International Journal of Pharmacy & Life Sciences
Open Access to Researcher

An Imperical study of Flavonoid Fraction of *Juglans regia* in Diabetic rats for their Wound Healing Activity

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Article info

Received: 19/12/2025

Revised:15/01/2026

Accepted: 09/02/2026

www.ijplsjournal.com

Abstract

The present work is deals with study of the wound healing activity of Flavonoid Fraction of *Juglans regia* Linn. (*FFJR*) in Diabetic Rats. From the fruits, the flavonoid fraction was prepared and flavonoid and phenolic compounds were determined. The diabetes was induced by the single administration of Streptozotocin in citrate buffer. The wound healing activity of flavonoid fraction was determined by Dead space, Incision and Excision wound models. During the experimental period the fasting blood glucose level in diabetic animals were higher than those of normal animals. However, the fasting blood glucose level was significantly reduced in treatment groups. Topical application of flavonoid fraction, ointment base and Povidone iodine on dorsal inter scapular region causes significant and faster rate of wound closure and reduced epithelialization period.

In incision wound model, there is a significant increase in skin tensile strength by 10% w/w and 20% w/w ointment of *FFJR* compared to control. The hydroxyproline content was increased significantly in dose dependent manner by 20% *FFJR*. FFJR significantly decreased the epithelialization period, hence have significant effect on the proliferative phase of wound healing in diabetic rats.

Keywords: *Juglans regia*, Wound, Diabetes

Introduction

Wound healing is a multifaceted and dynamic procedure of restoring cellular structures and tissue layers. There are two types of wounds: acute and chronic. Acute wounds, heal in normal, orderly succession of healing, are either traumatic or surgical and move from side to side the healing progression at a predictable phase from injury to complete wound closure.

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Chronic wounds fail to advancement through a normal, orderly and timely sequence of mend and that has not determined over a reasonable era of time and they do not development through the unsurprising stages of wound healing. These wounds may eventually pass through the repair procedure without restoring sustained anatomical and useful results.[1] Plants or chemical entities consequent from plants need to be identified and formulated for treatment and supervision of wounds. In this direction, a number of herbal products have been used in managing and treatment of wounds over the years.[2] The main aim of research, to augment the rate of healing of diabetic wounds using flavonoid fraction of *Juglans regia* (*FFJR*) in the shortest time possible, with minimal pain, discomfort and scarring to the patient. At the site of wound closure, a supple and fine scar with high tensile strength is desired.[3]

The wound healing activity of the flavonoid fraction of *Juglans regia* Linn. (commonly known as walnut) in diabetic rats has been explored to evaluate its potential therapeutic effects. *Juglans regia* is known for its rich flavonoid content, which exhibits strong antioxidant, anti-inflammatory, and antimicrobial properties. In the context of diabetes, wound healing is often delayed due to impaired immune function, oxidative stress, and poor circulation. Diabetic rats are commonly used in studies to simulate human diabetic conditions, which can provide insights into the efficacy of treatments in similar human pathologies.

The flavonoid fraction of *Juglans regia* is believed to promote wound healing by modulating several cellular and molecular pathways involved in tissue repair. These include enhancing collagen synthesis, stimulating angiogenesis (the formation of new blood vessels), and reducing inflammation at the wound site. In experimental models, various wound healing methods, such as excision, incision, and burn wound models, are utilized to assess the efficacy of the flavonoid fraction in diabetic rats. The results of these studies often demonstrate significant improvements in wound closure rates, reduction in wound size, and enhanced tissue regeneration, which could potentially be attributed to the antioxidative and anti-inflammatory actions of flavonoids.

Additionally, studies have shown that *Juglans regia* flavonoids exert their effects by reducing oxidative stress, a major factor in delayed wound healing in diabetic patients. These flavonoids work by neutralizing free radicals, improving cellular integrity, and supporting the body's natural healing processes. Furthermore, the application of *Juglans regia* flavonoids can enhance the activity of growth factors and cytokines that are crucial for proper wound healing. Thus, the flavonoid fraction of *Juglans regia* appears to hold promise as a natural adjunctive therapy for accelerating wound healing in diabetic conditions.

