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Research Article

Wound Healing and Cytotoxicity Effects of *Hilleria latifolia* and *Laportea Ovalifolia*

Susana Oteng Dapaah[1], Christian Agyare[1]*, Emelia Oppong Bekoe[2], Paul PokuSampane Ossei[3,4], Yaw Duah Boakye[1] and Emmanuel Akomanin Asiamah[3]

[1]Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

[2]Department of Pharmaceutics and Microbiology, School of Pharmacy, College of Health Sciences, University of Ghana, Legon, Accra, Ghana

[3]Department of Pathology, Komfo Anokye Teaching Hospital, Kumasi, Ghana

[4]School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

***Corresponding Author**: Christian Agyare, Department of Pharmaceutics, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

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Abstract

Wounds represent a major global health challenge and as a result the focus of scientists of today is shifted towards that angle. The study was therefore aimed at investigating the wound healing property of methanol leaf and root extracts of *Hilleria latifolia* (HLLE, HLRE) and methanol leaf extract of *Laportea ovalifolia* (LOLE), as well as their cytotoxic effect on Keratinocytes cell line. The excision wound model was used to investigate the wound healing activity of the extracts. Also the extracts effect on cell viability and LDH release were used to assess their cytotoxic effect. There was significant ($p < 0.0001$) increase in the rate of wound closure when treated with 5 and 10% of the extracts as compared to the untreated group. Histological studies on extracts-treated wound tissues revealed increase collagenation and re-epithelialisation. The viability of HaCaT-keratinocytes was reduced significantly ($p < 0.001$ and $p < 0.05$) when treated with 100 µg/mL of HLLE and HLRE respectively. There was also no significant release of LDH from the extracts-treated HaCaT-keratinocytes at the concentrations used. The extracts (HLLE, HLRE and LOLE) exhibited wound healing activity with increased collagenation and re-epithelialisation and had little or no cytotoxic effect on HaCaT-keratinocytes at the test concentrations.

Keywords: *Hilleria latifolia; Laportea ovalifolia; Excised wound; Aqueous cream; HaCaT-keratinocytes*

Abbreviations

HLLE: *Hilleria latifolia leaf extract*; HLRE: *Hilleria latifolia* root extract; LOLE: *Laportea ovalifolia* leaf extract; TGF: Transforming Growth Factor; FGF: Fibroblast Growth Factor; EGF: Epidermal Growth Factor; PDGF: Platelet-Derived Growth Factor; ECM: Extracellular matrix; LDH: Lactate dehydrogenase; HaCaT: Human adult high calcium low temperature; CO2: Carbon dioxide; PBS: Phosphate Buffer Solution; MTT:3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO: Dimethylsulfoxide; FCS: Foetal Calf Serum; HCL: Hydrochloric acid

Introduction

Wounds are physical, chemical or thermal break in continuity of the skin or its underlying tissues leading to disturbance in the normal skin anatomy and disruption of anatomical and functional integrity of living tissues [1]. Wounds normally serve as a passage or entry for infectious microbial agents into the inner tissues [1-3]. The occurrence of various forms of wounds has increased over the years and this

may be due to increased life expectancy in most countries especially the developed countries [4] and also in Ghana, taking into consideration the recent flood and fire disaster that hit the capital of our country.

Wounds can be healed through a natural complex process instigated immediately after injury, to repair damaged tissues or organs. The process consists of four (4) distinct but interconnected and overlapping phases, which occur in proper sequence and time frame to enhance successful healing [5,6]. These integrated phases are: haemostasis, inflammation, proliferation and tissue remodelling.

Haemostatic phase (coagulation) results in vascular constriction and fibrin clot formation to control active bleeding, leading to release of pro-inflammatory cytokines and growth factors like transforming growth factor (TGF)-β, fibroblast growth factor (FGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) to initiate the next phase [5].

Inflammatory phase is characterized by vasodilation of blood vessels and infiltration of neutrophils, which act to clear any invading microorganisms and cellular debris at the wound site with subsequent release of macrophages to continue the degradation of microbes and other tissue debris at the wound area [6].

Proliferative phase normally follows and overlaps with the inflammatory phase, with fibroblasts and endothelial cells being the most prominent cells present to enhance capillary growth, collagen formation, and the formation of granulation tissue at the injury site [5].

Tissue remodelling phase is characterised with extracellular matrix (ECM) remodeling to approach the normal or original tissue [7]. In this pahase regression of many newly formed capillaries takes place to cause the vascular density of the wound to return to normal. Also contractile fibroblasts (myofibroblasts) are believed to cause physical contraction of wounds. There is also an increase in tensile strength of tissues, with scar tissue becoming about 80% stronger as the original tissue as a result of intermolecular cross-linkage of collagen [5,6].

