



Wound Healing Mechanism by the Standardized Extracts of *Acalypha indica* and *Aristolochia bracteolata* on Human Cell Lines

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Abstract

Acalypha indica and *Aristolochia bracteolata* are two traditional herbal medicines used for treatment of various diseases particularly for skin disorders. They are being used in Siddha and Ayurvedic treatments. To investigate the action mechanism on wound healing process, the *in vitro* wound healing assays were carried out using Human dermal fibroblasts and Human epidermal keratinocytes. The aerial parts of *Acalypha indica* and *Aristolochia bracteolata* were extracted using ethanol and evaluated for its efficacy on viability and migration by MTT and Scratch wound assay. The genes associated with its wound healing property were evaluated by RT-PCR and angiogenic activity by CAM assay. Proliferative capability of the extracts was confirmed by MTT assay. Results showed that the wound healing mechanism involves the activation of wound healing related genes of MMP-2, Collagen type 1 and Collagen type 4 in HDF cells. The expression of MMP-2 facilitates essential events of wound migration, angiogenesis and tissue remodelling. Collagen 1 and 4 structures the ECM matrix during the tissue repair. The construction of new blood vessels (angiogenesis) by the plant extracts helps in supplying nutrients necessary for the cell proliferation and migrations.

Keywords

Acalypha indica, *Aristolochia bracteolata*, Cell viability, Scratch wound, CAM assay, Gene expression.

ABBREVIATIONS

A.indica – *Acalypha indica*

A.bracteolata – *Aristolochia bracteolata*

ECM – Extra Cellular Matrix

MMP – Matrix Metallo Proteinase

CAM – Chorio Allantoic Membrane

DMEM – Dulbecco's Modified Eagles Medium

INTRODUCTION

Wound healing is a general term for the repair response of the body which occur promptly after a disturbance in the skin integrity. It is followed by a complex multifactorial phase which includes inflammation, proliferation and remodelling of tissues. The proliferative phase is thought to be a rate-limiting factor in normal skin regeneration characterized by epithelialization, wound contraction and collagen deposition by fibroblasts and keratinocytes. A delay in the proliferative phase leads to more extensive scarring [1].

An agent that accelerates the above-cited process is a promoter for wound healing. Therapeutics that are in trend for wound healing applications has their own drawbacks of low efficiency, high cost for treatment and development of adverse effects. Medicinal plants on the other hand are well known for their rapid restorative wound healing abilities [2]. Over three quarters of the world population rely on plant and their extracts for wound care and over 400 species of plants have been identified as potential wound healers. Such wound healant herbs are conceived to have effect on wound management through debridement and disinfection thereby providing a moisturized environment which fastens the healing process [3]. These properties and traditional medicine claims for herbs as restorers of innate healing have inspired many researchers to validate the declarations and uncover the mechanisms of the medicinal plants which possibly could explain the potentiality on wound healing process [4].

Acalypha indica and *Aristolochia bracteolata* are two folklore acclaimed plants used in the treatment of wounds for decades in Asia. *Acalypha indica* (*A.indica*) (Euphorbiaceae) is a small erect herb distributed throughout India, Sri Lanka and Bangladesh. The whole plant has been proclaimed to possess purgative, diuretic, anti-inflammatory, anti-oxidant, anti-arthritis, anti-venom, anti-hyperlipidemic, anti-obesity, anti-cancer and analgesic properties [5]. The chemical constituents reported are acaindinin, acalyphin, acalyphamide, aurantiamide tannin, β -sitosterol acetate and succinimide. Phenolic phytochemicals like geraniin, corilagin, chebulagic acid, glucogallin, Ellagic acid, gallic acid, kauren-18-oic-acid, 16 α ,17-dihydroxy-ent-kauran 19-oic-acid and 4,4',5,5',6,6' hexahydroxy diphenic acid and flavonoids such as clitorin, mauritianin, nicotiflorin and biorobin are reported as anti-oxidants [6]. The extracts of the plant are used for dermal wound healing due to its anti-oxidant properties [7]. Topical application of *Acalypha indica*

has been validated scientifically for its ability on rat cutaneous wound. It has shown to augment the process of wound healing by up-regulating TGF- β 1 and increase in collagen 1 α and 3 α synthesis [8].

Aristolochia bracteolata (*A.bracteolata*) (Aristolochiaceae) is a perennial herb distributed throughout India and Malaysia. The whole plant has anthelmintic, anti-inflammatory, anti-pyretic properties. It has been validated scientifically for its anti-fertility and abortifacient effects [9]. The whole plant is used to treat snake bites and various skin disorders [10]. The phytochemical analysis revealed the presence of alkaloids, triterpenoids, steroids, sterols, tannins and glycosides. Apart from much researched aristolochic acid, aristolic and p-coumaric acids also contribute to the various activities of the plant. Fractionated methanolic extract of *A.b* by column chromatography resulted in three types of Aristolochic acids A, B and D [11]. *In vivo* wound healing study showed a positive effect by increasing the levels of two antioxidant enzymes, superoxide dismutase and catalase which resulted in better collagenation [12]. The two plants are commercially available for wound healing applications as topical dosage forms and ointments in traditional Siddha and Ayurvedic medicines.

