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Reinwardtia indica: phytochemical screening and evaluation of wound healing activity of the extracts in experimental model rats

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Abstract: *Reinwardtia indica* is traditionally used for wound healing. The main aim of this study was to evaluate the wound healing activity of leaves extracts of *R. indica* using the excision wound model in rats. The leaves of *R. indica* were collected from Gondrang, Chitwan, Nepal. Leaves were shade dried, extracted by double maceration and subjected to phytochemical screening. Then, the fusion method was used for the formulation of ointment and evaluated. Rats (n=24) were divided into four groups with 6 in each. Excision wound model was used, 2 cm diameter (314 mm^2), 2 mm depth wound was created. The treatment was given daily topically to all groups and the % mean wound contraction rate was calculated on days 4, 8, 12 and 16. The result was analyzed statistically using Graph pad prism version 5. Phytochemical test revealed the presence of alkaloid, flavonoid, tannin, phenol, terpenoid, carbohydrate, etc. All the evaluation parameters showed satisfactory results. The extract of *R. indica* ointment (2% w/w and 5% w/w) increased the wound contraction rate day by day. The % means wound contraction rate, on day 12, (80% and 88%), and on day 16, (97% and 100%) and statistically significant difference was at $p<0.0001$. The *R. indica* extract ointment showed an increased wound contraction rate. So, in further *R. indica* could be used for commercial production of wound healing ointment.

Key words: Excision wound model; Fusion method; Phytochemical screening; *Reinwardtia indica*; Wound healing.

Introduction

Wound is defined as disruption of cellular, anatomical and functional continuity of a living tissue. It may be produced by the physical, chemical, thermal, microbial or immunological insult to the tissue (1). Wound healing is a complex process in which the skins, and the tissues under it, repair themselves after injury (2). he wound healing process is divided into three phases as inflammation (0–3 days), cellular proliferation (3–12 days) and tissue remodelling (3–6 months) (3,4). During inflammatory phase, it involves the recruitment of leukocytes (neutrophils and macrophages) at the site of injury. The proliferative phase is involved by the migration and proliferation of keratinocytes and fibroblasts, collagen deposition, angiogenesis, epithelialization, tissue granulation and wound contraction (2). The remodelling phase involves the degradation of excess collagen in the wound by several proteolytic enzymes, leading to the completion of tissue repair (5). Many factors influences the wound healing such as infections, nutrition,drugs and hormones, type and sites of wound, and certain disease conditions (6).

The treatment of wounds includes the administration of drugs either locally (topical) or systemically (oral or parenteral) (7).The topical agents include antibiotics and antiseptics, desloughing agents (chemical debridement, e.g. hydrogen peroxide, collagenase ointment), wound healing promoters (e.g. Tretinoin, *Aloe vera* extract, honey extract)(8,9). Substances obtained from natural and synthetic bioactive materials having antioxidant, chelation and antimicrobial activities may promote the wound healing (10).

Plants are being used for treatment of various kinds of human diseases as wound healing property, anti-inflammatory, pain healing, antidiarrheal activity (11), Though many plants have wound healing property, most of the people use costly allopathic medicine for wound healing due to lack of knowledge and lack of documentation about the plants and their efficacy. People prefer allopathic medicine over plants (12, 13). But, many of allopathic medicine available have shown numerous unwanted side effects such as redness, swelling, blistering, draining, itching, ulcers, etc (14). Therefore, it is necessary to aware people for safer and effective medicinal plants for wound healing as well as documentation

of such plants for further study (15).

Reinwardtia indica known as Pyoli in Nepali is a small evergreen shrub growing to about 1 m tall. It is widely distributed from east India, Nepal, and China at an altitude of 450 m above sea level (16). Traditionally, juice of root has been used for the treatment of fever scabies, wound, and indigestion. The stem paste is applied on wounds, cuts, boils, and pimples. The pastes of aerial parts are applied on cuts to stop bleeding and for mouthwash and leaves are used in the treatment of paralysis and the leaves are found to be safe for herbal product formulation as per standard parameters (17, 18).

