Journal of Pharmaceutical Analysis 6 (2016) 389–395

HOSTED BY

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

journal homepage: www.elsevier.com/locate/jpa

Original Article

Wound healing activity of *Delonix elata* stem bark extract and its isolated constituent quercetin-3-rhamnopyranosyl-(1-6) glucopyranoside in rats☆

Pradeepa Krishnappa[a,b], Krishna Venkatarangaiah[a,*], Venkatesh[a,c], Santosh Kumar Shimoga Rajanna[a], Rebijith Kayattukandy Balan[d]

[a] *Department of Post Graduate Studies and Research in Biotechnology, Kuvempu University, Shankaraghatta 577451, Karnataka, India*
[b] *Department of Biotechnology, MS Ramaiah Institute of Technology, Bengaluru 560054, India*
[c] *Department of Biochemistry, Indian Institute of Science (IISc), Bengaluru 560012, India*
[d] *Division of Biotechnology, Indian Institute of Horticultural Research (IIHR), Bengaluru 560089, India*

ARTICLE INFO

Keywords:
Delonix elata
Quercetin-3-rhamnopyranosyl-(1-6) glucopyranoside (QRPG)
Wound healing
Hydroxyproline
Tensile strength

ABSTRACT

Delonix elata L. is a Ceasalpinaceae species and is traditionally used in India for treatment of skin diseases, liver diseases and rheumatic problems. However, systematic evaluation of its wound healing activity is lacking. Thus, in the present study, we aimed to assess the wound healing activity of *D. elata* stem bark extract (DSE) and its isolated constituent quercetin-3-rhamnopyranosyl-(1-6) glucopyranoside (QRPG) in rats. The formulations effects on wound healing were assessed by the wound contraction rate, epithelialization period, tensile strength, content of the hydroxyproline, hexosamine and uronic acid in granulation tissue, histopathological studies and Col 1 α (I) expression level in wound tissue by reverse transcription polymerase chain reaction (RT-PCR) study. The topical application of DSE ointment caused faster epithelialization, significant wound contraction (100%), and better tensile strength (710.5 ± 10.5 g/cm^2), while QRPG showed wound epithelialization with 98.2% contraction, better than that of the control group (78.18%). The biochemical analysis of granulation tissue revealed that DSE and QRPG significantly increased hydroxyproline, hexosamine and uronic acid content. A significant increase in the expression of Col 1 α (I) was observed in the wound tissue of DSE and QRPG treated rats. DSE and QRPG were shown to enhance wound healing by increasing collagen synthesis through up-regulation of Col 1 α (I), thus validating ethnomedicinal uses.

1. Introduction

Plants have immense potential for the management and treatment of wounds. A large number of plants are used by tribes and folklore in many countries for the treatment of wounds and burns. Several plants have been experimentally used as traditional medicines to treat skin disorders and wound injuries [1–4]. Efforts are made all over the world to discover therapeutic agents that can promote wound healing and thereby reduce the cost of hospitalization and save patients from amputation or other severe complications.

Delonix elata L. (Ceasalpinaceae) is a deciduous tree, known as Vathanarayani in Kannada and White Gulmohar in English. It grows in the dry forests of India and is also found in African countries. The plant is used by the local inhabitants of Chitradurga, India, for treatment of skin diseases, hepatic diseases, rheumatic problems and bronchitis in infants. In our previous studies, leaf extract of *D. elata* has shown significant antinociceptive activity [5] and stem bark extracts have shown potential antioxidant and hepatoprotective activity against carbon tetrachloride induced hepatotoxicity in rats [6]. We have also reported the antibacterial activity of *D. elata* against *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris, Salmonella typhi, Klebsiella pneumoniae and Salmonella paratyphi* [7].

Medicinal plants provide leads to find therapeutically useful compounds, thus in the present investigation, we made effort to isolate and characterize the active principle of *D. elata* stem bark extract and elucidate the relationship between its structure and the wound healing activity in rats.

2. Materials and methods

2.1. Chemicals

Povidone iodine ointment (Cipla, Bengaluru, India), white petro-

☆ Peer review under responsibility of Xi'an Jiaotong University.

* Corresponding author.
E-mail address: krishnabiotech2003@gmail.com (K. Venkatarangaiah).

http://dx.doi.org/10.1016/j.jpha.2016.05.001
Received 15 July 2015; Received in revised form 6 April 2016; Accepted 3 May 2016
Available online 04 May 2016

leum jelly (Oom Laboratories, Shimoga, India), petroleum ether, chloroform, ethanol, diethyl ether, HCl, NaOH, copper sulfate, H_2O_2, H_2SO_4, p-dimethyl aminobenzaldehyde L-hydroxyproline, acetylacetone, sodium carbonate, and carbazole reagent were procured from Merck (Mumbai, India). Glucuronic acid, hexosamine and sodium tetraborate were obtained from the Sigma-Aldrich Co. (Bengaluru, India). All the chemicals used were of analytical grade. Water was purified using the Milli-Q system from Millipore, Bedford, MA, USA.

2.2. Preparation of extracts

The stem bark of *D. elata* was collected from Chitradurga, Karnataka, India, in October 2012. Taxonomic identification of the plant was done by Dr. Manjunatha, compared with the voucher specimen deposited at Kuvempu University [8]. The plant material was pulverized and soxhlet extraction was carried out with ethanol to obtain crude extract, and named as *D. elata* ethanolic extract (DSE) (Yield: 9.67%).

