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Preclinical Assessment of the Wound Healing Efficacy of *Hemidesmus indicus* Hydroalcoholic Root Extract on *Cyprinus rubrofuscus*

Prodip Roy[1], Dwipannita Mukherjee[1*], Soumyadip Sahu[1], Mrityunjoy Majumdar[1], Sourav Roy[1], Shreya Sarkar[1], Gourav Pal[1], Subhajit Paul[1], Ruchismita Manna[2] and Arpan Chanda[3]

[1]Department of Pharmacology, Netaji Subhas Chandra Bose Institute of Pharmacy, Chakdaha, Nadia - 741222, West Bengal, India; dwipannitamukherjee2004@gmail.com

[2]Amity Institute of Pharmacy (Department of Pharmacology), Amity University Kolkata, Kadampukur, Newtown – 700135, West Bengal, India

[3]Seacom School of Pharmacy (Department of Pharmacology), Seacom Skills University, Kedradangal, Bolpur – 731236, West Bengal, India

Abstract

Background: Effectively managing wounds presents a significant challenge, as skin injuries frequently result in secondary bacterial infections and the limitations of existing treatments, including high cost and adverse reactions. As a result, there has been a growing interest in using herbal remedies. To improve animal welfare and experimental effectiveness, *Hemidesmus indicus,* which is well-known for its folklore and its antibacterial and antioxidant qualities, was evaluated for its capacity to heal wounds using a fish model. **Aim:** This study aims to explore the potential wound healing properties of *H. indicus* root hydroalcoholic extract on *Cyprinus rubrofuscus*. **Methods:** After anaesthesia, standardised excision wounds or burn wounds were created in the region between the pectoral fin and dorsal fin of each fish (*C. rubrofuscus)* in all experimental groups. Group I served as control, Group II (standard) received providone iodine solution (0.1%), Group III (Test I) received a test root extract of *H. indicus* (25 mg/L), and Group IV (Test II) received a test root extract of *H. indicus* (10mg/L). The healing progression was evaluated by noting the time for complete epithelialisation; 50% wound contraction, and histopathological analysis was also performed. These experimental findings revealed that animals treated with *H. indicus* root hydroalcoholic extract exhibited a significantly faster wound contraction rate compared to the control group in both models, in a dose-dependent manner. **Result:** In the excision wound model, complete wound contraction was observed within 15.833±0.401 days at 25 mg/L and 17.5±0.718 days at 10 mg/L, whereas in the burn wound model, complete wound contraction occurred within 18.333±0.666 days at 25 mg/L and 19.833±0.6 days at 10 mg/L. Both treatments showed statistically significant improvement compared to the control group ($p < 0.01$). **Conclusion:** This research indicates that the hydroalcoholic extract from the roots of *H. indicus* has strong wound-healing capabilities.

Major Findings: The hydroalcoholic root extract of *H. indicus* showed dose-dependent wound healing in excision and burn models on *C. rubrofuscus*. These findings highlight its strong wound-healing potential and support its traditional use.

Keywords: Burn Wound Model, *Cyprinus rubrofuscus*, Excision Wound Model, *Hemidesmus indicus*, Histopathology, Hydroalcoholic Extract

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1. Introduction

Wound healing refers to the process of restoring the skin and other soft tissues following an injury. An inflammatory response occurs after an injury, leading to inflammation in the cells beneath the affected area. The dermis, which is the deepest layer of the skin, starts to generate additional collagen, facilitating the synthesis

*Author for correspondence

of connective tissue. Subsequently, the epithelial tissue, or outer skin, begins to regenerate[1]. The wound healing process is categorised into four distinct yet overlapping phases: Hemostasis, inflammation (also known as the defensive phase), proliferation, and maturation[2].

