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Molecular evidence of wound healing potency of lyophilized powder of *Ziziphus rugosa* Lam. leaf extract

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Ziziphus rugosa Lam. (Fam. Rhamnaceae) is a medicinal plant that is commonly used in ethnobotanical and traditional practices for the treatment of wounds, burns, and ulcers. Despite traditional claims for medicinal efficacy of the plant in wound healing, there is no clear scientific data to support the claim, neither *in vivo* nor *in vitro*. Every traditional or folklore type of medicine requires extensive scientific research at cellular and molecular level in order to formulate potent pharmaceuticals suitable for human consumption. Here, we determined the wound healing potency of *Z. rugosa* using *in vitro* scratch wound assay experiments using rat L6 pre-myoblast cells treated with different concentrations of *Z. rugosa* leaf extract (ZLE). The viability of cells treated with test extract was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The concentration and expression fold of Transforming growth factor β1 (TGF-β1), an important marker gene in wound healing was analysed by qPCR based on the wound closure observed in scratch wounds. MTT assay revealed that cell viability is not affected on a notable manner on treatment with ZLE. The data obtained from scratch wound assay clearly indicates that ZLE treatment improved cell survival, proliferation and migration in scratch wounds, leading to wound closure. The rate of closure of wounds was significant (P <0.05) in tested concentrations of ZLE when compared to untreated control cells. When compared to control, the concentration of the marker gene TGF-β1 increased significantly (P <0.05) in cell line cultures of rat L6 cells treated with different concentrations of ZLE. Similarly, the expression fold of the gene was found to be significantly higher (P <0.05) in cells treated with ZLE than in control cells. ThisThis study at cellular and molecular level strengthens *Z. rugosa*'s potential as a herbal medicine for wound treatment. Further research is required to determine the optimal dosage for the treatment of wounds.

Keywords: β-Actin, Cheruthudali, Chunna fruit, Kottamullu, Malamthudali, Traditional medicine, Transforming growth factor β1, Wild jujube, Zunna berry

Plants hold an enormous amount of untapped potential for the management, and treatment of wounds. In the tribal medicine and folklore of many different countries, the treatment of wounds and burns frequently involves the application of various plants. These naturally occurring substances promote healing and regeneration of the damaged tissue via a variety of different pathways. Phytomedicines which have a variety of life-sustaining bioactive components are not only inexpensive and accessible, but they are also risk-free if used in correct concentrations[1]. Thus, since antiquity numerous medicinal plants and plant-based techniques have long been recognised for their important roles in skin regeneration, wound healing and as well other therapeutic applications[2].

A wound is a disruption of the cellular, anatomical, and functional integrity of living tissue brought on by physical, chemical, electrical, or microbiological agents[3,4]. Wound healing is a complex process which involves regeneration and restoration of the damaged tissue. This process proceeds through three phases namely haemostasis and inflammation, proliferation and maturation and finally remodelling[5]. Transforming Growth Factor Beta 1 (TGF-β1) is one of the important marker genes for studying wound healing potential at molecular level[6]. TGF-β1 acts as a potent chemoattractant and inflammatory mediator for a variety of immune cells[7].It also contributes to reepithelialisation, angiogenesis, and extracellular matrix (ECM) formation during the proliferative phase[8]. TGF-β1 is a potent inducer of the fibroblasts to myofibroblast transition and promotes tissue remodelling[9]. Thus TGF-β1 plays a significant role in the different phases of wound healing process.

Ziziphus rugosa Lam. is a straggling shrub or small tree of family Rhamnaceae growing in warm-temperate and subtropical regions throughout the world. The plant is also known as 'wild jujube' or 'Zunna berry' (English) and locally 'Malamthudali',

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'Cheruthudali' or 'Kottamullu'. Various parts of this plants are used in folk medicine for the treatment of, skin disease, cough, diarrhoea, hypotension, menorrhagia and broncho-pulmonary infections[10]. It is also specifically used for treating wounds, mouth ulcers and burns[11]. Despite the traditional knowledge there has not yet been a comprehensive scientific study at the biochemical, cellular and molecular level that focuses on the mechanism by which this herbal remedy work in healing wounds. In the present study, we have made an attempt to determine the wound healing potential of *Z. rugosa* through scratch wound assay and the effect in molecular level by TGF-β1 expression level studies.

