Original Article

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Synergistic Wound Healing Effects of *Hibiscus rosa-sinensis* and *Centella asiatica* Extracts Combination (HRSCA): in vivo and 3D Organotypic Models

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Abstract:

Objective: *Hibiscus rosa-sinensis* and *Centella asiatica* are medicinal plants used as an alternative therapy for wound healing, and many studies have reported evidence of their effectiveness in treating wounds. This study aimed to evaluate the histological and molecular changes observed in the healing properties of a *Hibiscus rosa-sinensis* and *Centella asiatica* extracts combination (HRSCA) on wounds.

Material and Methods: Twenty-four rats were randomly divided into 4 groups (untreated, 10% Povidone-Iodine solution; 5.0% and 10.0% HRSCA). The excisional wounds were inflicted using a tissue biopsy punch and treated once daily for 14 days. The wound healing properties and tensile strength test were measured. A 3D organotypic skin model using human keratinocytes and human dermal fibroblast cell lines in a collagen type 1 scaffold was developed to further evaluate the wound healing properties. Full-thickness wound was induced and was either treated with 5% HRSCA or left untreated. qRT-PCR was used to evaluate the affected genes involved in the wound healing pathway.

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Results: The in vivo model showed no significant differences in tensile strength between groups. However, HRSCA treatment significantly enhanced wound healing. The 3D assay revealed HRSCA modulated genes like COL53A, CSF2, CXCL11, CXCL5, IL6ST, ITGA5, PLAT, and WISP1.

Conclusion: HRSCA enhanced wound closure in *in vivo* and 3D organotypic skin models, potentially through several pathways, such as inflammatory as well as extracellular matrix and adhesion pathways. The findings from this study provide substantial evidence regarding the wound healing potential of HRSCA.

Keywords: 3D organotypic cell culture, *Centella asiatica, Hibiscus rosa-sinensis*, *in vivo,* wound healing

Introduction

Wound healing involves complex biological mechanisms. Traditionally, wound healing is defined by 4 phases: hemostasis, inflammation, proliferation, and remodeling[1]. These phases occur in a cascading and overlapping manner[2]. However, after many advancements, wound healing is much more complex than the 4 phases described previously. Instead, it is a complex series of interactions involving cells and mediators[2,3].

As the dominant component of the epidermis layer of the skin, keratinocytes play essential functions in skin repair. This includes a barrier that prevents the entrance of foreign bodies and filters UV radiation[4]. As the skin is injured, keratinocytes are the initiators of the re-epithelialization process by undergoing proliferation, migration, and differentiation in order to reconstruct the epidermis layer. This process is regulated by various cytokines, growth factors, chemokines, and matrix metalloproteinases (MMPs)[5]. Throughout the process, keratinocytes work together with fibroblasts in contracting the wound[6].

Fibroblasts, in contrast, are responsible for the deposition and remodelling of the extracellular matrix (ECM), as well as for contracting the wound[4]. Hence, fibroblasts are essential in the inflammatory, proliferation, and remodelling phase of skin wound healing[7]. Activation of dermal fibroblasts at the wound site is initiated by growth factors. Fibroblasts secrete new collagen fibres to fill the wound site. This can be observed 3 days after wound creation[8]. The collagen pattern secreted is important as densely packed collagen fibres lead to scar formation and, in some cases, excessive scarring (fibrosis), while insufficient collagen production can lead to dehiscence. In short, these conditions are not favourable as they result in altered or loss of structure and weakened tissue, and hence, loss of its function as a physical barrier to the external environment[4,9].

Hibiscus rosa-sinensis is a native of Southeast Asia, where it is wildly cultivated as an ornamental plant in tropical and sub-tropical regions[10]. The leaves and flowers are reported to be effective in promoting ulcer healing and hair growth[11]. Studies have also reported that ethanol extracts of *Hibiscus rosa-sinensis* flowers enhance wound healing[12-14]. Aqueous and methanol extracts of *Hibiscus rosa-sinensis* leaves have shown promising results in accelerating the wound healing process, as an increase in the skin's tensile strength and uniform collagen arrangement formation was observed[15].

Previous studies on *Centella asiatica* extracts have reported their potential as an antioxidant, antimicrobial agent, agent of collagen synthesis, and even as a wound healer[16-18]. Most studies report asiaticoside as the active constituent producing the mentioned effect. In various wound healing models, topical application (0.2-0.4%), injection (1 mg) or ingestion (40 µg/disk) of asiaticoside have been shown to increase hydroxyproline content, improve tensile

strength, increase collagen synthesis and remodeling of the collagen matrix, promote epithelialization, stimulate glycosaminoglycan synthesis, and elevate antioxidant levels[19–20].

Both *Hibiscus rosa-sinensis* and *Centella asiatica* have shown promising results in the wound healing process when given separately. *Hibiscus rosa-sinensis* has been reported to improve wounded skin strength[15], while *Centella asiatica* has been reported as a potent wound healer[17]. However, the effects of a combination of these 2 plants in the wound healing process have never been studied. The wound healing process is regulated by various pathways. Therefore, this project aimed to identify the histological changes of the wound healing properties in the *in vivo* model and reveal the potential molecular pathway mechanism by the *Hibiscus rosa-sinensis* and *Centella asiatica* (HRSCA) extracts combination using a 3D organotypic skin model.

Material and Methods

Plant material

Dried and powdered leaves of *Hibiscus rosa-sinensis* and the aerial part of *Centella asiatica* were purchased from Herbagus Sdn. Bhd. (794624-D). The plants were authenticated by botanists from the Institute of Bioscience, Universiti Putra Malaysia (IBS, UPM). Voucher specimens (MFI 0159/20 *Hibiscus rosa-sinensis* and MFI 0160/20 *Centella asiatica*) were deposited at the Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns).

