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Evaluation of wound healing action of *Delonix regia* leaf extract in rats

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Abstract

The objective of the present investigation was to evaluate the wound healing action of *Delonix regia* leaf extract in rats. Successive solvent extraction of the leaves of Delonix regia was perfomed using soxhlet method and the extraction abilities of different solvents for recovering extractable components from leaves followed the order: methanol>ethylacetate>water>pet ether. The findings of preliminary phytochemical screening suggest the presence of alkaloids, saponins, phenolics, tannins, terpenoids, sterols, and flavonoids in the leaf of the plant. The acute toxicity study reveals a LD_{50} of more than 2000 mg/Kg for tannin rich ethyl acetate extract of the plant. The ethyl acetate extract of *Delonix regia* leaves were evaluated for the *in vivo* wound healing effect by the excision model. The topical application of 5 % w/w of the *Delonix regia* resulted in an enhanced and statistically significant ($p < 0.001$) wound healing activity *in vivo*. The plant extract exhibited 78.47 ± 5.86 % contraction of wound on the 20th day whereas only 52.07 ± 5.46 % contraction of wound was found in the control animals.

Keywords: Wound healing, *Delonix regia*, flavonoids, extract, in vivo, excision model

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Introduction

Plants have been a rich source of medication in various pathological ailments and have been used since ages for treatment of several diseases. The folkloric use of plants in treatment of ailments though doesnot have much scientific evidence and needs to be scientifically validated for their pharmacological potentials.

Delonix regia (Caesalpiniaceae) has been used widely as medicine in Ayurveda .The chemical constituents reported from this plant belong to different classes such as carbohydrates, amino acids, tannins, flavonoids, phytosterols, alkanes, esters, and anthocyanin pigments. It has number of medicinal uses, many of which have been verified by scientific methods. 23-27 The presence of tannins and flavonoids has been associated with healing of wound. Hence the present investigation was taken with an objective to the wound healing capability of the tannin rich extract of *Delonix regia* leaves.

Material and Methods

The chemical and reagent used in the present study were procured from various scientific suppliers and were used as obtained without any further purification. The instruments used were available in the laboratory of the institution and were used without calibration. The leaves of *Delonix regia* were collected from the local surrounding of Gwalior, Madhya Pradesh in the month of January.

Solvent extraction of phytoconstituents[47]

Powdered plant material (250 g) was evenly packed in the extractor of the soxhlet apparatus and extracted successively with various solvents of increasing polarity including petroleum ether, ethyl acetate and methanol by hot continuous extraction process for about 15 h. The aqueous extraction was carried out by cold maceration process after completion of the solvent extraction process. The extracts were concentrated by distillation to reduce the volume and transferred to 100 mL beaker and the remaining solvents were evaporated on water bath. The extracts obtained collected and placed in desiccators to remove the excessive moisture**.** The dried extracts were stored in desiccators for further investigation.

Qualitative Phytochemical Screening[48]

All the four extracts were evaluated by phytochemical qualitative reactions for identifying the presence or absence of usual plant secondary metabolites. The screening was performed for triterpenes/steroids, alkaloids, glycosides, flavonoids, saponins, tannins, and phenolic acids. The color intensity or the precipitate formation was used as analytical responses to these tests.

Preparation of test sample

A small quantity of the extracts were dissolved in 5 mL of distilled water and filtered. The filtrate was tested to detect the presence of various phytochemical constituents in the sample.

Test for carbohydrates

Molisch's test

Few drops of Molisch's reagent were added to 2-3ml of filtrate, followed by addition of concentrated sulphuric acid along the sides of the test tube. Formation of violet colour ring at the junction of two liquids indicates the presence of carbohydrates.

Test for alkaloids

Small amount of extract mixed with few ml of dilute hydrochloric acid. Shaken well and filtered. Following tests were performed with the obtained filtrate.

Dragendorff's test

A few drops of Dragendorff's reagent (potassium bismuth iodide solution) were added to 2-3 mL of filtrate. Occurrence of orange red precipitate indicates the presence of alkaloids.

Mayer's test

A few drops of Mayer's reagent (potassium mercuric iodide solution) were added to 2-3ml of filtrate. Formation of cream (dull white) precipitate indicated alkaloids.

