Development of Herbal Composite and Evaluating its Antibacterial potential ability

***Syamili[1], E, Dr. Manju[2], R, and Dr. Lali Growther[3]**

[1]Research Scholar, Department of Microbiology, Hindusthan College of Arts and Science, Coimbatore, Tamil Nadu, India, 7907257740, syamumol88@gmail.com

[2]Assistant Professor, Department of Microbiology, Hindusthan College of Arts and Science, Coimbatore, Tamil Nadu, India, 8825490672, rajumanju1985@gmail.com

[3]Head, Associate Professor, Department of Microbiology, Hindusthan College of Arts and Science, Coimbatore, Tamil Nadu, India, 9843225410, lalijps@gmail.com

*Corresponding author

ABSTRACT

The objective was framed to analyse whether the plant extracts could support for the development of tissue engineered wound dressing materials and fight against pyogenic organisms. The present study was aimed to develop herbal composite using *Hemigraphis colorata* and *Cyanthillium cinereum* extracts. Fibroblast cell adhesion on herbal composite coated cotton materials were recorded using microscopic studies with an aim of developing a wound dressing material based on tissue engineering process. The engineered material was evaluated for its antibacterial potential against wound pathogens; and to assay the wound healing ability using a standard *in vitro* wound scratch method. Tissue engineered materials were developed using L_{929} fibroblast cells. L_{929} fibroblast cells attachment and its stage wise development on cotton wound dressing mesh materials were microscopically observed. Antibacterial activity results revealed that the herbal composites contain different types of phytochemical constituents that can affect the multiple target sites against the bacterial cells and its components. *In vitro* wound healing assay revealed that the developed composite (containing methanol extracts of *Hemigraphis colorata* and *Cyanthillium cinereum*) exhibited cell migration and proliferation after 12^{th} hour of incubation indicating the wound healing ability of herbal composite. Hence, it can conclude that the phytochemical compounds present in the developed herbal composites are considered highly significant in treating diabetic wounds. The results showed that the developed tissue engineered wound dressing has commercial interest in pharmaceuticals companies for the manufacturing of such materials in near future with different types of drugs coating on it.

Keywords: Herbal composite, *Hemigraphis colorata, Cyanthillium cinereum,* Tissue engineering, wound healing.

INTRODUCTION

Disruption of skin cells physiologically arranged is termed as wound (Mehmet Evren Okur et al., 2020). The disruption and damage of skin tissues and surrounding layer was caused by accidental cut, boils, diabetic wounds, carcinoma wounds and ulcers (Boateng et al., 2008). Wound healing is a physiological process, by which the living body repairs tissue damages, or influenced by a number of intrinsic and extrinsic factors (Eming et al., 2007). Delay of wound healing in the patients occurs due to different factors, like multiplication of bacteria causing interruption in wound healing associated with an exacerbation of pain (Guo and DiPietro, 2010). Diabetic foot ulceration is an example for delayed wound healing. Some of the bacteria associated with colonization in diabetic foot infection include *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Proteus species*, *Escherichia coli* and *Klebsiella pneumoniae* (Richard et al., 2012). In order to reduce the bacterial colonization in wounds and to increase the rate of wound healing, antimicrobials loaded dressings were processed (Mariana et al., 2019). Recently, Rodrigues et al., (2018) described wound healing based on cellular perspective model. Antimicrobial wound dressings can fasten the process of wound healing by preventing the bacterial infection. Antimicrobial dressings can be used on acute or chronic wounds which are critically colonised, or when local and/or systemic infection is already compromising the wound or could compromise wound healing (Lei et al., 2013). One such antimicrobial wound dressing materials were designed with herbal composite extracts using tissue engineering applications in the present research.

The therapeutic efficacies of many indigenous plants, for various diseases have been described by traditional herbal medicine practitioners. They are still the primary health care system in some parts of the world (Farah et al., 2000). The presence of various

life sustaining constituents in plants has urged scientists to examine these plants with a view to determine potential wound healing properties (Kumara et al., 2007). Based on this concept, two herbs were selected in the present research to develop a tissue engineered wound dressing materials.

Hemigraphis colorata (Murikootti) is a prostrate herb. It is claimed in folk medicine that the plant has very good wound healing activity (Subramaniam et al., 2007). *Cyanthillium cinereum* (poovamkurunnila) is a species of perennial plants in the sunflower family. A paste made from the plant is used as a poultice on cuts, wounds and skin diseases. They are used as an antidote to poison and in the treatment of leprosy and chronic skin diseases (Suresh et al., 2015). As the selected medicinal plants were considered to possess wound healing properties, the objective was framed to analyze whether the plant extracts could support for the development of tissue engineered wound dressing materials and fight against pyogenic organisms (Inada and Wildermuth, 2005).

