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Research Article

Effect of Withania Coagulans Dunal on Genomic Analysis of Nociceptive Threshold in DRG of STZ Induced Rats Followed by Histopathology of Paw Skin

Dr. Trupti Deshpande[1*], Dr. H.D. Une[2], Dr. Arulmozhi S[3]., Dr. P.D.Ghode[1], Dr. Atul Sayare[1], Prof. Vrushali Kakad[1], Dr. Shweta Ghode[4], Dr. Vrunda Sheth[5]

[1]*JSPM's Rajarshi Shahu College of Pharmacy and Research, Tathawade, Pune, Maharashtra, India*
[2]*Y.B. Chavan College of Pharmacy, Aurangabad, Maharashtra, India*
[3]*Poona College of Pharmacy, Pune*
[4] *Rasiklal Makinchand Dhariwal Institute of Pharmaceutical Education & Research, Chinchwad, Pune 411019, Maharashtra, India*
[5]*Suburban Diagnostics Reference Laboratory, Mumbai*

Abstract

Background: The most prevalent kind is distal symmetrical neuropathy, which affects sensory nerves. Both small and large nerve fibres suffer. Following foot ulceration and amputation, this neuropathy causes death and morbidity. Up to 50% of type 1 and 2 diabetics are affected. Insulin and other growth hormones help neurons survive and develop. Insufficient signalling from these factors and nerve regeneration can cause neuropathy.

Objective: To examine how *Withania* Coagulans Dunal fruit extract affects diabetic neuropathy in streptozotocin-induced diabetic rats utilizing behavioural models, genomic analysis with RTPCR, and hind paw skin histology of epidermal nerve fibres.

Methods: A single intraperitoneal injection of freshly produced Streptozotocin (55mg/kg body weight, in 0.1M citrate buffer (pH 4.5)) caused diabetes in Sprague Dawley male rats (180-250 g). After one week of STZ injection, hyperglycemia developed. Six groups of moderately diabetic rats with glycosuria and hyperglycemia (blood glucose levels > 250 mg/dl) were studied. Diabetes-induced rats received 50mg/kg and 100mg/kg oral Ethyl acetate fraction of *Withania Coagulans* (EAWC). Results were presented as mean ± SD. Analyses included ANOVA and Bonferroni post hoc tests. In week 5, all groups nociceptive thresholds were assessed using Rotarod motor in coordination, hot plate thermal allodynia, cold water tail immersion, Von Frey hair test Mechano-tactile test, and Randall–Selitto paw pressure test mechanical hyperalgesia.

Results: The dose-dependent reduction in nociceptive threshold was considerably reduced by *Withania Coagulans* fruits extract. Epidermal nerve fibres grow with epidermis thickness. Pain threshold genes also increase in L4-L6 DRG. The study found that *Withania Coagulans* fruits reduced diabetic and neuropathic pain in mice.

Conclusion: Present study shown that Ethyl acetate fraction of *Withania Coagulans* fruits, rich in flavonoids and able to penetrate BBB, may help streptozotocin-induced diabetic rats with neuropathy. This study also proposes molecular techniques targeting these alterations to cure incurable illnesses.

Keywords: *Withania Coagulans*, Streptozotocin, Diabetic neuropathy, Mechano-tactile allodynia, Mechanical hyperalgesia, RTPCR, Epidermal nerve fibers.

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INTRODUCTION

Diabetes is a highly challenging health condition to effectively control and is experiencing a worldwide increase. There are no signs or indications suggesting that this epidemic will decelerate. Diabetes induces detrimental alterations in the blood vessels of individuals, hence increasing their susceptibility to illness and mortality. Chronic renal failure and end-stage renal diseases are the prevailing vascular outcomes, often associated with alterations in metabolism and hemodynamics. Chronic elevation of blood sugar levels, known as hyperglycemia, leads to increased oxidative stress, which is a significant factor in the initiation, advancement, and worsening of diabetes and its associated vascular complications. The cause of kidney damage in long-term diabetes has been demonstrated to include immune-mediated low grade chronic inflammatory processes, as well as oxidative stress. Extensive research suggests that kidney damage is the result of several mechanisms that are strongly linked to immunological inflammatory processes and oxidative stress (1).

The objective of controlling diabetic kidney complications is to delay their start and progression by strict efforts to manage blood pressure or plasma glucose levels. Although not optimal, it is necessary to manage blood pressure and plasma glucose levels in order to prevent renal complications. This shortcoming underscores the necessity for more sophisticated treatments that may specifically target the oxidative stress-inflammatory cytokine signaling cascade, a process intimately associated with renal complications in diabetes, and impede the progression of these challenges. Consequently, medicinal plants, which are believed to be a crucial source of drug discovery from natural sources, have undergone extensive research in order to identify new therapeutic compounds (2).

Therefore, the use of natural substances derived from plants may have a positive impact on the treatment of diabetic kidney issues by decreasing oxidative stress and reducing the levels of proinflammatory and immunoregulatory cytokines. The challenging aspect will be identifying the most promising compounds and evaluating their protective mechanism. The fruits of *Withania coagulans*, a member of the Solanaceae family, have garnered significant attention for their possible benefits in the treatment of chronic degenerative conditions such as diabetes. There have been several reported ethnomedical uses for the herb *Withania coagulans*, which is also known as Indian Rennet, Vegetable Rennet (in English), or Panir dodi (in Hindi) (3).

