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ORIGINAL ARTICLE

Evaluation of *Acacia auriculiformis* Benth. Leaves for Wound Healing Activity in Type 2 Diabetic Rats

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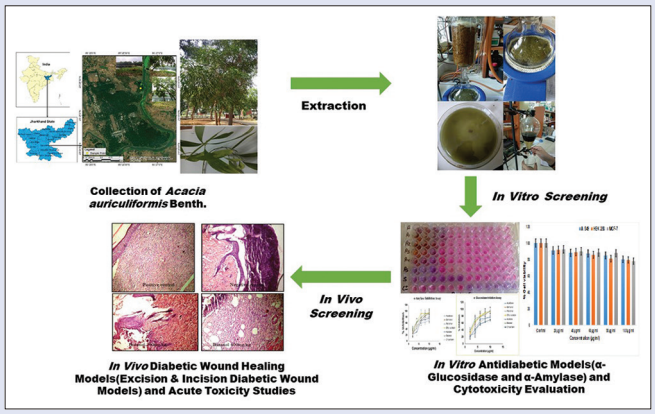
ABSTRACT

Background: *Acacia auriculiformis* Benth. is a perennial shrub which has been traditionally known to treat various medical complications such as sore eyes, allergy, rheumatism, sore eyes, and rashes. **Objectives:** The current study is designed to decipher a shred of clinical confirmation and biochemical support for wound-healing efficacy of methanolic leaves extract of *A. auriculiformis*. **Materials and Methods:** We prepared the leaves extract in different solvents, i.e., petroleum ether, chloroform, ethyl acetate, acetone, butanol, and methanol. The *in vitro* studies were carried out on the above leaf's extracts using α-glucosidase and α-amylase inhibition *in vitro* antidiabetic assays. Animal model of streptozotocin-induced diabetes was used in the 0-, 7-, and 15-day studies, respectively. The comparison study was carried out in diabetic wound control in respect of the period of epithelialization, %wound contraction, and hydroxyproline content in the excision wound model. However, the breaking strength parameter was used to calculate healing potential in the incision wound model. The bioactive methanol extract was subjected to LC-MS/MS analysis to characterize the phytoconstituents responsible for pharmacological activity. **Results:** The methanolic leaves extract showed the highest percentage inhibition of 94.259% and 95.259% in α-glucosidase and α-amylase *in vitro* antidiabetic assays, respectively. The high content of collagen fibers and stronger epithelial cells growth were observed in histopathological studies of hydrogel containing methanolic leaves extract as compared to the diabetic wound control and standard. Subsequently, for investigating the biological impact upon live cells, cytotoxicity study was tested in different cell lines (A549, HEK-293, and MCF-7). Cytotoxic results showed that greater than 75% of cells were visible in all the cell lines, which gives the confirmation of biocapability of the extract. The LC-MS/MS results revealed the presence of compounds such as β-sitosterol, lupeol, stigmasterol, and quercetin. **Conclusion:** *A. auriculiformis* is a potent medicinal plant that can be further utilized as a complementary and alternative therapy for the treatment of diabetes-induced wounds and the management of oxidative stress and diabetes.

Keywords: *Acacia auriculiformis* Benth., cytotoxicity, *in vitro* antidiabetic activity, LC-MS/MS, methanolic extract of *Acacia auriculiformis* leaves, streptozotocin-induced diabetes, type 2 diabetic wound healing

SUMMARY

- Various leaves extract of *Acacia auriculiformis* Benth. were investigated for *in vitro* antidiabetic and *in vivo* diabetic wound-healing activity on rats
- The pharmacological activities were supported by acute toxicity and cytotoxicity studies
- The LC-MS/MS analysis was performed to know the bioactive compounds present in the methanolic extract
- The methanolic leaves extract exhibited significant results in overall pharmacological evaluations.



Abbreviations used: *A. auriculiformis: Acacia auriculiformis*; ECM: Extracellular matrix; p-NPG: 4-Nitrophenyl-β-D-glucopyranoside; STZ: Streptozotocin; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; CNS: Central nervous system; OECD: Organization for Economic Co-operation and Development; g: Gram; mg: Milligram; ml: Milliliter; mM: Millimolar; nm: Nanometer; g: Gram; cm^2: Centimeter square; h: Hour; µL: Microliter; M: Molar; SEM: Standard error of the mean; ANOVA: Analysis of variance.

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INTRODUCTION

Chronic wounds are one of the most frequently reported diabetic complications that often lead to amputation in patients.[1] The everlasting stimulation of oxidative stress, swelling, and sepsis generated by the hyperglycemic microenvironment at the wound spot traps chronic nonhealing diabetic wounds. It also results in deferred reepithelialization, inadequate vascular bruises, and extracellular matrix (ECM) fusion of diabetic wounds, along with impaired growth factor expression.[2] Modern treatments approach offer reasonable control over glycemic levels, although they are limited to prevent complications. Several drugs such

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as insulin, metformin, specific sulfonylureas, thiazolidinedione, and DPP-4 inhibitors that have been approved for the management of type 2 diabetes are associated with unwanted clinical side effects.[3] Irrespective of their therapeutic effects, side effects and nonhealing wound effects limit its application. The mechanism of wound healing comprises the coordination of different cells, growth factors, and cytokines. Therefore, an intense strategy for dealing with diabetic wounds by antioxidative stress, anti-inflammatory approach, or else antibacterial approach is of great significance.[4]

Phytochemicals have tremendous potential for managing and treating diabetic wounds beyond conventional medical therapies as a good alternative. Phytochemicals to facilitate blood clotting, fight against infections, and improve wound healing promote antimicrobial, antioxidants and wound healing.[5-7] It is reported that medicinal plants rich in polyphenols have remarkable antioxidant activity. Phenolics encourage wound healing primarily because of their astringent, antimicrobial, and free radical scavenging effects. Eventually, polyphenolic constituents such as flavonoids can enhance wound-healing properties, apparently through antimicrobial and antioxidant features. By inhibiting lipid peroxidation, cell damage prevention gets promoted and viability of collagen fibrils gets increased.[8]

Acacia auriculiformis A. Cunn. Ex Benth., often called "Australian acacia," a member of *Fabaceae* family, is a perennial shrub which is widely dispersed all over India and other parts of the world. The Australian acacia shrub is an ornamental shade tree with greenish-white–shaded clustered flowers usually seen in parks and roadsides. The phytochemical investigations disclose tannins, flavonoids, anthocyanidins, carbohydrates, and triterpenoid saponin glycosides in the plant extracts.[9] This acacia tree was traditionally used as a remedy for the cure of sore eyes, rheumatism, itching, allergy, and aches.[10,11] Other species of *Fabaceae* including *A. auriculiformis* have been scientifically confirmed for wound-healing ability. The various phytochemical extracts and phytoconstituents from *A. auriculiformis* tree parts have been investigated for different pharmacological activities such as spermicidal,[12] antidiabetic activity,[13] hepatoprotective,[13] antioxidant,[14] antimutagenic,[15] cestocidal,[16] central nervous system-depressant activity,[15,17] antifilarial,[18] antimalarial,[19] antimicrobial,[20] and chemopreventive.[15] Based on ethnopharmacological evidence on *A. auriculiformis* shrub and related species, the present investigation was targeted to validate the wound-healing potentials of *A. auriculiformis* in diabetic rats.

MATERIALS AND METHODS

Chemicals

All the chemicals including solvents and buffers used for the extraction (petroleum ether, chloroform, ethyl acetate, acetone, butanol, and methanol), *in vitro* (α-amylase, α-glucosidase, and 4-nitrophenyl-a-D-glucopyranoside [p-NPG]), and *in vivo* study (streptozotocin [STZ], nicotinamide) were of analytical class procured from Sigma-Aldrich.

Extraction, fractionation, and phytochemical screening of *Acacia auriculiformis* leaves

In September 2015, leaves were collected from Mesra, Ranchi, Jharkhand (India) region. The satellite image of plant collection site (Obtained from ArcGIS software Version 10.7) is shown in Figure 1. It was further identified and authenticated by Dr. V. P. Prasad (Senior Principal Scientist-E and HoO) and with specimen voucher (CNH/Tech. II/2015/42/32111). A voucher specimen (CNH/Tech.II/2015/42/32111) has been deposited in the herbarium of the Botanical Survey of India, Kolkata, India. Different extracts (petroleum ether, chloroform, ethyl acetate, acetone, butanol, and methanol) were prepared as per previously reported method of maceration. Thereafter, all the extracts were dried under Vacuum for lyophilization. All extracts were stored in the dark and under refrigeration.

Different leaves extract of *A. auriculiformis* were further screened to know the existence of secondary metabolites such as alkaloids, glycosides, flavonoids, tannins, steroids, and saponins through the procedure depicted by Rangra *et al.*[21]

Total phenolic content

The phenolics in the various leaf extracts were determined using Folin-Ciocalteu reagent spectrophotometric method.[22] Samples dissolved in methanol (1 mg/mL, 500 µL, three replicates) were placed into the test tubes containing 2.5 mL of 10% Folin-Ciocalteu reagent (prepared in distilled water) and 2.5 mL of 7.5% $NaHCO_3$. The blank solution used was 0.5 mL methanol, 2.5 mL of 10% Folin-Ciocalteu reagent distilled in water, and 2.5 mL of 7.5% of $NaHCO_3$. Thereafter, samples were incubated in a thermostat at 45°C for 45 min, and the absorbance was measured using a spectrophotometer (Shimadzu 2450, Japan) at 765 nm. The total phenolic content was proclaimed as gallic acid equivalents (GAE) in gram per kg dry leaves material.

