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Comparative analysis of tender and mature *Hemigraphis colorata* leaves on TGF-β activity in wound healing and molecular characterisation of plant extracts

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With rising demand for effective and sustainable measures in wound healing researchers are compelled to explore diverse methods that combine folk medicine with existing wound care to double the impact. *Hemigraphis colorata* (Blume) H G Hallier, an exotic plant in the Acanthaceae family, is a perennial herb that has potential therapeutic benefits. In the present investigation, a comparative study was conducted between the effect of tender and mature leaf extracts of *H. colorata* as well as the differentially expressed genes of both leaves using four upstream primers and one downstream ($HT_{11}C$) primer. The rate of cell migration was studied by creating a scratch wound in HaCaT monolayer culture. The finding indicated that the application of tender leaf extract resulted in a higher rate of wound closure, whereas the mature leaf extract led to a two-fold increase in the secretion of TGF-β protein, as measured by ELISA. This demonstrates the therapeutic effectiveness of tender leaf extract in promoting wound healing compared to mature leaf extract. Additionally, the results of DDRT-PCR unveiled a diverse range of differentially expressed genes, which were registered as molecular markers (ESTs) and did not show homology with any known gene sequences in the NCBI database.

Keywords: DDRT-PCR, Differentially expressed genes, Expressed sequence tags, HaCaT (Human keratinocytes) cells, RNA profiling, Sequence analysis

Human skin is a highly adaptive multifunctional organ that shields humans from a myriad range of radiation, and chemical and physical threats. Skin has an intricate and highly developed healing mechanism involving a sequence of cellular processes, including hemostasis, inflammation, proliferation, and remodelling, to facilitate wound healing[1]. The process is mediated by an elaborate signalling network that includes various growth factors, cytokines, and chemokines. The epidermal growth factor (EGF) family and transforming growth factor beta (TGF-β) are two major growth factors that play a vital role in the wound healing process[2-4]. Certain conditions like diabetes[5], asthma, sex hormones[6], hereditary elements, autoimmune disorders, psychological stress[7], smoking, obesity, and aging[8] can disrupt the reparative process, causing healing impairment and leading to chronic wounds[9]. Due to their high incidence and recurrence[10] it is essential to develop wound-healing solutions that are affordable, efficient, and widely available[11]. Traditional Asian healthcare relies heavily on indigenous medicine, drawing from plants and natural products. The integration of traditional and modern medicine is an evolving approach that seeks to combine the strengths of both systems for more comprehensive healthcare solutions[12,13]. Literature reveals the existence of a large number of plants[14–16] and plant-derived products for wound healing process[17–19].*Hemigraphis colorata*, is a perennial prostrate herbal plant belonging to Acanthaceae family[20]. The plant has the power to soothe fresh wounds, cuts, ulcers, inflammations, and skin disorders, and to halt bleeding, the leaf juice is placed directly into the wound[21]. The leaves are used internally in traditional medicine to treat a variety of conditions, including anaemia, haemorrhoids, bloody diarrhoea, diabetes mellitus gallstones, and heavy periods, and as a diuretic and contraceptive[22–25]. Saponins, flavonoids, terpenoids, coumarins, carbohydrates, carboxylic acids, xanthoproteins, tannins, proteins, alkaloids, steroids, and sterols are some important secondary metabolites found in the extract[26,27]. The leaf extracts show increasing reducing power and DPPH radical scavenging activity with increased concentration due to the presence of phenolic compounds[28]. The leaf paste, induced wound contraction and epithelialisation in mice[29,30] than povidone iodine ointment. The leaves and stem extracts showed excellent antibacterial effects against various pathogens[31,32]. Despite real-life examples, the bioactive compounds in *H. colorata* and the factors stimulating the response of growth factors at the site of the wound are yet to be elucidated. In the present study, we attempted to identify the wound healing

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capacity in *in vitro* cell line wound models using both tender and mature leaves of *H. colorata* and to quantify the levels of TGF-β protein produced at the wound site upon interaction with the leaf extract. The study was further extended to analyse differentially expressed genes of mature and tender leaves of *H. colorata* using differential display reverse transcriptase PCR (DDRT-PCR).

