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# Evaluation of *in vivo* Wound Healing Activity of Ursolic Acid Rich Chloroform Extract of *Hedyotis herbacea Linn* Ointment

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***Authors' contributions***

*This work was carried out by author KAB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SS managed the analyses of the study. Author SJ managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aim:** This study evaluates the effect of *in vivo* wound healing potential of Ursolic acid rich chloroform extract of *Hedyotis herbacea Linn* ointment using incision and excision wound model.
**Study Design:** Wound healing potentials of *Hedyotis herbacea* were analysed by Incision and excision wound model.
**Place of Study:** Nandha College of Pharmacy, Erode, Tamilnadu.
**Methodology:** *Hedyotis herbacea* was subjected to extraction (cold maceration), with solvents of increasing polarity. All the extracts were estimated for the presence of phytoconstituents by HPTLC. As the study has been focused on the phytoconstituent based biological activity, the Ursolic acid rich chloroform extract was chosen for the study of wound healing activity. The Chloroform extract of *Hedyotis herbacea* was incorporated into ointment base, to prepare the ointment. The ointments prepared with Chloroform extract (2.5% and 5%) of *Hedyotis herbacea* (CEHH) were subjected for evaluation of excision and incision wound model. Wistar albino rats were divided into four groups each consisting of six animals; group I (left untreated) considered as

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untreated control, group II treated with 5% w/w povidone-iodine ointment served as standard, group III treated with CEHH 2.5% w/w ointment, and group IV treated with CEHH 5% w/w ointment were considered as test groups. All the treatments were given once daily. Wound healing effect was assessed by the rate of wound contraction, level of total protein, Hydroxy proline, Hexosamine and Hexuronic acid and histopathology studies in an excision wound model. Tensile strength was also measured in both excision and incision wound.

**Results:** Wound healing activity of CEHH 5% w/w ointment treated group was greater than CEHH 2.5% w/w and untreated groups in both excision and incision wound model. The high rate of wound contraction (*P< 0.001), high tensile strength (*P< 0.001), and elevated total protein, Hydroxyproline, Hexosamine, and Hexuronic acid content were observed in animals treated with CEHH ointments when compared to the untreated control group of animals. Histopathological studies of the CEHH ointments treated groups also revealed the effectiveness in wound healing.

**Conclusion:** These results justified the claimed traditional use of the *Hedyotis herbacea* as wound healing plant.

*Keywords: Hedyotis herbacea Linn; chloroform extract; wound healing; incision wound and excision wound.*

## 1. INTRODUCTION

A wound is defined as a break in the epithelial integrity of the skin or outlined as a loss or breaking of cellular and anatomic or functional continuity of living tissue. As per the Wound Healing Society, wound contraction is a parameter used to measure physical injuries that lead to a gap or break of the skin that causes disturbances within the traditional skin anatomy and performance. They lead to the loss of continuity of epithelium with or without the loss of underlying connective tissue [1-2]. Current estimates indicate that nearly half-dozen million individuals suffer from chronic wounds worldwide. Long lasting wounds perpetually release inflammatory mediators that produce pain and swelling at the wound site. Wound contraction is a parameter used to measure the degree of infection and recovery rate of injured patients. Chronic wounds can also lead to multiple organ failures and ultimately lead to death. Healing of wounds is crucial for the restoration of discontinuous anatomical continuity and disturbed skin integrity [3-5]. In order to measure the wound healing, an acceptable methodology of wound contraction is used as a parameter to measure the physical injuries which created a gap or breaking of the skin.

In recent years, many studies are applied on herbal medication to elucidate their potential in wound management, and these natural remedies have proven their effectiveness as an alternate treatment to the modern medication for the treatment of wounds. Several natural herbs are pharmacologically proven to possess potent wound healing activity [6].