Plants serve various purposes, including food, fuel, herbs, and more and play a vital role[3]. Since ancient times, humans have depended on plants for medicinal purposes. Historical records indicate that the intrigue with plants existed even before the advent of humanity. The plant kingdom is an extensive source of organic compounds, many of which have been utilised for medicinal purposes and could serve as a foundation for the future creation of innovative agents effective against numerous pathological conditions[4]. The fundamental healthcare offered by herbal plants is crucial for society. The primary challenge to the acceptance of herbal medicines lies in the insufficient research and quality control. Therefore, it becomes exceedingly important to standardise the plant materials intended for medicinal use[3]. More infections and diseases seem to impact humans than any other species of animals. Nature has provided us with a comprehensive array of remedies for all human ailments and the diseases associated with them[5]. One of the plant materials that is commonly utilised in herbal medicine is "*Anantmool*" (*Hemidesmus indicus*). It is an Indian traditional medicinal plant, also referred to as Indian *Sarsaparilla*, from the *Apocynaceae* family[6], native to the Indian subcontinent[7]. It is mostly produced in South Asian countries such as India, Bangladesh, Pakistan, Iran and Maldives[8]. The plant has been predominantly located across India, extending from the Indo-Gangetic plains to the eastern regions up to Assam, and throughout Southern India in both mesophytic and semidry habitats. It thrives in damp deciduous forests, shrublands, and degraded zones characterised by wild soils. Typically, it is cultivated at elevations of 600 meters[9]. Phytochemical studies indicate the presence of flavonoids, steroids, saponins, terpenoids, lignin, phenolic compounds, tannins, cardiac glycosides, carbohydrates, and proteins in the roots of *H. indicus*[10]. The plant has been traditionally utilised for a range of ailments, including diabetes, leprosy, skin conditions, urinary disorders, sexually transmitted infections, syphilis, snakebites, scorpion stings, dyspnea, inflammation, rheumatism, menorrhagia, pyrosis, abdominal colic, dysentery, diarrhoea, anorexia, respiratory issues, fever, cough, bronchitis, and cancer. It has already been established that plants exhibiting anti-microbial, analgesic and anti-inflammatory activity demonstrate potential healing effects on wounds. In this context, *H. indicus* has been chosen to investigate its wound healing activity in a preclinical evaluation[11,12].

2. Materials and Methods

2.1 Plant Materials

The fresh roots of *H. indicus* were collected during the month of January-February from the local market of Netaji Subhas Chandra Bose Institute of Pharmacy, Chakdaha, Nadia, West Bengal, India.

2.2 Preparation of Extract[13,14]

The roots of *H. indicus* were collected, carefully washed with distilled water, and the roots were manually chopped into small pieces. Subsequently, they were dried in the shade for 5 days. Finely ground, shade-dried roots of *H. indicus* were extracted using 70% hydroalcoholic solution for 72 hrs, using a Soxhlet extractor. An air-drying method was employed to dry the extract. The extract was then wrapped in aluminium foil and stored at 4°C.

2.3 Phytochemical Screening[15]

For the purpose of identifying the various active ingredients, such as alkaloid, carbohydrate, flavanoid, saponin, glycoside, tannin, polyphenol and steroid, the crude extracts underwent qualitative analysis. Standard practises were used to conduct phytochemical tests.

2.4 Animals

Cyprinus rubrofuscus, also known as the amur carp fish, weighing an average of 80-100gm were used to perform this research work. The *C. rubrofuscus* is a freshwater fish widely found in Eastern Asia[16,17]. The *Cyprinus* genus is well known for its high tolerance to different environmental conditions[18]. *Cyprinus rubrofuscus* is notably characterised by its exceptional resistance to cold temperatures, elevated alkalinity, and low oxygen environment[19]. During this study, the fish were kept in a suitable and hygienic environment, maintained at a standard temperature and with a continuous air supply.

For the skin irritation study, New Zealand white rabbits were used, which were purchased from a

Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)-approved breeder. The rabbits were housed in CPCSEA-approved Netaji Subhas Chandra Bose Institute of Pharmacy (NSCBIP)) animal house under standard laboratory conditions with 12 hours light/12 hours dark cycles and a room temperature of 22 °C with appropriate food and water supply.

2.5 Drug Solution Preparation[20-22]

2.5.1 Standard Drug

0.1% Povidone iodine solution was prepared by adding 1ml 10% (marketed Betadine solution) Povidone iodine solution into 99ml of water.

2.5.2 Test Drug

- 10 mg/Litre: For preparing a 10 mg/L test drug, 10mg of *Hemidesmus indicus* extract was added to 1000ml water.
- 25 mg/Litre: For preparing a 25 mg/L test drug, 25 mg of *Hemidesmus indicus* extract was added to 1000ml water.

2.6 Wound Healing Models

2.6.1 Excision Wound Model[23-25]

Amur carp, each weighing between 80 and 100 grams, were utilised for this study. The subjects were divided into four distinct groups, with six fish in each group. Before the creation of the wounds, the fish were anaesthetised by immersing into the clove oil solution (0.1ml/L). Excision wounds were inflicted on the fish; the scale of the fish between the pectoral fin and dorsal fin on one side was cleared by using forceps, and the marked place was. A full-thickness excision wound measuring 50mm² was created along the predetermined markings using forceps and a surgical blade. The wound was left entirely exposed. After that, the drugs were administered twice daily by soaking a sterile cotton swab in the prepared solution and gently applying it directly to the wound area of the respective experimental groups for ≤ 60 sec.