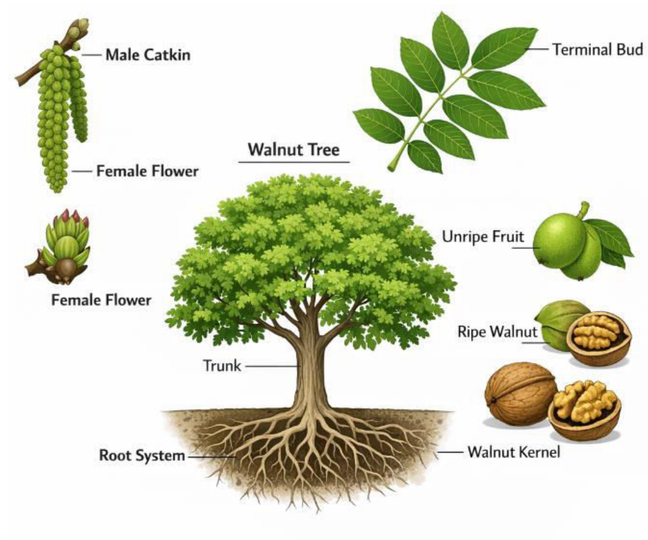

Fig. 1: *Juglans regia Linn*

Material and Methods

Procurement of Material

Fruits of *Juglans regia* were procured freshly from the local market. were taxonomically authenticated by Dr. S. N. Dwivedi, Botanist, Janta PG college, APS University, Rewa. The specimen herbarium sheets were submitted and preserve in Department.

Flavonoid fraction preparation

Fruits of *J. regia* were dried under shade and subjected to coarse powder for extraction process. Accurately weighed quantity of bark powder was macerated with 70% methanol in dark and filtered to harvest a viscous supernatant, dried under vacuum below 40°C. The viscous residue was collected, weighed and kept at 4°C. Methanolic extract was dissolved in water and taken in a separating funnel for fractionization and pet ether, ethyl acetate and butanol were added as per their polarity. A butanol fraction was unruffled and dried under vacuum to acquire flavonoid fraction.

Animals[11,12,13.14.15]

Male Wistar albino rats between 2 and 3 months of age weighing 150-200 gm were selected. All animals were housed under normal condition of 25±1^{0}C, 12 hr light and dark cycle, were fed with palatable rat feed and water *ad libitum*. All trials were carried out according to the conditions of the Institutional Animal Ethical Committee.

Determination of preliminary phytochemicals [16,17]

The chemical tests were performed for the presence of flavonoids, which could be responsible for the wound healing movement in diabetic animals.

Acute Toxicity Studies [18,19,20]

The acute toxicity study was carried out in adult Wistar albino rats by OECD guideline no. 425. The animals were fasted overnight and extracts of the plant (suspended in 0.5 % w/v sodium CMC) were administered orally at dose level of 2000 mg/kg. Then the animals were observed continuously for three hour for general behavioral, neurological, autonomic profiles and then after every 30 min for next three hour and finally for mortality after 24 hour till 14 days.

Induction of Diabetes [21,22,23]

Diabetes was induced by a single *i.p.* injection of streptozotocin (STZ) (50 mg/kg body. wt) dissolved in 0.1 M of cold citrate buffer (pH 4.5) 15 min after the *i.p.* administration of Nicotinamide (110 mg/kg body. wt) in overnight fasted rats.[4] Initiation of diabetes was inveterate by tail vein blood glucose estimation using glucometer after 72 h. After two weeks, rats with blood glucose level greater than 250 mg/dl were deemed as diabetic and used for the experiment.

