Indian Journal of Traditional Knowledge
Vol 22(4), October 2023, pp 739-745
DOI: 10.56042/ijtk.v22i4.7197

Assessment of *in vivo* wound healing potentiality of *Trapa natans* L. leaves on different wound models in rat

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Received 31 December 2020; revised & accepted 20 January 2023

The primary goal of this study was to assess the wound healing potentiality of *Trapa natans* L. Excision and incision models were used to evaluate wound healing activity. For the excision model experiment was conducted for 21 days and for the incision model experiment continued for 10 days. Experimental animals were divided into four groups, the control group received simple ointment, the animal of the standard group treated with 0.2% w/w soframycin ointment, and the test groups received *T. natans* extract ointment (2.5% w/w & 5% w/w). Wound healing was determined by the rate of contraction, the time required for epithelization, collagen cell formation, the amount of hydroxyproline present in the wound site, and wound-breaking strength. Experimental results of the test showed a comparatively higher rate of contraction, lesser epithelization period, and higher hydroxyproline amount present in the wound area. These results were strengthened by the histopathological studies of the healing area tissues. When the standard and test groups were compared to the control group, statistically significant data were found ($p<0.001$). The test group had better wound-healing properties as compared to the control group. From the result of this study, it was revealed that *T. natans* L. is an excellent herbal alternative for treating wounds.

Keywords: Excision model, Hydroxyproline, Incision model, *Trapa natans* L.

IPC Code: Int Cl.[23]: A61K 36/00, A61K 36/185

The wound is an injury of the external or internal body tissues occurring due to violence, accident, or surgery. The four stages of the wound-healing process are homeostasis, inflammation, proliferation, and maturation. To restore the skin's damage, an appropriate healing treatment is required[1,2]. The focus of research in the field of wound healing has changed recently. Although ethnopharmacology recommended he herbal plant as the essential drug for recovery of the wound but still very little progress has been done. Herbal drugs show the most important role in the treatment of wounds, and Lythraceae family is one of them[3,4]. Lythraceae is a worldwide spread family including 31 genera and 600 species. One of the most significant plants in this species is *T. natans*[5]. The plant has been found to be effective in treating diarrhea, ophthalmology, and ulcer-related disorders[6,7]. Additionally, it has been observed from the literature that this plant possesses antibacterial, anti-inflammatory, and wound-healing properties[8,9]. It was noticed that this herb has traditionally been used to heal external wounds[10,11]. So, the main objective of the current study was to assess the ability *T. natans* for wound healing.

Materials and Methods

Identification of the drug

The *T. natans* plant was collected from Purulia, West Bengal, in the month of July 2018. The plant was identified and authenticated by Dr. Anjula Pandey, NBPGR, New Delhi, India, and voucher no. NHCP/NBPGR/2013-7 was allotted for this plant species.

Preparation of extract

T. natans L. leaves were shade dried and coarsely powdered. After that 50 g of powder was extracted with 1 L methanol with the help of Soxhlet apparatus for 48-60 h at 50◦C temperature. The yield of the extract was 24.5%.

Preparation of ointments

Wool fat (5%), hard paraffin (6%), cetostearyl alcohol (5%), and 84% yellow soft paraffin were used to make the ointment. The ingredients were melted with continuous stirring. An alcohol-free *T. natans*

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extract was prepared and used for the preparation of ointment[12].

In this study, four different types of ointments (I–IV) were taken. Batch I was blank control ointment, Batch II was marketed drug (0.2% w/w Soframycin ointment) and batches III-IV contained varying concentrations of the *T. natans* L. extract (2.5 g and 5 g per 100 g of the ointment base, respectively)[13].

Evaluation of ointment[14,15]

Organoleptic property

The organoleptic property of the ointment such as color, odor, texture, phase separation, and homogeneity of all ointment was determined.

pH test

For the test of pH, 1 g ointment was dispersed in 100 mL distilled water. Using the specified buffer solutions 4 and 7, the pH of the dispersion was checked using a pre-calibrated pH meter. The pH measurement process was repeated three times.

Wash ability

After applying the ointment to the skin, the time it took for it to be washed using water was recorded.