Materials and Methods

Collection of plant material

Healthy and mature leaves of *Z. rugosa* was collected from various localities in Idukki, Kottayam and Thrissur in Kerala. The taxonomic status of the plant was authenticated by Botanical Survey of India (BSI) Coimbatore as *Ziziphus rugosa* Lam. and a voucher specimen of the plant BSI/SRC/5/23/2022/ Tech/650 were deposited.

Preparation of *Ziziphus rugosa* leaf extracts

One kg of fresh mature leaf of *Z. rugosa* was surface sterilized by washing in tap water and rinsed with sterile water and ground in a waring blender with equal volume of RO water. The aqueous extract was then filtered through a two layered cheese cloth followed by centrifugation (Remi, India) at 10000 rpm for 10 min for efficient separation of residual particles. The supernatant obtained after the process was lyophilized by freeze-drying (VirTis Genesis, USA). The freeze-dried powder was reconstituted (mg/mL) in sterile water. Two different concentrations of the reconstituted ZLE (10 µg and 100 µg) were used for the study.

In vitro cell line cultures

Establishment of cell line cultures Rat L6 cells

Rat L6 pre-myoblast cells were procured from cell repository of National Centre for Cell Science (NCCS), Pune. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Himedia, Mumbai) supplemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin, 250 ng/mL amphotericin B and 10% heat-inactivated FBS(Fetal Bovine Serum) (PAN Biotech). Cultures were maintained in 5% CO_2 at 37°C in a 95% humidified CO_2 incubator (FormaSteri-Cycle i160, Thermo Scientific) until subculturing[12]. The cells were seeded at a density of approximately 2×10^4 cells/mL for experiments[13,14].

Cell viability assay

The cell viability of rat myoblasts L6 cells treated with ZLE was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay[15]. Freshly harvested cells from the culture flask were used for the assay. 100 µL of cell suspension (1.5×10^4 cells/mL) were seeded in a 96-well plate. After the incubation, 10 µL of MTT reagent of concentration 5 mg/mL in PBS(Phosphate Buffer Saline) was added to the cultures and incubated again at 37°C for 2-4 h. During incubation, the formation of formazan crystals was observed periodically. After the formation of formazan crystals, 100 µL solubilisation solution (1N HCl in isopropanol) was added to each well. The plate was then incubated for 2 h in dark. The absorbance was read at 570 nm. Percentage of viable cells at particular concentrations of extract was calculated by using the following formula: Viability (%) = (AT /AC) × 100 where the AT and AC are the absorbance of treated and control cultures, respectively at 570 nm.

Scratch wound assay

For the scratch wound experiment, rat L6 pre-myoblast cells were seeded onto 6-well culture plates. Cell cultures were incubated overnight at 37°C and 5% CO_2. The cells grown in uniform monolayers were scratched with a sterile 200 µL tip. The wounded cells were rinsed thrice with filter sterile PBS of pH 7.2 to remove the detached cells. The wounded cells were incubated with 1.9 mL of serum free medium containing DMEM with 1% antibiotic and antimycotic solution[16]. The wounded monolayers were treated with two different concentrations of ZLE (10 and 100 µg). Sterile water was added in untreated control wounds. The experiment was done in duplicate wells for each trial. The wound closure was monitored for 24 h using an inverted phase-contrast microscope (Optika, Italy) equipped with a camera to analyse the wound healing rate using the software Optika Pro View and the wound closure rate was determined[17].

qPCR assay of molecular marker genes

Designing of Primers

Gene-specific primers of the wound marker gene transforming growth factor-β1 (TGF-β1) and housekeeping gene rat β-actin were designed from the

reference gene accessions NM_011577.2 and NM_031144.3, respectively, based on a previously published mRNA sequence using Vector NTI (Invitrogen) and synthesized at IDT, USA. Appropriate primers were designed by maintaining the quality as given below.

TGFβ1: FP- 5′-CTGCTGGCAGTAGCTCCCC TATTT-3′ (GC 54%, Tm 60°C, Bp 24)

TGFβ1: RP- 5′-GGTGGGGTCTCCCAAGGAAAG GTA-3′ (GC 58%, Tm 61.9°C, Bp 24)

βACT: FP- 5′-TGTCACCAACTGGGACGATA-3′ (GC50.0%, Tm 55.7°C, Bp20)

βACT: RP- 5′-GGGGTGTTGAAGGTCTCAAA-3′ (GC50.0%, Tm 55.0°C, Bp20)

The synthesized primers were dissolved in sterile double distilled water to get a concentration of 100 pM/µL. The primer stock solutions were stored in −20°C.