To the best of our knowledge, although *in vivo* wound healing studies on rats are available on the selected plant extracts, *in vitro* wound healing mechanistic studies have not been reported. Thus *in vitro* assessment of these plant extracts upon epidermis and dermis layer of human skin is carried out using HaCaT cells [human-derived immortalised keratinocytes cell line] and HDF cells [Human dermal skin fibroblasts cell line] as the epidermis layer is composed of keratinocytes and dermis layer consists of fibroblasts cells.

Our study aims to investigate the wound healing efficacies and predict the possible proliferative, migratory and angiogenic actions of *Acalypha indica* and *Aristolochia bracteolata* in HDF and HaCaT cell lines. The genes associated with the wound healing property have also been evaluated by RT-PCR.

MATERIALS AND METHODS

2.1 Chemicals, Reagents and Cell lines

Gallic acid, aristolochic acid, Folin-Ciocalteu's reagent and Epidermal Growth Factor were procured from Sigma Aldrich (Missouri, USA). Potassium acetate, aluminum chloride and sodium bicarbonate were procured from Thermo fisher scientific (Massachusetts, USA). Human Dermal Fibroblasts (HDF) and Human dermal keratinocytes (HaCaT) cell lines were purchased from National Centre for Cell

Science (NCCS), Pune. Dulbecco's Modified eagle's Medium (DMEM), antibiotics (100 U Penicillin and 100 U/ml Streptomycin) and Phosphate Buffer Saline (PBS) were procured from Lonza (Basel, Switzerland). Fetal Bovine Serum (FBS) was procured from HiMedia, (Mumbai, India) and Trizol Reagent was procured from Gibco BRL.

2.2 Authentication of the plant material

The aerial parts of *Acalypha indica* and *Aristolochia bracteolata* were gathered from Kayathar township of Tuticorin district. The botanical identities of the plants were confirmed by Dr. V. Chelladurai - Botanist, Government Siddha Medical College, Palayamkottai. A voucher specimen was generated for the herbarium samples [*Acalypha indica* No: A.I/19/20.02.2015 and *Aristolochia bracteolata* No: A.B/20/15.03.2015] and deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Sri Ramachandra Institute of Higher Education and Research, Chennai, India.

2.3 Extraction of the Plant materials

The aerial parts of the plants (*A.indica* and *A.bracteolata* -200 g each) were shade dried, coarsely powdered and subjected to cold maceration for 72, 48 and 24 hrs with absolute ethanol. The collected extracts were filtered and concentrated using a rotary evaporator at 45°C.

2.4 Determination of Total Phenolic Content

The total phenolic content of *A.indica* and *A.bracteolata* were determined by a spectrophotometric method reported by Pourmorad *et al.* 2006 (mg of GA/g of extracts) [13].

2.5 Determination of Total Flavonoid Content

The total flavonoid content was determined by Aluminium chloride calorimetric method reported by Pourmorad *et al.* 2006. [13] A standard curve of quercetin was plotted and the results are quantified and expressed as quercetin equivalents (mg of Quercetin/g of extract).

2.6 High-Performance Thin-Layer Chromatography

HPTLC was executed by applying the sample solution (*A.indica* extract - 25 mg/ml, Standard -Gallic acid-100 mg/ml. *A.bracteolata* - 25 mg/ml, Standard-Aristolochic acid-500 mg/ml) on pre-coated silica gel G60 F254 plates (10×10 cm with 250 µm thickness, E.merck) with 100 µl Hamilton syringe using Linomat V Applicator. The mobile phase Toluene: Ethyl acetate: Formic acid: Methanol at 3:3:0.8:0.2 ratio for *A.indica* and Hexane: Chloroform: Methanol at 1:6:4 for *A.bracteolata* was saturated in Camog glass twin chamber at room temperature. After development, the plates were dried and scanned in absorbance mode at 254 nm for *A.indica* and at 254 and 366 nm for *A.bracteolata* using a Camog TLC

scanner III with deuterium source in the reflection mode, slit dimension setting of length 6 and width 0.45 mm and a scanning rate of 20 mm/sec. The peaks of the chromatograms were determined by winCAT software.

2.7 Cell viability assay

Briefly, HDF and HaCaT cells were seeded separately into two 96 well plates at a density of 2×10^4 cells/well in DMEM containing 10% FBS. Upon confluency, cells were treated with plant extracts at a different concentration ranging from 1-100 µg/ml and were incubated for 24 h. Cells without plant extract treatment served as control. After incubation for the indicated time, cells were treated with MTT (3-(4-5-dimethyl thiazol- 2yl) - 2,5- diphenyltetrazolium bromide) (5 mg/ml) and incubated in the dark for 4 hrs. DMSO was added to the wells for the product dissolution and the absorbance was taken in an ELISA reader at 570 nm [14].