Only a few pharmacological activities have been studied in *R .indica* till the date as in previous studies, water, carbinol, methanol leave extract showed the presence of phytochemicals as alkaloids, glycosides, steroids, flavonoids, terpenoids, carbohydrates, saponins, and possessed antibacterial activity, antioxidant activity against nitric oxide radical, DPPH free radicals and ferric ion reducing antioxidant activity, anion scavenging activity (15,16,19, 20). The alcoholic leaf and flower extract had shown antibacterial activity and antioxidant activity against NO and DPPH free radicals (21).The hydro-alcoholic leaf extracts and hydro-alcoholic stem extract have shown anti-oxidant, anti-microbial, cytotoxicity against cervical cancer SiHa cells and antimicrobial against human pathogen (18), *R. indica* leaf extract has shown neuroprotective effect against scopolamine induced memory impairment in rat by attenuating the oxidative stress (22), polyherbal formulation including root of *R. indica* has shown antidepressant activity in rat by increasing the monoamino level (serotonin, dopamine, norepineprine, monoamine oxidase, gamma amino butyric acid (GABA), thus beneficial in management of mild to moderate depression (23).

By oral administration, study done for 28 days for the safety evaluation of polyherbal formulation containing hydroalcoholic extract of *R. indica* in rodents showed no adverse effect of polyherbal formulation in a dose of acute (upto 5,000 mg), sub-acute (up to the maximum tested dose of 800 mg/kg/day for 28 days) (24). From, acute and sub-acute toxicity study of hydro-alcoholic leaves extract of *R. indica* in rat found non-toxic up to 5000 mg/kg in acute study whereas up to 2000 mg/kg dose level in the sub-acute study done in 28 consecutive days. So, the leaves are found to be safe for herbal product formulation as per standard parameters. It will be helpful for further preclinical and clinical studies (25).

Since, *R. indica* is used for wound healing traditionally (21), thus the hypothesis of the present study is that plant exerts wound healing activity scientifically. So, this study aimed to evaluate the wound healing activity of *R. indica* in rats. From this study, we can prove the traditional use of this plant for wound healing activity scientifically. The proven scientific evidence on this plant for wound healing activity will also support future marketed formulations of safer and effective medicine for wound healing.

Materials and Methods

Drug and chemicals

Povidone-iodine ointment (Amtech med Pvt. Ltd.) was acquired through chemical suppliers. Other required analytical grade reagents and chemicals were obtained from authorized suppliers through the laboratory of Shree Medical and Technical College (SMTC), Bharatpur, Chitwan.

Plant material, collection and, authentication

Leaves of *Reinwardtia indica* were collected from Gondrang, Chitwan. Verbal consent from the local's area was taken during collection. A specimen of the plant was used to prepare herbarium and then authenticated by botanist Mr. Bishnu Bhattarai, Birendra Multiple Campus, Bharatpur, Chitwan, Nepal. The crude plant sample and herbarium of collected plant specimens were submitted to the Pharmacognosy lab of SMTC (Voucher No. PUCD-2019-002 and PUH-2019-03 respectively).

Method of extraction

The shade dried leaves were extracted by double maceration method. For this, 100g of coarsely powdered, air-dried plant material was soaked in 700ml water for 24 hr, followed by filtration. The filtrate (menstruum) was collected and the residue (marc) was again macerated on the same volume of fresh solvent for 24 hr followed by filtration. Both the filtrate were combined and evaporated to obtain dried crude extract. After evaporation, the extractive value for each extract was calculated by using the formula given below;

$$\%\text{Extractive value (w/w)/ \% Yield} = \frac{\text{final weight of crude extract}}{\text{weight of sample}} \text{x100} \ldots\ldots (1)$$

Preliminary phytochemical screening

Phytochemical screening of the aqueous extract was performed for different constituents like alkaloids, terpenoids, tannins, flavonoids, phenol, glycosides, steroids, carbohydrates using methods modified from previous studies (26, 27).