Group I: Control group: the fish in this group did not receive any treatment.

Group II: Standard group: the fishes in this group received povidone-iodine solution (0.1% PI) twice daily.

Group III: Test group: the fish in this group were treated with *H. indicus* 25mg/L solution until complete epithelialisation occurred, administered twice daily.

Group IV: Test group: the fishes in this group were treated with *Hemidesmus indicus* 10mg/L solution until complete epithelialisation occurred, administered twice daily.

The measurement of wound contraction was conducted by outlining the wound using cellophane paper and a permanent marker on days 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 post-wounding.

On graph paper with a millimetre scale, the areas of the wound were documented and quantified. Considering the time required for complete epithelialisation was established and documented.

2.6.2 Burn Wound Model[26-29]

Amur Carp (*Cyprinus rubrofuscus)* fish weighing between 100gm were divided into four groups. After anaesthesia, partial thickness burn wounds were induced on descaled animals by pouring hot molten wax at 80^{0}C into a cylinder with a 50 mm² circular aperture placed on the area between the pectoral fin and dorsal fin of the animal until the wax became solid. After the wax-adhered cylinder was removed, a clearly defined, roughly 50mm² circular burn wound with a significant partial-thickness injury was visible. As soon as the injury occurred, the drugs or vehicles were applied topically.

Group I: Control group: the fish in this group did not receive any treatment.

Group II: Standard group: the fishes in this group received povidone-iodine solution (0.1% PI) twice daily.

Group III: Test group: the fishes in this group were treated with *H. indicus* 10mg/L solution until complete epithelialization occurred, administered twice daily

Group IV: Test group: the fishes in this group were treated with *H. indicus* 25mg/L solution until complete epithelialization occurred, administered twice daily.

The measurement of wound contraction was conducted according to the excision wound model. Considering the time required for complete epithelialization was established and documented.

2.7 Skin Irritation Study

Healthy, 10–12-month-old New Zealand white rabbits were taken, and a small dorsal area of 6 cm² was shaved for test topical formulation application. 10mg/Litre and

25 mg/Litre of *H. indicus* solution were applied to the shaved area of the rabbit. The animals were observed for 3 days for any symptoms of redness, oedema, inflammation[30].

2.8 Statistical Analysis

The results are presented as mean ± S.E.M. A statistical analysis was conducted using ANOVA followed by Dunnett's test, where a P value of <0.01 compared to the control group was considered significant[31].

3. Results and Discussion

3.1 Extract Preparation

The *H. indicus* roots were extracted by the cold Soxhlet method for 72 hours, using 70% hydroalcoholic solution, and 8.33 % yield was obtained.

3.2 Phytochemical Investigation

Through the different phytochemical investigations, the hydroalcoholic extract of *H. indicus* roots showed the presence of different phytochemicals such as alkaloids, steroids, flavonoids, tannins, saponins, polyphenols, glycosides, and carbohydrates (Table 1).

3.3 Impact of *Hemidesmus indicus* Root Extract on Wound Healing

3.3.1 Excision Wound Healing

In the present study, the excision wound model was utilised to assess the wound healing capabilities of the hydroalcoholic extract derived from *H. indicus* roots at dosages of 10 mg/L and 25 mg/L. Key parameters assessed included the duration of 50% of wound contraction and the duration of epithelialisation. The findings were compared against a control group and a group treated with a standard medication.

Animals treated with the hydroalcoholic extract of *H. indicus* exhibited a dose-dependent enhancement in wound healing activity. The 25 mg/L dosage resulted in the quickest epithelialization time (15.833±0.401 days), followed by the 10 mg/L dosage (17.5±0.718 days), while the control group took the longest to achieve complete epithelial coverage (19.666±0.666 days). The standard drug facilitated the most rapid epithelialization (15.333±0.614 days), although the higher extract dosage (25 mg/L) closely approached its effectiveness as compare the standard (Table 2 and Figure 1).

