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Effective Diabetic Foot Ulcer Healing Potential of Isolated Asperesocoumarin Compound from Fungal Endophyte

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ABSTRACT:

Diabetes was a metabolic disorder characterized by prolonged hyperglycemia that is affecting approximately 500 million populations leading to over 5millions deaths. DM results in complications like retinopathy, nephropathy, neuropathy and especially foot ulcer that are leading to amputation and disability to majority of population. Natural products are ideal to treat the foot ulcers, and fungi serve as critical sources of the molecules that treat diabetic foot ulcers effectively. Thus this research is designed to isolate coumarin molecule from fungal endopytes and to investigate the anti-diabetic activity of Asperesocoumarin-B (Ac-B) and related healing of foot ulcers in STZ+NIC induced diabetic model. The isolated molecule showed better anti-diabetic activity in terms of lowering the blood glucose levels, oral glucose tolerance, controlling the body weight, as well as normalizing liver and kidney function parameters. The isolated molecule also showed significant normalization of antioxidant enzymes and lowering of oxidative LPO. The isolated molecule (AC-B) showed increase in wound healing of the diabetic rats in terms of reduction of the wound size over 12 days and also increase in total protein content. Histopathological studies on liver tissue and wound tissue showed significant healing and preserved tissue architecture and integrity. Healing effects were attributed in part to reduced free radical damage, the promotion of antioxidant status, the rapid deposition of collagen, and the anti-diabetic activity.

Introduction

Deficiency or resistance to insulin, impaired insulin secretion, or excessive hepatic glucose production results in diabetes mellitus (DM). There are 415 million diabetics worldwide, 8.3% of the adult population, according to IDF. By 2035, the number of diabetics will reach 592 million. It is estimated that 90% of diabetics are type 2 diabetics, with the same rates affecting both men and women at the same time [1], and 1.5 to five million people died from diabetes between 2012 and 2015.

In addition to lipid peroxidation, protein inactivation, and protein glycation, hyperglycemia produces reactive oxygen species (ROS). Complications of hyperglycemia, including retinopathy, nephropathy, and coronary heart disease, can be linked to these events [1]. In addition to diabetic foot, neuropathy, and cardiovascular complications, DM is known to cause complications. Diabetes often causes impaired wound healing, which leads to chronic, non-healing ulcers that require costly treatment [2]. Amputation is required in approximately 14-24% of diabetic foot ulcer patients even when they receive adequate treatment and care [3]. Diabetic patients with foot ulcers are 2.5 times more

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likely to die than diabetic patients without foot ulcers [4]. DUFs are estimated to affect 9.1-26.1 billion people annually by the International Diabetes Federation. In recent years, DFU prevalence increased significantly, reaching an average global prevalence of 6.4% [5].

Natural products would be ideal if they could combat such diseases without causing secondary health issues. There has been extensive research on the potential anti-diabetic properties of secondary metabolites from plants, especially with regard to wound healing. Therefore, compounds derived from fungi could be developed as diabetes therapeutics [6]. A variety of natural products can be synthesized by fungi with an intriguing chemical diversity, making them one of the richest natural product sources among living organisms [7]. Penicillins (antibacterial), echinocandin B (antifungal), cyclosporin A (immunosuppressive), and lovastatin (cholestrol-lowering) are also fungal-derived drugs. Additionally, these factors indicate that fungi can provide new pharmaceuticals. Coumarin compounds isolated from aspergillus were proven to control diabetes [8]. Thus this current research had been designed to investigate the healing ability of natural compound isolated from fungal endophyte in streptozotocin and nicotinamide induced diabetic foot ulcer model.

Materials and methods

Isolation of compound from endophytic fungus from Acanthus

Acanthus ilicifolius leaves were collected from the forest near Nilgiri hills. Plants were carefully selected for sampling if they were healthy and mature. Plants were collected randomly in sterile plastic bags and brought to the laboratory for examination. [9], leaf surfaces were sterilized for 1 minute in 70% ethanol, 5 minutes in NaOCl 5.3%, and 2 minutes in sterile distilled water. Surface sterilization was validated by imprinting the plant part onto nutrient media was used to validate the surface sterilization procedure.

After drying, leaf segments were sliced into approximately 0.5cm squares and placed on petri plates containing potato dextrose agar medium (PDA). In order to prevent bacteria from growing, streptomycin sulphate was added (100mg/L). After that, it was monitored every day for the growth of endophytic fungal colonies. The fungi growing out of the samples were then transferred to fresh PDA plates. At 28°C, pure cultures of isolated fungal (Aspergillus) strains were maintained in PDA slants. Several fungal endophytes were isolated from the cut ends and subcultured. From isolates with different morphologies, pure cultures were prepared. The isolates were mass cultured for 7-10 days at 28ºC in potato dextrose broth. Whatman No.1 filter paper was used to separate mycelia.