Ointment formulation

The fusion method was used for the preparation of ointment. Simple ointment B.P. was prepared using hard paraffin, cetostearyl alcohol, white soft paraffin, and wool fat. The master formula used for the preparation of ointment was taken from British Pharmacopoeia. The master formula used for the preparation of ointment was taken from British Pharmacopoeia (28), shown in Table 1.

The 100g of simple ointment base was prepared by placing hard paraffin (5g) in a beaker and melted over water bath. The other ingredients such as cetostearyl alcohol (5g), white soft paraffin (85g), and wool fat (5g) were added by continuous stirring, all the ingredients were melted over a water bath with constant stirring until they became homogeneous. The mixture was removed from the heat and stirred until cold.

To prepare aqueous extract ointment, 2 and 5g of the extract was incorporated into 100 gm of simple ointment base to prepare 2% and 5% w/w ointment, respectively by using mixing stirrer until the formation of homogenous ointment. Finally, the ointment was transferred in a suitable container with proper labeling. Povidone-iodine ointment (5% w/w) was used as a standard drug for comparing the wound healing potential of the extract.

Table 1. List of ingredients required for ointment formulation.

Ingredients	Master Formula (M.F.)	Reduced Formula (R.F.)
Wool fat	50g	5g
Hard paraffin	50g	5g
White soft paraffin	850g	85g
Cetostearyl alcohol	50g	5g
Total	1000g	100g

Physical evaluations of ointment formulation

Physical evaluations of ointment was done according to the previous method with some modifications (29, 30).

Visual appearances

The color and odor of various ointment formulations were observed.

Homogeneity

Homogeneity was observed by pressing a small quantity of the formulated ointment between the thumb and index finger. They were tested for their appearance with no lumps and no grittiness. The consistency of the formulations and the presence of coarse particles were used to evaluate the texture and homogeneity of the formulations.

Stability studies

All the formulations were subjected to accelerated stability testing for about 5 weeks. Room temperatures were maintained as per (ICH guidelines 1993). The stability studies were carried out in all formulations at different temperature conditions (refrigerator and room temperature).

Measurement of pH

The pH of various ointment formulations was determined by using digital pH meter. For this, 0.5 gm of the weighed formulation was dispersed in 50 ml of distilled water.

Ethical statement

The ethical approval for this experiment was taken from the Nepal Health Research Council (NHRC), Kathmandu, Nepal (Ref. No. 3000).

Experimental design

Animal

Healthy adult rats (150–250g) of either sex, in total 24 were used for the study. The animals were available from SMTC, which in turn had acquired from Banaspati Bivag, Kathmandu, Nepal. They were housed in cages in groups of 4-5 inside the premises of SMTC, Bharatpur, Chitwan, Nepal. The animals were kept at the monitored condition of humidity, temperature, light and dark cycles of 12 hr (25 ± 1 °C, 12-h light/dark cycle). Animals were provided with food and water ad libitum (31, 32) and acclimatized for one week before the study. During the experiment animals were housed individually in their cages so as to avoid biting and possible wound scratch among each other. Animals were periodically weighed before and after experiments. The procedures and animal handling protocols were authorized from the ethics committee, Nepal Health Research Council (NHRC), Kathmandu, Nepal (Reference. No. 3000).

Grouping and dosing of animals

Animals were divided into 4 groups, a negative control, positive control groups and two test groups. Six rats were used in each group using a stratified randomization technique. Negative Control (NC) group were treated with ointment base, Positive Control (PC) group were treated with standard drug Povidone -ointment (5% w/w), and Test A and B (TA and TB) groups were treated with *R. indica* extract ointment 2%w/w, and 5%w/w respectively.