2.3. Isolation and characterization of bioactive compound

The DSE (20 g) was dissolved in 100 mL water and subjected to liquid-liquid fractionation with ethylacetate (100 mL). Aqueous layer containing precipitate was filtered using Whatman No.1 filter paper and dried on water bath. The dried extract fraction was subjected to silica gel column chromatography (60 cm×4 cm, 60–120 mesh, 200 g), and eluted with a stepwise gradient of chloroform and chloroform-methanol combination (9:1, 8:2, 7:3, and 6:4, v/v). A total of 110 fractions (10 mL each) were eluted. Fractions 37–45 yielded a residue of about 0.68 g. This residue was further purified by preparative TLC (silica gel) using the solvent system chloroform-methanol with the ratio of 8:2 to afford pale yellow color compound (322 mg). Characterization of the isolated compound was performed by subjecting it to qualitative analysis followed by IR, ^{1}H NMR, ^{13}C NMR, and mass spectral (MS) studies.

2.4. Acute toxicity study

Acute toxicity study was conducted for the stem bark extract and isolated compound by the up-and-down procedure [9]. Dimethyl sulfoxide (DMSO) (1%, v/v) was used as a vehicle to suspend the drugs and administered orally. Animals were observed individually for first 30 min after dosing, periodically at the intervals of 4 h during day first and thereafter, observed for 14 days for changes in their behavioral pattern and mortality.

2.5. Drug formulations

Two types of topical formulations were prepared, 5% (m/m) ointment formulation of ethanolic extract of stem bark and 0.5% (m/m) QRPG ointment formulation. White petroleum jelly was used as the ointment base. For oral administration, suspensions of 300 mg/kg DSE and 50 mg/kg QRPG were prepared with 1% (m/v) DMSO according to the acute toxicity study. The drug formulations were freshly prepared every third day. The povidone-iodine ointment was used as a standard reference.

2.6. Experimental animals

Wistar albino rats of either sex, weighing 180–200 g were used for the study. The animals were housed at (25 ± 1) °C, with humidity of 55%–60% in the Department of Biotechnology, Kuvempu University, Karnataka, India. They were fed with a standard commercial pellet diet (Sai Durga feeds and foods, Bangalore) and water *ad libitum* during the experiment. The study was permitted by the Institutional Animal Ethical Committee (Reg. No. NCP/IAEC/CL/13/12/2010-11), India.

2.7. Wound healing activity

Excision, incision and dead space wound models were used to evaluate the wound healing activity. The animals were divided into four groups, each containing six animals, for excision and incision wound models. 50 mg of formulated ointments were applied topically to each animal once a day. The animals of group 1 received the ointment base (control), whereas group 2 was treated with a 5% (m/m) povidone–iodine ointment. Groups 3 and 4 were treated with ointments of 5% (m/m) DSE and 0.5% (m/m) QRPG, respectively.

For the dead space wound model, animals were grouped into four groups, each containing six animals. The animals of group 1 (control) were treated with 1% DMSO. Group 2 was treated with DSE (300 mg/kg) and group 3 with QRPG (50 mg/kg). Group 4 was treated with 5% (m/m) povidone-iodine ointment (standard).

The animals were anaesthetized with diethyl ether prior to and during the infliction of the wound. All the animals were closely observed for any infection so that the infected animals could be excluded from the study.

2.7.1. Excision wound creation

The animals were anaesthetized prior to and during the creation of experimental wounds with diethyl ether. The rats were then inflicted with an excision wound as described by Morton and Malone [10]. The dorsal fur of the animals was shaved with an electric clipper and full thickness of excision wound of 500 mm^2 was created along the marking using toothed forceps, a surgical blade and pointed scissors. The entire wound was left open. All the groups of animals were treated in the same manner as mentioned above. The wound tissue was removed from the rats by sacrificing the animals on the 7th post-wound day for reverse transcription-polymerase chain reaction (RT-PCR) analysis.

2.7.2. Incision wound creation

The test rats were anesthetized with diethyl ether prior to and during the creation of the experimental wounds. The dorsal fur of the animals was shaved with an electric clipper and two para-vertebra along an incision of 6 cm length were made through the skin at a distance of about 1.5 cm from the midline on each side of the depilated back of the animals as described earlier by Ehrlich and Hunt [11]. After incision, the parted skin was stitched together at intervals of one centimeter using surgical thread (No. 000) and a curved needle (No. 11). The wounds were then left undressed. All groups of animals were treated as described above.

2.7.3. Dead space wound creation

Dead space wounds were created by the subcutaneous implantation of sterile cylindrical grass pith (2.5 cm×0.3 cm), on either side of the lumbar region on the ventral surface of each rat [12]. On the 10th post-wound day, the animals were sacrificed under diethyl ether anesthesia, and the granulation (wound) tissues formed on the grass piths were excised.

2.8. Biophysical parameters

The rate of wound contraction in the excision model was determined as a percentage reduction of the wound size and the surface area was measured on the 1st, 4th, 8th, 12th, 16th and 21st post-wounding days by tracing the wound on a transparent graph sheet. The period of epithelialization was also noted [13]. The tensile strength of the incision wound tissues was measured on the 10th day by Lee's method [14] after removing the sutures on the 8th post-wound day.