MATERIALS AND METHODS

The present research work was carried out in the Department of Microbiology, Hindusthan College of Arts and Science, Coimbatore, Tamil Nadu, india. The work was done during the period of September 2019 to March 2020. All the bacterial samples were procured from a diagnostic laboratory, Coimbatore, Tamil Nadu, India.

Collection and processing of plant leaves (Lin et al., 2012)

Plant **(***Hemigraphis colorata* and *Cyanthillium cinereum*) leaves were collected from a nursery farm in Coimbatore, Tamil Nadu, India. The leaves were washed in water and dried under shade in a bright light room for 7 consecutive days. The dried leaves were broken into small pieces and powdered using a blender under standard conditions. Powdered leaves were sieved and placed in screw cap bottles at room temperature.

Soxhlet extraction of plant leaf extracts (Saohin et al., 2007)

For the study, infusion method of Soxhlet Extraction had been adopted. The powdered herbs of Plant leaf were filled in the thimble and placed in the soxhlet extractor. The extractor had been filled with solvent solution of methanol and the temperature of 60^0C was set and left for 6hours. Slowly and steadily the temperature was increased upto 100C. The extract from the thimble was collected in the round bottom flask kept in the heating mantle below by passing through a side arm tube. For all the other solvents (ethanol, petroleum ether and water) the extraction method was used for both herbs separately. Thus collected extract was taken in a petridish kept at 40 – 50°C till get dried. Dried powder was crushed using pestle and mortar to obtain fine particles. Finally the samples were stored at room temperature prior to testing.

Development of herbal composite

Development of Herbal composite was done using the methanol extracts of *Hemigraphis colorata* and *Cyanthillium cinereum* in the ratio of 1:1 (10ml + 10ml). During the composite preparation process, the *Cyanthillium cinereum* extract was kept under stirring conditions using a magnetic stirrer (for 110 rpm, at 40°C). First *Hemigraphis colorata* extract (10ml) was drop wise to the *Cyanthillium cinereum* extract (10ml) at the rate of 1ml per minute. The magnetic stirring condition was kept constant for atleast 2 hours for complete composite formation. Developed composite was stored at 4°C for further studies.

Cell adhesion assay: To develop and analyse tissue engineered wound dressing material using *in vitro* test method (Houreld and Abrahamse, 2007)

Non-adhering wound dressing gauze made of cotton cellulose was used in the study. L_{929}dermal fibroblasts were cultured according to the standard culture method described by

Houreld and Abrahamse, (2007). Briefly, cells were cultured in Dulbecco´s modified Eagle's Medium (DMEM) supplemented with and 10% fetal calf serum, 1mM sodium pyruvate, 2mM L-glutamine, 0.1mM non-essential amino acids, 1% Penicillin-Streptomycin and 0.2% Amphotericin-B. The cells were cultured for 7 days in 75cm^2 cell culture flasks at 37°C and in humidified atmosphere containing 5% CO_2 atmosphere.

Wound dressing samples (coated with herbal composites) were cut aseptically (size - 1.5 cm × 1.5 cm) and stabilized with a stainless steel ring. Cultured fibroblasts were harvested through trypsin-EDTA (Gibco) treatment and seeded at 2×10^4 cells/cm^2 on to the wound dressing samples by placing the material in a cell culture dish. Fibroblasts were incubated directly with the test specimen in complete DMEM (containing 1% antibiotic - antimycotic solution and 10% fetal calf serum) for 24h and the culturing process was carried out using rotary cell culture system. During the incubation period, cell morphology and cell proliferation on the wound dressing samples were recorded.

Rotary cell culture system was used for the proliferation of fibroblast at a revolution rate 15-20 rotation per min. The proliferation was carried out at 37°C and in humidified atmosphere containing 5% CO_2 atmosphere. The increase in the cell number and attachment on the surface and interstices of the fibres were recorded at 0^{th}hr, 6^{th}hr, 12^{th}hr and 24^{th}hr. These developed wound dressing material shall be used to promote diabetic wound healing in diabetic ulcers and other chronic wounds. Developed materials with attached fibroblasts were visualized after thawing under a field emission scanning electron microscope (FESEM).