On the other hand, a 50% reduction in wound contraction is a crucial factor that can indicate the quality of epithelialization, or the healing rate. A quicker healing rate suggests a more rapid proliferation of the injured cells. The standard drug facilitated the most rapid 50% wound contraction (8.833±0.6 days). The group treated with the 25 mg/L extract showed a significantly shorter 50% wound contraction time (9.166±0.477 days) from the initial stages of healing compared to the control group (11.333±0.683 days). The 10 mg/L group also demonstrated notable improvement (9.833±0.792 days), although the effect was less pronounced than that of the higher dosage. The standard drug facilitated the most rapid epithelialization, although the higher extract dosage closely approached its effectiveness (Table 2 and Figure 1).

3.3.2 Burn Wound Healing

In the burn wound model, topical application of *H. indicus* at doses of 10 mg/L and 25 mg/L, as well as

Table 1. Phytochemical investigation results of the hydroalcoholic root extract of *Hemidesmus indicus*

Phytoconstituent	Test	Observation	Result
Alkaloid	Dragendorff test	Reddish Brown Precipitate	Present
Flavonoid	Ferric chloride test	Blackish Red Colour	Present
Saponin	Foam test	Formation of Stable Foam	Present
Glycoside	Keller-Kiliani test	Blue Colour at Junction	Present
Tannin	Lead Acetate test	White Precipitate	Present
Carbohydrate	Molisch's test	Purple Colour	Present
Polyphenol	Ferric Chloride test	Red Colour	Present
Steroid	Salkowski Test	Reddish Brown Colour	Present

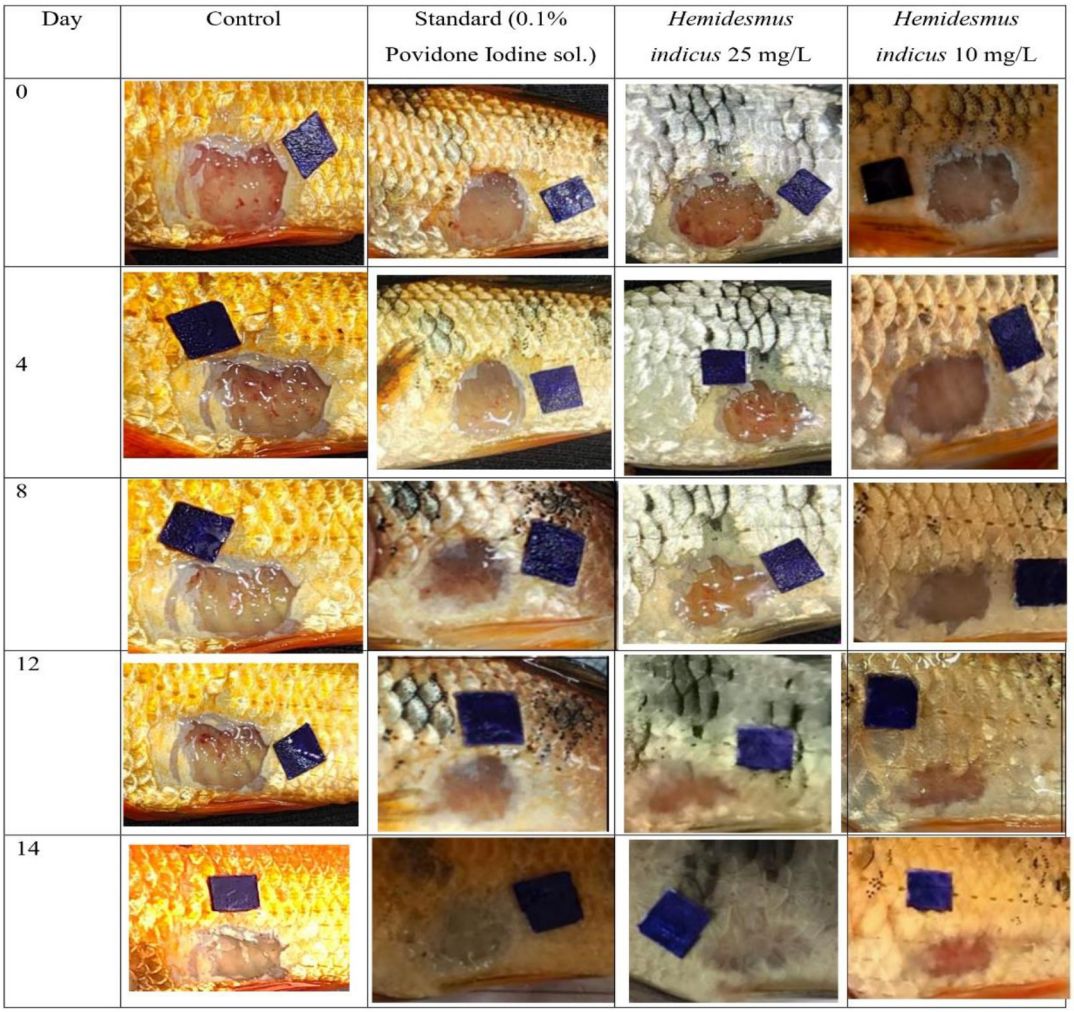


Figure 1. Images of healing in the excision wound model.

