Indian Journal of Experimental Biology
Vol. 58, April 2021, pp. 236-245

Wound healing potency of *Hemigraphis alternata* (Burm.f) T. Anderson leaf extract (HALE) with molecular evidence

Divyaa Sreekumar[1], Salini Bhasker[1]*, Ponnuswamy Renuka Devi[2] & Mohankumar C[1]

[1]Molecular Biology Division, SCMS Institute of Bioscience and Biotechnology Research and Development, South Kalamassery, Cochin-682 033, Kerala, India
[2]Department of Biotechnology, Anna University, Regional Campus, Coimbatore-641 046, Tamil Nadu, India

Received 04 February 2019; *revised 15 September 2020*

Hemigraphis alternata (Burm.f) T. Anderson, commonly called as Red Ivy or Purple waffle plant and locally, *Murikootti* (Malayalam), is well known traditionally for its therapeutic effect in wound healing. Besides the analytical and biochemical evidence, more investigations at molecular level is inevitable for highlighting the healing effect of the herbal drug. In the present study, we have demonstrated the wound healing effect of *H. alternata* by *in vitro* cell line and animal model experiments using the *Hemigraphis alternata* leaf extract (HALE). The cell survival ability, cell proliferation and migration of cells in scratch wounds leading to wound closure were evident from the data by HALE treatment. Based on the wound closure observed in scratch wounds, the marker genes of wound healing PAI-1 and TGF-β1were analyzed by qPCR. The concentration and the expression fold were determined in 3T3-L1 and L6 cell lines treated with HALE. The concentration of marker gene of wound healing PAI-1 and TGF-β1showed significant increase in cell line cultures of 3T3-L1 and L6 cells treated with different concentrations of HALE (P <0.05). Similarly, the expression fold of both the genes was found higher in cells treated with HALE than the control. The molecular data showing the concentration and the expression of marker genes during wound healing in scratch wounds provide strong support for using HALE as a herbal medicine with the anti-inflammatory property and the antioxidant activity.

Keywords: Anti-inflammatory, Antioxidant activity, *Murikootti*, Plasminogen Activator Inhibitor 1, Purple waffle plant, Red Ivy, Traditional medicine, Transforming growth factor beta 1

Bioactive compounds are secondary metabolites synthesized as products of biochemical side tracks in the plant cells with several pharmacological and toxicological effects in man and animals[1,2]. The search for natural remedies for wound management and healing has led to screening of plants including *Acrtium lappa*[3,4], *Aloe vera*[5,6], *Astragalus propinquus*[7,8], *Bauhinia variegata* L.[9], *Calendula officinalis*[10], *Celtis timorensis* Span[11], *Juglans regia* L.[12], *Verbascum inulifolium*[13], etc. as potential source of phytomedicine. Therapeutic potential of plant secondary metabolites remains as the main source of herbal medicine[14]. *Hemigraphis alternata* (Burm.f) T. Anderson (Fam. Acanthaceae), commonly called the Red Ivy or Purple waffle plant, and locally known as *Murikootti* (Malayalam), is a tropical herb used as a folklore medicine for wound healing traditionally[15]. Its aqueous leaf extract shows significant healing effect in fresh wounds, particularly in wound closure[16]. Despite the traditional knowledge of the medicinal effect of *H. alternata*, in depth scientific analysis at biochemical and molecular level focusing towards the mechanism of herbal action in wounds is still lacking[17]. The application of the aqueous leaf extract of *H. alternata* in fresh wounds exhibits a wonderful healing effect in wound closure[18]. In folk medicine, it is used to heal ulcers, to promote urination, to cure anemia, gall stones and diabetes and as a contraceptive[19].

Previous reports substantiate the wound healing property of *H. alternata* with analytical and phytochemical evidences[16]. With the presence of active components such as phenols, flavonoids, terpenoids, saponins, coumarins, carboxylic acid, cinnamic acid and tannins in *H. alternata*, which vouches for the wound healing property, the clinical acceptance of *H. alternata* leaf extract (HALE) in wound closure requires molecular evidence[20]. Healing of wounds is a well orchestrated process that involves several cellular and molecular events[21,22]. From the

*Correspondence:
Phone: +91 484 2556315; (Mob.): +91 9847865609
E-Mail: dr.salinibhasker@gmail.com (SB);
divyaa.sreekumar5@gmail.com (DS)

earlier level of hemostasis and inflammation of the wound to the later stages of healing like angiogenesis, cell proliferation, regeneration and functional restoration, different growth factors and cytokines of the body systems are actively involved[23,24]. Hence, investigation on the involvement of these innate growth factors of the system during wound healing at molecular level may provide a new scientific outlook and thereby vindicate application of traditional herbal medicine. In the present study, we have made an attempt to determine the effect of Transforming Growth Factor beta 1 (TGF-β1) and Plasminogen Activator Inhibitor 1, (PAI-1) genes during healing process in scratch wound of cell line and in animal models under the treatment of HALE. An attempt was also made to understand the biochemical factors that trigger the healing process by HALE.

Materials and Methods

Collection of plant material

Since *Hemigraphis alternta* is an ornamental plant of mesophytic habitat, we maintained a garden at our premises for the experimental purpose. The taxonomic status of the plant was authenticated by Botanical Survey of India (BSI) Coimbatore as *Hemigraphis alternata* (Burm.f.) T. Anderson, and a voucher specimen of the plant BSI/SRC/5/23/2016/ TECH/1554 were deposited. Fresh healthy and mature leaves of *H. alternata* were taken from the basal nodes of the plant was used for the study.

Preparation of *H. alternata* leaf extracts (HALE) powder

One kg of fresh tender leaf of *H. alternata* 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 filtered through a two layered cheese cloth and was centrifuged (Remi, India) at 10000 rpm for 10 min as the standardized conditions for efficient separation of residual particles. The supernatant obtained after the centrifugation 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 HALE (10 µg and 100 µg) were used for the study.

Biochemical evaluation of HALE

Anti-inflammatory activity using HALE

Human Red Blood Cells (*HRBC*) *membrane stabilization assay*

RBC was separated from human blood using the standard procedure and suspended in 10% normal saline solution[25]. The reaction mixture (3 mL) consists of 1.5 mL aqueous solution containing 100 µg HALE, 1.5 mL of 10% RBCs suspension. Saline was added to the control tubes as blank. Aspirin was used as a standard drug (250 µg/µL) instead of test sample as positive control[26,27]. The reaction mixture was incubated in water bath at 56°C for 30 min. At the end of the incubation, the tubes were cooled under running tap water. The cooled mixture was centrifuged at 3200 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples, and percentage inhibition of hemolysis was calculated using OD values.