The mycelia were collected carefully and dried under shade and powdered. These were extracted with MeOH using soxhlet apparatus. This process was performed 3 times and the filtrates were pooled. The filtrates were evaporated under reduced pressure to yield 50 g of extract (MEF). First, the extract was eluted with pet ether (100%), then with a mixture of pet ether and ethanol (1:0-0:1) and then finally by Ethanol (100%). Fractions were collected and combined with TLC after the column was monitored. Fractions (F1-F5) that generated identical colour or Rf were combined and subjected for antibacterial activity. The second step of column chromatography was eluted for fraction 3 (F3) using chloroform and methanol (1:1) to yield 4 sub fractions (SF1-SF4) which are again subjected to antibacterial activity. The second fraction SF2 was eluted again using pet ether and ethanol (7:3) to yield sub fraction SSF1 and SSF2 [10]. SSF1 was utilized for the study and the compound was identified as Asperiso coumarin B (Ac-B) from literature.

Experimental Animals

Swiss rats weighing 150-250 gm were acclimated to a room with a temperature of 23 ± 2 °C, humidity (50-55%) and a 12 h light/12 h dark cycle. In addition to feeding the animals standard pellets (Kamadenu Enterprises, Bangalore) and providing water ad libitum, they were caged in polypropylene cages.

Acute oral toxicity studies

Each group contains 3 animals and will be housed individually. The test compound (Ac-B) had been given as a single dose. The compound was given at the dose of 5, 50, 300, and 2000 mg/kg body weight. If the 1st group animals survived, the further group animals will receive a higher dose as per OECD Guidelines 423 [11-13]. The animals were monitored for their physical activity, tremor, writhing, respiration, convulsions, salivation and diarrhea, sense of touch and sound.

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Induction of hyperglycemia

A Study [14] reported the results of this test which was conducted on normal rats that had been fasted overnight. Five groups of six animals each were present.

Group I: Control rats were given normal saline (5ml/kg, p.o.)

Group II: rats (control) were administered with Ac-B at 50mg/kg

Group III: rats (disease control) received vehicle only

Group IV: Ac-B 50 mg/kg body weight was administered to rats.

Group V: 0.25 mg/kg glibenclamide was administered to rats.

An intraperitoneal (i.p.) injection of streptozotocin (STZ) at a dose of 50 mg/kg body weight was administered to overnight-fasted rats in groups III, IV and V which is followed by nicotinamide (NIC) at a dosage of 120 mg/kg in citrate buffer (pH 4.5) in a volume of 0.5 ml/kg .wt, as per the protocol established [15]. The confirmation of diabetes was carried out by measuring fasting blood glucose levels in STZ + NIC-treated rats 48 hours post-induction, following the method described [16]. To prevent hypoglycemic mortality, rats were given a 5% w/v glucose solution (2 ml/kg b.w.) 24 hours after the STZ and NIC injection.

Anti-diabetic activity

Oral glucose tolerance test

Normal animals were fasted overnight (18 hours) for the oral glucose tolerance test. 30 minutes after vehicle and drug administration, glucose (2 g/kg) was administered. A blood sample was drawn from the tail vein at 0, 30, 60, and 120 min. of drugs administration. Blood glucose levels were measured using a digital glucometer [17].

Investigation of anti-diabetic activity

A cannula was used to administer the treatments once daily for 21 days. At 1, 7, 14 and 21 days, tail vein blood was collected to determine glucose levels. Glucose levels were measured using a digital glucometer. Plasma insulin was estimated using a radioimmunoassay kit.

Investigation of liver and kidney function parameters

Aspartate aminotransferase (AST), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) are determined by spectrophotometry according to [18-19]. In sera, creatinine, urea, and uric acid were measured using spectrophotometry in accordance with the methods described [20]

Investigation of diabetic ulcer healing activity

This study used a punch biopsy needle to create wounds on all rats on the tenth day after DM induction. Ketamine (30 mg/kg, ip) was used to anesthetize rats and a 6mm2 area was marked on the right foot's dorsal surface. The marked skin was then carefully removed [21]. traced the wounds on graph paper after wounding and every four days thereafter until the 12th day. Using the formula below, the area of the wound was calculated regularly and the rate of wound contraction was calculated.

$$\%wound\ contraction = \frac{(initial\ wound\ area - current\ wound\ area)}{Initial\ wound\ area} \text{x}100$$

A comparison of wound areas on respective days between the test and control groups is conducted to determine the significance of wound healing between the groups.

***In-vivo* Antioxidant Activity**

On 21stday, liver and wound tissue were removed, washed with normal saline, and preserved in 10% formalin for histopathological analysis. The other parts of the kidney and liver were homogenized in ice-cold Tris-HCl buffer to measure superoxide dismutase (SOD), catalase, reduced glutathione (GSH), glutathione peroxidase (GPx), and malondialdehyde MDA. This is a direct indicator of lipid peroxidation (LPO) measured by TBA reaction (at 532 nm) [22].

Histopathological studies

After 21 days of treatment, the animals were anesthetized with diethyl ether and sacrificed by cervical dislocation. The liver and tissues near the wound were immediately dissected, excised, and rinsed in ice-cold saline solution. The tissues were processed as follows. For four days, liver tissue was fixed in a 10% formalin fixative solution. A rotary microtome was used to cut solid transverse sections 4-5 mm thick after tissues had

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been fixed in ethanol (70-95%), cleared in xylene, and embedded in paraffin. A light microscope (40x) was used for histopathological observations of the sections stained with haematoxin eosin.