2.9. Biochemical parameters

The granulation tissue was dried at 60 °C for 12 h in an oven to obtain a constant dry weight [15]. Simultaneously, the dried tissue was

hydrolyzed with 6 M HCl (5.0 mL) for 24 h at 110 °C, and then neutralized (pH 7). The neutralized hydrolysate was used for biochemical estimations.

The total collagen content of the granulation tissues was estimated based on the hydroxyproline index by the method of Neuman and Logan [16]. Hexosamine was estimated by the method of Elson and Morgan [17]. Uronic acid in the wound tissue was determined by the carbazole method with slight modifications as described by Bitter and Muir [18].

2.10. Col 1 α (I) expression analysis by RT-PCR

The total RNA was isolated from the wound tissue excised on the 7th post-wound day using an animal total RNA isolation mini kit (BIO-52043, Bioline, UK) as per the manufacturer's protocol. The isolated RNA was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The single-stranded cDNA was prepared from the isolated RNA by using a First Strand cDNA synthesis kit (cat# 1611, Fermentas, USA) as per the manufacturer's protocol with Oligo[dT]$_{18}$ primers.

The primer pairs (Table S1) were designed using the Bio Edit version 7.0.9.0 for the Col 1 α (I) gene (GenBank accession No. NM_053304) and the internal control β-actin gene (GenBank accession No. NM_031144) sequences retrieved from the NCBI repository [19]. The primer sequences were cross validated with Integrated DNA technologies for secondary structures including hairpins, self-dimers, and cross-dimers in primer pairs. All the primers were synthesized at Sigma-Aldrich, India with the optimum parameters set as a melting temperature (Tm) of 60–65 °C, a primer size of 18–26 nucleotides, a GC content of 35%–55% and a product size of 100–200 bp for target gene amplification. The specificity of primer pairs was confirmed by using BLAST analysis in NCBI against *Rattus norvegicus* sequences.

PCR was performed to amplify Col 1 α (I) in a thermal cycler (ABI-Applied Biosystems, Veriti, USA) using the following cycling parameters: an initial denaturation step at 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, an annealing step at 47 °C for 45 s, an extension step at 72 °C for 45 s and a final extension step at 72 °C for 20 min using Col 1 and β-actin primers separately. The amplified products were resolved on a 3.50% agarose gel, stained with ethidium bromide (10 μg/mL) and visualized in a gel documentation system. The images were captured and subjected to densitometric analysis using the Bio-Rad Gel Doc™ XR gel documentation system equipped with Quantity One™ Software. The values were expressed as the band intensity of the Col 1 α (I) relative to the level of the reference mRNA for the house-keeping gene, β-actin.

2.11. Histopathological evaluation

On the 10th post-wounding day, granulation tissue was excised from the sacrificed animals. A part of a wet tissue was preserved (10% formalin), dehydrated through a series of alcohol, cleared in xylene and embedded in paraffin wax. 5 μm sections were cut and stained with Hematoxylin and Eosin (HE) [20], and finally evaluated by histopathological examination.

2.12. Statistical analysis

The results are presented as mean ± standard errors (SE). Duncan's test was used to evaluate the significance of the differences between the groups. The differences in values at $P < 0.05$ or $P < 0.01$ were regarded as statistically significant. GraphPad Prism 5 software was used for statistical analysis.

Table 1
^{13}C NMR spectral data of QRPG.

Carbon position	Signal (δ)	Carbon position	Signal (δ)
1			
2	157.48	1″	101.28
3	113.72	2″	70.82
4	181.91	3″	
5	163.33	4″	70.24
6	99.08	5″	68.65
7	164.42	6″	66.73
8	94.26	1‴	100.80
9	160.00	2‴	73.34
10	104.13	3‴	75.93
1′	124.91	4‴	71.71
2′	116.09	5‴	71.08
3′	148.58	6‴	18.02
4′	146.98		
5′	118.65		
6′	128.31		

3. Results

3.1. Isolation and characterization of bioactive compound

The pale yellow amorphous compound isolated from DSE was confirmed as flavonoid in qualitative group testing (lead acetate solution test, alkaline reagent test, ferric chloride test, and Shinoda's test). The melting point of the compound was 175–177 °C and IR, ^{1}H NMR, ^{13}C NMR and ESI–MS spectra are provided in Figs. S1–S4. The IR spectrum showed the peak values at (KBr) V_{max} (cm^{-1})=3300 (br –OH), 2927.61, 2402.31, 1614.11, 1518.2 and 1458.11 (aliphatic C–H), 1655 (αβ-unsaturated C=O), 1615 (C=C), 1066 (C–O). ^{1}H NMR showed the peak values with (400 MHz, DMSO-d6)δ 13.0 (1H, OH), 11.0 (1H, OH), 9.0 (1H, OH), 8.03 (1H, OH), 7.53 (1H, Ar-H), 7.50 (1H, Ar-H), 6.88 (1H, Ar-H), 6.19 (1H, Ar-H), 6.50 (1H, Ar-H), 5.40 (1H, Anomeric-H), 5.25 (1H, Anomeric-H), 0.98 (1H, CH3). ^{13}C NMR details are provided in Table 1. ESI–MS at m/z=595 [M^{+}H]$^{+}$, analyzing for $C_{27}H_{30}O_{15}$, together with two anomeric protons indicated the presence of two sugar residues in the molecule. Based on the spectral details, the isolated compound was characterized as quercetin-3-rhamnopyranosyl-(1-6) glucopyranoside (Fig. 1).