Plant extraction

Hibiscus rosa-sinensis was extracted from its leaves, according to the method in Ali et al.[15]: 200.0g of plant sample was macerated in 1000ml of 95.0% ethanol for approximately 24 hours. The extract was filtered through muslin cloth before being filtered through Whatman filter paper no 1. Filtered extract was evaporated and lyophilized.

Ethanolic extract of *Centella asiatica* was prepared using the reflux method[16]: 180.0g of powdered sample was refluxed in 1800.0 ml of 95.0% ethanol at 60 °C for 1h. The extract was filtered through Whatman filter paper, evaporated and freeze dried; 2.5 mg and 5.0 mg of the powdered extracts were added into 0.05ml of distilled water to obtain the 5.0% w/w and 10.0% w/w concentration.

Experimental animals

Twenty-four healthy male Wistar albino rats (8 weeks) weighing 250.0±50.0 g were used in this study. Animals were housed in plastic cages in a 12 h day/light cycle environment at room temperature and were given standard chow and water ad libitum. Four rats were housed in a single plastic cage and acclimatised for 5 days. These animal experiments were performed at the animal facilities in Universiti Teknologi MARA (UiTM) and were conducted in accordance with the Guide for Care and Use of Laboratory Animals of UiTM. The protocol was approved by the UiTM Committee of Animal Research and Ethics (UiTM CARE: 375/2022).

Excision wound and treatment

All 24 rats were anesthetized with intraperitoneal ketamine 100mg/ml and xylazine 100.0mg/ml 0.1ml/100.0mg IP prior to the excisional wound infliction. The hair located at the dorsal area of the rats was removed using a hair clipper; 70.0% ethanol was used to disinfect the surgical area, and then 8.0mm^2 in diameter circular excisional wounds were made using a tissue biopsy punch. Three full-thickness excision wounds were made using a biopsy tissue puncher with a diameter of 8.0mm.

The wound-inflicted rats were divided equally (n=6) into 4 groups as follows: (i) Untreated, (ii) Positive control (10.0% w/w Betadine® solution), (iii) 5.0% w/w of HRSCA, (iv) 10.0% w/w HRSCA. The rats were treated once daily for 14 days, and were monitored every day for any behavioural

changes or abnormal changes, infection and adverse effects after the topical application. After 14 days of treatment, the rats were sacrificed in a CO_2 chamber.

Wound contraction

The wound healing progress can be assessed by measuring the rate of wound contraction, which is manifested as a percentage reduction of the original wound size. The wound contraction of the rats was measured every alternate day on days 0, 2, 4, 6, 8, 10, 12, and 14 using a transparent polythene sheet on the wounded margin, which was then placed on a 1.0 mm^2-scale graph paper. The percentage of wound size contraction was calculated using the formula:

Period of Reepithelization

Reepithelization is the process of resurfacing a skin wound with a new epithelium. The reepithelization period is the time required for the resurfacing process to be completed and for the scabs to start falling off the wound site. The period of reepithelization of each rat from all the groups was closely observed and recorded from day 0 to day 14.

Tensile strength

Tensile strength was measured using a tensiometer (XT-plus Texture Analyzer); 5.0X1.5cm skin stripes were excised carefully from the rats. The strips were then clipped tightly onto the bottom and top clamp of the tensiometer machine. The distance between the 2 clamps was 5.0 cm, and the test was set with a constant crosshead speed of 100.0 mm/min. The machine was programmed to run until the skin sample tore. The extensibility (mm) of the skin and the tensile strength (g/mm2) were recorded.

Histological evaluation

At the end of the study, 1.5x1.5 cm of the wound skin was excised for biopsy samples, and then they were fixed in 10.0% formalin for 24 hours. The samples were paraffin-embedded and sectioned into 5.0µm thickness. The slides were stained in Hematoxylin and Eosin (H&E) and special staining Masson's Trichrome (MT). The histological changes were examined under a light microscope with ×20 and ×40 objective lens. For H&E staining, the collagen fiber orientation was observed, while for MT staining, the differentiation between mature and immature collagen was observed based on the color density of the stained collagen fibers.

Cell culture

Human Dermal Fibroblast (CRL2522) and Human Keratinocyte Cell (HaCaT) were purchased from the American Type Culture Collection (ATCC) and Thermo Fischer, respectively. The cells were maintained according to providers' instructions. Briefly, the cells were cultured in 50ml tissue culture flasks in 10.0 ml complete media (Dulbecco's Modified Eagle Medium (DMEM)), 10.0% Fetal Calf Serum, 1.0% Streptomycin and 1.0% Penicillin), incubated in 5.0% CO_2 at 37 °C. All media and supplements were supplied by Sigma–Aldrich (Aston Manor, South Africa) and Nacalai Tesque (USA), unless otherwise stated.

3D organotypic co-culture skin model

For 3D organotypic co-culture, CRL2522 were seeded in a collagen mixture (PureCol collagen, 10x Phosphate Buffered Saline (PBS), distilled water in an 8:1:1 ratio. pH was adjusted to pH7-7.4 with 0.1M NaOH) and allowed to gel. Keratinocyte cells were then cultured on top of the jellified fibroblast layer. Next, the 3D organotypic co-culture skin model was incubated in 5.0% CO_2 at 37 °C for 2 weeks until stratification of the layers could be observed before proceeding to assays.