The extract has shown potential in exhibiting anticancer, wound-healing,immunomodulating, antihyperglycemic, and hypolipidemic properties. Despite the numerous reports of the benefits of *Withania coagulans* in diabetes and its potential to target the complex interplay of oxidative stress and inflammatory and immunoregulatory cytokines in diabetic renal complications, it is important to investigate its effect on the kidneys of streptozotocin (STZ) induced diabetes. The present study examined the impact of *Withania coagulans* on lipid peroxidation, immunoregulatory and proinflammatory cytokines, and antioxidant defense in order to gain a better understanding of the process. Therefore, the use of natural substances derived from plants may have a positive impact on the treatment of diabetic kidney issues by decreasing oxidative stress and reducing the levels of proinflammatory and immunoregulatory cytokines (4).

The challenging aspect will be identifying the most promising compounds and evaluating their protective mechanism. The fruits of *Withania coagulans*, a member of the Solanaceae family, have garnered significant attention for their possible benefits in the treatment of chronic degenerative conditions such as diabetes. There have been several reported ethnomedical uses for the herb *Withania coagulans*, which is also known as Indian Rennet, Vegetable Rennet (in English), or Panir dodi (in Hindi). The extract has shown potential in exhibiting anticancer, wound-healing, immunomodulating, antihyperglycemic, and hypolipidemic properties. Despite the numerous reports of the benefits of *Withania coagulans* in diabetes and its potential to target the complex interplay of oxidative stress and inflammatory and immunoregulatory cytokines in diabetic renal complications, it is important to investigate its effect on the kidneys of streptozotocin (STZ) induced diabetes. The present study examined the impact of *Withania coagulans* on lipid peroxidation, immunoregulatory and proinflammatory cytokines, and antioxidant defense in order to gain a better understanding of the process (5).

MATERIAL AND METHODS

Plant Material Collection, Authentication

Withania Coagulans D. dried fruits, known by several names such as vegetable rennet (English), Indian rennet, Indian cheese maker, Panirkephool, Panir band, Paneer bandh, and Punirdodi (Hindi) (6). Furthermore, the name of this plant corresponds with the latest revision in "The Plant List." The fruits sourced from Sri Venkateswara University in Tirupati, India, were collected and authenticated.

Figure 1 Dried fruits of *Withania Coagulans*

Chemicals and Equipment/Apparatus

The list of chemicals used and equipment/apparatus are tabulated in table 1.

Table 1 The list of chemicals and equipment/apparatus used in the work

Sr. No.	Chemical Name	Manufacturer/Vendors
1	Streptozotocin (STZ)	HiMedia Laboratories Pvt. Ltd., Mumbai, India.
2	Metformin	Abbott Healthcare Pvt. Ltd. Himachal Pradesh, India.
3	Gabapentin	Alkem Labs, Mumbai
4	Methanol	S. D. Fine chemicals, Mumbai, India.
5	Ethyl acetate	S. D. Fine chemicals, Mumbai, India.
6	Petroleum Ether	S. D. Fine chemicals, Mumbai, India.
Equipment/Apparatus		
1	Soxhlet Apparatus	
2	Separating Funnel	
3	Aluminum plates Precoated with silica gel 60F254 of 0.2 mm thickness (E. Merck, Darmstadt, Germany)	
4	Electronic balance [Denver instrument, India].	
5	Iodine/UV Chamber	
6	Glucometer- OneTouch	

Preparation of Plant Extracts

The fruits of *Withania Coagulans* D. were dried in the shade, pulverized into a fine powder, and soaked in petroleum ether for a complete day each fruit. The extraction of each residue was performed using methanol through the utilization of the Soxhlet apparatus (7) subsequent to filtration. The resulting residue was separated into fractions using a separating funnel with a mixture of ethyl acetate and water at a ratio of 1:8:2 (1 milliliter residue, 8 milliliters ethyl acetate, and 2 milliliters water). In order to get the dry mass of the ethyl acetate fraction (EAWC) of *Withania Coagulans*, each filtrate was subjected to concentration.

Induction of Chronic Diabetes & Experimental Design

A group of rats that had fasted overnight were given a single intraperitoneal injection of newly manufactured streptozotocin (STZ) at a dose of 55 mg/kg body weight. The injection was administered in 0.1M citrate buffer with a pH of 4.5. The purpose of the injection was to induce hyperglycemia. The rats were administered a 5% glucose solution overnight in order to avoid drug-induced hypoglycemia. Hyperglycemia was confirmed on the third day after the STZ injection by testing glucose levels using a Glucometer (8). For the study, rats having a blood glucose concentration of 300 mg/dl were used.

Table 2 Experimental design of the animal activity

Group ID	Group Details	Treatment (Dose & Route)	No. of animals	Animal ID
G1	Normal Control	Saline, 10ml/kg/day, Oral Route	6	1-6
G2	Diabetic control	Saline, 10ml/kg/day, Oral Route	6	7-12
G3	Diabetic + EAWC1	EAWC,50mg/kg/day, Oral Route	6	13-18
G4	Diabetic + EAWC2	EAWC,100mg/kg/day, Oral Route	6	19-24
G5	Diabetic + Metformin	Metformin,120 mg/kg/day, Oral	6	25-30
G6	Diabetic + Gabapentin	Gabapentin,100 mg/kg/day, Oral	6	31-36

Parameters estimated at the end of 28 days dosing

This section provides the rats' estimated body weight and blood glucose levels following a 28-day period. Two specific doses were selected: 50 mg/kg and 100 mg/kg of body weight. The initial body weight was determined and compared to rats housed under normal circumstances. The Glucometer was utilized to assess the blood glucose levels prior to and during the four-week therapy duration (10).