Statistical analysis

The statistical analysis was carried out using GraphPad Prism 5.0 (San Diego, USA). The results were calculated as the mean ± SEM of triplicate studies. Data were analyzed by ANOVA followed by Dunnets test. $p<0.001$, 0.01 were considered significant at respective levels of significance.

Results

Acute toxicity of the test compound

The acute toxicity studies revealed that there is no signs of toxicity that is noted till 300mg/kg body weight but at 2000mg/kg animals died in 24 hrs duration. Thus the LD_{50} values of all the tested compounds was noted to be more than 300mg/kg. Hence, $1/10^{th}$ of the dose that is 50mg/kg was decided as remedial dose of the compounds.

Effect of Ac-B on the oral glucose tolerance

The study investigates the effects of Asperesocoumarin (Ac-B) on glucose tolerance in diabetic rats induced by streptozotocin (STZN) and nicotinamide (NIC). The groups compared include control rats, control rats treated with Ac-B, diabetic rats, the diabetic rats treated with Ac-B, and the diabetic rats treated with glibenclamide, a standard diabetes medication. The oral glucose tolerance was determined by measuring the blood glucose levels (in mg/dL) of various groups of rats measured at different time points (initial, 30 minutes, 60 minutes, and 120 minutes) after glucose administration. Ac-B at 50 mg/kg significantly improved glucose tolerance in diabetic rats and rats that were not diabetic. In diabetic rats, it reduced glucose levels substantially within 30 minutes and maintains these reductions through 120 minutes, showing similar effectiveness to glibenclamide as shown in table 1. These results indicated that Ac-B is effective in improving glucose tolerance.

Table 1: Effect of Ac-B on the oral glucose tolerance of the STZ+NIC induced diabetic rats

Groups	**Blood glucose level (mg/dL)**			
	Initial	**30min**	**60min**	**120min**
Normal	89.49±5.43	120.43±7.55	108.85±6.04	90.39±5.95
Normal + Ac-B (50mg/kg)	85.98±5.05	89.42±5.01	85.66±5.31	81.35±4.19
Diabetic (STZN)	198.54±15.32	249.78±16.32	254.31±17.47	267.59±20.45
STZN+ Ac-B 50mg/kg	211.55±15.55	164.55±14.38	123.28±15.48	95.56±6.32
STZN+ Glibenclamide 0.25mg/kg	207.55±13.98	153.49±15.44	111.42±13.33	82.29±5.43

A mean standard deviation is calculated based on the results (n=6); *** indicates significance at $P<0.001$, **$P<0.01$, *$P<0.05$ compared to control group; ### indicates significance at $P<0.001$, ##$P<0.01$, #$P<0.05$ compared to diabetic group

Effect of Ac-B on the Blood glucose levels of STZ+NIC induced diabetes

Table 2 presents the blood glucose levels (in mg/dL) of different groups of rats measured over a 21-day period, investigating the long-term effects of Asperesocoumarin (Ac-B) on blood glucose levels in diabetic rats induced

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by streptozotocin (STZN) and nicotinamide (NIC). Ac-B at 50 mg/kg significantly improved blood glucose levels in diabetic and normal rats over a 21-day period as illustrated in table 2. Compared to glibenclamide, it reduced glucose levels substantially, showing effectiveness comparable to glibenclamide. These results suggested that Ac-B has potential as an antidiabetic agent with long-term benefits.

Table 2: Effect of Ac-B on the levels of glucose in STZ+NIC-induced diabetic rats

Groups	**Blood glucose level (mg/dL)**			
	0th day	**7th day**	**14th day**	**21st day**
Normal	90.43±5.44	92.33±5.18	91.47±5.01	93.97±6.28
Normal+Ac-B (50mg/kg)	92.21±5.02	89.08±5.08	83.48±4.32	76.22±4.34
Diabetic (STZN)	204.55±14.71	240.47±17.27	289.75±19.42	361.63±21.44
STZN+Ac-B 50mg/kg	211.55±14.21	164.55±13.25	129.82±8.32	90.48±5.33
STZN+Glibenclamide 0.25mg/kg	209.55±13.27	155.26±12.95	115.62±7.48	84.39±5.32

The data are presented as mean±SEM (n=6); *** indicates significance at P<0.001, **P<0.01, *P<0.05 compared to diabetic group; ### represents significance at P<0.001, ##P<0.01, #P<0.05 in comparison with diabetic group.