Antibacterial activity of developed tissue engineered materials (El-Rehewy et al., 2009)

In this method, a modified disc diffusion method was carried out using developed wound dressing materials. Herbal composite and fibroblast cell adhered cotton mesh materials were developed and its antibacterial potential was evaluated under *in vitro*

conditions. (Fibroblast cell adhesion procedure on to cotton mesh materials was described in previous section). Antibacterial activity was determined using the diffusing ability of the wound dressing mesh coated with herbal composite and fibroblast cells on MHA plates.

Briefly, Mueller-Hinton agar (MHA) plates were prepared by pouring 15ml of media into sterile Petri dishes. The plates were allowed to solidify for 5min and 0.1ml inoculum was swabbed uniformly and allowed to dry for 60s. Premeasured disc shaped (size - 20mm in diameter) of herbal composite and fibroblast cell adhered cotton mesh materials were selected for the study. The materials were placed on the MHA plates (seeded with bacterial inoculum) and gently pressed to attach on the agar surface firmly using sterile conditions. A plain mesh without drugs and carrier was also kept in the plate as control. All the plates were incubated at 37ºC for 24 - 48h. At the end of incubation, the zone of inhibition formed around each material was measured in millimetre. Experiments were carried out in triplicates and antibacterial activity was expressed in Standard deviation values using Statistical Package for Social Sciences (SPSS – 9 for Windows 7.0) software.

Wound scratch assay – healing abilities (Liang et al., 2007)

L_{929} mouse fibroblast cells grown in 24 well plates at a density (1 X 10^5 cells per ml) with ~80% confluence was taken for the analysis. After developing a confluent monolayer of fibroblast cells, a linear scratch was created gently using a scrapper. The cell migration and proliferation of fibroblast cells at the wound site was observed till the wound gets closed. The cell development was evaluated for 100µg concentrates of herbal composite at different time periods (0^{th} hour, 12^{th} hour and 24^{th} hour). Migration of cells between the scratch site which emphasize the wound healing was observed using Phase contrast microscope for each time period.

RESULTS AND DISCUSSION

Cell adhesion assay

Tissue engineered materials were developed using L_{929} fibroblast cells. L_{929} fibroblast cells attachment and its stage wise development on cotton wound dressing mesh materials were microscopically observed. Inverted microscopic images showing the extent of cell attachment and proliferation of L_{929} cells on the fibres and interstices were noted from 0^{th}hr to 24^{th}hr. The increase in the cell number and attachment on the surface and interstices of the fibres were observed at 0^{th}hr, 6^{th}hr, 12^{th}hr and 24^{th}hr. No growth was observed on 0^{th}hr (Fig. 1). Partial cell growth was observed on 6^{th} hr (Fig. 2). Cell attachment was found to be evident on 12^{th}hr (Fig. 3) and proliferated cell covering the entire surface and interstices of the fibres was observed at 24^{th}hr (Fig. 4). With increasing cultivation period of time, fibroblasts showed a preference for aligning in parallel to one another. The present findings indicate multiple layers of cells on mesh were resulted from intense matrix production by fibroblasts in the developed conditions. Similar research analysis was carried out by Sarker et al., (2014). They evaluated fibroblasts adhesion and proliferation on Alginate-Gelatin cross-linked (ADA-GEL) hydrogel for diabetic wound ulcer treatment.

In agreement with the results from the literature survey, the present study revealed that the selected wound dressing mesh materials have supported well for the attachment and development of cells during the specified test period. Cell adhesion studies carried out in the present research revealed that the method has a wide range of important applications in tissue engineering and biocompatibility studies for wound healing and other biomaterials. The future potential of cell adhesion characterization is an emerging field for diagnosing and treating chronic diseases in the early stage at the cellular level (Zhong et al., 2012).

Antibacterial activity of developed tissue engineered materials

Antibacterial activity of developed tissue engineered wound healing materials showed promising results emphasizing the need for the materials in diabetic wound ulcer cases. About 31.3 ± 0.27mm and 30.1 ± 1.25mm of inhibitory zone was observed for *Escherichia coli* and *Klebsiella pneumoniae*. Followed by, 29.3 ± 1.25mm and 28.3 ± 0.57mm of inhibitory zone was observed for *Staphylococcus epidermidis* and *Staphylococcus aureus;* also *Enterobacter* sp showed 25.8 ± 0.57mm (Table-1).