the marketed standard Betadine solution, significantly reduced the period of epithelialisation compared with the control group. The standard-treated group showed complete epithelialisation within 17.166±0.703 days. While the epithelialization period was 19.833±0.6 days and 18.333±0.666 days for the 10 mg/L and 25 mg/L treatment groups, compared to 21.166±0.654 days in the control group. Similarly, all treatment groups showed a significant reduction in the time required for 50% wound contraction. The 10 mg/L and 25 mg/L groups achieved 50% contraction in 10.166±0.792 days and 9.8±0.532 days, respectively, whereas the standard-treated group reached this level in 8.4±0.465 days, compared to 12.666±0.421 days in the control group (Table 3).

Furthermore, the study demonstrated a clear dose-dependent response, where the higher dose (25 mg/L) produced better results compared to the lower dose (10 mg/L) in both the parameters, namely epithelialisation period and 50% wound contraction time, as compared to the control group (Figure 2).

3.4 Skin Irritation Test

The study on acute dermal irritation was conducted by observing the skin areas of the rabbit one hour after the application of the drug solution. At the 24-hour observation, two of the treated skin areas appeared normal, while the remaining treated site showed normalcy at the 72-hour observation (Figure 3).

3.5 Histopathology

Histopathological examination of skin tissues from both the excision and burn wound models showed significant improvement in the treated groups as compared to

Table 2. Effect of *Hemidesmus indicus* on the period of epithelialisation and 50% wound contraction in the excision wound model

Treatments	Period of Epithelialisation (days)	Wound Contraction 50% (days)
Control	19.666±0.666	11.333±0.683
Povidone Iodine Solution (0.1% PI)	15.333±0.614***	8.833±0.6***
Hemidesmus indicus (25mg/L)	15.833±0.401***	9.166±0.477***
Hemidesmus indicus (10mg/L)	17.5±0.718***	9.833±0.792***

All values are mean ± SEM, n=6, ***p<0.01 vs control

Table 3. Effect of *Hemidesmus indicus* on the period of epithelialisation and 50% wound contraction in the burn wound model

Treatments	Period of epithelialisation (days)	Wound Contraction 50% (days)
Control (No treatment)	21.166±0.654	12.666±0.421
Standard (0.1% PI)	17.166±0.703***	8.4±0.465***
Hemidesmus indicus (25 mg/L)	18.333±0.666***	9.8±0.532***
Hemidesmus indicus (10 mg/L)	19.833±0.6***	10.166±0.792***

