Ameliorative effect of watercress (Nasturtium officinale) aqueous extract on gene expression of Glut4 and Ampk in diabetic rats

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Abstract

Background: One of the most prevalent chronic illnesses in the nation and the world is diabetes. It remains as one of the leading cause of death so it's important to create cutting-edge, potentially cost-effective co-treatment techniques derived from plants that don't have any negative impacts. Previous research has shown that watercress (Nasturtium officinale) possesses anti-inflammatory, antioxidant, and anti-diabetic properties.

Objectives: to investigate the effects of watercress aqueous extract on gene expression of glucose transporter 4 (GLUT4) and 5' AMP-activated protein kinase (AMPK) in streptozotocin induced diabetic rats.

Materials and methods: Sixty healthy male albino rats were used in this study. Animals were split into four equal groups, each of 15 rats. Group 1: (Negative Control), Group 2: (Diabetes Positive Control injected with (45mg/kg body weight) Streptozotocin intraperitoneally), Group 3 (diabetic rats received 100 mg of watercress aqueous extract /Kg body weight for 8 weeks, daily) and Group 4 (diabetic rats received 200 mg of watercress aqueous extract /Kg body weight for 8 weeks, daily). Blood was collected after scarification on weeks 2, 4, 6, and 8 of the experiment for serum separation and pancreatic tissues were collected on weeks 4 and 8 of the experiment. Fasting blood glucose as measured by glucometer, insulin hormone was measured by ELISA kit.Triglyceride, LDL, HDL, creatinine, urea, AST and ALT were measured by spectrophotometer. SYBR Green qPCR Master Mix was used for measurement of GLUT4, AMPK and Beta-actin.

Results: The treated group exhibited a significant and progressive decrease in fasting blood glucose, triglycerides, total cholesterol, LDL, HDL, creatinine, urea, ALT, and AST and a significant and progressive increase in insulin hormone at all experiment times compared to the controls. The 4th group exhibited a significant and progressive up regulation gene expression of GLUT4 and AMPK genes at 4 and 8 weeks compared to the controls considering watercress as an alternative treatment for diabetes.

Conclusion. Watercress aqueous extract had positive effects on lowering and maintaining normal glucose levels.

Keywords: Diabetes, Watercress, Gene expression, GLUT4, AMPK.

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Introduction

Diabetes mellitus is one of the most common metabolic illnesses, is spreading alarmingly around the world. The annual budget for health care is mostly allocated to diabetes and related conditions each year. Over 382 million individuals worldwide were expected to have diabetes in 2013. According to WHO projections, by 2030, diabetes would rank as the seventh leading cause of death **(Alam et al.,2021).**

A prevalent chronic metabolic disorder that poses a major threat to human health is diabetes mellitus **(Galicia-Garcia et al. ,2020).** In patients with diabetes mellitus, microvascular and macrovascular problems are regarded as significant pathophysiologic manifestations **(Llauradó et al.,2022).** Vascular endothelial damage brought on by hyperglycaemia is thought to be one of the earliest signs of cardiovascular problems in diabetes mellitus, and it is thought to be the primary causative factor of the pathologic alterations of DM **(Mazrouei et al., 2022).**

Metabolic diseases characterized by hyperglycaemia resulting from insulin resistance, insufficiency, or both can be linked to diabetes mellitus, a complex metabolic disease **(Al-Saeedi et al., 2021**). DM comes in four primary common forms, Type 1, Type 2 diabetes, Gestational diabetes mellitus (GDM), and monogenic diabetes **(Alam et al.2021).** Long-term blood glucose increases are associated with macro- and microvascular disorders that can lead to renal disease, heart disease, stroke, and other serious illnesses. In addition to hyperglycaemia, other factors that contribute to the pathophysiology of diabetes include hyperlipidaemia and oxidative stress, which increase the risk of diabetic complications. **(Kangralkar et al., 2010).**

Apart from the total loss or destruction of pancreatic β cells, which results in insulin-dependent Diabetes mellitus (type1), streptozotocin has also been shown to cause peripheral insulin resistance or decrease the release of insulin from these cells. Among other factors, the dosage, age, strain, nutritional status, and mode of administration of STZ can result in mild to severe hyperglycaemia in animals **(Hayashi et al., 2006).**