Effect of Ac-B on the body weight changes

In this study, Ac-B's effects on body weight changes (in grams) in different groups of rats from the initial measurement to the final measurement were measured. The study examines the effect of Asperesocoumarin (Ac-B) on body weight in diabetic rats induced by streptozotocin (STZN) and nicotinamide (NIC). Comparing the normal group with the normal+Ac-B group, both groups start with similar initial body weights (189.74±6.17 g vs. 182.51±5.28 g). By the final measurement, the normal group shows an increase in body weight to 218.49±6.24 g, while the normal+Ac-B group also shows an increase, reaching 206.65±4.21 g. This indicates that Ac-B does not adversely affect body weight in normal rats and supports healthy weight gain. Glibenclamide group shows an increase in body weight to 199.55±5.82 g, which is less pronounced than the weight gain observed in the STZN+Ac-B group but still indicates an improvement compared to the untreated diabetic group. While diabetic rats without treatment experience weight loss, those treated with Ac-B or glibenclamide gain weight, indicating improved health and metabolic status. These results suggest that Ac-B has potential benefits in managing not only blood glucose levels but also preventing weight loss associated with diabetes

Table 3: Effect of Ac-B on the changes in body weight of the STZ+NIC induced diabetic rats

Groups	**Body weight (gm)**	
	Initial	**Final**
Normal	189.74±6.17	218.49±6.24

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Normal+Ac-B (50mg/kg)	182.51±5.28	206.65±4.21
Diabetic (STZN)	190.29±6.98	175.48±5.09
STZN+Ac-B 50mg/kg	184.27±5.27	208.77±6.12
STZN+Glibenclamide 0.25mg/kg	187.55±5.32	199.55±5.82

Study expressed the values as mean± SEM (n=6); *** indicates significance at P<0.001, **P<0.01, *P<0.05 compared to control group; ### indicates significance at P<0.001, ##P<0.01, #P<0.05 in comparison to diabetics

Effect of Ac-B on the Biochemical Parameters of STZ+NIC Induced Diabetic Rats

Table 4 presents the biochemical parameters, including the level of plasma insulin (IU/ml), the level of hemoglobin (Hb) in the blood (mg/dL), the level of glycated hemoglobin (HbA1C) in the blood (mg/g of Hb), and total protein (mg/dL), in different groups of rats. Results showed that Ac-B significantly improved biochemical parameters such as plasma insulin, hemoglobin, HbA1C, and total protein in diabetic rats. These improvements are significant compared to those observed with glibenclamide.

Table 4: Biochemical parameters of diabetic rats induced by STZ+NIC and Ac-**B**

Groups	**Plasma insulin (µIU/ml)**	**Hb (mg/dL)**	**HbA1C (mg/g of Hb)**	**Total protein (mg/dl)**
Normal	15.49±1.03	12.95±1.28	0.24±0.03	7.42±0.54
Normal+Ac-B (50mg/kg)	16.95±2.53	13.27±2.18	0.23±0.04	8.46±0.76
Diabetic (STZN)	5.88±1.02	7.04±1.95	0.75±0.08	4.25±0.31
STZN+Ac-B 50mg/kg	15.02±1.04	12.62±1.78	0.41±0.04	7.05±0.48
STZN+Glibenclamide 0.25mg/kg	15.44±0.95	12.11±1.24	0.44±0.04	7.88±0.53

Value expressed as mean±SEM (n=6); *** indicates significance at P<0.001, **P<0.01, *P<0.05 compared to control group; ### indicates significance at P<0.001, ##P<0.01, #P<0.05 in comparison to diabetics

Effect of Ac-B on the hepatic enzymes

Comparing the normal group with the normal+Ac-B group, both groups have similar levels of ALP, with the normal group at 90.48±3.54 U/L and the normal+Ac-B group slightly lower at 87.59±3.86 U/L. ALT levels are significantly lower in the normal+Ac-B group (30.57±2.34 U/L) compared to the normal group (44.39±3.21 U/L), indicating that Ac-B may have a protective effect on liver function. AST levels are comparable between the two groups, with the normal group at 82.48±7.42 U/L and the normal+Ac-B group at 81.06±6.58 U/L. Albumin levels are also similar, with the normal group at 4.59±0.21 mg/dL and the normal+Ac-B group at 4.32±0.18 mg/dL, suggesting that Ac-B does not adversely affect these liver function parameters in normal rats. In the diabetic (STZN) group, ALP levels are significantly higher at 179.49±12.96

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U/L compared to the STZN+Ac-B group, which shows a marked reduction to 105.43±9.47 U/L. This indicates that Ac-B significantly lowers ALP levels in diabetic rats. ALT levels are also much higher in the diabetic group at 99.44±5.44 U/L, whereas the STZN+Ac-B group shows a significant reduction to 37.54±2.35 U/L. AST levels in the diabetic group are elevated at 185.63±10.32 U/L, but the STZN+Ac-B group shows a reduction to 105.44±9.44 U/L, indicating improved liver function with Ac-B treatment. Albumin levels are lower in the diabetic group at 2.33±0.09 mg/dL, while the STZN+Ac-B group shows improved levels at 3.64±0.17 mg/dL, suggesting that Ac-B helps in maintaining better protein synthesis and liver function in diabetic rats.