Total flavonoid content

The total flavonoid content in the leaves extract was examined by spectrophotometric method.[22] Samples were prepared by dissolving various leaf extracts in methanol solution having a concentration of 1 mg/ml. Thereafter, 1 ml each of the extracts and 2% of the $AlCl_3$ solution was taken in a test tube, and methanol was added to it. The sample containing test tube was shaken well and incubated at room temperature for an hour. The absorbance was observed at 415 nm using spectrophotometer (Shimadzu 2450, Japan).

In vitro antidiabetic assay

α-Amylase inhibition assay

In vitro α-amylase inhibition assay was executed as per the method depicted by Bernfeld.[23] The leaves test extract (100 µL) having concentration 2, 4, 6, 8, and 20 mg/mL were permitted to contact with 200 µL of the α-amylase enzyme (Sigma-Aldrich) and 100 µL of 2 mM of phosphate buffer having pH 6.9. The acarbose was utilized as a positive control standard. The mixture was incubated for 20 min, and then, the starch solution (100 µL) having concentration 1% was added. The control was prepared by the procedure followed in the previous step. In this preparation, 200 µL of buffer was used instead of the enzyme. The resultant solution was incubated for 5 min at room temperature; thereafter, dinitro salicylic acid (DNS) reagent was added to both control and test solutions. After that, both the solutions were placed on a water bath for 5 min. Later absorbance was measured at 540 nm with spectrophotometer (Shimadzu 2450, Japan) and percentage α-amylase enzyme inhibition of test and control was determined using the equation (i):

$$\%\alpha - \text{Amylase Inhibition} = 100\left(\frac{\text{Control - Test}}{\text{Control}}\right) \text{(i)}$$

α-Glucosidase inhibition assay

The α-glucosidase inhibition assay of the leaves test extract of *A. auriculiformis* was carried out using the method narrated by Dinparast *et al.*[24] with some slight alterations. The solution of α-glucosidase enzyme (20 mL of 0.5 U/mL) was mixed with potassium phosphate

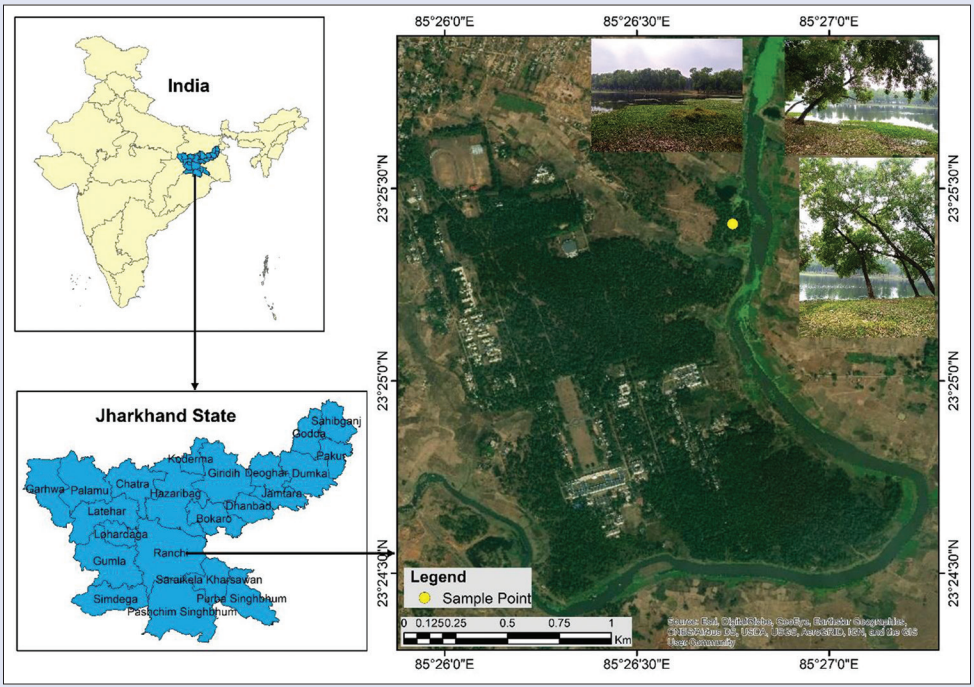

Figure 1: Satellite image of plant collection site (Obtained from ArcGIS software Version 10.7)

buffer (120 mL of 100 mM) at pH 6.9. Thereafter, to this reaction mixture, the test extract sample (10 µL) having concentrations 2, 4, 6, 8, and 20 mg/mL was put in and incubated for 15 min at 37°C. The acarbose was used as a standard positive control in this assay. The enzymatic reaction was started by the addition of 20 mL of 5 mM p-NPG in buffer solution and incubated the plates again for 15 min at 37°C. Thereafter, the termination of the reaction was done by adding sodium carbonate solution (80 mL of 0.2 M). The absorbance of 4-nitrophenyl liberated from p-NPG was determined at wavelength 405 nm. The solution mixture without α-glucosidase enzyme blank was used for adjustment of absorbance in the background. To calculate inhibitory activity, the increasing order of absorbance was observed and compared with control (buffer solution in place of the test sample). The rate of α-glucosidase enzyme inhibition was measured by applying the formula in equation (ii) as:

$$\%\alpha\text{ - Glucosidase Inhibition} = 100\left(\frac{\text{Control - Test}}{\text{Control}}\right) \text{(ii)}$$

Preparation of hydrogel formulation

The hydrogel was prepared by dissolving sodium metabisulfite, propylparaben sodium, and methylparaben in water. Further addition of carbopol 934 (a gelling agent) was done by continuously stirring until the dispersion was fully swollen. To get the stiff gel, triethanolamine was added slowly with consistent stirring. The final step was to add the test leaves extract (10%) and stir continuously for 15 min, followed by making up the volume of the mixture with water. To achieve uniformity in gel, a continuous stirring of the mixture was performed.[25,26]

Evaluation of hydrogel

pH

The pH of the hydrogel was determined with a digital pH meter (CHEMLAB-Digital PH meter). One gram of gel was dissolved in 100 mL of Millipore water.[25]

Viscosity

The viscosity of the hydrogel was measured using dial reading viscometer (Brookfield AMETEK) using spindle 4 at 0.2 g and without any dilution in the formulation.[25]

Homogeneity

The homogeneity of the prepared hydrogel was evaluated by observing the aggregate formation and physical texture or appearance.[25]

Skin irritancy test (patch test)

The hydrogel was applied to the skin (approximate area of 6 cm^2) and covered loosely with the skin by semi-occlusive dressing means using a gauze patch for 4 h. After 4 h, the patch was removed and skin was observed for visible skin reaction or irritation, erythema, intense erythema with edema, and vesicular erosion.[25]

Experimental animals

The Wistar rats (145–175 g) of either sex were used for the study. The rats were maintained at the standard experimental laboratory, animal house (conditions 25°C ± 2°C, 50% ± 15% RH, and normal photoperiod [12-h dark/12-h light]), Department of Pharmaceutical Sciences and Technology, Birla Institute of Technology, Mesra, Ranchi, Jharkhand, India. Water and commercial pellet diet were administered *ad libitum* to all the in-house rats. The experimental animal protocol was authorized by the Institutional Animal Ethics Committee (IAEC), Department of Pharmaceutical Sciences and Technology, Birla Institute of Technology, Mesra, Ranchi, Jharkhand, India, with a protocol number PROV/BIT/PH/IAEC/08/2016.

Acute toxicity study

The acute toxicity study was executed according to the Organization for Economic Co-operation and Development guidelines 425 (acute oral toxicity: up-and-down procedure).[27] The Wistar rats (150–200

g) of either sex were used for this study. The five rats were selected and fasted overnight to conduct the toxicity study. The test leaves extracts were given orally to rats up to a single dose of 2000 mg/kg body weight. The mortality, visible lesions, and abnormal behavior symptoms were studied for 14 consecutive days. No rats were found to be dead by the end of the 7th day in all the groups. Hence, the given leaves extract of plant *A. auriculiformis* were found to be innocuous up to a dose of 2000 mg/kg. Therefore, based on results obtained from acute toxicity studies and exhaustive literature search, 200 and 400 mg/kg doses were selected for *in vivo* wound-healing activity in diabetic rats.

Animal experimental protocol

The rats were categorized into four groups, and each group comprised six rats for *in vivo* wound healing in the diabetic rat study. The first group was denoted as normal control and received hydrogel base only. However, the second group was designed as wound control and was served with only a hydrogel base. The third group was marked as standard and received mupirocin. The fourth group received test extract hydrogel (applied daily) and was considered as a test. The wound-healing activity was evaluated concerning physical, biochemical, and histopathological examinations.