RNA isolation and cDNA synthesis

The wounded cell lines of and L6 rat pre-myoblast monolayers were treated with ZLE for 24 h, along with the untreated cells as the control. Total RNA was isolated using TRI-reagent (Sigma, Bangalore)[18]. RNA was electrophoresed on 1% formaldehyde agarose gel and the gel documentation was performed using gel documentation system (BIO RAD, USA). RNA isolated was stored at −80°C. The concentration of RNA was determined spectrophotometrically and about 4 µg of total RNA was transcribed to cDNA with 100 oligo-(dT) primers using Verso cDNA synthesis Kit (Thermos Scientific, California) cDNA amplification was carried out in a total volume of 20 µL in the presence of 2 pM each of the forward primer and reverse primer of rat origin for Transforming growth factor Beta 1 (TGF-β1) using RNA isolated form cell cultures[19].

PCR assay for amplification of TGF-β1

The partial TGF-β1 gene was PCR amplified from the cDNA using gene-specific forward and reverse primers using a thermal cycler. The PCR reaction was carried out using GoTaq Green Master Mix (Promega, USA) in 25 µL reaction mixture containing 12.5 µL of PCR master mix, 2 µL of forward primer, 2 µL of reverse primer, 5 µL of cDNA and 3.5 µL of water. The amplification of the gene was standardized at following cycling condition 95°C for 2 min followed by 35 cycles of 95°C for 1 min, 58°C, for 1 min, 72°C for 1 min with final extension of 72°C for 2 min and held at 4°C. Agarose gel electrophoresis of the PCR products were carried out in 2% gel containing 0.05 mg/mL ethidium bromide along with TrackIt 1 Kb Plus DNA ladder as the molecular weight marker[20].

Construction of standard curve of TGF-β1 gene

Tenfold serial dilution series of the gene TGF-β1 ranging from 50 ng to 0.05 ng reactions was carried out to construct the standard curve[21]. The threshold cycle (Cq) value was plotted against the logarithm of their initial template copy concentration. The efficiency curve was generated by a linear regression of the plotted points. From the slope of the curve, the PCR efficiency (E) was calculated according to the following equation: E= 10[(-(-1)/slope)]-1.

Absolute quantification of wound marker gene TGFβ 1

The real-time PCR amplification of TGF-β1 genes were run separately on a Real time PCR instrument (BIO RAD, USA) and the threshold cycle (Cq) was determined using the software. All the PCR runs were performed in triplicate and each reaction mixture was prepared using a 2X SYBER mix (ABI) in a total volume 20 µL containing 50 ng of template cDNA, 2 pM sense, and antisense primer and 1X SYBR Green and 11.6 µL PCR-grade water. The reaction was set for 40 cycles with the conditions: initial de-naturation 95°C for 10 min, denaturation 95°C for 15 s, annealing 58°C for 15 s, and elongation 72°C for 45 s. After completion of the cycling process, samples were subjected to melting analysis by the following conditions as 95°C for 15 s, 60°C for 15 s and 95°C for 15 s[22].

Relative quantification of TGF-β1 in wounded monolayers of L6 cells

The relative expression of wound healing marker gene TGF-β1 in *Z. rugosa* treated wounded monolayers of rat myoblasts L6 cells were analyzed using qPCR using 2-ΔΔCT method[23]. All samples in triplicate were run in a single reaction. The primers taken for the qPCR were same as that used for the absolute quantification of the gene. The real-time PCR amplification was run on a Real time instrument (BIO RAD, US) and the threshold cycle (Cq) was determined using the software. The wounded L6 rat myoblast monolayers were treated *Z. rugosa* extracts for 24 h, along with the untreated cells as the control. The RNA isolation, cDNA synthesis, PCR assay for amplification of TGF-β1and Quantitative PCR (Real time PCR) wound marker gene TGF-β1 were carried out using the procedures mentioned above.