Table 5: Effect of Ac-B on the hepatic enzymes of the STZ+NIC induced diabetic rats

Groups	**ALP (U/L)**	**ALT (U/L)**	**AST (U/L)**	**Albumin (mg/dl)**
Normal	90.48±3.54	44.39±3.21	82.48±7.42	4.59±0.21
Normal+Ac-B (50mg/kg)	87.59±3.86	30.57±2.34	81.06±6.58	4.32±0.18
Diabetic (STZN)	179.49±12.96	99.44±5.44	185.63±10.32	2.33±0.09
STZN+Ac-B 50mg/kg	105.43±9.47	37.54±2.35	105.44±9.44	3.64±0.17
STZN+Glibenclamide 0.25mg/kg	110.55±8.56	65.41±3.41	95.26±7.43	3.41±0.19

The value expressed as mean±SEM (n=6); *** represents significance at a P<0.001, **P<0.01, *P<0.05 in comparison to the control group; ### signifies significance at P<0.001, ##P<0.01, #P<0.05 in comparison to diabetics

Effect of Ac-B on the renal parameters

The study evaluated the effects of Asperesocoumarin (Ac-B) on these renal parameters in diabetic rats induced by streptozotocin (STZN) and nicotinamide (NIC). Uric acid levels in the normal+Ac-B group (4.86±0.39 mg/dL) are slightly lower compared to the normal group (5.42±0.43 mg/dL). Urea levels are also reduced in the normal+Ac-B group (82.17±4.11 mg/dL) compared to the normal group (95.76±4.39 mg/dL). Creatinine levels remain similar between the two groups, with the normal group at 0.84±0.1 mg/dL and the normal+Ac-B group at 0.82±0.07 mg/dL. These results indicate that Ac-B does not adversely affect renal function in normal rats and may even have a slight protective effect. Overall, Ac-B significantly improved renal parameters in diabetic rats, reducing uric acid, urea, and creatinine levels.

Table 6: Effect of Ac-B on the renal parameters of the STZ+NIC induced diabetic rats

Groups	**Uric Acid (mg/dl)**	**Urea (mg/dl)**	**Creatinine (mg/dl)**
Normal	5.42±0.43	95.76±4.39	0.84±0.1
Normal+Ac-B (50mg/kg)	4.86±0.39	82.17±4.11	0.82±0.07
Diabetic (STZN)	10.53±0.74	143.28±6.84	1.25±0.18

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STZN+Ac-B 50mg/kg	6.43±0.41	118.83±5.55	0.88±0.08
STZN+Glibenclamide 0.25mg/kg	5.98±0.37	117.47±5.39	0.83±0.09

The values were expressed as mean±SEM (n=6); *** indicates significance at P<0.001, **P<0.01, *P<0.05 compared to control group; ### indicates significance at P<0.001, ##P<0.01, #P<0.05 compared to diabetic group

Oxidative stress markers and Ac-B

Table 7 compares levels of oxidative stress markers. As part of this, there is superoxide dismutase (SOD) activity, catalase (CAT) activity, glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, and glutathione reductase (GPx) activity.), and lipid peroxidation (LPO) levels (nM/mg protein), in different groups of diabetic rats induced by streptozotocin (STZN) and nicotinamide (NIC). Results indicated that Ac-B significantly improves oxidative stress markers in diabetic rats, including enhancing antioxidant enzyme activities and reducing lipid peroxidation levels. These effects are significant to those observed with glibenclamide, suggesting that Ac-B has potent antioxidant properties in addition to its anti-diabetic effects.

Table 7: Effect of Ac-B on the oxidative stress markers of the STZ+NIC diabetic rats

Groups	**SOD (U/mg protein)**	**CAT (U/mg protein)**	**GPx (µmol/mg protein/min)**	**GR (µmol/mg protein/min)**	**LPO (nM/mg protein)**
Normal	7.33±1.08	10.856±0.96	18.84±2.17	54.56±3.73	21.07±1.20
Normal+ Ac-B (50mg/kg)	8.53±1.02	13.938±0.63	21.2±1.81	57.86±4.70	14.36±1.19
Diabetic (STZN)	3.58±0.50	5.002±0.33	7.46±1.12	33.98±2.47	29.31±3.77
STZN+ Ac-B 50mg/kg	6.88±0.86	10.488±0.89	17.76±2.03	56.71±4.87	23.05±1.36
STZN+ Glibenclamide 0.25mg/kg	6.32±0.85	9.303±0.81	18.44±2.12	49.95±5.07	23.01±1.19

The values were expressed as mean ± SEM (n=6); *** indicates significance at P<0.001, **P<0.01, *P<0.05 compared to control group; ### indicates significance at P<0.001, ##P<0.01, #P<0.05 compared to diabetic group

Wound healing effect of Ac-B in diabetic rats

The rats were induced with diabetes by administering streptozotocin (STZ) and nicotinamide (NIC). They were divided into various groups, including normal rats and diabetic rats, each further subdivided into those treated with Ac-B, glibenclamide, or left untreated. Initially all the groups had similar initial wound areas, indicating consistent wound creation. However, starting from the 4th day, the rats treated with Ac-B exhibited a notable reduction in wound area compared to the control group. This trend continued throughout the observation period, with the Ac-B treated group consistently showing smaller wound areas on the 8th and 12th days. These findings suggest that Ac-B enhances wound closure in normal rats, indicating its potential as a wound healing agent even in non-diabetic

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conditions. In diabetic rats the STZN + Ac-B and STZN+ Glibenclamide groups showed progressively smaller wound areas over time, indicating sustained enhancement in wound healing with treatment. This suggests that Ac-B, similar to glibenclamide, effectively mitigates the impaired wound healing associated with diabetes, potentially through its anti-diabetic and/or other therapeutic properties.