Induction of type 2 diabetes (streptozotocin-nicotinamide-induced diabetes rat model)

Type 2 diabetes was produced in overnight-fasted Wistar albino rats (14–175 g), by injecting the freshly processed STZ (50 mg/kg, intraperitoneally) solution in citrate buffer (0.5 M. pH 4.5). Fifteen minutes before the STZ injection, nicotinamide (NA) (120 mg/kg) dissolved in normal saline was injected intraperitoneally. Blood was withdrawn from the tail vein, and the blood glucose level was measured through a digital glucometer (Dr. Morepen Glucose Monitor BG-03). Rats showing a rise in blood glucose level (>250 mg/dL) were preferred for evaluating wound-healing potential.[25,28]

Wound-healing evaluation in diabetic rats

Excision diabetic wound model

The selected diabetic rats were anesthetized by injecting ketamine (5 mg/kg) and xylazine (50 mg/kg). An excision wound was created on the shaved depilated back of diabetic rat by cutting 100 mm^2 full thickness areas and left undressed. The percent wound contraction was determined on alternate days, and the epithelialization period was determined on the complete scar tissue removal. After that, hydroxyproline content was determined, and on the 12th day, and histopathological examination was carried out after wounding.[25]

Incision diabetic wound model

In the incision wound model, the body weight of rats (145–175 g) was carefully maintained and observed, as demonstrated in Figure 2. After that, to the anesthetized diabetic rats, two paravertebral incision wounds having 1 cm distance from the midline on each side were created at the shaved hairless back of rats. Stitching of incision wound was done with a curved needle (No. 11) and surgical thread (black silk, No. 000) by keeping together both the edges. The sutures were detached from the wound after the 3rd day of wounding, and breaking strength was calculated using continuous water flow technique.[25]

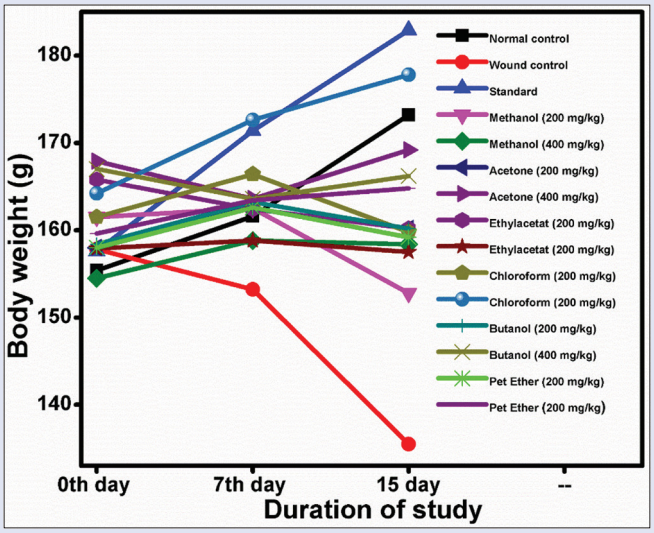

Figure 2: Body weight of rats used in diabetic incision wound model

Parameters evaluated in wound-healing activity

Percentage of wound contraction

The percentage of wound contraction was measured in 2 days with the help of a tracing paper sheet and the wound-healing rate was expressed as % wound contraction equation (iii).[28]

$$\%\text{Wound contraction} = \frac{\text{The initial day wound size - Specific day wound size}}{\text{Initial day wound size}} \times 100 \quad \text{(iii)}$$

Epithelialization period

Epithelialization period was calculated by monitoring the total number of days taken by eschar to diminish and having no wounding mark on the wound.[28]

Hydroxyproline content

On the 12th day, wound tissues were examined for hydroxyproline content (a basic collagen component).[28] Tissues were subjected to vacuum drying until a steady weight was achieved and after that hydrolyzed (6 N HCl, 130°C, 3 h) in closed tubes. The hydrolysate was then neutralized (pH 7) and then treated with chloramine-T oxidation for 20 min. The reaction was stopped by adding 0.4 M perchloric acid. After that, the color generated by the Ehlrich reagent at 60°C was observed in a UV spectrophotometer (557 nm) (Shimadzu 2450, Japan).

Breaking strength

Tensile or breaking strength of the rat skin was obtained using a continuous water flow procedure.[29] On the 3rd post-wound day, sutures of the incision wound were pulled off, and the breaking strength was calculated.

Histopathological studies

Histopathology of tissue was studied by collecting tissue samples kept in 10% formalin solution on the 12th post-wound day. The tissue sample was dehydrated with an ethanolexylene solution and filtered. Thereafter,

Table 1: Qualitative phytochemical screening results of different leaves extracts of *Acacia auriculiformis* Benth

Phytochemical tests	Leaves extracts					
	Petroleum ether	Chloroform	Ethyl acetate	n-Butanol	Acetone	Methanol
Alkaloids	++	+	-	+++	+	+
Flavonoids	-	-	+++	+++	+++	+++
Steroids	+++	++	+	+	+	+
Tannins	-	+	++	++	+++	++
Glycosides	-	+	+++	+++	++	+++
Saponins	-	+	++	+++	+++	++

+: Slightly present, ++: Moderately present, +++: Highly present, -: Absent

Table 2: Total phenolic and flavonoid content of methanol leaves extract of *Acacia auriculiformis*

Leaf extract	Total phenolic content[a,c]	Total flavonoid content[b,d]
Methanol	48±1.8[a]	45.5±2.3[b]

[a]Data expressed as mg GAE/g of leaves extract, [b]Data expressed as mg quercetin equivalent (GAE)/g of leaves extract, [c]Data expressed as µg GAE/mg of plant fraction, [d]Data expressed as µg quercetin equivalent (GAE)/mg of plant fraction. Values are means of three biological replicates. GAE: Gallic acid equivalent

tissue samples with microtome sections having a 5 mm thickness fixed with paraffin were used for the histopathological study. These microtome sections were prepared in alcoholexylene and splashed with hematoxylin-eosin dye. The histological tissues were observed in Digital Microscope (Leica DMS1000 B).[25]

In vitro cytotoxicity

The methanolic extract of *A. auriculiformis* was screened for its cytotoxicity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on the embryonic kidney cells (HEK-293), human breast cancer cells (MCF-7), and adenocarcinoma human alveolar basal epithelial cells (A549) cell lines. At a concentration of 2 × 10^6 cells/well, the cells were cultured and sown in 96-well cell culture plates (Greiner CELLSTAR® 96-well plates, Merck, South Africa). Various concentrations of methanolic extract of *A. auriculiformis* (20, 40, 60, 80, and 100 µg/mL) were preceded into the wells sown with cells. Positive control wells (with culture medium constituting only cells) and negative control wells (with culture medium beyond cells) were also incorporated. The test samples were incubated for 48 h, after which the sample-burdened medium was counterfeited by with 100 µL of fresh culture medium and 20 µL of MTT solution (5 mg/mL in PBS) in individual well. An 85 µl aliquot was removed from the wells. Thereafter, 50 µl of DMSO was added in individual well and blended simultaneously with the pipette and incubated at 37°C for 10 min. The cell viability was arbitrated at 540 nm in microplate reader (Spectrostar Nano, Germany). The absorbance spectrum was used to obtain the number of viable cells. The MTT assay is derived from the principle that particular potentiality of viable cells to lower the tetrazolium elemental part of MTT into purple-colored formed crystals.[30] The percentage cell viability was computed by utilizing the equation (iv).

$$\% \text{ Cell Viability} = (\text{A540 nm treated cells})/(\text{A540 nm untreated cells}) \times 100 \quad \text{(iv)}$$

Statistical analysis

All the *in vivo* activity outcomes were represented as mean ± standard error of the mean, and the significance of statistics was analyzed using one-way analysis of variance (ANOVA) accompanied by Dunnett's test.

LC-MS/MS analysis

The LC-MS/MS analysis was performed through Waters ACQUITY FTN with Waters Alliance 2695 HPLC Pump having Auto-Sampler, quaternary pump system, and a PDA detector type UPLC LG 500 nm. The ESI detector set at positive ionization mode was used for mass spectroscopy. The reverse-phase C_{18} column (SUNFIRE) with specifications as 250 mm × 4.6 mm × 5 µm, 3D channel range (200–450 nm), resolution (1.2 nm), and compensation reference 2D parameter range (310–410 nm) was used. The methanol leaves extract was exposed to LC-MS/MS using mobile phase as acetonitrile: formic acid in water (5:95) with the effective gradient run at a flow rate of 1.5 ml/min for 20 min of run time.

RESULTS

Phytochemical evaluation

The phytochemical tests of the leaf's extracts reported the inhabitance of glycosides, flavonoids, saponins, and tannins, as shown in Table 1.

Total phenolic and flavonoid content

The total phenolic content is the measure of amount of phenolic content, whereas thetotal flavonoid content is the measure of amount of flavonoid content present in the given sample. The methanol leaves extract of *Acacia auriculiformis* was imposed to total phenolic content and total flavonoid content assay by applying the Folin-Ciocalteu reagent and spectrophotometric method, respectively. The polar methanol leaves extract revealed remarkable results, which are summarized in Table 2.

In vitro antidiabetic assay

The leaf extracts showed significant inhibitory action against α-glucosidase and α-amylase enzymes. The ethyl acetate leaves extract showed significant %inhibition of 66.546% against α-glucosidase at a concentration of 4 µg/ml in comparison to standard acarbose (72.857). The methanolic and butanolic leaves extract showed %inhibition of 60.207 and 65.466 at 4 µg/mL concentration, respectively." On the other side, the petroleum ether, acetone, and chloroform leaves extract showed 47.682%, 30.245%, 41.643% percentage inhibition respectively. The results of these leaves extracts showed weak inhibition at 4 µg/ml, as shown in Figure 3.

However, in α-amylase assay, the butanol leaves extract showed a significant % inhibition of 64.266% as compared to standard acarbose (62.857%) at the same concentration of 4 µg/mL. The methanol, ethyl acetate, and chloroform leave extracts showed %inhibition of 50.20, 63.54, and 51.64, respectively, at the same concentration. The petroleum ether (43.682%) and acetone (30.043%) leaves extract showed reduced inhibition at 4 µg/mL [Figure 3].

