REPORT

Wound healing activity of *Hymenocallis littoralis* - Moving beyond ornamental plant

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Abstract: Spider lily (*Hymenocallis littoralis)* belongs to *Amaryllidaceae* family is a well-known plant species for its medicinal properties. The inhibitory effects of *H. littoralis* methanol sonication extracts were evaluated for wound healing activity. This is the first report on the wound healing activity of Malaysian origin *H. littoralis.* The bulb, flower, root, anther, stem and leaves of *H. littoralis* methanol sonication extracts were used for scratch-wound assay. The cell line was treated with two different concentrations; 1 and 10µg/ml of extracts. The extracts were prepared freshly by dissolving in sterile phosphate saline buffer (PBS) and the healing activity was observed from 2, 4, 8, 12, 24, 36 and 48 h. The bulb, root, stem and anther methanol extracts demonstrated active wound healing activities at 1 µg mL^{-1}at 36 h of treatment. At the low concentration the bulb, root, stem and anther methanol extracts heals the wound compared to leaf and flower extracts. It's demonstrated that these extracts contain effective phytochemical substances which are responsible for wound healing process. This finding suggests the potential application of *H. littoralis* methanol extract in wound healing activity.

Keywords: *Hymenocallis littoralis*, Methanol sonication, Root, Bulb, Hs27 cell line.

INTRODUCTION

Wound healing comprises continues cell-cell and cell-matrix interactions in three overlapping phases such as inflammation (0-3days), cellular proliferation (3-12 days) and finally remodeling of the wound (3-6 months) (Schmidt *et al*., 2009). A thick actin bundles of myofibroblasts expressed in wounded dermis (Houghton *et al*., 2005; Gurtner *et al*., 2008). Nevertheless in the presence of oxygen free radicals, microbial infections and UV rays could reduce the wound recovery mechanism process (Houghton *et al*., 2005; Kumar *et al*., 2007; Schmidt *et al*., 2009). Traditional herbal medicine with effective pharmacological activities such as antioxidant and antimicrobial often used in broad area in various skin diseases (Suntar *et al*., 2010) includes cuts, wounds and burns (Kumar *et al*., 2007). Numerous herbs were scientifically proven to be used as remedies to cure the wounds in animal based studies (Dalazen *et al*., 2005).

Wound healing property of *Hymenocallis littoralis* (*Amaryllidaceae*) extract was evaluated for the first time using cell based techniques. *Hymenocallis littoralis* an ornamental plant exhibits numerous therapeutic properties such as anti-Candida, antioxidant, cytotoxicity and wound healing activities (Abou-Donia *et al*., 2008). Fascinatingly, this exploration was carried out because *Aloe vera (Xanthornrhoeaceae)* is the only plant which was used cell cultured-based technique to evaluate the wound healing activity (Krishnan, 2006). Currently, there are reports on anticancer property of *H. littoralis* extract but there are no reports on the wound healing activity (Idso *et al*., 2000; Ingrassia *et al*., 2008). Thus, bulb, anther, stem, leaves, flower and root's crude extracts were subjected to the wound healing assessment using human foreskin fibroblast cell line (Hs27). The human fibroblast *in vitro* model is essential to correlate the contractile events of wound (Marharet *et al*., 1998; Theim and Grosslinka, 2003).

MATERIALS AND METHODS

Samples preparation

H. littoralis plants were bought from Penang Botanical Gardens and the authenticity work was carried out by Mr. Shanmugam (Curator) from School of Biological Sciences, Universiti Sains Malaysia. Each of the plant parts (leaves, stem, root, bulbs, flowers, and anther) were cut into small pieces, washed in running tap water and dried at 40°C in a sterile oven for a week to remove the moisture content. The samples were powdered using blender (Panasonic, 380V) and extracted using methanol solvent by sonication technique to obtain the crude extracts. The sonication was run for 5 min and the extract

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was filtered through filter paper (Whatman No.1). The technique is repeated for three times using the residues. The filtrate was collected and concentrated in a rotary evaporator (RII0 Buchi, Switzerland) at 40°C. The concentrated extract was dried in an oven at 40°C for three days to obtain consistent weight and freeze dried for 2 days (Masoko and Eloff, 2007). The stock solution of *H. littoralis* methanolic extract of flower, root, bulb, leaf, stem and anther were prepared in 0.5% (v/v) dimethylsulfoxide (DMSO). The working solutions for every extracts were prepared at 1 and 10µg mL^{-1}concentrations via dilution technique and diluted in complete medium