Wagner's test

A few drops of Wagner's reagent (solution of iodine in potassium iodide) was added to 2-3ml of filtrate. Reddish brown precipitate represents alkaloid.

Hager's test

A few drops of Hager's reagent (Picric acid) were added to 2-3ml of filtrate. Yellow precipitate indicated alkaloid.

Test for glycosides

Legal's test

1ml of pyridine and 1 mL of sodium nitroprusside was added to 1 mL of extract. Pink to red colour indicates the presence of glycosides.

Keller-Killiani test

Glacial acetic acid was added to 2 mL extract, followed by the addition of trace quantity of ferric chloride and 2 to 3 drops of concentrated sulphuric acid. Reddish brown colour appears at the junction of two liquid indicates the presence of cardiac glycosides.

Baljet test

2 mL of extract was added to sodium picrate solution. Yellow to orange colour formation indicates the presence of glycosides.

Test for triterpenoid

Libermann-Burchard test

A small quantity of extract was treated with few drops of acetic anhydride, followed by a few drops of concentrated sulphuric acid. A brown ring was formed at the junction of two layers and the upper layer turns green colour, infers the presence of phytosterols and formation of deep red colour indicates the presence of triterpenoids.

Salkowski test

A small quantity of the extract was treated with chloroform and few drops of concentrated sulphuric acid and allowed to stand for few minutes. Yellow colour at the lower layer indicates the presence of triterpenoids.

Test for steroids and sterols

Liebermann- Burchard reaction

2 mL of extract was mixed with chloroform. To that mixture added 1-2 mL of acetic anhydride and 2 drops of concentrated sulphuric acid along the sides of the test tube. If the solution becomes red, then blue and finally bluish green colour, sterols are present in the extract.

Salkowski reaction

2 mL of extract was mixed with 2 mL chloroform and 2 mL concentrated sulphuric acid and shaken well. If the chloroform layer appears red and acid layer shows greenish yellow fluorescence, it indicates sterols and steroids.

Test for phenols

Ferric chloride test

1 mL of the alcoholic solution of the extract was added to 2 mL of distilled water followed by few drops of 10% ferric chloride. Formation of blue or green colour indicates the presence of phenols.

Lead acetate test

1 mL of alcoholic solution of extract was diluted with 5 mL distilled water and to this few drops of 1% aqueous solution of lead acetate was added. Formation of yellow colour precipitate indicates the presence of phenols.

Test for tannins

Lead acetate test

A few drop of lead acetate was added to 5 mL of aqueous extract. Formation of yellow or red colour precipitate indicates the presence of tannins.

Test for saponins

Foam Test

1 mL of test sample was diluted with 20 mL of distilled water and shaken it in a graduated cylinder for 3minutes. Foam of 1 cm after 10 min indicates the presence of saponins.

Froth test

5 mL of test sample was added to sodium bicarbonate solution. After vigorous shaking the mixture, kept it for 3minutes. A honey comb like froth formation indicates the presence of saponins.

Test for flavonoids

Alkaline reagent test

A few drops of sodium hydroxide solution was added to the extract. Formation of an intense yellow colour, which turns to colourless on addition of few drops of dilute hydrochloric acid, indicates the presence of flavonoids.

Shinodas test [Magnesium hydrochloride reduction test

Alcoholic solution of extract was treated with a small piece of magnesium ribbon and a few drops of concentrated HCl was added and heated. Appearance of crimson red or occasionally green to blue colour indicates the presence of flavonoid.

Test for proteins and amino acids

Biuret test

3 mL of test solution was added to 4% sodium hydroxide and few drops of 1% copper sulphate solution was added to it. Formation of violet colour indicates the presence of proteins.

Ninhydrin test

A mixture of 3 mL test solution and 3 drops of 5% Ninhydrin solution was heated in a boiling water bath for 10 min. Formation of purple or bluish colour indicates the presence of free amino acids.

Pharmacological Evaluation

Animals

Healthy male Wistar male rats weighing 180-250g were used for the study. The animals were housed in cages during the course of experimental period and maintained at 12 day and night schedule with a temperature [17-26°C] maintained at standard experimental condition. The animals were fed with standard rodent pellet feed and water *ad libitum*. The animals were fasted 12 hours before the experiment with free access to only water.