Surgical Wound Models [24,25,26]

Preparation of ointment

Simple ointment of *FFJR* (5%, 10%, 20% w/w) was prepared using white soft paraffin ointment base. Povidone iodine ointment (5%w/w) was used as the standard drug for comparing the wound healing potential of the extract in different animal models.[5]

Excision Wound

The Animals were depilated and wounded under light ether anesthesia, semi aseptically. Screening for wound healing activity was performed by excision wound model and incision wound model without infection. All the test sample and standard drug were applied topically just the once a day after cleansing with surgical cotton wool.

Procedure for wound creation

Each animal was anesthetized by open mask method by mild anesthetic ether. A predetermined area of 500mm^2 full thickness skin was excised in the dorsal inter scapular region. The areas of the wounds were measured (sq.mm) without delay placing a transparent polythene graph paper over the wound and then tracing the area of wound on it. This was taken as the initial wound area reading. All the test samples were applied formerly daily. The wound area of each animal was measured on days 0, 2, 4, 6, 8, 10, 12 and 14 after inflicting the wound.[6]

Incision Wound Model

The animals were anaesthetized under light ether anesthesia. One full thickness paravertibral incision of 1.5cm length was made including the cutaneous muscles depilated back of each rat. After the incision the parted skin was kept together and stitched with black silk at together the end of the created wounds. The test sample was applied in a similar manner as in excision wound model.[7]

Parameters for wound healing activity

The parameters for wound healing activity was determined by the reported methods[6-9]

Statistical analysis [27,28,29]

The results were analyzed by ANOVA followed by Dunnet's "t" test to decide the statistical significance. $p<0.05$ was selected as the level of significance.

Results and Discussion

Determination of preliminary phytochemicals

The qualitative analysis confirmed the presence of flavonoids in the given sample. All chemical tests, including Shinoda test, alkaline reagent test, lead acetate test, ferric chloride test, hot water extract test, TLC, and UV spectroscopy, showed positive results. The characteristic color changes, precipitate formation, chromatographic spots, and UV absorption peak around 350 nm collectively indicate that the sample is rich in flavonoid compounds.

ISSN: 0976-7126
Chhajed *et al.,* 17(1):11-18, 2026

Acute Toxicity Studies

The study revealed administered orally. Mild behavioral changes were observed during the initial 24-hour period, but the animals fully recovered within the 14-day observation period.

Statistical analysis

The results indicate that FFJR treatment produced a significant, dose-dependent improvement compared to the diabetic control group. The diabetic control (DC) group showed persistently elevated values throughout the study period, confirming disease progression. In contrast, FFJR at 200 mg/kg caused a gradual and significant reduction over 7, 14, and 28 days, while 400 mg/kg produced a more pronounced and faster reduction. By day 28, the higher dose brought the values closer to normal control levels. Overall, the findings demonstrate that FFJR exhibits significant protective/therapeutic activity, with 400 mg/kg being more effective than 200 mg/kg.

Table 1: Details of animal employed in the study

S. No.	**Parameters**	**Details**
1	Animal Species	Male Wistar Albino Rats
2	Age	2–3 months
3	Weight	150–200 g
4	Housing Conditions	Normal conditions
5	Temperature	25 ± 10°C
6	Light/Dark Cycle	12-hour light/dark cycle
7	Diet	Palatable rat feed
8	Water Supply	Ad libitum (free access)
9	Ethical Approval	Animal Ethical Committee
10	Trial Conduct	ethical committee guidelines

Table 2: Parameters for the induction of diabetes

S. No.	Parameters	Details
1	Animal Species	Wistar albino rats
2	Fasting	Overnight fasting
3	Induction Method	**Single *i.p.* injection** of streptozotocin (STZ)
4	STZ Dose	50 mg/kg body weight
5	Citrate Buffer	0.1 M cold citrate buffer (pH 4.5)
6	Nicotinamide Dose	110 mg/kg body weight
7	Time Between Nicotinamide and STZ	15 minutes
8	Diabetes Confirmation Method	Blood glucose estimation using a glucometer via tail vein
9	Post-Administration Period	72 hours for initial glucose level measurement
10	Diabetes Diagnosis	Blood glucose level > 250 mg/dL after 2 weeks
11	Outcome	Rats with blood glucose levels above 250 mg/dL were classified as diabetic and used for the experiment.