Biopsy punch

Cell migration was assessed using punch biopsy assay. Wound was generated by punching 2.0mm punch

at full depth in the gel layer. The punched cells were then incubated for another 16 hours and 24 hours at 37°C with 5.0% CO2. The co-cultures were treated with the HRSCA (3:2 ug/ml) in order to evaluate their effects on re-epithelialization. Tissue samples were taken and frozen at -80 °C for gene expression profiling analysis.

RNA isolation and cDNA synthesis

Tissues were homogenised with a TissueRuptor® II homogenizer (Qiagen, Germany) and processed for RNA extraction using RNeasy plus mini kit (Qiagen, Germany), according to the manufacturer's instructions. Eluted RNA was quantified on a Quantus Fluorometer using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA) and stored at -80°C until further use.

Human Wound Healing RT^2 PCR Profiler arrays (Qiagen, Germany) were performed according to the manufacturer›s instructions. Briefly, 400.0 ng of RNA template was added to the genomic DNA elimination mix (RT2 First Strand kit, Qiagen, Germany) to a total volume of 10.0 µL and incubated for 5 min at 42 °C, followed by 1-minute incubation on ice. The reverse transcription mixture was added to a total volume of 20.0 µL, and the mix was incubated for 15 min at 42 °C and 5 min at 95 °C. The samples were diluted by adding 91 µL RNase-free water, and frozen at –30 °C. cDNA product was mixed with SYBR Green Mastermix, RNase-free water and amplified using LightCycler 480 (Roche, Indianapolis, IN, USA).

Gene expression profiling

The Qiagen human wound healing RT2 Profiler PCR Array System (Qiagen, PAHS-121ZA) was used to evaluate 84 genes (Table 1) that include genes involved in the ECM, remodelling enzymes, cellular adhesion, cytoskeleton,

Table 1 Genes evaluated using the human wound healing RT2 Profiler PCR Array System. Wells A1 through G12 contained genes involved in wound healing. Wells H1 through H5 contained housekeeping genes, H6 contained a human genomic DNA control (HGDC), H7 through H9 contained replicate reverse transcription controls (RTC), and H10 through H12 contained replicate positive PCR controls (PPC)

Pathways	Genes
Extracellular matrix & cell adhesion	
ECM components	COL14A1, COL1A1, COL1A2, COL3A1, COL4A1, COL4A3, COL5A1, COL5A2, COL5A3, VTN
Remodelling enzymes	CTSG, CTSK, CTSV, CTSL2, F13A1, F3 (Tissue Factor), FGA (Fibrinogen), MMP1, MMP2, MMP7, MMP9, PLAT (tPA), PLAU (uPA), PLAUR (uPAR), PLG, SERPINE1 (PAI-1), TIMP1
Cellular adhesion	CDH1 (E-cadherin), ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGAV, ITGB1, ITGB3, ITGB5, ITGB6
Cytoskeleton	ACTA2 (a-SMA), ACTC1, RAC1, RHOA, TAGLN
Inflammatory cytokines & chemokines	CCL2 (MCP-1), CCL7 (MCP-3), CD40LG (TNFSF5), CXCL1, CXCL11 (ITAC/IP-9), CXCL2, CXCL5 (ENA-78/LIX), IFNG, IL10, IL1B, IL2, IL4, IL6
Growth factors	ANGPT1, CSF2 (GM-CSF), CSF3 (GCSF), CTGF, EGF, FGF10, FGF2, FGF7, HBEGF (DTR), HGF, IGF1, MIF, PDGFA, TGFA, TGFB1, TNF, VEGFA
Signal transduction	
TGFßx	TGFB1, TGFBR3, STAT3
WNT	CTNNB1, WISP1, WNT5A
Phosphorylation	MAPK1 (ERK2), MAPK3 (ERK1), PTEN
Receptors	EGFR, IL6ST (GP130)
Other	PTGS2
Housekeeping genes	B2M, HPRT1, RPL13A, GAPDH, ACTB

ECM=extracellular matrix, TGFßx=transforming growth factor beta, WNT=Wnt pathway

inflammatory cytokines and chemokines, growth factors and signal transduction.

Briefly, cDNA was thawed and added to the master mix (containing ROX as a reference dye and SYBR green); 25.0 µl of the mixture was added to each well. Real-time qPCR was performed on the Bio-Rad CFX96 (BioRad Laboratories, USA) using the following cycles: 1 cycle at 95 °C for 10 min, 40 cycles at 95°C for 15 s, and 1 min at 60 °C. A dissociation (melting) curve was performed at 95 °C for 1 min and 65 °C for 2 min, and readings were taken at 65 to 95 °C at 2 °C per minute intervals. A single peak at temperatures greater than 80 °C had to be obtained.

Threshold cycle (Ct) values greater than 35 were considered negative. Ct values were exported to an Excel-based Data Analysis Template (available from the GeneGlobe website) with the appropriate pathway-focused genes. Results were normalised against an average of all 5 housekeeping/reference genes. The program carried out all the necessary calculations (delta-delta Ct method) and interpretations of the control wells. Fold-change was also calculated and reported as fold up-regulation if greater than 1, and fold down-regulation if less than 1. Untreated normal organotypic skin cultures were used as a control.

Results

Wound contraction

Starting from treatment day 0 to the end day 14, a significant reduction in the size of the excised wound was recorded (Figure 1). Rats treated with 10.0% HRSCA showed significantly smaller wound contraction at day 4, 12, and 14 compared to the untreated group, whilst rats treated with 5.0% HRSCA had significantly smaller wound contraction at day 4 and 14 (Table 2).

