effect of QI on wound closure

5. DISCUSSION

Impaired wound healing is the one of the serious problem and to find the proper remedy to overcome this issue is a major concern of the practiceners and researchers. The general objective in wound management is to enable wound healing in the shortest time possible with minimal pain, discomfort and scarring by means of either topical or systemic antimicrobial agents. In chronic wounds, delay in wound healing can be associated with number of factors including free radicals generation. Impairment at the cellular level may be prevented by different antioxidants, which reduce damage caused by free radicals.[20] Plant products are believed to be effective in promoting wound healing since they are composed of various antioxidant and anti-inflammatory molecules.

In the present study, use of Quercus infectoria treatment accelerated the healing process probably due to the presence of antioxidants and tannins in both hydrolysable and condensed form. Tannin exhibits efficient antioxidant, antibacterial and wound healing properties. Plant phenolic compounds flavonoid and triterpenoids acts as a reducing agent (either by donating hydrogen atom or quenching the singlet oxygen) and thus acts as antioxidant. In the present study, considerable amount of phenols and lesser amounts of flavonoids and triterpenoids were observed. The reducing power of the plant extracts increased with increasing concentration and can reduce the oxidized intermediates of the lipid peroxidation process.[21] In wound healing a significant amount of reactive oxygen species (ROS) is generated during inflammation associated oxidative burst in neutrophils and macrophages. The ROS generated by neutrophils and macrophages can lead to considerable peroxidation of cell membrane and cell organelles. Although at micromolar concentrations, oxidants such as hydrogen peroxide may favorably influence signal transduction processes that support healing, at millimolar concentrations, hydrogen peroxide and other oxidants are likely to overwhelm the antioxidant defense system, triggering tissue damage.[22,23] This is turn may affect wound healing adversely.[24] Consistent with this phenomena, treatment of wounds with antioxidants such as flavonoids have beneficial effects on wound healing. Flavonoids content promote wound contraction and reduce epithelialization time due to their astringent, antimicrobial, anti-inflammatory and antibreaking properties.[25]

It is known that there is generally a reduction in wound area of 10-15 % by first week, but during the first days, the wound is largest, inflammation along with most of the discomfort and pain is manifested. Novel therapies are therefore, intended to speed up the healing process, particularly during the first 2–3 days of wound formation. Accurate and early assessment of the therapeutic efficacy is necessary to define the treatment frequency and schedule. Though histology is the standard method to assess inflammation, new epithelium layer formation at cellular level cannot be carried out repeatedly and leads to random sampling. Non invasive assessment techniques will allow early assessment of herbal extract efficacy during the first few days. This is important since the anti inflammatory effect of the extracts in initial days would lead to reduction in inflammation and edema formation. In previous studies, OCT has been used for monitoring the effect of Helium-Neon Laser irradiation on hair follicle growth cycle[26] and healing of bacteria infected wounds[19], effect of antimicrobial photodynamic therapy on wound collagen remodeling[27] and ionizing radiation induced skin alteration etc.[19] In the present study, OCT enabled non invasive assessment of wound healing process in control and QI extract treated animals was performed. The signatures of cutaneous wound healing such as inflammation, edema, crust, epidermal migration, new epithelium formation, dermal –epidermal junction and

reorganization of the collagen matrix was assessed. The presence of new epithelium layer, edematic regions on wound bed could be detected as early as day 2 in the treated group. The decreased imaging depth and increased scattering in the treated group on day 9 suggests a more compact extracellular matrix. The OCT data also corroborated well with the structural alterations observed from histology such as new epithelium layer formation. Hence, OCT allows early as well as repetitive evaluation of morphological changes, thereby helping in timely assessment of the treatment effect and further treatment planning. However, for adverse pathological situations like that observed in case of diabetic or burn wounds, there is an overwhelming level of oxidative stress and concurrent reduction in antioxidants, followed by delayed wound closure. The results of a recent study suggest that QI extract treatment may lead to improvement of wound healing in diabetic mice.[28]

6. CONCLUSION

The results of our study based on OCT, histology and wound area analysis, thus corroborate previous studies and extend the view that QI extract treatment might prove beneficial for wound healing. This suggests that QI has a potential of faster epithelialization and wound contraction, probably due to the presence of major phytochemicals and their antioxidant scavenging activity. The results of this study would help in the quantitative assessment of the healing potential of the herbal extracts and may find application in treatment of wounds healing, which show poor healing due to excessive oxidative stress and inflammation.

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