WOUND HEALING POTENTIAL OF THE HYDROALCOHOLIC LEAF EXTRACT OF *RHUS CHINENSIS* MILL.

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ABSTRACT

Rhus chinensis is a well-known herb used as antiulcer, antibacterial and in healing bleeding piles, etc. The present study was aimed for wound healing potential of hydro-alcoholic leaf extract of *Rhus chinensis* in the form of simple ointment using three types of wound models in rats as incision wound, excision wound and dead space wound. The results were comparable to standard drug povidone iodine ointment, in terms of wound contraction, tensile strength, biochemical parameters such as hydroxyproline and hexosamine content.

KEY WORDS: *Rhus chinensis*, incision, excision, dead space wound, tensile strength, hydroxyproline.

INTRODUCTION

Rhus chinensis belongs to the genus *Rhus* and the Family *Anacardiaceae (Miller et al., 2001).* Commonly called sumac, *Rhus* consists of approximately 250 individual species of flowering plants. Like most sumacs, *Rhus chinensis* is a dioecious shrub that can reach 8 m in height. It bears odd pinnately compound leaves and creamy white flowers. The fruits (drupes) are orange or red in color at maturity and contain one seed (Miller et al., 2001; Djakpo and Yao, 2008). Numerous curative properties are ascribed to different parts of this tree, namely root, bark, stem, leaf, fruit, flowers, seed and gall (Duke and Ayensu, 1985; Kao, 1985). A decoction is used in the treatment of hemoptysis, inflammations, laryngitis, snakebite, stomach-ache and traumatic fractures. The seed is used in the treatment of coughs, dysentery, fever, jaundice, malaria and rheumatism. The root bark is cholagogue. Galls on the plant are rich in tannin. They are used internally for their astringent and styptic properties to treat conditions such as diarrhoea and haemorrhage (Duke and Ayensu, 1985). It is used in the treatment of persistent cough with blood, chronic diarrhoea, spontaneous sweating, night sweats, bloody stool, urorrhoea and bloody sputum. It is applied externally to burns, in bleeding due to traumatic injuries, haemorrhoids and ulcers in the mouth (Him-Che, 1985). It is a frequent ingredient in polyherbal prescriptions for diabetes mellitus (Duke and Ayensu, 1985). It is also used to treat rectal and intestinal cancer, prolapse of the rectum, seminal enuresis and hemorrhoids (Him-Che, 1985). Phytochemical studies on *Rhus* species have been reported earlier and

resulted in the characterization of several compound groups such as flavonoids (Taniguchi et al., 2000), triterpenoids (Lee et al., 2005). The galls on *Rhus chinensis* leaves are rich in gallotannin (50–70%), a type of hydrolysable tannin (Huang, 1998; Yuan and Lin, 2000). R*hus chinensis* is rich in phenolic compounds, gallic acid and methyl gallate (Ahn et al., 1998; Choi et al., 2009). The high level of gallotannins along with phenolic compounds, gallic acid and methyl gallate, known antimicrobial agents make *Galla chinensis* very useful in bacterial control (Wu-Yuan et al., 1988; Ahn et al., 1998; Kang et al., 2008). Tian et al tested the antioxidant activity of gallotannins with different polarities and found that all of the consecutive extracts of *Galla chinensis* possessed remarkable antioxidant activity (Tian et al., 2009). *Galla chinensis* has also shown the anticariogenic activity. The chemical compounds of *Galla chinensis* on combined effects with fluoride enhances the mineralization of dental enamel (Cheng et al., 2008).

In the present study, *Galla chinensis* was found to be effective in healing external wounds. As this plant has been reported to have gastric and duodenal ulcer healing activity, it was hypothesized that it should also be able to heal an external wound. We have selected different wound models using Wistar rats. Preliminary phytochemical studies of the extracts were done. Successive hydroalcoholic solvent extraction of the leaf was done and 5 % and 10% extract ointments were made. The extracts were then studied for its wound healing property on different animal models.