2.8 In vitro Scratch assay

The migration property of HDF and HaCaT cells were accessed using scratch assay [15]. High density of HDF and HaCaT cells (5×10^4 cells/ml) were seeded separately in two six-well plates in DMEM supplemented with 10% FBS. Once cell culture monolayer was formed, the layer was scratched with a sterile 1ml pipette tip. PBS wash was given for the removal of formed cellular debris. Fresh medium deprived of FBS, containing *A.indica* and *A.bracteolata* (25 µg/ml and 50 µg/ml each) for test and Epidermal Growth Factor for positive control was replaced with old medium. Cells without any treatment served as negative control. Photographs were taken using digital camera (Nikon, Ti Eclipse Tokyo, Japan) connected to the inverted microscope on day 0 and successively on day 1 and day 2. Quantitative analysis of the acquired images was done using Image J 1.49. The percentage of scratch closure was determined by comparing the images from day 0 to day 2. Ten readings were taken from each scratch. The assay was performed in triplicates per sample.

Wound closure = [Average distance between scratches at day 1(day 2) – Average distance between scratches at day 0] / [Average distance between scratches at day 0]

2.9 Analysis of mRNA expression of remodelling enzyme MMP-2 and extracellular matrix components (Collagen 1, Collagen 4) associated with wound repair by RT-PCR analysis

Approximately 6×10^6 HDF cells were seeded in one 6-well plate and incubated at 37°C for 48h. After the cells reached confluence, scratch wound was induced as described in the above section. Cells were

treated with 25 µg/ml and 50 µg/ml of *A.indica* and *A.bracteolata* extracts. Total RNA was isolated using Trizol Reagent following the manufacturer's instruction. The concentrations and purity were measured with a spectrophotometer (Bio photometer plus, Eppendorf, USA). For reverse transcription (RT), 1 µg of total RNA was used in a final volume of 10 µL containing 250 pmol of random hexamers, 1 µL buffer, 25mM of dithiothreitol, 10 mM of each dNTP, 100 U of Super-script II reverse transcriptase enzyme and then transcribed into cDNA using the Verso cDNA Synthesis Kit (Roche, Mannheim, Germany). The reaction was carried out at 42°C for 45 min, then at 92°C for 5 min. PCR amplification was carried out using 10 µL of the RT reaction in a final volume of 50 µL containing 1x PCR buffer, 0.2 mM of each dNTP, 1.5mM MgCl₂, 1.5 U of Taq DNA polymerase, 1 µM of primer forward (F) and 1 µM of primer reverse (R). The PCR cycling profile was as follows: 94°C for 3 min, then 30–36 cycles of 94°C for 45 sec, 56–65°C (T_m) for 30 sec, 72°C for 1 min and a final extension at 72°C for 10 min (Mastercycler gradient thermocycler, Eppendorf). PCR samples were amplified using specific primers, PCR conditions were optimized and linear amplification range was determined for each primer by varying annealing temperature and cycle number. Products were separated on 2% agarose gels and visualized by using safe dye (Biolegend, USA). The PCR amplification for all primers was performed for 45 cycles. The expression levels of genes of interest were normalized against the housekeeping gene GAPDH. Negative control reactions containing samples without cDNA were used.

2.10 In vivo Chick Chorioallantoic Membrane Assay

Fertilized E6 chicken eggs (45±5 g) were incubated at 37 °C in 80% humidity for ten days. On the 10th day, the surface of the eggs was disinfected. 1×1 cm window was sectioned to expose the vascular zone. Sterilized filter paper discs were loaded with *A.indica* and *A.bracteolata* extracts (50 µg/ml and 100 µg/ml each), β-sitosterol (10µg/ml) as standard angiogenic drug and eggs without treatment served as control. The windows were sealed with clinical tape and eggs were returned to the incubator for another one day. Images of the CAM in the standard, test and untreated eggs were shot using Sony Cyber-shot DSC-TX-30. Quantitative morphometric analysis of the density of blood capillaries in before and after images of the CAM were analyzed by calculating the number of red pixels per unit area using Image J 1.49.

2.11 Statistical Analysis

Significant differences among untreated and treated groups were evaluated by Student's t-test. Values are

reported as mean ±SEM. *p<0.01, **p<0.05, ***p<0.001

RESULTS

3.1 Percentage yield of extracts

The yield of ethanolic extract of *A.indica* and *A.bracteolata* was 6.34% and 15.68% (w/w) respectively. The semi-solid mass thus obtained was used for further studies.

3.2 Total phenolic and flavonoid content of the ethanolic extracts

The total phenol content was found to be 27.43 mg/g for *A.indica* and 46.69 mg/g for *A.bracteolata* in terms of gallic acid equivalent. The total flavonoid content was 87.5 mg/g for *A.indica* and 45.93 mg/g for *A.bracteolata* as quercetin equivalent.

3.3 High Performance Thin- Layer Chromatography

Prepared extracts were standardized by HPTLC to quantify gallic acid and Aristolochic acid respectively from *A.indica* and *A.bracteolata*. The HPTLC chromatograms and 3D histograms of the standard Gallic acid and *A.indica* at 254 nm are shown in Figure 1(A). Similarly, the HPTLC chromatograms and 3D histograms of the standard Aristolochic acid and ethanolic *A.bracteolata* are shown in Figure 1(B). A calibration curve was plotted for the standards and the amount of standard present in the extracts was calculated. The results showed that 0.2% w/w of Gallic acid was present in *A.indica* and 2.5% w/w Aristolochic acid was present in *A.bracteolata*.