Inhibition of albumin denaturation

The anti-inflammatory activity of the HALE was studied using inhibition of albumin denaturation technique[28]. The reaction mixture consists of 50 µL HALE extract (100 µg) µL) and 5% aqueous solution of bovine albumin fraction (450 µL). The pH of the reaction mixture was adjusted to 6.3 using 1N HCl. The mixture was incubated at 37°C for 20 min and heated at 57°C for 30 min. After cooling, 2.5 mL phosphate buffer saline (pH 6.3) was added to the sample, the absorbance was measured spectro-photometrically (ELICO, India) at 660 nm. The experiment was performed in triplicates with the drug aspirin (250 µg/µL) as control[29]. The Percentage inhibition of protein denaturation was calculated as

$$\text{Percentage inhibition} = A_{\text{control}} - A_{\text{sample}} / A_{\text{control}} \times 100$$

Proteinase inhibition

About 3 mL reaction mixture was prepared with a composition of 2 mL 25 mM Tris-HCl buffer (pH 7.4) with the enzyme trypsin (0.06 mg) and 1.0 mL HALE sample. The mixture was kept at 37°C for 5 min and l.0 mL of 0.8% (w/v) casein was added as substrate. The total mixture was incubated for 20 min at 37°C and 2 mL of 70% perchloric acid was added to arrest the reaction. After incubation, the suspension was centrifuged and the absorbance of the supernatant was read spectrophotometrically at 210 nm against buffer as blank[30]. For the positive control, 1.0 mL aspirin (250 µg/µL) was taken instead of HALE extract. The percentage inhibition of proteinase activity was calculated from the absorbance value.

Antioxidant test

DPPH free radical scavenging assay

The free radical scavenging activity was tested as per the protocol[31]. The reaction mixture contained 50 µL of HALE (100 µg) extract, 1.5 mL DPPH reagent

(1.0 mM) and 1.5 mL of 80% methanol. The mixture was incubated in dark for 30 min and the absorbance was read at 517 nm. The scavenging potency was measured from the OD value[31].

Nitric oxide free radical scavenging assay

Aqueous HALE extract (mg/mL) was prepared and the final volume was made up to 10 mL. One mL above extract containing 100 µg was taken along with 1.0 mL (100 mM) sodium nitro prusside (SNP). The mixture was incubated for 2.5 h at room temperature (25°C), added with 1.0 mL of Griess reagent and the OD was measured at 540 nm[32] and calculated as

Percentage of Scavenging = $A_{control}$- A_{sample}/$A_{control}$ X100

***In vitro* cell line cultures**

Establishment of cell line cultures 3T3-L1 and L6 cells

Mouse 3T3-L1 fibroblasts cells and 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 (Himedia, Mumbai) supplemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin, 250 ng/mL amphotericin B and 10% heat-inactivated FBS (PAN Biotech). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. The cells were seeded at a density of approximately 2×10^3 cells/mL for experiments.

Scratch wound assay

The 3T3-L1 fibroblast cells and Rat L6 pre-myoblast cells were seeded onto 6-well culture plates for scratch wound assay. 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[33,34]. The wounded cells were incubated with 1.9 mL of serum free medium containing DMEM with 1% antibiotic and antimycotic solution. The wounded monolayers were treated with two different concentrations of HALE (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 inverted phase contrast microscope (Lynx, Lawrence& Mayo, India) equipped with camera and measured using software View 7 version 7.1.1.6.

Cell viability assay

The effect of the HALE on cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay following the method[35]. 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 470 nm.

qPCR assay of molecular marker genes

Absolute quantification of PAI-1 and TGF-β 1 gene in cell lines

Gene specific oligonucleotide primers for PAI-1 gene of rat (NM_012620) and TGF-β1 gene of mouse (NM_011577.2) were designed using Vector NTI (Invitrogen) and synthesized at Sigma-Aldrich, Bangalore. The wounded cell lines both 3T3-L1 mouse fibroblast monolayers and L6 rat pre-myoblast monolayers were treated with HALE extracts for 24 h, along with the untreated cells as the control. Total RNA was isolated using TRI-reagent (Sigma, Bangalore)[36]. About 4 µg of total RNA was transcribed to cDNA using 100 ng Poly T primers (Fermentas, Canada). cDNA amplification was carried out in a total volume of 20 µL in the presence of 2 pM each of the forward primer (5'-TCAGCCCTCA CTTGCC TCAC-3') and reverse primer (5'-ATAGCC AGCACCGAGGACAC-3') of rat origin Plasminogen Activator Inhibitor 1 (PAI-1) and 2pM each of the forward primer (5'-CTGCTGGCA GTATCCAG GGCTCTCCG-3') and reverse primer (5'-GGTGGG GTCTCCCAAGGAAAGGTA-3') of mouse origin for Transforming growth factor Beta 1 (TGF-β1) using RNA isolated form cell cultures. The PCR assay for amplification of PAI-1 and TGF-β1 gene were standardized at following cycling condition 94°C for 4 min followed by 34 cycles of 94°C for 1.0 min, 60°C for 30 s and 72°C for 45 s, with a final extension of 72°C for 10 min and held at 4°C. The real time PCR amplification of PAI-1 and TGF-β1 and gene was run separately on a Realplex instrument with Realplex 1.5 software (Eppendorf, Germany) and the threshold cycle (CT) was determined using the software. All the PCR runs were performed in triplicate and each reaction mixture was prepared using 2X SYBER mix (ABI) in a total volume 20 µL containing 50 ng of template cDNA, 2 pM sense and antisense primer and 1X SYBER Green. The reaction was set for 40 cycles with the conditions: initial denaturation 95°C for 10 min, denaturation 95°C for 15 s, annealing 62°C for 15 s and elongation 72°C for 45 s. After completion of cycling process, samples were subjected for melting analysis by following

conditions as 95°C for 15 s, 60°C for 15 s and 95°C for 15 s. Tenfold serial dilution series of the gene PAI-1 and TGF-β1 ranging from 50 ng to 0.05 ng reactions was carried out to construct the standard curve[31]. The CT 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.