Similar, qualitative antibacterial activity of drug coated biomaterials collected from published articles showed that, the increase in antibacterial activity was due to the synergistic behaviour of two drugs in combination rather alone (El-rehewy et al., 2009). This is the reason why combination of drugs was used to combat bacteria instead of using a single herbal drug. In the present research also, significant antibacterial activity was measured against test organisms, *E. coli*, *K. pneumoniae*, *Enterobacter* sp, *S. epidermidis* and *S. aureus* using two different herbal extracts in combination. Throughout the study, herbal composite has been used instead of evaluating the efficiency of individual herbal extracts. Many research reports emphasized the need for two or more drugs in combination to combat against the infection causing bacteria, because the causative agents have the ability to gain resistance when exposed to single type of herbal extract.

Wound healing ability of herbal composite - Wound scratch assay

In vitro wound healing assays have commonly been applied to measure cell migration, cell proliferation and wound closure in response to stimulation with specific agents. In this study, the herbal composite used for the cell adhesion studies was determined for its ability to improve wound healing by acting directly on L_{929} mouse fibroblast cells.

After creating a scratch on L_{929} mouse fibroblast cell lines, the cell migration, cell proliferation and wound closure was measured for a known concentration (100µg) of herbal

composite extract at three different time periods (0^{th} hour, 12^{th} hour and 24^{th} hour). In Control group natural wound healing happened. But in treated group the wound healing is delayed due to addition of developed samples. However, the samples did not restrict the healing process instead it aids in wound healing after the specified incubation period. The same was proved and presented in the images (Fig. 5).

Fig. 5 corresponding to self-wound healing ability of the developed herbal composite (containing methanol extracts of *Hemigraphis colorata* and *Cyanthillium cinereum*) showed that, at 0^{th} hour, no cell migration and proliferation was observed for the known concentrate (100µg) including control (Distilled water). At 12^{th} hour, herbal extract showed positive cell migration and cell proliferation when compared to the control sample. After 24hours, more cell proliferation was evident and thus indicating the wound healing ability of herbal composite.

In vitro scratch assay could be recorded as an appropriate and inexpensive method for the wound healing potential of herbal composite used in the present research.

Similar *in vitro* wound scratch assay method was recorded from the literature survey. During the survey, many research works were found highly correlated with the results of the present research. Some recent study on these tissue engineered wound dressing materials have been reported. Won et al., (2019) developed a potential dermal substitute using decellularized pig dermis and human dermal fibroblasts. In another study, electrospun polycaprolactone based scaffolds for wound healing and skin bioengineering applications was studied by Joseph et al., (2019). As there was similar wound healing abilities were noted for many herbal compounds from the literature survey on L_{929} mouse fibroblast cell lines, it was proved that the developed herbal composite could be used for the development of novel tissue engineered wound healing materials.

Based on the obtained positive wound healing ability of concentrations, it can be concluded that the developed tissue engineered wound dressing material could be useful to treat diabetic wounds. The fibroblast migration and proliferation of cells could able to favour in increasing the levels of cytokines, growth factors and keratinocytes at the wound site which results in effective wound healing in diabetic wounds.

CONCLUSION

The cell adhesion assay, antibacterial activity and in vitro wound healing assay of developed tissue engineered wound dressing materials revealed that need for such a novel product for treating complicated diabetic foot ulcer and equivalent wound infective cases. The biological properties of the herbal extracts thus described in the present research were attributed that the developed herbal composite could be used for the development of novel tissue engineered wound healing materials based on the obtained antibacterial activity and wound healing abilities. Hence, it can conclude that the phytochemical compounds present in the developed herbal composites are considered highly significant in treating diabetic wounds. The results showed that the developed tissue engineered wound dressing has commercial interest in pharmaceuticals companies for the manufacturing of such materials in near future with different types of drugs coating on it.

CONFLICT OF INTEREST

Authors declare no conflict of interest in the present research

REFERENCES

Boateng JS, Matthews KH, Stevens HN, Eccleston GM. Wound healing dressings and drug delivery systems: A Review. *J Pharm Sci*, 2008; 97(8):2892-2923.

El-Rehewy MK, El-Feky MA, Hassan MA, Abolella HA, Abolyosr A, El-Baky RM and Gad GF. In vitro Efficacy of Ureteral Catheters Impregnated with Ciprofloxacin, N-acetyl cysteine and their Combinations on Microbial Adherence. *Clin Med*, 2009; 3:1–8.

Eming SA, Krieg T, Davidson JM. Inflammation in wound repair molecular and cellular mechanisms. *J Invest Dermatol*, 2007; 127:514–525.