Behavioral Models of Diabetic Neuropathy

After a four-week experiment, an EAWC behavioral evaluation was performed utilizing many animal models to study diabetic neuropathy.

Motor in coordination study

Over a period of three consecutive days, rats were trained daily using the rotarod apparatus, which maintained a constant rotation speed of 10 revolutions per minute. During each training session, the animals were subjected to a repetitive process of being placed on a rotating rod for a duration of three minutes. A comprehensive inspection was place one day after the completion of the final training trial. The rats were given either the test chemical or a vehicle one hour before to the rotarod test on the last day of testing. The animals were subsequently subjected to a test employing a rotarod that spun at a speed of 10 revolutions per minute. The inability to maintain balance on the rotating rod for a duration of one minute, as well as the average time it took to lose balance, were both indicative of motor impairments (11).

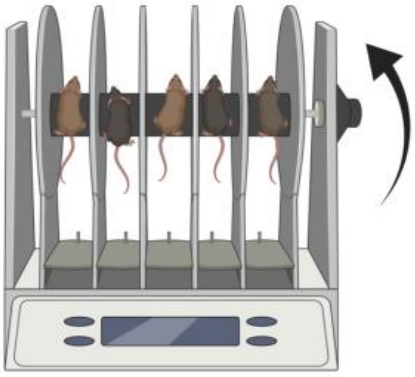

Figure 2 Rotarod apparatus

Thermal Allodynia

Eddy utilized a hotplate to sequentially place each animal, with the temperature being precisely regulated at 55±1°C. The pain threshold was assessed by monitoring the time it took for the first evidence of paw licking or leap reaction to occur. To avoid any harm to the paw, a cutoff limit of 10 seconds was implemented. The duration of the animal's jump or hind paw licking was recorded using a timer (12).

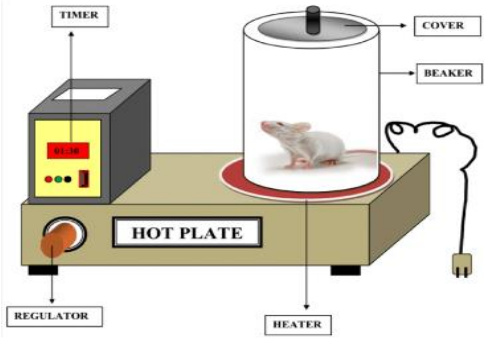

Figure 3 Thermal Allodynia

Cold allodynia

The animal's tail withdrawal reflexes were utilized to measure cold allodynia, which was seen when the animal's tail was immersed in ice water at a temperature of 4±20C. Tail retraction delay was seen in all groups (13).

Mechano-tactile allodynia (Von Frey hair test)

The study focused on rats exhibiting mechano-tactile allodynia responses. The withdrawal threshold of the hind paw was measured in response to mechanical stimuli of different strengths. This was done using a flexible Von-Frey hair device that applies calibrated bending forces (in grams) of variable intensities. The requirement for the threshold amount stated in grams. After four weeks of STZ injection, the withdrawal threshold for tactile responses was evaluated (14).

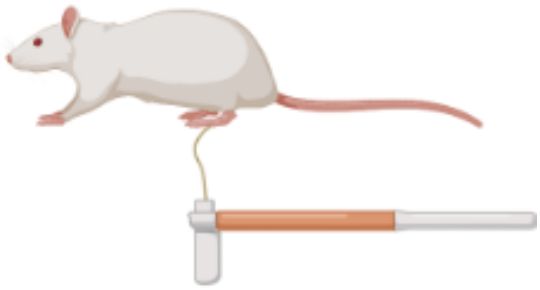

Figure 4 Von-Frey hairs apparatus

Mechanical hyperalgesia (Randall–Selitto paw pressure test)

After four weeks of STZ injection, the mechanical nociceptive threshold of each group was evaluated using the Randall and Selitto method from 1957, which measures mechano-hyperalgesia. The baseline nociceptive threshold was assessed in the fourth week after the onset of diabetes caused by STZ. The Randall-Selitto Paw Pressure Test Apparatus was utilized to assess the nociceptive threshold of rats. This was achieved by gradually applying pressure to their hind paws (15).

Figure 5 Randall–Selitto paw pressure test

RT-PCR Study

The rats were euthanized in the fifth week after evaluating the neuropathy parameters and nociceptive threshold. A total of three to six rats in each group were administered with DRGs and spinal cords from the L4-L6 region. Total RNA was then extracted using Triazole reagent, following the manufacturer's suggested methodology for total RNA extraction. Prior to reuse, the RNA samples were correctly labeled and stored at temperatures ranging from -20 to -40oC. In accordance with the manufacturer's instructions, RNA was extracted from the samples using the QI Aamp Mini spin method. After elution, the RNA was stored at a temperature of -18°C or below, and the creation of cDNA was immediately initiated in the PCR clean room (16).

Table 3 The composition of master mix

Name	Volume	Final concentration
5X cDNA synthesis buffer	4ul	1X
dNTP Mix	2ul	500uM each
RNA Primer	1ul	-
RT Enhancer	1ul	-
Verso Enzyme Mix	1ul	-
Template (RNA)	1-5ul	500 ng
Nuclease free water	To 20ul	-
Total Volume	20uls	-

The master mix was thoroughly mixed and aliquot 20µl into the appropriate number of Optical Flat Cap 8/Strip or plates. Reaction parameters were set as described in table 4.

