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Research Article

### Wound healing promoting activity of Earthworm, *Eutyphoeus gammiei* (Beddard): *in vitro* studies on human skin keratinocyte cell line (HaCat).

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#### ABSTRACT

Earthworm, *Eutyphoeus gammiei*, homogenate (EGH) was screened for wound healing activity on human keratinocyte cell line, HaCat, by cell proliferation and migration assays. The maximum proliferation and migration of keratinocyte cells were observed at the dose of 25µg/ml. As cell proliferation and migration are key factors for wound healing, the study clearly suggests the potential role of earthworm species *Eutyphoeus gammiei* on wound healing.

**Keywords:** *Eutyphoeus gammiei*, Keratinocyte, MTT assay, scratch assay.

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#### INTRODUCTION

Skin protects us from various environmental factors such as ionizing radiation, temperature, pressure, UV ray etc<sup>1</sup>. However, when this protective function of skin is lost due to physical, chemical or thermal injuries, it is called coetaneous wound<sup>2</sup>. After injury the wounded tissue initiates a cascade of biochemical events to repair the damaged region, known as wound healing process<sup>3</sup>. Wound healing is a well-regulated complicated process that involves interactions among resident and recruited cells such as epithelial cells, fibroblasts, endothelial cells, inflammatory cells, and interactions of those cells with extracellular matrix molecules, growth factors, cytokines, and chemokines<sup>4</sup>.

During healing process keratinocytes first migrates without proliferating<sup>5</sup>. After that keratinocyte proliferate in order to provide more cells for migration<sup>6</sup>. Before they begin migrating, keratinocytes change shape, becoming longer and flatter and extending cellular processes like lamellipodia and wide processes that look like ruffles<sup>7</sup>. Actin filaments and pseudopodia form<sup>8</sup>. During migration, integrins on the pseudopod attach to the ECM, and the actin filaments in the projection pull the cell along<sup>9</sup>. The interaction with molecules in the ECM through integrins further promotes the formation of actin filaments, lamellipodia, and filopodia<sup>9</sup>. All these promote proliferation and migration of keratinocyte.

In China, Japan, Indonesia and the Far East, earthworms have been used to treat various chronic diseases since 1340 AD<sup>10</sup>. The use of earthworm species as wound healing agent has been reported in various studies<sup>11-15</sup>. The medicinal property of earthworm may vary depending on the species and living environment of that organism<sup>16-19</sup>. Antibacterial and anti-inflammatory activities of the earthworm, *E. gammiei*, have been reported<sup>20, 21</sup>. However, there is no scientific report about the wound healing activity of *E. gammiei*. Therefore, the present study was designed to evaluate the wound healing promoting activity of earthworm extract (*E. gammiei*) using human skin keratinocyte cell line.

#### MATERIALS AND METHODS

##### Sample collection

Adult earthworm *Eutyphoeus gammiei* was collected by hand sorting and digging method by spade from Agartala, Tripura at early morning.

##### Preparation of tissue homogenate

The tissue homogenate of *Eutyphoeus gammiei* (EGH) was made in 1X phosphate buffer solution (pH 7.4) according to the biochemical protocol. Specific amount of tissue was weighted to prepare 10% (w/v) tissue homogenate using Potter Elvenjem glass homogenizer. Homogenate was filter sterilized and stored at -20°C unit analyses<sup>20</sup>.

### Protein estimation

Protein content was estimated following Lowry's method<sup>22</sup>.

### Cell lines

Human skin keratinocyte (HaCat) cell line was kind gift from Prof. Parimal Karmakar, Department of Life Science and Biotechnology, Jadavpur University, Kolkata, India.

### Cell culture

Human skin keratinocyte (HaCat) cells were maintained in DMEM and supplemented with 10% FBS, 100 IU/mL penicillin and 100µg/mL streptomycin, at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> (CO<sub>2</sub> incubator, ESCO, Singapore).

### Cell proliferation assay

Cells were cultured in 96 well plates with or without different concentration of EGH for 24 h time duration. The cells viability was assessed using MTT assay method. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was dissolved in phosphate-buffered saline at a concentration of 5 mg/mL. MTT was added to each well, and plates were incubated at 37°C for 3 h. The medium was replaced with 100 µL DMSO, and the absorbance for each well will be measured at 570 nm on a microplate reader (BioTek, USA). Total viable cell count was taken at different time periods of 24h, 48h and 72h<sup>23, 24</sup>.