*Hedyotis herbacea Linn* (family- Rubiaceae) is an erect, glabrous annual shrub found in temperate and tropical regions of Africa and Asia. A paste of leaves has been used as emollient and applied to abscesses and wounds [7-9]. The aqueous extract of the plant has been found to possess wound healing activity [10]. Our earlier study reveals the presence of Ursolic acid in the chloroform extract [11]. Ursolic acid has been already proven to possess wound healing properties [12] and no pharmacological activity was carried out in non-polar extracts. Hence the present study was performed to evaluate the wound healing activity of Ursolic acid rich chloroform extract of *Hedyotis herbacea,* against excision and incision wound model.

## 2. MATERIALS AND METHODS

### 2.1 Collection and Authentication of Plant Material

Leaves of *Hedyotis herbacea Linn* was collected from the Tirunelveli district, Tamil Nadu, India. The specimen was authenticated by V. Chelladurai, Retired, Research Officer-Botany (Scientist-C), Centre Council for Research in Ayurveda & Siddha, Government of India.

### 2.2 Preparation of Plant Extract

Leaves were dried in shade and ground to obtain a fine powder. The crude powder was subjected to extraction with various solvents with increasing polarity such as Petroleum ether, Chloroform, Ethyl acetate and Ethanol. The crude powder 1.5

kg was kept in contact with respective solvents for 7 days at room temperature with occasional shaking by the maceration technique. Then the extracts were filtered and the filtrates were concentrated at reduced pressure to obtain the corresponding residue. Yield of the extracts were found to be Petroleum ether 27.26gm, Chloroform extract 32.85gm, Ethyl acetate 18.23gm and Ethanol 8.72gm. All the extracts were estimated for the presence of phytoconstituents by HPTLC. As the study has been focused on the phytoconstituent based biological activity, the Ursolic acid rich chloroform extract was chosen for the study of wound healing activity.

## 2.3 Preparation of Herbal Ointment

The wound healing ointment was prepared by incorporating residue of chloroform extract of *Hedyotis herbacea* at the concentration of 2.5% w/w and 5% w/w into ointment base.

## 2.4 Wound Healing Activity Testing

### 2.4.1 Animals

To carry out the *in vivo* wound healing experiments, female Wistar rats of 6-8 weeks old and 160-180g body weight were purchased from Biogen Laboratory Animal Facility, Bangalore. The animals were maintained at the standard laboratory conditions in cross ventilated animal house at 25 ± 2C and light and dark cycle of 12:12 hours and fed with standard diet and water *ad libitum* during the study. The animals were used for experiment after 15 days of acclimatization. All the experiments were conducted in accordance with the internationally accepted guideline for laboratory animal use and care [13].

*2.4.1.1 Grouping of animals*

In the present study, healthy, adult female Wistar rats (160-180g), and 6–8 weeks of age were used. Four groups, each containing six rats, were used for the excision model. Animals in group I was left untreated and considered control, group II was treated with Povidone iodine ointment (5 % w/w) served as a standard, Group III and group IV were treated with Chloroform extract of *Hedyotis herbacea* ointment (CEHH) 2.5% w/w and 5% w/w, respectively. The animals were grouped in the above said manner for both the excision and incision wound models.

### 2.4.2 Induction of Excision wound [14]

All animals were anesthetized by intra-peritoneal injection of 1% sodium pentobarbital (i.p.,60 mg/kg body weight). Four groups of animals were depilated on the back and a predetermined area of 500 $mm^2$ full-thickness skins was excised in the dorsal interscapular region. All the surgical interventions were carried out under sterile conditions. Rats were left undressed in the open environment. The area of newly excised wounds on day zero had no significant difference among the three groups by statistical analysis. Each animal was placed in an individual cage with food and water *ad libitum* and maintained good health. Digital camera (Canon computer 1262, Japan) was employed for the photograph to measure the area of the wounds at different time intervals. After 24h of wound creation, groups II, III and IV was treated with povidone-ointment and CEHH 2.5% w/w and 5% w/w respectively, once daily until the complete healing.

### 2.4.3 Measurement of wound contraction [14]

Wounds were traced on 1$mm^2$ graph paper on the day of wounding and subsequently at a gap period of 3 days till the 12th day. The modification in the wound area was measured every day and the rate of wound contraction calculated by using below mentioned formula. Significance in wound healing of the test groups is derived by comparing healed wound area on respective days with healed wound area of the untreated control group.

$$\% \text{ Wound contraction} = \text{Healed area/Total wound area} \times 100$$

Where, Healed area = Original wound area - Present wound area.