These days, novel drugs and many modern medications originate from plants **(Shakya, 2016)**. Traditionally, watercress leaves have been used as a stimulant, hypoglycaemic, expectorant, depurative, diuretic, and stomachic. Meanwhile, it has been used to treat calculi, scurvy, tuberculosis, asthma, bronchitis, jaundice, and tuberculosis. Glucosinolates, carotenoids, polyphenols, vitamin C, vitamin A, and α-tocopherol are abundant in N. officinale. It serves as the primary source of folic acid, calcium, iodine, and iron **(Chaudhary et al., 2018).**

Watercress, or Nasturtium *Nasturtium officinale* a native of Western Asia, India, Europe, and Africa. It belongs to the Brassicaceae family, is a high-value, wild herb that is perennially aquatic or semi-aquatic and used in cooking by people almost everywhere. Its distribution is now nearly worldwide. It is full of vitamins, has strong flavour, and gorgeous dark green leaves **(Yamuna et al. ,2018).**

The purpose of this study is to examine how the watercress aqueous extract affects the expression of the glucose transporter 4 (GLUT4) and 5' AMP-activated protein kinase (AMPK) genes in streptozotocin-induced diabetic rats.

Materials and methods

Ethical Considerations: Approved by the Veterinary Medical Research Ethics

Committee, animal handling and rights, Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt. (Approval number: Soh.un.vet /00029R1).

Materials

Animals: Sixty Healthy Male Albino Rats, 3 months old, weighting about 320 ±20-gram, were purchased from the faculty 0f science, sohag university and housed in Animal Laboratory House, Faculty of Science, Sohag University, Sohag, Egypt at temperature of 23±2 °C with a 12 h–12 h dark/light cycle and were allowed free access to food and water ad libitum. Rats were kept in eight 75 x 50 x 35 cm metal cages in groups. Prior to the trial, the rats were kept under observation for one week. The animals were not fed for eighteen hours prior to the experiment's start.

Chemicals: We bought streptozotocin, trisodium citrate dihydrate, and citric acid monohydrate from the Sigma-Aldrich Company, which is located in St. Louis, Missouri, in the United States

Analytic kits

1. ABT Total RNA Mini Extraction kit (spin column) (Catalog No, ABT002) (Applied Biotechnology Company, Ismailia, Egypt).
2. ABT H-minus cDNA Synthesis kit (Catalog No, ABT009) (Applied Biotechnology Company, Ismailia, Egypt).
3. Maxima SYBR Green qRT-PCR Master Mix (Catalog No, k0251) (Thermo Fisher Scientific Company, US) was purchased for measurement of GLUT4, AMPK and Beta-actin.
4. Primers: The specific primers of (GLUT4), 5' AMP- (AMPK) and Beta-actin were used for amplification of different genes in real-time PCR analysis- Thermo Fisher Scientific Company, are showed in (**Table .1**).

Table .1. Forward primer and reverse primer of GLUT4, AMPK and Beta-actin genes (Abdelrahman et al. ,2024):

Gene name	Forward primer	Reverse primer
GLUT4	GCAACGTGGCTGGGTAGGCA	CCCACAGAGAAGATGGCCACGG
AMPK	CAGGCATATGGTGGTCCATAGAG	TCATGGGATCCACCTGCAGC
Beta-actin	ACTCTGTGTGGATTGGTGGC	CGCAGCTCAGTAACAGTCCG

Experimental design

The experiment was designed in 4 groups (n=15) to finalize the aims of this study as shown in (**Fig. 1**):

Group 1: (Negative Control): Control healthy rats this group of rats received standerd rat ration and drinking water

Group 2: (Diabetes Positive Control): Streptozotocin (STZ)-induced diabetic rats intraperitoneally injected with as 45 mg streptozotocin /kg body weight without administration of watercress

Group 3: Eight weeks of daily administration of one dosage of 100 mg watercress/kg body weight was given to STZ-induced diabetic rats administrated orally using oral gavage.

Group 4: For eight weeks, 200 mg of watercress per kilogram of body weight was administered orally using oral gavage to STZ-induced diabetic rats once daily, as shown in (**Fig. 1**).