Wound healing activity

Excision wound model

After one weeks of acclimation, animals were inflicted with excision wounds under light inhalation of diethyl ether (Emplura Chemical) anesthesia by chamber induction method (33). For this, saturated the cotton ball with anesthetic diethyl ether, kept inside a desiccator with a tightly closed lid. Then rats were placed inside desiccators, observed its activity and respiration to determine the depth of anesthesia. Then, the hairs on the skin of the back, shaved with sterilized razor blades. A circular area was marked and the surface of the marked area was carefully excised by using sharp sterilized scissors and circular wound of about 2cm diameter (circular area=314mm^2) and 2mm depth was excised on the dorsal thoracic region using toothed forceps, a surgical blade and pointed scissors. Animals were closely observed for any infection, those which showed signs of infection were separated and excluded from the study. After 24 hr of wound creation, 10mg ointments were gently applied to each animal to cover the wounded area once per day for 16 days (34-36).

Measurement of wound contraction rate

In the excision wound model, the wound area was measured by using semitransparent tracing paper and a permanent marker on 1, 4, 8, 12 and 16 days for all groups. Wound contraction was measured every 4th day until complete wound healing and represented as a percentage of the healing the wound area. Percentage of wound contraction was calculated taking the initial size of the wound as 100% using the following formula:

$$\% \text{ Wound contraction rate: } \frac{\text{Initial wound area} - \text{Specific day wound area}}{\text{Initial wound area}} \times 100 \ldots . (2)$$

Statistical analysis

The data obtained in the studies were subjected to ANOVA followed by Tukey's multiple comparison tests for determining the significant difference using Graph pad Prism 5. The analysis was done at a 95% confidence

level and P-value <0.05 was considered to be significant.

Results

Extractive value

The extractive value of leaf extracts of *R. indica* was observed 19.84%.

Phytochemical screening

The phytochemical screening tests of aqueous extracts of *R. indica* indicated the presence of alkaloid, flavonoid, tannin, phenol, terpenoid, carbohydrate, etc, shown in Table 2.

Evaluation of ointment formulation

The stability studies were carried out in all formulations at different temperature conditions (refrigerator and room condition). All the evaluation parameters showed satisfactory results, shown in Table 3.

Baseline data on experimental animals

The basic baseline data including number of animals and average body weight in various experimental groups were as shown in Table 4.

Excision wound model

Wound contraction rate

The wound healing activity was assessed by calculating the % wound contraction rate for all animal groups and increased wound contraction rate was observed by *R. indica* extract than the normal control group. On day 4, the % mean wound contraction rate on animal groups by TA, TB, and PC was 22%, 29%, 23%, respectively. On day 8, the % mean wound contraction rate on animal groups on TA and TB was 59% and 65% respectively. On day 12, the % mean wound contraction rate on animal groups TA, TB, PC were 80%, 88%, 83%, respectively. On day 16, the % mean wound contraction rate on animal groups NC, PC, TA and TB were 88%, 99%, 97%, and 100%, respectively. A statistically significant difference was obtained in the wound contraction rate between the four groups (NC, PC, TA, and TB) at $p<0.0001$, shown in Table 5 and (Figures 1, 2, 3, 4). Figure 5 showed the increased in wound contraction rate of treatment group from day 0 to day 16 when compared with negative control group.

Table 2. Phytochemical screening of R. indica extracts.

Phytochemical Test	Test Performed	Inferences	*R. indica* extract
Alkaloids	Mayer's test	Pale yellow ppt.	+
	Dragondroff's test	Orange red ppt.	+
	Wagner's test	Brown ppt.	-
Flavanoids	Alkaline reagent test	Intense yellow colour	+
	Lead acetate test	Yellow colour ppt.	+
Carbohydrates	Molisch's test	Red-violet ring	-
	Benedict test	Green, yellow or red	+
	Fehling's test	Brick red ppt.	+
Tannins	Ferric chloride test	Brownish green	+
Terpenoids	Salwoskii test	Reddish brown ppt.	+
Phenol	Ferric chloride test	Bluish black ppt.	+
Glycosides	Keller killiani test	Blue colour in acetic layer	-
	Legal test	Blood red colour	-

Note: "+" sign indicates the presence and "-" sign indicates the absence of phytochemicals

Table 3. Evaluation of various ointment formulations.