3.4 Cell viability assay

MTT assay determines the cytotoxicity of any of the plant extracts towards the studied cell line. Morphological changes such as cell shrinkage, granulation formation and reduced cell density were looked for. In HDF cell line, *A.indica* showed dose dependant toxicity at higher concentrations (25 – 100 µg/ml). *A.bracteolata* showed a consistent dose dependant proliferation of cells [Figure 1(C)]. In HaCaT cell line, all the tested concentrations of *A.indica* and *A.bracteolata* maintained the viability of cells by more than 95±3% [Figure 1(D)].

In view of the observed toxicity of *A.indica* at higher concentrations in HDF cell line and proliferation of cells in HaCaT cell line, 25 and 50 µg/ml were taken up for scratch wound and 50 and 100 µg/ml were taken up for CAM assay.

3.5 In vitro scratch assay

The migration potential of HDF and HaCaT cells was assessed using optimized *A.indica* and *A.bracteolata* concentrations. As shown in Figure 2(B), 50 µg/ml of *A.indica* and *A.bracteolata* restored the HDF cells to a fully confluent state similar to Standard Epidermal growth factor, whereas 25µg/ml of both the extracts

attracted $60 \pm 5\%$ of the cells to the denuded area at the end of 48 h. However, the migration of HaCaT cells to the denuded area at the end of 24 h was $55 \pm 2\%$ by *A.indica* and $72 \pm 5\%$ by *A.bracteolata* at 25 $\mu\text{g/ml}$ concentration, At 50 $\mu\text{g/ml}$, the migration was $45 \pm 3\%$ by *A.indica* and $15 \pm 4\%$ for *A.bracteolata*. They remained the same post 48 h [Figure 3(A)]. The motility of HaCaT cell line was less relative to HDF cells upon treatment with plant extracts.

3.6 Analysis of mRNA expression of remodelling enzymes, extracellular matrix components associated with wound repair by RT-PCR analysis

As *A.indica* and *A.bracteolata* extracts completely closed the denuded area at the end of 48 h in HDF cell line, the RT-PCR analysis was thus performed in HDF cells to investigate the relative mRNA expression levels of selected remodelling enzyme MMP-2 and extracellular matrix components collagen I and IV which are associated with wound repair. Figure 3[C] represents a significant up regulation of the extracellular matrix components in treatment group compared with control, while that of GAPDH

remained unaffected. Significant expression of matrix metalloprotease protein 2 (MMP2) was observed in the treatment group control but not higher than control group. Dose response relationship of mRNA expression of ECM proteins has also been observed.

3.7 In vivo Chick chorio allantoic Membrane assay

We evaluated the biological effects of *A.indica* and *A.bracteolata* in an *in vivo* CAM model to monitor de novo blood vessel formation. CAM treated with 100 $\mu\text{g/ml}$ of *A.indica* showed $45 \pm 3\%$ greater vascularity than the control and 1.5 times greater than the effect produced by the standard angiogenic drug beta-sitosterol [Figure 4]. Whereas *A.bracteolata* showed an anti-angiogenic effect at 50 $\mu\text{g/ml}$ and at 100 $\mu\text{g/ml}$ there has been an increase of $18 \pm 2\%$ angiogenesis, relative to the 0th-day effect. The quantification is based on arbitrary points given for de novo small vessel formation up to reorganization of existing vessels. These results suggest that both the extract has a significant direct effect on the angiogenesis in an *in vivo* model system.

Figure 1: HPTLC fingerprinting of *Acalypha indica*(A) and *Aristolochia bracteolata* (B), Graphical representation of the percentage of Human Dermal Fibroblasts(C) and Human dermal keratinocytes (D) surviving the treatment with plant extracts after 24 h

