



Evaluation Of Effect Of Aqueous And Ethanolic Extracts Of *Pithecellobium Bijeninum* Leaves On Wound Healing

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Abstract

The objective of the present investigation was to evaluate the wound healing action of *Pithecellobium bijeninum* leaf extract in rats. Successive solvent extraction of the leaves of *Pithecellobium bijeninum* was performed using petroleum ether and ethanol by soxhlet method and distilled water by cold maceration method. The acute toxicity study suggests a LD₅₀ of more than 2000 mg/Kg for both the ethanolic and aqueous extracts of the plant. The ethanolic and aqueous extracts of *Pithecellobium bijeninum* leaves were evaluated for the *in vivo* wound healing effect by the excision model. The topical application of 5 % w/w of the *Pithecellobium bijeninum* resulted in an enhanced and statistically significant ($p < 0.05$) wound healing activity *in vivo* when using the ethanolic extract. The wound healing by the aqueous extract was not significant in comparison to the control group. The ethanolic extract exhibited 82.1861 ± 5.86 % contraction of wound on the 20th day whereas only 49.2866 ± 5.46 % contraction of wound was found in the control animals. On the other hand, the maximum coverage of wound by the aqueous extract on the 20th day of treatment was 67.4664 ± 3.011 %. The standard drug (povidone) was able to contract 89.1449 ± 4.20 % of the wound in comparison to the 1st day and exhibited significant action ($p < 0.01$).

Keywords

Excision, wound, healing, *Pithecellobium*, extract

Introduction

Wounds have affected humans since pre-historic times and the treatment and healing of wounds is an art as old as humanity.¹ Research on wound healing drugs is a rapidly developing area in modern biomedical sciences. Plant-derived natural products are significant as sources of medicinal agents and models for the design of new remedies.² Lately, research has been directed to traditional folk medicines as they are generally characterized by high acceptability and good toleration. The healing potential of phytomedicines is often associated with angiogenesis, which is a critical step of wound healing. The healing efficacy seen in phytomedicine treated wounds shows great promise although for most natural products no well controlled scientific data are available.³⁻⁶

Pithecellobium bijeninum is a flowering species native to Mexico, Central America, and northern part of South America. The various parts of the plant are reported to be rich in tannins, fixed oil, quercetin, olein, steroids, saponins, high protein content in seeds, and several steroidal compounds have been isolated from the plant.⁷ Leaves yield quercetin, kaempferol, dulcitol and afezilin. Fatty acid analysis of seed extract yielded 9 saturated and 17 unsaturated fatty acids. Total protein content was highest in the seeds (50.3-67.1%), followed by stems, roots, leaves, flowers, and fruits. GC-MS study of leaves yielded bioactive constituents namely phytol, anthracene, 9-(3-butenyl), diisooctyl phthalate, 13-docosenamide, 3,6,9-triethyl-3,6,9-trimethyl formic acid, cyclotetrasiloxane, octamethyl, l(+) ascorbic acid 2,6dihexadecanoate. The leaves of the plant have been associated with anti-inflammatory, anti-oxidant, anti-microbial, adulticidal and other pharmacological actions.^{7, 8-10}

Considering the above facts, it was decided upon to explore and scientifically validate the wound healing properties of *Pithecellobium bijeninum* leaf extracts. The objective of the present investigation is to evaluate the wound healing capability of the tannin rich extract of *Pithecellobium bijeninum* leaves.

Material and Methods

Collection and pharmacognostic investigation of plant

The leaves of *Pithecellobium bijeninum* have been collected from the surroundings of Bhopal, Madhya Pradesh and authenticated with the botany department. The macroscopic features of the plant material was observed for and compared to that of previously reported literature in order to study the pharmacognostical features of the plant. The cross section of the leaves were also examined and studied for the presence of type of cells in them. The presence of epidermal cells, palisade, parenchyma, vascular bundles etc was observed for in the cross section of the leaves.¹¹

Preparation of the plant material and extraction

The leaves of the plant were washed with distilled water and dried in shade (preventing from direct sunlight). The dried leaf has been powdered using slow speed blender and is kept in closed airtight container. Powdered plant material (110 g) was evenly packed in the extractor of the soxhlet apparatus and extracted successively with various solvents of increasing polarity including petroleum ether and ethanol 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.¹¹ The dried extracts were stored in desiccators for further investigation.