3.2. Acute toxic studies

Acute toxicity studies revealed that rats administered with QRPG at the concentration of 500 mg/kg showed 50% of lethality and hence, it was considered as the LD_{50} value. One tenth of the dose, i.e., 50 mg/kg, was considered as safer dose for oral administration. LD_{50} of DSE was 3000 mg/kg and hence 300 mg/kg was considered as a safer dose for oral administration.

3.3. Wound contraction and epithelialization

The progressive reduction in the wound area of the different groups

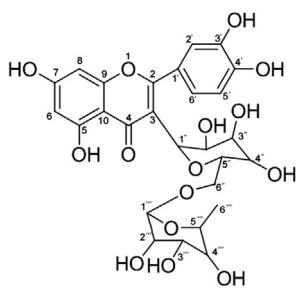

Fig. 1. Structure of quercetin-3-rhamnopyranosyl-(1-6) glucopyranoside.

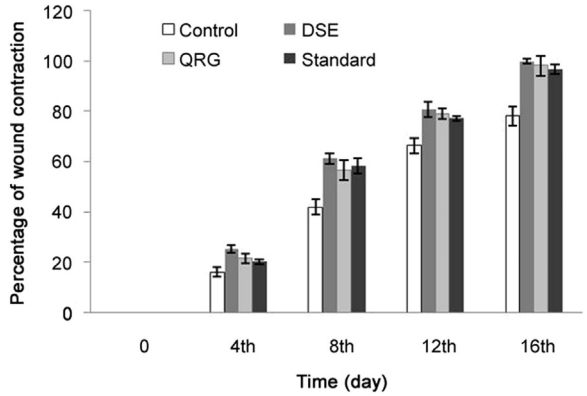

Fig. 2. Effect of topical application of DSE and QRPG on the wound area expressed as percentage of wound contraction. Values are expressed as mean ± SE of six animals in each group.

of animals over 16 days is presented in Fig. 2 and in supplementary file. On the 16th post-wound day, the fastest healing of the wound was observed in animals which had received DSE ointment (100% wound contraction, Fig. S2b) as compared with the animals treated with standard 5% (m/m) povidone-iodine ointment (96.7% wound contraction, Fig. S2d). The topical application of QRPG at the wound site in an excision wound healing model showed significant wound healing activity ($P < 0.05$) (98.2% wound contraction on 16th post-wound day, Fig. S2c). The lowest rate of wound healing was observed in control group (78.18% wound contraction on the 16th post wound day, Fig. S2a). Fig. S2a shows delay in the wound contraction in control animals. Table 2 summarizes the epithelialization period of different animal groups and it was found that the rate of epithelialization in DSE ointment treated animals (17 days) was on par with the effect of the standard povidone-iodine ointment (18 days). It was observed that QRPG significantly reduced the epithelialization time of wounds (18 days) as compared to the control animals (22 days, $P < 0.05$).

3.4. Measurement of tensile strength

The wound healing measured by the tensile strength of the healing skin, treated with different formulations on the 10th day evealed that the wound treated with the ointment base had the least strength (488 ± 12 g/cm^2) (Table 2). The tensile strength of the wound tissue treated with the DSE formulation (710.5 ± 10.5 g/cm^2) was substantially higher than that of the control group. The tensile strength of the wound treated with the povidone–iodine ointment was comparable (694.5 ± 5.5 g/cm^2) to that the of ointment formulation of QRPG (668.5 ± 21.5 g/cm^2). This observation confirms that DSE as well as QRPG, possesses excellent wound healing properties.

3.5. Content of hydroxyproline, hexosamine and uronic acid

Table 2 depicts the results of the content of hydroxyproline, hexosamine and uronic acid of the wound tissue of the experimental and control animals. The results revealed that treatment with DSE significantly increased the collagen content of the wound tissue (4.77 ± 0.03 mg/100 mg dry tissue) as compared to the control animals (2.84 ± 0.07 mg/100 mg dry tissue) ($P < 0.05$). The collagen content of the granulation tissue of DSE treated animals was on par with that of the animals that received povidone–iodine ointment (4.76 ± 0.07 mg/100 mg dry tissue). The ground substances for collagen synthesis, namely, the hexosamine (0.62 ± 0.01 μg/100 mg dry tissue) and uronic acid (0.24 ± 0.01 μg/100 mg dry tissue) levels of the granulation tissue of DSE treated animals were also increased significantly as compared to the control group ($P < 0.05$).

3.6. Col 1 α (I) gene expression analysis by RT-PCR

The mRNA level of Col 1 α (I) was semi-quantitatively measured using RT-PCR and expressed as a densitometric band intensity of the target gene relative to the level of the reference mRNA for the housekeeping gene, β-actin. The total RNA extracted from the wound tissue of different groups of animals showed an absorption ratio of 2.0 ± 0.1 and 1.5–2.0 at an optical density of 260/280 and 260/230 nm, respectively, in a Nanodrop ND-1000 spectrophotometer. They also showed good integrity and purity when loaded on an agarose gel. The amplified products of Col 1 α (I) and β-actin genes were subjected to 3.5% agarose gel electrophoresis with a 100 bp ladder. The agarose gel electrophoresis (Fig. 3) showed very clear and intense DNA bands of Col 1 α (I) and β-actin genes which yielded a specific sized (102 bp and 120 bp, respectively) amplicon as predicted. A significant increase in the expression of Col 1 α (I) in the 7th day wound tissue of DSE and QRPG treated rats is shown in Fig. 4.