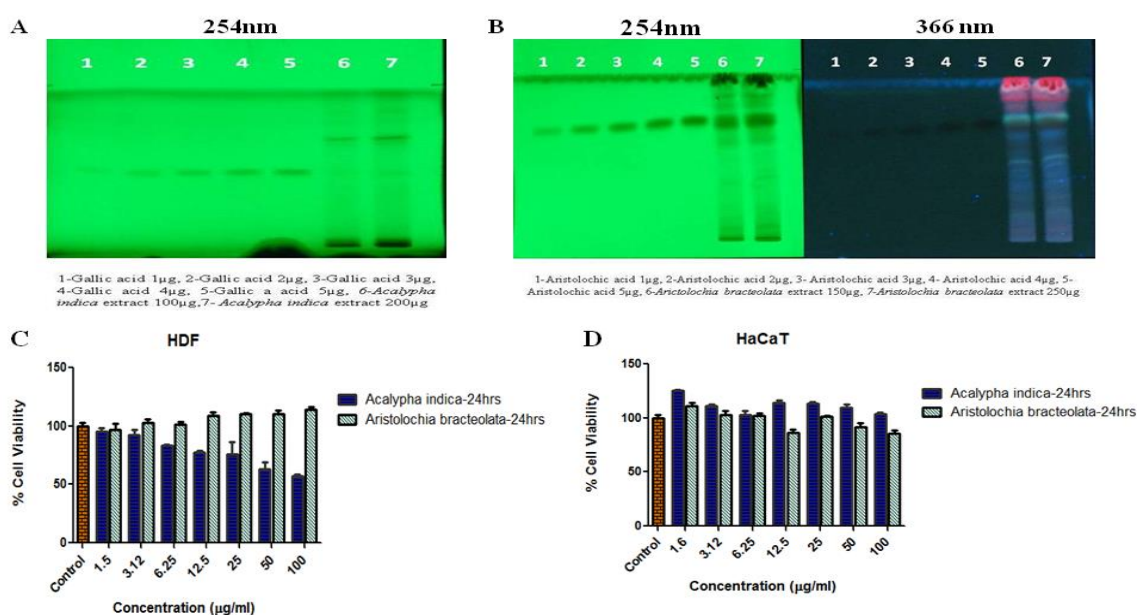


Figure 1: HPTLC fingerprinting of *Acalypha indica*(A) and *Aristolochia bracteolata* (B), Graphical representation of the percentage of Human Dermal Fibroblasts(C) and Human dermal keratinocytes (D) surviving the treatment with plant extracts after 24 h

Figure 2: Phase contrast images of Scratch wound assay on HDF cells after 24 hrs (A) and 48 hrs (B). Magnification – 20X

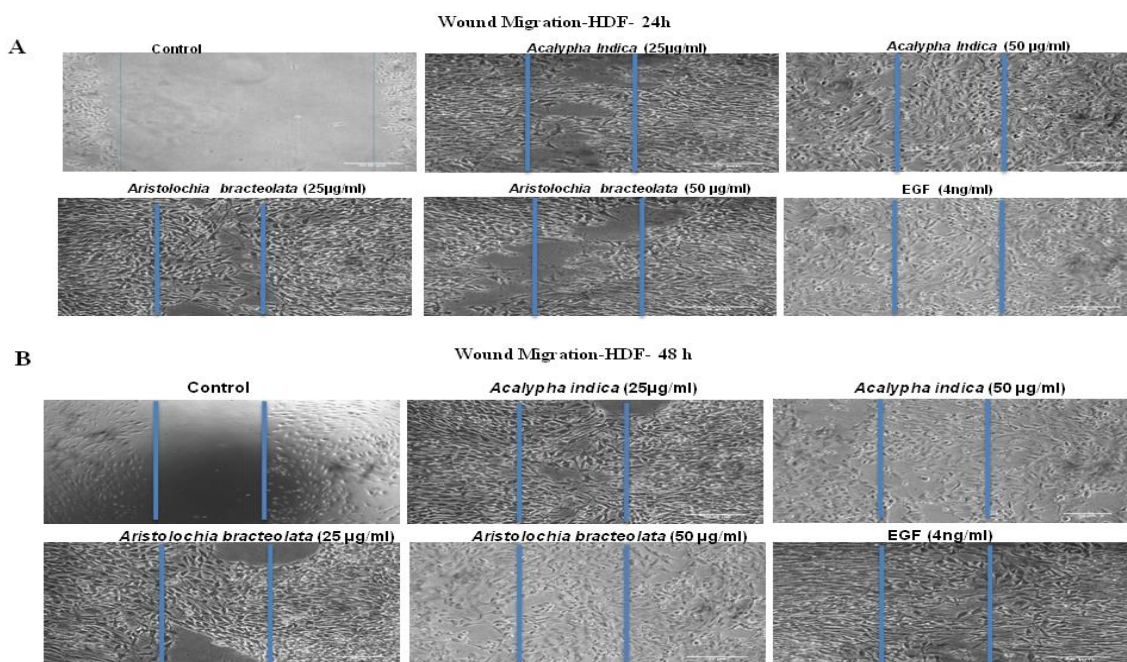


Figure 2: Phase contrast images of Scratch wound assay on HDF cells after 24 hrs (A) and 48 hrs (B). Magnification – 20X

Figure 3: A) Phase contrast images of scratch wound assay on HaCaT cells after 24 & 48hrs. B) Graphical representation of rate of wound closure. Magnification – 20X. C) Analysis of mRNA expression of remodelling enzymes, extracellular matrix components associated with wound repair by RT-PCR analysis