Materials and Methods

Aqueous extraction and the cell model

The taxonomic status of the plant was authenticated by Botanical Survey of India (BSI) Coimbatore as *Hemigraphis colorata* (Burm.f.) T. Anderson, and a voucher specimen of the plant BSI/SRC/5/23/2016/ TECH/1554 were deposited. Mature and tender leaves of *H. colorata* were collected from the neighbourhood places, rinsed under running water, and wiped thoroughly.0.5 g of fresh leaf tissue was ground well in a mortar and pestle with 5 mL distilled water and was then centrifuged at 10,000 rpm for 10 min. The aqueous layer collected was used for the complete set of experiments. The undifferentiated HaCaT fibroblast monolayer culture procured from the National Centre for Cell Sciences (NCCS); Pune was used as a model to study the wound-healing activity of the leaf extract.

Scratch wound assay

The scratch wound assay is a widely adapted method to study the effects of growth and migration at various experimental conditions. The typical scratch wound assay[33] involves the creation of a wound gap in a monolayer cell culture. The healing rate in the presence of a therapeutic factor is determined by examining the wound gap closure rates by cell migration[34]. Uniform monolayer cultures of HaCaT cells were used for the assay. Wounds are created when the cultured cells cover the entire surface area of the 6 well culture plates. A 200 µL yellow tip (Tarson) was used to meticulously scratch the confluent monolayer. The wounded cells were rinsed thrice with filter sterile PBS of pH 7.4 to remove the detached cells and were then incubated with 1.9 mL of medium containing Dulbecco's modified Eagle's medium (DMEM, Himedia) with 1% antibiotic and antimycotic solution (Himedia) and 0.05% FBS (PAN Biotech). 100 µL aqueous extracts of tender and mature leaves were introduced into the medium separately. The experiment was done in duplicate wells for each trial. In controls, 100 µL of sterile water was added. The plates were then gently swirled and incubated at 37°C in a 5% CO_2 incubator for the next 48 h to analyse the wound healing properties. The wounded area of the HaCaT cell monolayer treated with the leaf extract was observed for any histological changes. The wound model plates were monitored daily under an inverted phase contrast microscope to determine the wound closure rate using the IS software.

TGF-β quantification

The HaCaT cells were seeded to 6 well plates to obtain an optimal cell density of 1 x 10^6 cells/mL, the cells were incubated for 24h to ensure cell attachment. After incubation, the medium was replaced with fresh DMEM containing 0.5% FBS and 1% antibiotic and antimycotic solution. Along with fresh culture medium, 100 µL of tender and mature leaf extracts of *H. colorata* were added to respective wells, and sterile water was used as a control. The treated cells were then incubated in a CO_2 incubator for 48h before protein isolation. The cell lysis and protein isolation experiments were carried out according to the protocol described by Hong Ji[35]. The culture medium in HaCaT cells treated wells was decanted and washed twice with PBS without disturbing the cells. 1 mL of pre-chilled cell lysis buffer containing 50 mM Tris HCL (pH 8.0), 50 mM NaCl, 1 mM $MgCl_2$, and 1% Triton X-100, along with 1 X protease inhibitor cocktail (5 mg PMSF and 100 µg Aprotinin dissolved in 100% ethanol) was added to each well and was then incubated for 30 min on ice with occasional mixing. The cell lysate was collected in a fresh vial and centrifuged at 12000 g for 15 min. After centrifugation, the supernatant was collected in a fresh tube and the pellet was discarded. The supernatant was stored at 4^0C. Lowry method is used to determine the total protein present in the given sample using Folin's reagent which gives a coloured complex[36].

Quantification of TGF-β protein using solid phase ELISA technique

TGF-β isoform proteins have been quantitated in such tissue extracts using custom sandwich ELISA assays[37]. 100 µg proteins in PBS (pH 7.4) were coated in each well of the microtiter strip. To the blank wells, 100 µL of PBS (pH 7.4) was added. The microtiter strip was incubated for 24h at 4^0C after wrapping it with adhesive plastic tape. The plates were washed using PBS-Tween 20 (PBS-T) buffer 5 times. Then