Even though wounds may be healed naturally through these phenomenon, it can be delayed and enter into the chronic state especially when infected with harmful microorganisms and complicated with prolonged inflammatory process. As a result, orthodox medications have been employed to shorten the duration of the healing process and minimize complications associated with natural wound healing process (such as wound infection due to bacterial contamination). However, the cost of these orthodox medications are outrageous and wound contaminating bacteria are becoming more resistant to their effect [8]. The search for newer, effective and affordable alternatives has then become the focus of a lot of scientist now.

The search cannot be completed without investigating into natural products, especially medicinal plants, since they have been widely used and accepted as more efficacious in traditional medicine for the treatment of wounds among past and present generations [8]. Reports by Agyare., *et al*. [9,10] reveal medicinal plants that have been reported to exhibit effective wound healing activities. An ethnopharmacological study by Agyare., *et al.* [11] into plants used locally as wound healing agents in Ghana revealed that, a wide range of medicinal plants are being used in the management of wounds in the country. Although these plants are effective in wound healing in the traditional setting, there is no scientific evidence on their healing properties, as well as their toxic effects on the skin cells on which they are applied to.

This study, therefore sought to prove scientifically the wound healing activity and determine the cytotoxic effect of two medicinal plants (*Hilleria latifolia* and *Laportea ovalifolia*) used locally for the management of wounds.

Hilleria latifolia (Lam.) H. Walt is a perennial herb of 30 to 120 cm high, belonging to the family *Phytolaccaceae*. It is locally known as '*Avegboma*' or '*Kukluigbe*' by the Ewes and '*Anafranaku*' by the Asantes. It has ovate-elliptic leaves of 15 cm long and 6 cm broad, with numerous short hair-like structures on lower surface. Flowers are pink or white in slender racemes up to 13 cm long [12,13]. In Ghana, the leaves of the plant are used for the management of rheumatism, boils and wounds [11]. They are also used in Congo to treat some skin diseases [12,13]. The presence of secondary metabolites such as, saponins, tannins, glycosides, steroids, terpenoids, flavonoids and alkaloids in the leaves and aerial parts of the plant has been reported by Dapaah., *et al*. and Woode., *et al.* [14,15]. The leaf and root extracts of

H. latifolia have been reported to demonstrate antidepressant-like effects, antimicrobial, antioxidant and anti-inflammatory properties [14, 16-18], anti-nociceptive and some neurobehavioral properties [19], as well as their ability to modify the activity some antibiotics [15].

Laportea ovalifolia (Schumach.) Chew is a herbaceous weed which belongs to the family *Urticaceae*. It is known as 'akyekyenwonsa', 'abrewa nom taa' or 'Kumasi otuo' by the Asantes. It is densely covered with stinging hairs and more often creeping than erect. The perennial stems are cylindrical, greenish to sometimes reddish or brownish in colour often prostrate with erect shoots [20]. Locally, the leaves of *L. ovalifolia* are used in the management of wounds [11] whereas its fruits are used as a poison antidote [21] and the roots boiled in water is taken to prevent excessive menstrual bleeding [22]. Leaf extract of the plant has been screened phytochemically to reveal the presence of glycosides, sterols and terpenoids, saponins, tannins, flavonoids, phlobatanins and cardiac glycosides [15,23]. *L. ovalifolia* has been reported to possess antimicrobial, antihyperglycemic, antidiabetic, antioxidant, anti-inflammatory activities and it is also effective in reducing oxidative stress in diabetes as well as modify the activity of selected antibiotics [15,18,24-26].

The aim of this study was therefore to investigate the wound healing properties of the leaf and root extracts of *H. latifolia* and leaf extract of *L. ovalifolia*, as well as assess their cytotoxic effects on skin cell lines.

Materials and Methods

Plant collection and extraction

Plant parts of *H. latifolia* (leaves and roots) and *L. ovalifolia* (leaves) were collected from Aburi (longitude 0.1729°W and latitude 5.8512°N) in the Eastern region of Ghana in February, 2014. The plants were authenticated by Dr. Alex Asase of the Department of Botany, University of Ghana, and voucher specimen AA 63 and AA 71, respectively deposited in the Ghana Herbarium, Department of Botany, University of Ghana, Legon, Accra. The various plant materials obtained were thoroughly washed under running tap-water to remove dirt particles and dried at a temperature of 25 to 28°C for two weeks. Well-dried plant materials became very crispy and were powdered using the laboratory milling machine (Christy and Norris, Chelmsford, England). Each of the powdered plant materials (800 g) were soaked in 70% v/v methanol (2.5 L) and extracted using the ultra-turrax (T 25 Janke and Kunkel, Labortenik, Germany) under ice-cooling at a speed of 24000 rpm for 3 to 5 min. The laboratory sieve (Retsch, Haan, Germany) of mesh number 200 with aperture of 75 µm and Whatmann filter paper Number 1 were then employed to filter, and filtrates were concentrated using the rotary evaporator (Rotavapor BÜCHI R-200 with heating bath B-490, Büchi, Konstanz, Germany) at 40°C under reduced pressure. The concentrated filtrates were then dried in the hot air oven (Gallenkamp, London, UK) at 40°C to obtain solid mass which were labelled and stored in air tight containers at 4 to 8°C in a refrigerator. The percentage yields of the extracts, *H. latifolia* leaf extract (HLLE), *H. latifolia* root extract (HLRE) and *L. ovalifolia* leaf extract (LOLE) were calculated in relation to the quantity of the dried powdered materials used and were recorded as 17.49, 7.50 and 11.29%, w/w, respectively.