Table 4 The reaction parameters for RT-PCR

Reaction	Temp.	Time	Number of Cycles
cDNA synthesis	42^0C	30min	1 cycle
Inactivation	95^0C	2min	1Cycle

After the run, tubes were carefully stored at -80^0C. PCR was performed for each sample in duplicate and each gene with Negative control. The reaction was performed using 2X Power SYBR™ Green PCR Master Mix Cat. #4367659) ** in a 20.0µl final volume reaction at Nirav Bio Solutions Pvt. Ltd., Pune. The sequence of primers for RT-PCR is tabulated in table 5.

Table 5 The sequence of primer for RT-PCR

Gene title	Symbol	Primer sequence (from 5′- to 3′)
Fibroblast growth factor	FGF	Forward 5'-TGTTCCCTTGACCATTGGCT-3'
		Backward5'-AATTCAGGCTCTGTGGGCTG-3'
Insulin-like growth factor	IGF	Forward 5'GCTGGTGGACGCTCTTCAGT-3'
		Backward5'-TTGAAGTAAAAGCCCCTTGGTC-3'
Nerve Growth Factor	NGF	Forward 5'-GATCGGCGTACAGGCAGAAC-3'
		backward 5'-TCTCCCTCTGGGACATTGCT-3'

Histopathological Studies

Isolation of Paw Skin: To conduct histological investigation, the plantar skin from both hind paws will be extracted and stored in a solution of 10% neutral formalin. After undergoing the Hematoxylin-Eosin staining technique and being mounted with DPX mountant, the slides were analyzed using a 40X microscope. The slides were analyzed using a computerized microscope fitted with a camera (17).

Statistical Analysis

The mean ± standard error of the mean was utilized to describe the findings. The statistical analysis was performed on Graph Pad Prism. The One Way Analysis of Variance (ANOVA) is employed for analysis, followed by the Bonferroni test for post-hoc multiple comparisons among treatment groups. Statistical significance was considered at a significance level of $P<0.05$.

RESULT AND DISCUSSION

Pharmacognostical Evaluation

Following the extraction process, the methanolic extract was obtained and subsequently separated into ethyl acetate fractions. We own a 2.9% share of the EAWC (ethyl acetate-water combination) and a 1.1% share of the ethyl acetate component. The findings of the Pharmacognostic assessment of *Withania coagulans* D. are presented in table 6.

Table 6 The Pharmacognostic evaluation of plants ethyl acetate fractions

Parameters	*Withania coagulans* D.
1) Colour	Dark Brown
2) Odour	Aromatic
3) Taste	Bitter
Ash Values	
1) Total Ash	19.53
2) Acid-insoluble ash	12.93
Extractive Values	
1) Alcohol-soluble extractive	6.40
2) Water Soluble extractive	28.07

Parameters estimated at the end of 28 days dosing

Following a period of 28 days, an assessment was conducted to determine the blood glucose level and body weight of the rats. Two specific doses were selected: 50 mg/kg and 100 mg/kg of body weight. Table 7 presents the results of several variables. The mean± SEM is employed to represent all values. Figures 6 and 7 depict the graphical depiction of the blood glucose level and body weights for each EAWC group, respectively. The rats that were administered demonstrated significantly greater body weights compared to the DC group. However, when given a dosage of 100 mg/kg, their weights were equivalent to those of the groups treated with metformin. When comparing the groups treated with EAWC to the group treated with DC, there was an increase in body weights. However, this increase was not superior to the improvement shown in the group treated with metformin. All EAWC and treated groups exhibited reduced blood glucose levels as compared to the DC and metformin treated groups.

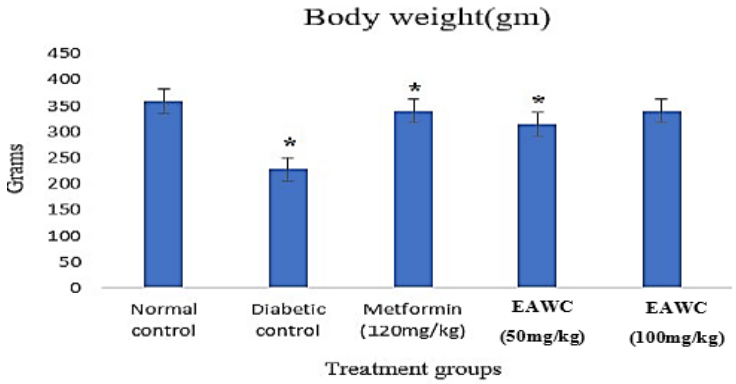

Figure 6 The graph of body weights calculated in each group of EAWC

Table 7 The body weight and blood glucose level were estimated in rats after 28 days

Normal Control	Diabetic Control	Metformin (120mg/kg)	EAWC (50mg/kg)	EAWC (100mg/kg)
Body Weight (Gms)				
357.5 ±36.97	226.16± 14.65*	338.33± 38.02*	260.66± 10.22	284± 12.88
Blood Glucose Level mg/Dl				
71.33±3.23	483.83±5.80****	207.33±24.37****	272.50± 11.43****	247.66±11.09****

Results are expressed as means ± SEM;(n=6), One-way ANOVA followed by Bonferroni test; Vs. respective controls; *p< 0.05, ****p< 0.0001.