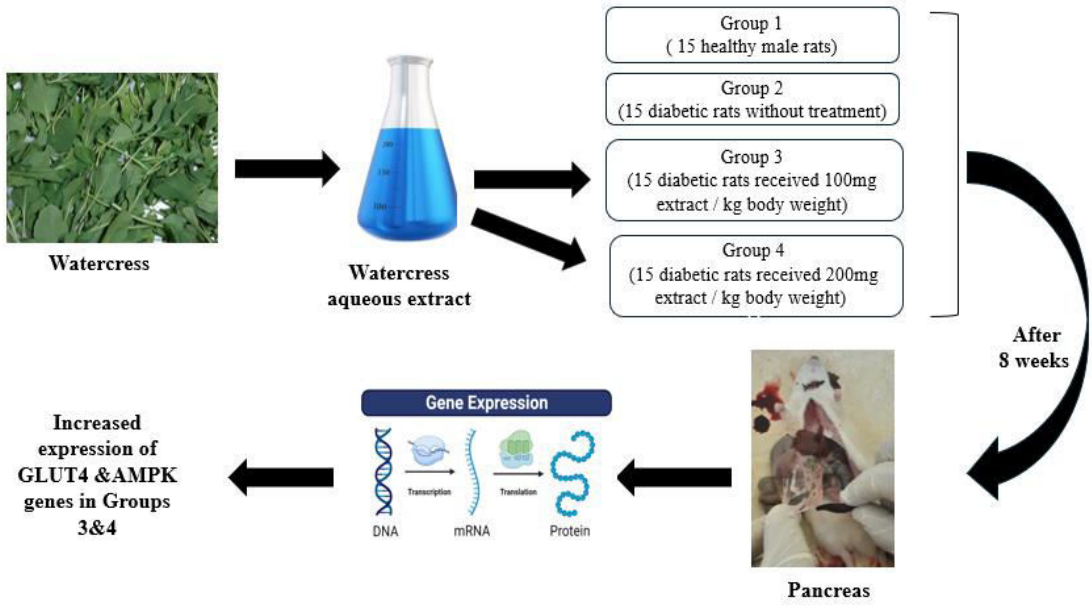

Fig.1. Summarized figure for the experiment

Methods

Induction of diabetes: To develop diabetes in the overnight fasting rats, a single intraperitoneal injection of 45 mg/kg streptozotocin (Sigma-Aldrich, USA). To develop diabetes in the overnight fasting rats, a single intraperitoneal injection of 45 mg/kg streptozotocin (Sigma-Aldrich, USA) freshly dissolved in 0.1 M citrate buffer (pH 4.5) was given,. **(Salah-Eldin et al.2015).**

Forty-eight (48) hours after STZ injection, tail was pricked, blood samples were collected, then by using a glucometer, blood glucose levels were measured to diagnose diabetes and by looking for polydipsia and polyuria.

For the following experiment, Diabetic animals were exclusively identified from STZ-injected rats whose blood glucose levels were 250 mg/dl or above. After receiving STZ, rats were allowed to have their normal diet and water free to prevent hypoglycaemic shock, a 15% glucose solution was also added to the drinking water. Day 0 was the day that the presence of hyperglycaemia had been confirmed.

plant material and aqueous extract: -

Aerial parts of the watercress (Nasturtium officinale) were obtained from an accredited supplier, in Akhmim city (Sohag, Egypt). A botanist from the Division of Botany, Faculty of Science, Sohag University, Egypt, identified plant samples. The plant was dried in shadow (green watercress contains 10% dry matter and each 1-gram dry matter contains 0.375-gram active principle) then boiled in distilled water, filtered and the filtrated solution was the extract.

Administrating the plant extract: For eight weeks, rats in groups 3 and 4 were daily orally given the plant extract by using oral gavage (o.g) at a dose of 1 ml/rat (equivalent to 100 and 200 mg/kg body weight) (Shahrokhi et al., 2009). The rats in diabetic (group 2) and the control healthy rats (group 1) were given the same volume of distilled water daily oral using oral gavage (o.g).

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(group 2, n = 15) and the control healthy rats (group 1, n = 15).