Determination of wound healing activity of extracts

The excision wound model was employed to investigate the extracts ability to promote *in vivo* wound healing.

Experimental animals

Sprague Dawley male rats (150 to 200g) were obtained from the animal house of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana, and kept in stainless steel cages with soft wood shavings as bedding. They were maintained under standard environmental conditions of temperature (30 ± 2°C) and adequate humidity, with a twelve hour cycle of light and darkness. The animals were fed with standard pellet diet and provided with water *ad libitum*.

Ethical approval

All animals were humanely handled in the experimental period according to the guidelines of the Animal Welfare Regulations (USDA 1985; US Code, 42 USC § 289d) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002). Approval

from the Animal Ethical Committee (FPPS-AEC/CA01/13), Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

Formulation of extracts into cream

Aqueous cream base was prepared following the protocol described in the British Pharmacopoeia [27], without the preservative (phenoxyethanol). HLLE, HLRE and LOLE were incorporated into the aqueous cream to give different concentrations of 5 and 10% w/w. The formulated creams were stored in the refrigerator (Sharp Corporation, UK) at 25°C and constantly monitored for physical changes like phase separation, colour, odour, and texture.

Excision wound model

The excision wound model described by Morton and Malone [28] and modified by Agyare., et al. [29] was employed in this study. The dorsal fur of rats was completely removed using new and clean razor blades. The area that was shaved was neatly wiped with 70% v/v ethanol and the rats were anaesthetized with an intramuscular injection of ketamine hydrochloride (Pfizer, New York, USA) (50 mg/kg body weight). Sterile surgical scissors and toothed forceps (J J Int. Instruments, Kerala, India) were used to make full thickness wounds of about 20 to 25 mm diameter at the back of the rats.

The rats were randomly selected into different treatment groups of five (5) rats. The treatment groups were as follows: Group A received no treatment; Group B was treated with aqueous cream base only; Group C treated with silver sulphadiazine (1% w/w) (Ayrtondrugs, Accra, Ghana); Group D and E received 5 and 10% w/w HLLE aqueous creams, respectively; Group F and G received 5 and 10% w/w HLRE aqueous creams, respectively; Group H and I were also treated with 5 and 10% w/w LOLE aqueous creams, respectively.

Treatment of wounds and wound diameter assessment

Wounds were not treated for the first 24h period, after which they were treated daily for fifteen (15) days by topical application of 0.1g of the respective creams. All the wounds were cleansed with normal saline (0.9% w/v) (Intravenous infusions, Koforidua, Ghana) before treatment.

Diameter of the excised wounds was measured with the aid of a pair of divider and meter rule on every other day to assess the rate of wound contraction. Percentage wound closure was calculated as a percentage of the original wound from day 1 post wounding and result expressed as mean ± standard error mean (SEM). The equation below was used to calculate the percentage wound closure:

$$\% \text{ wound closure} = [(\text{wound size}_{initial} - \text{wound size}_{final}) / \text{wound size}_{initial}] \times 100$$

Histological studies

Treated and untreated wound tissues from all the groups were excised and kept in 10% neutral buffered formalin (Sigma Aldrich, Michigan, USA) on the 15th day post wounding for histological examination to assess the degree of tissue repair (re-epithelisation, angiogenesis, collagenation and granular tissue formation). The tissues stored in buffered formalin were dehydrated several times in different concentrations of ethanol (Merck BDH, Poole, UK) (50%, 70%, 95% and 100%), after which they were rinsed a number of times in xylene (Merck, BDH Poole, UK) to remove ethanol and then embedded in paraffin (Paraplast Plus, Sherwood, St. Louis, USA) to strengthen them for easy dissection. These tissues were sectioned into a thickness of about 5 µm and later 'deparaffinised'. The tissues were then mounted on a clean glass slides and stained with haematoxylin and eosin (Sigma Aldrich, St. Louis, Missouri, USA), they were finally observed under the microscope (Carl Zeiss Microscopy, Thornwood, USA) [30-32].

***In vitro* cell toxicity studies of extracts**

The effect of the extracts on cell viability and LDH release was investigated using HaCaT-keratinocytes (skin cell line) which was a kind donation from Professor N. E. Fusenig, German Cancer Research Center (Deutsche Krebs for schungszentrum) in Heidelberg, Germany.