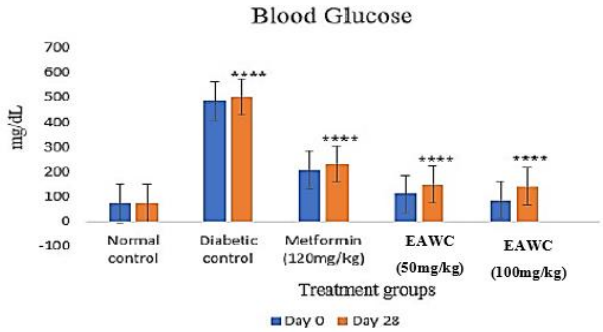

Figure 7 The graph of blood glucose level calculated in each group of EAWC

Behavioral Models of Diabetic Neuropathy

Motor in Co-ordination Study (Rotarod)

The incapacity to maintain balance on the rotating rod for a duration of one minute, as well as the mean duration before falling off, were used as indicators of motor impairments. Table 8 displays a compilation of the results from the motor coordination research. Figure 8 displays the motor graphs obtained from the EAWC coordination tests. The treated group has shown significant results in comparison to the group treated with gabapentin. The motor and coordination activities of the rats have improved as a consequence. Although EAWC2 showed promising results, it did not match the outcomes of the groups getting gabapentin therapy.

Table 8 The motor in coordination studies of ethyl acetate fractions, latency recorded in seconds

Normal Control	Diabetic Control	Gabapentin (100mg/kg)	EAWC (50mg/kg)	EAWC (100mg/kg)
211.3333±27.28	18.83333±3.96****	185.3333±20.77****	107.8333±19.47*	156.8333±15.55***

Results are expressed as means ± SEM;(n=6), One-way ANOVA followed by Bonferroni test; Vs. respective controls; *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001.

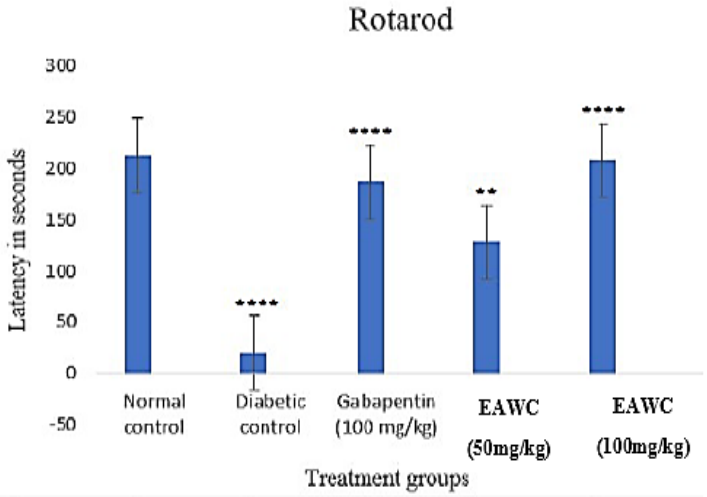

Figure 8 The graph of motor in coordination studies of EAWC

Thermal Allodynia (Eddy's hotplate)

A timer was employed to measure the duration until the animal engaged in either licking its hind paws or leaping. Table 9 displays the thermal allodynia data for both ethyl acetate fractions (EAWC). Figure 9 displays the graphs obtained from the thermal allodynia experiments carried out by EAWC. Although group 2 also did better than the group treated with gabapentin, the groups treated with EAWC2 showed exceptional outcomes that surpassed even those of the gabapentin-treated group.

Table 9 The results of thermal allodynia of both the ethyl acetate fractions Paw withdrawal latency recorded in seconds

Normal Control	Diabetic Control	Gabapentin (100mg/kg)	EAWC (50mg/kg)	EAWC (100mg/kg)
42.6666±5.95	8.6666±1.78****	44.3333±5.79***	26.8333±3.45*	46.6666±4.04****

Results are expressed as means ± SEM;(n=6), One-way ANOVA followed by Bonferroni test; Vs. respective controls; *p< 0.05, ***p< 0.001, ****p< 0.0001.

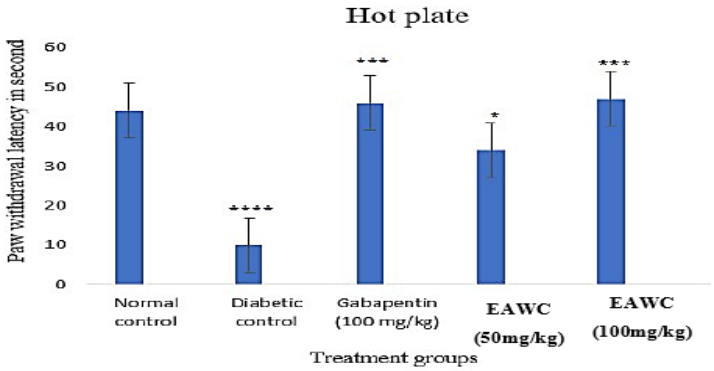

Figure 9 The graph of thermal allodynia studies of EAWC

Cold allodynia (Cold Water Tail immersion)

Tail pullout delay was seen in each group. Table 10 presents the results of the cold allodynia analysis. Figure 10 displays the results of the EAWC's cold allodynia graphs. Significant tail withdrawal delay was observed in both the EAWC and treatment groups. However, the delay showed improvement in a manner that was dependent on the dosage. The tail pullout latency was measured in seconds.

Table 10 The results of cold allodynia of both the ethyl acetate fractions.