MATERIALS AND METHODS

Plant material

Leaves of *Rhus chinensis* Mill were collected in the month of August 2012 from Sikkim. The collected leaves were made free from aerial parts and thoroughly washed with running water to remove the earthy material or adherent impurities and dried in the shaded area for 3-4 weeks. Voucher specimen of the plant has been deposited in the WEED HERBARIUM of Assam Agricultural University (AAU), Jorhat (Accession No.-5164) in the form of herbarium specimen following Radford (1986). Another set is made ready to deposit in the Botanical Survey of India, Eastern Regional Centre, Shillong.

Extraction

Air dried plant leaves (300gm) were powered in a mechanical grinder and the plant materials were extracted by hydro-alcoholic (70%) solvent using Soxhlet extraction apparatus. After completion of the extraction, solvent was completely removed under reduced pressure and the extracts were stored in vacuum desiccators.

Phytochemical analysis

The concentrated plant extracts were subjected to preliminary screening for the detection of various plant constituents present. Extracts showed the presence of flavonoids (Pourmorad et al., 2006), tannins (Scalbert, 1992), phenols (Pourmorad et al., 2006) and triterpenoids (Ali-Shtayeh and Abu Ghdeib, 1999) along with other phytochemicals.

Drug formulation

Two different strengths (5% and 10% w/w) of ointments were prepared from the extract, where 5 g and 10 g of extracts were incorporated in 100 g of simple ointment base B.P. respectively (from Register, 1953).

Evaluation of wound healing activity

The wound healing efficacy of *Rhus chinensis* Mill.leaf extract were evaluated employing three animal models viz., excision ,incision and dead space wound model.

Animals

Healthy Wistar albino rats of either sex, weighing (150–220) g and New Zealand albino rabbits 2.9 ± 0.3 kg were used. All animals were housed, fed and treated in accordance with the in-house guidelines for animal protection. The animals were housed under standard environmental conditions of temperature and humidity (25±0.50 °C) and were fed with standard pellet diet and water *ad libitum* throughout experimentation period. Ethical clearance for handling the animals was obtained from the Institutional animal ethical committee (**Reg No. HPI/2012/60/IAEC/**) prior to the beginning of work.

Acute dermal toxicity test

Skin irritation test for the test substances were conducted on rabbits by using occluded dermal irritation test (Robinson and Perkins, 2002). The hair was removed from the back region of the rabbit using depilator before 24 hours of sample application. This procedure was carefully done to avoid skin injury which could alter its permeability. The total shaved skin area was 40mm x 30mm, detected by millimeter rule. 0.5 g of 70% hydro alcoholic leaf extract ointment (10% w/w) of *R. chinensis* was topically applied on free surface of the skin.

Grouping of animals

For incision, excision and dead space wound model, animals of either sex weighing between 150 and 200 g were divided into four groups, each group consisting of five animals as follows: Group A-simple ointment base 0.5 g; Group B-*Rhus chinensis* Mill.5% w/w ointment 0.5 g; Group C-*Rhus chinensis* Mill 10 % w/w ointment 0.5 g and Group D-Povidine iodine ointment 0.5 g, 5% w/w.

Excision wound model

The rats were inflicted with excision wounds as described by (Morton and Malone, 1972)**.** In this model a standard wound is made by cutting a circular skin in dorsal thoracic region of the experimental animals. The hair was removed from the dorsal thoracic region of the rats using depilator. An area of 500 mm^2 was marked on the shaved area with an indelible ink and rubber seal. The area was washed with normal saline. A full thickness excision wound of circular area of 500 mm^2 was created along the markings under light ether anaesthesia. The rats were kept individually in separate cages. The physical attributes of wound healing *viz.* wound closure (contraction) and epithelialisation were recorded. The wound contraction was studied by tracing the raw wound area on a transparent paper on 4th, 8^{th} and 12^{th} day. The criterion for complete epithelialisation was fixed as formation of scar with absence of raw wound area. The wound area was measured planimetrically with the help of sq.mm scale graph paper. The percentage wound closure was calculated by using the following formula:

Percentage of wound contraction

$$= \frac{\text{wound area on day "0"} - \text{n (wound area on days)}}{\text{wound area on day "0"}} \times 100$$

Where, *n* = number of days (4^{th}, 8^{th} and 12^{th} day).