Determination of the influence of the extracts on cell viability

The method as described by Mosmann [33] and modified by Agyare., *et al.* [34] was employed to assess the effect of HLLE, HLRE and LOLE on cell viability. Different concentrations of HLLE, HLRE and LOLE (0.1, 1.0, 10.0 and 100.0 µg/mL) were prepared in HaCaT keratinocytes medium (PAA Laboratories Pasching, Austria) in 96-well micro-titre plate (Sarstedt, Nümbrecht, Germany) and seeded with 10^5 cells per well. The plates were then incubated in 5% CO2 at 35oC after which the medium was removed and cells washed with 100 µL of phosphate buffer (Sigma Aldrich, St. Louis, Missouri, USA) solution (PBS). This was followed by the addition of 50 µL MTT (Sigma Aldrich, Taufkirchen, Germany) solution and re-incubated to allow the formation of insoluble formazan crystals, which were then dissolved in 50 µL of DMSO (Applied Chem GmbH, Darmstadt, Germany) to give a violet colour. The intensity of the violet coloured dissolved formazan crystals was measured by taking absorbance at 595 nm against 690nm, which correlates with the number of viable cells. HaCaT keratinocyte cells treated with 1% foetal calf serum (FCS) (Invitrogen, Karlsruhe, Germany) and untreated cells served as positive and negative controls respectively.

LDH cell toxicity assay

HaCaT keratinocytes (10^5 cells per well) were incubated at 35°C for 24h in the presence of different concentrations (0.1, 1.0, 10.0 and 100.0 µg/mL) of HLLE, HLRE and LOLE. Twenty-five micro-litres of the reaction mixture (Lactate, NAD+, INT and diaphorase) was then added and incubated in the dark at 20°C for 30 min. The reaction was terminated by the addition of 10 µL 1M HCL (Sigma Aldrich, Michigan, USA) solution to each well. The absorbance of the resultant solutions was measured at 490 against 690 nm. Untreated cells and cells treated with lysis solution (10% Triton X-100 (Sigma Aldrich, St. Louis, Missouri, USA) in 5% FCS (Invitrogen, Karlsruhe, Germany) were used as negative and positive controls respectively [35].

Statistical analysis

Data were presented as the mean ± standard error mean (SEM) in the studies. Analysis of results was done using one-way and two-way ANOVA followed by the Dunnett's and Bonferroni's post hoc tests respectively. Graphs were plotted with Graph Pad Prism for windows version 6 (Graph Pad, San Diego, CA, USA).

Results

Wound healing activity of extracts

HLLE, HLRE and LOLE at 5 and 10% significantly ($p<0.0001$) reduced wound size from day 5 to 15 after injury (Figures 1 A1, B1, C1). Also, their respective area under the curve (AUC) revealed a significant reduction ($p < 0.0001$) in wound size when compared to the untreated group (Figures 1 A2, B2, C2).

Histological studies of wound tissues treated with HLML, HLMR and LOML

The images from the histological study revealed that untreated wound tissues had persistent inflammation with marked tissue necrosis and little proliferation indicating poor wound healing rate (Figure 2A). Wound tissues treated with aqueous cream only (Vehicle) revealed presence of persistent inflammation and little proliferation which indicated poor wound healing rate (Figure 2B). Silver sulphadiazine (1%w/w)-treated wound tissues were characterised with granulation tissue formation and angiogenesis with evidence of fibroblast proliferation and collagen synthesis indicating re-epithelialisation and wound healing (Figure 2C). HLLE, LOLE and HLRE (10%)-treated wound tissues showed high rate of wound healing with increased collagenation and re-epithelialisation, which indicates high rate of wound healing (Figures 2D, 2F, 2H), while HLLE, LOLE and HLRE (5%)-treated wound tissue showed appreciable angiogenesis and fibroblastic activity with evidence of substantial collagen deposition in wounds (Figures 2E, 2G, 2I).