Spreadability test

The spreadability of the prepared ointment was determined by spreading 1 g of ointment in between two horizontal plates of diameter 20 cm × 20 cm.

Stability analyze

The stability of the prepared ointment was assessed for a month at various temperatures, including 2°C, 25°C, and 37°C. It was found that the herbal ointment was stable in a wide range of environment.

Skin irritability test

The ointment was applied to the human skin and results were recorded.

Preparation of animal for this study

Wister albino rats of either sex weight (100-150 g each) were procured from the Central Animal House, NIET, Greater Noida. All of the experimental animals were housed in the animal room in polypropylene cages with six animals per cage under standard laboratory settings (12 h light & 12 h dark 24°C). The animals had free access to food and water *ad libitum* for 14 days. Animals fasted for 12 h before the experiment but had free access to water. The Institutional Animal Ethical Committee gave approval of experimental protocols vide Reg. No. 1845/PO/Re/S/16/CPCSEA, 12/01/2016.

Various treatment groups for the wound healing study

The wound-healing potentiality of the above-mentioned plants was conducted using the following methods. Four animal groups, each with six animals, were created for each model. Animals from group I was considered as a control group whereas group II was identified as the standard (soframycin ointment). Animals in groups III and IV had treatments with ointments containing 2.5% and 5% *T. natans* extract, respectively[16].

Excision model

Ketamine hydrochloride was administered intravenously at a dose of 50 mg/kg body weight to anesthetize rats. After that, the backs of all the test groups' animals (rats) were depilated. Excision wounds were created by cutting about 500 mm^2 of rat back skin of the prearranged area. Rats were kept undressed in the open atmosphere. After that, the drugs, *i.e.,* the standard drug (soframycin), ointment without drug (control), and the ointments containing test drug (*T. natans* extract 2.5 & 5%) were administered topically until the wound was entirely cured. Wound concentration and epithelization time were observed using this wound model. The number of days needed for the wound to close up or disappear is used to calculate the epithelization period. According to the reduction in the wound area, wound contraction was determined. Tracing the wound margin and area on graph paper, the progressive change in wound area was evaluated and expressed as a unit (mm^2)[17,18].

Measurement process of wound contraction

By tracing the wound border on graph paper at regular intervals over the period of four days, the changes in the wound area were measured. The reduction of wounds on graph paper, which represents the change in the healing process for the created wound, was depicted as a unit (mm^2). By determining the total wound area and the healing area, wound contraction was determined[19].

Healed area (% wound contraction) = (healed area/total area) × 100

Estimation of hydroxyproline

On the day 10^{th} and 20^{th} after wounding, a small skin piece was collected from each healed wound area and further analyzed to evaluate for amounts of hydroxyproline, an important component of collagen. In the starting point, wound tissues were dried out in a hot-air oven at 65 to 75°C until a constant weight was

reached. The dried skin tissue was then hydrolyzed for four hours at 130°C in a sealed tube with hydrochloric acid (6N). After that, the neutralization process was done with KOH (0.1N) and then the oxidation reaction process was carried out with the help of chloramine-T, continuing the reaction for twenty minutes. The introduction of perchloric acid (0.4 M) completed the process. The endpoint was found when an Ehrlich reagent was added at 60°C temperature. The resulting solution's absorbance was measured with a double beam UV-VIS spectroscopy (Shimadzu 1700) selecting the wavelength 557 nm[20,21].

Histopathology study of excision wound

Rats were anesthetized on 20th day, and the injured area's middle region's skin tissues were collected. Collected tissues were fixed in 10% formalin solution. Histology was performed for the evaluation of the wound cells[22,23].

Incision model

Ketamine hydrochloride was used to anesthetize the animals. On the skin, a paravertebral-long cut approximately 2.5 cm in length was done. Full aseptic measures were taken. The treatment methods used for each group were similar to those described in the excision model study. A curved surgical needle (No. 11) and black color silk surgical string (No. 000) was used to sew the cut skin back together 0.5 cm apart after the incision. All pre-decided medication was given topically to the animal wound once every day for up to 10 days. After the incisions had fully recovered, the stitches were pulled out, and the tensile test was assessed using a Tensiometer-13 that was manufactured locally[24,25].