Evaluation parameter		Formulations		
		2% w/w	5% w/w	Ointment base
Physical evaluation	Color	Light brown	Dark brown	White
	Odour	Characteristic	Characteristic	Characteristic
	Homogeneity	Smooth and homogenous	Smooth and homogenous	Smooth and homogenous
Measurement of pH		6.7-7.1	6.7-7.1	6.7-7.1

Table 4. Baseline data on experimental groups.

Animal group	Number of study animals			Body weight in gm (Mean ± SEM)		
	Male	Female	Total	Male	Female	Total
NC	3	3	6	191.00 ± 5.77	155.33 ± 7.22	173.17 ± 9.38
PC	3	3	6	180.00 ± 2.31	190.00 ± 3.46	185.00 ± 8.26
TA	3	3	6	181.00 ± 0.58	170.67 ± 5.93	175.84 ± 3.38
TB	3	3	6	182.33 ± 6.06	150.67 ± 4.37	166.50 ± 9.48

Table 5. Effects of extracts of *R. indica* on the excision wound model in rat.

Group	Treatment	Wound contraction rate (%)			
		Day 4	Day 8	Day 12	Day 16
NC	Ointment base	14.66±1.535	40.41±1.191	63.20±1.568	88.07±1.203
PC	Povidone ointment	23.35±1.590	62.12 ±4.271**	83.88±2.553***	99.11±0.1626***
TA	*R. indica* extract ointment (2% w/w)	22.34±6.527	59.53±4.070**,#	80.89±2.468***,#	97.69±0.3421***,#
TB	*R. indica* extract ointment(5% w/w)	29.53±3.630	65.74±4.434***,#, @	88.03±2.593***,#, @	100±0.0***,#, @

Note: Values are expressed in mean ± SE (n=6). **p<0.01, ***p<0.001 as compared to Negative Control (NC), # p>0.05 as compared to Positive Control (PC), @ p>0.05 as compared to TA.

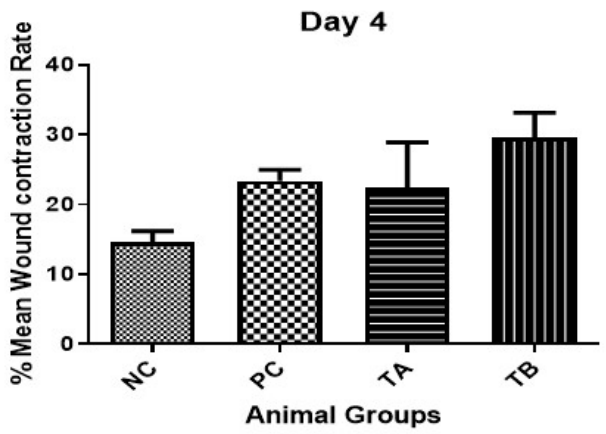

Figure 1. % Mean wound contraction rate on day 4.

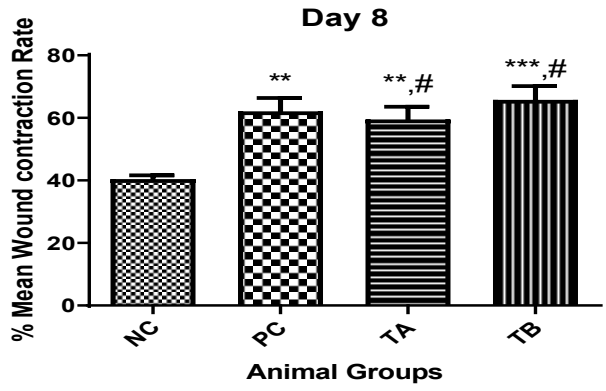

Figure 2. % Mean wound contraction rate on day 8. (Results are expressed as mean ± SE, **p<0.01, ***p<0.001 as compared to NC, # p>0.05 as compared to PC, @p>0.05 as compared to TA).