### 2.4.4 Measurement of Tensile Strength [15]

The tensile strength of a healing skin wound indicates the degree of wound healing. It represents how much the healed tissue resists breaking under tension and identify the quality of healing tissue. On the $12^{th}$ day, all the animals were anesthetized by injecting ketamine hydrochloride (50mg/kg, i.p.,), and the healed tissue was excised from all animals. The tensile strength of the samples was tested using DAK SYSTEM BENCH. The speed was set at 100mm per minute and the load cell used was 500kg. The jaws of the tensile tester were set at 50mm, apart for the samples. The test specimen was

clamped in the jaws and the machine was run at the rate of 100±2mm/min, until the specimens tore apart. The highest load reached was recorded when the sample was broken. The distance between the jaws when rupture of the test specimen occurred was noted.

#### 2.4.5 Parameter Studied

After the experimental period, the animals were sacrificed by cervical dislocation and the blood and tissue samples were collected for analyzing biochemical parameters such as tissue protein [16], hydroxyproline [17], hexosamine [18], and hexuronic acid [19].

#### 2.4.6 Incision wound model [20]

The animals were grouped, anaesthetized, and treated the same as in the excision model. Paravertebral incision of 6 cm length was made through the entire thickness of shaved skin and subcutaneous tissue at a distance of about 1.5 cm away on either side of the vertebral column of the rats with the help of a sharp scalpel. After complete haemostasis, the parted skin was kept together and stitched at 0.5 cm intervals as interrupted sutures using surgical thread and a curved needle. The perpetual threads on both wound edges were tightened for good closure of the wound. After stitching, the wounds were left undressed, and all the groups were treated as follows. Group -I was kept as untreated control, Group-II with povidone-iodine ointment served as standard, Group III and IV with CEHH 2.5% w/w and 5% w/w ointment respectively daily for 10 days. When wounds were cured thoroughly, the sutures were removed on day 11 and the tensile strength of cured wound skin was measured, as described in the excision wound model, using a tensiometer on day 12.

#### 2.4.7 Histological evaluation of healed wounds [21]

On day 12 granulation tissue was excised and isolated from the healed skin of each group of rats and fixed in 10% formalin before histologically processed. Sections were made with Haematoxylin and Eosin (H and E) staining, and observed under a light microscope (10 and 40X) for assessment of histological features.

### 2.5 Statistical Analysis

All the results were expressed as Mean ± SEM. The data were statistically analyzed by one – way analysis of variance (ANOVA) followed by dunnet test. P values <0.001 were considered significant.

## 3. RESULTS AND DISCUSSION

Wound contraction, the process of shrinkage of the area of the wound depends on the reparative abilities of the tissue, type, and extent of the damage, and general state of the health of the tissue [22]. The process of mobilizing healthy skin surrounding the wound to cover the denuded area, involve complex and superbly orchesterated interactions of cells, extracellular matrix and cytokines. This centripetal movement of wound margin is believed to be due to the activity of myofibroblast [23]. *Hedyotis* species such as *Hedyotis diffusa Hedyotis auricularia, Hedyotis philippensis, and Hedyotis insularis, Hedyotis herbacea* traditionally claimed as wound healing plant [24]. It was provoked us to explore the wound healing efficacy of *Hedyotis herbacea* species. Pentacyclic triterpenoids have been studied for two decades and their pharmacological importance has increased significantly. In addition to their low toxicity, they are also described as having wound healing properties [25]. The wound healing process is effectively enhanced by triterpenoids due to their astringent and antimicrobial properties which are responsible for wound contraction and a higher rate of epithelialization [12]. Previous study showed that Pentacyclic triterpenoid, Ursolic acid has been isolated from the *Hedyotis herbacea* [26]. Moreover In silico studies have shown that Ursolic acid is a potential wound healing promoter. Molecular dynamic studies have shown that Ursolic acid had minimum potential energy and was more stable than the standard compound, nitrofurazone when compared with the target protein (GSK-3 β) [27]. Based on the significance of Ursolic acid in wound healing activity the research was aimed to study the effect of Ursolic acid enriched fraction in the wound healing process. Moreover in our previous study, all the extracts were screened by HPTLC for estimation of Ursolic acid [11]. The results suggested that the chloroform extract consists of higher amount Ursolic acid than other extracts. So, the chloroform extract was chosen for ointment preparation. The topical application of 5% CEHH ointment treated group showed better wound healing activity than the untreated control group in both excision and incision wound model. Efficacy of CEHH ointment in wound contraction might be due to increase in proliferation and