Results

Physical evaluation of the prepared ointments such as simple ointment, *T. natans* L. extract ointment 2.5% (TNEO 2.5%), and 5% (TNEO 5%) were done (Table 1).

Excision model

The extent of contraction of wounds for the control groups in the excision wound model increased from 18.26% to 41.73% from 4th to 8th day and 65.35% to 95% contraction was observed 12th to 20th day. It takes 25 days for the complete epithelization.

Rats treated with the standard drug had 63.03% wound contraction up to day 8, and 87% to 100% wound contraction from 12 to 20 days. For the complete epithelization with both test drugs (TNEO 2.5% and 5%), 20 days were required (Table 2, Fig. 1 & Fig. 2). The proliferation of the fibroblast and neutrophil-forming granulation was visible in the histogram obtained from the excision wound[26,27]. The changes were clearly observed in 5% *T. natans* ointment and the standard group (Fig. 3). Significant levels of hydroxyproline contents were found in the test groups and standard group as compared to the control group (Table 3)[28,29].

Table 1 — Physiological evaluation of the prepared ointment

Parameters	Simple ointment	Ointment containing 2.5% *Trapa natans* L. extract	Ointment containing 2.5% *Trapa natans* L. extract
Colour	Light Yellow	Greenish	Greenish
Odour	Characteristic	Characteristic	Characteristic
pH	7.2	6.42	6.5
Homogeneity	Smooth	Smooth	Smooth
Spreadability (g cm/sec)	11.88	11.76	10.54
Washability	Good	Good	Good
Stability (2°C, 25°C and 37°C)	Stable	Stable	Stable
Irritability	Not irritable	Not irritable	Not irritable

Table 2 — Healing rates in various groups of rats over a 20 day period

Post injuries day	Contraction rate (%) of the wound area after applying the drug			
	Group- I	Group- II	Group-III	Group-IV
4th	18.26±4.53	34.03±3.18 *	25.19±2.87*	27.48±5.02 *
8th	41.73±3.56	63.92±2.22 *	54.23±3.02 *	60.01±3.71 *
12th	65.35±1.66	87.16±1.1*	80.11±1.6*	83.08±2.48*
16th	80.05±0.71	98.29±0.22 *	94.98±0.43*	97.05±0.40 *
20th	95.14±0.59	100	100	100

The data represents mean ± SEM (where n = 6). * Denoted $p<0.001$ when compared with the control group (One-way ANOVA was applied to determine the statistical analysis, and then Dunnett's test for comparison was performed)

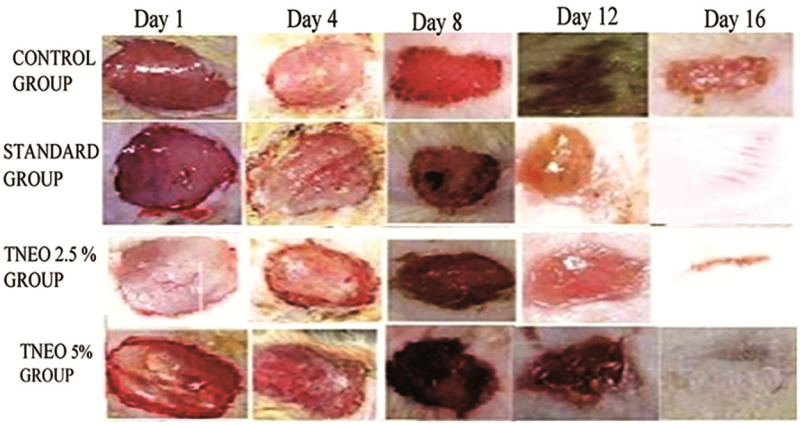

Fig. 1 — The contraction rate of wound areas on different post-excision day rats treated with a controlled drug (simple ointment) standard drug (soframycin ointment), and test drug (TNEO 2.5% & TNEO 5%)