### Cell Migration assay

Human skin keratinocyte (HaCat) cells were treated with EGH and an artificial wound was created on culture plates by scratching the plates through microtips. Microscopic images were taken immediately after wounding and during an incubation period of upto 72 hours. Finally the activity of the extract was compared with the control and measured by calculating the percentage of closed area<sup>25</sup>.

### Statistical analysis

All data were represented as mean ± SEM. Differences among groups were evaluated using one-way ANOVA to determine statistical significance. P<0.05 was accepted as statistically significant. P<0.05, P<0.01 are represented by \*, \*\* and \*\*\*, respectively.

## RESULTS

### EGH stimulates keratinocyte proliferation

MTT assay was used to examine the effect of EGH on human skin keratinocyte (HaCat) cells proliferation. The cells were treated with 3.125µg/ml, 6.25 µg/ml, 12.5µg/ml, 25µg/ml, 50 µg/ml and 100µg/ml concentrations of EGH, and the cell viability was determined by MTT assay at 24 h and 48 h. As seen in Table 1, 25µg/ml dose was found to be the optimal dose. At this dose after 24h and 48h, the proliferation was increased up to 135% (p < 0.01) and 136% (p < 0.001). Thus EGH possesses the property of inducing proliferation of keratinocytes in dose and time dependent way.

Table 1: Effect of EGH on keratinocyte proliferation at different concentration

Percentage of keratinocyte (HaCat) cell proliferation							
	Cntrl	3.125µg	6.25µg	12.5µg	25µg	50µg	100µg
<b>24 Hour</b>	100±0.1	110±1.6	120±1.7*	124±2.1*	135±1.7**	120±1.8**	108±1.9
<b>48 hour</b>	100±0.2	110±1.4	120±1.4*	127±1.8**	136±2.5***	117±1.9	108±2.3

Data are expressed as the means ± SD of three independent experiments, and the statistical significance was calculated using one way ANOVA, n=3. \*\* (p<0.05), \* (p<0.01) and \*\*\* (p<0.001) in comparison to control (without extract).

### EGH increases Keratinocyte Migration

In the *in vitro* wound model scrape wounds were generated in nearly confluent monolayer of cells and the migration of the edge of the wound was monitored with a phase contrast microscope. Cells with or without EGH treatment were allowed to migrate into the void area for 0–72 h. The keratinocyte cells started to migrate into the void area at

24 h after EGH treatment and wound area closes up to 80% compared to respective wound area, and the void area of the cells was almost closed at 48 h. In contrast, the migration of untreated cells was slower at the corresponding time points. The gap width at each time point after treatment with EGH was measured and plotted in Fig 1.