Normal Control	Diabetic Control	Gabapentin (100mg/kg)	EAWC (50mg/kg)	EAWC (100mg/kg)
36.1666±4.68	10.8333±3.60****	36.8333±2.86****	32.5±2.86***	37.3333±2.33****

Results are expressed as means ± SEM;(n=6), One-way ANOVA followed by Bonferroni test; Vs. respective controls; ***p< 0.001, ****p< 0.0001.

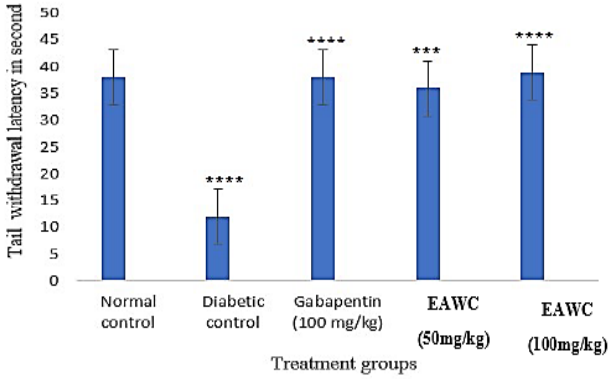

Figure 10 The graph of results of cold allodynia of EAWC

Mechano-tactile allodynia (Von Frey Hair Test)

After four weeks of STZ injection, the groups were evaluated for withdrawal threshold (tactile) reactions. The results for mechano-tactile allodynia for both ethyl acetate fractions is presented in Table 11. Figure 11 displays the graphical representations of the findings from the Von Frey Hair Test for the EAWC. EAWC exhibited a dose-dependent rise in the withdrawal threshold response, surpassing the effectiveness of gabapentin treatment. The threshold for paw withdrawal, measured in grams, is presented.

Table 11 The results of mechano-tactile allodynia of both the ethyl acetate fractions.

Normal Control	Diabetic Control	Gabapentin (100mg/kg)	EAWC (50mg/kg)	EAWC (100mg/kg)
9.1666±0.88	4.7533±0.38****	7.8433±0.59*	7.4833±0.35*	8.8833±0.73***

Results are expressed as means ± SEM;(n=6), One-way ANOVA followed by Bonferroni test; Vs. respective controls; *p< 0.05, ** p< 0.01, *** p< 0.001, ****p< 0.0001.

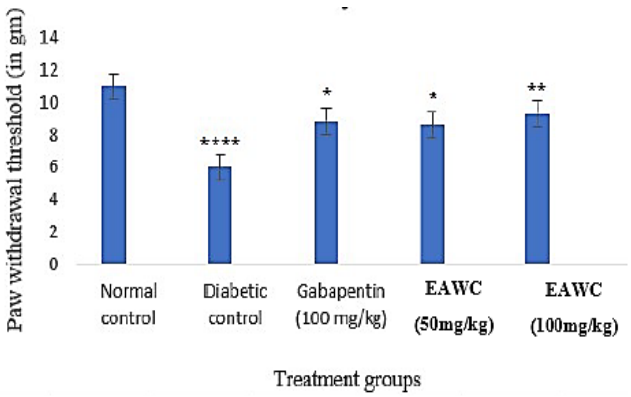

Figure 11 The graph of results of Von Frey Hair Test of EAWC

Mechanical Hyperalgesia (Randall–Selitto Paw Pressure Test)

After four weeks of STZ injection, the mechanical nociceptive threshold, which is a measure of mechano-hyperalgesia, was evaluated in each group. Table 12 presents the results of studies on mechano-hyperalgesia. Figure 12 displays the graphs representing the results of the EAWC mechano-hyperalgesia experiments. The EAWC (50mg/kg) and EAWC (100mg/kg) treated groups had a significantly elevated mechanical nociceptive threshold in comparison to the group treated with gabapentin. The paw withdrawal threshold, measured in grams, is provided.

Table 12 The results of mechano-hyperalgesia studies of both the ethyl acetate fractions

Normal Control	Diabetic Control	Gabapentin (100mg/kg)	EAWC (50mg/kg)	EAWC (100mg/kg)
183.6666±12.26	42.1666±2.89****	155±11.41****	135.1666±10.94****	156.5±11.16****

Results are expressed as means ± SEM;(n=6), One-way ANOVA followed by Bonferroni test; Vs. respective controls; ****p< 0.0001.

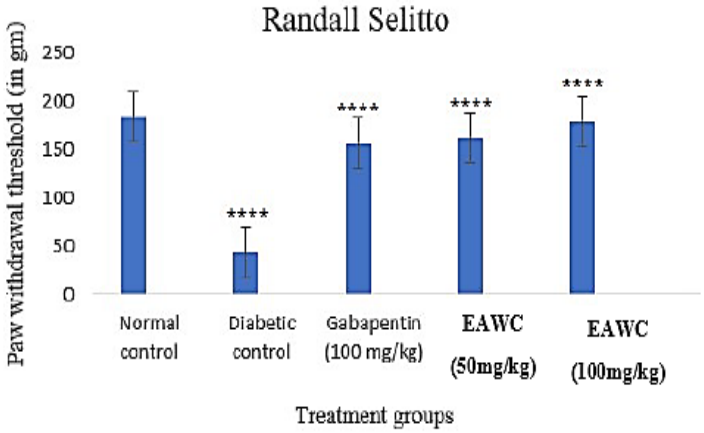

Figure 12 Results obtained through mechano-hyperalgesia studies of EAWC

RT-PCR Study (DRG from L4-L6 Region)

Insulin-like Growth Factor (IGF)

The IGF was quantified in each group of animal models using the RT-PCR technique for both ethyl acetate fractions. Table 13 provides the computed IGF values for the animal models of the two ethyl acetate fractions. Figure 13 displays the graphs illustrating the results of the IGF measurements in the animal models treated with both ethyl acetate fractions (EAWC). The treatment groups exhibited significant and dose-dependent increases in IGF when compared to the group treated with gabapentin. The groups who received EAWC therapy did not exhibit a substantial rise in their IGF levels.