the unbound protein binding sites were blocked by adding 100 µL of blocking buffer containing 5% BSA in PBS (pH 7.4) and it was incubated at 4^0C for 24h followed by washing using PBS-T buffer. 1:1000 dilution of primary antibody, Anti-transforming growth factor β mouse monoclonal antibody (Pierce) dissolved in a diluent buffer containing 2% BSA.0.01% sodium azide and PBS were added to each well. The strip was then covered with adhesive plastic tape and incubated in a platform shaker at 37^0C for 30 min. 1:5000 dilution of the secondary antibody, Rabbit IgG Anti-human HRP (Genei, Bangalore) in conjugate buffer (pH 7.6) containing 0.01 M sodium phosphate, 0.25 M NaCl, and 15 mg/mL BSA was added to each well after thorough washing with PBS-T buffer. The strip was again incubated for 30 min at 37^0C after covering with adhesive plastic tape. After washing, 100 µL of TMB solution (substrate) was added to each well using a multichannel pipette and was kept for 5 min for the development of colour. 1 N sulphuric acid was used as the stop solution. The absorbance was measured at 450 nm using an ELISA reader.

DDRT-PCR analysis of differentially expressed genes in *H. colorata*

RNA was isolated from tender and mature leaves of *H. colorata* by following the GTC method of extraction[38]. RNA was observed as two distinct bands representing 28S and 18S rRNA respectively in formaldehyde gel. The primers for DDRT analysis of tender and mature leaves of *H. colorata* were designed using vector NTI® (Invitrogen) software. Both the downstream polyT primer (5' [6FAM] AAGCTTTTTTTTTTTC 3') and the four upstream random primers were designed with a Hind III restriction site at the 5' position for easy cloning. The primers were synthesised at Sigma, Bangalore, and were dissolved in sterile double distilled water to make the concentration up to 100 pM/µL. The primer stocks were then stored at -20^0C until used. The first strand synthesis (cDNA) of RNA isolated from tender and mature leaves of *H. colorata* was done in a total volume of 25 µL containing 10 µL of RNA, 5 µL of 5X buffer, 25 U RNase inhibitor, 10 mM dNTPs, 100 ng oligodT primer (5' [6FAM] AAGCTTTTTTTTTTTC 3') and 20 units of MMuLV reverse transcriptase enzyme.The amplification of genes in tender and mature leaves of *H. colorata* was carried out using a thermal cycler (Eppendorf, Germany). For the amplification of the genes, four arbitrary forward primers LMM5, MOB1, HAP13, HAP14, and the reverse primer (5' 6[FAM] AAGCTTTTTTTTTTTC 3') were used. The PCR reaction was carried out in 20 µL reaction mixtures containing 2 µL of 10X reaction buffer containing MgCl2, 2.5 µL of cDNA, 10 mM dNTP, 10 pM of forward primers and reverse primer, and 5 units of Taq DNA polymerase. The PCR-amplified gene products were separated on 6% denaturing urea polyacrylamide gel electrophoresis[39]. Sample preparation was done by adding 20 µL of 2X formamide gel loading dye (90% formamide, 0.5% EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) to 20 µL of PCR products which were amplified using four arbitrary forward primers and the reverse primer ($HT_{11}C$) of both tender and mature leaf of *H. colorata*. The sample mix was subjected to heat denaturation at 90^0C for 2 min and was then snap chilled in ice and then loaded into the wells of denaturing urea polyacrylamide gel along with 100 bp plus DNA ladder followed by electrophoretic separation of DNA bands at 500 V, 20 W for about 2h. The gel was carefully transferred to a petri dish containing 10 µL of 10 mg/mL ethidium bromide dissolved in 100 mL of 1X TBE buffer and was kept to stain for approximately 30 min by covering the petri dish to prevent the exposure of ethidium bromide. After incubation, the gel was subjected to UV exposure for the analysis of differentially expressed genes among tender and mature leaves of *H. colorata.* The differentially expressed bands were eluted and re-amplified using gene-specific forward primers and $HT_{11}C$ reverse primers. The re-amplified differentially expressed gene products were excised quickly from agarose gel over long wavelength UV light to avoid nicks. DNA was extracted from the gel slices using the gel extraction kit (Thermo Scientific TM, Lithuania).