PCR assay for amplification of housekeeping gene

The PCR reaction was carried out using GoTaq Green Master Mix (Promega, USA) in 25 µL reaction

mixture containing 12.5 µL of PCR master mix, 2 µL of forward primer, 2 µL of reverse primer, 5 µL of cDNA and 3.5 µL of water. The amplification of the gene was standardized at following cycling condition 95℃ for 2 min followed by 35 cycles of 95℃ for 1 min, 58℃, for 1 min, 72℃ for 1 min with final extension of 72℃ for 2 min and held at 4℃. The specificity of primers was evaluated by melting curve analysis, showing a single amplified product for housekeeping gene β-actin.

Estimation of amplification efficiency

The standard curve was constructed using tenfold serial dilution of cDNA ranging from 50 to 3.125 ng for both TGF-β1 and β-actin. Relative quantitative PCR reactions were performed whereby amplification of TGF-β1 gene was normalized with the house-keeping β-actin gene. From the slope of the curve, the PCR efficiency (E) was calculated according to the following equation: E= 10[(-(-1)/slope)]-1. The relative expression level of TGF-β1 gene in *Z. rugosa* treated was measured by taking untreated control samples as the calibrator. The reaction mixes for the amplification contained 50 ng cDNA (1 µL), 2 pM each of the forward and reverse primer of TGF-β1and β-actin (0.25 µL), 5 µL 2X Power SYBR green PCR Master Mix (Invitrogen FG, USA) and 3.5 µL PCR grade water in a total volume 10 µL. The reaction was set for 40 cycles with the conditions: initial denaturation 95℃ for 10 min, denaturation 95℃ for 15 s, annealing 58℃ for 15 s, and elongation 72℃ for 45 s. After completion of the cycling process, samples were subjected for melting analysis by following conditions as 95℃ for 15 s, 60℃ for 15 s, and 95℃ for 15 s. The PCR reaction was carried out in triplicate for each gene. At the end of PCR, the melting curve analysis was run to determine the presence of any primer — dimer artifacts or co-amplified nonspecific product. To omit the sampling differences such as RNA quality, normalization was carried out with the housekeeping actin gene.

Statistical analysis

All the experiments were done in triplicate. The whole data were statistically evaluated for determining the arithmetic mean, Standard deviation and the Standard error. The results are expressed as Mean ± Standard deviation (SD). The significance of difference was assessed using one-way analysis of variable (ANOVA) using SPSS software. P <0.05 was considered significant.

Results

Cell viability

The effect of ZLE in inducing viability and proliferation of cells was estimated by MTT assay using cell line cultures of rat pre-myoblast L6 cells (Fig. 1). For the purpose of the study, two distinct concentrations, namely 1 and 5 µg were utilized. When the treatment was administered at a concentration of 1µg, the percentage of cell survival ability L6 cells was 90.87±0.33; however, this value decreased to 86.69±0.42% when the concentration was increased to 5 µg. The result showed a slight decrease in cell viability with increasing concentration of extract.

***In vitro* scratch wound assay**

The potential of ZLE for wound healing was examined in scratch wounds created in cell culture monolayers of L6 pre-myoblast cells. The wounded monolayers were treated with lower (10 µg) and higher concentration (100 µg) of ZLE and incubated. Untreated wounded cells served as control. The histomorphological changes of the wound were monitored for 24 hours and documented under phase contrast microscope. An active tendency of cell proliferation and migration of cells was observed in the scratch wounds treated with 10 µg concentration (70.56±0.91%) but the rate of wound closure decreased at 100 µg concentration (68.21±0.49%). In the untreated wounds the cell migration occurred in a very slow pace (6.07± 0.22%) (Fig. 2). Thus, the treated ZLE extracts significantly increased (P <0.05)

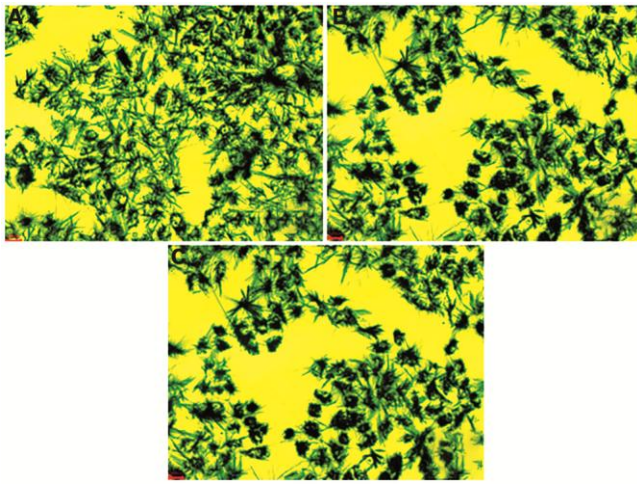

Fig. 1 — MTT Assay-Formazan crystal formation. (A) control cells; (B) Cells treated with 1 µg ZLE; and (C) Cells treated with 5 µg ZLE.