Phytochemical Testing of extracts

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.¹²

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

A total of three animals were used which received a single oral dose (2000mg/kg) of ethanolic and aqueous extracts of *Pithecellonium bijenum*. 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.¹³

Preparation of test samples and standard drugs

The test samples were prepared by formulating the dried ethanolic and aqueous 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

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.¹⁴

Experimental procedure for wound healing by excision model

Experiment Design

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

Table 1 Experimental design for excision model

Group	Nomenclature	Treatment
Group I	Control	Untreated
Group II	Vehicle Control	Simple ointment base
Group III	Standard	Povidone iodine ointment (5% w/w)
Group IV	Test	<i>Pithecellonium bijenum</i> ethanolic extract ointment (5% w/w)
Group V	Test	<i>Pithecellonium bijenum</i> aqueous 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 wound¹⁵

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 cm²) 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 contraction¹⁵

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.

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

Results and Discussion

Pharmacognostic Study

The plant leaf was dark green on the outer surface and light green on the lower surface. The leaves are bipinnate, having 2 pairs of kidney shaped leaflets. The length of leaf from base to the tip of the leaflet ranged from 2-4 cm. A clear midrib and entire margin was present in the leaves (Figure 1). The transverse section revealed epidermal cells, parenchymatous cells and starch granules. Epidermal cells were visible on both the lower and upper surface of the leaf.



Figure 1 Leaf of *Pithecellobium bijenum* and transverse section of leaf

Extraction Yields

The extraction abilities of different solvents for recovering extractable components from leaves followed the order: ethanol>water>pet ether.

Phytochemical Testing

All the extracts were tested for the presence of various categories of phytochemicals and the results are presented in Table 2.

The findings suggest the presence of alkaloids, saponin glycosides, phenolics, terpenoids, sterols, and flavonoids in the leaf of the plant. The presence of glaucin and annonaine, linoleic acid, Phytol and Acetate camphor (+)-2-bornanone in the leaves of the plant has also been reported by Vanitha and Manikandan¹⁶ in their review on *Pithecollonium bijenum*.

Table 2 Phytochemical screening of *Pithecellobium bijenum* leaf extracts

Chemical Tests	Observation to be tested	Pet. Ether extract	Ethanollic extract	Aqueous extract
Alkaloids				
<i>Mayer's reagent</i>	cream colour precipitate	-	+	-
<i>Hager's reagent</i>	yellow colour precipitate	-	+	-
<i>Wagner's reagent</i>	reddish brown precipitate	-	+	-
<i>Dragendorff's reagent</i>	reddish brown precipitate	-	+	-
Glycosides				
<i>Legal Test</i>	Pink or red color formation	-	-	-
<i>Baljet Test</i>	Orange or yellow color formation	-	-	-
<i>Keller-Kiliani</i>	Reddish brown color in acid layer	-	-	-
Phenolics				
<i>Ferric chloride Test</i>	Blue or green color	-	+	+
<i>Lead acetate Test</i>	Yellow color	-	+	+
Flavonoids				
<i>Shinoda test</i>	red color	-	+	-
<i>Alkaline reagent test</i>	Yellow color that turns red on acidification	-	+	+