Incision wound model

The method of incision wound model was adapted from Ehrlich and Hunt (Ehrlich and Hunt, 1969). In the incision wound model, the rats were anaesthetized by ether and two longitudinal paravertebral incision of 6 cm length were made through the skin and cutaneous muscle at a distance of about 1.5 cm from the midline on each side of the depilated back. After the incision, the parted skins were closed with interrupted sutures of 1cm apart using surgical thread (no. 000) and sterilized curved needle (no. 9). The wounds were left undressed. The wounds of animals in different groups were treated with topical application of ointments as described above, for the period of 10 days. The wounding day was considered as day '0'.

When wounds were cured completely, the sutures were removed on the 8th day post-wounding and the tensile strength of the skin, i.e. the weight required to break or open the wound was measured by tensiometer on the 10th day.

Dead space wound model

Dead space wounds were inflicted by implanting sterile cylindrical grass piths (2.5 cm x 0.3 cm) s.c. in the groin and axilla by the technique of D′Arcy *et al*. as described by (Turner, 2013).

On the 10th (post-wound) day, the granulation tissues formed on the implanted tubes were carefully detached from surfaces of the tubes. The wet weight of the granulation tissue was noted. Thereafter, the granulation tissues were collected, dried at 60 °C for 24 h and their dry weights were noted. The dried tissue was added to 5 ml of 6M HCl and kept at 110 °C for 24 hrs. The neutralized acid hydrolysate of the dry tissue was used for the determination of hydroxyproline and hexosamine**.**

Wound healing evaluation parameters

Measurement of wound breaking or tensile strength

When wounds were cured completely, the sutures were removed on the 8th day post-wounding and the tensile strength of the skin i.e. the weight required to break or open the wound was measured by tensiometer on the 10th day according to the continuous water flow technique (Lee, 1968) "figure 1".

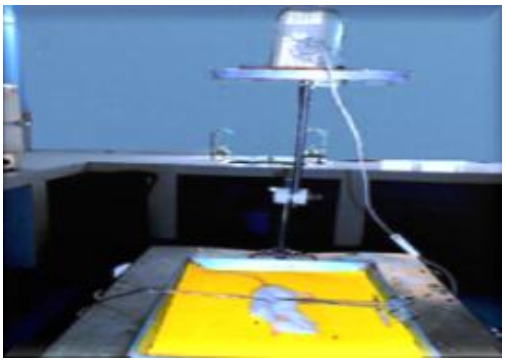

Figure 1: Tensiometer

Briefly, the anaesthetized rat was placed on operation table. The Allis forceps were firmly applied on the lines, facing each other. The forceps on one side was hooked to a metal rod, fixed firmly to the operation table; while the other forceps was fixed to a light polythene container through a string which runs over a pulley. Water was allowed to flow at a constant rate into the polythene container so as to build a gradual pulling force necessary to disrupt the wound. The flow of water was regulated by means of an occlusion clamp on rubber tubing connected to a reservoir, kept at a suitable height. As soon as the gapping of the wound was observed, the water flow was stopped. The volume of water in the polythene container was measured and converted to the corresponding weight. The tensile strength was expressed as the minimum weight of water necessary to bring out the gapping of the wound.

Estimation of hydroxyproline (Woessner, 1961)

For the preparation of protein hydrolysate, 50 mg of tissue sample in 1 ml hydrochloric acid was weighed and sealed in screw-capped glass tube. The tubes were autoclaved at 15 kilograms per cm^2 for 3 hours. The hydrolysate was neutralized to pH 7.0 and brought to appropriate volume. Test tubes were marked as sample, standard and blank. One ml of test sample was added to test tubes marked as sample, 1 ml Milli-Q water to test tubes marked as blank and 1 ml standard solutions to test tube marked as standard. 1 ml of 0.01 M copper sulphate solution was added to all the test tubes followed by addition of 1 ml of 2.5 N sodium hydroxide and 1 ml 6% hydrogen peroxide. The solutions were occasionally stirred for 5 min and then kept for 5 min in water bath at 80 °C. Tubes were chilled in ice-cold water bath and 4.0 ml of 3.0 N sulphuric acid was added with agitation. 2 ml of *p*-(dimethylamino) benzaldehyde was added and heated in water bath at temperature 70 °C for 15 min. The absorbance was measured at 540 nm using UV spectrophotometer. The hydroxyproline content of the samples were determined by interpolating the O.D. values on the standard graph.