Table 3: Evaluation parameters of hydrogel formulation

Sample number	Hydrogel of extract	Parameters			
		pH	Viscosity (Pa.s)	Homogeneity	Skin irritancy test
1	Methanol	4.43±0.05	2.794	Good	Negative
2	Acetone	3.99±0.05	2.892	Good	Negative
3	Ethyl acetate	4.21±0.05	2.972	Good	Negative
4	Chloroform	3.79±0.05	2.688	Good	Negative
5	Butanol	4.40±0.05	2.894	Good	Negative
6	Petroleum ether	4.12±0.05	2.942	Good	Negative

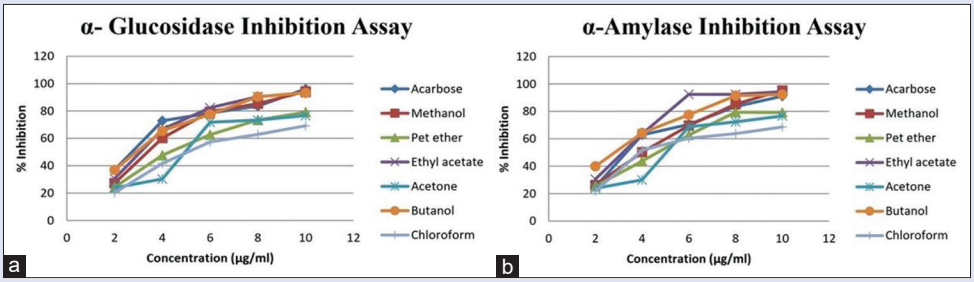

Figure 3: The percentage inhibition shown by different leaves extract of *Acacia auriculiformis* in (a) α-glucosidase and (b) α-amylase *in vitro* antidiabetic assay

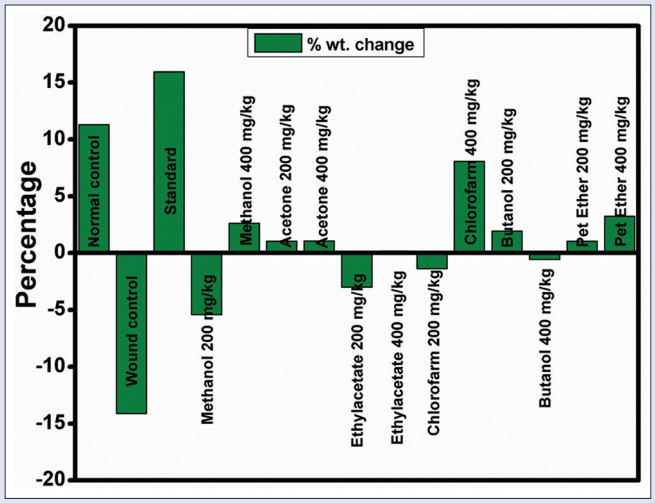

Figure 4: Changes in the body weight of animals in incision wound model

Evaluation of hydrogel

The hydrogel formulations of test leave extracts were evaluated for various physicochemical parameters such as pH, viscosity, homogeneity, and physical appearance or skin irritancy test or patch test, as given in Table 3.

Acute toxicity

The acute toxicity study of leaves extracts was tested on rats for 14 days. Outcomes of the results showed no signs of physical lesions and abnormal behavior. At the end of the 7th day, no rats showed mortality signs. The leaves extract of *A. auriculiformis* Benth. were found to be non-toxic up to the dose of 2000 mg/kg. Based on the above study and literature reports, the dose of 200 and 400 mg/kg body weight of the rats was selected for the further desired pharmacological study.

Evaluation of wound-healing activity in diabetic rat parameters

Blood glucose level (using streptozotocin-induced diabetes model)

The blood glucose level of overnight-fastened rats in bodyweight range of 145–175 g was determined using a glucometer before wound creation. After 2 days of STZ injection, rats consistently showed elevated blood glucose levels (≥250 mg/dl), and it was selected for wound creation [Table 4]. The changes in the bodyweight of animals were also observed, as shown in Figure 4.

Percentage of wound contraction

In the present experiment, the hydrogel formulation of test leaves extracts topically applied on excision-induced wound in diabetic rats. Results depicted that, there is a notable ($P < 0.05$) increase in percentage rate wound contraction on the 15th day of given treatment as compared to normal control. A significant rise in the wound surface area with increment in a time-dependent manner was observed in methanol and ethyl acetate leaves extracts at a dose of 400 mg/kg. The normal control also depicted significant ($P < 0.05$) results in comparison to diabetic wound control, which displayed deprived wound healing on the 16th post-wound day [Table 5].

Weight of dry granulation tissue

The granulation tissue primarily contains collagen, fibroblasts, edema, and small new blood vessels. It is developed in the final stage of the proliferative phase. The elevation in weight of dry granulation tissue in test formulations and standard treated rats indicates high protein content, and it supports the effectiveness of the given test extracts [Table 6 and Figure 5].

Epithelialization period

The epithelialization period exhibited significant results ($P < 0.05$) in hydrogel formulation of methanol leaves extract and normal wound control-treated rats as compared to diabetic wound control which showed deprived epithelialization period, as given in Table 7.

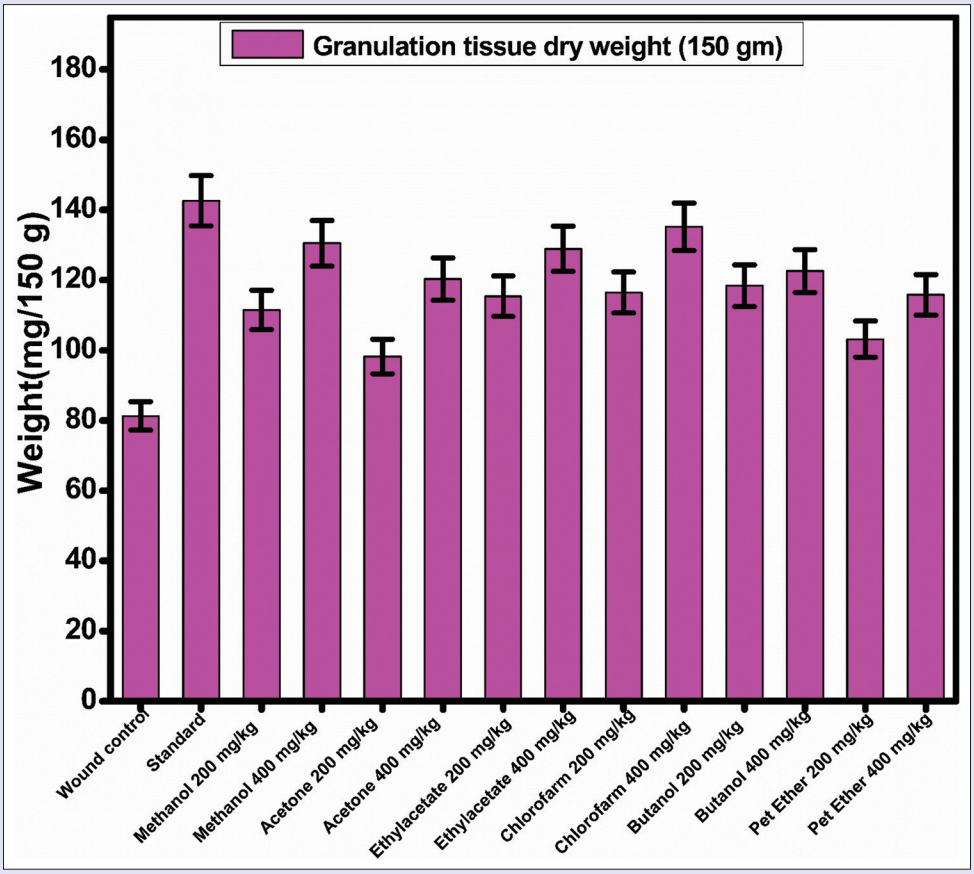

Figure 5: Effect of different extract of *Acacia auriculiformis* on granulation tissue dry weight (mg/150 g) in excision wound model

Table 4: Blood glucose level (using streptozotocin-induced diabetes model)

Groups/treatment (mg/kg)	0th day	7th day	15th day
Normal control	85.6±3.15	92.4±4.42	85.6±3.31
Wound control	270.8±4.45	298.6±5.14$^{a,\#}$	310.4±6.12$^{a,\#}$
Standard	268.6±3.73	148.2±3.16$^{b,\#}$	125.9±2.44$^{a,*,b,\#}$
Methanol (200)	261.5±3.03	186.5±2.37$^{b,\#}$	152.4±3.16$^{b,\#}$
Methanol (400)	263.5±4.44	178.2±3.38$^{b,\#}$	143.4±4.24$^{b,\#}$
Acetone (200)	288.0±4.57	213.2±4.11$^{b,\wedge}$	180.2±4.06$^{b,\#}$
Acetone (400)	280.2±3.52	190.6±3.30$^{b,\#}$	152.2±3.16$^{b,\#}$
Ethyl acetate (200)	275.8±3.23	192.4±2.23$^{b,\#}$	171.2±3.56$^{b,\#}$
Ethyl acetate (400)	258.9±3.07	192.6±4.49$^{b,\#}$	170.5±2.63$^{b,\#}$
Chloroform (200)	266.5±3.22	186.4±3.22$^{b,\#}$	159.8±3.88$^{b,\#}$
Chloroform (400)	265.2±4.29	180.6±4.88$^{b,\#}$	152.8±3.11$^{b,\#}$
Butanol (200)	278.4±2.57	193.2±3.11$^{b,\#}$	162.2±3.80$^{b,\#}$
Butanol (400)	272.6±5.03	185.6±5.30$^{b,\#}$	160.2±3.09$^{b,\#}$
Petroleum ether (200)	273.1±2.13	189.6±3.45$^{b,\#}$	169.2±4.98$^{b,\#}$
Petroleum ether (400)	275.6±5.10	208.4±6.06$^{b,\#}$	178.8±4.22$^{b,\#}$

*P<0.05, ^P<0.01, #P<0.001. Values are represented as mean±SEM (n=6). In statistical analysis, P<0.05 was considered to be significant. [a]Versus normal control, [b]Versus wound control. SEM: Standard error of mean

Hydroxyproline content

The hydroxyproline content found in the hydrogel of test of leaves extract-, normal wound control-, and diabetic wound control-treated wound tissue is given in Table 7. The hydrogel formulation of methanol leaves extract-treated diabetic rats and normal wound control-treated rats exhibited significant results (P < 0.05) in comparison to diabetic wound control-treated rats.