Cloning and transformation

The purified and eluted differentially expressed fragments were ligated into the linearised TA cloning vector pTZ57R/T (Fermentas, Germany). Overnight grown culture of *E. coli* DH5-α was used to prepare competent cells. 10 µL of ligation mixture was transformed into E. coli DH5-α using heat shock method at 42^0C for 2 min. The transformed cells were plated on LB agar with 50 µg/mL ampicillin, 0.1 mM of IPTG, and 40 µg/mL of X-gal. The resulting colonies were selected by blue-white screening for the insert. The white colonies representing recombinant plasmids were selected for further confirmation. The pTZ57R/T-plasmid clones were confirmed by

restriction digestion and colony PCR. The confirmed plasmid clones were subjected to automated sequencing at Sci Genome, Ernakulam. The sequences were analysed using the bioinformatics tool Bioedit and were examined for sequence similarity in NCBI's BLAST search[40]. The sequences were submitted to NCBI as Expressed Sequence Tags (ESTs).

Results and Discussion

A comparison study was done on the efficacy of tender and mature leaves of *Hemigraphis colorata* in wound closure. TGF-β1 is released in large amounts from platelets after wounding. This acts as a chemo-attractant for neutrophils, macrophages and fibroblasts and in turn these cells enhance TGF-β1 levels in various cell types. However, the effect of TGF-β on re-epithelialisation appears paradoxical; its expression by keratinocytes after wounding with the inhibitory effect of TGF-β on keratinocyte proliferation. Thus, the amount of TGF-β secreted at the time of proliferation is also studied in HaCaT cell line wound model cells. Additionally, an attempt to profile the RNA of both tender and mature leaves of *H. colorata* by differential display reverse transcriptase PCR (DDRT-PCR) was performed. The classical scratch wound method was adopted for creating wounds on the surface of a confluent monolayer grown on culture plates. The wounded monolayer was treated with freshly prepared 100 µL of aqueous extracts of both tender and mature leaf extracts of *H. colorata* along with culture medium (0.5% FBS) in corresponding wells. The cells around the wounded area were observed for responses including survival of cells from acute damage at the wound edge, re-attachment of cells to the substrate, the extension of cells to the wounded area, and enhanced migration and proliferation rate to the wound site in 12-24 h post-treatment. The migration rate was monitored every 24 h for 2 days post-treatment. After the first 24 h, it was observed that the tender leaf extract-treated cells showed a higher rate of proliferation toward the wounded area under an inverted phase contrast microscope. The size of the wound on Day 0 was an average of 1.71 µm and it reduced to 0.86 µm in Control cells on Day 2, whereas the wound has almost closed in the cells treated with tender and mature leaf extract. The wound closure observed in the cells treated with the aqueous extracts of tender and mature leaves might be due to both cell proliferation and migration (Fig. 1).