3.7. Histopathology

The granulation tissue provided further evidence of the wound healing efficacy of the DSE and QRPG. The granulation tissue of the control animals showed lower epithelialization and collagen formation with a greater aggregation of macrophages, indicating the incomplete healing of wounds (Fig. 5A). The granulation tissue obtained from the DSE treated animals showed a significant increase in collagen deposition, a few macrophages and more fibroblasts (Fig. 5B). A high deposition of collagen and a significant reduction of macrophage infiltration were noticed in the wound tissue treated with QRPG (Fig. 5C). The animals treated with povidone-iodine ointment showed increased collagenation and depletion in the accumulation of macrophages at the site of injury (Fig. 5D).

4. Discussion

Phytochemical analysis of *D. elata* stem bark extracts revealed the presence of flavonoids [6]. In the present study, a flavonoid was isolated from the DSE based on bioassay guided separation. Characterization of the isolated compound was carried out by IR, ^{1}H NMR, ^{13}C NMR and MS analysis. The IR spectra exhibited characteristic absorption bands at 3300 cm^{-1} for OH group and 1655 cm^{-1} for α,β-unsaturated carbonyl group, 1615 cm^{-1} for C=C group, and 1066 cm^{-1} for C–O group. The ^{1}H NMR spectrum (400 MHz,

Table 2
Effects of DSE and QRPG on epithelialization time, tensile strength and content of hydroxyproline, hexosamine and uronic acid.

Parameters	Ointment base (control)	DSE (5.0%, m/m)	QRPG (0.5%, m/m)	5% povidone-iodine ointment (standard)
Epithelialization time (days)	22 ± 0.25	17 ± 0.25*	18 ± 0.48*	18 ± 0.29*
Tensile strength (g/cm^2)	488 ± 12	710.5 ± 10.5*	668.5 ± 21.5*	694.5 ± 5.5*
Hydroxyproline content (mg/100 mg dry tissue)	2.84 ± 0.07	4.77 ± 0.03*	4.54 ± 0.1*	4.76 ± 0.07*
Hexosamine content (μg/100 mg dry tissue)	0.37 ± 0.02	0.62 ± 0.01*	0.61 ± 0.02*	0.58 ± 0.02*
Uronic acid content (μg/100 mg dry tissue)	0.12 ± 0.01	0.24 ± 0.01*	0.24 ± 0.01*	0.22 ± 0.01*

Values are expressed as mean ± SE.
* $P < 0.05$ as compared to control.

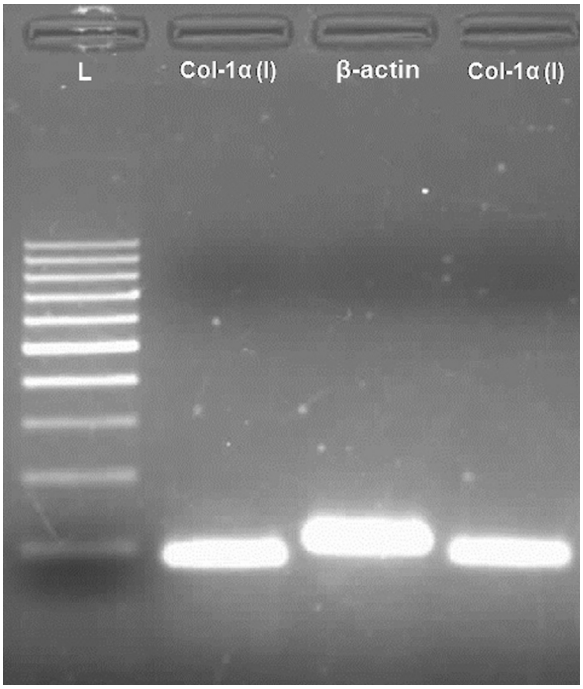

Fig. 3. RT-PCR analysis of Col 1 α (I) mRNA expression in 7th day wound tissue of DSE and QRPG treated rats. β-actin was co-amplified as an internal control (Lane 1: 100 bp ladder; Lane 2: DSE treated; Lane 3: control; Lane 4: QRPG treated).

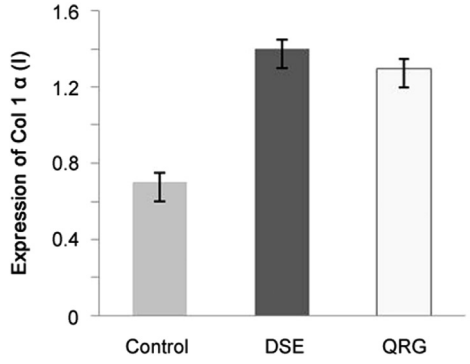

Fig. 4. The values were normalized to β-actin expression and represented as Col 1 α (I)/ β-actin band intensity for the 7 th day wound tissue. Values are expressed as mean ± SE (n=6).