Breaking strength

The hydrogel of methanol leaves extract-treated rats and normal wound control-treated rats demonstrated significantly (P < 0.05) good breaking strength in comparison to diabetic wound control-treated rats, which depicted poor breaking strength [Table 8 and Figure 6]. Different extracts were found effective and showed improved breaking strength in an incision wound model. The comparative effectiveness of different extracts was found as methanol > acetone > ethyl acetate > chloroform > butanol > petroleum ether.

Histopathological studies

On the 15th day, a skin tissue sample from each rat was isolated and stained with eosin and hematoxylin. It was kept in 10% of formalin solution. The stained tissue sections were qualitatively analyzed and observed for cell functioning features such as edema, congestion, necrosis, infiltration of polymorphonuclear leukocytes and monocytes, collagen formation, fibroblast proliferation, epithelialization, and angiogenesis under the light microscope. The histopathological evaluation of the tissue extracted from the test extract-treated rats revealed epithelial cells with high collagen contents. However, in the diabetic- and normal control-treated rat's tissue sample showed less composition of fibroblasts and macrophages [Figure 7].

LC-MS/MS analysis

The bioactive methanol fraction was subjected to LC-MS/MS [Figure 8] with an effective gradient run for the identification of bioactive compounds present. The fragmentation pattern of the

Table 5: Percentage contraction in wound area in excision wound-healing model

Groups/treatment (mg/kg)	Percentage contraction in wound area		
	0th day	7th day	15th day
Normal control	-	43.8±1.16	80.4±2.01
Wound control	-	22.5±2.15	58.5±2.12[a,*,c,^]
Standard (mupirocin)	-	56.4±1.16	91.2±1.54[b,^,c,^]
Methanol (200)	-	36.4±1.37	72.4±2.16[b,*,c,*]
Methanol (400)	-	38.6±1.75	78.5±1.24[b,*,c,*]
Acetone (200)	-	33.2±2.10	70.2±2.06[c,*]
Acetone (400)	-	39.2±1.51	75.2±1.25[b,*,c,*]
Ethyl acetate (200)	-	32.4±1.23	71.2±1.56[b,*,c,^]
Ethyl acetate (400)	-	33.6±1.30	82.4±1.30[b,^,c,^]
Chloroform (200)	-	26.2±1.22	79.2±1.08[b,*,c,^]
Chloroform (400)	-	30.6±1.08	72.8±1.11[b,*,c,^]
Butanol (200)	-	29.2±1.18	62.2±1.80[c,*]
Butanol (400)	-	25.6±1.30	68.2±1.19[c,^]
Petroleum ether (200)	-	28.1±2.15	69.2±1.98[c,^]
Petroleum ether (400)	-	28.4±2.06	70.8±1.42[b,*,c]

Compared wound control group and treatment groups ([a]Versus normal control; [b]Versus wound control; **P*<0.05, ^*P*<0.01, #*P*<0.001). Compared 7th day and 15th day ([c]Versus 7th day percentage contraction in wound area, **P*<0.05, ^*P*<0.01, #*P*<0.001)

Table 6: Granulation tissue dry weight in excision wound-healing model

Groups/treatment (mg/kg)	Granulation tissue dry weight (mg/150 g)
Normal control	-
Wound control	81.3±3.07
Standard	142.6±2.87[b,#]
Methanol (200)	111.5±2.83[b,*]
Methanol (400)	130.5±3.09[b,#]
Acetone (200)	98.2±1.57
Acetone (400)	120.3±2.42[b,^]
Ethyl acetate (200)	115.4±2.13[b,*]
Ethyl acetate (400)	128.9±2.27[b,^]
Chloroform (200)	116.5±2.80[b,*]
Chloroform (400)	135.2±2.29[b,#]
Butanol (200)	118.4±2.67[b,^]
Butanol (400)	122.6±3.22[b,^]
Petroleum ether (200)	103.2±2.83[b,*]
Petroleum ether (400)	115.8±3.11[b,*]

[a]Versus normal control, [b]Versus wound control. In statistical analysis, *P*<0.05 was considered to be significant.**P*<0.05, ^*P*<0.01, #*P*<0.001. Values are represented as mean±SEM (*n*=6). SEM: Standard error of mean

Table 7: Epithelialization period and hydroxyproline content observed in wound tissue treated with various leaves extracts

Treatment groups (mg/kg)	Epithelialization period (mean time of day)	Hydroxyproline content (mg/g tissue)
Normal control	8.3±0	0.127±0.005
Wound control	15.5±1.1	0.071±1[a,#]
Standard	17.2±1.3[b,#]	0.139±0.52[a,#,b,#]
Methanol (200)	11±1.3[b,*]	106.20±0.001[a,^,b,#]
Methanol (400)	14±2.3[b,#]	0.235±0.00[a,^,b,#]
Acetone (200)	6.1±1.1[b,^]	0.019±0[a,^,b,#]
Acetone (400)	5.7±1.1[b,^]	0.132±0.005[a,^,b,#]
Ethyl acetate (200)	10.3±0[b,*]	0.045±0.004[a,^,b,#]
Ethyl acetate (400)	11.2±1.5[b,^]	0.129±1.001[a,^,b,#]
Chloroform (200)	8.1±3.2[b,*]	0.096±1[a,^,b,#]
Chloroform (400)	11±1.2[b,#]	0.009±0.001[a,^,b,#]
Butanol (200)	9.1±2[b,^]	0.111±0.002[b,^]
Butanol (400)	10±1.5[b,^]	0.122±0.005[b,^]
Petroleum ether (200)	9.4±0[b,*]	0.091±1.1[b,^]
Petroleum ether (400)	11.7±0[b,*]	0.095±0.001[b,^]

[a]Versus normal control, [b]Versus wound control, **P*<0.05, ^*P*<0.01, #*P*<0.001. Values are represented as mean±SEM (*n*=6). In statistical analysis, *P*<0.05 was considered to be significant. SEM: Standard error of mean

Table 8: Breaking strength observed in excision wound healing model

Group (mg/kg)	Breaking strength
Normal control	240.5±3.86
Wound control	213.4±7.05[a,#]
Standard	317.1±4.55[a,#,b,#]
Methanol (200)	293.8±6.60[a,^,b,#]
Methanol (400)	302.6±5.58[a,#,b,#]
Acetone (200)	290.2±8.57[a,^,b,#]
Acetone (400)	295.7±6.44[a,^,b,#]
Ethyl acetate (200)	280.8±6.90[a,*,b,#]
Ethyl acetate (400)	288.1±7.39[a,^,b,#]
Chloroform (200)	275.2±5.37[a,*,b,^]
Chloroform (400)	287.7±6.23[a,^,b,#]
Butanol (200)	260.8±7.43[b,^]
Butanol (400)	261.2±8.41[b,^]
Petroleum ether (200)	259.3±7.36[b,^]
Petroleum ether (400)	262.9±6.58[b,^]

[a]Versus normal control, [b]Versus wound control, **P*<0.05, ^*P*<0.01, #*P*<0.001. Values are represented as mean±SEM (*n*=6). In statistical analysis, *P*<0.05 was considered to be significant. SEM: Standard error of mean

compound (β-sitosterol) at retention time 9.414 min is shown in Figure 9. The MS result showed molecular ion peak 414 [M + H] + which is comparable to the molecular weight of β-sitosterol (413 amu) reported earlier. The base peak was found to be at 413 (*m/z*). The resulting fragment peaks are appearing as follows (*m/z*) 396 [M + H-H_2O], 381 [M + H-H_2O-CH_3], 354 [M + H-H_2O-CH_3-CH-CH_2], 329 [M + H-H_2O-CH_3-CH-CH_2-C-CH], 303 [M + H-H_2O-CH_3-CH-CH_2-C-CH-CH-CH], 288 [M + H-H_2O-CH_3-CH-CH_2-C-CH-CH-CH-CH_3], 273 [M + H-H_2O-CH_3-CH-CH_2-C-CH-CH-CH-CH_3-CH_3], 255 [M + H-H_2O-CH_3-CH-CH_2-C-CH-CH-CH-CH_3-CH_3-H_2O], 213 [M + H-H_2O-CH_3-CH-CH_2-C-CH-CH-CH-CH_3-CH_3-H_2O-CH_2-CH_2], 199 [M + H-H_2O-CH_3-CH-CH_2-C-CH-CH-CH-CH_3-CH_3-H_2O-CH_2-CH_2-CH_2-CH_2], 173 [M + H-H_2O-CH_3-CH-CH_2-C-CH-CH-CH-CH_3-CH_3-H_2O-CH_2-CH_2-CH_2-CH_2-CH-CH], 159 [M + H-H_2O-CH_3-CH-CH_2-C-CH-CH-CH-CH_3-CH_3-H_2O-CH_2-CH_2-CH_2-CH_2-CH-CH_2].