transformation of fibroblast cells into myofibroblast.

### 3.1 Effect of CEHH Ointment on Percentage of Wound Contraction by Excision Wounded Animals

The rate of wound contraction on post-wound days was presented in the table and figures. The size of the wound was measured on 3rd day, 6th day, 9th day, and 11th day. 2.5% w/w CEHH ointment treated groups showed effective contraction against untreated control where as 5% w/w CEHH ointment treated animals showed higher rate of wound contraction compared to 2.5% w/w CEHH ointment treated with untreated control group. It confers that the healing process of 5% w/w CEHH ointment is more effective than 2.5% w/w CEHH ointment. The rate of wound contraction on post-wound days was presented in the table and figures.

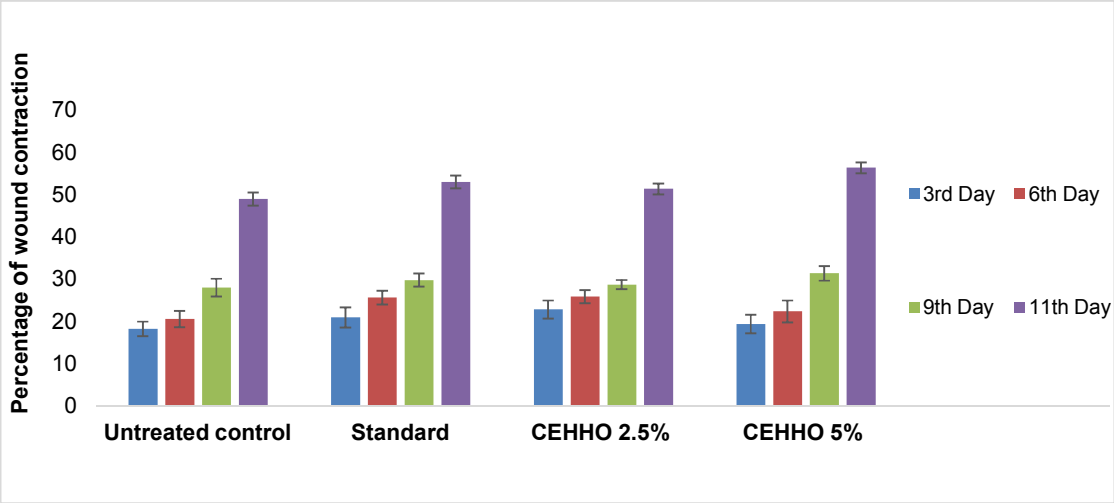
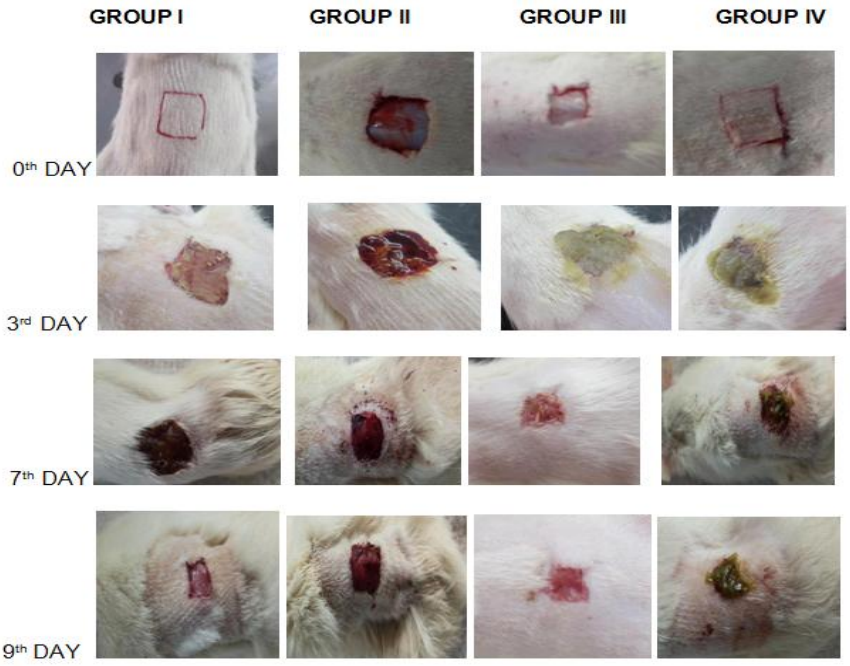