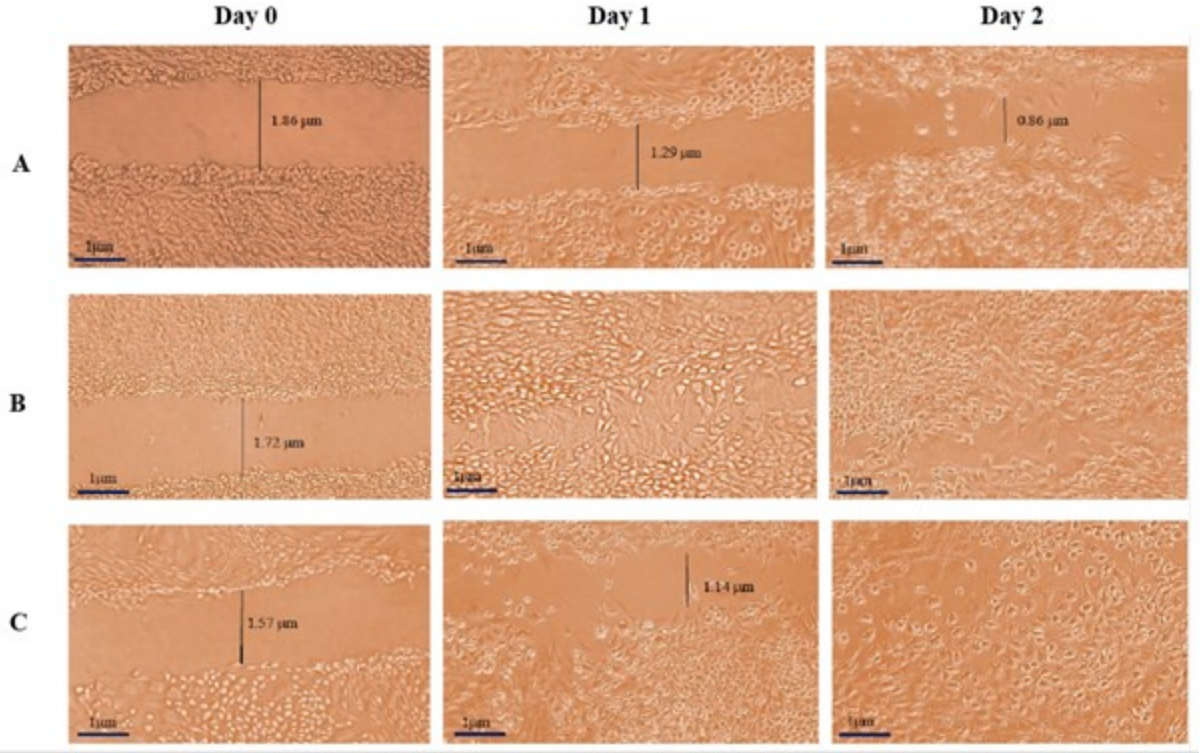

Fig. 1 — The wound healing effect of (A) control, (B) *H.colorata* tender leaf extract, and (C) *H.colorata* mature leaf extract on a wounded monolayer of HaCaT cells under 10X magnification.

*H. colorata*induced expression of TGF-β was quantified using the ELISA. The cells treated with aqueous leaf extracts of *H. colorata* along with DMEM containing 0.2% BSA were incubated for 48 h, and protein was isolated. The TGF-β antigen was quantified by ELISA using anti-TGF-β mouse monoclonal antibody. The data obtained imply that the amount of TGF-β protein produced by the HaCaT cells in the presence of aqueous mature leaf extract is higher. Fig. 2 shows that there has been a 2 fold increase in the amount of TGF-β produced in mature leaf extracts treated cells when compared to tender leaf extracts treated cells. Cell proliferation is typically regulated by CDKs through a sequence of processes known as the cell cycle[41]. In most cells, TGF-β inhibits proliferation, meaning it induces cytostasis by causing cell cycle arrest in the G1 phase. In epithelial and glial cells, TGF-β reduces CDK activity by stimulating the transcription of CDK inhibitors (CKIs) like p15 and p21, leading to cytostasis[42]. Treatment of various cell types with TGF-β results in a wide range of biological effects such as growth inhibition, growth promotion, escape from contact inhibition, and induction of growth factor and extracellular matrix production. Alan in 1987 described that the TGF-β does not stimulate fibroblast proliferation at 24 and 48h, which implied that after a prolonged pre-replicative interval cell did not enter the S phase of the cell cycle[43]. In the present investigation, there was a higher expression of TGF-β in cells treated with mature leaf extracts compared to cells treated with tender leaf extracts. This can be explained based on cell proliferation, which indicates that the higher expression of TGF-β in cells treated with mature leaf extracts showed inhibition of cell proliferation. While in cells treated with tender leaf extracts, the expression of TGF-β was comparatively lower but the cell proliferation rate was higher. TGF-β acts as a negative regulator of re-epithelialisation since it inhibits keratinocyte proliferation after wounding *in vitro* and *in vivo*[44]. Inhibition of cell proliferation by TGF-β is found at the G1 phase of the cell cycle. A possible explanation for this data is that the TGF-β blocks progress through the G1 phase by preventing phosphorylation and function at the inactivation of pRb since pRb is expressed in the growth-suppressing form in such cells[45]. Thus, further studies on the molecular characterisation and effect of the phytocompounds present in *H. colorata* in other *in vitro* and *in vivo* models are essential.