Relative quantification of PAI-1and TGF- β1gene in cell lines

The relative quantification of PAI-1 and TGF-β1 gene and their expression in HALE treated samples were determined using 2-ΔΔCT method[36,37]. 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 genes. The real time PCR amplification was run on a Realplex instrument with Realplex 1.5 software (Eppendorf, Germany) and the threshold cycle (CT) was determined using the software. The wounded 3T3-L1 mouse fibroblast monolayers and L6 rat myoblast monolayers were treated with HALE extracts for 24 h, along with the untreated cells as the control. Total RNA was isolated using TRI-reagent (Sigma, Bangalore). About 4 µg of total RNA was transcribed to cDNA using 100 ng Poly T primers (Fermentas, Canada). cDNA amplification was carried out in a total volume of 20 µL in the presence of 2 pM sense and antisense primers. The PCR assay was standardized at following cycling condition 94°C for 4 min followed by 34 cycles of 94°C for 1.0 min, 60°C for 30 s and 72°C for 45 s and a final extension of 72°C for 10 min and held at 4°C for amplifying the partial PAI-1 gene and TGF-β1 gene. The reaction mix for the amplification of the house keeping gene actin included 50 ng cDNA, 2 pM primer mix each of the forward primer (5'GTCCCTCACCCTC CCAA AAG-3') and reverse primer (5'-GCTGCCTCAACA CCTCAA CCC-3') of Actin gene were used. 8 µL PCR grade water and 1X SYBER Green (ABI) in a total volume 20 µL. The reaction was set for 40 cycles with the conditions: initial denaturation 95°C 10 min, denaturation 95°C for 15 s, annealing 62°C for 15 s and elongation 72ºC for 45 s. After completion of cycling process, samples were subjected for melting analysis by following conditions as 95°C 15 s, 60°C for 15 s and 95°C for 15 s. The PCR reaction was carried out in triplicate for each gene. At the end of PCR, the melting curve application was run to determine the presences of any primer –dimer artifacts or co-amplified nonspecific product. In order to omit the sampling differences such as RNA quality, normalization was carried out with housekeeping actin gene.

Estimation of amplification efficiency

The standard curve was constructed based on the procedure[38]. Tenfold serial dilution series of cDNA with PAI-1 and TGF-β1 gene specific primers ranging from 50 to 3.125 ng reaction was used to construct the standard curve for TGF-β1 gene and PAI-1 gene. For the generation of standard curve for the housekeeping gene, actin five different concentrations of the first strand cDNA were used. PCR efficiency (E) was calculated according to the equation E= 10[(-(-1)/slope)] -1. Relative quantitative PCR reactions were performed whereby amplification of PAI-1 gene and TGF-β1 gene was normalized with housekeeping actin gene. The relative expression was level of TGF-β1 gene and PAI-1 gene in HALE treated was measured by taking untreated control samples as the calibrator.

Animal model studies

Efficacy of HALE in rat excision wound model

The wound model studies were done at Central Animal Facility, Karnataka College of Pharmacy, Bangalore. Female Wistar rats (200-250 g) used for the study was procured from NIMHANS Bangalore, Karnataka. Ethical clearance for performing the experiments on animals was obtained from the Institutional Animal Ethics Committee (IAEC). The animals were acclimatized for ten days under laboratory conditions and maintained at 27±2°C with a relative humidity of 65±7.5% under 12 h light/dark cycle. The animals were fed with rodent pellet diet (Gold Mohur Lipton India Ltd.) and water *ad libitum*. The animals were grouped in to three categories: Gr. I, the untreated control; Gr. II, standard drug soframycin treated; and Gr. III, HALE treated. The animals were anaesthetized prior to and during creation of wounds. Excision wounds were inflicted by cutting away 500 mm^2 full thicknesses along the marking on the depilated back 5 cm away from the ears. Animals in Gr. I were not given any treatment. Gr. II animal wounds were treated with 2% w/v soframycin (Himedia, India) and for Gr. III, the wounds were treated with HLAE (100 µg/1 mL) dose for 10 days. The wounded area was left undressed and

exposed to its environment. The degree of wound closure was studied and measured on 4th day and 10th day. Histological analysis was done to analyze the newly formed epidermis layer, collagen content, period of re-epithelization and inflammatory changes that occurred during the early post wound period, confirmed the depth of wounds and examined the pathological changes and the rate of recovery.

Statistical analysis

All the experiments were done in triplicate reactions. The whole data was statistically evaluated for determining the mean, standard deviation and the standard error. The results of the excision wound model are expressed as mean ± S.D from n=6 rats in each group. The significance of difference among the groups was assessed using one-way analysis of variance (ANOVA) followed by Tukey's test compared between normal control (Untreated) *vs*. all groups $P < 0.05$ were considered significant.

Results

Anti-inflammatory effect of HALE

The anti-inflammatory property of HALE was analyzed by three means— HRBC membrane stabilization, albumin denaturation, and inhibition of protein denaturation. Under the treatment of HALE with human RBC (HRBC) showed the suppression of hemolysis i.e. the rupture of RBC cells or erythrocytes. Table 1 shows the percentage of inhibition of hemolysis of RBC by the effect of HALE compared with the control drug aspirin. The inhibitory effect of HALE in rupturing the erythrocytes is evident from the data indicating its effect in keeping the stability of RBC membrane. Since the activation of wound inflammation has a coordination with the denaturation of protein especially albumin, the inhibitory effect of albumin denaturation and proteinase was estimated colorimetrically using HALE. The data showed high percentage of inhibiting albumin denaturation in the presence of HALE similar to the effect of the standard drug (Table 1). The inhibition of proteinase, the key enzyme of protein denaturation was also measured by using trypsin as enzyme in presence of HALE (Table 1).

Table 1 — Anti-inflammatory and antioxidant activity using HALE

	Cont. (aspirin 100 µg) (%)	HALE 100 (µg) (%)
Inhibition of hemolysis	60.945±0.947	34.975±0.708
Inhibition of albumin denaturation	96.92± 0.536	91±3.33
Inhibition of proteinase activity	33.4±0.432	21.5±0.543
Scavenging of DPPH		65.90±6.432
Scavenging of NOX		39±0.449

Antioxidant activity

The antioxidant activity of HALE was evaluated by DPPH and NOX free radical scavenging assay. Both the assay value exhibits the scavenging potency at the level of 35% and 65% for NOX and DPPH respectively. Table 1 displays the free radical scavenging potential of HALE using DPPH and NOX. The nitric oxide generated from SNP reacts with oxygen to form nitrite. As an anti-oxidant, HALE donate protons to the nitrite radical and a decrease in OD value was observed. The decreased absorbance was taken to measure the nitrite radical scavenging potency.