Proteins				
<i>Biurets Test</i>	Voilet color	-	-	+
<i>Ninhydrin Test</i>	Purple or bluish color	-	-	+
Triterpenoids				
<i>Lieberman-Burchard Test</i>	Deep red color	+	+	-
<i>Salkowski Test</i>	Yellow color in lower layer	+	-	-
Cabohydrates				
<i>Molisch's test</i>	Violet color ring	+	+	-
Steroids				
<i>Lieberman-Burchard Test</i>	Brown ring at junction Upper layer turns green	+	+	-
<i>Salkowski Test</i>	Red color in chloroform layer	+	-	-
Tannins				
<i>Lead acetate Test</i>	Yellow or red color	-	+	-
Saponins				
<i>Foam Test</i>	Foaming of 1 cm	-	-	+
<i>Froth Test</i>	Honey comb froth	-	-	+

Acute Toxicity Study

The acute toxicity test was performed by using the dried ethanolic and aqueous extracts 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 LD₅₀ 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 ethanolic and aqueous extracts of *Pithecellobium bijenum* leaves were tested to determine the *in vivo* wound healing effect by the excision model (n=5). The topical application of 5 % w/w of the *Pithecellobium bijenum* extract containing ointments on the wound resulted in an enhanced and statistically significant ($p < 0.05$) 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.

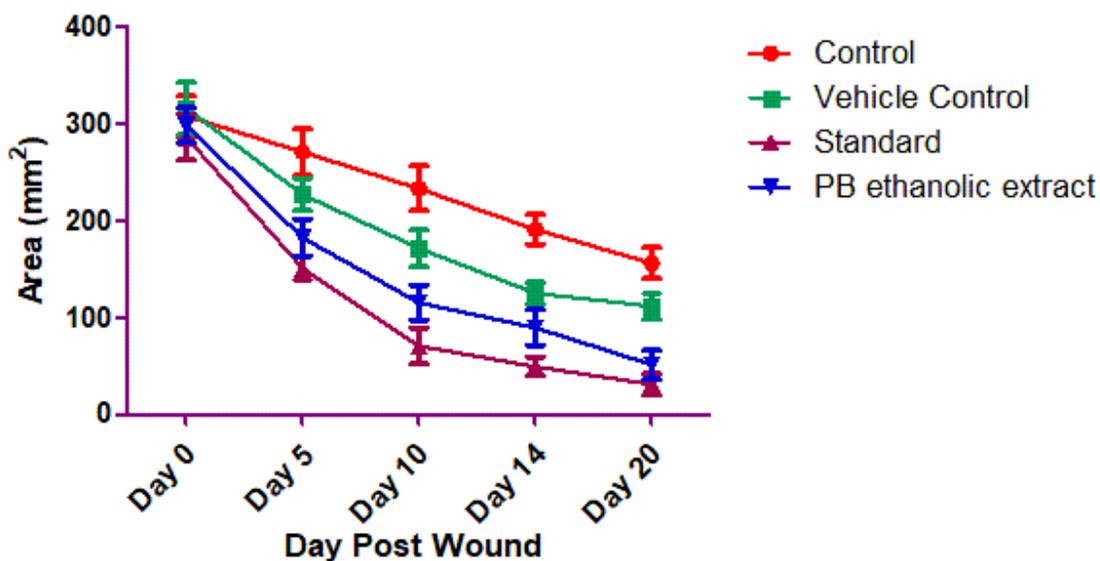


Figure 2 Wound healing efficacy of ethanolic extract of *Pithecellobium bijeninum* by *in vivo* excision model