Table 13 A determined IGF for animal models of both the ethyl acetate fractions Relative gene expression level was measured by using RT-PCR

Normal Control	Diabetic Control	Gabapentin (100mg/kg)	EAWC (50mg/kg)	EAWC (100mg/kg)
0.0491±0.007	0.0016±0.0003 ****	0.0098±0.02	0.0144±0.0003*	0.0087±0.0002**

Results are expressed as means ± SEM;(n=6), One-way ANOVA followed by Bonferroni test; Vs. respective controls; *p< 0.05, **p< 0.05, ****p< 0.0001.

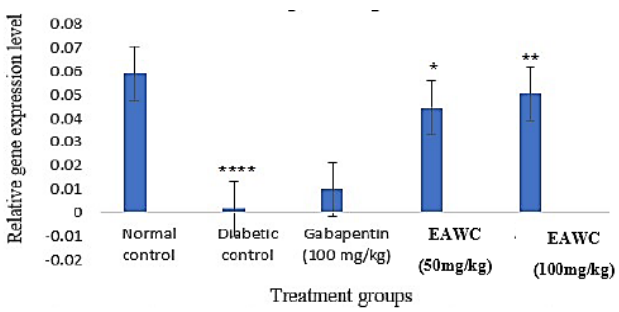

Figure 13 The graph of results obtained from determinations of IGF in the animal model of EAWC&

Nerve Growth Factor (NGF)

The NGF in both ethyl acetate fractions was assessed using the RT-PCR technique in each group of animal models. Table 14 presents the computed NGF values for both ethyl acetate fractions in animal models. Figure 14 displays the graphs depicting the results of the NGF measurements conducted on the animal models treated with both ethyl acetate fractions (EAWC). The treatment groups demonstrated an increase in NGF levels that was dependent on the dosage, surpassing the group treated with gabapentin

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Table 14 A determined NGF for animal models of both the ethyl acetate fractions Relative gene expression level was measured by using RT-PCR

Normal Control	Diabetic Control	Gabapentin (100mg/kg)	EAWC (50mg/kg)	EAWC (100mg/kg)
0.00993±0.002	0.0015±0.0002** **	0.00375±0.0004	0.0059±0.001*	0.0153±0.0019***

Results are expressed as means ± SEM;(n=6), One-way ANOVA followed by Bonferroni test; Vs. respective controls; *p< 0.05, ***p< 0.001.

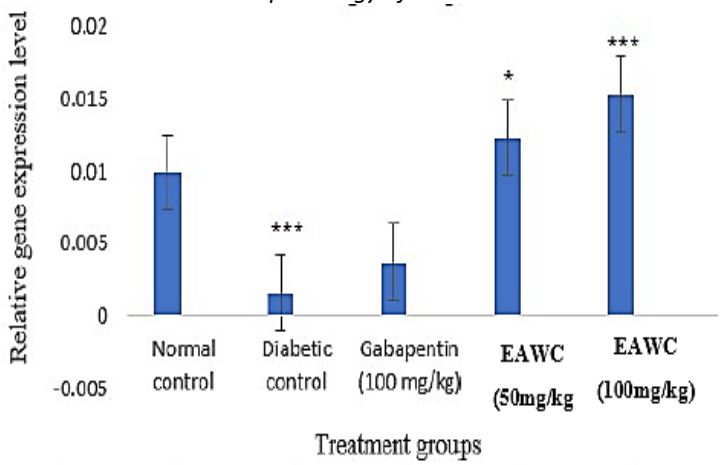

Figure 14 The graph of results obtained from determinations of NGF in the animal model of EAWC&

Fibroblast Growth Factor (FGF)

The FGF was determined using the RT-PCR technique for both ethyl acetate fractions in each group of animal models. Table 15 presents the computed FGF values for the ethyl acetate fractions in animal models. Figure 15 displays the graphs depicting the results of FGF assays in animal models treated with both the ethyl acetate fractions (EAWC and). Both plant ethyl acetate fractions significantly increased FGF levels in a manner that depended on the dosage. However, FGF showed more improvement compared to EAWC.

Table 15 A determined FGF for animal models of both the ethyl acetate fractions Relative gene expression level was measured by using RT-PCR

Normal Control	Diabetic Control	Gabapentin (100mg/kg)	EAWC (50mg/kg)	EAWC (100mg/kg)
0.0440±0.004	0.00315±0.0004 ****	0.0140±0.002	0.0166±0.002*	0.0263±0.0008***

Results are expressed as means ± SEM;(n=6), One-way ANOVA followed by Bonferroni test; Vs. respective controls; *p< 0.05, ***p< 0.001, ****p< 0.0001.