**Fig. 1. Percentage of wound contraction of CEHH ointment treated group and Standard compared with untreated group**



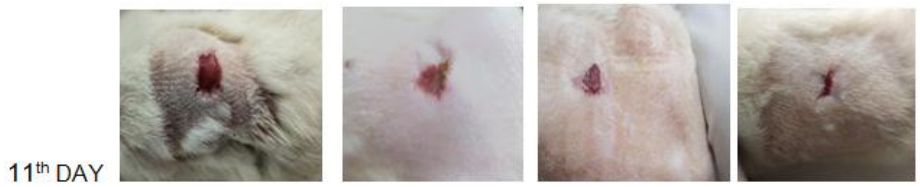



**Fig. 2. Photographs of wound repair at different time intervals in excision wound model in rats**

**Table 1. Effect of extract on wound contraction in excision wound Percentage of wound closure (% contraction)**

| Group | Untreated control | Standard | CEHHO 2.5% | CEHHO 5% |
|---|---|---|---|---|
| 3rd Day | 18.89±1.732 | 20.89±2.38 | 22.78±2.138 | 19.34±2.184 |
| 6th Day | 20.53±1.943 | 25.59±1.642$^{ns}$ | 25.82±1.532$^{ns}$ | 22.32±2.597$^{ns}$ |
| 9th Day | 27.97±2.096 | 29.76±1.574$^{ns}$ | 28.68±1.089$^{ns}$ | 31.34±1.722$^{ns}$ |
| 11th Day | 48.93±1.558 | 52.97±1.506$^{ns}$ | 51.32±1.265$^{ns}$ | 56.32±1.296$^{*}$ |

*Values are expressed as the mean ± S.D; Statistical significance (p)calculated by one way ANOVA followed by dunnett's test. ns- no significant, $^{*}$P< .001 comparing treated group with untreated control group*

### 3.2 Effect of CEHH Ointment on Tissue Protein, Hydroxy Proline, Hexosamine and Hexuronic Acid by Excision Wound

CEHH ointment treated group showed concentration-dependant action as shown by the increased levels of biochemicals such as tissue protein, Hydroxy proline, Hexosamine, and Hexuronic acid compared to untreated control. The results were depicted in the Table 2. Protein contributes the development of granulation tissue which is responsible for wound healing. Increased levels of protein signified the wound healing process [28]. Hydroxyproline concentration is a measure of collagen concentration. The higher concentration of hydroxyproline, increases the faster wound healing. Biochemical analysis showed a higher content of hydroxyproline, which reflects increased cellular proliferation and higher collagen synthesis.Hexosamine and hexuronic acid are matrix molecules, which can be used as the basic substrates for the synthesis of a new extracellular matrix. Glycosaminoglycans are known to stabilize collagen fibers, enhance electrostatic and ionic interactions with them, and possibly control their alignment and characteristic size. They are capable to bind and modify protein-protein interactions. It is an important determinant of cellular response in development, homeostasis, and disease [29-30]. Hexosamine and Hexuronic acid were found in enhanced levels in wound tissue, which confers additional confirmation for wound healing. All the biochemical parameters are elevated in the chloroform extract (CEHH), which can be considered as the biological markers in the wound healing process. The high content of Ursolic acid in the chloroform extract may be responsible for the above said ethnopharmacological activity.