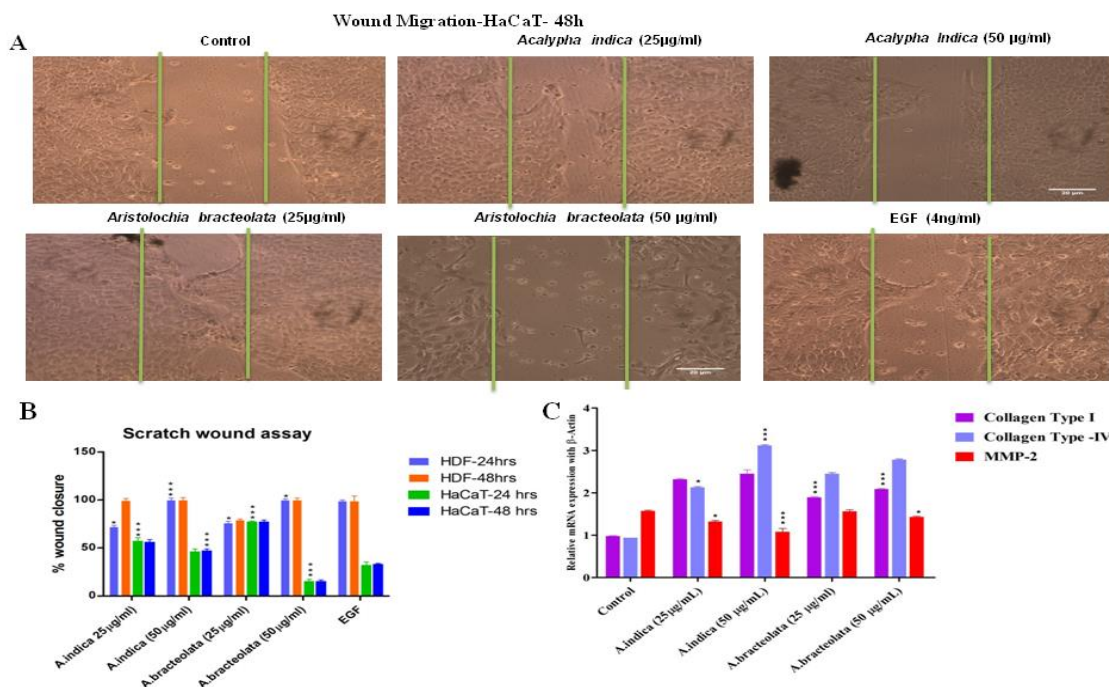


Figure 3: A)Phase contrast images of scratch wound assay on HaCaT cells after 24 & 48hrs. B) Graphical representation of rate of wound closure . Magnification – 20X. C) Analysis of mRNA expression of remodelling enzymes, extracellular matrix components associated with wound repair by RT-PCR analysis

Figure 4: Images of CAM before and after the treatment of *Acalypha indica* (A), *Aristolochia bracteolata* (B), Control and standard beta-sitosterol (C) and Graphical representation of percentage of angiogenesis (D).

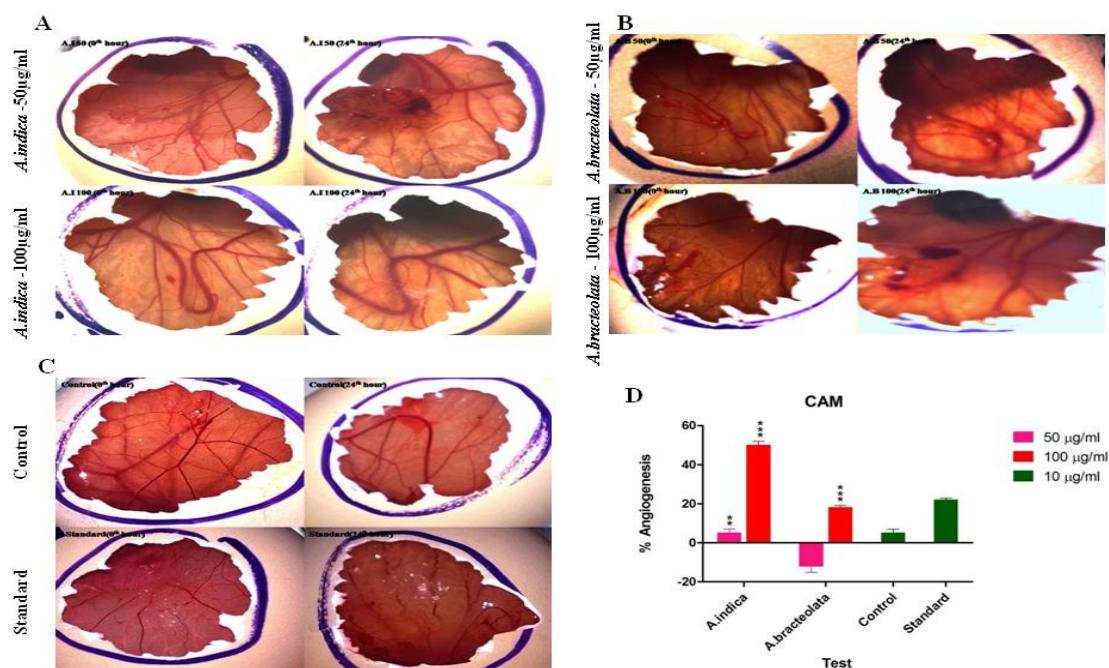


Figure 4: Images of CAM before and after the treatment of *Acalypha indica* (A), *Aristolochia bracteolata* (B), Control and standard beta-sitosterol (C) and Graphical representation of percentage of angiogenesis (D).