Period of reepithelization

10.0% HRSCA was shown as the fastest period of epithelization as compared to the negative control, the positive control, and 5.0% HRSCA, which was 10.8±0.9 days (Table 3). There was no significant difference between 5.0% HRSCA, and 10.0% HRSCA in the epithelialization period. However, both extract treatments elicited a significant (p-value<0.05) shortening of the epithelialization period, as compared to the negative control group.

control, the positive control (10.0% w/w Betadine® solution), 5.0% w/w of HRSCA and 10.0% w/w HRSCA treated groups across post-wounding days in the excision model.

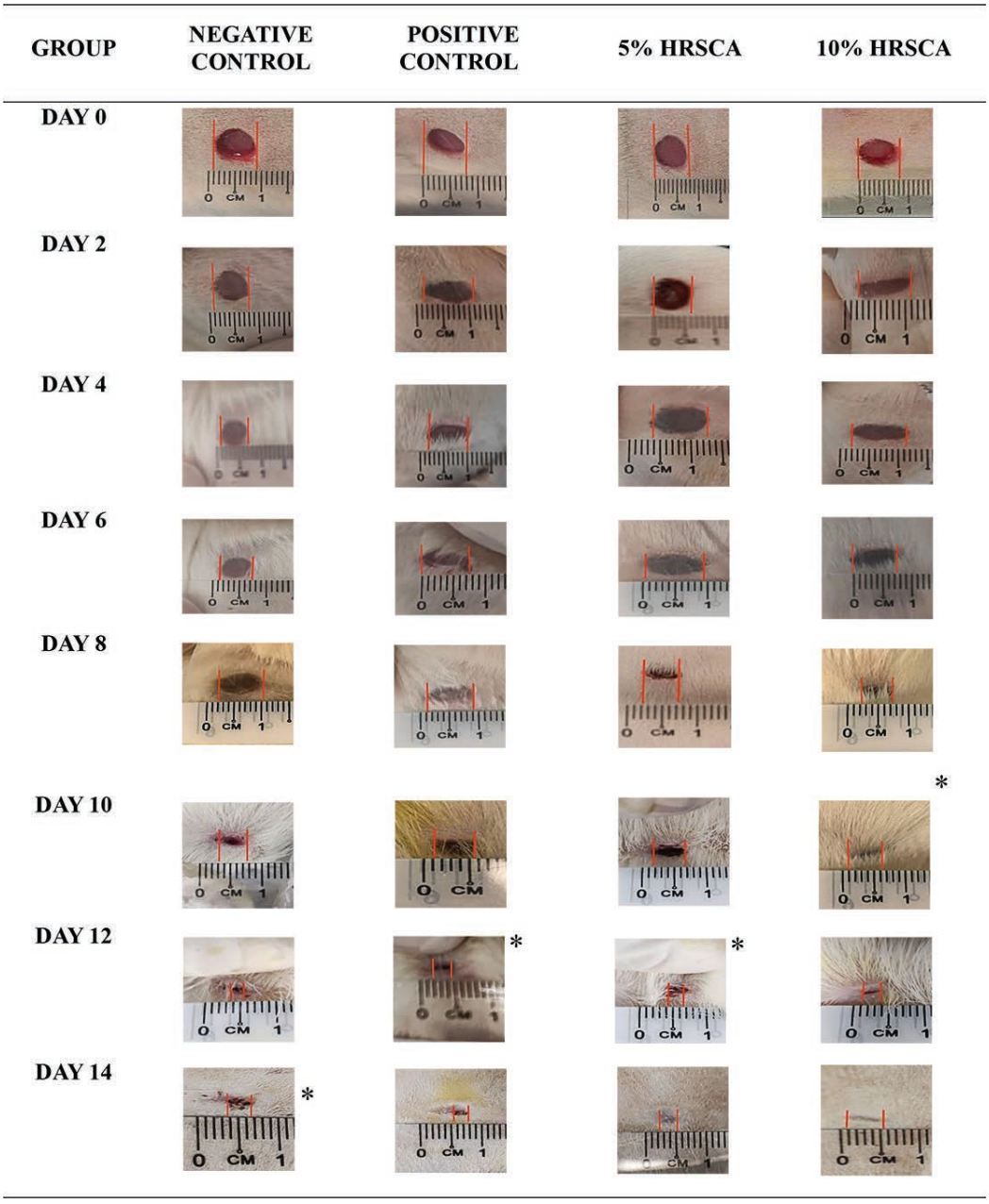
Tensile strength

Both 5.0% and 10.0% HRSCA extract produced higher tensile strength in wound healing in comparison with the negative control groups, which was 62.7±6.1 g/mm^2 and 68.4±33.8 g/mm^2, respectively (Figure 2). Although the tensile strength showed improvement, the difference between the 2 concentrations in terms of their effect on wound healing was not significant compared to the control groups.

There is a limitation to this study as the measurement was done only on day 14. During this period almost all the skin had fully recovered. Besides that, due to the small sample size, individual differences could heavily impact the standard deviation, making it appear larger than it might be in a larger, more uniform sample.

Histologic changes

The amount of granulation tissue observed in the slides stained with H&E staining was scant in tissues from rats treated with HRSCA, moderate in the positive control group and profound in the negative control group. The presence of a substantial amount of granulation tissue in the untreated and positive control groups on day 14 suggests a delayed wound healing process. This finding indicates that the wounds remain open and have not progressed to the remodeling phase, which is essential for wound closure and tissue restoration.