Cell line and culture conditions

The human foreskin fibroblast (Hs27; ATCC CRL-1634) was used to study wound healing property of *H. littoralis*. The cell line is gifted by Dr. Ong Ming Thong from Institution for Research in Molecular Medicine (INFROMM), Universiti Sains Malaysia. The stock culture was maintained in a 25 cm^2 flask in Dulbecco's modified Eagle's medium high glucose (DMEM-H) (GIBCO, Invitrogen, United States of America) supplemented with 10% (volume/volume) fetal bovine serum (FBS) (GIBCO, Invitrogen, USA) and 1% (v/v) anti-mycotic antibiotic (GIBCO, Invitrogen, USA). The cells were incubated at 37°C in a humidified carbon dioxide incubator (5% CO_2). Cells at passages 10 and 11 were used in this study (Yue *et al*., 2010).

Scratch-wound assay

Hs27 cells culture were seeded at density of 2,500 cells per well into the 96 well flat-bottom micro plates (Nunc) with incomplete medium (DMEM-H and 1% (v/v) antibiotic-antimycotic) for 2 days to synchronize the cell growth. The cells were observed daily under inverted microscope (MOTIC, AE 31 model, USA) to confirm the growth condition of the cells. On 48h, the cells were wounded using sterile p-200 pipette tips (Axygen T- 300) and the cells washed gently twice using sterile phosphate buffer saline (PBS, pH 7). The wounded cells were treated with 120µL of *H. littoralis* plant extracts with 10 and 1µg ml^{-1} concentrations. The cells were observed at 0, 2, 4, 8, 12, 24, 36 and 48 h to analysis the cell migration and the images were captured for each observation time points (Liang *et al*., 2007). Cells treated with complete medium denoted as control while cells treated with 0.05% DMSO solvent stand as negative control. The complete medium is consists of 5% FBS, 1% antibiotic and basal media (DMEM-High glucose) to provide complete nutrients for cell growth.

Image capture and data analysis

Images at time zero (t= 0) were captured to record the initial diameter of the wounds and the recovery of the wounded due to cell migration toward the denuded diameter was evaluated at 2, 4, 8, 12, 24, 36 and 48 h. The images were captured using an Apotome microscope (Axio Vision 4, Carl Zeiss, USA). The diameter of wound was measured by Axio Vision Release 4.8.1 software (Carl Zeiss, USA). The migration of the cells was expressed as percentage of wound closure:

$$\text{Percentage (\%) of wound closure} = \left[\frac{[(\text{At - 0h}\ \ \text{At - Äh})}{\text{At - 0h}}\right]\times 100\%$$

Where, $A_{t\text{-}0h}$ is the length of wound measured immediately after scratching and $A_{t\text{-}\Delta h}$ is the length of wound measured at interval time points (4, 8, 12, 24, 36, 48 hr) after scratching (Yue *et al*., 2010).

STATISTICAL ANALYSIS

Each experiment was performed with four replicates. The data were expressed as mean ± standard error. One way Anova and Dunnet *post hoc* test was employed for statistical analysis. Statistical significance was established at $P<0.05$ (Yue *et al*., 2010).

RESULTS

Table 1 shows the wound closure for the all the extracts at tested concentrations, negative and control groups in fixed time points. As an early preliminary study only two concentration of plant extract were used in the treatment. The diameters of the created wound were calculated using recommended Axio Vision Release 4.8.1 software. Among all the extracts, bulb and root produced better cure for the created wound. The bulb extract 1µg mL^{-1} produced 100% improvement at 36 h, 92.11% is a control group, 88.90% is a negative control group and 79.04% 10 µg mL^{-1} of bulb extract. Even though the wound healed percentage is nearly similar to bulb extract yet the density of cell growth is more obvious in bulb compared to control group. The picture shown clearly the intense growth of cells compared to control group. Methanolic root extract displays 100% heals at 36h, while control group was 79.45%, negative control group 83.74% and 10 µg mL^{-1} concentration of root extract was 74.80%. Both these bulb and root extracts significantly repaired the injured cells within 36h. The anther, stem, flower and leaves cured the wound only at 48h. Anther and stem extracts repairs wound 100% at 1µg mL^{-1} at 48h; meanwhile flower extract only heals 95.822% and leaves extract 91.44%. Hence, both bulb and root extracts were more effective in wound healing activity compared to other methanolic extracts.