Cell viability

The effect of HALE in inducing viability and proliferation of cells was estimated by MTT assay using cell line cultures of 3T3-L1 and L6 cells. Under the treatment of HALE, both the cells showed a higher percentage of cell survival ability 97% in 3T3L1 and 95% in L6, respectively) indicating the level of active proliferation of cells.

***In vitro* scratch wound closure by HALE**

The effect of HALE was examined in scratch wounds created in cell culture monolayers of 3T3-L1 fibroblast and L6 pre-myoblast cells. The detached cells were removed from the scratch area and the wounds were made intact prior to the treatment of HALE. The wounded monolayers were treated with lower (10 µg) and higher concentration of HALE (100 µg) and incubated. The histomorphological changes of the wound were monitored for two days and documented under phase contrast microscope. An active tendency of cell proliferation and migration of cells was observed in the scratch wounds treated with HALE leading to the closure of wounds. An increasing phase of cell migration and wound closure was seen in scratch wounds treated with higher concentration of HALE (Fig. 1). The untreated wounds i.e. without HALE, the cell migration occurred in a very slow pace (Fig. 2).

qPCR data of wound healing marker genes

Total RNA was isolated for scratch wound cells treated with HALE and from untreated wound cells of 3T3-L1 and L6 cell lines. Fig. 3A demonstrates the RNA isolated from the cells both test and control. The presence of two discrete bands as 28S rRNA and 18S rRNA indicates the purity of RNA. Aliquot of RNA from both the cell lines were subjected to cDNA synthesis using Poly T primers and cDNA was used

for the amplification of PAI-1 and TGF-β1 gene fragments, using the designed primers and quantified by qPCR. In the case of PAI-1 gene, a fragment of 191 bp was amplified for qPCR from the scratch wound model of cells (Fig. 3B). Similarly, a fragment size of 180 bp was amplified for TGF-β1 gene from both the cell lines (Fig. 3C).

Gene concentration of PAI-1 and TGF-β1 in 3T3-L1 and L6 cells

The concentration of marker genes PAI-1 and 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 was highly linear R2 ^ 0 in the range tested by triplicate reaction. The slope of the curve was −3.237 and the amplification efficiency was determined form the slope as 1.04 for the PAI-1 gene, whereas the slope of the curve and the amplification efficiency was −3.264 and 1.02 for TGF-β1 (Fig. 4 A & B). With respect to the standard curve of the marker gene, concentration of PAI-1 and TGF-β1 was calculated. Fig. 5 A & B demonstrates the concentration of PAI-1 and TGF-β1 genes in cell line cultures treated with HALE at 10 µg and 100 µg. It is obvious from the assay data that the concentration of PAI-1 and TGF-β1 genes was more in HALE treated cells than the control untreated cells. Both the genes showed 2 to 3 fold increase by the effect of HALE indicating the functional role of PAI-1 and TGF-β1 genes in wound closure. Moreover, the concentration was found higher in treated cells with 100 µg HALE than 10 µg.

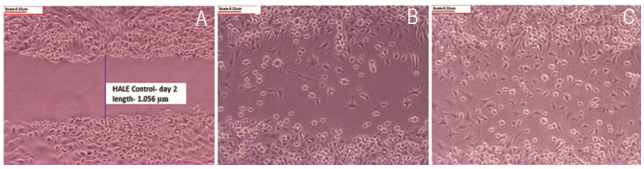

Fig. 1 — Wound healing of L6 myoblast cells after treatment with aqueous HALE extracts of *Hemigraphis alternata.* (A) 0 h; (B) 24 h of 10 µg; and (C) 24 h of 100 µg concentration.

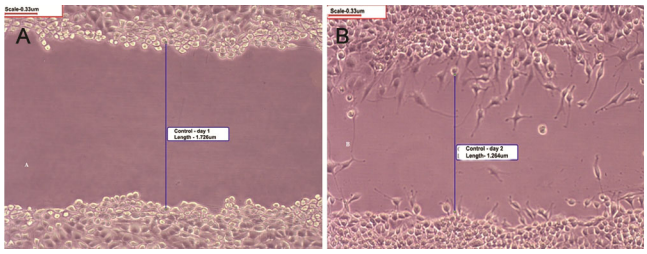

Fig. 2 — Control L6 cells after scratch wound. (A) 0 h; and (B) 24 h.

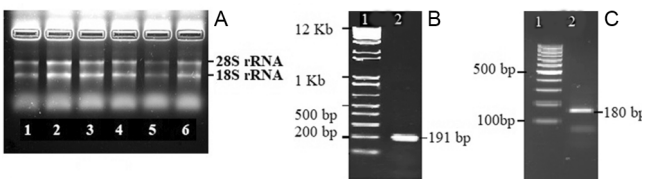

Fig. 3 — (A) RNA isolated from 3T3-L1 and L6 cell lines. Lane 1 and 2 control, lane 3and 4 HALE treated RNA from 3T3-L1, lane 4 and 6 HALE treated RNA from L6 cell lines; (B) PCR amplification of wound healing marker genes. a. Plasminogen Activator Inhibitor 1 (PAI-1) gene (191 bp) and from L6 myoblast. Lanes - 1: 12 Kb Molecular ladder, Lane 2: PAI-1 gene (191 bp); and (C) Transforming Growth factor β 1 gene (TGF-β1) gene (180 bp) from 3T3-L1 cells. Lanes - 1: 100 bp Molecular ladder, Lane 2: TGF-β1 gene (180 bp).

Expression fold of PAI-1 and TGF-β1 genes

Based on the variations observed in the gene concentration of PAI-1 and TGF-β1 related to the treatment of HALE in scratch wounds, the expression fold of both the genes was measured in cell line cultures treated with HALE. Relative quantification of the gene was done by qPCR using mouse Actin as the house keeping gene. A gene fragment of 180 bp was amplified (Fig. 6A). The expression fold of PAI-1 and TGF-β1 was calculated by normalizing with the house keeping gene actin using Realplex software. Fig. 6 B & C demonstrates the expression fold of PAI 1 and TGF-β1 in HALE treated wound cells. A clear demarcation was seen in the expression level of PAI-1 and TGF-β1 in HALE treated cells under lower (10 µg) and higher concentration (100 µg). The increased expression level of PAI-1 and TGF-β1 at higher concentration of HALE clearly matches with concentration of both the genes.