4. DISCUSSION

Wound healant herbs are classically applied extemporaneously as aqueous pastes. But aqueous extract serves as a source for the growth of micro-organisms which is attributed to the enzyme polyphenol oxidase that degrades polyphenols in water [16]. Thus for modern day proprietary herbals, the dried plant material is solvent extracted with ethanol for a potent representative remedial portion having a much longer shelf life.

Flavonoid and phenolic anti-oxidant composition is presumed to be largely contributory to the wound healing efficacy of the prepared extracts by preventing and protecting the cells from oxidative damage. The same extracts were quantified by HPTLC for Gallic acid content (0.2% w/w) for *A. indica* and for marker Aristolochic acid (2.5% w/w) in case of *A. bracteolata*. Gallic acid being reported from the ethanolic extract of the leaves, [6] its HPTLC quantification is taken in lieu of specific marker of *A. indica* as acalyphine and acalyphamide, not being commercially available. Gallic acid is presumed to be a potent antioxidant that directly upregulates the expression of genes associated with the antioxidant property. They also accelerate the migration of Keratinocytes and fibroblasts [17].

Proliferation and migration of HDF and HaCaT cells are the critical components of restorative wound healing. Proliferative phase is initiated by macrophages and progressed by fibroblasts. MTT assay established the cytocompatible dose range and indicated proliferative capability of the tested extracts towards HDF and HaCaT cells [1], [15]. The wound closure capability assessed by scratch wound assay, a rapid, convenient and cost effective method demonstrated the mediation of second phase of wound healing by *A. indica* and *A. bracteolata*. *A. indica* and *A. bracteolata* at the concentration of 50 µg/ml stimulated the HDF cells to close the wounded area entirely and 25 µg/ml of both the extracts closed HaCaT cells partially. The complete closure action on HDF is a positive inductive event because fibroblasts are involved in tissue granulation, collagen metabolism and restoration of the structure. As reported by Zubair M *et al.* 2012 Stimulation of cellular migration and repair of injured dermis are facilitated by polyphenol rich ethanol extracts [18]. Ours being the flavonoid and phenol rich ethanolic extract, might be the reason for the rapid HDF migration and wound closure.

The process of cellular migration involves various extracellular proteinases, particularly those belonging to the matrix metalloproteinase (MMP)

families. In a study reported by Ganeshkumar *et al.* 2012, Increase in the expression of Col 1a and Col 3a has been observed in the animal models when treated with *A.indica* [8]. In our study mechanistic behaviour of the extracts towards the complete closure of HDF scratch was evaluated by monitoring the genetic expression of MMP and Collagen families. There has been a significant expression of MMP-2 in HDF cell line by *A.indica* and *A.bracteolata* relative to control. MMP-2 localizes mainly in fibroblasts and plays a role in tissue granulation. They also participate in the basement membrane remodelling during angiogenesis, their activation is a key factor in cell migration. Our RT-PCR results confirmed the upregulation of type I and type IV collagen transcripts in the *A.indica* and *A.bracteolata* treated group. During the disruption of tissues following injury, collagen I and IV repairs the defect and restores the anatomic structure and function.

Reepithelization of tissues is associated with angiogenesis, which involves the process of blood vessel formation to supply the tissues with sufficient nutrients essential for proliferation, growth and migration of cells. Complete reconstruction of the tissue fails with improper angiogenesis. CAM assay showed strong angiogenic activity of *A.indica* at 100µg/ml, whereas biphasic angiogenic effect has been observed for *A.bracteolata* in which, at higher concentration (100 µg/ml) it showed significant angiogenic activity and at a lower dose (50µg) it showed anti-angiogenic activity. The anti-angiogenic activity might be due to the inhibition of the FGF-2 and VEGF growth factors which play a role in angiogenesis. Many flavonoids, polyphenols and terpenoids present in the plant extracts have shown to inhibit tumorigenesis, carcinogenesis and angiogenesis *in vitro* whereas saponins are thought to act on the angiogenic activity [19]. To elucidate the exact concentration and mechanism underlying the biphasic property of *A.bracteolata* further studies are needed to be carried out. For *in vitro* scratch wound assay, a lower dose (50µg/ml) was sufficient to heal the wound, whereas blood vessel formation (angiogenesis) occurred only at a higher dose (100µg/ml) as the assay involves the use of a whole living organism.

Apart from flavonoids, phenols and saponins, the metals present in the plant extracts plays significant roles in all the aforesaid processes. *A.indica* has been reported to have higher Iron content (159.59 ppm) followed by Calcium (69.7 ppm) whereas *A.bracteolata* has higher Nickel content (168 ppm) followed by Chromium (82.75 ppm).^[6] Higher iron content of *A.indica* helps in inducing unrestrained

proinflammatory macrophage activation, whereas calcium has an established role in homeostasis and serves as a modulator in keratinocyte migration and proliferation. The role Chromium in *A.bracteolata* is multifactorial involving in collagen synthesis, cell proliferation and immune function.