The fragmentation pattern of compound (lupeol) at retention time 22.556 min is shown in Figure 10. The MS result showed molecular ion peak 426 [M + H] + which is comparable to the molecular weight of lupeol (426 amu) reported earlier. The base peak was found to be at 426 (*m/z*). The resulting fragment peaks are appearing as follows (*m/z*) 411[M + H-CH_3], 393[M + H-CH_3-H_2O], 344 [M + H-CH_3-H_2O-CH-C-C-C], 315 [M + H-CH_3-H_2O-CH-C-C-C-CH_2-CH_3], 272 [M + H-CH_3-H_2O-CH-C-C-C-CH_2-CH_3-CH_2-CH_2-CH_3], 257 [M + H-CH_3-H_2O-CH-C-C-C-CH$_2$-CH_3-CH_2-CH_2-CH_3-CH_3], 218 [M + H-CH_3-H_2O-CH-C-C-C-CH_2-CH_3-CH_2-CH_2-CH_3-CH_3-CH-CH-CH], 189 [M + H-CH_3-H_2O-CH-C-C-C-CH$_2$-CH_3-CH_2-CH_2-CH_3-CH_3-CH-CH-CH-CH_2-CH_3], 175[M + H-CH_3-H_2O-CH-C-C-C-CH_2-CH_3-CH_2-CH_2-CH_3-CH_3-CH-CH-CH-CH_2-CH_3-CH_2].

The fragmentation pattern of compound (stigmasterol) at retention time 10.605 min is shown in Figure 11. The MS result showed molecular ion peak 413 [M + H] $^+$ which is comparable to the molecular weight of stigmasterol (412 amu) reported earlier. The base peak was found to be at 413 (*m/z*). The resulting fragment peaks are appearing as follows (*m/z*) 395 [M + H-H_2O], 311 [M + H-H_2O-CH_2-CH_3-CH-CH-CH_2-CH_3], 297 [M + H-H_2O-CH_2-CH_3-CH-CH-CH_2-CH_3-CH_2], 283 [M + H-H_2O-CH_2-CH_3-CH-CH-CH_2-CH_3-CH_2-CH_2], 268 [M + H-H_2O-CH_2-CH-CH-CH_2-CH_3-CH_2-CH_2-CH_3], 255 [M + H-H_2O-CH_2-CH_3-C

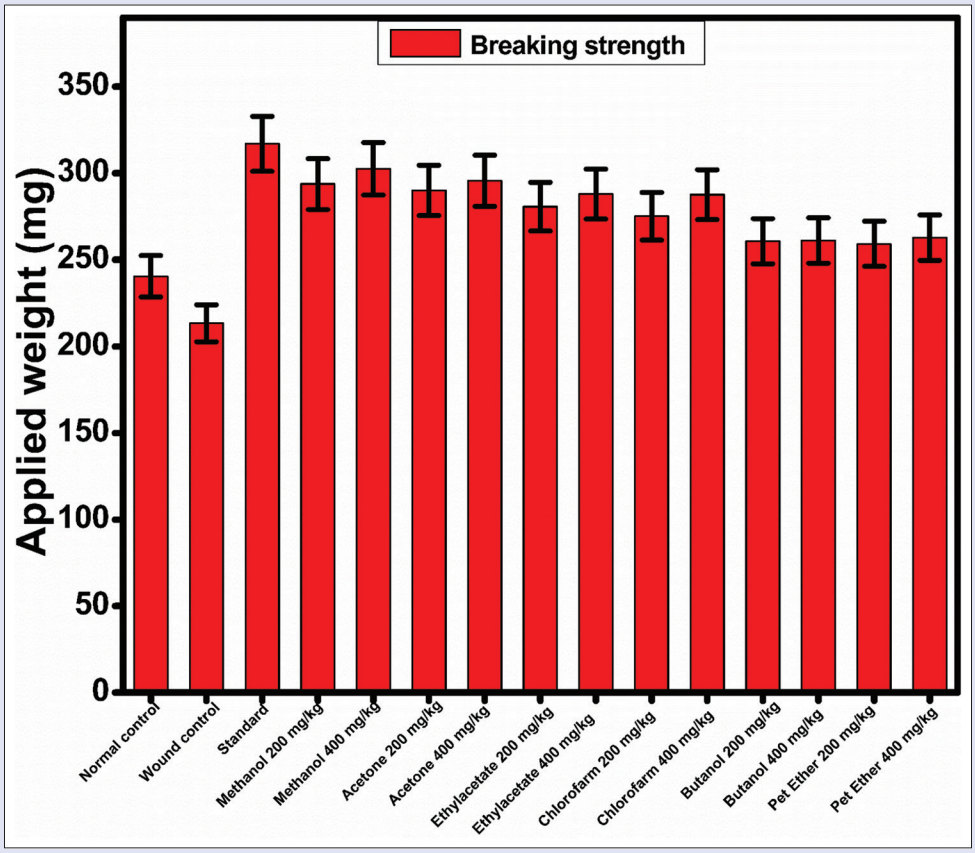

Figure 6: Effect of different extract of *Acacia auriculiformis* on the breaking strength in incision wound model

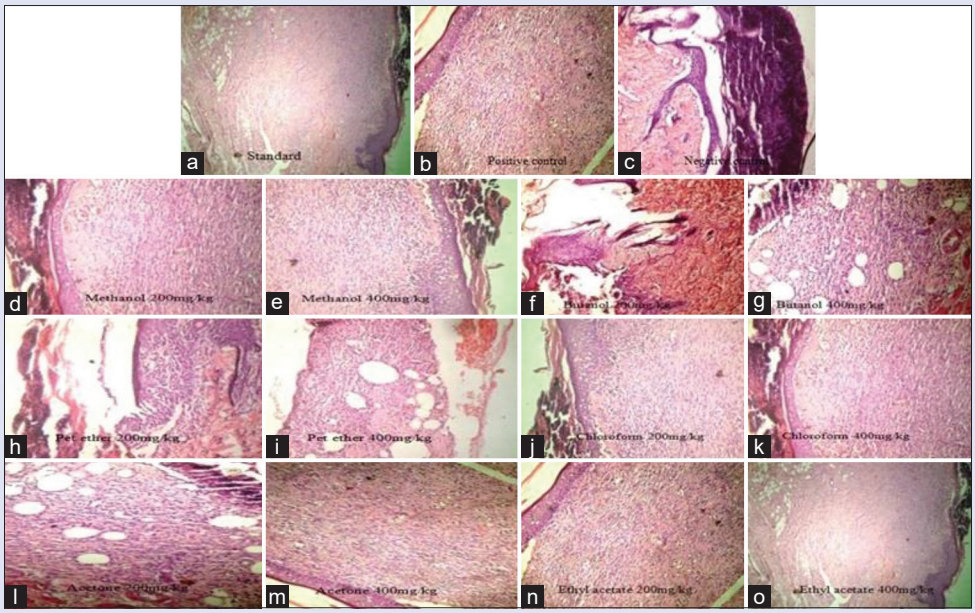

Figure 7: Histological images of the tissue obtained from the group treated with (a) positive control, (b) negative control, (c) standard drug, (d and e) methanol extracts (200 mg/kg and 400 mg/kg), (f and g) butanol extracts (200 mg/kg and 400 mg/kg), (h and i) petroleum ether (200 mg/kg and 400 mg/kg), (j and k) chloroform extracts (200 mg/kg and 400 mg/kg), (l and m) acetone (200 mg/kg and 400 mg/kg), and (n and o) ethyl acetate extracts (200 mg/kg and 400 mg/kg). The significant rise in macrophages, fibroblasts, collagen deposition, and tissue edema was observed in methanol-treated group as compared to negative control-treated group

H-CH-CH_2-CH_3-CH_2-CH_2-CH_3-CH], 241 [M + H-H_2O-CH_2-CH_3-C H-CH-CH_2-CH_3-CH_2-CH_2-CH_3-CH-CH_2], 227 [M + H-H_2O-CH_2-C H_3-CH-CH-CH_2-CH_3-CH_2-CH_2-CH_3-CH-CH_2-CH_2], 215 [M + H-H $_2O$-CH_2-CH_3-CH-CH-CH_2-CH_3-CH_2-CH_2-CH_3-CH-CH_2-CH_2-C],

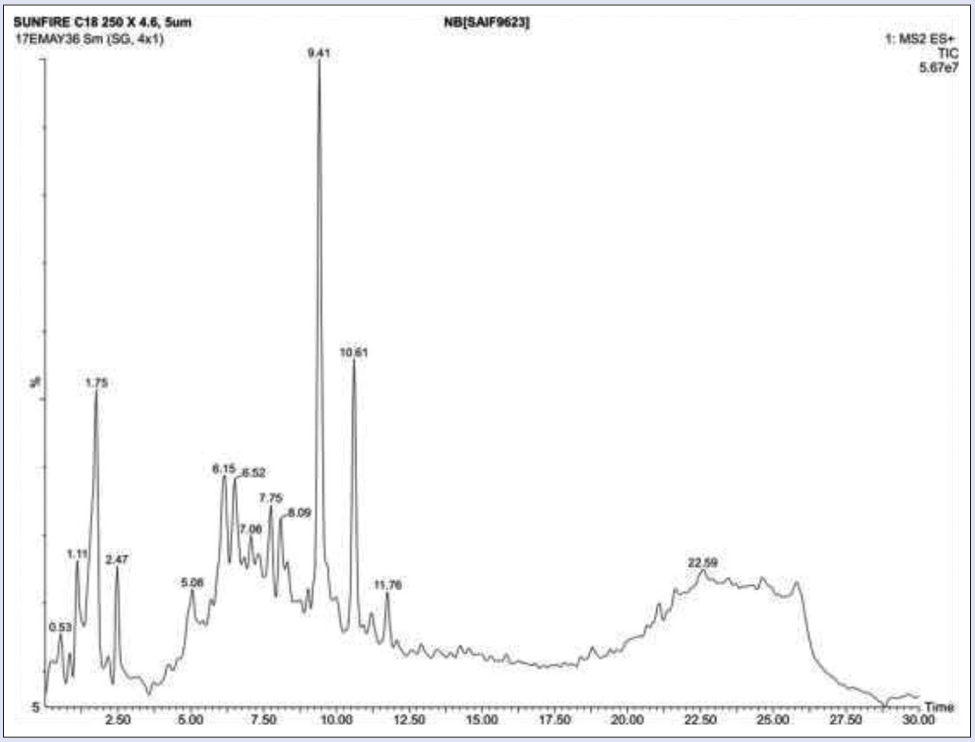

Figure 8: LC-MS/MS profile of methanolic extract of *Acacia auriculiformis* leaves