Effect of HALE in rat wound models

In the light of the biochemical and molecular data, on the effect of HALE in *in vitro* wound models, the study was extended to animal model using female

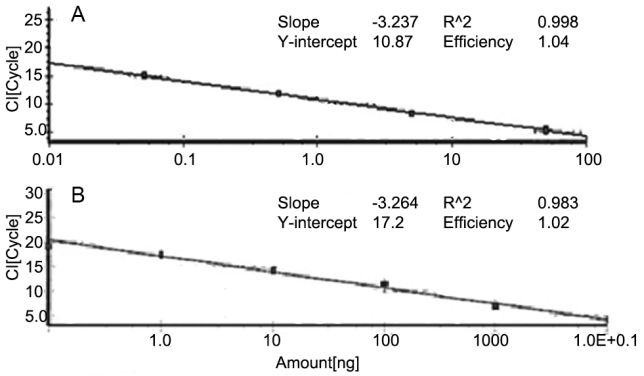

Fig. 4 — (A) Standard curve of PAI-1 gene using PCR eluted PAI-1 products; and (B) Standard of TGF- β1 gene (180 bp).

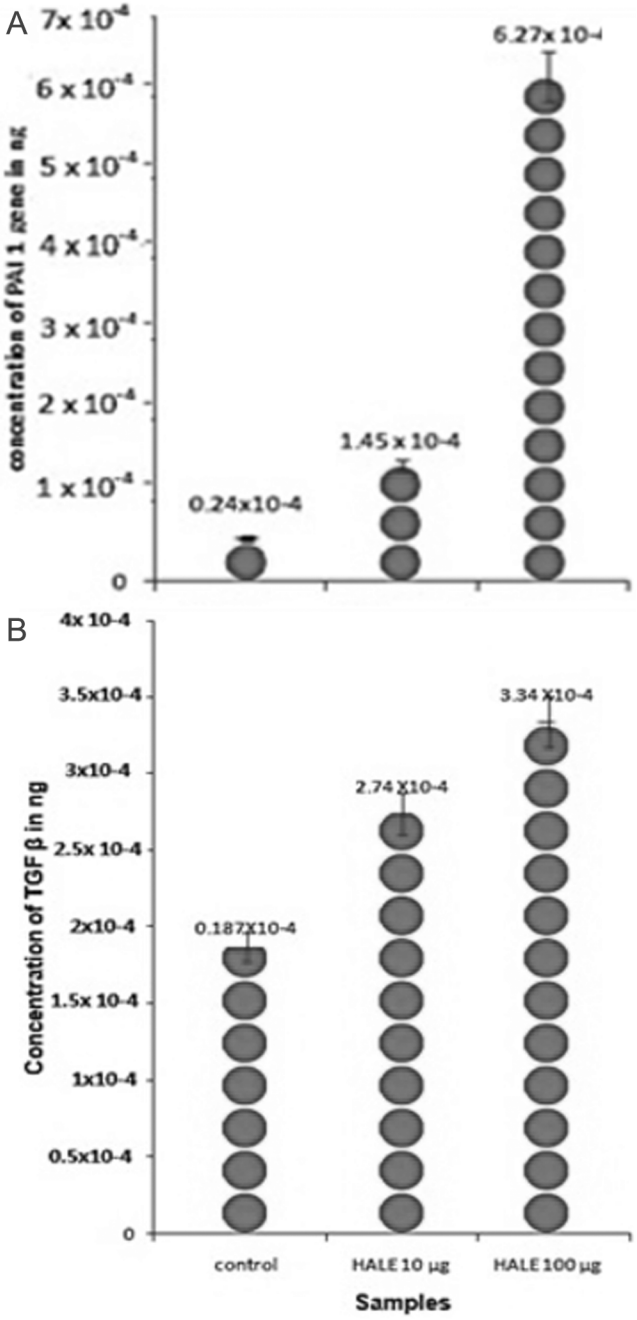

Fig. 5 — (A) Concentration of PAI-1 gene transcripts in cell line cultures treated with HALE at two concentrations of 10 µg/µL and 100 µg/µL; and (B) Concentration of TGF-β1 gene transcripts in cell line cultures treated with HALE in two concentrations of 10 µg and 100 µg.

Wister rats. The study was done in three groups of animals- normal HALE treated group and soframycin treated. Fig. 7 displays the effect of wound closure in excision wounds treated with HALE. A moderate level of wound closure was observed in HALE treated tissue compared to the standard drug soframycin

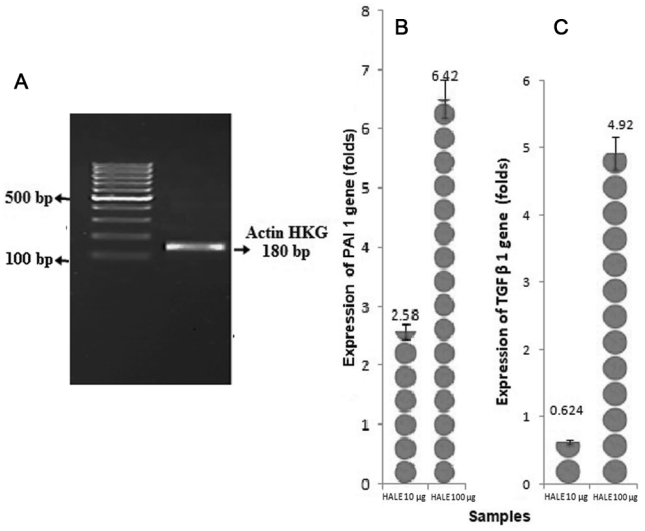

Fig. 6 — (A) PCR amplification of housekeeping gene Actin (180 bp) from cell lines. Lanes - 1: 100 bp Molecular ladder, Lane 2: Actin gene (180 bp); (B) Expression fold of PAI-1 gene transcript in cell line cultures treated HALE in concentrations of 10 µg/ µL and 100 µg/µL; and (C) Expression fold of TGF-β1 gene transcript in cell line cultures treated with HALE in concentration of 10 µg/ µL and 100 µg/µL.

(Fig. 7 A & B). Soframycin treated wounds exhibited maximum healing effect (Fig. 7 C & D). The active phase of wound closure for HALE treated showed 50% wound closure and the standard drug soframycin showed 70% closure of wounds. Histopathological analysis of the sacrificed animals provides strong indication of active wound contraction and epithelization of HALE treated wounds similar to the drug soframycin treated group. In wounds treated with HALE, epithelium formation was complete with more number of blood vessels and connective tissue than the untreated control (Fig. 8 A-C). A similar trend was seen in soframycin treated wound indicating the therapeutic effect of HALE as a wound healing drug. In control group, the wound contraction and epithelization was found in a slow pace.