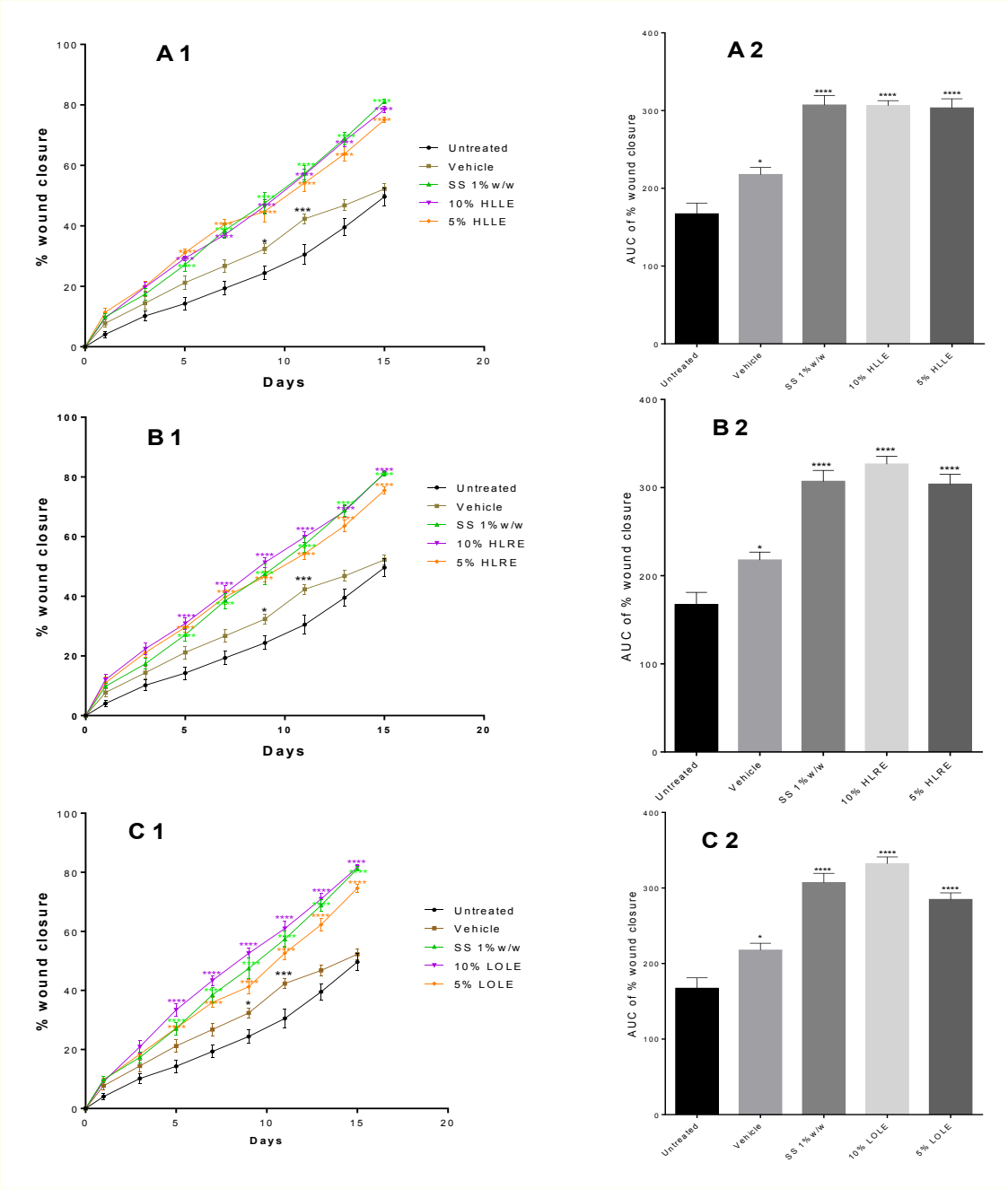

Figure 1: *Influence of HLLE, HLRE and LOLE on wound closure. A1, B1, C1: time-course curves of HLLE HLRE and LOLE respectively, A2, B2, C2: AUC of time course curve of HLLE, HLRE and LOLE respectively, SS: Silver sulphadiazine, HLLE: Hilleria latifolia leaf methanol extract, HLRE: Hilleria latifolia root methanol extract, LOLE: Laportea ovalifolia leaf methanol extract. Values are mean ± SEM (n = 5). ****p < 0.0001; ***p < 0.001; *p < 0.05 compared to untreated group (One-way and Two-way ANOVA followed by Dunnett's and Bonferroni's post hoc tests, respectively).*