(*) indicates the reepithelization period: the day of scabs fell off

HRSCA=*Hibiscus rosa-sinensis* and *Centella asiatica* extracts combination

Figure 1 Wound contraction progress in the negative control, the positive control (10.0% w/w Betadine® solution), 5.0% w/w of HRSCA and 10.0% w/w HRSCA treated groups across post-wounding days in excision model

Table 2 Percentage of wound contraction among the groups from day 0 until day 14

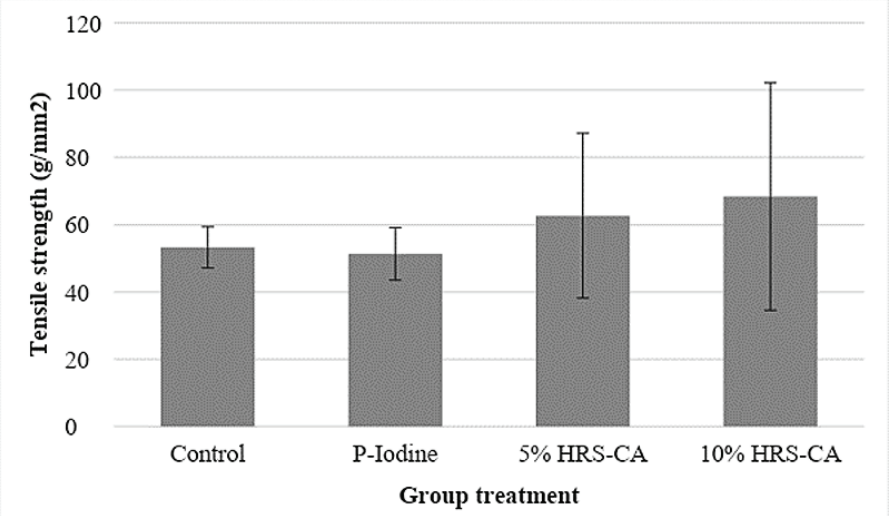
	Wound contraction (mm^2) (%)			
Treatment day	Negative Control	Positive Control	5% HRSCA	10% HRSCA
0	8±0	8±0	8±0	8±0
2	7.17±0.47 (10.3)	6.50±0.65 (18.8)	6.50±0.65 (18.8)	6.25±0.25 (21.9)
4	6.08±0.53 (24.0)	5.67±0.37 (29.1)	4.92±0.45 (38.5)**	5.17±0.47 (35.4)*
6	4.92±0.79 (38.5)	4.67±0.69 (41.6)	4.17±0.47 (47.9)	4.17±0.37 (47.9)
8	3.92±0.84 (51.0)	3.83±0.47 (52.1)	3.58±0.34 (55.3)	3.17±0.55 (60.4)
10	3.17±0.85 (60.4)	2.92±0.34 (63.5)	2.50±0.41 (68.8)	2.17±0.55 (72.9)
12	2.50±0.58 (68.8)	2.42±0.34 (69.8)	1.83±0.37 (77.2)	1.42±0.45 (82.25)**
14	1.92±0.34 (76.0)	1.67±0.24 (79.1)	1.33±0.37 (83.4)*	0.75±0.25 (90.6)**

Values are Mean±standard deviation (S.D.) (n=6),*p-value<0.05 as compared to negative control by ANOVA (Tukey's test), **p-value<0.01 as compared to negative control by ANOVA (Tukey's test), HRSCA=*Hibiscus rosa-sinensis* and *Centella asiatica* extracts combination

Table 3 Effect of topical application of 5% *Centella asiatica* extracts combination (HRSCA) and 10% HRSCA on period of epithelialization (number of days) post-wound creation in rats

	Period of re-epithelization (day)		
Negative Control	Positive Control	5% HRSCA	10% HRSCA
13.8±0.37	12.2±0.37	11.83±0.37*	10.8±0.9*

Values are Mean±standard deviation (S.D.) (*n*=6), *p-value<0.05 as compared to negative control by ANOVA (Tukey's test), HRSCA=*Hibiscus rosa-sinensis* and *Centella asiatica* extracts combination



HRSCA=*Hibiscus rosa-sinensis* and *Centella asiatica* extracts combination

Figure 2 Tensile strength of the rats' skin after 14 days of different treatment. Results are presented as mean±standard deviation (S.D.)

In 10x magnification, telocytes (red arrows) were present in the untreated and positive control groups; meanwhile, they were absent in groups treated with HRSCA (Figure 3aa and 3ab). Telocytes are involved in the formation of new blood vessels in the proliferative stage[21].

Groups treated with HRSCA showed little inflammatory filtrate, whilst positive and untreated groups showed moderate and plenty of inflammatory filtrate, respectively. Inflammatory mediators were abundant during the inflammation stage. These inflammatory mediators release reactive oxygen species (ROS) in order to kill the invading pathogens, and prolonged exposure to ROS in the later stages of wound healing will contribute to the formation of scars[22]. Scar formation may impair the normal wound healing process.

Collagen fibres observed in the group treated with HRSCA showed horizontal orientation (yellow arrow) (Figure 3bc and 3bd), closer to the epidermis layer; meanwhile, collagen fibres were vertically oriented (brown arrow) (Figure 3ba and 3bb) at the epidermis in control groups. Fascicle collagen (blue arrow) (Figure 3bc and 3bd) was also noted in the HRSCA treated group, which indicates faster healing activity whilst reticular formation of collagen (black arrow) (Figure 3ba and 3bb) was present in the control groups, which indicates delayed healing.