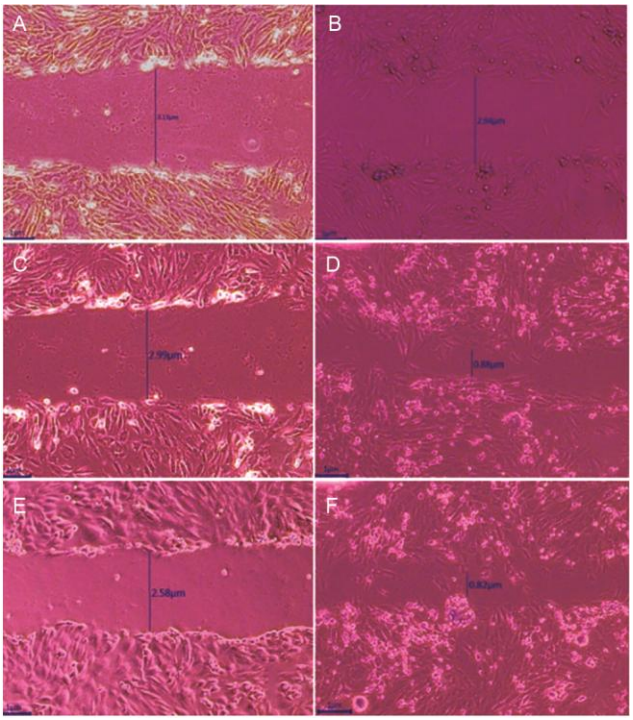

Fig. 2 — Scratch wound assay on L6 myoblast cells. (A & B) Control 0 & 24 h; (C& D) 0 & 24 h of 10 µg ZLE; and (E & F) 0 & 24 h of 100 µg ZLE.

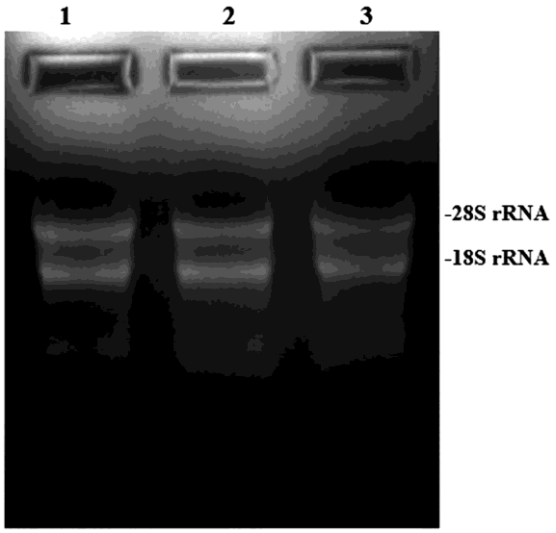

Fig. 3 — RNA isolated from scratch wound of L6 myoblast cells. [Lane 1: Control cells; Lane 2: 10 µg of ZLE treated cells; Lane 3: 100 µg ZLE treated cells]

the proliferation and migration of L6 myoblasts than control leading to wound closure.

qPCR data of wound healing marker genes

Total RNA was extracted from scratch wound cells of L6 cell lines that had been treated with ZLE (10 and 100 µg) as well as the untreated wound cells.