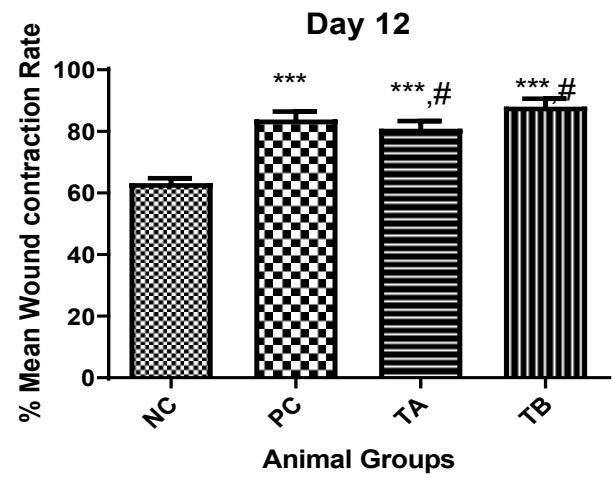

Figure 3. % Mean wound contraction rate on day 12. (Results are expressed as mean ± SE, **p<0.01, ***p<0.001 as compared to NC, # p>0.05 as compared to PC, @p>0.05 as compared to TA).

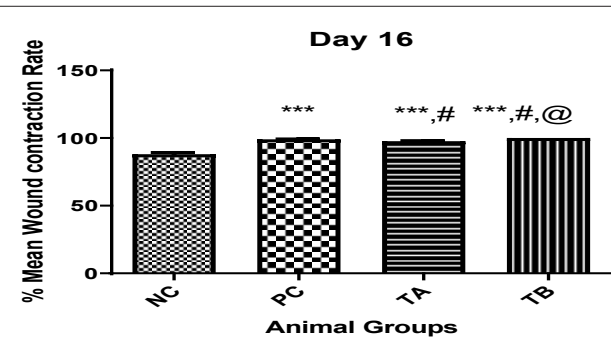

Figure 4. % Mean wound contraction rate on day 16. (Results are expressed as mean ± SE, **p<0.01, ***p<0.001 as compared to NC, # p>0.05 as compared to PC, @p>0.05 as compared to TA).

Discussion

Cuts, mechanical abrasions, surgical procedures, burns, infectious diseases and other pathological conditions results wound. All type of wounds follows roughly the same healing process (37). Although many wounds heal naturally, healing processes need to accelerate because different types of complications may arise when wound healing is delayed (38). Wound healing is a complex cellular process by which damaged cellular structures and tissue layers are restored to its normal stage. The length of the healing process depends on the extent of the injury and the regenerating ability of the tissue (39).

Ointment is necessary to achieve a sustained drug release at the application sites because applying the extract directly on the affected wound cannot bring the desired effect as it does not stay longer on the wounded skin of the experimental animals (40).The use of the ointment base has additional roles like formation of occlusive barrier for moisture by hard and soft paraffin, thickeners and stabilization of ointment by the use of wool fat and cetostearyl alcohol (41). So, for the evaluation of wound healing activity of *R. indica* extract, 2% w/w, and 5% w/w and simple ointment was formulated. Before the animal study, quality evaluation of the ointment was done and the result showed that in extract ointments (2% w/w and 5% w/w) and simple ointment, visual appearances (color and odor), homogeneity and pH were same as observed on the day of preparation and were stable in room temperature and refrigerator,