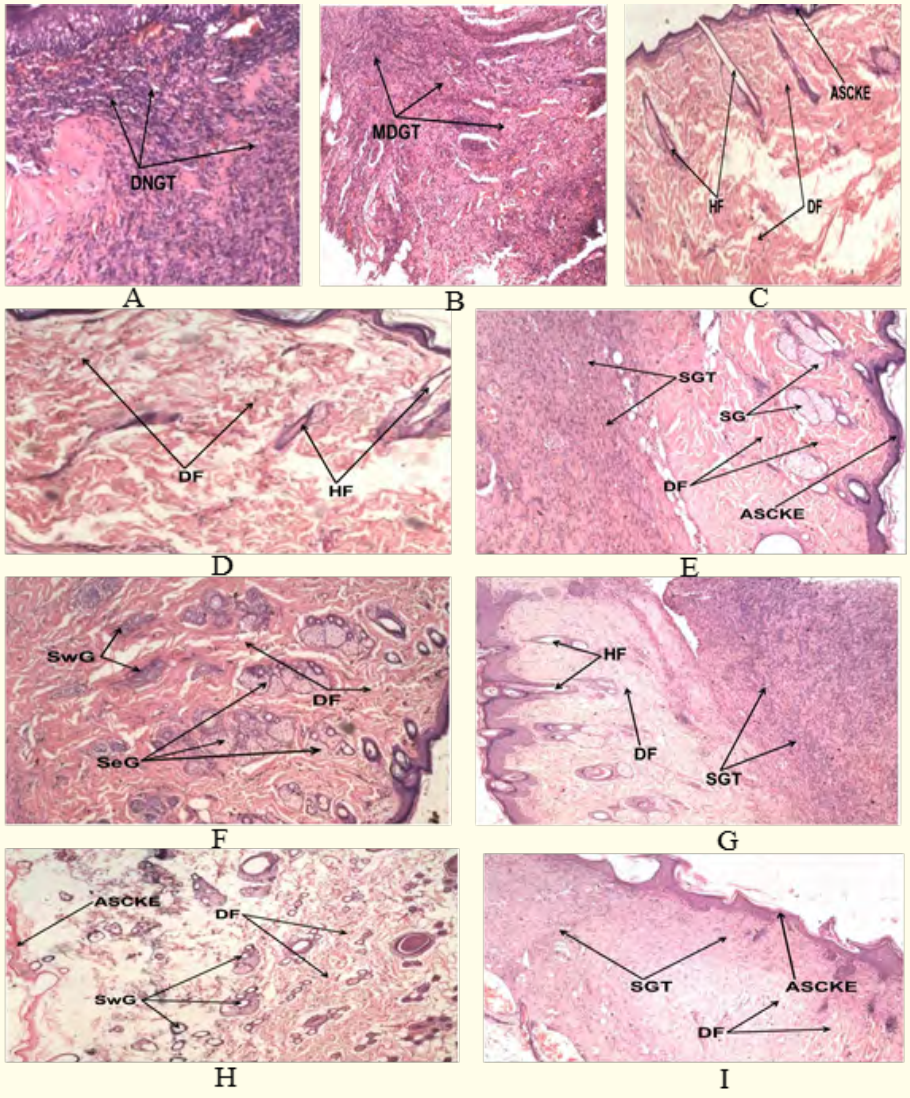

Figure 2: *Histological images (x400) of wound tissues from HLLE, LOLE and HLRE-treated and untreated wounds. A: Untreated wound tissues, B: vehicle-treated (aqueous cream only) wound tissues, C: 1% w/w silver sulphadiazine-treated wound tissues, D: 10% w/w HLLE-treated wound tissues, E: 5% w/w HLLE-treated wound tissues, F: 10% w/w LOLE-treated wound tissues, G: 5% w/w LOLE-treated wound tissues, H: 10% w/w HLRE-treated wound tissues, I: 5% w/w HLRE-treated wound tissues, DNGT: Diffuse necrotic granulation tissue, MDGT: Moderate diffuse granulation tissue, HF: hair follicle, DF: Dense fibrous tissue, ASCKE: Atrophic squamous cell keratinized epithelium, SGT: Scanty granulation tissue, SeG: Sebaceous gland, SwG: Sweat gland.*

Cell toxicity studies of extracts

Influence of HLLE, HLRE and LOLE on cell viability

Treatment with 100 µg/mL of HLLE ($p < 0.001$) and HLRE ($p < 0.05$) significantly reduced the viability of HaCaT-keratinocytes compared to the untreated cells. However, at the lower test concentrations (0.1, 1.0 and 10.0) and LOLE-treated cells, there was no significant ($p > 0.05$) difference when compared to the HaCaT-Keratinocyte untreated cells, whereas the cells treated with 1% foetal calf serum (positive control) significantly ($p < 0.0001$) increased the cells viability compared to the untreated cells (Figure 3).