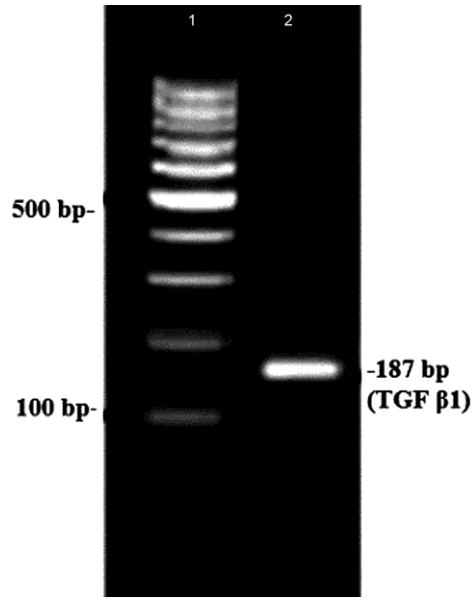

Fig. 4 — PCR amplification of Transforming Growth factor β 1 gene (TGF-β1) from cell lines. [Lane 1: 1000 bp Molecular ladder; Lane 2: TGFβ 1 gene (187 bp)]

Fig. 3 illustrates the RNA, that was extracted from the test cells as well as the control cells. The level of purity of the RNA can be identified by the two distinct bands, known as 28S rRNA and 18S rRNA. A portion of the RNA from the sampes was used as a template for the synthesis of cDNA with Poly T primers. The cDNA that was produced was then utilised in the amplification of TGF-β1 gene fragments with the primers that were designed for the purpose, and the results were measured using quantitative polymerase chain reaction (qPCR). In rat pre-myoblast L6 cell lines, the TGF-β1 gene was amplified to produce a fragment with a size of 187 base pairs (Fig 4).

Melting curve analysis of TGFβ1

The melting peak of the samples showed a clear melting of DNA duplex at 83.0±0.5ºC. As SYBR green dye binds to the double stranded DNA in the sequence in independent way non-specific amplification would be absent. Melting point analysis was done to distinguish target amplicons from PCR artifacts such as primer dimer or misprimed products. Melting point analysis is also useful for optimizing primer-annealing temperature. In the melting process of the double stranded DNA causes a sharp reduction in the fluorescence signal around the melting temperature (Tm) of the PCR product, resulting in a clear peak in the negative derivation of the melting curve (-dE/dT). Thus, different fragments with different melting

temperatures appears as separate peak. Nonspecific peak was not detected for any reaction.

Gene concentration of TGF-β1 in L6 cells

The concentration of marker gene TGF-β1 was determined by absolute quantification using the standard curve of the purified gene fragments at the concentration range of 50-0.05 ng. The curve is highly linear $R^{\Lambda}2$ is 0.967 in the range tested by the triplicate reaction. The slope of the standard curve was –3.281 with an amplification efficiency of 100.1% (Fig. 5). Figure 6 demonstrates the concentration of TGF-β1 genes in cell line cultures treated with ZLE at 10 and 100 µg. Wounded monolayers of L6 myoblasts treated with 10 µg of ZLE showed the highest copy number of 3.301 ng of TGF-β1. While 100 µg ZLE treated L6 wounded monolayers showed a copy number of 3.049 ng which indicate a reduction in gene copy number when the concentration of extract is increased. The untreated control cells exhibited lowest copy number of 2.606 ng TGF-β1gene.

Amplification efficiency of β-actin

The melting peak of the samples showed a clear melting of DNA duplex at 82.5°C. No other specific products were seen to be amplified above the threshold. Moreover, the amplified products showed only a single band of expected size 180 bp of β-actin gene (Fig. 7). The concentration of the primer was also adequate. The Efficiency curve of housekeeping gene β-actin is highly linear $R^{\Lambda}2$ is 0.995 in the range tested by the triplicate reaction. The slope of the standard curve was –3.301 with an amplification efficiency of 100.9% (Fig. 8).

Expression fold of TGF-β1 gene

The expression fold of of TGF-β1 gene was measured in the cell lines treated with extracts of 10 µg and 100 µg concentration of ZLE. Relative quantification of the gene was done by qPCR using mouse β-actin as the house keeping gene. The expression fold of TGF-β1 was calculated by normalizing with the house keeping gene actin. Highest level of TGF-β1 gene expression is observed for cell lines treated with10µg (2.5-fold) followed by