Farah MH, Edwards IR, Lindquist M, Leon C, Shaw D. International monitoring of adverse health effects associated with herbal medicines. *Pharmacoepidemiol. Drug Saf,* 2000; 9 105–112.

Guo S and DiPietro LA. Factors Affecting Wound Healing *J Dent Res*, 2010; 89(3):219-229.

Houreld N and Abrahamse H. *In vitro* exposure of wounded diabetic fibroblast cells to a helium-neon laser at 5 and 16 J/cm^2. *Photomed Laser Surg*, 2007; 25:78–84.

Inada N, and Wildermuth MC. Novel tissue preparation method and cell-specific marker for laser microdissection of *Arabidopsis* mature leaf. *Planta,* 2005; 221, 9–16.

Joseph B, Augustine R, Kalarikkal N, Thomas S, Seantier B and Grohens Y. Recent advances in electrospun polycaprolactone based scaffolds for wound healing and skin bioengineering applications. Mater Today Commun, 2019;19:319-335.

Kumara B, Vijayakumara M, Govindarajan R and Pushpangadan P. Ethnopharmacological approaches to wound healing- Exploring medicinal plants of India. Journal of Ethnopharmacology, 2007; Vol. 114: 103-113.

Lei J, Sun L, Li P, Zhu C and Lin Z. The Wound Dressings and Their Applications in Wound Healing and Management. *Health Sci J*, 2019; 13(4):662-667.

Liang CC, Park AY, Guan JL. *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*. Nat Protoc, 2007; 2(2):329-33.

Lin L, Lei F, Sun D, Dong Y, Yang B, Zhao M. Thermal inactivation kinetics of Rabdosia serra Hara leaf peroxidase and polyhenol oxidase and comparative evaluation of drying methods on leaf phenolic profile and bioactivities, Food Chem, 2012; 134 (4) pp. 2021-2029.

Mariana AM, Finn LA, Anne T, Havard S and Vasile O. Chitosan as a Wound Dressing Starting Material: Antimicrobial Properties and Mode of Action. *Int J Mol Sci*, 2019; 20(23):5889-5895.

Mehmet Evren Okur, Ioannis D. Karantas, Zeynep Senyigit, Neslihan Ustundag Okur and Panoraia I. Siafaka. Recent trends on wound management: New therapeutic choices based on polymeric carriers. Asian J Pharm Sci, 2020;15(6):661-684.

Melanie Rodrigues, Nina Kosaric, Clark A. Bonham, and Geoffrey C. Gurtner. Wound Healing: A Cellular perspective. Physiol Rev, 2019;99:665-706.

Richard JL, Lavigne JP, Sotto A. Diabetes and foot infection: more than double trouble. *Diabetes Metab Res Rev*, 2012; 28:46–53.

Saohin W, Boonchoong P, Iamlikitkuakoon S, Jamnoiprom I, Mungdee W. Effects of drying temperature and residual moisture content of Fa-Tha-Li (*Andrographis paniculata* (Burm.f.) Nees) crude powder for capsule preparation. Thai. J. Pharm. Sci, 2007; 31 pp. 28-35.

Sarker B, Singh R, Silva R, Roether JA, Kaschta J. Evaluation of Fibroblasts Adhesion and Proliferation on Alginate-Gelatin Crosslinked Hydrogel. PLoS ONE, 2014; 9(9): 952-959.

Srinivasa Rao Bolla, Abeer Mohammed Al-Subaie, Reem Yousuf Al-Jindan, *In vitro* wound healing potency of methanolic leaf extract of *Aristolochia saccata* is possibly mediated by its stimulatory effect on collagen-1 expression, Heliyon, 2019 May; 5(5): 641-648.

Subramaniam A, Rajasekharan DA, Sreekandan S, Nair G. Effect of Hemigraphis colorata (blume) H.G.Hallier leaf on wound healing and inflammation in mice, Ind j Pharmacol, 2001;33: 283-285.

Suresh SN, Varsha V, Prejeena V. Phytochemical screening of *Cyanthellium cinereum* leaf extracts. International Journal of Medicine and Pharmaceutical Research, 2015; 3(6), 1238-1241.

Won JY, Lee MH, Kim MJ, Min KH, Ahn G, Han JS, Jin S, Yun WS and Shim, JH. A potential dermal substitute using decellularized dermis extracellular matrix derived bio-ink. Artif Cells Nanomed Biotechnol, 2019;47:644-649.