Acute Toxicity Study[49]

A total of three animals were used which received a single oral dose (2000mg/kg) of tannin rich ethylacetate extract of *Delonix regia*. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h and daily thereafter for a period of 14 days. Once daily observations were made for changes in skin and fur, eyes and mucous membrane (nasal) and also respiratory rate, circulatory (heart rate and blood pressure), autonomic (salivation, perspiration, urinary incontinence, and defecation) and central nervous system (drowsiness, tremors and convulsion) changes. Mortality, if any, was also observed over the period of 2 weeks.

Preparation of test samples and standard drugs

The test samples were prepared by formulating the dried ethylacetate free plant extract in simple ointment base (cetostearyl alcohol, wool fat, white paraffin, and hard paraffin) as a 5 % w/w ointment. Commercially available Povidone Iodine Ointment (5 %) was used as standard drug for comparison of action.

Preparation of simple ointment base[50]

Hard paraffin (5 g) and cetostearyl alcohol (5 g) were taken in a porcelain dish maintained on water-bath at 70°C. Wool fat (5 g) and white soft paraffin (85 g) are added to this mixture and stirred until all the

ingredients were in molten state and mixed. The mixture was stirred until cold and packed in suitable container.

Experimental procedure for wound healing by excision model

Experiment Design

The animals were divided in to 4 groups of 5 rats each and the experiment was designed as per table 1.

Table 1 Experimental design for excision model

Group	Nomenclature	Treament
Group I	Control	Untreated
Group II	Vehicle Control	Simple ointment base
Group III	Standard	Povidone iodine ointment (5% w/w)
Group IV	Test	*Delonix regia* extract ointment (5% w/w)

All the test samples; vehicle and standard drug samples were applied topically on the wound of each of the animals daily, under sterile conditions.

Induction of wound[51]

On the day of inducing wound, each animal was anesthetized by the open mask method using short exposure to diethyl ether. The hair (fur) on the back of each rat was removed by shaving using an electric shaver. The area of the wound to be created was marked on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of 1.5 cm in width (circular area 2.25 cm^2) created along the markings using toothed forceps, a surgical blade and pointed scissors. The entire wound left open. All the surgical procedures were carried out under sterile condition. After 24 h of wound creation, the ointments were applied gently to cover the wounded area once daily until complete healing. Wound area and wound contraction, were monitored on each day.

Measurement of wound contraction[51]

The progression of wound healing was judged by the periodic assessment of the contraction of excision wounds. Wound contraction was monitored by tracing the outline of the wound on tracing sheet and then using graph sheet to calculate the area of the wound size. All animals in each group were monitored until complete healing of wounds occurred and the day at which each wound healed was recorded.

$$\text{Percent wound contraction} = \frac{Healed\ area}{Total\ area}\ X\ 100$$

Results and Discussion

The extraction abilities of different solvents for recovering extractable components from leaves followed the order: methanol>ethylacetate>water>pet ether. Shabir et al[52] also reported that methanol provided maximum yield among various solvents used for extraction. The findings of phytochemical screening suggest the presence of alkaloids, saponin glycosides, phenolics,

terpenoids, sterols, and flavonoids in the leaf of the plant. The presence of phenolic acids, beta sitosterol and lupeol the leaves of the plant has also been reported by Singh and Kumar[53] in their review on *Delonix regia*.

Acute Toxicity Study

The acute toxicity test was performed by using the dried ethylacetate extract at concentration of 2000 mg/kg to the test animal, administered orally. No animal died and hence the dose of upto 2000 mg/Kg was considered to be safe. As none of the animals died, the LD_{50} was considered to be more than 2000 mg/Kg and any dose less than 2000 mg/Kg would be considered for evaluation of wound healing action.

Wound Healing action

The ethyl acetate extract of *Delonix regia* leaves were evaluated to determine the *in vivo* wound healing effect by the excision model (n=5). The topical application of 5 % w/w of the *Delonix regia* resulted in an enhanced and statistically significant ($p < 0.001$) wound healing activity *in vivo*. The wound area measurements and the percent wound contraction results of the progressive healing of the excision wounds for the control; vehicle control; standard reference drug and plant extract are presented in table 2 (Figure 1 & 2). From the results it can be clearly seen that the ethyl acetate extract of the plant had an excellent wound healing potential with almost complete closure of the wound of the animals by 20 days. The plant extract exhibited 78.47 ± 5.86 % contraction of wound on the 20th day whereas only 52.07 ± 5.46 % contraction of wound was found in the control animals. It is apparent that the *Delonix regia* ethyl acetate leaf extract showed good activity.