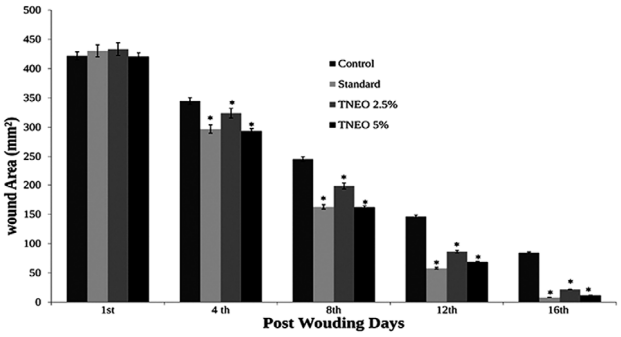

Fig. 2 — Graphical presentation of the excision wound area (mm 2) of various rat groups over a 20-day period treated with the respective drug. The data represents mean ± SEM (where n = 6). * denoted $p<0.001$ when compared with the control group

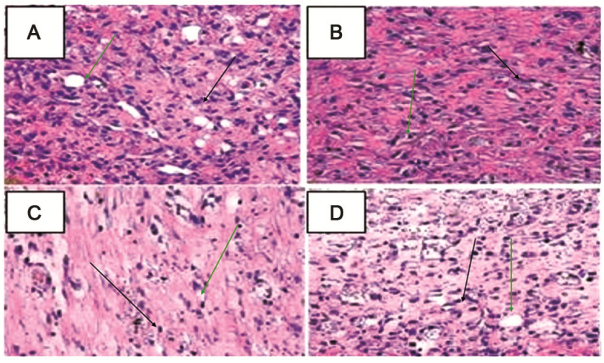

Fig. 3 — Histopathology of wound skin of rat at 20 days with pigment with H&E (100×), A. control group, B. Standard Group C. *Trapa natans* L. 2.5% group, D. *T. natans* L. 5% group (Black arrow indicating collagen and green arrow indicating inflammatory cell)

Incision model

In comparison to the control group, the test groups TNEO (2.5% and 5%) revealed a faster and better healing process in the incision model. The results of

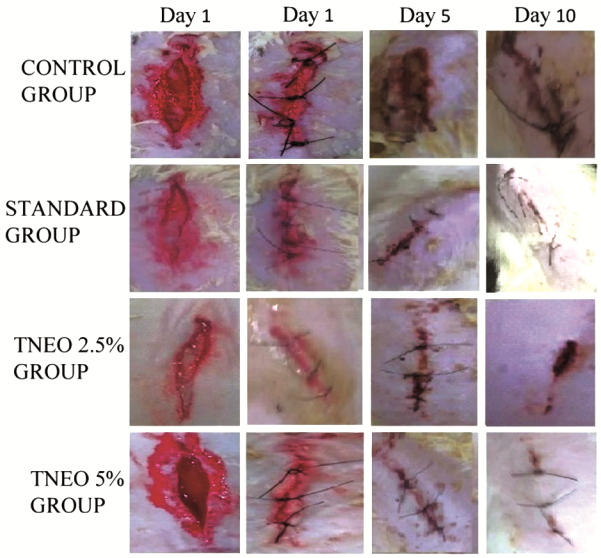

Fig. 4 — Day wise gradual healing of incision wound in Wister albino rats treated with controlled drug (simple ointment) standard drug (soframycin ointment), and test drug (TNEO 2.5% & TNEO 5%)

TNEO 5% are similar to that of the standard drug (Fig. 4). The test groups were treated with TNEO 2.5% and 5% *T. natans* showed wound-breaking strength of 430.16±5.23g and 465.42±7.11, respectively as depicted in (Table 4). 5% *T. natans* ointment showed approximately the same result as the standard drug.

Discussion

Wound is a very common problem for all of us. A large number of drugs are available in the market which may help to accelerate the wound healing process, but day by day the side effects of synthetic drugs gradually increase, and cost also becomes high.

Table 3 — Level of hydroxyproline development in rats in an excision wound

Treatment groups	Hydroxyproline	
	10^{th} day	20^{th} day
Group I (Control)	41.8±1.96	143±3.96
Group II (Standard)	60.2±1.83*	185±7.58*
Group III (TNEO 2.5% w/w)	53.5±1.59*	174.16±5.7*
Group IV (5% w/w TNEO)	57.4±2.83	182.16±7.05*

The data represents mean ± SEM (where, n = 6). *denoted $p<0.001$ when compared with the control group