DMSO-d6) indicated a 5, 7, 3′, 4′-tetraoxygenated flavone. The ^{1}H NMR spectrum exhibited a characteristic proton signal at δ 13.00, 11.0, 9.0 and 8.03 corresponding to four free hydroxyl at C-5, C-7, C-3′ and C-4′ carbon atoms. The aromatic protons exhibited one ABX coupling system at δ 7.53(1H, dd, J=8.4, 2.0 Hz) for H-6′, δ 7.50 (1H, d, J=2.0 Hz) for H-2′, and δH 6.88 (1H, d, J=8.4 Hz) for H-5′. The other AX coupling system at δH 6.19 (1H, d, J=2.0 Hz) and δH 6.50 (1H, d, J=2.0 Hz) was assigned to H-6 and H-8 protons, respectively. The ^{1}H NMR spectrum also supported the presence of two rhamnose moieties with the anomeric proton signals at δ5.40 (1H, br.s) and at δ 5.25 (1H, d, J=7.0 Hz). The ^{13}C NMR spectrum (100 MHz, DMSO-d6) gave 31 carbon signals which indicated the presence of 15 carbon signals due to the flavonol skeleton. By comparison with the ^{13}C NMR spectral data of quercetin, it was revealed that both C-2 (δ 157.48) and C-4 (δ 181.91) were downfield shifted while C-3 (δC 113.72) was upfield shifted, demonstrating C-glycosylation at C-3. The ^{13}C NMR spectral features were very similar to those of quercetin glycosides. In 3′,4′-dihydroxyl flavone system, the δC value for 4′ appeared further downfield than that of 3′. Therefore, the δC 146.98 and 148.58 belonged to C-3′ and C-4′, respectively. The compound showed a molecular ion peak at m/z 595[M+H]$^{+}$ in the ESI–MS, analyzing for C_{27} $H_{30}O_{15}$, together with two anomeric protons, indicating the presence of two sugar residues in the molecule. The methyl protons of the sugar rhamnose appeared at δ 0.98 and rest of the sugar protons appeared in the range δ 3.04–4.00. In ^{13}C NMR, signals corresponding to the anomeric carbon of glucose were found at 100.58 ppm and those corresponding to rhamnose were seen at 101.02 ppm. The attachment of the rhamnose to C-6 of the glycosyl moiety was evidenced by the downfield shift of the glycosyl C-6 carbon resonance to δ 66.73. The chemical shift values of the sugar carbon resonances confirm the presence of pyranose form of the two sugar moieties in the quercetin-3-rhamnopyranosyl-(1-6) glucopyranoside.

Wound healing represents an innate response to tissue injury. Although the healing process takes place by itself and does not require much help, various risk factors such as infection and delay in healing showed in this process [21]. The healing of wounds is a complex process that involves the activation and synchronization of coagulatory and inflammatory events, fibrous tissue accretion, deposition of collagen, epithelialization, wound contraction, tissue granulation and remodeling [22]. In the present investigation, three different models were used to assess the wound healing effect of DSE and QRPG on various phases of wound healing.

Wound contraction indicates the rate of reduction of the unhealed area during the healing process. Thus, a fast rate of wound contraction indicates better efficacy of medication. Wound contraction plays an important role in the closure of full thickness wounds, where the surrounding skin is pulled in by forces that develop within the granulation tissue [23]. In the present study, treatment with DSE and QRPG significantly enhanced the rate of wound contraction and epithelialization, and provided strength to the regenerated tissue as evidenced by the increased levels of collagen. Collagen is the predominant extracellular protein in the granulation tissue and contains a high concentration of the amino acid 4-hydroxyproline [24]. The concentration of hydroxyproline has been used as an estimate of collagen content. The enhanced wound healing activity has been attributed to increased collagen formation [25,26]. Earlier reports have indicated an increase of basic ground substances like hexosamine and uronic acids which are responsible for extra cellular matrix synthesis during the earlier phases of wound healing [27]. Similarly, treatment with DSE and QRPG increased the levels of these substances which were found to be high in the early phases of wound healing. The histopathological evaluation strongly supported the biochemical results which indicated a significant increase in collagen and fibroblastic deposition in DSE and QRPG treated rats as compared to control animals.

In wound healing, type I collagen gene expression is found in every phase of the repair process [28]. Its synthesis coincides with increased wound-breaking strength [29]. Ultimately, in wound healing, the rather cellular but fiber-rich scar tissue contains, predominantly, fibrils derived from type I collagen molecules [30]. Type I collagen can directly promote the adhesion and migration of numerous cell types, including keratinocytes and fibroblasts [31]. Type I collagen thus gradually replaces the other collagen types when the wound matures to a scar. In the present investigation, a significant increase in the expression of Col 1 α (I) was observed in the wound tissue of DSE and QRPG treated rats.

The study revealed the effect of the stem bark ethanol extract and its constituent quercetin-3-rhamnopyranosyl-(1-6) glucopyranoside on collagen synthesis and their modulatory role in Col 1 α (I) gene expression which is a significant factor contributing to the normal wound healing process. DSE and QRPG showed promising and somewhat stronger wound healing promoting activity than the standard povidone-iodine ointment. The study provided a clear insight into the biochemical and molecular mechanisms underlying the wound healing promoting activity of *D. elata* using a rat model and provided pharmacological evidence to the ethnomedicinal claim.