Table 3: Determination of preliminary phytochemicals

S. No.	Test	Observation	Result (Flavonoids)
1	Shinoda Test	Appearance of red color on addition of magnesium ribbon and dilute hydrochloric acid	Positive
2	Alkaline Reagent Test	Formation of intense yellow color, which becomes colourless on adding acid	Positive
3	Lead Acetate Test	Formation of yellow precipitate	Positive
4	Ferric Chloride Test	Formation of blue-black or green color	Positive
5	Hot Water Extract Test	Observation of yellow color in hot water extract	Positive
6	TLC (Thin Layer Chromatography)	Presence of flavonoid spots on chromatogram	Positive
7	UV Spectroscopy (Absorption in UV-Vis Range)	Presence of peak at around 350 nm	Positive

Table 4: Acute Toxicity Studies

S. No.	Parameters	Details
1	Animal Species	Adult Wistar albino rats
2	Guideline Followed	OECD guideline no. 425
3	Fasting	Overnight fasting
4	Dose Administered	2000 mg/kg (suspended in 0.5% w/v sodium CMC)
5	Administration Route	Oral (gavage)
7	Observation Period	Continuous for the first 3 hours
8	Subsequent Observations	Every 30 minutes for the next 3 hours, and daily for 14 days
9	Behavioral & Neurological Profiles	Monitored for signs of distress, sedation, or abnormal behavior
10	Autonomic Profile	Monitored for signs of salivation, urination, defecation, etc.
11	Mortality	Observed after 24 hours and up to 14 days
12	Results	No mortality or severe toxicity observed in the rats after 14 days
13	Signs of Toxicity	Mild behavioral changes like slight lethargy (if any) observed for the first 24 hours
14	Recovery	All rats recovered fully without any lasting effects by the end of the study

Table 5: Changes in blood glucose level (mg/dl) during study period.

S. No.	Group	Treatment	Days			
			0	7	14	28
1.	NC	0.9% NS	110.3±2.21	112.4±3.45	121.2±2.31	121.4±3.12
2.	DC	--	321.1±3.22	352.3±3.44	345.5±3.29	344.2±2.21
3.	FFJR	200 mg/kg	321.3±4.31	301.8±4.13**	280.8±3.14***	210.2±4.22***
4.	FFJR	400 mg/kg	319.5±3.22	270.2±4.22***	220.2±5.41***	160.5±3.38***

*NC: Normal Control. DC: Disease control. NS: Normal Saline, Values are expressed as mean±SEM, n=6 in each group; * p <0.05, ** p<0.01, *** p <0.001, compared to disease control*

Table 6: Effect of *FFJR* on wound area of excised uninfected wound, on tensile strength and by hydroxyproline content of excised uninfected wound in rats

Group	Treatment	Wound area in mm²								Tensile strength (g/cm²)	Hydroxyproline (µg/mg tissue)
		0th	2th	4th	6th	8th	10th	12th	14th		
Control		389.12±3.33	387.34±4.22	386.34±9.22	385.37±4.73	384.55±5.44	385.16±9.46	382.83±5.55	383.56±8.77	87.50±7.15	0.73±0.04
Std	*5% w/w*	390.33±4.91	388.83±8.34	380.22±8.44	375.16±6.77	361.16±5.34	355.66±6.66*	312.16±5.13**	301.50±5.24*	220.20±9.41**	0.89±0.02***
FFJR	5% w/w	390.33±3.15	380.33±9.22	375.16±4.34	370.50±6.22	360.66±7.29*	351.16±6.19**	312.50±3.19**	305.83±7.86**	158.31±5.22*	0.68±0.05
FFJR	10% w/w	383.50±6.80	371.66±7.54	365.16±8.14	355.33±8.42*	340.33±4.57**	320.16±6.51**	310.33±7.09**	290.16±8.50**	190.83±9.44**	0.72±0.03*
FFJR	20% w/w	388.00±2.30	370.33±4.33	365.83±8.45	350.50±8.78*	340.33±9.18**	331.66±5.61***	307.16±7.96***	280.50±8.46***	215.16±8.56**	0.80±0.06***