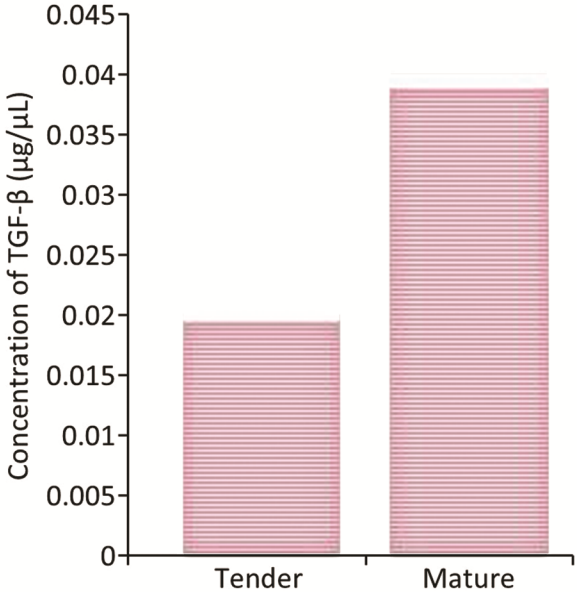

Fig. 2 — The concentration of TGF-β protein (µg/µL) production in *H.colorata* mature and tender leaf extract treated HaCaT.

RNA profiling of tender and mature leaves of *H. colorata*

The RNA isolated from tender and mature leaves of *H. colorata* by the GTC method was of appreciable quality. 4 µg of the total RNA was used for the synthesis of cDNA using 100 ng of one base anchored oligodT reverse (5' 6[FAM] AAGCTTTTTTTTTTTC 3') primer and 20 units of MMuLV reverse transcriptase enzyme. 100 ng of the cDNA synthesised was subjected to PCR amplification using 10 pM for each of the four arbitrary forward primers and one base-anchored reverse primer. The PCR amplification showed a differentially expressed band of 123 bp and 195 bp size in tender leaf by using the primer combination $HT_{11}C$ and LMM5 (TL4, TL3). A primer combination of $HT_{11}C$ and MOB1 (ML1) produced a differentially expressed band size of 278 bp size in mature leaves of *H. colorata*. A gene fragment of 177 bp was differentially expressed in the tender leaf using primer combination $HT_{11}C$ and HAP13 (TL1). $HT_{11}C$ and HAP14 (TL2) primer combination produced a band of 316 bp size in tender leaf (Table 1, Fig. 3A & 3B). The differentially

Table 1— PCR amplified differentially expressed genes in tender and mature leaves of *H. colorata*

Primer combination	Tender leaf	Mature leaf
$HT_{11}C$ + LMM5	123 bp (TL4)	-
$HT_{11}C$ + LMM5	195 bp (TL3)	-
$HT_{11}C$ + MOB1	-	278 bp (ML1)
$HT_{11}C$ + HAP13	177 bp (TL1)	-
$HT_{11}C$ + HAP14	316 bp (TL2)	-

expressed genes were eluted from urea polyacrylamide gel using 2X PCR buffer and incubated for 90 min at 94^0C in a thermocycler. 5 µg of the eluted product was used for reamplification using the differentially expressed gene specific forward primers and one base anchored oligodT ($HT_{11}C$) reverse primer. Each differentially expressed gene fragment showed the expected sizes during reamplification PCR. The PCR-amplified differentially expressed genes were purified using a Gel extraction kit (Thermo scientificTM, Lithuania). The eluted products ML1, TL1, TL2, TL3, and TL4 were ligated to TA cloning vector pTZ57R/T using T4 ligase. The ligation mixture was transformed into the *E.coli* DH5α by heat shock method. The positive transformants were selected by blue-white screening. The presence of differentially expressed genes was confirmed by *BamH1* and *Xba1* digestion of the pTZ57R/T- recombinant plasmids as well as the PCR amplification method. Expected size amplicons were obtained for all the clones in PCR amplification (Fig. 4A) and showed an expected size release in