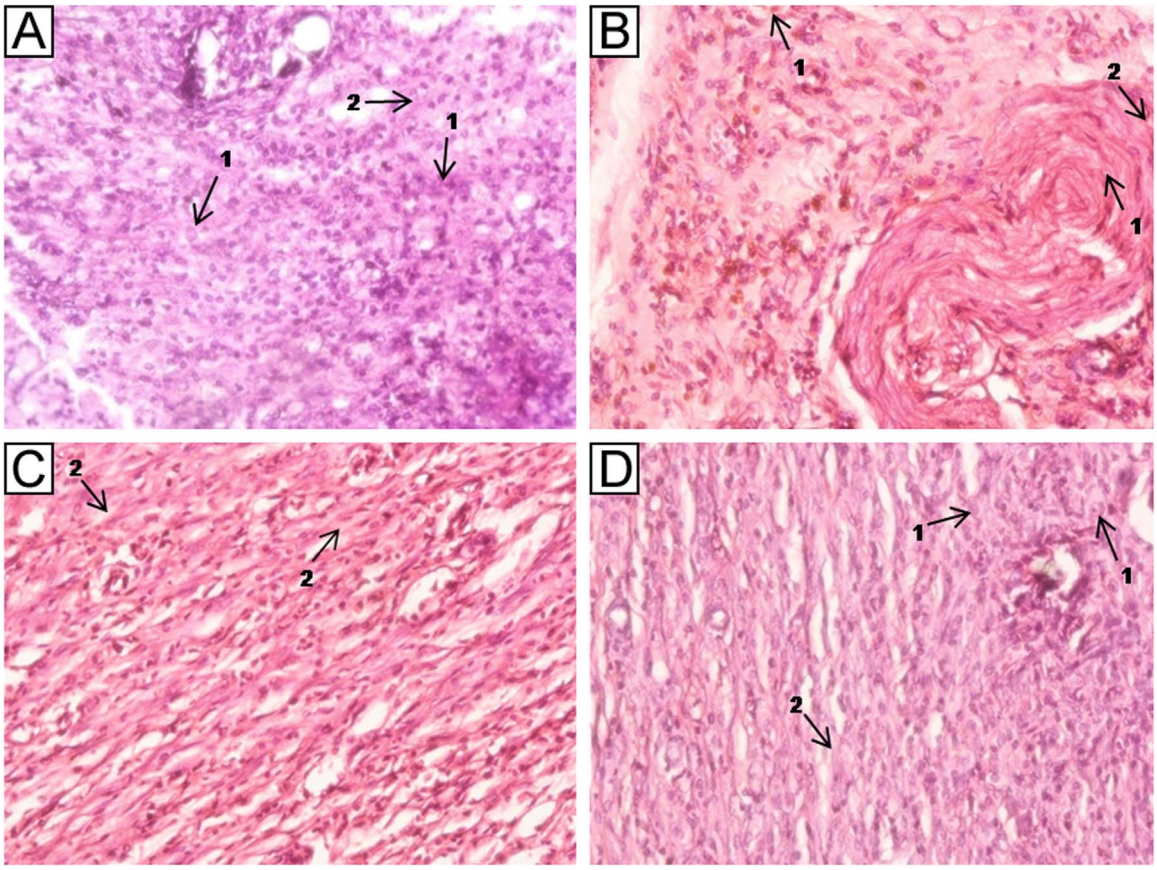

Fig. 5. Histological examination showing control, DSE, QRPG and standard reference treated wound tissues on the 10th post-wound day (1. Macrophages; 2. Collagen fibers). (A) Wound tissue of control animals, (B) wound tissue of DSE treated animals, (C) wound tissue of QRPG treated animals, and (D) wound tissue of povidone-iodine ointment treated animals.

Acknowledgments

The Registrar of Kuvempu University, India, the Principal of the National College of Pharmacy, Shivamogga, India, and the Principal of MSRIT, Bengaluru, India are acknowledged for providing all the necessary facilities. This work is partially supported by the Department of Biotechnology, New Delhi, India (Grant no. BT/PR11505/SPD/24/337/2008; BT/PR9128/INF/22/190/2013).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jpha.2016.05.001.