Table 8: Effect of Ac-B on the wound area of the excision wound of STZ+NIC induced diabetic rats

Groups	Area of wound (mm^2)			
	Initial	**4th day**	**8th day**	**12th day**
Normal	5.97±0.02	4.48±0.35	2.87±0.21	1.05±0.11
Normal+ Ac-B (50mg/kg)	5.89±0.05	4.02±0.29	2.08±0.19	0.47±0.05
Diabetic (STZN)	6.05±0.04	5.33±0.44	4.39±0.17	3.22±0.36
STZN+ Ac-B 50mg/kg	6.01±0.03	5.01±0.29	3.81±0.19	1.62±0.33
STZN+ Glibenclamide 0.25mg/kg	5.99±0.08	5.17±0.31	4.01±0.21	2.01±0.29

The values were expressed as mean ± SEM (n=6); *** indicates significance at P<0.001, **P<0.01, *P<0.05 compared to control group; ### indicates significance at P<0.001, ##P<0.01, #P<0.05 compared to diabetic group

Regarding ulcer contraction percentages, in the normal groups, a consistent progression of wound contraction percentage is observed over time, indicating efficient wound healing. Notably, rats treated with Ac-B display significantly higher percentages of wound contraction at all-time points compared to untreated normal rats, suggesting that Ac-B accelerates ulcer healing in healthy individuals. These findings support Ac-B's potential for treating diabetic wounds, as it effectively accelerates wound healing in diabetic rats, which is significantly greater than that seen with the standard anti-diabetic drug glibenclamide.

Table 9: Effect of Ac-B on the wound healing of the STZ+NIC induced diabetic rats

Groups	% wound contraction		
	4th day	**8th day**	**12th day**
Normal	24.95±2.94	51.92±2.18	82.41±4.28
Normal+ Ac-B (50mg/kg)	31.74±2.99	64.68±3.21	92.02±5.33
Diabetic (STZN)	11.90±2.04	27.43±1.88	46.77±3.28
STZN+ Ac-B 50mg/kg	16.63±1.14	36.60±1.76	73.04±4.11
STZN+ Glibenclamide 0.25mg/kg	13.68±1.21	33.05±2.17	66.44±4.21

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Values expressed as mean ± SEM (n=6); *** denotes significance at P<0.001, **P<0.01, *P<0.05 in comparison to the control group; ### denotes significance at P<0.001, ##P<0.01, #P<0.05 in comparison to the diabetic group.

Ac-B showed elevated levels of antioxidant enzymes including SOD, CAT, GPx, and GR compared to the STZ+NIC treated groups which indicate robust antioxidant defence. Treatment with Ac-B elevates these levels, suggesting that Ac-B exhibits antioxidant enzyme activity in the wound area of normal rats. Conversely, the diabetic group displays diminished levels of antioxidant enzymes compared to normal, indicating compromised antioxidant defense. However, Ac-B treatment reverses this sequence, leading to increased levels of SOD, CAT, GPx, and GR, akin to the group treated with glibenclamide. This implies that Ac-B effectively restores antioxidant enzyme activity in the wound area of diabetic rats.

Table 10: Effect of Ac-B on the antioxidant enzymes in the wound area of the STZ+NIC induced diabetic rats

Groups	**SOD (U/mg protein)**	**CAT (U/mg protein)**	**GPx (µmol/mg protein/min)**	**GR (µmol/mg protein/min)**
Normal	6.38±0.94	9.44±0.84	16.39±1.89	47.45±3.25
Normal+ Ac-B (50mg/kg)	7.42±0.89	12.12±0.55	18.44±1.58	50.32±4.09
Diabetic (STZN)	3.12±0.44	4.35±0.29	6.49±0.98	29.55±2.15
STZN+ Ac-B 50mg/kg	5.99±0.75	9.12±0.78	15.45±1.77	49.32±4.24
STZN+ Glibenclamide 0.25mg/kg	5.48±0.74	8.09±0.71	16.04±1.85	43.44±4.41

The values were expressed as mean ± SEM (n=6); *** indicates significance at P<0.001, **P<0.01, *P<0.05 compared to control group; ### indicates significance at P<0.001, ##P<0.01, #P<0.05 compared to diabetic group

Ac-B treatment showed to induce mild oxidative stress in both normal and diabetic rats without significant alterations in total protein levels as shown in table 11. Further research is needed to understand the implications of these findings for wound healing outcomes in diabetic rats and to elucidate the mechanisms underlying Ac-B's effects on oxidative stress and protein metabolism in the wound area.