All values are mean ± SEM, n=6, ***p<0.01 vs control

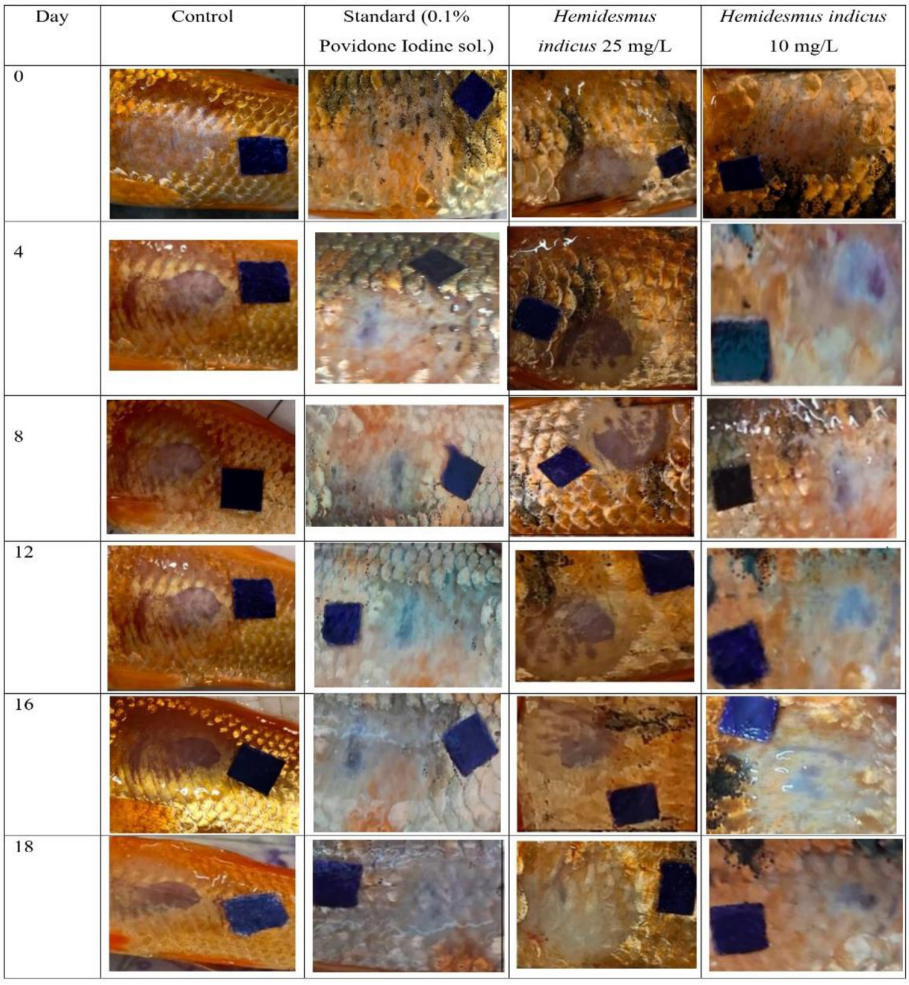


Figure 2. Images of healing in the burn wound model.

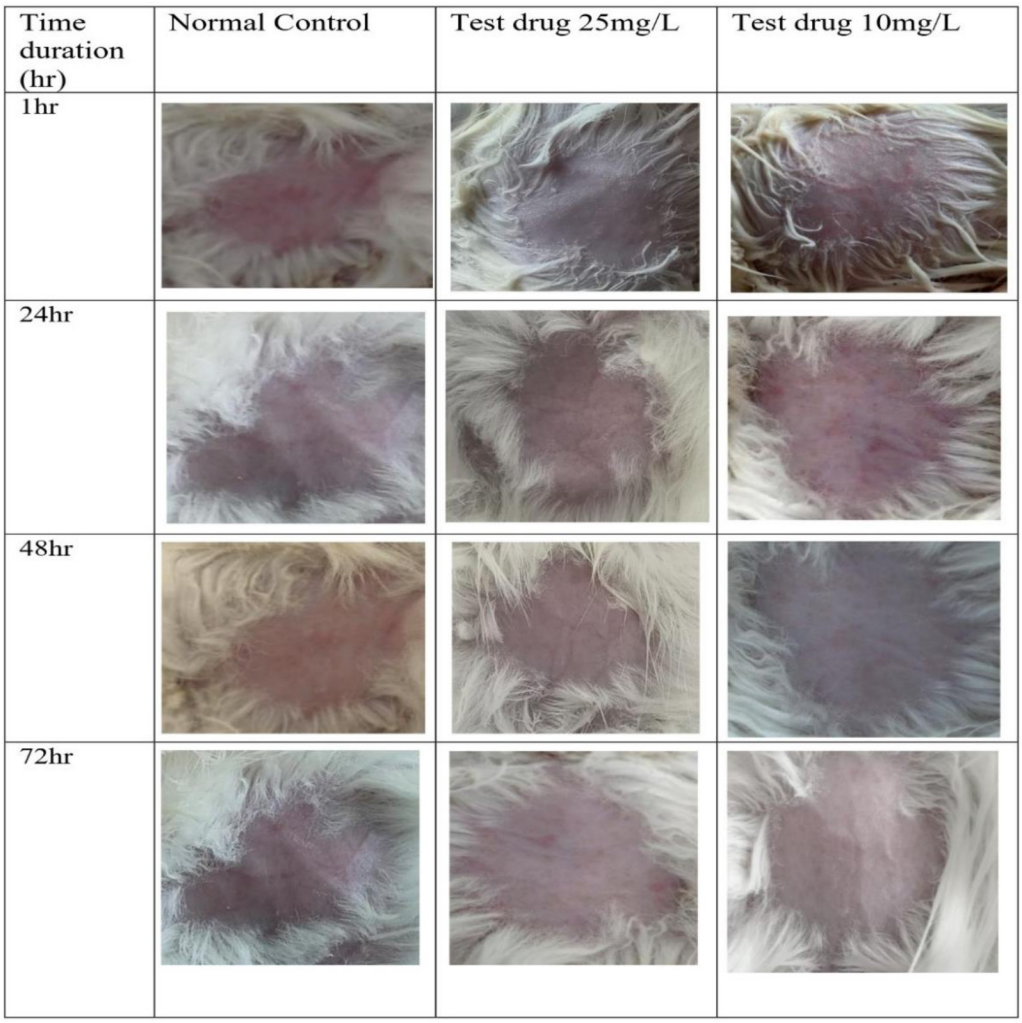
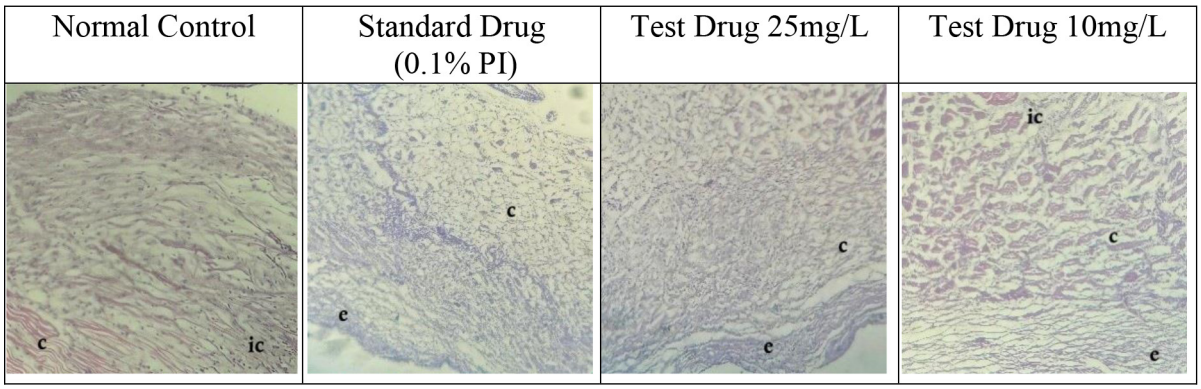