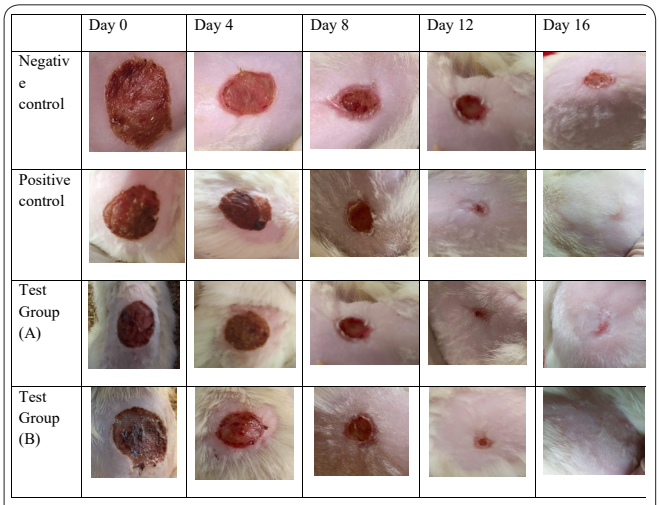

Figure 5. Photograph of excision wounds showing wound contraction.

which was similar to observation done in previous studies (29,30).

In the excision wound healing model, the aqueous extract showed increased wound contraction rate as compared to the negative control group. On day 4, the % mean wound contraction rate on animal groups by extract (2%w/w, 5%w/w and, standard drug) were 22%, 29%, and 23% respectively. Similarly, on day 16, the % mean wound contraction rate was normal control (88%), positive control (99%), and extract 2% w/w (97%) and, extract 5% w/w (100%), respectively. A statistically significant difference was obtained in the wound contraction rate between the four groups at $p<0.0001$.

The *R. indica* extract (5% w/w) showed a better wound contraction rate than the standard drug povidone ointment 5% w/w. It might be due to the presence of phytochemicals such as flavonoids, terpenoids, steroids, tannin, and phenolic compounds which were identified to promote wound healing activity mainly because of their antibacterial and antioxidant activity (10, 16-17, 21,39). Many studies has reported the role of phytochemicals in wound healing as tannins are seen to be active detoxifying agents and inhibit bacterial growth (42), terpenoids have astringent and antimicrobial property which promote the wound healing process (43), flavonoids and polyphenols shows the antioxidants activity by scavenging the free radical and possess anti-inflammatory properties and have antimicrobial activities (44-49). Therefore, phytochemicals present in the crude extract may contribute to wound healing activities independently or synergistic effects. And, our study also showed that extract of *R. indica* revealed the presence of alkaloids, flavonoids, carbohydrates, phenol, steroids, terpenoids and tannins. The result was almost similar to the previous result (16).

So, the ointment of aqueous leaves extract of *R. indica* (2% w/w and 5% w/w) showed the wound healing activity by increasing the wound contraction rate. This can be supported by the fact that the greater the increase in the rate of wound contraction, the better the efficacy of the medication and the wound will close at a faster rate if the medication is more efficient (50).

The results showed that *R. indica* extract ointment (5% w/w) showed increased wound contraction rate than 5% w/w povidone-ointment which might be due to presence of phytoconstituents. The phytoconstituents shows individual or additive effect that fastens the wound healing processes. This validates the ethnomedicinal use of the leaves of *R. indica* for treatment of wounds as claimed in the folklore literature. So, in further *R. indica* could be used for commercial production of wound healing ointment. However, it needs further evaluation that which phytoconstituents are responsible for wound healing activity, their mechanism of action and further in clinical settings before consideration for the treatment of wounds.

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Conflict of Interests

The authors declare that there are no conflicts of interest.

Authors' contributions

RG: Supervision, designed the project, helped in data analysis, finalized manuscript draft. PG, BMT, SC, BB, and TP: Performed experimental work and data analysis. NK and SA: Advisors, data curation, manuscript revision and publication. Funding acquisition required was done by G.E.-S.B, M.N, M.A and N.K. Final approval of manuscript was done by all the authors.

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