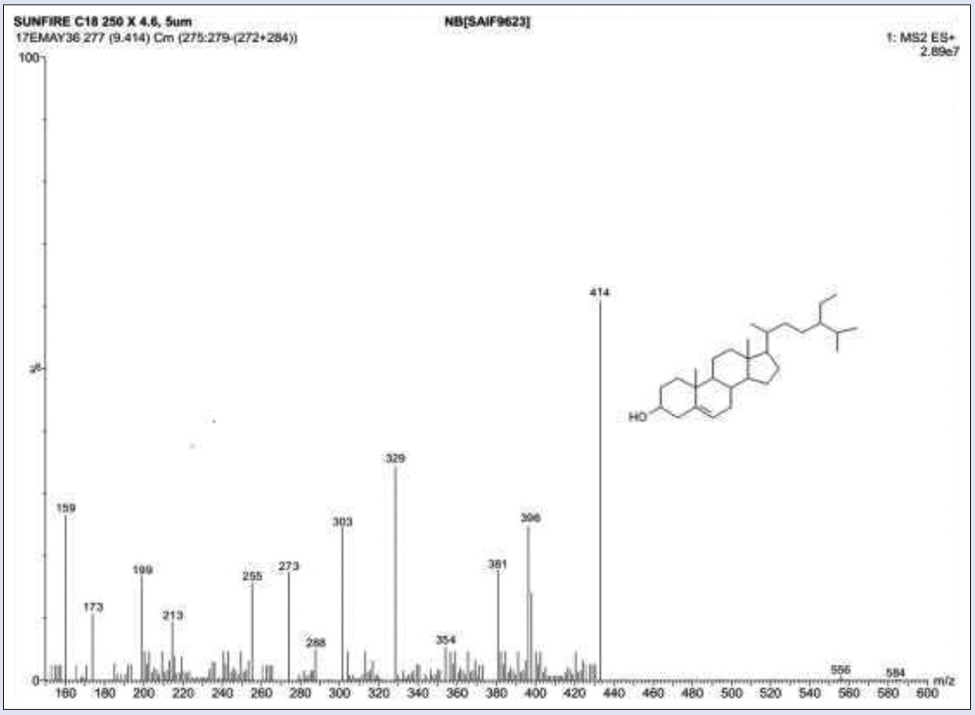

Figure 9: Mass fragmentation pattern of beta-sitosterol

201 [M + H-H_2O-CH_2-CH_3-CH-CH-CH_2-CH_3-CH_2-CH_2-CH_3-CH-CH_2-CH_2-C-CH_2], 186 [M + H-H_2O-CH_2-CH_3-CH-CH-CH_2-CH_3-CH_2-CH_2-CH_3-CH-CH_2-CH_2-C-CH_2-CH_3], 173 [M + H-H_2O-CH_2-CH_3-CH-CH-CH_2-CH_3-CH_2-CH_2-CH_3-CH-CH_2-CH_2-C-CH_2-CH_3-CH].

The fragmentation pattern of compound (quercetin) at retention time 8.086 min is shown in Figure 12. The MS result showed molecular ion peak 303 $[M + H]^+$ which is comparable to the molecular weight of quercetin (302.236 amu) reported earlier. The detailed fragmentation pattern of quercetin is given in Figure 13.