Figure 3. Acute dermal irritation assessment in rabbits.

the untreated control. Incomplete epithelialization, ongoing inflammatory cell infiltration, and inadequate collagen deposition were observed in the control group. The control exhibited poor collagen deposition, chronic inflammatory cell infiltration, and inadequate epithelialisation. At 25 mg/L, *H. indicus* treatment showed nearly identical histostructural restoration to the group that had standard 0.1% povidone-iodine treatment. This group's characteristics included dense collagen fibres, well-formed epidermis, and decreased inflammatory cells. Significant improvement was also observed in the 10 mg/L dose; however, not as much as in the 25 mg/L group (Figures 4 and 5).

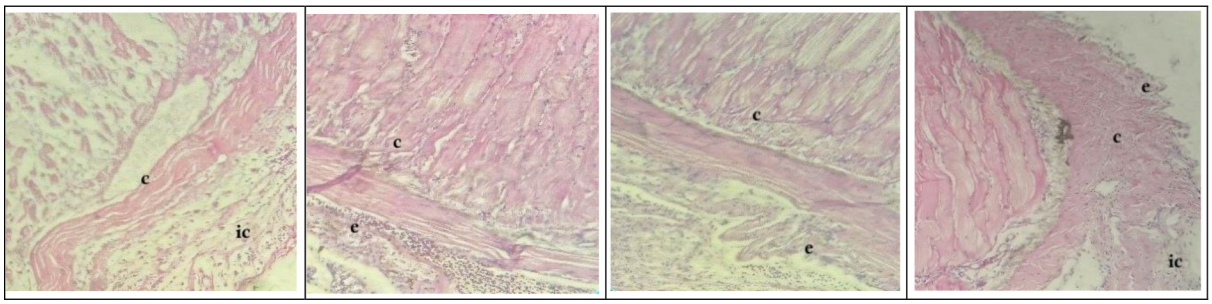
Wound healing is a multifaceted and coordinated series of biological processes that includes inflammation, proliferation, collagen synthesis, angiogenesis, and tissue remodelling. After an injury, these phases work together to restore tissue integrity and functional continuity.

The present research investigation used an excision wound model and Burn wound model on *C. rubrofuscus* fish to assess the hydroalcoholic extract of root of *H. indicus* ability for healing wounds. In comparison to the control and standard drug-treated groups, the duration of wound contraction and epithelialization was measured. The basis of taking Betadine as a standard in the study is common. Povidone-iodine is a broad-spectrum antiseptic that works effectively against a variety of mycobacteria and bacteria. Free iodine is progressively released at the application site to produce its antibacterial effect. The released iodine breaks down structural elements, penetrates microbial cell membranes, and obstructs vital biological functions, finally resulting in cell death[32].