5. CONCLUSION

the mechanism of *A.indica* and *A.bracteolata* on wound healing process involves the activation of wound healing related genes of MMP-2, Collagen type 1 and Collagen type 4 in HDF cells. The elevated expression of MMP-2 facilitates essential events of wound migration, angiogenesis and tissue remodelling. Collagen 1 and 4 structures the ECM matrix during the tissue repair. The construction of new blood vessels (angiogenesis) by the plant extracts helps in supplying nutrients necessary for cell proliferation and migration. Enriched amount of antioxidants like phenols, flavonoids and metal components of *A.indica* and *A.bracteolata* like calcium, iron, nickel and chromium are thought to play a role in the aforesaid processes. Identification, isolation and fractionalisation of purified compounds may further elucidate the role of each compound in wound healing mechanism. *A.indica* and *A.bracteolata* were all favourable for wound healing both *in vitro* and *in vivo* [8, 11] and can surely be developed as therapeutic agent.

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CONFLICT OF INTEREST

The authors report no conflicts of interest

REFERENCES

- Schäfer M, Werner S. Transcriptional control of wound repair. Annu. Rev. Cell Dev. Biol. 2007 Nov 10; 23:69-92.
- Balekar N, Katkam NG, Nakpheng T, Jehtae K, Srichana T. Evaluation of the wound healing potential of *Wedelia trilobata* (L.) leaves. Journal of Ethnopharmacology. 2012 Jun 14;141(3):817-24.
- Karodi R, Jadhav M, Rub R, Bafna A. Evaluation of the wound healing activity of a crude extract of *Rubia cordifolia* L. (Indian madder) in mice. International Journal of Applied Research in Natural Products. 2009 Jun 2;2(2):12-8.
- Esimone CO, Nworu CS, Jackson CL. Cutaneous wound healing activity of an herbal ointment containing the leaf extract of *Jatropha curcas* L.(Euphorbiaceae).

- International Journal of Applied Research in Natural Products. 2008 Dec;1(4):1-4.
5. Kirtikar KR, Basu BD. Indian Medicinal Plant, vol. III. International Book Distributors. 1999. Dehradun, India.
 6. Nor, S.Z., Saidin, S., Zulkifli, R.M., Muhamad, I.I., Ya'akob, H., Nur, H., 2017. A review of *Acalypha indica* L. (Euphorbiaceae) as traditional medicinal plant and its therapeutic potential. J. Ethnopharmacol.
 7. Deep, M., 2001. J. Soc. Ethnobotany. 13-16.
 8. Ganeshkumar M, Ponrasu T, Krithika R, Iyappan K, Gayathri VS, Suguna L. Topical application of *Acalypha indica* accelerates rat cutaneous wound healing by up-regulating the expression of Type I and III collagen. Journal of Ethnopharmacology. 2012 Jun 26;142(1):14-22.
 9. Nataraj SK, Puvvada PK, Badami S, Patil SB, Kannan E, Thillainayagam S, Kodiyalam C, Bhojraj S. Pre-coital and post-coital anti-implantation and abortifacient activities of *Aristolochia bracteolata* Lam. aerial parts. Journal of Natural Medicines. 2007 Jul 1;61(3):302-6.
 10. Angalaparameswari S, Saleem TM, Alagusundaram M, Ramkanth S, Thiruvengadarajan V, Gnanaprakash K, Chetty CM, Pratheesh G. Anti-microbial activity of aristolochic acid from root of *Aristolochia bracteata* Retz. Inter. J. Bio. Life Sci. 2012; 8:2-4.
 11. Nandhini DU, Rajasekar M, Venmathi T. A review on worm killer: *Aristolochia bracteolata*. J. Pharmacog and Phytochem. 2007; 6, 6-9
 12. Shirwaikar A, Somashekar AP, Udupa AL, Udupa SL, Somashekar S. Wound healing studies of *Aristolochia bracteolata* Lam. with supportive action of antioxidant enzymes. Phytomedicine. 2003 Jan 1;10(6/7):558.
 13. Pourmorad F, Hosseini-mehr SJ, Shahabimajd N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. African journal of biotechnology. 2006;5(11).
 14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 1983 Dec 16;65(1-2):55-63.
 15. Liang CC, Park AY, Guan JL. *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nature protocols. 2007 Feb;2(2):329.
 16. Bharathajothi P, Bhaaskaran CT. Phytochemical and Pharmacological evaluations of *Aristolochia bracteolata* Lam. Asian Journal of Plant Science and Research. 2014;4(6):15-9.
 17. Yang DJ, Moh SH, Son DH, You S, Kinyua AW, Ko CM, Song M, Yeo J, Choi YH, Kim KW. Gallic acid promotes wound healing in normal and hyperglucidic conditions. Molecules. 2016 Jul 8;21(7):899.
 18. Zubair M, Ekholm A, Nybom H, Renvert S, Widen C, Rumpunen K. Effects of *Plantago major* L. leaf extracts on oral epithelial cells in a scratch assay. Journal of Ethnopharmacology. 2012 Jun 14;141(3):825-30.
 19. Jadhav J, Mane A, Kanase A. Antiangiogenic properties of *Boerhaavia diffusa* extracts in chick Chorioallantoic Membrane (CAM). Int. J. Drug. Dev. Res. 2011; 3:307-317.