Fig. 1 displayed images of wound healing at 36h for various *H. littoralis extracts* at 1 µg mL^{-1}. The images were corresponding with the table 1 information. figs. 1B and 1C shown a clear healing property for root and bulb extracts compared to control (fig. 1A). The other extracts were shown a slow healing activity for the created wound compared to root and bulbs. This exhibits the root and

Table 1: Percentage of wound closure (%) for each extracts at fixed interval time for *Hymenocallis littoralis*

Percentage of wound closure (%)					
Extracts	Hour	Control	Negative control	1 µg mL^{-1}	10 µg mL^{-1}
Anther	4	9.11±5.63	13.44±8.62	3.29±1.77	2.23±1.41
	8	12.79±9.31	15.08±9.80	6.14±3.24	5.24±4.40
	12	21.40±10.54	24.97±13.29	17.84±2.56	11.53±8.01
	24	35.81±19.74	33.32±14.95	36.84±10.07	21.69±14.99
	36	70.53±11.26	49.21±17.25	83.29±4.88	34.91±14.99
	48	100.00	90.07±19.86	100.00	54.79±9.26
Flower	4	6.69±4.28	6.71±2.88	7.38±5.90	6.98±2.40
	8	11.60±5.42	15.66±2.99	5.99±3.06	8.30±3.78
	12	17.26±8.15	15.26±8.37	13.79±2.31	10.46±4.73
	24	45.67±8.57	52.17±13.41	34.71±12.39	12.21± 4.15
	36	66.13±7.41	77.45±6.83	74.34±4.71	48.12±4.58
	48	83.46±11.05	94.09±11.81	95.82±8.34	46.58±8 87
Bulb	4	6.82±7.84	10.56±2.95	7.75±8.47	2.60±2.76
	8	12.18±7.16	13.15±4.00	8.17±5.71	5.10±2.04
	12	19.28±5.67	38.04±14.99	17.17±10.31	11.62±3.59
	24	61.27±11.59	64.86±2.69	69.52±2.77	65.70±2.74
	36	92.11±15.79	88.90±13.24	100.00	79.04±4.94
	48	100.00	100.00	100.00	100.00
Leaf	4	4.94±6.95	11.40±15.90	4.92±6.91	0.13±4.22
	8	12.35±5.82	9.42±4.03	5.57±6.40	6.67±8.68
	12	15.94±6.08	24.23±20.04	11.08±7.56	5.88±8.00
	24	54.10±11.03	48.26±17.11	24.48±9.98	17.92±6.42
	36	70.58±8.38	82.41±11.82	68.95±10.43	41.30±10.40
	48	89.25±12.73	100.00	91.44±10.84	71.89±5.86
Root	4	5.50±1.95	7.06±7.68	16.06±5.49	7.71±2.53
	8	9.00±4.45	11.49±8.00	13.89±4.44	8.04±5.60
	12	20.65±6.15	22.95±5.00	23.33±5.41	20.32±5.10
	24	56.82±10.17	66.08±9.33	69.00±5.23	56.76±15.65
	36	79.45±13.95	83.74±12.53	100.00	74.80 ± 17.41
	48	100.00	100.00	100.00	100.00
Stem	4	12.37±11.33	7.97±2.74	5.80±2.42	9.35±10.20
	8	15.72±11.18	12.06±5.50	10.85±7.91	10.84±9.03
	12	25.32±13.29	18.12±3.55	23.56±6.75	16.67±8.25
	24	56.11±9.96	59.60±5.49	61.94±8.04	52.55±5.49
	36	78.14±16.26	80.33±13.92	92.89±8.21	74.15±8.27
	48	95.10±9.81	100.00	100.00	100.00

Note: Experiment was conducted in quadruplicates (n=4)

bulb extracts possess wound healing property compared to other extracts. fig. 2 shows the wound healing activity in high concentration 10µg mL^{-1}. Nevertheless at this concentration the plant extracts fails to express the wound healing activity. There was no significant closured area for the created wounds.