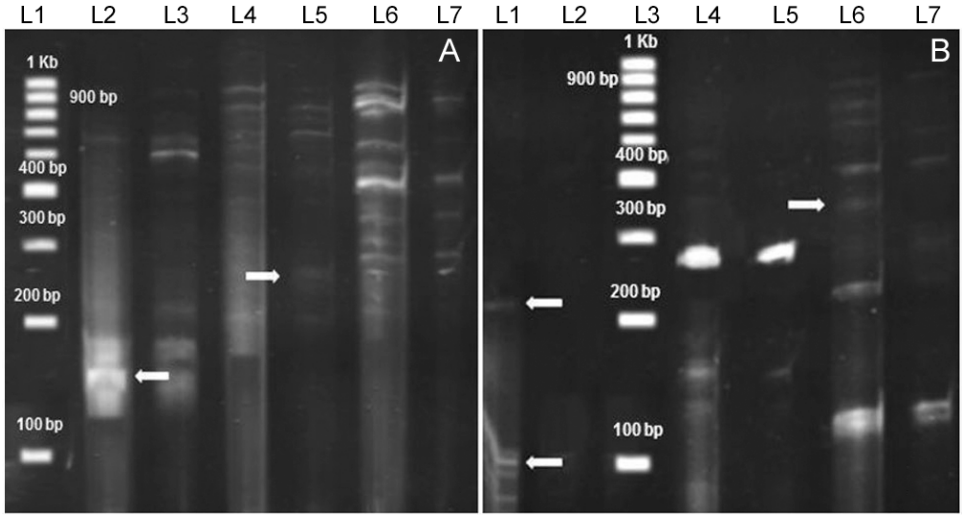

Fig. 3 — (A) 6% Denaturing urea polyacrylamide gel electrophoresis. Lane 1 -100 bp plus DNA ladder, Lane 2–Tender ($HT_{11}C$ + MOB1), Lane 3–Mature ($HT_{11}C$ + MOB1), Lane 4–Tender ($HT_{11}C$ +HAP 13), Lane 5–Mature ($HT_{11}C$ + HAP 13), Lane 6–Tender ($HT_{11}C$ + LMM3), Lane 7–Mature ($HT_{11}C$ + LMM3). (B) 6% Denaturing urea polyacrylamide gel electrophoresis. Lane 1–Tender ($HT_{11}C$+LMM5), Lane 2–Mature ($HT_{11}C$ + LMM5), Lane 3–100bp ladder, Lane 4 & 5–Tender & Mature ($HT_{11}C$ + LMM4), Lane 6–Tender ($HT_{11}C$ + HAP 14), Lane 7–Mature ($HT_{11}C$ + HAP 14).

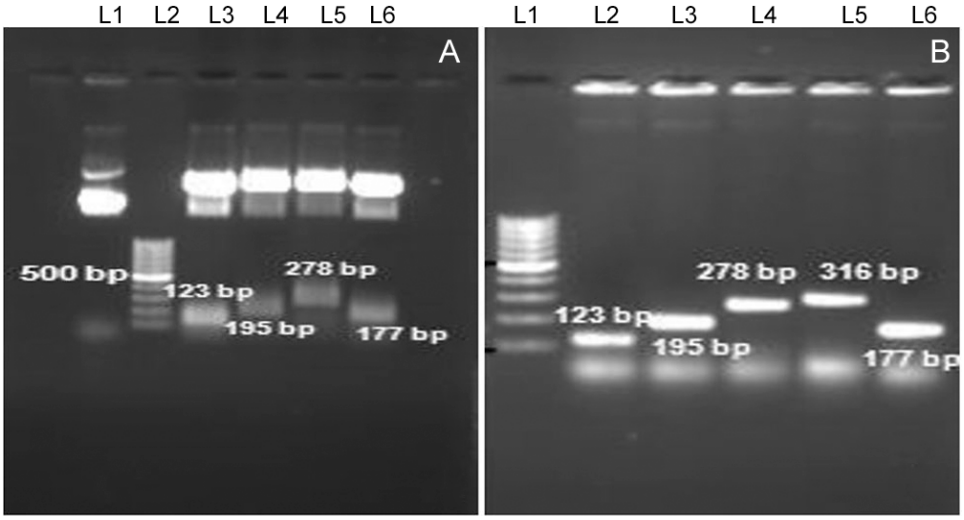

Fig. 4 — (A) Confirmation of recombinant plasmids by restriction digestion using *BamH1* and *Xba1* enzymes. Lane 1–undigested plasmid, Lane 2–100 bp plus DNA ladder, Lane 3–Digested TL4, Lane 4–digested TL3, Lane 5–digested ML1, Lane 6–TL digested TL1.B) confirmation of recombinant plasmids by colony PCR. Lane 1–100 bp plus DNA ladder, Lane 2–TL4, Lane 3–TL3, Lane 4–ML1, Lane 5–TL2, Lane 6–TL.