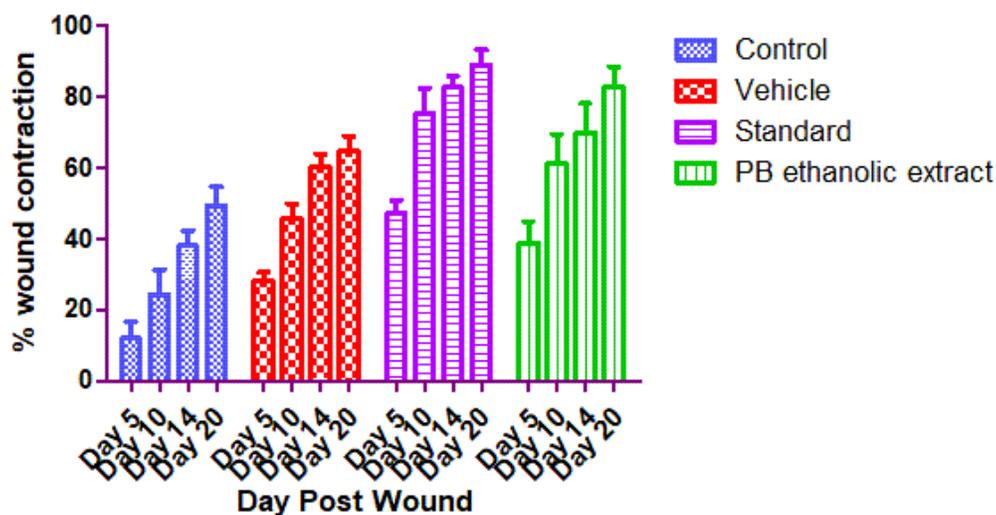


Figure 3 % contraction of wound exhibited by ethanolic extract of *Pithecellobium bijeninum* by *in vivo* excision model

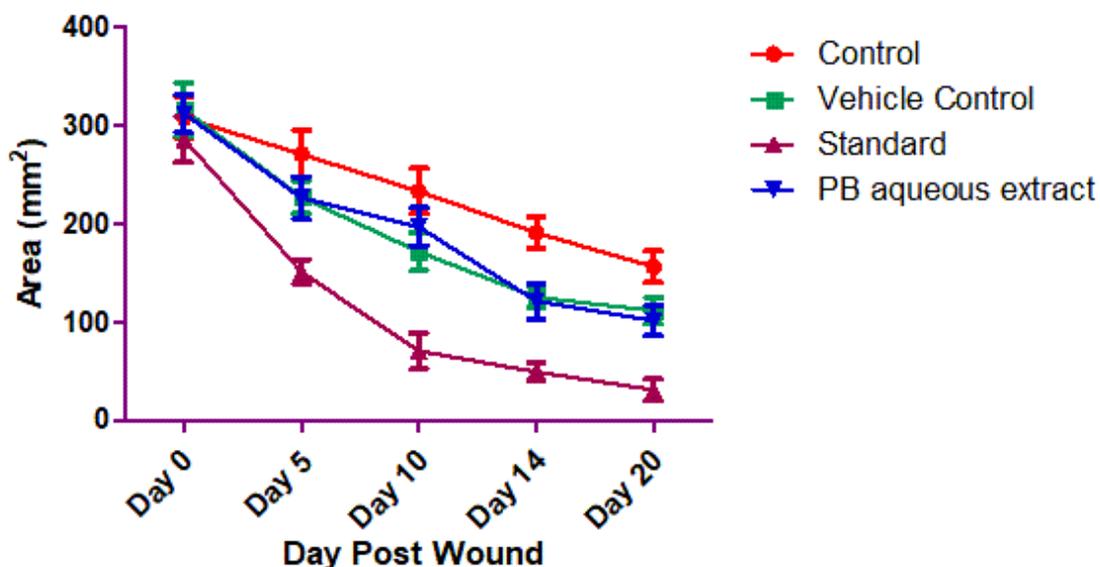


Figure 4 Wound healing efficacy of aqueous extract of *Pithecellobium bijeninum* by *in vivo* excision model