Estimation of hexosamine

For estimation of hexosamine, the weighed granulation tissues were hydrolyzed in 6N HCl for 8 h at 98 °C, neutralized to pH 7 with 4N NaOH and diluted with Milli-Q water. Hexosamine contents of granulation tissues were estimated with minor modifications (Johansen et al., 1960). The diluted solution was mixed with acetyl acetone solution and heated to 96 °C for 40 min. The mixture was cooled and 96% ethanol was added, followed by the addition of p-dimethylamino-benzaldehyde solution (Ehrlich's reagent). The solution was thoroughly mixed, kept at room temperature for 1 h and the absorbance was measured at 530 nm using a double beam UV-Vis spectrophotometer (Shimadzu). The amount of hexosamine was determined by comparing with a standard curve. Hexosamine content has been expressed as mg/g dry tissue weight.

Statistical analysis

The data obtained from each experiment *i.e.* means of wound area measurement, epithelization period, wound breaking strength/tensile strength, wet and dry weight; hydroxyproline and hexosamine of the granulation tissue between different groups (Control, Standard, Test treated) were subjected to one-way ANOVA followed by Dennett's Multiple Comparison tests. The 'P' values were analyzed and recorded in respective tables.

RESULTS

Skin-irritation test

In skin irritation test, no irritation symptoms were developed over the test period. Neither erythema formation nor skin swelling were developed during 72 h time period for all test substances. This indicates that the test substances from the leaves of *R. chinensis* Mill.do not have irritant property.

Wound healing activity

Effect of topical application of 70% hydro alcoholic leaf extract ointment of *R. chinensis* in excision wound model:

Topical application of different concentrations of *R. chinensis* extract ointment (5% and 10% w/w extract in simple ointment) has shown high rate of

wound contraction and decrease in period of epithelialisation time when compared to control group. The 10% ointment treated group demonstrated greater wound healing promoting property than the 5% ointment treated group. However, the results indicated that wound healing potency of the 10% extract ointment was found lesser than the reference standard ointment. Results are given in table 1

Table 1: Effect of topical application of ointments containing hydro alcoholic extracts of *R. chinensis* leaves on excision wound parameters

Group	Mean percentage of wound contraction ± SEM			Period of epithelialisation (days)
	4th day	8th day	12th day	
A(Control)	21± 1.871	37±2.894	47±4.823	23 ± 1.225
B (*R. chinensis* 5% (w/w) ointment)	23± 2.249	54±4.301	70±7.906*	20±1.393
C (*R. chinensis* 10% (w/w) ointment)	28± 3.813	67±7.473**	85±5.000***	16 ± 1.594**
D (Standard Povidone iodine 5% (w/w) ointment)	35± 3.536*	73±6.442***	87±5.148***	14 ±1.068***

The values are expressed as Mean ± SEM, n=5 in each group. * P<0.05, **P<0.01 and ***P<0.001, when treated groups compared with normal control group.

Table 2: Effect of hydroalcoholic leaf extracts ointment of *R. chinensis* on tensile strength (g) in incision wounds

Group	Tensile strength in gram (mean ± SEM)
A(Control)	313 ± 12.08
B (*R. chinensis* 5% (w/w) ointment)	385 ± 8.936**
C (*R. chinensis* 10% (w/w) ointment)	429 ± 17.99***
D (Standard Povidine iodine5% (w/w) ointment)	495 ± 16.36***

The values are expressed as Mean ± SEM, n=5 in each group. * P<0.05, **P<0.01 and ***P<0.001, when treated groups compared with normal control group.

Effect of topical application of 70% hydroalcoholic leaf extract ointment of *R. chinensis* in incision wound model:

The results of incision wound model are presented in table 2. The skin breaking strength/tensile strength of the 10% extract ointment treated group and standard ointment treated group were comparable to each other. The 5% extract ointment treated animals exhibited a lesser but significant increase in the skin breaking strength compared to control group.