Samples collection: Blood samples were obtained by scarification of both the normal and STZ-induced diabetic rates at 2,4,6 and 8 weeks in three clean dried tubes without anticoagulant, then placed them in liquid nitrogen and kept at -80 ^{o}C to protect their mRNA until they were extracted. Quantitative (real-time PCR) was then used to measure the expression levels of GLUT4 and AMPK in pancreatic tissue at 4 and 8 weeks.

Quantitative real time PCR(Qrt-PCR) analysis of GLUT 4 and AMPK

A) RNA extraction: Using Total RNA Mini Extraction kit (spin column) (Catalog No, ABT002), according to the enclosed instructions. Using Nanodrop Spectrophotometry (Quawell 5000, Taiwan), the concentration and purity of the extracted RNA were evaluated. Because extracted RNA is susceptible to RNAase degradation, it needs to be stored frozen at -80 oC and converted to cDNA as soon as possible.

B) Reverse transcription: Using (H-minus cDNA Synthesis kit; Catalog No. ABT009), the extracted RNA was converted to cDNA. 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and 4°C for 20 hours were the temperature settings set for the thermal cycler. The produced DNA was frozen at -20 C and allowed to cool before being used.

C) Real time PCR: The qRT-PCR analyser (Step One, Applied Bio systems, Singapore) was utilized with prepared cDNA, and the MAXIMA SYBR Green qPCR Master Mix (Catalog No., K0251) was used to choose the specific primers. Table (1) shown the forward and reverse primer sequences used. In with the following program: 1 cycle at 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds; one cycle at 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds.

Each PCR run was conducted in three separate runs. Competitive threshold cycle analysis was used to determine relevant gene expressions. The cycle threshold (Ct) was employed by the 2-ΔΔCt Method (**Livak and Schmittgen, 2001**).

Based on fold changes in Glut4 and AMPK gene levels relative to levels in Negative Control samples, gene expression was assessed.

(ΔCt) Delta Ct for samples = (ΔCt) of target gene (Glut4 or AMPK) ¬(ΔCt) of reference gene (β-actin).

(ΔCt) Delta Ct for the controls = (ΔCt) of target gene (Glut4 or AMPK) ¬(ΔCt) of reference gene (β-actin).

(ΔΔCt) Delta delta Ct = delta Ct of samples - delta Ct of control.

Mean fold change of the target gene = 2- ΔΔCt

Statistical analysis

GraphPad Prism 9 (GraphPad, Inc., San Diego, CA) was used to analyse the data. For multiple comparisons, one-way ANOVA (Tukey and Duncan tests) was utilized to identify group differences. The standard deviation of the mean (SD) ± mean was used to express all the data. At the level of $P < 0.05$, statistical significance was taken into account.

Results

The results obtained in this study were statistically analysed, the mean and standard deviation values of the gene expression of (GLUT4) and (AMPK) of the Streptozotocin-induced diabetic rats in the treated and control groups were presented in (**Tables 2- 4** and **Figs 2 – 6**).

Our results indicate that the aqueous extract of watercress has an ameliorative effect on gene expression of GLUT4 and AMPK as follow:

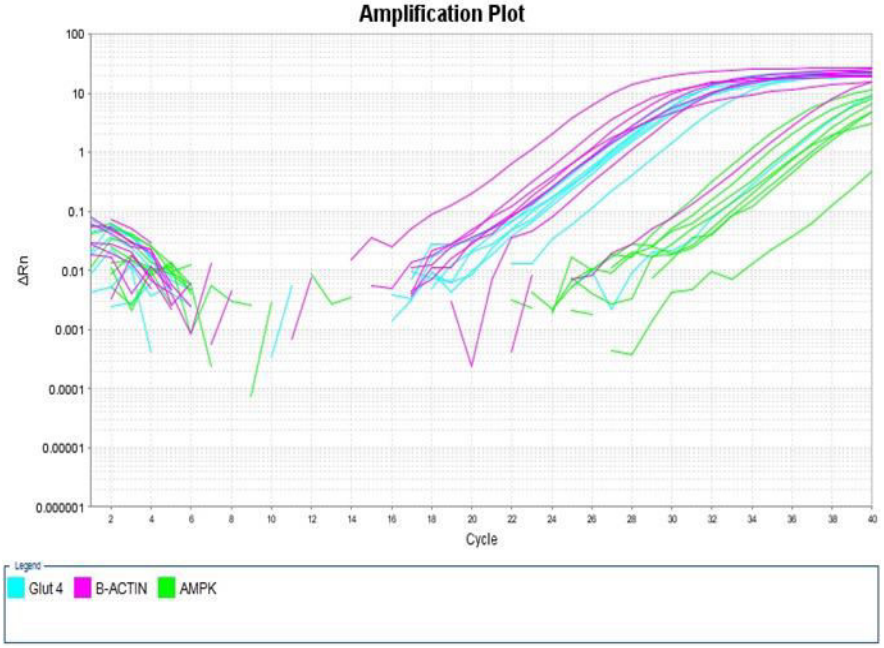
The data represented in (**Table.2**) and **Figs. (2&3)** showed that expression of GLUT4 gene non significantly up regulated (p =0.1) and