DISCUSSION

Wounds have an incredible influence on the healing healthcare economy (Agyare *et al*., 2013). In general the purpose of wound healing management is to heal the wound as fast as possible with minimal pain and scar formation (Clark, 1991; Suntar *et al*., 2010). Wound healing is a complicated process of cellular and biochemical interactions involving various cells such as keratinocytes, fibroblasts and endothelial cells (Tao *et al*., 2007; Krishnamoorty 2012). A significant wound healing is vital for the reestablishment of disrupted functional status of the skin and disturbed anatomical continuity (Ramesh and Shanoy, 2013). An agent could treat a wound in shortest time without scar is always required in market (Suntar *et al*., 2010). The *H. littoralis* showed significant promotion of wound healing activity in fibroblast Hs27 cell line treated with root and bulb methanolic extracts in 36 h.

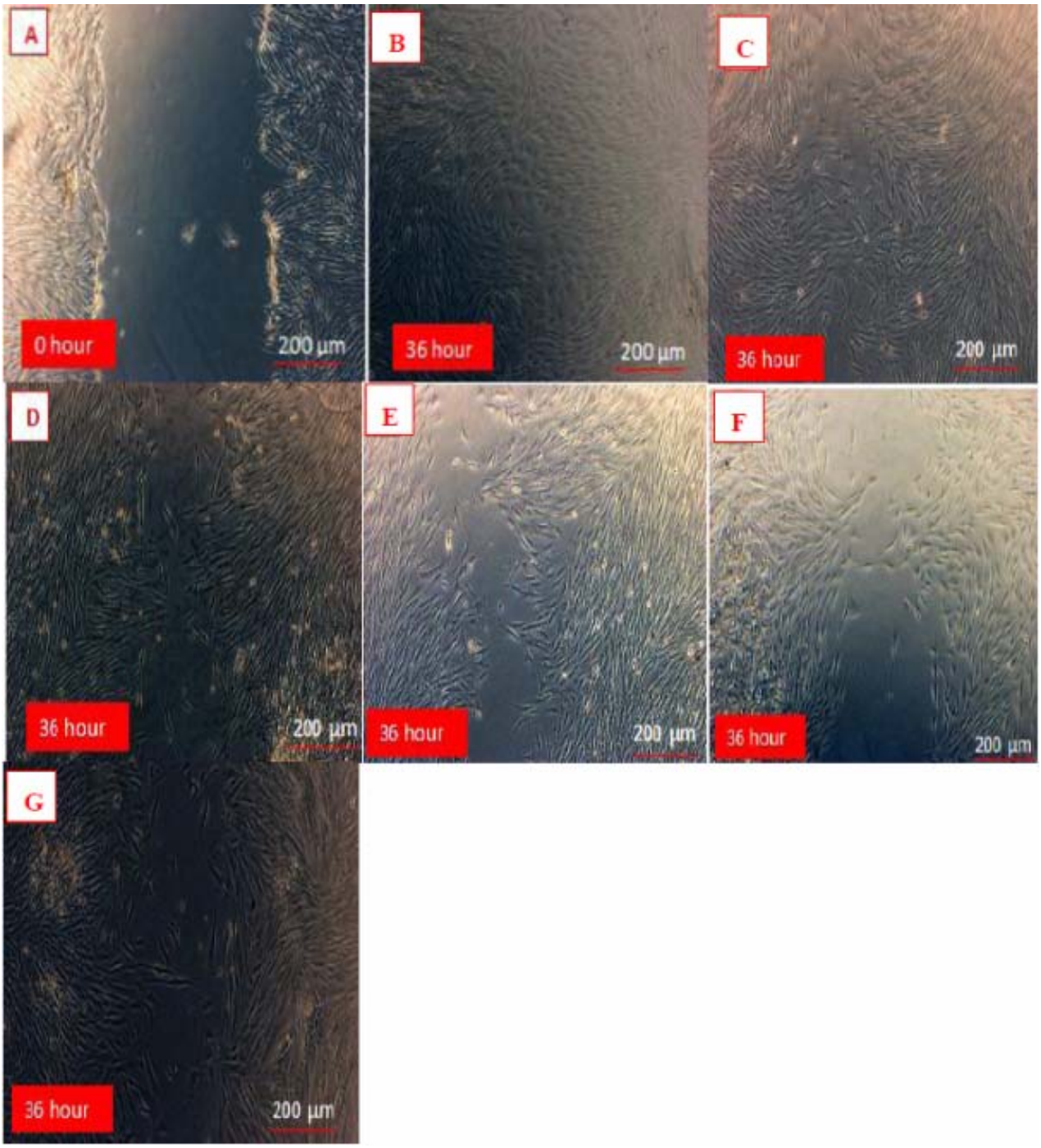

Fig. 1: Wound healing of methanolic extracts *Hymenocallis littoralis* at 36 hours for 1 µg mL^{-1}. [A] Wounded cells at 0 hour; [B] Bulb extract [C] Root extracts [D] Anther extract [E] Flower extract [F] Leaf extract [G] Stem extract