*Std-Povidone Iodine Ointment 5%w/w, NC: Normal Control. DC: Disease control. NS: Normal Saline, Values are expressed as mean±SEM, n=6 in each group; * p <0.05, ** p<0.01, *** p <0.001, compared to disease control*

The present study was designed to evaluate the wound healing potential of the flavonoid fraction of *Juglans regia* Linn. (FFJR) in diabetic rats using various wound models. The results demonstrated that the topical application of FFJR, at different concentrations (5%, 10%, and 20% w/w), significantly accelerated wound closure, reduced the epithelialization period, and improved skin tensile strength compared to the disease control group.

In the excision wound model, a clear reduction in wound area was observed with the treatment of FFJR, suggesting its potential to promote faster wound healing. The incision wound model further validated this, as the rats treated with FFJR showed increased skin tensile strength, which is a measure of wound strength and healing. A similar effect was observed in the hydroxyproline content assay, where a dose-dependent increase in hydroxyproline, a major component of collagen, was noted. This indicates enhanced collagen synthesis and tissue repair, which is crucial in wound healing.

The underlying mechanism of the wound healing effect can be attributed to the antioxidant, anti-inflammatory, and collagen-synthesizing properties of flavonoids. In diabetic conditions, wound healing is typically impaired due to oxidative stress, poor circulation, and increased inflammation. FFJR helps mitigate these effects by reducing oxidative stress and promoting angiogenesis and collagen deposition at the wound site. This is consistent with the results observed in the study, where FFJR treatment led to quicker epithelialization and stronger scar tissue formation.

The diabetic rats in this study exhibited delayed wound healing, which is a common complication in diabetes due to impaired immune function and delayed tissue repair. The findings from this study demonstrate that FFJR can significantly accelerate the proliferative phase of wound healing, making it a promising candidate for topical wound healing treatments in diabetic patients.

Moreover, the observed reduction in blood glucose levels (Table 5) following the treatment with FFJR further supports its potential in managing wound healing in diabetic conditions, as elevated blood glucose levels often impair immune response and delay tissue regeneration

Conclusion

There was significant wound contraction by flavonoid fraction. It increases the number of fibroblasts, collagen tissue and causes complete Epithelialization. The percentage of wound contraction significantly decreases when the extract were applied on infected wound. Diabetic rats show delayed Epithelialization period compared to non-diabetic rats. In our study, flavonoid fraction significantly decreased the epithelialization period. So, flavonoid fraction may have significant effect on the proliferative phase of wound healing in diabetic rats. An increase in tensile strength and hydroxyproline content of treated wounds in the present study may be due to increase in collagen concentration and stabilization of fibers.

Future Scope**:**The active phytoconstituents responsible for the observed effects of FFJR can be isolated, characterized, and quantified using advanced analytical techniques such as HPLC, LC–MS, and NMR .Further studies can be carried out to elucidate the exact mechanism of action, particularly its role in antioxidant activity, insulin secretion, or glucose uptake pathways. Chronic toxicity and long-term safety studies are needed to establish the safe therapeutic dose of FFJR. The efficacy of FFJR can be evaluated in different experimental models and compared with standard drugs to validate its therapeutic potential.

Acknowledgements

Authors are thankful to Management of Oxford International College, Indore

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Cite this article as:
Chhajed M., Lodha R. J., Chhajed A. J., Rathore A. S., Bukkawar A., Wathore S., Patil P. and Sinha P. (2026). An Imperical study of Flavonoid Fraction of *Juglans regia* in Diabetic rats for their Wound Healing Activity. *Int. J. of Pharm. & Life Sci.,* 17(1):11-13.

Source of Support: Nil
Conflict of Interest: Not declared
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