Effect of topical application of 70% hydroalcoholic leaf extract ointment of *R. Chinensis* in dead space wound model:

The *R. chinensis* extract ointment caused increased strength in the dead space wound parameter viz. wet and dry weight of granuloma tissue respectively. Topical application of the 5% ointment increased the dry and wet weights of the tissue, whereas, the 10% ointment significantly increased ($p<0.001$) the wet and dry weights respectively when compared with the control group as shown in table 3. The hydroxyproline content of granulation tissue were significantly ($P < 0.01$) increased in extract treated groups at different concentrations (5% and 10% w/w extract in simple ointment) when compared with control as depicted in table 4.

Table 3: Effect of hydroalcoholic leaf extracts ointment of *R. chinensis* on wet and dry weight of granuloma tissue (mg) in dead space wound

Group	Wet Weight(mg)	Dry Weight (mg)
A Control	167 ± 17.29	49 ± 4.496
B 5% (w/w) *R. chinensis* ointment	226 ± 12.27*	84 ± 5.685*
C 10 % (w/w) *R. chinensis* ointment	260 ± 12.75***	111 ± 9.438***
D Standard Povidine iodine 5% (w/w) ointment	304 ± 14.44***	151 ± 13.81***

The values are expressed as Mean ± SEM, n=5 in each group. * P<0.05, **P<0.01 and ***P<0.001, when treated groups compared with normal control group

Table 4: Effect of hydroalcoholic leaf extracts ointment of *R. chinensis* on hydroxyproline content of granulation tissue (mg/g tissue) in dead space wound

Group	Hydroxyproline (mg/g tissue)
A (Control)	24 ± 2.379
B (5% (w/w) *R. chinensis* ointment)	41 ± 5.636*
C (10 % (w/w) *R. chinensis* ointment)	50 ± 4.986**
D (Standard Povidine iodine 5% (w/w) ointment)	56 ± 4.802***

The values are expressed as Mean ± SEM, n=5 in each group. * P<0.05, **P<0.01 and ***P<0.001, when treated groups compared with normal control group

Standard Povidine Iodine (5%) also showed significant results as compared with control (P < 0.001). The results of hexosamine content of the granulation tissue on dead space wound model were presented in the table 5**.** The hexosamine content was significantly (P < 0.05) increased in 10% extract treated group when compared with control group. The 5% extract ointment treated animals exhibited a lesser increase in the hexosamine content compared to control group**.**

Table 5: Effect of hydroalcoholic leaf extracts ointment of *R. chinensis* on hexosamine content of granulation tissue (mg/100mg tissue) in dead space wound

Group	Hexosamine (mg/100 mg tissue)
A (Control)	0.41±0.04583
B (5% (w/w) *R. chinensis* ointment)	0.53±0.04637
C (10 % (w/w) *R. chinensis* ointment)	0.72±0.08602*
D (Standard Povidine iodine 5% (w/w) ointment)	0.88±0.09695***

The values are expressed as Mean ± SEM, n=5 in each group. * P<0.05, **P<0.01 and ***P<0.001, when treated groups compared with normal control group.

Standard Povidine Iodine (5%) also showed significant results as compared with control (P < 0.001) (Anicuta, Dobre et al. 2010). In the formulation F2, F3 and F4 shows the broad peak in the range 1400-1600 cm-1 this may be attributed due to the presence of –CONH2, -COO- and –OSO3- groups. F5 shows broad peak around 1200 cm-1 this may be due to the presence of H and SO3H groups.

DISCUSSION

Wound healing process consists of different phases such as granulation, collagenation, collagen maturation and scar maturation which are concurrent but independent to each other. Hence in this study three different models were used to assess the effect of herbal ointment on various phases.

Phytochemical studies on *Rhus* species have been reported earlier and resulted in the characterization of several compound groups such as flavonoids (Taniguchi et al., 2000; Lee et al., 2005), triterpenoids (Lee et al., 2005), phenolics (Huang, 1998), tannins (Yuan and Lin, 2000). Wound healing activity of hydro-alcoholic leaf extract of *R. chinensis* was confirmed by its effects on excision, incision and dead space wound models. Preliminary phytochemical investigation of *R. chinensis* was carried out and found to contain tannins, flavonoids and saponin. Toxicity studies i.e. skin irritation study on rabbit skin indicates that all the test substances from the leaves of *R. chinensis* do not have irritant property.