Zhong Y, He S, Ji B. Mechanics in mechanosensitivity of cell adhesion and its roles in cell migration. *Int. J. Comp. Mater. Sci. Eng*, 2012; 1: 1-7.

Fig. 1: L_{929}fibroblast cell development on wound dressing mesh at 0^{th} hour

0th hour (No cell growth)

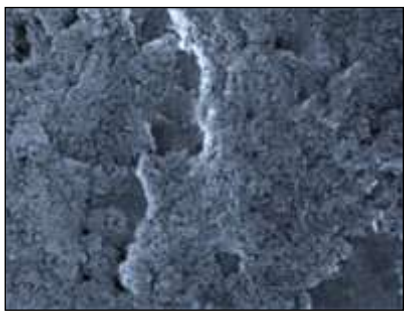
Fig. 2: L_{929}fibroblast cell development on wound dressing mesh after 6^{th} hour

6th hour (Partial cell growth)

Fig. 3: L_{929}fibroblast cell development on wound dressing mesh after 12^{th} hour

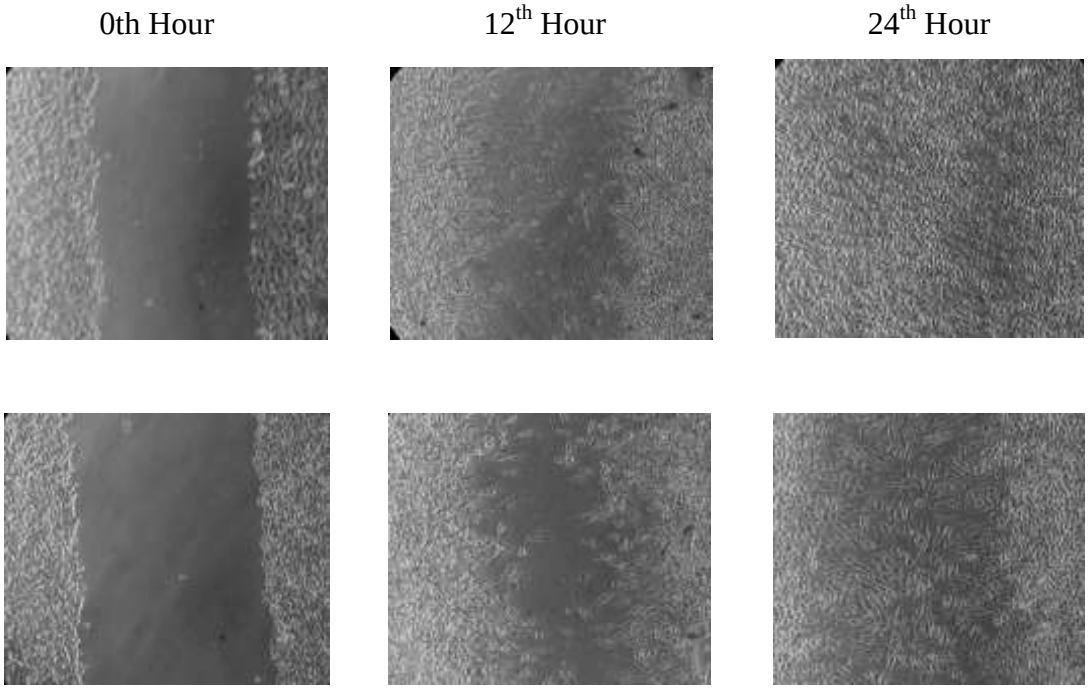
18th hour (Cell attachment evident)

Fig. 4: L_{929}fibroblast cell development on wound dressing mesh after 24^{th} hour



24th hour (Proliferated cell covering the entire surface and interstices of the fibres

Fig. 5: Self-wound healing scratch assay: *In vitro* Wound Scratch Assay



A: Control (Distilled water) [Top three pictures]
B: Herbal extract sample (100µg) [Bottom three pictures]

Table-1: Antibacterial activity of developed tissue engineered materials

S. No	Test organism	Zone of inhibition (mm)	
		Uncoated	Coated*
1	*Escherichia coli*	0	31.3 ± 0.27
2	*Klebsiella pneumoniae*	0	30.1 ± 1.25
3	*Enterobacter* sp	0	25.8 ± 0.57
4	*Staphylococcus aureus*	0	28.3 ± 0.57
5	*Staphylococcus epidermidis*	0	29.3 ± 1.25

*Tissue engineered wound dressing materials coated with two herbal composites