Discussion

The pharmaceutical acceptance of the therapeutic property of folk medicines using modern analytical methods has become a research trend today for the development of herbal drugs. The data of the present study demonstrates the wound healing effect of the medicinal herb *Hemigraphis alternata* at biochemical and molecular level. Naturally in wounds, the inflammation was initiated by hemolysis of RBC membrane, thereby releasing the content in blood plasma. It was noticed that *H. alternata* leaf extract

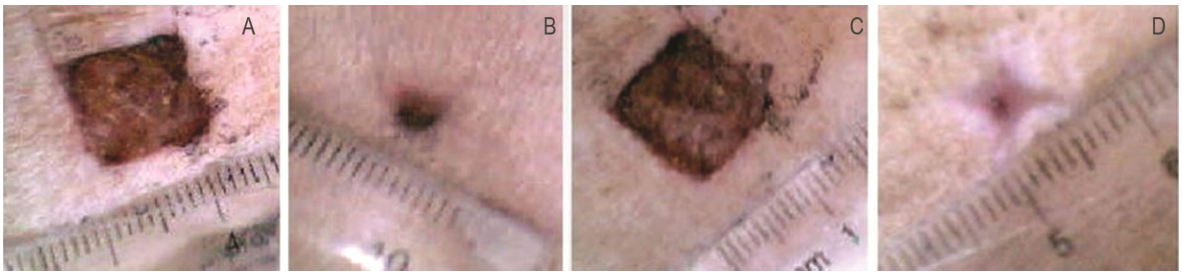

Fig. **7** — Rat wound closure on 4th and 10th day (A & B) in standard drug soframycin treated control group; and (C & D) in HALE extract treated group.

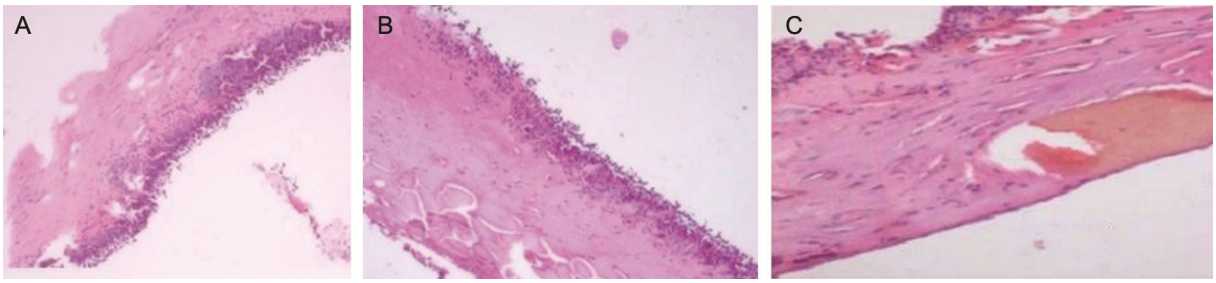

Fig. 8 — Histopathological studies on wound contraction and epithelization in excision rat wound model. (A) HALE extract treated (100 µg/1 mL) dose/10 days; (B) soframycin treated; and (C) Untreated control

(HALE) suppressed hemolysis by rupturing the RBC membrane supporting the anti-inflammatory property of HALE. Moreover, the ability of HALE to inhibit denaturation of albumin further supports its anti-inflammatory activity. The positive effect of HALE in proteinase inhibition reveals its capacity in preventing the denaturation of the protein which is essential for suppressing the infection. Thus, the stable nature of RBC membrane, inhibition of albumin denaturation and the inhibition of proteinase activity in the presence of HALE confirms the anti-inflammatory activity. Anti- oxidants normally help to control wound oxidative stress and there by accelerates wound healing[39,40]. Since molecular oxygen has a pertinent role in the therapy of wounds, the production of free radicals and other ROS by oxidation results in creating cytological effect for delayed wound healing[41]. Here, the presence of *H. alternata* leaf extract has induced the scavenging of free radicals and other ROS like H_2O_2 from the wound environment. Plants have several compounds with strong anti-oxidant activity that capture and neutralize free radicals[42]. In the case of molecular data, the active phase of proliferation and migration of cells during MTT assay using HALE showed the nontoxic nature of HALE. The triggering effect of HALE in healing the scratch wounds by cell multiplication and migration was evident from the *in vitro* cell line culture. Since the wound closure observed in scratch wound models indicates the healing effect by the treatment of HALE, the study was extended for determining the expression and concentration of marker genes of wound healing PAI-1 and TGF-β1 in cell line cultures treated with HALE. Subsequent to the cell migration and proliferation observed in the scratch wounds treated with HALE leading to wound closure, the concentration PAI-1 and TGF-β1 gene involved in wound healing was determined in the cells of the scratch wounds treated with HALE by absolute quantification assay using qPCR. This is for detecting the healing effect of HALE involved in wound closure at molecular level. A correlation was observed between the concentration and the expression level of both genes in HALE treated cells towards wound healing. As the marker gene of wound healing, it is well established that the TGF-β1 has many cellular responses like re-epithelization a prominent phase of wound healing process[43,44]. TGF-β1 is also seen to have efficient activity in process of angiogenesis, proliferation and production of extra cellular matrix during healing[45,46]. Similarly, the potential role of PAI-1, the serine protease inhibitor in suppressing the conversion of plasminogen activator (PA) to active plasmin which in turn activates fibrolysis and extracellular remodeling during wound healing[47,48]. PAI 1 is also

involved in cutaneous wound healing[49,50]. The qPCR data of absolute and relative quantification of PAI-1 and TGF-β1 showed the concentration and the expression fold of both the genes and it clearly indicates the molecular evidence of wound healing effect of HALE. Along with the *in vitro* cell line data, animal model experiments using HALE in excision wound model in rats have provided enough evidence of epithelialization, formation of blood vessels and connective tissues. Thus the biochemical and molecular results obviously confirm the healing efficacy of the medicinal herb *Hemigraphis alternata* and the data highlights the therapeutic merits of the plant beyond the level of a folk medicine.