Two different concentrations of *R. chinensis* (5% w/w and 10% w/w) were selected for the preparation of ointment. In excision wound model, both *R. chinensis* (5% w/w) and *R. chinensis* (10% w/w) showed significant effect in period of epithelialisation and wound contraction, which is comparable to standard. In incision wound model, both *R. chinensis* (5% w/w) and *R. chinensis* (10% w/w) showed significant increase in breaking/tensile strength when compared with control; the effect is comparable to standard. In dead space wound model, *R. chinensis* (5% w/w) and *R. chinensis* (10% w/w) both showed significant increase in the wet and dry weight of the granuloma tissue. Hydroxyproline and hexosamine content of granulation tissue were significantly increased in extract treated groups at both 5% (w/w) and 10% (w/w) concentrations when compared with control.

Collagen is the predominant extracellular protein in the granulation tissue of wounds (Chithra et al., 1998a). Immediately following injury, there is an increase in the synthesis of collagen in the wound area. Collagen plays a role in haemostasis and in providing strength and integrity to the wound matrix. It is also essential for re-epithelialisation and cell–cell and cell–matrix interactions (Raghow, 1994; Chithra et al., 1998b). Hydroxyproline is the major constituent of collagen and is found almost exclusively in collagen. The estimation of hydroxyproline is an accepted method of biochemically evaluating the total collagen content of a sample (Lin et al., 2003) and is also used as a marker of collagen synthesis (Rasik et al., 1999). A biochemical analysis of the granuloma wound tissue of both the extract treated wounds demonstrated a significant increase in the hydroxyproline content compared to that of the untreated wounds. Since hydroxyproline is the direct estimate of collagen synthesis, it supports the wound healing activity of *R. chinensis*. Glycosaminoglycan's and proteoglycans are synthesised by fibroblasts in the wound area. These substances form a hydrated gel-like ground substance (the provisional matrix) on which collagen is deposited. As the collagen content increases, hexosamine levels decrease (Dunphy and Udupa, 1955; Chithra et al., 1998b). Estimation of hexosamine therefore, estimates the amount of ground substance in a wound (Chithra et al., 1998a). From the biochemical estimation of hexosamine in the present study, it was found that treatment with *R. chinensis* resulted in a maximal concentration of hexosamine on day 10 compared to untreated group. This was associated with a

concomitant increase in total collagen content in both treated groups (10% w/w and 5% w/w) on day 10. This indicates replacement of granulation tissue in the wound area by collagen.

Moreover it is well established that the free radicals play an important role in the pathogenesis of certain diseases and ageing. It is also evident that the antioxidant supplement helps in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases (Sanwal and Trevithick, 1982). Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. It is consented that the reactive oxygen species (ROS) are deleterious to wound healing process due to the harmful effects on cells and tissues. The granulation tissue consisting of new capillaries and fibroblast may be replaced by hematoma within the wound. In order to study the wound healing abilities, an attempt has been made by employing the topical treatment of extracts on the excised wounds. We have clearly observed an enhanced wound contraction induced by the hydro-alcoholic leaf extract of both the concentrations (5% w/w and 10% w/w). This could be attributed to the enhanced contractile property of myofibroblast resulting in the increase of epithelialization. (Thiem and Goslinska, 2004) have reported that topical application of compounds with free radical scavenging properties in patients have been shown to improve wound healing significantly and protect tissues from oxidative damage. Cha BC and co- workers established that antioxidative property of *R. chinensis (BaeCheon et al., 2000)*. The preliminary phytochemical screening of *R. chinensis* leaf extract showed the presence of flavonoids, tannins, and triterpnoids along with other phytochemicals. Phytochemical constituents like triterpenoids (Scortichini and Rossi, 1991), flavonoids (Tsuchiya et al., 1996) are known to promote the wound healing process mainly due to their astringent, anti-microbial and free radical scavenging activity. From our experimental data, it is demonstrated that *R. chinensis* showed wound healing activity partly by enhancing the collagen synthesis, probably due to the presence of a mixture of phytoconstituents.

CONCLUSION

The observations and results obtained in this study indicate that the leaf extract of *Rhus chinensis* possesses potent wound healing activity. Wound contraction, increased tensile strength, increased hydroxyproline and hexosamine content explains the reputed wound healing observed.

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