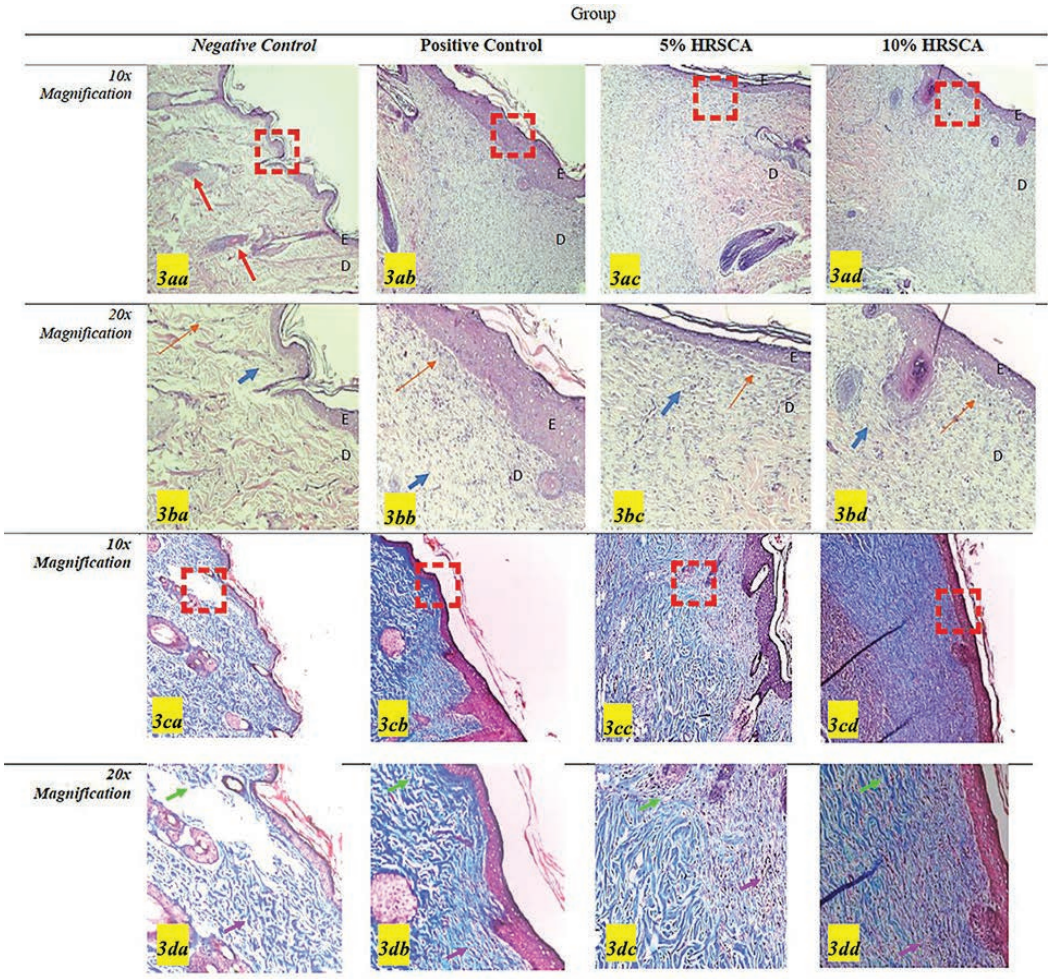
The skin samples were also stained with Masson's Trichrome stain to observe mature (green arrow) (Figure 3da–3dd) and new collagen fibres (purple arrow) (Figure 3da–3dd). In normal skin, the presence of a high amount of mature collagen was noted; however, there was an absence of early (immature) collagen. Groups treated with HRSCA showed a high amount of mature collagen, which indicates accelerated wound healing activities. The untreated and positive control groups showed a low and moderate presence of mature collagen, respectively.

Gene expression profiling

The profile of 84 genes involved in the wound healing of the 3D organotypic skin model treated for 14 days with HRSCA (5% w/v) was determined by RT–qPCR. Results were compared to the wounded untreated construct as the control group. A total of 12 genes were found to be overexpressed, while 34 genes were underexpressed.

A total of 12 genes were upregulated, reflecting enhanced cellular responses, specifically in inflammation, extracellular matrix (ECM) remodelling, cellular adhesion, and growth factor activity. Notably, the CD40LG gene, part of inflammatory cytokines and chemokines, was upregulated. This finding suggests that HRSCA treatment might activate immune responses, potentially leading to the increased recruitment of immune cells that could aid in clearing infection and supporting tissue repair at the wound site. The upregulation of CTSV and F3, both remodelling enzymes, indicates active ECM remodelling, a critical process in wound healing, where old or damaged tissue is replaced with new ECM components to restore tissue structure.

Furthermore, the increased expression of cellular adhesion molecules, such as ITGA6, ITGB6, and CHD1, suggests improved cell–matrix interactions. These molecules play essential roles in facilitating keratinocyte migration and adhesion to the ECM, which is fundamental for re–epithelialization. Upregulated growth factors, including HBEGF, IGF1, PDGFA, TGFA, and VEGFA, point to active cellular proliferation and angiogenesis. These factors are critical for forming new tissue and blood vessels, essential steps in wound closure and nutrient supply to the regenerating tissue. Only WISP1, a signal transducer involved in cell survival and proliferation, was significantly upregulated, suggesting a more targeted influence of HRSCA on specific signalling pathways that promote tissue repair.