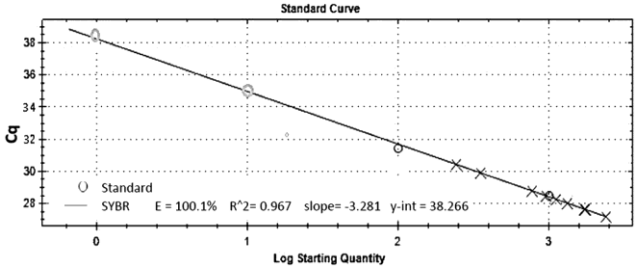

Fig. 5 — Standard curve of TGF-β1 using PCR eluted TGF-β1 products

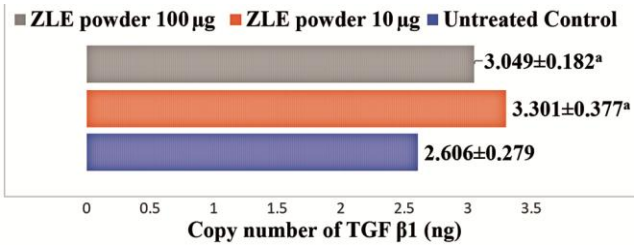

Fig. 6 — Concentration of TGF-β1 gene transcripts in cell line cultures treated with ZLE in two concentrations of 10 µg and 100 µg. [Value expressed as Mean± Standard deviation. 'a' indicates *P* <0.05 when compared to untreated control]

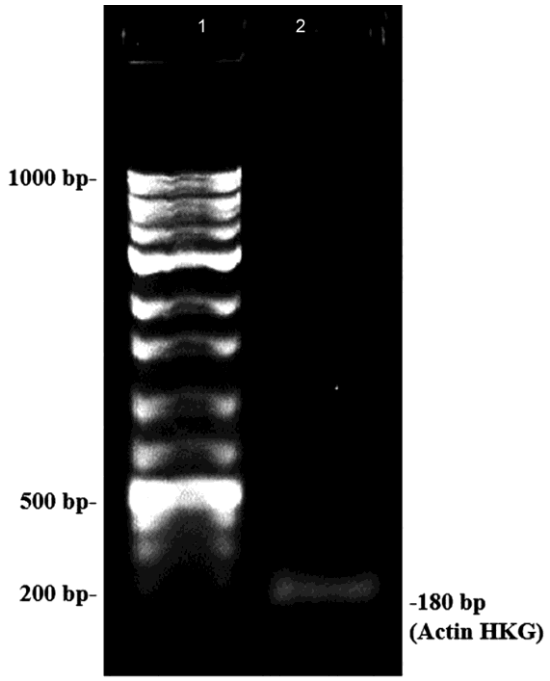

Fig. 7 — PCR amplification of housekeeping gene β Actin (180 bp) from cell lines. [Lane 1: 1000 bp Molecular ladder; Lane 2: β Actin gene (180 bp)]

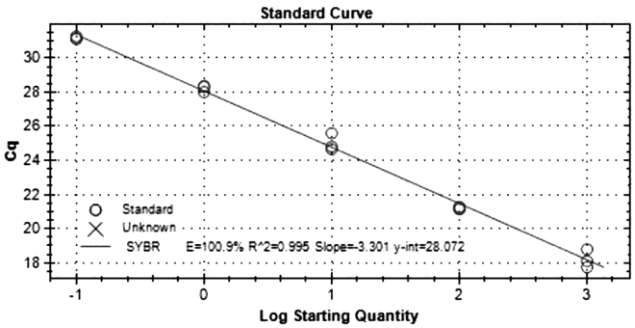

Fig. 8 — Efficiency curve of housekeeping gene β Actin

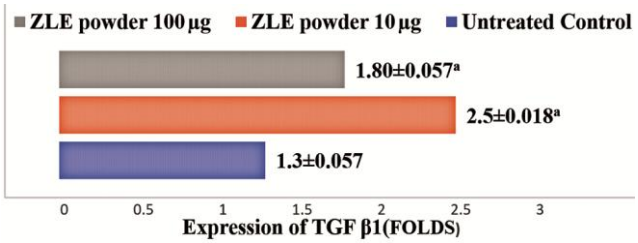

Fig. 9 — Expression fold of TGF-β1 gene transcript in cell line cultures treated with ZLE in concentration of 10 and 100 µg. [Value expressed as Mean± Standard deviation. 'a' indicates P <0.05 when compared to untreated control]

100 µg concentration (1.8-fold). In the untreated cell lines the gene expression level was the lowest (1.3-fold) (Fig. 9).