Table 2 Area of wound and % contraction of wound by excision model

Group	Control	Vehicle Control	Standard	*Delonix regia* extract
Day	**Area mm² (% contraction)**			
Day 0	311.5 ± 26.1	336.4 ± 21.7	268.5 ± 24	281.1 ± 19.7
	-	-	-	-
Day 5	269.2 ± 27.18	237.4 ±14.69	141.3 ± 12.21	172.8 ± 15.39
	(13.57 ± 4.68)	(29.72 ± 2.55)	(47.37 ± 3.89)**	(38.52 ± 6.18)**
Day10	224.1 ± 23.21	177.9 ± 18.81	60.8 ± 18.42	101.5 ± 19.28
	(28.05 ± 7.18)	(47.11 ± 4.27)	(77.35 ± 7.32)**	(63.89 ± 8.23)**
Day 14	174.2 ± 12.36	136.5 ± 12.01	39.6 ± 9.59	84.7 ± 17.58
	(44.07 ± 4.42)	(59.42 ± 3.65)	(85.25 ± 3.28)**	(69.86 ± 8.16)**
Day 20	149.3 ± 17.82	112.7 ± 14.79	21.1 ± 11.07	60.5 ± 12.58
	(52.07 ± 5.46)	(66.49 ± 4.39)	(92.14 ± 4.20)**	(78.47 ± 5.86)**

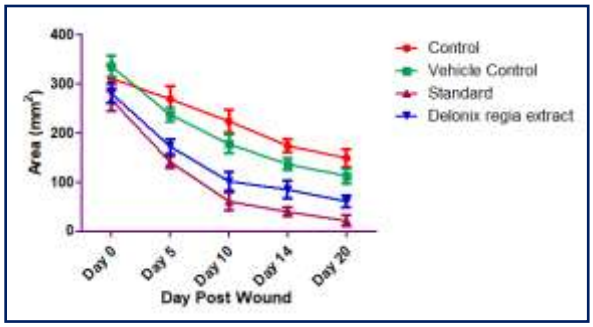

Figure 1 Wound healing efficacy of *Delonix regia* by *in vivo* excision model

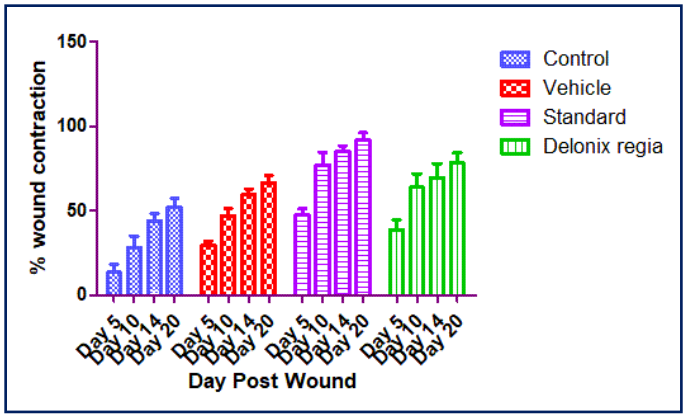

Figure 2 % contraction of wound exhibited by *Delonix regia* by *in vivo* excision model

Conclusion

Wound healing is a complex and continuous process that begins immediately after injury, followed by homeostasis, blood clotting, inflammation, proliferation and remodeling phases. The present investigation had thrown light on the remarkable potential of commonly available plant *Delonix regia* in terms of its pharmacological benefits it offers. The ethyl acetate extract of the leaves of Delonix regia was found to be effective in the functional recovery of the wound. The strength of the investigation lies in establishing and reporting for the first time the wound healing efficacy of this plant. The result may be attributed to the phytoconstituents such as flavonoids, tannins and phenolics present in the extract which may be due to their individual or cumulative effect that enhanced wound healing. This plant can be explored further as a source of an economical therapeutic agent for wound management as a pro-healer, as well as to facilitate faster wound healing processes without formation of residual scar tissues.

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