HRSCA=*Hibiscus rosa-sinensis* and *Centella asiatica* extracts combination

Figure 3 Histological changes of wounded skin post-treatment using hematoxylin and eosin (H&E) (3a-3b) and Masson's trichrome (MT) (3c-3d) staining. E, epidermis; D, dermis; Red box, focus area in 20x magnification; Red arrow, telocytes (blood vessels); Yellow arrow, collagen fibres orientation (horizontal); Brown arrow, collagen fibres orientation (vertical); Blue arrow, fascicle collagen; Black arrow, reticular collagen, Green arrow, mature collagen; Purple arrow, new (immature) collagen

Meanwhile, 34 genes were downregulated, which may suggest a suppression of certain cellular functions or a regulatory shift in the wound healing response. Among these, the reduced expression of several cellular adhesion genes (ITGA1, ITGA5, ITGB1, and ITGB3) may imply a selective modulation of adhesion processes, potentially favouring only specific integrins necessary for effective cell migration and wound closure in this model. Downregulation of cytoskeletal components, ACTA2 and TAGLN, could indicate a less rigid cytoskeletal structure, which might facilitate cell migration over rigidity in the wound healing environment.

The downregulation of ECM components, such as collagen genes (COL1A1, COL1A2, COL3A1, COL5A1, COL5A2, and COL5A3), suggests that HRSCA may reduce the deposition of new collagen fibres, potentially influencing the stiffness of the wound bed. This might modulate scar formation by minimizing excessive ECM accumulation, which could lead to more pliable and natural tissue repair outcomes. The significant downregulation of genes such as COL5A3 could be particularly relevant in balancing collagen synthesis and degradation, possibly leading to an optimized remodelling phase without excessive fibrosis.

The reduction in growth factors (ANGPT1, CSF2, CSF3, EGF, FGF2, and FGF7) might suggest that HRSCA selectively activates only a subset of pathways required for wound healing, reducing potentially redundant or counterproductive signalling. Additionally, the downregulation of inflammatory chemokines (CCL2, CXCL1, CXCL11, CXCL2, CXCL5, and IL6) and receptor (IL6ST) could indicate a controlled inflammatory response, which is critical to preventing prolonged inflammation that can impair wound healing.

Of all these genes, only one gene was significantly upregulated (WISP1) while 7 were significantly downregulated (COL5A3, CSF2, CXCL11, CXCL5, IL6ST, ITGA1 and ITGA5) (Table 4).

Discussion

Wound healing is an automated and naturally occurring process in returning injured skin as close as possible to its original skin appendages and functions. Keratinocytes, fibroblasts, monocytes and endothelial cells are among the various cells that are involved in the wound healing mechanisms that play distinct roles in haemostasis, inflammation, cell proliferation, and the remodelling stages in order to restore the integrity of injured skin. In the early stages of wound healing, the exposed blood vessels will be closed to prevent excessive exsanguination, followed by the release of chemokines at the wound sites by keratinocytes

Table 4 Genes significantly regulated in 3D skin culture treated with *Hibiscus rosa-sinensis* and *Centella* asiatica extracts combination (HRSCA) compared to untreated skin culture (p-value<0.05)

Gene	Fold regulation	p-value	Summary
WISP1	7.02	0.016	This gene encodes a member of the WNT1 inducible signalling pathway (WISP) protein subfamily, which belongs to the connective tissue growth factor (CTGF) family
COL5A3	-4.38	0.023	Encodes alpha 3 chain for type V collagen
CSF2	-6.92	0.047	Encodes cytokine that controls the production, differentiation, and function of granulocytes and macrophages
CXCL11	-2.05	0.049	Mediate epidermal- dermal communication during wound repair (re-epithelialization)
CXCL5	-4.43	0.015	Promotes angiogenesis
IL6ST	-3.06	0.016	The protein encoded by this gene is a signal transducer shared by many cytokines, including interleukin 6 (IL6), ciliary neurotrophic factor (CNTF)
ITGA1	-4.62	0.012	This gene encodes the alpha 1 subunit of integrin receptors. This protein heterodimerizes with the beta 1 subunit to form a cell-surface receptor for collagen and laminin. The heterodimeric receptor is involved in cell-cell adhesion and may play a role in inflammation and fibrosis.
ITGA5	-8.68	0.001	The product of this gene belongs to the integrin alpha chain family. Integrins are heterodimeric integral membrane proteins composed of an alpha subunit and a beta subunit that function in cell surface adhesion and signalling