References

[1] H.M. Kumara Swamy, V. Krishna, K. Shankarmurthy, et al., Wound healing activity of embelin isolated from the ethanol extract of leaves of *Embelia ribes* Burm, J. Ethnopharmacol. 109 (2007) 529–534.
[2] B.G. Harish, V. Krishna, H.S. Santosh Kumar, et al., Wound healing activity and docking of GSK3-β with lupeol isolated from *Celastrus paniculatus* Willd, Phytomedicine 15 (2008) 763–767.
[3] R. Sharath, B.G. Harish, V. Krishna, et al., Wound healing and protease inhibition, activity of bacoside-A, isolated from *Bacopa monnieri* Wettest, Phytother. Res. 24 (2010) 1217–1222.
[4] G.M. Lingaraju, V. Krishna, H. Joy Hoskeri, et al., Wound healing promoting activity of stem bark extract of *Semecarpus anacardium* using rats, Nat. Prod. Res. 26 (2012) 2344–2347.
[5] K. Pradeepa, V. Krishna, Venkatesh, et al., Antinociceptive activity of *Delonix elata* leaf extract, Asian Pac. J. Trop. Biomed. 2 (2012) S229–S231.
[6] K. Pradeepa, V. Krishna, Venkatesh, et al., Antioxidant and prophylactic effects of *Delonix elata* L. stem bark extracts and flavonoid isolate quercetin against carbon tetrachloride induced hepatotoxicity in rats, Biomed. Res. Int. (2014) ID 507851.
[7] K. Pradeepa, V. Krishna, B.G. Harish, et al., Antibacterial activity of leaf extract of *Delonix elata* and molecular docking studies of Luteolin, J. Biochem. Technol. 3 (2012) S193–S197.
[8] B.K. Manjunatha, V. Krishna, T. Pullaiah, Flora of Davanagere District, Karnataka, India, Regency Publication, New Delhi, 2004.
[9] A.A. Adeneye, O.P. Ajagbonna, T.I. Adeleke, et al., Preliminary toxicity and phytochemical studies of the stem bark aqueous extract of *Musanga cecropioides* in rats, J. Ethnopharmacol. 105 (2006) 374–379.
[10] J.J.P. Morton, M.H. Malone, Evaluation of vulnerary activity by an open wound procedure in rats, Arch. Int. Pharmacodyn. Ther. 196 (1972) 117–126.
[11] H.P. Ehrlich, T.K. Hunt, The effect of cortisone and anabolic steroids on the tensile strength of healing wounds, Ann. Surg. 170 (1969) 203–206.
[12] R.A. Turner, Inflammatory Agent in Screening Methods of Pharmacology, 2nd ed., Academic Press, New York, 1965.
[13] S. Ghosh, A. Samatha, N.B. Mandal, et al., Evaluation of the wound healing activity of methanol extract of *Pedilanthus tithymaloides* (L.) Poit leaf and its isolated active constituents in topical formulation, J. Ethnopharmacol. 142 (2012) 714–722.
[14] K.H. Lee, Studies on the mechanism of action of salicylates III. Effect of vitamin A on the wound healing retardation action of aspirin, J. Pharm. Sci. 57 (1968) 1238–1240.
[15] G. Dipasquale, A. Meli, Effect of body weight changes on the formation of cotton pellet induced granuloma, J. Pharm. Pharmacol. 17 (1965) 379.
[16] R.E. Neuman, M.A. Logan, The determination of hydroxyproline, J. Biol. Chem. 184 (1950) 299–306.
[17] L.A. Elson, W.T. Morgan, A colorimetric method for the determination of glucosamine and chondrosamine, Biochem. J. 27 (1933) 1824–1828.
[18] T. Bitter, H.M. Muir, A modified uronic acid carbazole reaction, Anal. Biochem. 4 (1962) 330–334.
[19] T.A. Hall, BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT, Nucleic Acids Symp. Ser. 41 (1999) 95–98.
[20] A.E. Galigher, E.N. Kozloff, Essential of Practical Microtechnique, Lea and Febiger, Philadelphia, 1971.
[21] K. Shanmuga Priya, A. Gnanamani, N. Radhakrishnan, et al., Healing potential of *Datura alba* on burn wounds in albino rats, J. Ethnopharmacol. 83 (2002) 193–199.
[22] P.K. Ghosh, A. Gaba, Phyto-extracts in wound healing, J. Pharm. Pharm. Sci. 16 (2013) 760–820.

[23] M. Ganeshkumar, T. Ponrasu, R. Krithika, et al., Topical application of *Acalypha indica* accelerates rat cutaneous wound healing by up-regulating the expression of Type I and III collagen, J. Ethnopharmacol. 142 (2012) 14–22.
[24] D.J. Etherington, T.J. Sims, Detection and estimation of collagen, J. Sci. Food Agric. 32 (1981) 539–546.
[25] E. Trabucchi, F. Preis-Baruffaldi, C. Baratti, et al., Topical treatment of experimental skin lesions in rats: macroscopic, microscopic and scanning electron-microscopic evaluation of the healing process, Int. J. Tissue React. 8 (1986) 533–544.
[26] A. Shukla, A.M. Rasik, B.N. Dhawan, Asiaticoside-induced elevation of antioxidant levels in healing wounds, Phytother. Res. 13 (1999) 50–54.
[27] M. Nithya, L. Suguna, C. Rose, The effect of nerve growth factor on the early responses during the process of wound healing, Biochim. Biophys. Acta 2003 (1620) 25–31.
[28] J.K. Makela, E. Vuorio, Type I collagen messenger RNA levels in experimental granulation tissue and silicosis in rats, Med. Biol. 64 (1986) 15–22.
[29] J. Viljanto, Biochemical basis of tensile strength in wound healing. An experimental study with viscose cellulose sponges on rats, Acta Chir. Scand. 333 (1964) 1.
[30] E.J. Miller, S. Gay, Collagen Structure and Function, Wound Healing: Bio-chemical and Clinical Aspects, WB Saunder Company, Philadelphia, USA, 1992.
[31] K. Scharffetter-Kochanek, C.E. Klein, G. Heinen, et al., Migration of a humankeratinocyte cell line (HACAT) to interstitial collagen type I is mediated by the alpha 2beta 1-integrin receptor, J. Invest. Dermatol. 98 (1992) 3–11.