Table 4 — Observed incision wound breaking strength data

Treatment group	Tensile strength of healed skin (g)
Group -I (Control)	305.54±3.20
Group- II (Standard)	490.15± 4.8 *
Group III (TNEO 2. 5%)	430.16±5.23 *
Group IV (TNEO 5%)	465.42±7.11*

The data represents mean ± SEM (where, n = 6). * denoted $p<0.001$ when compared with the control group

The major problem occurs in the proper and timely healing of the wound[30]. Traditionally, *T. natans* plant is used for wound healing activity. Plant extract does not have the desired impact when applied directly to the skin because it does not stay there for a long period of time. But when the plant extract is applied on the skin as an ointment form, it stays for a long time. Ointment prolongs the drug release at the area of the wound. White soft paraffin and hard paraffin in the ointment also provide an occlusive barrier that guards the skin against moisture. The desired outcomes of this study are to speed up the healing of wounds. The wound healing process consists of various stages, the first step being granulation and then collage formation followed by collagen maturation and scar maturation[31,32]. Soframycin ointment was chosen in this investigation as the standard medication because of its effective antibacterial, anti-inflammatory, and ability to boost collagen synthesis[33]. It was found that ointments containing *T. natans* extract had a significantly greater ability to constrict wounds than the control group. The TNEO 5% treatment group and standard group (0.2% w/w soframycin ointment) exhibited results that were nearly identical. As the dose of the test drug increased, the time of wound healing was lesser and the rate of wound contraction increased. On the 18^{th} day TNEO 5% treated group showed 100% contraction, which was similar to that of Soframycin ointment treated group. The active ingredient present in the *T. natans* plant may be responsible for the faster rate of wound area constriction due to its increased collagen formation, higher cell proliferation, and antibacterial properties.

Neutrophils are very important native immune cells that give the first response for inflammation in the progression wound healing process and play an active role in the defense mechanism. Neutrophils actively destroy germs and protect the open wound at the wound site. Thus, the effective action of neutrophils decreases the chance of infection. On the 10^{th} day, migration of neutrophils on the wound site was observed[34]. On the 20^{th} day, in the wound area, collagen fibers, and fibroblast cell formation were observed. In the case of the standard drug and test drugs TNEO (2.5% and 5%), collagen fibers, and fibroblast cells were observed more as compared to that in the case of control dug (simple ointment), which gave a clear indication that wound healing was better in case of the test and standard drug[35,36].

In the incision wound model, the tensile wound strength of different groups treated with simple ointment, TNEO (2.5% w/w and 5% w/w), and standard drug were observed after 10 days of treatment. From the experimental results, it was observed that the wounds treated with the test groups showed more tensile wound strength as compared to that of the control group ($p<0.001$), and the 5% ointment showed the same result as that of the standard drug. An increase in tensile strength indicates better collagen synthesis, and the formation of intramolecular which is one of the major components of wound healing. The wound-breaking strength also links with the formation of intramolecular cross-linkage cell migration, and matrix deposition[37,38]. In collagen, the formation of hydroxyproline is considered a very important component for wound healing. The increased hydroxyproline level in the wound regions and the improved breaking strength of the test drug-treated wounds suggest faster collagen synthesis and healing. The production of hydroxyproline in collagen is regarded as a crucial step in the healing process. The greater breaking strength of the treated wound and the higher levels of hydroxyproline in the wound regions point to quicker collagen formation and healing[39,40].

Conclusion

This reported study concerned two different wound models, which take into account physical parameters, histopathological parameters, and biochemical parameters. In this study, significant differences have been observed between the responses offered by the control group and test groups with regard to the potentiality of wound healing properties. Test groups have shown better results as compared to the control groups in the aspect of the rate of contraction of the wound, wound breaking strength, and hydroxyproline concentration in the site of the wound area ($p<0.001$). From this investigation, it is concluded that *Trapa natans* L. is a very good alternative as a wound healing drug.

Acknowledgment

The authors are thankful to the NIET Pharmacy Institute, Greater Noida, Uttar Pradesh for giving the facilities for the research.

Conflict of Interest

The authors declare that they do not have any conflict of interest.

Authors' Contributions

CM: Perform the research work, data analysis, and original draft preparation; RM: Guidance in research and manuscript preparation; ANC: Guidance in experiment design and analysis.

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