### 3.3 Effect of CEHH Ointment on Tensile Strength of the Wound

The wound healing agent requisite the property of increasing the viability of collagen fibrils around the wound area that increases the tensile strength of the wound. This was assessed by evaluating the tensile strength of the healed wound using a tensiometer. The CEHH ointment treated group was found to possess significant concentration-dependent action by increasing the tensile strength as compared to untreated control in both excision and incision wound model. Increased tensile strength in the CEHH ointment treated group in both excision and incision model, suggests increased collagen formation. The results were depicted in the Table 3 and Fig. 3.

### 3.4 Histopathology Study

The histopathological studies were performed on the tissue of the excision wound on the 11th day. The histopathological features of the tissue of all groups of animals are showed in Figs. 4a–4d. Group I (Untreated control) animal showed fragments of tissue lined by stratified squamous

epithelium with adjacent areas showing neutrophilic abscess and karyorrhectic or necrotic debris. There is no evidence of healing of wound (Fig. 4(a)). Group II (Standard) showed fragments of tissue showing proliferation of thin-walled vessels. The dermis showed granulation tissue with mild lymphocytic infiltration. Adjacent areas showed dense fibro collagenous tissue (Fig. 4(b)). Group III (CEHH 2.5% w/w ointment) showed focal areas of granulation tissue with inflammatory infiltrates. In the view of 40X, edema with congested vessels along with focal inflammation was observed (Fig. 4(c)). Group IV (CEHH 5% w/w Ointment) showed focal areas of granulation tissue with the proliferation of the vessels and focal inflammation in the dense area of fibrosis (Fig. 4(d)). The 5% CEHH ointment showed a better healing response to the wound.

**Table 2. Effect of extraction protein, hydroxy proline, hexosamine and hexuronic acid levels in excision wound**

| Group | Protein (mg/gdry tissue) | Hydroxyproline (µg/mg of protein) | Hexosamine (µg/mgof protein) | Hexuronic acid (µg/mg of protein) |
|---|---|---|---|---|
| **Untreated control** | 45.67±1.45 | 43±1.08 | 81.77±0.5141 | 17.82±0.5371 |
| **Standard** | 57.61±0.78* | 81.87±0.8817*** | 130.6±1.084*** | 54±1.155*** |
| **CEHH 2.5% Ointment** | 48.9±3.194ns | 57.75±6.225* | 113.1±3.769*** | 28.46±2.571** |
| **CEHH 5% Ointment** | 55.5±3.31* | 71.93±0.9961** | 124.3±2.501*** | 49.62±0.4174*** |

*All values are expressed as mean± S.E.M; (n=6) *(P<.001), **(P=.01), ***(P=.05), all treated groups compared with untreated control*

**Table 3. Effect of CEHH ointment on tensile strength of Excision and Incision wound**

| Group | Type of Wound | Untreated control | Standard | CEHH 2.5% w/w Ointment | CEHH 5% w/w Ointment |
|---|---|---|---|---|---|
| Tensile stregth (µg/g of tissue) | Excision | 328.13±1.93 | 1069.91±4.8 | 481.3±3.69 | 912.5±3.48 |
| | Incision | 339.77±6.7 | 1084.50±3.32 | 857.91±2.65 | 939.27±3.15 |