Table 11: Effect of Ac-B on the oxidative stress in the wound area of the STZ+NIC induced diabetic rats

Groups	**LPO (nM/mg protein)**	**Total protein (mg/dl)**
Normal	6.38±0.94	78.33±6.49
Normal+ Ac-B (50mg/kg)	7.42±0.89	89.48±9.55
Diabetic (STZN)	3.12±0.44	63.29±7.53

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STZN+ Ac-B 50mg/kg	5.99±0.75	76.55±5.68
STZN+ Glibenclamide 0.25mg/kg	5.48±0.74	75.42±6.56

Values were expressed as mean±SEM (n=6); *** indicates significance at P<0.001, **P<0.01, *P<0.05 compared to control group; ### indicates significance at P<0.001, ##P<0.01, #P<0.05 compared to diabetic group

Effect of Ac-B on the histopathology of liver

Histopathological analysis of liver tissue from rats with STZ-NIC induced diabetes revealed clear pathological changes, including hepatocellular necrosis, vacuolation, and inflammatory infiltrates, indicative of diabetic hepatopathy. This pronounced degeneration underscored the degenerative impact of the STZ on liver morphology. However, the administration of aperesocoumarin-B (Ac-B) particularly at alongside with STZ and NIC, exhibited a significant protective effect on liver. At 50mg/kg Ac-B demonstrated a significant reduction in hepatocellular degeneration, preserving the normal hepatic architecture. Moreover, a decrease in inflammatory infiltrates suggested an anti-inflammatory effect, further contributing to the observed hepatoprotection. Importantly, these protective trends were notably comparable to the effects observed with Glibenclamide, confirming the hepatoprotective potential of Ac-B.

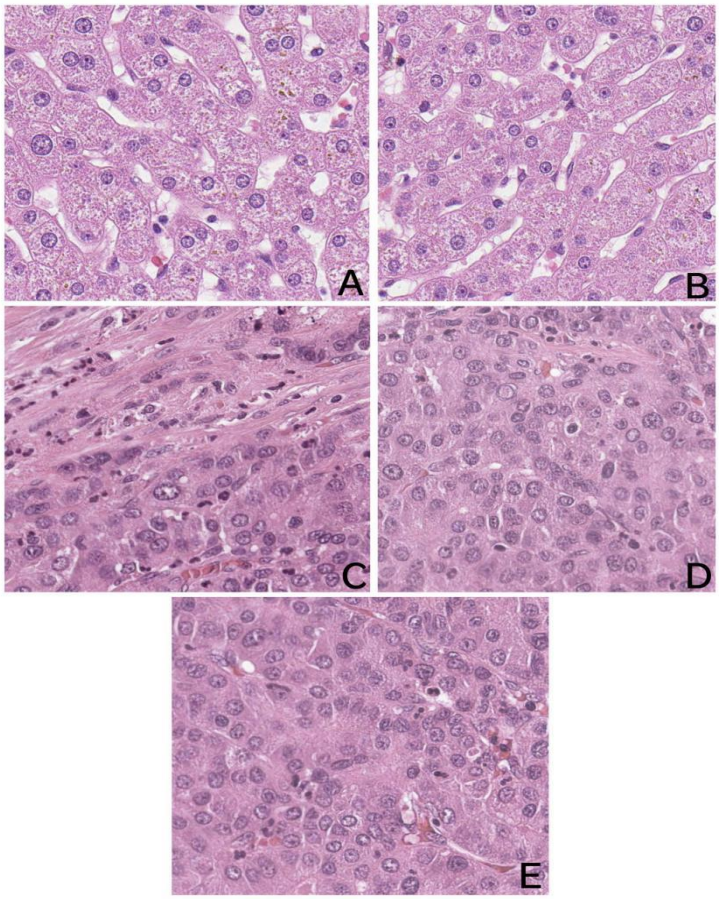

Figure 1: Histopathology of Liver tissue treated with Ac-B in STZ+NIC induced diabetic rats

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A. Normal B. Ac-B 50mg/kg C. STZ+NIC D. Ac-B+STZ+NIC E. Gliben+STZ+NIC

Effect of Ac-B on the wound area

The histopathological evaluation conducted in this study offers valuable insights into the mechanisms through which the administered extracts facilitate accelerated wound closure. The observed histological changes correspond to the enhanced healing observed in the Ac-B treated groups as compared to the control. Histological examination revealed a notable increase in cellular proliferation and angiogenesis in the wound bed of the treated groups. Both Ac-B and Ac-B along with STZ+NIC extracts, exhibited a significant promotion of fibroblast proliferation and neovascularization compared to the control. Additionally, histopathological analysis revealed a reduction in inflammatory infiltrates and microbial colonization in the wound beds of the treated groups. The extracts demonstrated potent anti-inflammatory properties by attenuating inflammatory cell infiltration and promoting a more controlled inflammatory response. Moreover, the extracts exhibited remarkable antimicrobial activity, as evidenced by the reduced microbial load observed in the histological sections. These effects are crucial in preventing infection and creating a favorable environment for efficient wound healing.