Discussion

Wound healing is a complex, sequential process of bringing back tissue construction in damaged tissues close to its probable ordinary state[24]. This requires three phases; an inflammatory stage that involves variety of proinflammatory mediators and suppression of the immune system, a proliferative stage in which fibroblast proliferation, aggregation of collagen, and formation of new blood vessels occur, and a remodelling stage that involves wounded tissue regeneration and reconstruction. Therefore, enhancing each of these steps can hasten wound healing and reduce the risk of complications[25,26]. TGF-β1 is one of the most important marker gene for the wound healing process[27]. It plays a very critical and important role in all stages of the wound healing[28].

Wound repair requires complicated interactions between cells as well as numerous growth factors[29], with TGF- β playing a vital throughout all phases of wound healing. In the haemostasis and inflammatory phase TGF-β1 acts as a potent chemoattractant and inflammatory mediator for a variety of immune cells[30]. Reepithelialisation, angiogenesis, and extracellular matrix (ECM) formation during the proliferative phase are also mediated by the TGF-β isoforms[31]. Tissue remodelling which is the final phase in the wound healing process involves active participation of TGF-β1 like in apoptosis of resident cells, vascular regression, granulation tissue remodelling, wound contracture, replacement of type III collagen by type I collagen[32] and fibroblasts[33] to myofibroblast transition[34]. Chronic wounds usually exhibit diminished TGF-β1signalling[35]. Shultz and Wysocki demonstrated that TGF-β1 reduces collagenase expression, which weaken collagen and ECM[36].

The well-known *in vitro* cell scratch assay, which is a common technique to evaluate the wound healing potential of plant extracts, was used to measure the wound healing activity. It involves developing a scratch on a thick monolayer of appropriate cells and observing the cell migration and wound healing under a microscope[37]. Since decreased levels of cell proliferation could have an impact on the results, the potential cytotoxic effect of the extract on the rat L6 pre-myoblast cells was examined using MTT assay before the wound healing assay. Additionally, toxicity evaluation is a crucial factor in the quality control of pharmaceutical preparations[38,39]. The cell scratch assay, which uses fibroblasts, myoblasts, and keratinocytes, is a popular technique for determining the activity of substances and natural products in an *in vitro* wound healing model[40,41].

The data of the present study demonstrates the wound healing effect of the ethnomedicinal plant *Z. rugosa* Lam. at cellular and molecular level. The viability percentage of untreated control cells is considered as 100%. The rat L6 cell lines showed more than 85% survival ability during MTT assay on treating with tested concentrations of ZLE extract indicating its non-toxic nature to be used as a medicine. The appropriate concentration of treatment should be fixed with higher level studies. ZLE extract showed a stimulating effect in wound healing as evident from *in vitro* scratch wound assay. The treated cell lines showed an active proliferation and migration of cells when compared with the untreated cell lines. Histological observation of the scratch wound monolayers showed an increasing rate of wound closure either due to cellular migration or multiplication in lower concentration (10 µg) than higher concentration (100 µg). To the best of our knowledge, this is the first account of the wound healing potential of the studied species. Since the closure of the wound that was observed in scratch wound models is an indication that the treatment of ZLE has a healing effect, the study was extended to determine the expression and concentration of marker gene of wound healing TGF-β1 in cell line cultures that had been treated with ZLE.

Using absolute and relative quantification experiment by qPCR, we were able to quantify the concentration and expression fold of the TGF-β1gene that was present in the ZLE treated scratch wound cells. The results were compared with the untreated cells which were taken as the control. This is for the

purpose of identifying the healing effect of ZLE, which is engaged in the molecular level process of wound closure. The results of absolute and relative quantifications are clear molecular evidence for the wound healing potential of the plant which highlights the therapeutic advantages of the plant beyond the level of a traditional medicine. This is demonstrated by the findings of the cellular and molecular tests which conclusively confirm the curative potential of the medicinal herb *Ziziphus rugosa* Lam.

Conclusion

Above study on the wound healing properties of the leaf extract of *Ziziphus rugosa*, particularly ulcers, has evidently been demonstrated. The scratch wound assay using the rat L6 pre myoblast cells and the increase in expression level of the marker gene TGFβ-1, which was assessed using qPCR, provide evidence at cellular and molecular level that ZLE has a positive influence on the healing of wounds. Additional research is required for standardising the concentration of treatment and the development of a potential wound healing medicine from the plant.

Conflict of interest statement

Authors declare no competing interests.

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