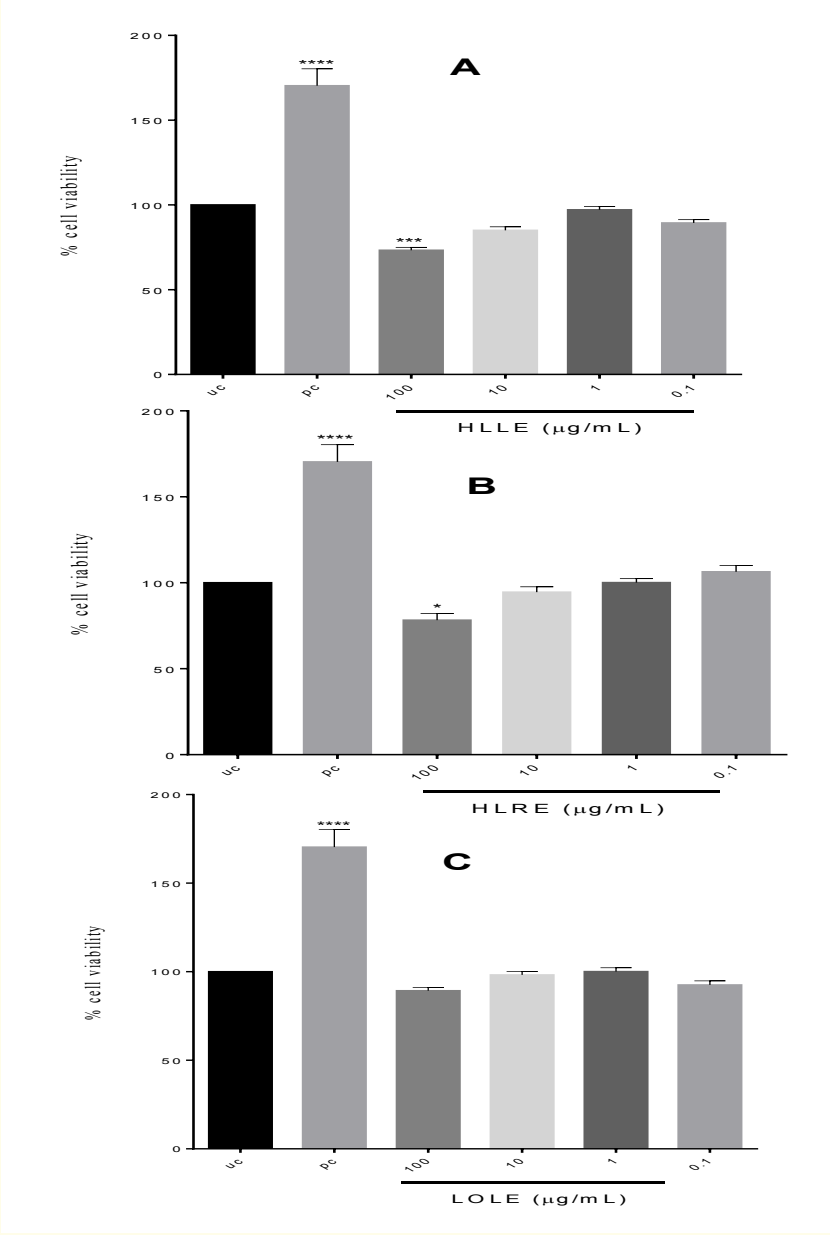

Figure 3: *Influence of HLLE (A), HLRE (B) and LOLE (C) on HaCaT-keratinocytes viability. uc: untreated cells; pc: positive control (1% foetal calf serum); HLLE: Hilleria latifolia leaf methanol extract, HLRE: Hilleria latifolia root methanol extract, LOLE: Laportea ovalifolia leaf methanol extract. Values are mean ± SEM. ****p < 0.0001; ***p<0.001; *p<0.05.*

Influence of HLLE, HLRE and LOLE on LDH release

HLLE, HLRE and LOLE-treated cells at the test concentrations showed no significant ($p > 0.05$) LDH release from HaCaT-keratinocytes compared to the untreated cell (Figure 4).