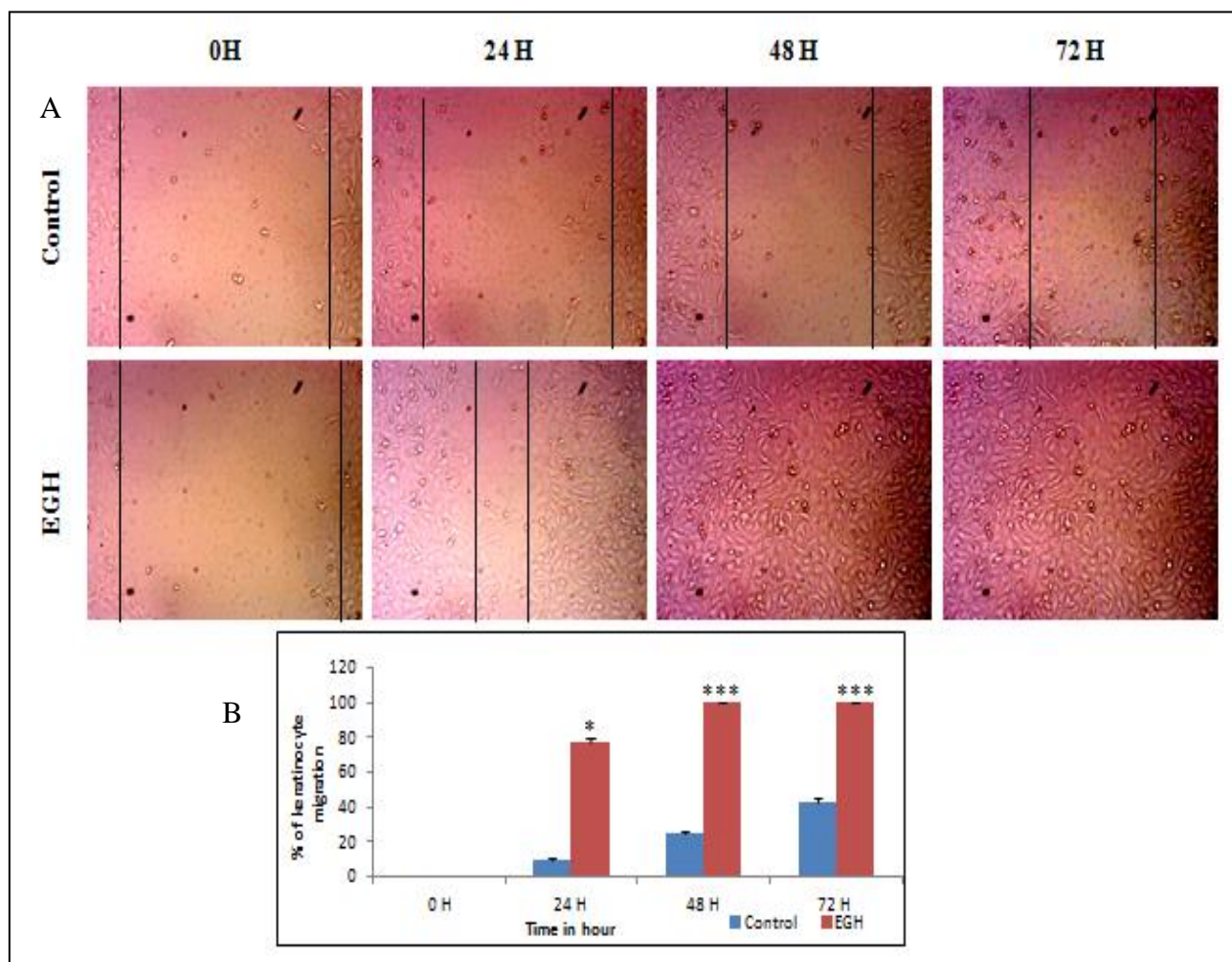


Figure 1: EGH induced migration of keratinocyte in an *in vitro* wound model. The wound is generated in monolayer cells and (A) photographed with a phase contrast microscope (10X) immediately after wounding (0 h) and 24, 48 and 72 h later. Gap of the wound areas were measured using ImageJ Software, and (B) % of wound closure with time is calculated. Data are expressed as the means  $\pm$  SD of three independent experiments, and the statistical significance was calculated using one way ANOVA. n=3 \*\* (P<0.05), \* (P<0.01) and \*\*\* (P<0.001) in comparison to control (without extract). h, hour.

## DISCUSSION

Nonhealing wounds are a major health problem worldwide<sup>26</sup>. The number is likely to grow due to age related conditions and pathologies such as diabetes, obesity, and cardiovascular diseases<sup>27</sup>. In China, Japan, Indonesia and the Far East, earthworms have been used to treat various chronic diseases since 1340 AD<sup>10</sup>. The use of earthworm as, anti-microbial, anti-inflammatory, anti cancer, anti-pyretic, analgesic, nerve diseases have been reported in various studies. The medicinal property of earthworm may vary depending on the species and living environment of that organism<sup>16-19</sup>. The earthworm, *E. gammiei* possess the antibacterial and anti-inflammatory activity<sup>20, 21</sup>. Therefore, the present study was designed to evaluate the wound healing property of *E. gammiei*.

Human keratinocyte cell line was used for this study. Keratinocytes are the major cellular component of the epidermis and are responsible for barrier restoration upon injury through a process known as epithelialisation<sup>28</sup>. The cellular and molecular processes involved in initiation, maintenance and completion of epithelialisation are essential for successful wound closure<sup>29</sup>. During regeneration of epidermis after wounding, activation, proliferation and migration of keratinocytes towards wounded area from the surrounding epidermis takes

place<sup>30</sup>. As depicted in Table 1 and Fig 1, EGH time and dose dependently increased the rate of proliferation and migration into wounded area and 25 $\mu$ g/ml found to be the optimum dose for both proliferation and migration of keratinocyte. Therefore, the present work shows potent wound healing activity of *E. gammiei* and provides convincing scientific basis regarding the age old ethnomedicinal use of earthworm for wound healing.

## CONCLUSION

This study provides scientific basis for the wound healing property of the earthworm *E. gammiei*.

## COFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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