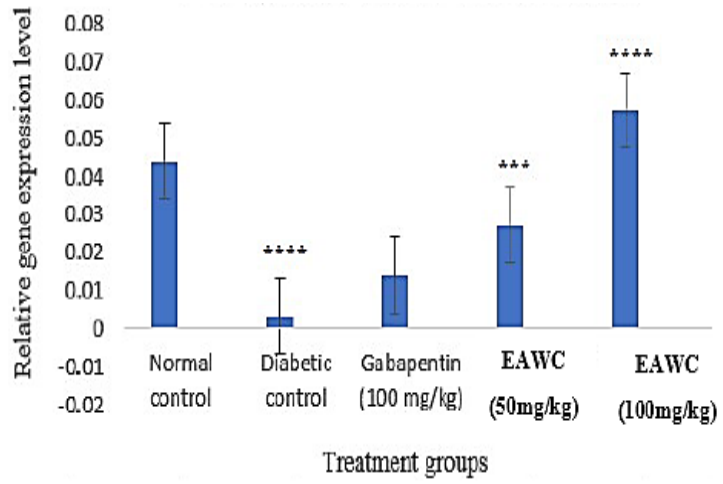

Figure 15 The graph of results obtained from determinations of FGF in the animal model of EAWC

Histopathological Studies

Table 16 displays the histopathology of the paw skin in animal models treated with ethyl acetate fractions. The normal control rat has a distinct layer of keratinocytes (k) on its outer surface, which serves as the typical boundary between the epidermis and dermis. In contrast, the diabetic control rat exhibits a significant decrease in the thickness of the epidermis. The outer surface of the diabetic rat treated with a low dose of EAWC (50 mg/kg) shows a slight increase in epidermis thickness, while the diabetic rat treated with a high dose of EAWC (100 mg/kg) displays an epidermis that is nearly normal in thickness and comparable to that of a rat treated with normal saline.

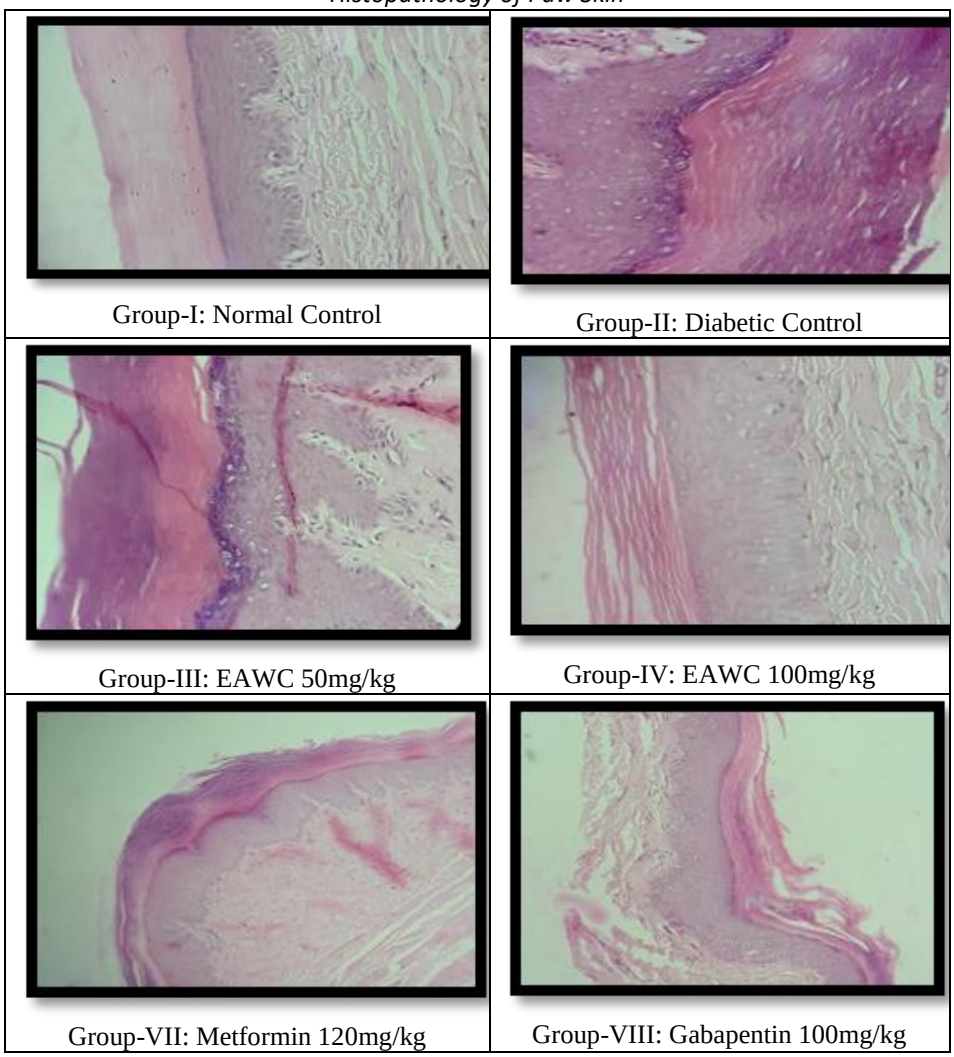

Table 16 The histopathology of the paw skin

SUMMARY AND CONCLUSION

Administering a daily dose of ethyl acetate fraction of W. *coagulans* at 50mg/kg of body weight for 30 days resulted in the restoration of plasma glucose, the findings of this study indicate that regular intake of ethyl acetate fraction of *W. coagulans* extract for 28 days significantly improved glycemic status and almost normalized plasma glucose concentrations (18-20). Thus, it can be concluded that ethyl acetate fraction of W. *coagulans* extract contains active components that have antihyperglycemic effects. Further research is being conducted to investigate the mechanism of action of these active components in exerting their antidiabetic/antihyperglycemic effects (21).

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