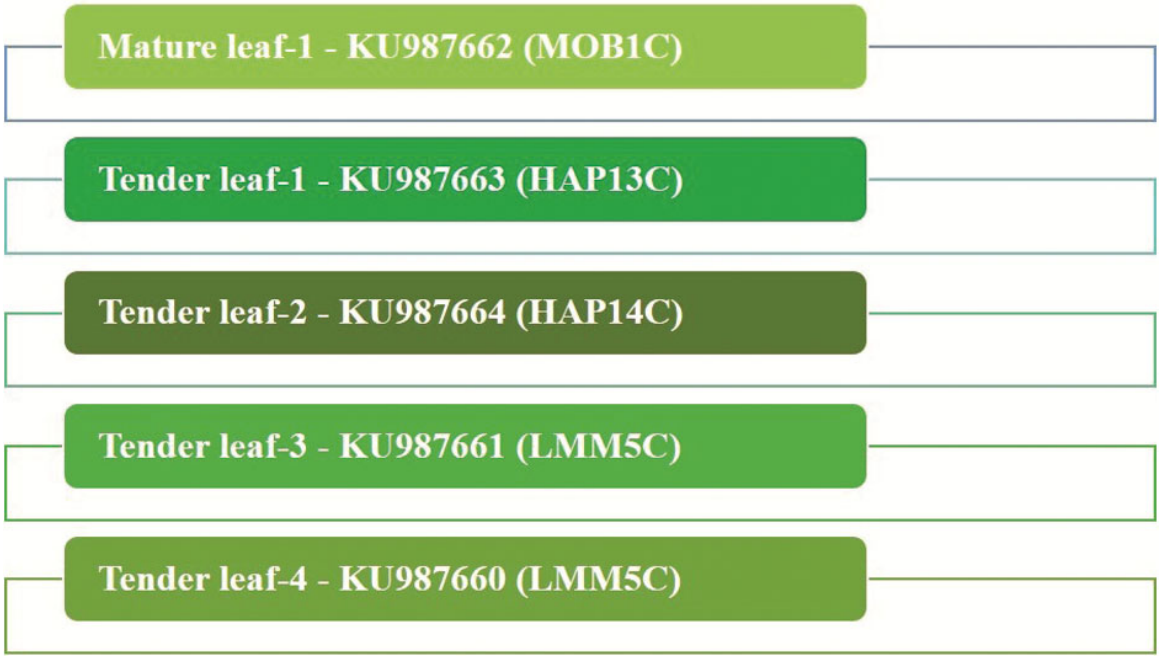

Fig. 5 — Gene-Bank accession numbers for ESTs.

restriction digestion (Fig. 4B). The confirmed plasmids were purified using a Plasmid purification kit (BioLine) and sequenced. All the sequencing reads were clear and non-overlapping. Further, the sequences were analysed using Vector NTI® software (Invitrogen). Homology search of the sequences with other known sequences in the public domain was done using BLAST search of NCBI. The differentially expressed gene fragments ML1, TL1, TL2, TL3, and TL4 did not show any similarity with other known genes in the public domain hence submitted in NCBI as expressed sequence tags (ESTs) and ESTs were registered with accession number (Fig. 5).

Conclusion

The results from the scratch wound assay indicated that the tender leaf extract of *Hemigraphis colorata* significantly accelerated cell migration, suggesting its potential to enhance wound closure through mechanisms that promote cellular movement and proliferation. In contrast, the mature leaf extract was associated with elevated levels of TGF-β protein, which is known for its role in limiting excessive cell proliferation and promoting tissue remodeling. This finding suggests that the mature leaf extract may play a role in modulating fibroblast activity and tissue remodeling during wound healing. Additionally, DDRT-PCR analysis revealed distinct differentially expressed genes between the tender and mature leaves, highlighting the complex molecular mechanisms involved. These findings suggest that the tender and mature leaf extracts influence different biological pathways, with the tender leaves promoting proliferation and migration, while the mature leaves regulate key processes in wound repair.

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Conflict of interest

The authors declare that they have no conflicts of interest related to the publication of this article.

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