Conclusion

The usage of folk medicines for curing human diseases has increased their market value in herbal industry. As a traditional medicine, the leaf extract of *Hemigraphis alternata* has been used for wound healing for decades. But the absence of proper scientific evidence for revealing the drug action of herbal medicine diminishes its wider acceptance in clinical therapy. Results of the present study at the molecular level substantiate the wound healing effect of *H. alternata*. The cell line data substantiates the drug action if *H. alternata* at molecular level in scratch wounds. The expression of the marker gene PAI1 and TGF-β1 quantified by qPCR highlights healing effect of *H. alternata*. Being a fast growing herb, it would be possible for exploiting the wound healing property of *H. alternate* for developing a herbal ointment in modern medicine. Hence further studies are warranted for developing a cost effective healing drug from this herb.

Acknowledgement

The corresponding author is extremely grateful to DST, Government of India, New Delhi, for the financial support provided under Women Scientist Scheme (WOS-A).

Conflict of interest

The authors declare no conflicts of interest.

References

1 Yuan H, Ma Q, Ye L & Piao G, The traditional medicine and modern medicine from natural products. *Molecules*, 21 (2016) 559.

2 Wani ZA & Pant S, Ethnomedicinal study of plants used to cure skin diseases and healing of wounds in Gulmarg Wildlife Sanctuary (GWLS), Jammu & Kashmir. *Indian J Tradit Know*, 19 (2020) 327.

3 Schell A, Copp J, Bogie KM & Wetzel R, Honey based salve and burdock leaf dressings as an alternative to surgical debridement of a traumatic wound eschar. *Adv Wound Care*, 8 (2019) 101.

4 Kolacz NM, Jaroch MT, Bear ML & Hess RF, The effect of burns & wounds (b&w)/ burdock leaf therapy on burn-injured Amish patients. *J Holist Nusr*, 32 (2014) 327.

5 Bagyaraj S, Karthick AS, Gomathi S, Sandini S, Sowmiya R, Devi B & Vaithiyanathan D, Preparation and Characterization of Silver Nanoparticle/*Aloe vera* Incorporated PCL/PEO matrix for wound dressing application. *Indian J Biochem Biphys*, 58 (2021) 35.

6 Govindarajan S & Noor A, Carbohydrate fraction of Aloe *vera* ameliorates inflammation through suppression of pro-inflammatory mediators and oxidative stress *in vitro* and rats with Freund's adjuvant induced paw edema. *Indian J Exp Biol*, 59 (2021) 177.

7 Tam JC, Ko CH, Lau KM, To MH, Kwok HF, Chan YW, Siu WS, Etienne-Selloum N, Lau CP, Chan WY, Leung PC, Fung KP, Schini-Kerth VB & Lau CBS, A Chinese 2-herb formula (NF3) promotes hind limb ischemia-induced neovascularization and wound healing of diabetic rats**.** *J Diabetes Complicat*, 28 (2014) 436.

8 Tam JC, Lau KM, Liu CL, To MH, Kwok HF, Lai KK, Lau CP, Ko CH, Leung PC, Fung KP & Lau CB, The *in vivo* and *in vitro* diabetic wound healing effects of a 2-herb formula and its mechanisms of action. *J Ethnopharmacol*, 134 (2011) 831.

9 Rajput RT & Gohil KJ, Development and evaluation of herbomineral ointment from *Bauhinia variegata* L. for wound healing effects. *Indian J Nat Prod Rep*, 11 (2020) 96.

10 Parente LM, , Lino Junior Rde S, Tresvenzol LM,Vinaud MC, de Paula JR & Paulo NM, Wound healing and anti-inflammatory effect in animal models of *Calendula officinalis* L growing in Brazil. *Evid Based Complement Alternat Med*, 2012 (2012) 375671.

11 Kumar PM, Suba V & Reddy RB, Wound healing activity of *Celtis timorensis* Span. (Cannabaceae) leaf extract in Wistar albino rats. *Indian J Exp Biol*, 55 (2017) 688.

12 Huo J, Zhao Z, Hua Z, Fan J, Du J & Bo Guo, Evaluation of *Juglans regia* L., root for wound healing via antioxidant, antimicrobial and anti-inflammatory activity. *Indian J Biochem Biphys*, 57 (2020) 304.

13 Ozay Y, Guzel S, Ozkorkmaz EG, Yıldız A, Yıldırım Z, Erdogdu IH, Darcan S, Pehlivanoglu B & Kumas M, Effects of methanolic extract of *Verbascum inulifolium* Hub.-Mor. on incisional and excisional skin wounds in diabetic and non-diabetic rats. *Indian J Exp Biol*, 57 (2019) 157.

14 Thakur R, Jain N, Pathak R & Sandhu SS, Practices in wound healing studies of plants. *Evid Based Complement Alternat Med*, 2011 (2011) 438056.

15 Anitha V, Antonisamy JM & Jeeva S, Anti–bacterial studies on *Hemigraphis colorata* (Blume) HG Hallier and *Elephantopus scaber* L. *Asian Pac J Trop Med*, 5 (2012) 52.

16 Edwin BT & Nair PD, In vitro evaluation of wound healing property of *Hemigraphis alternata* (Burm. F) T. Anders using fibroblast and endothelial cells. *Biosci Biotechnol Res Asia*, 8 (2011)185.

17 Akhil TT & Prabhu P, Evaluation of anti-oxidant, anti-inflammatory and cytotoxicity potential of *Hemigraphis Colorata. Int J Pharm Sci Res*, 4 (2013) 3477.

18 Annapoorna M, Sudheesh Kumar PT, Lakshman LR, Lakshmanan VK, Nair SV & Jayakumar R, Biochemical properties of *Hemigraphis alternata* incorporated chitosan hydrogel scaffold. *Carbohydr Polym,* 92 (2013)1561.

19 Skaar I, Adaku C, Jordheim M, Byamukama R, Kiremire B & Andersen M, Purple anthocyanin colouration on lower (abaxial) leaf surface of *Hemigraphis colorata* (Acanthaceae). *Phytochemistry*, 105(2014) 141.

20 Budovsky A, Yarmolinsky L & Ben-Shabat S, Effect of medicinal plants on wound healing. *Wound Repair Regen*, 23(2015)171.

21 Yang M, Sheng L, Zhang TR & Li Q, Stem cell therapy for lower extremity diabetic ulcers. Where do we stand?. *Bio Med Res Int*, 2013 (2013) 462170.

22 Maver T, Maver U, Stana Kleinschek K, Smrke DM & Kreft S, A review of herbal medicines in wound healing. *Int J Dermatol*, 54 (2015) 740.