In vitro scratch-wound assay is an economical and direct technique to study cell migration in cell lines (Liang *et al*., 2007). This assay is very useful since the technique is very quick to work, can be used to screen wide variety of samples simultaneously and easily compared to *in vivo* wound healing technique (Thakur *et al*., 2011). Commonly in *in vitro* model fibroblast cell growth will be stimulated and create wound to observe the healing properties (Adetutu *et al*., 2011). In tissue repairing mechanism, the skin fibroblast proliferation is crucial since it involves migration, proliferation, contractions and collagen production (Mimura *et al*., 2004; Adetutu *et al*., 2011). Inflammatory cells releases cytokines and growth factor induce the dermal fibroblast and myofibroblasts in the wound to initiate the tissue repair mechanism (Schmidt *et al*., 2009).

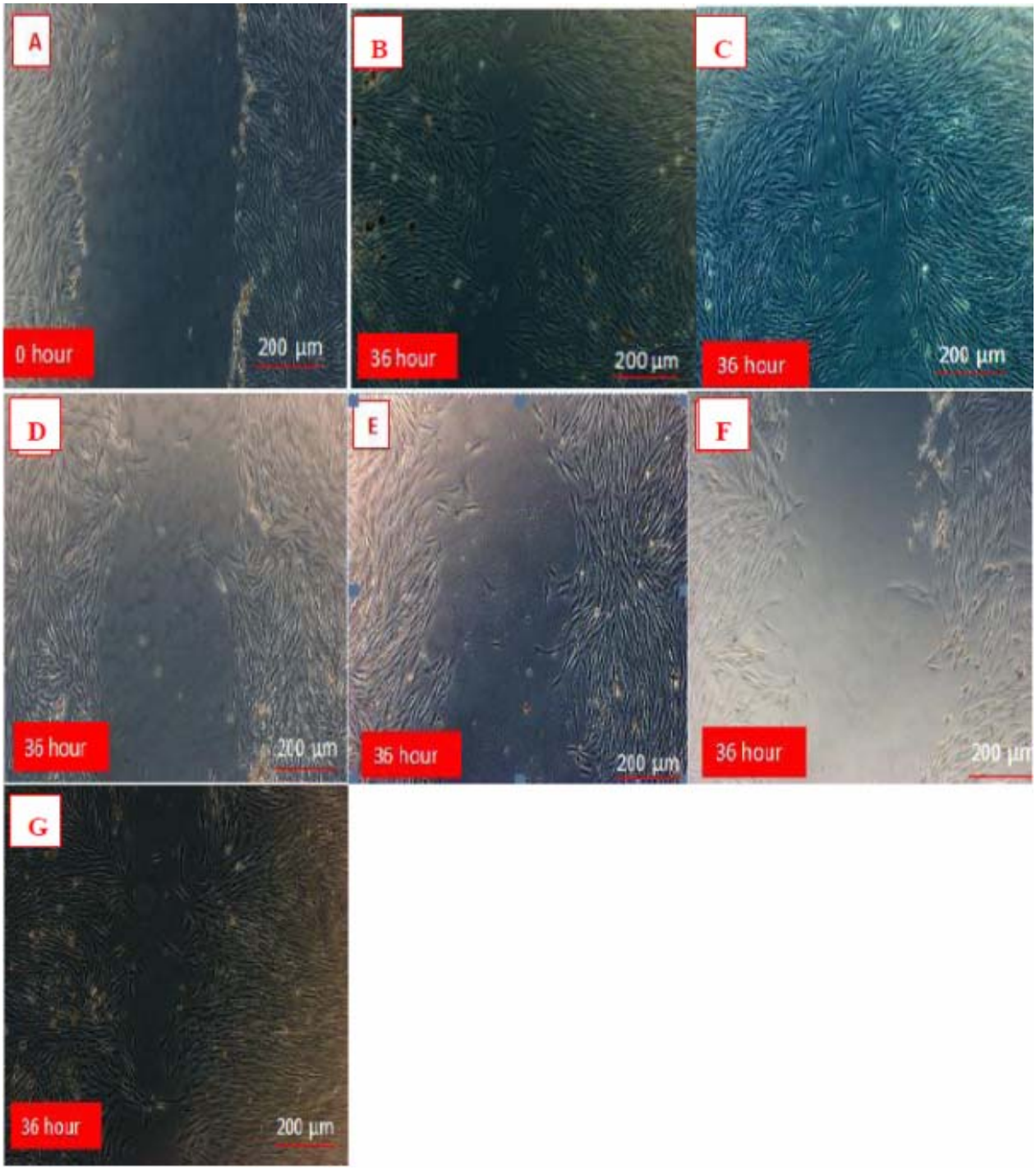

Fig. 2: Wound healing of methanolic extracts *Hymenocallis littoralis* at 36 hours for 10µg mL^{-1}. [A] Wounded cells at 0 hour; [B] Bulb extract [C] Root extracts [D] Anther extract [E] Flower extract [F] Leaf extract [G] Stem extract

The wound healing activity exhibited by *H. littoralis* is as per in descending order with the bulb > root > stem > anther > flower > leaf. The findings were presented in table 1. fig. 1 displayed images of wound healing at 36 h for various *H. littoralis extracts* at 1µg mL^{-1}. The *H. littoralis* mean IC_{50} antitumor activity was 75.72µg mL^{-1} (Yue *et al*., 2010) [10]. Thus for wound healing activity 1 and 10µg mL^{-1} concentration was chosen to be studied. The root and bulb extracts exhibit wound healing activity. Saponins, flavonoids, tannins (Obi *et al*., 2011), alkaloids and triterpenoids (Udegbunam *et al*., 2011) are the plant phytoconstituents attributes for the wound healing activity. *H. littoralis* plant extracts possess numerous isolated alkaloids phytoconstituents from the bulb and

root. Hence, alkaloids constituents in the extracts influence the wound healing process at tested concentrations.

Fig. 2 explains the wound healing activity of *H. littoralis extracts* images at 36 h for 10µg mL^{-1}. This exhibits that, higher the concentration of extracts lower curing effects on the wound. These may be due to high content of bioactive compounds that lead to reduction of proliferation activity. Previously, reported presences of anti-proliferation bioactive substances in *H. littoralis* (Thakur *et al*., 2011). The brine shrimp lethality assay's results (data not presented here) revealed that the *H. littoralis* leaf extract possess a highest cytotoxicity activity compared to other plant extracts. Thus, this could explain the reason for slow healing process of the leaf extract for both 1 and 10µg mL^{-1} (fig. 1F and 2F). Therefore, the cytotoxicity effects of the extracts might influences the wound healing capability.

In the wound healing process the reactive oxygen species (ROS) are very deleterious due to the harmful effects on cells and tissues. The ROS degrade the absorbable synthetic biomaterials in cells (Aliyeva *et al*., 2004; Kumar *et al*., 2007). Cytoprotective enzymes known as free-radical-scavenging enzymes play a crucial role in regulating the wound healing mechanism by reducing, de-activating and removing the ROS from cells (Kumar *et al*., 2007). Antioxidant may become therapeutic agents in wound healing process. The application of antioxidant compounds is proven to improve significantly the wound healing activity (Aliyeva *et al*., 2004; Kumar *et al*., 2007). *H. littoralis* root and bulb show a promising wound closure percentage at 36 hours of treatment. Bulb has high phenolic content (28.97mg GAE) and alkaloids may cause for the favorable wound cures at 36h at 1µg mL^{-1}. Moreover, this plant has an antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Pseudomonas aeruginosa* (Thiem and Grosslinka, 2003) and demonstrated antifungal activity against *Candida albicans* (Sundarasekar *et al*., 2012) for this plant. Thus, the *Hymenocallis littoralis* bulb and root extracts could be used as wound healing remedy at lower concentration of 1µg mL^{-1}.

CONCLUSION

This finding demonstrated that *Hymenocallis littoralis* bulb and root exhibits a promising wound healing activity for the scratch assay with Hs27 cell line. These methanolic extracts displays a closure wound healing activity at 36h at treated concentration 1µg mL^{-1}. Methanolic extracts possess phytoconstituents which are responsible to induce the release of cytokines and growth factors responsible for the tissue repairing mechanism in Hs 27 cell line. Further molecular studies currently been conducted to investigate the involvement of various mediators such as eicosanoids, prostaglandins, cytokines, responsible growth factors and transcription factors protein kinases.

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