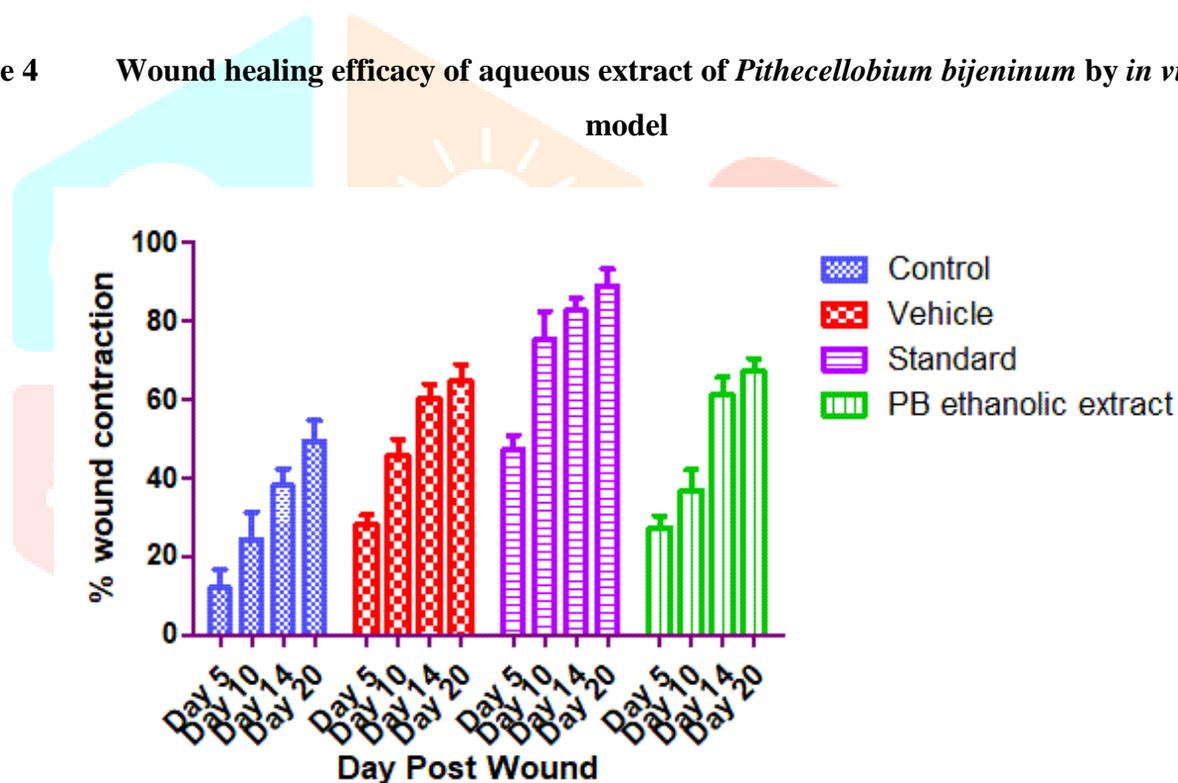


Figure 5 Wound healing efficacy of aqueous extract of *Pithecellobium bijeninum* by *in vivo* excision model

From the results it can be clearly seen that the ethanolic 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 82.1861 ± 5.86 % contraction of wound on the 20th day whereas only 49.2866 ± 5.46 % contraction of wound was found in the control animals (Figure 2 & 3).

As shown in the results above (Figure 3 & 4) the aqueous extract was unable to treat the wound significantly. The maximum coverage of wound by the aqueous extract on the 20th day of treatment was 67.4664 ± 3.011 %.

In comparison to the aqueous extract, the ethanolic extract exhibited better wound healing capability when used as a 5% w/w ointment for topical application on the wound. The standard drug (povidone) was able to contract 89.1449 ± 4.20 % of the wound in comparison to the 1st day and exhibited significant action ($p < 0.01$).

The occurrence of tannins in the ethanolic extract could be attributed for the effective wound healing action by the ethanolic extract. Previous studies on wound healing action of the plant extracts have also linked the presence of tannins in the extract to its wound healing property.

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 *Pithecellobium bijenum* in terms of its pharmacological benefits it offers. The ethanolic extract of the leaves of *Pithecellobium bijenum* 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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