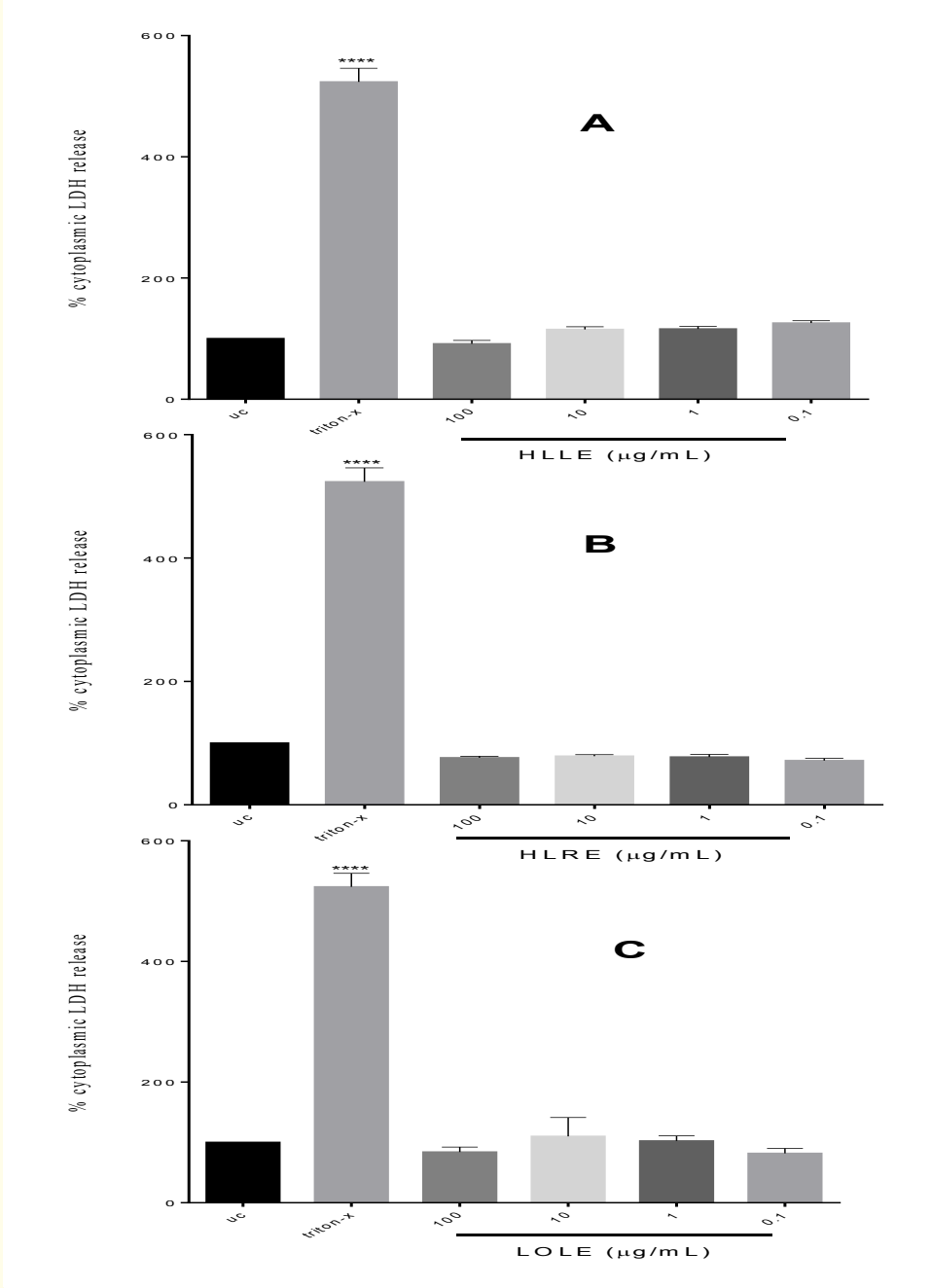

Figure 4*: Influence of HLLE (A), HLRE (B) and LOLE (C) on release of LDH from HaCaT-keratinocytes. uc: untreated cells. LDH: Lactate dehydrogenase. HLLE: Hilleria latifolia leaf methanol extract, HLRE: Hilleria latifolia root methanol extract, LOLE: Laportea ovalifolia leaf methanol extract. Values are mean ± SEM. ****p < 0.0001.*

Discussion

Wound contraction leading to wound healing is indicated by centripetal movement of the edges of a full-thickness wound and enhanced re-epithelialisation, granulation, angiogenesis, fibroblast proliferation, keratinocyte differentiation and proliferation [36,37].

Wounds treated with HLLE, HLRE and LOLE formulated aqueous creams exhibited high rate of wound contraction and the histological investigations of the treated wound tissues revealed high fibroblast proliferation, angiogenesis and granulation, collagenation, tissue formation and re-epithelisation compared to the untreated wound tissues. This is an indication of the extracts ability to heal wounds and hence confirms the ethnomedicinal use of *H. latifolia* and *L. ovalifolia* as wound healing agents [11]. The wound healing activity of HLLE, HLRE and LOLE may be attributed to their antioxidant and anti-inflammatory properties as reported by Dapaah., et al [18]. Over-production of ROS and prolonged inflammation can result in impaired wound healing [38,39]. Therefore an agent's ability to mop up excess ROS or free radicals can minimize the inflammatory response at wound site which can also enhance the wound healing process.

Highest administered doses of HLLE and HLRE reduced the viability of HaCaT-keratinocytes and could be suggestive of their cytotoxicity at higher concentrations. Also, HLLE, HLRE and LOLE, though not significant, inhibited the release of LDH from the cytoplasm of HaCaT-keratinocytes compared to the untreated cells. Low LDH release from the cytoplasm by the extracts suggests that HLLE, HLRE and LOLE may not be cytotoxic agents at the concentrations used. During development of human skin towards an intact barrier system, keratinocytes will undergo cellular proliferation followed by a switch to cellular differentiation to restore the breakage in the intact skin [34]. For this reason, any cytotoxic effect on the skin cells may cause a delay or inhibit the healing process. The little or no cytotoxic effect of HLLE, HLRE and LOLE, at the test concentrations, on HaCaT-keratinocytes may be contributing to their wound healing activity.

Conclusion

The leaf and root extracts of *H. latifolia* and the leaf extract of *L. ovalifolia* exhibited wound healing activity which confirms scientifically their local use as wound healing agents, with no or little cytotoxic effect on HaCaT-keratinocytes at the test concentrations.

Conflict of Interest

The authors declare no conflicts of interest.

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