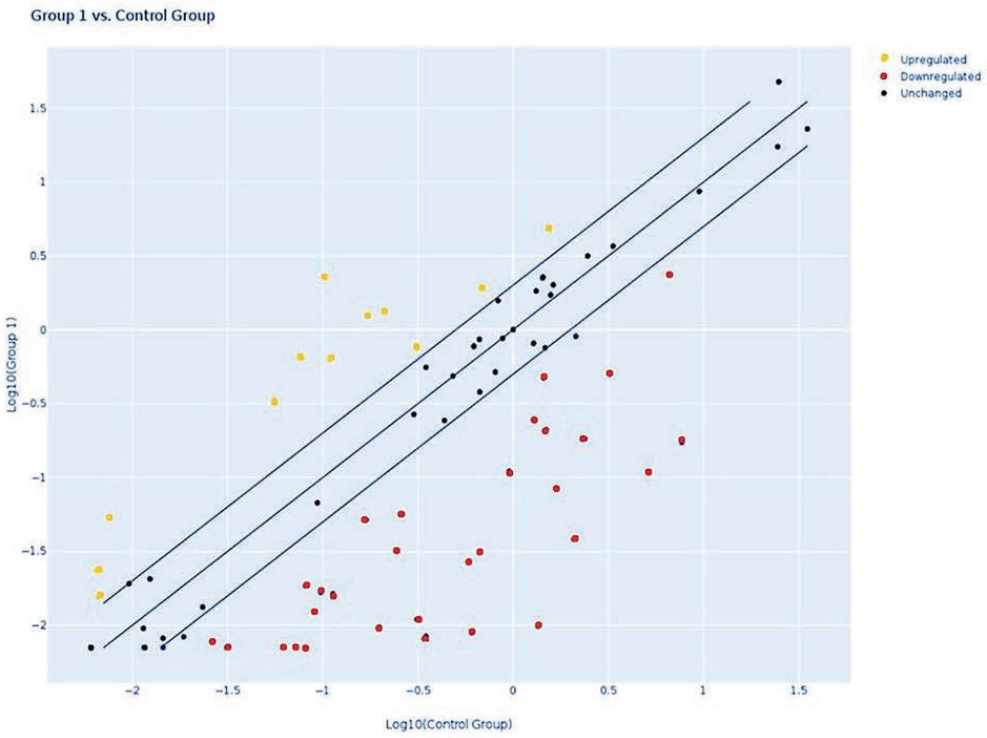


Figure 4 Summary of the gene expression of skin cultures treated with *Centella asiatica* extracts combination (HRSCA) compared to untreated via quantitative real-time polymerase chain reaction (qRT-PCR) Yellow dots represent the overexpressed gene, black dots represent genes neither over- or under-expressed, and blue dots represent genes underregulated

and other immune cells that will kill any invasive bacteria and foreign particles[5,23]. Vascular epithelial growth factors (VEGF) and basic fibroblast growth factors (bFGF) and other chemotactic factors will be released by keratinocytes, macrophages, neutrophils, and monocytes, which are essential for the healing processes during the proliferative stage[5,23]. Endothelial cells neighbouring the damaged blood vessels form new sprouts of blood vessels in the presence of VEGF. The formation of new blood vessels thus provides sufficient nutrients for cell growth and tissue regeneration in wound sites[9]. Fibroblast also proliferates in the presence of growth factors from platelets and macrophages. During the proliferative stage, the wound starts to shrink macroscopically as the fibroblast starts to synthesize high amounts of granulation tissue and collagen in the wound sites, where some of the fibroblasts are also differentiated into myofibroblasts, which help to contract the wound. The extracellular matrix (ECM) also develops during this stage. Maturation of collagen takes place during this stage until the wound is healed. In the later stage of wound healing, ECM remodelling occurs to replace the current ECM at the wound site, which is rich in type III collagen, with a stronger collagenous scar composed primarily of type I collagen.

HRSCA extract promotes the maturation of collagen in granulation tissue, which will be converted into contemporary ECM layers in the wound bed, resulting in less granulation tissue, as well as angiogenesis being present at the end of the treatment. The early collagen maturation

observed in the HRSCA-treated group promoted the wound healing process, with 83% of the wound closed. The HRSCA-treated group was also graded as good healing. Histologically, HRSCA-treated groups were shown to have faster wound contraction, neovascularization, and collagen reorganization, with a smaller size of wound contraction and shorter period of epithelization, as well as less granulation tissue post-treatment.

Furthermore, the gene profiling analysis showed growth factor genes that were responsible for kick-starting the cell proliferation, migration, and differentiation of fibroblast and keratinocyte cells that were upregulated in the HRSCA-treated wound[25,26]. Growth factors play important roles in both the proliferation and remodelling phase of wound healing[27]. The findings from this study suggest that the HRSCA-treated wound is at the proliferation phase of healing. CSF2 and CSF7 genes, on the contrary, were moderately down-regulated, most likely due to the same factor[28].

Genes involved in inflammatory cytokines and chemokines, as well as the cytokine signal transducer (IL6), were downregulated. Keratinocyte cells release C-X-C chemokine ligands, which are important for neutrophil recruitment, suggesting the difference in the wound healing stages between HRSCA and the control treatment[29]. As expected, ITGA5 and ITGB1, which were essential for IL1B signalling, were also downregulated[30]. In comparison to HRSCA-treated wounds, genes associated with the inflammatory phase are substantially expressed in the control wound, indicating that the wound is in the inflammatory phase of healing.

Interestingly, genes involved in collagen synthesis were either under-expressed or did not change in the expression level, indicating the degradation of collagen[31]. Collagen degradation is an important process of wound healing[32]. Collagen fragments act as angiogenic signals during angiogenesis, encouraging the growth of new blood vessels[32]. During the inflammation phase, immune cells, such as macrophages, that patrol the wound for the clearance of pathogens and devitalized tissue are attracted by the degraded collagen fragments that facilitate the transition into the proliferative stage[24]. Matrix metalloproteinases (MMPs) are collagenases and gelatinases that are responsible for degrading collagen fibres[33]. Fibroblast cells, in contrast, are responsible for synthesizing collagen fibres[7]. Regulation of collagen production by fibroblast cells and degradation by the enzymes is important for keeping balance in the turnover rate in order to avoid chronic wounds[7,34]. Excessive collagen degradation could result in impaired tensile strength and flexibility, while exaggerated collagen production, conversely, could lead to abnormal scarring, fibrosis, or keloid[32].

Conclusion

The rats treated with HRSCA had greater healing activities compared to the control groups and these findings are supported by the gene profiling analysis in the 3D organotypic co-cultured skin model, which showed the upregulation of growth factor genes, indicating that the wound was at the proliferation phase of healing.

To ensure the sustainability and applicability of HRSCA's wound healing effects, further studies are required. Long-term research should evaluate scar formation, tissue strength, and wound recurrence by extending the in vivo study duration and assessing tensile strength and elasticity at the various post-healing stages. Mechanistic studies using RNA sequencing or proteomics could reveal molecular pathways influenced by HRSCA, focusing on key genes like WISP1, COL5A3, and CXCL11.

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Conflict of interest

There are no conflicts of interest to declare.

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