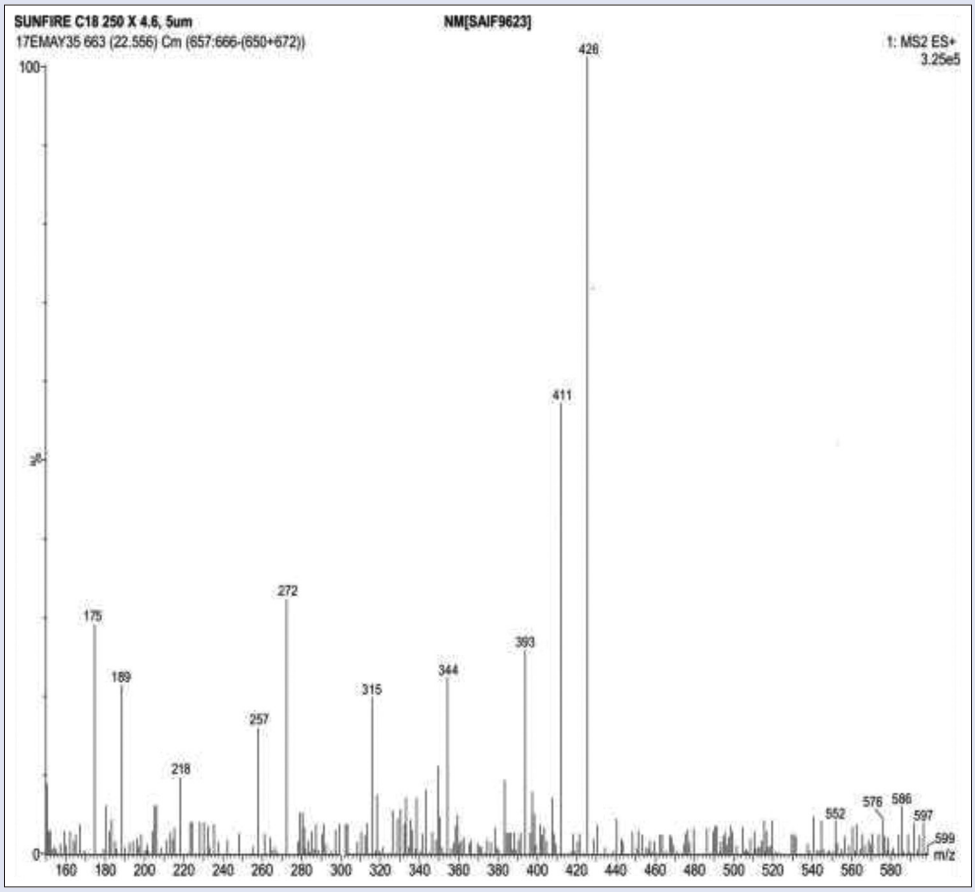

Figure 10: Mass fragmentation pattern of lupeol

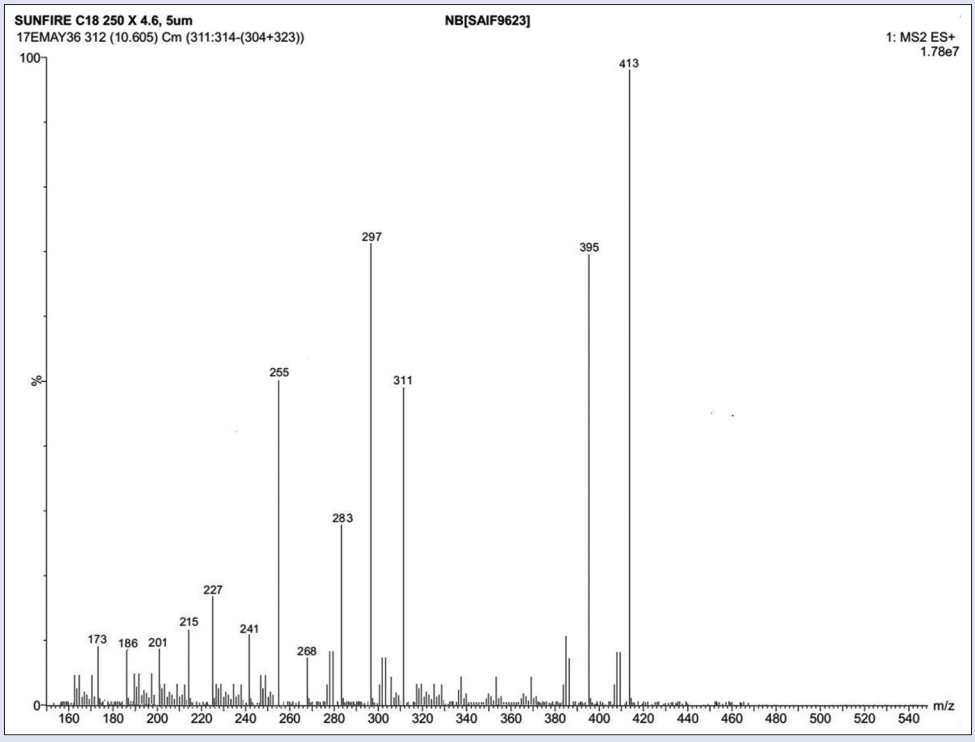

Figure 11: Mass fragmentation pattern of stigmasterol

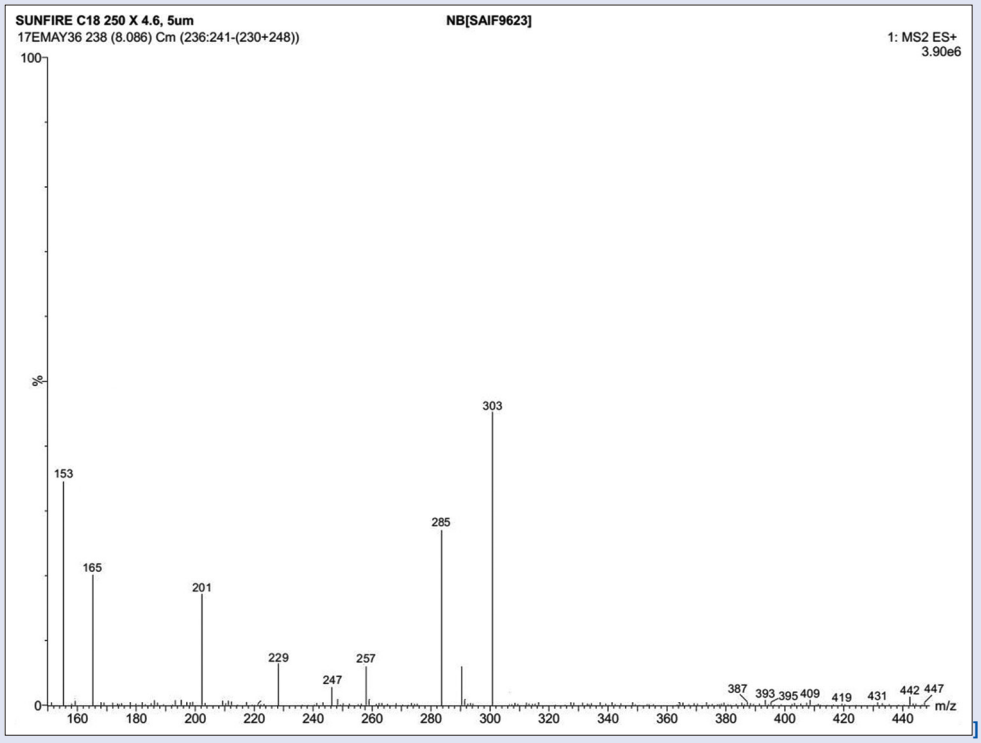

Figure 12: Mass fragmentation pattern of quercetin

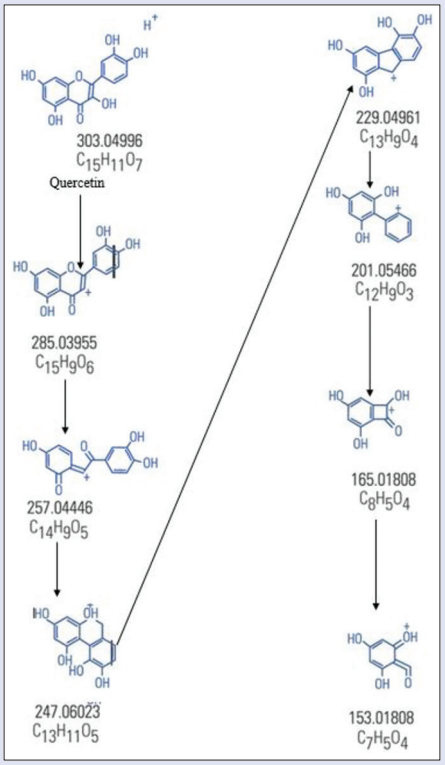

Figure 13: The detailed fragmentation pattern of quercetin

DISCUSSION

Our findings demonstrated that methanolic extract of leaves was successfully able to protect wound healing in diabetic-induced rats. In view of the heterogeneous pathological state at the diabetic wound spot, the impacts of most of the therapies are limited. Inflammation, infection, and hyperoxidative stress level on the wound sites in the hyperglycemic microenvironment have been reported to cause nonhealing wounds in diabetes. To overcome the pathological symptoms, there is a need to alleviate the oxidative stress, inflammation, and infection at the wound spot will be an efficient approach for diabetic wound healing. Methanolic extract of *A. auriculiformis* was proved to be a potent alternative for wound healing in diabetes due to its antioxidant, anti-inflammatory, and antibacterial efficiencies.

In the present study, *A. auriculiformis* extract was selected as an active constituent for highly potent diabetic wound healing and is also effective in diabetes management also.[13] STZ-induced diabetes model is the universally accepted model to evaluate diabetes mellitus. Therefore, diabetic rats induced with streptozotocin were used in this research for wound-healing assessment.

The outcomes of the present study disclosed that the topical application of the hydrogel of various leaf extracts on wounds in STZ-induced diabetes treated rats accelerated wound-healing process and decreased epithelialization tenure. The phytochemical screening results reported the presence of phytoconstituents such as glycosides, flavonoids, tannins, and saponins which are in aligned with the previous reported result to elevate wound-healing process.[31] The antioxidant and anti-inflammatory activities of flavonoids are reported to be one of the major mechanism behind the wound-healing process.[32] The existence of tannins ameliorated the restoration and coordination of fresh tissue and quickened the wound-healing process.[33]

The histopathological evaluation of negative control-treated rat's skin specimens did not show any significant elevation in collagen deposition, macrophages, fibroblast, and tissue edema proliferation. The poor wound healing indicated lesser infiltration of inflammatory cells, fewer fibroblasts proliferation, and blood vessel formation. However, the standard mupirocin cream-treated rats showed well-healed skin structures such as

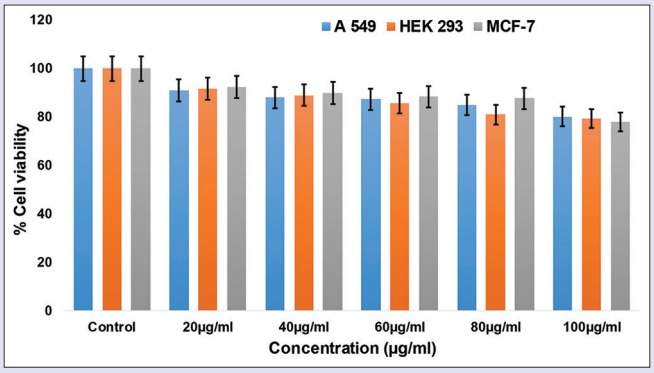

Figure 14: The cytotoxicity evaluation of the methanolic leaves extract of *Acacia auriculiformis* Benth. using adenocarcinoma human alveolar basal epithelial cells (A549), embryonic kidney cells (HEK-293), and human breast cancer cell line (MCF-7) cell lines

restoration of the adnexa, well-formed near to normal epidermis, collagen tissue formation within the dermis, and extensive fibrosis.

The histopathological studies on tissue obtained from methanolic extract-treated rats indicated a significant rise in macrophages, fibroblasts, collagen deposition, and tissue edema in comparison to the negative control-treated group. There was a substantial rise in the infiltration of inflammatory cells, increased proliferation of fibroblasts, and elevated blood vessel formation, indicating significant healing. The tissue architecture in the group treated with acetone showed mild-to-moderate leukocyte infiltration, fibroplasia, neovascularization, and no/minimal collagen deposition and epithelial regeneration. The recovery of tissue architecture was similar for chloroform and ethyl acetate, showing enhanced leukocyte infiltration, fibroplasia, neovascularization, and collagen deposition, which was comparable to standard mupirocin cream.

The cytotoxicity of the methanolic extract was assessed to inculcate and verify the biological compatibility of the extracts by MTT assay, as depicted in Figure 14. Results reveal that cells to the concentrations up to 200 µg/mL did not inflict distinct toxicity as the cell viability remained above 75%. On increasing the concentration, the order does not exhibit a continuous increase or decrease in cell viability. Anyhow, the results display that the determined concentrations are within the safe dosage range to be enforced for additional biological observations.

The LC-MS/MS analysis of methanol extract of *A. auriculiformis* revealed the presence of compounds such as β-sitosterol, lupeol, stigmasterol, and quercetin. Quercetin is a flavonoid which is reported as a potent antidiabetic agent[34] and is also known to cure diabetic wounds.[35] β-sitosterol is a bioactive phytosterol which is reported as antioxidant, antidiabetic agent.[36] It is also reported to promote wound healing.[37] Lupeol is a pentacyclic triterpenoid which promotes wound healing[38] and is evidenced to cure diabetes.[39] Stigmasterol is an unsaturated phytosterol which can treat diabetes[40] and promotes wound healing.[41] The phytoconstituents characterized through LC-MS/MS results support the evidence that they may be responsible for desired pharmacological activity, i.e., diabetic wound-healing activity.

CONCLUSION

Here, we conclude that after methanolic-treated group showed excellent therapeutic potential in healing diabetic wounds. Wound-healing properties of methanolic extract are linked with the presence of phenolic compound present in extract along with antioxidant properties, which are responsible for decreasing the oxidative stress associated with diabetes. This is our first report on wound-healing properties of *A. auriculiformis* leaves extracts. The methanolic extract should be accentuated due to its noteworthy antioxidant and anti-inflammatory activities. The LC-MS/MS analysis results also support the desired pharmacological effect of the methanol extract. Further, we will elaborate this work on mechanism of wound healing in diabetes and modes of action of the key most active plant metabolites. Overall, this *A. auriculiformis* is an auspicious and a treasured source of different bioactive compounds that could have immense health benefits.

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Conflicts of interest

There are no conflicts of interest.

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