(e- *epithelium, c- collagen* fibres, ic- inflammatory cells)

Figure 4. Histopathological assessment of skin in the excision wound model.



(e- *epithelium, c- collagen* fibres, ic- inflammatory cells)

Figure 5. Histopathological assessment of skin in the burn wound model.

When the extract was administered, there was a significant dosage-dependent improvement in wound healing; the 25 mg/L dose resulted in significantly quicker wound contraction from the beginning of healing than the control. At the 10 mg/L dose, healing was likewise notably improved; however, the effect's magnitude was moderate and slightly less apparent than at the higher dosage. Histopathological data confirm these findings, exhibiting that the 25 mg/L group had improved re-epithelialization, dense and regulated collagen deposition, decreased inflammatory cell infiltration, and increased angiogenesis, all of which were comparable to the standard therapy. Additionally, no skin irritation was noted, suggesting that the extract is safe to apply topically. The wound healing effect exerted by *H. indicus* may be due to its anti-inflammatory, antimicrobial, and antioxidant activity. It is well-established that agents with these pharmacological properties minimise oxidative stress at the wound site, limit microbial infection, and reduce inflammation, all of which have a major impact on the wound-healing process[33-36]. Through chemical analysis, *H. indicus* showed the presence of different phytochemicals like alkaloids, steroids, flavonoids, tannins, saponins, polyphenols, glycosides, and carbohydrates. Some of these constituents may contribute to the healing properties of *H. indicus*.

The steroids exhibit anti-inflammatory effects by inhibiting phospholipase A2, which is crucial for the production of inflammatory substances[37]. Alkaloids, characterised by the presence of nitrogen in their structure, typically demonstrate anti-inflammatory, antimicrobial, and antioxidant properties[38]. Tannins are recognised for their antioxidant capabilities, and the drugs that exhibit antioxidant effects are known to promote wound healing to varying degrees. Proteins serve as the building blocks of the body, with amino acids being the fundamental units of proteins. Amino acids can enhance the healing process by accelerating cell proliferation and also aid in the maturation of eschar. Additionally, flavonoids stimulate endogenous antioxidant systems, which in turn inhibit the biosynthesis of eicosanoids, leading to reduced production of inflammatory metabolites and thereby producing marked anti-inflammatory effects[39]. *Hemidesmus indicus*

has demonstrated anti-inflammatory, antioxidant, and antimicrobial properties. The antioxidant effects may be attributed to the presence of alkaloids. Additionally, the presence of steroids, tannins, and alkaloids may account for its anti-inflammatory properties. It is already established that tannins are the most active components of herbs, responsible for their wound healing effects. Based on all the findings mentioned above, it is evident that the potent wound healing effect observed in *H. indicus* may be attributed to the presence of its chemical constituents, including tannins, alkaloids, steroids, and proteins, which simultaneously render *H. indicus* a unique medicinal herb.

4. Conclusion

The present study was employed to evaluate the wound-healing potential of hydroalcoholic root extract of *H. indicus* in Amur carp (*Cyprinus rubrofuscus*) using an experimentally induced excision wound model and burn wound model. Phytochemical screening of the hydroalcoholic root extract revealed the presence of biologically active components, such as carbohydrates, flavonoids, tannins, alkaloids, steroids, phenolic compounds, saponins, and proteins, that are known to participate in tissue repair and regeneration. The Soxhlet extraction method was used to create the hydroalcoholic extract, which was then tested for its ability to heal wounds *in vivo* at two different concentrations (25 mg/L and 10 mg/L). When compared to the control group, both treatment doses showed a notable improvement in wound contraction and healing. Remarkably, the wound-healing activity of the *H. indicus* root extract (25 mg/L) was comparable to that of the standard treatment, povidone-iodine diluted solution.

These findings demonstrate that the hydroalcoholic extract of *H. indicus* roots has promising wound-healing properties and may serve as a potential natural therapeutic agent for managing external wounds. These encouraging findings warrant further investigation to elucidate the precise mechanisms of action and broaden its range of potential uses.

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Author Contribution

Mr PR, Ms DM, Mr SS, Dr MM and Mr SR contributed to the conception, design, and execution of the research work. Mr SP, Mr GP, Ms SS, Ms RM. Mr AC contributed to manuscript writing, discussion, and editing. All authors have read and agreed to the published version of the manuscript.