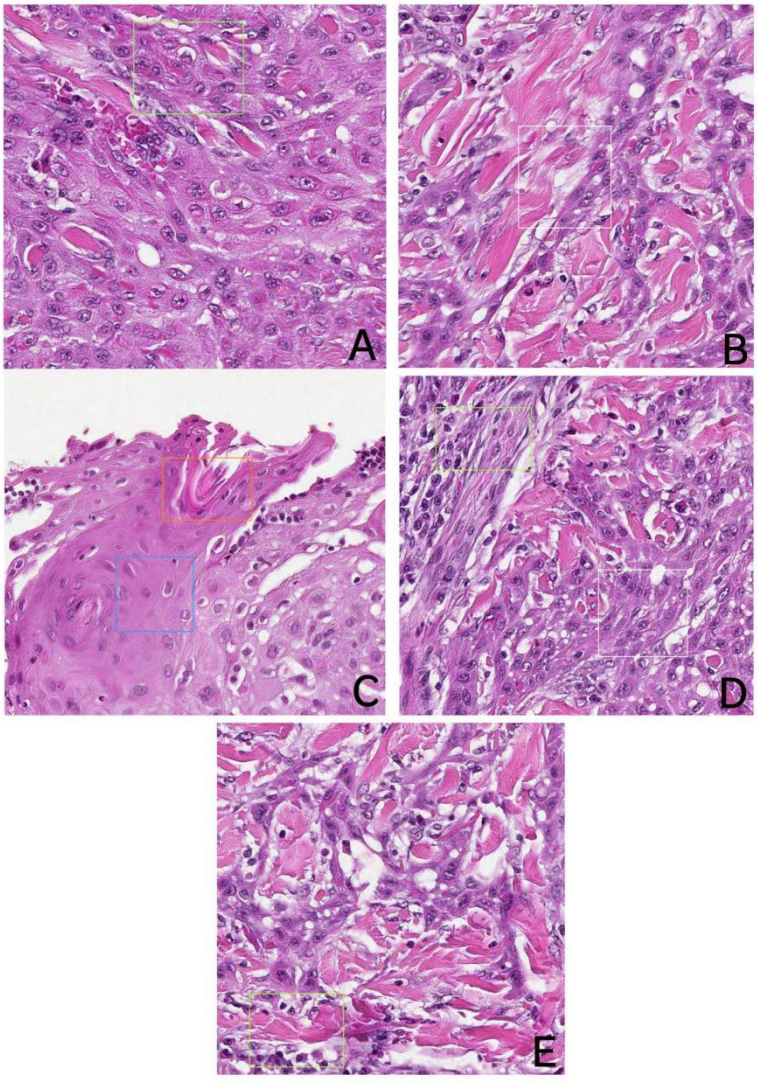

Figure 2: Histopathology of wound tissue treated with Ac-B in STZ+NIC induced diabetic rats

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A. Normal B. Ac-B 50mg/kg C. STZ+NIC D. Ac-B+STZ+NIC E. Gliben+STZ+NIC

Discussion

In any experiment involving DM, STZ serves as an excellent model. STZ causes damage to ß pancreatic islets [23]. As a result, this substance is capable of producing diabetogenic effects with symptoms that are identical to human hyperglycemic non-ketotic DM, such as hyperphagia, polyuria, and weight loss [24]. As a result, it was used in this model to produce diabetes. A diabetic's glucose degradation produces free radicals, and non-enzymatic protein glycation leads to oxidative stress and lipid peroxidation in the body [25]. Conversely, it has been reported that wounding of the cutaneous skin can decrease the antioxidant status due to ROS production. Diabetes ulcers lasting over 4 weeks typically result in worse outcomes and may require amputation [26]. Studies showed that coumarins from aspergillus have showed significant anti-diabetic activity and hence this study was designed to isolate the coumarin compounds from aspergillus and investigate for its activity aginst the STZ+NIC induced the diabetic foot ulcer [27].

A significant magnification of serum glucose, hepatic enzyme activity (ALP, AST, and ALT), renal function (urea, uric acid, creatinine), as well as oxidative stress parameters (H_2O_2 and MDA) was observed in this study after STZ injection compared with a negative control. Additionally, insulin, antioxidant enzyme activities (GR, GST, GPx, CAT, and SOD) as well as GSH levels significantly decreased. In the meantime, Ac-B administered at 50 mg/kg of body weight per day improved all apparent abnormalities. Gluconeogenesis, or the catabolism of proteins and fats, caused a significant loss in weight as a result of muscle wasting and a loss of protein in tissues [28]. According to it has been suggested that the increase in body mass in rats treated with Ac-B may be caused by the increment of insulin and the decrease of glucose, which controls muscle loss.

Wounds represent a significant health problem, both in terms of morbidity and mortality. Epithelization, contraction, and connective tissue deposition occur during wound healing. Collagen deposition and maturation are crucial to the healing process [29]. A significant faster rate of wound closure was observed in the diabetic Ac-B treated group as compared to the diabetic untreated group on day 4 after wound creation, followed by similar results on day 12 after wound creation. Diabetes treated group wound closure rate was significantly higher than that of diabetic untreated group on the 12th day of the study. Early on in wound healing, Ac-B showed better results than the control group, till the last phase where Ac-B alone treated group showed over 90% of the healing significant improvement over the control group.

Conclusion

Thus, in our study, which involves the STZ+NIC model of diabetic wounds induced by STZ+NIC administration, a variety of physical, histological, and biochemical parameters were observed, showing wound healing activity of isolated coumarin compound Asperesocoumarin-B (Ac-B). Healing occurred as a result of reduced free radical-induced tissue damage, enhanced antioxidant status, and faster collagen deposition.

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