23 Gantwerker EA & Hom DB, Skin: histology and physiology of wound healing. *Clin Plast Surg***,** 39 (2012) 85.

24 Ghosh PK & Gaba A, Phyto-extracts in wound healing. *J Pharm Pharm Sci*, 16 (2013) 760.

25 Boniface PK, Verma S, Shukla A, Khan F, Srivastava SK & Pal A, Membrane stabilization: a possible anti-inflammatory mechanism for the extracts and compounds from *Spathodea campanulata*. *Nat Prod Res*, 28 (2014) 2203.

26 Hossain MM, Ahamed SK, Dewan SM, Hassan MM, Istiaq A, Islam MS & Moghal MM, In vivo antipyretic, antiemetic, in vitro membrane stabilization, antimicrobial, and cytotoxic activities of different extracts from *Spilanthes paniculata* leaves. *Biol Res*, 47 (2014) 45.

27 Nambiar SS, Shetty NP, Bhatt P & Neelwarne B, Inhibition of LDL oxidation and oxidized LDL-induced foam cell formation in RAW 264.7 cells show anti-atherogenic properties of a foliar methanol extract of *Scoparia dulcis*. *Pharmacogn Mag*, 10 (2014) 240.

28 Rajarathinam G & Dronamraju SVL, Anti-inflammatory activity of dimethyl octenol and oleanene tetrol isolated from *Trianthema decandra L. Mol Bio Rep*, 45 (2018) 547.

29 Chandra S, Chatterjee P, Dey P & Bhattacharya S, Evaluation of in vitro anti-inflammatory activity of coffee against the denaturation of protein. *Asian Pac J Trop Biomed*, 2 (2012) 178.

30 Chinnadurai GS, Krishnan S & Perumal P, Studies on detection and analysis of proteases in leaf extract of medicinally important plants. *Phytomedicine*, 40 (2018) 176.

31 Ndhlala AR, Aderogba MA, Ncube B & Van Staden J, Anti oxidative and cholinesterase inhibitory effects of leaf extracts and their isolated compounds from two closely related croton species. *Molecules*, 18 (2013)1916.

32 Shorinwa OA, Ebong OO, Obianime AW & Siminialayi IM**,** Evaluation of *in-vitro* antioxidant and antiplasmodial activities of the acetone extract of *Crinum jagus* bulbs (Liliaceae) Linn. *Int J Pharm Sci* Res, 9 (2018) 2990.

33 Esposito D, Rathinasabapathy T, Schmidt B, Shakarjian MP, Komarnytsky S & Raskin I, Acceleration of cutaneous wound healing by brassinosteroids. *Wound Repair Regen*, 21(2013) 688.

34 Zhang C, Zhai W, Xie Y, Chen Q, Zhu W & Sun X, Mesenchymal stem cells derived from breast cancer tissue promote the proliferation and migration of the MCF-7 cell line in vitro. *Oncol Lett*, 6 (2013) 1577.

35 Kim M, Lee H**J**, Randy A, Yun JH, Oh SR & Nho CW, *Stellera chamaejasme* and its constituents induce cutaneous wound healing and anti-inflammatory activities. *Sci Rep*, 7 (2017) 42490.

36 Bhasker S, Madhav H & Mohankumar C, Molecular evidence of insulinomimetic property exhibited by steviol and stevioside in diabetes induced L6 and 3T3L1 cells. *Phytomedicine***,** 22 (2015) 1037.

37 Kozera B & Rapacz M, Reference genes in real-time PCR. *J Appl Genet*, 54 (2013) 391.

38 Kheirelseid EA, Chang KH, Newell J, Kerin MJ & Miller N, Identification of endogenous control genes for normalization of real-time quantitative PCR data in colorectal cancer. *BMC Mol Biol*, 11 (2010) 12.

39 Cano Sanchez M, Lancel S, Boulanger E & Neviere R, Targeting oxidative stress and mitochondrial dysfunction in the treatment of impaired wound healing: A systematic review. *Antioxidants,* 7 (2018) 98.

40 Annegowda HV, Nee WC, Mordi MN, Ramanathan S & Mansor SM. Evaluation of phenolic content and antioxidant property of hydrolysed extracts of *Terminalia catappa* leaf. *Asian J Plant Sci*, 9 (2010) 479.

41 Kurahashi T & Fujii J, Roles of anti-oxidative enzymes in wound healing. *J Dev Biol*, 3(2015) 57.

42 Karimzadeh S & Farahpour MR, Topical application of *Salvia officinalis* hydro ethanolic leaf extract improves wound healing process. *Indian J Exp Biol*, 55 (2017) 98.

43 Gopalakrishnan A, Ram M, Kumawat S, Tandan SK & Kumar D, Quercetin accelerated cutaneous wound healing in rats by increasing levels of VEGF and TGF-β1. *Indian J Exp Biol*, 54 (2016) 187.

44 Le M, Naridze R, Morrison J, Biggs LC, Rhea L, Schutte BC, Kaartinen V & Dunnwald M, Transforming growth factor beta 3 is required for excision wound repair *in vivo*. *PLoS One*, 7 (2012) 48040

45 Ponugoti B, Xu F, Zhang C, Tian C, Pacios S & Graves DT, FOXO1 promotes wound healing through the up-regulation of TGF-β1 and prevention of oxidative stress. *J Cell Biol*, 203 (2013) 327.

46 Toraldo G, Bhasin S, Bakhit M, Guo W, Serra C, Safer JD, Bhawan J & Jasuja R, Topical androgen antagonism promotes cutaneous wound healing without systemic androgen deprivation by blocking β-catenin nuclear translocation and cross-talk with TGF-β signaling in keratinocytes. *Wound Repair Regen*, 20 (2012) 61.

47 Ghosh AK & Vaughan DE, PAI-1 in tissue fibrosis. *J Cell Physiol*, 227 (2012) 493.

48 Rebalka IA, Raleigh MJ, D'Souza DM, Coleman SK, Rebalka AN & Hawke TJ, Inhibition of PAI-1 via PAI-039 improves dermal wound closure in diabetes. *Diabetes*, 64 (2015) 2593.

49 Simone TM, Longmate WM, Law BK & Higgins PJ, Targeted inhibition of PAI-1 activity impairs epithelial migration and wound closure following cutaneous injury**.** *Adv Wound Care*, 4(2015) 321**.**

50 Simone TM, Higgins CE, Czekay RP, Law BK, Higgins SP, Archambeault J, Kutz SM & Higgins PJ, Serpine1: A molecular switch in the proliferation/migration dichotomy in wound- "activated" keratinocytes. *Adv Wound Care*, 3 (2014**)** 281.