*All values are expressed as mean± S.E.M; (n=6)*

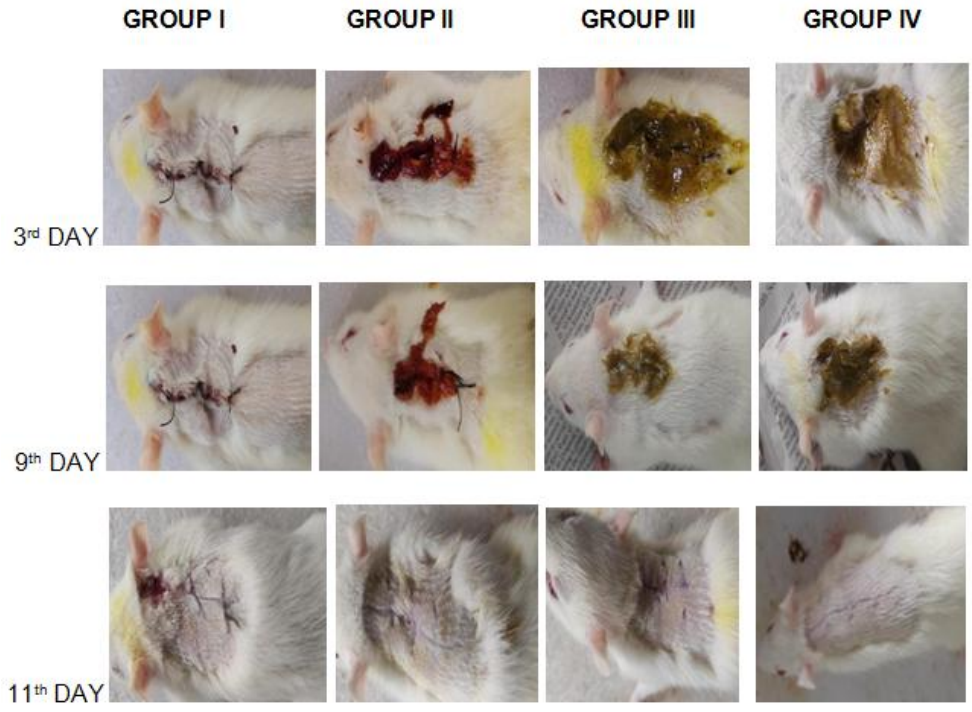



**Fig. 3. Photographs of wound repair at different time intervals in incision wound model in rats**

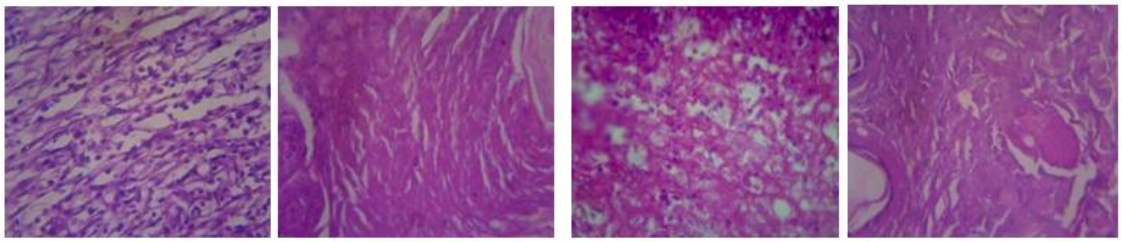



**Fig. 4. Photomicrograph of histopathological section of wound tissue of rats (stained with H&E, 40x magnification)**

Histopathological study of the ointments treated rat wound tissues also revealed the effectiveness of CEHH in improved wound healing. However, the other secondary metabolites such as steroid, alkaloids, quinones, phenolic compond and saponin has been found in minor quantities. These consituents may also contribute to the wound healing activity.

## 4. CONCLUSION

In conclusion, the results of the present study revealed that the ointment prepared from chloroform extract of *Hedyotis herbacea* contains the phytoconstituents that promote the natural healing process and it could be effectively used as a wound healing agent. CEHH 5% w/w ointment efficiently stimulates wound strength and increases the tensile strength around the wound area. Elevated levels of tissue protein, Hydroxy proline, Hexosamine and Hexuronic also provided additional confirmation for wound healing efficiency. Histopathological results also supports the wound healing ability of CEHH. The presence of Ursolic acid in the chloroform extract of may be responsible for the wound healing property. Further studies are planned to explore the active compounds other than Ursolic acid which may be responsible for wound healing activity. The biological activity of explored active compounds will be compared with crude extract to get the confirmation of wound healing agent present in the *Hedyotis herbacea.*

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

All the studies were conducted according to the ethical guidelines of CPCSEA after obtaining necessary clearance from the committee (IAEC approval No:68/PO/Re/S/02/CPCSEA).

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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