expression of AMPK gene significantly up regulated ($p < 0.05$) at 4 weeks in group 3 compared to the controls.

Table 2. Effects of watercress aqueous extract on gene expression (RQ) of GLUT4 and AMPK in experimental rats of group 1, 2 and 3 (at 4 weeks)

Experimental periods	Groups			P value
	Group 1	Group 2	Group 3	
GLUT 4	1± 0	6.1± 0.6	7.5± 5.4	0.1
AMPK	1± 0 [a]	6.33± 1.6 [b]	15.03± 1.1 [c]	0.000

[A, b, c] in the same row means that there were significant differences between the collected samples at $p < 0.05$.



Fig,2. Amplification plot of a run of 9 samples showing GLUT4 and AMPK expression to β-actin in experimental rats of group 1, 2 and 3 (at 4 weeks).

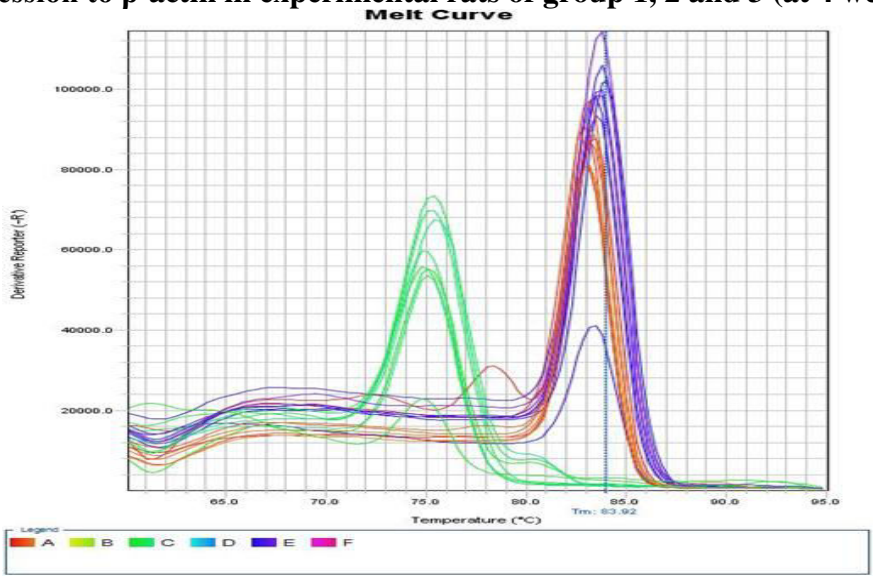


Fig.3. Melting curve of a run of 9 samples showing GLUT4 and AMPK expression to β-actin in experimental rats of group 1, 2 and 3 (at 4 weeks).

The data represented in (**Table .3, Figs. 4&5**) showed that expression of GLUT4 and AMPK genes significantly increased ($p < 0.05$) at 8 weeks in both treated groups compared to the controls.

Table 3. Effects of watercress aqueous extract on gene expression (RQ) of GLUT4 and AMPK in experimental rats (at 8 weeks)

Experimental periods	Groups			P value
	Group 1	Group 2	Group 4	
GLUT 4	1± 0 [a]	4.8± 1.35 [b]	14.17± 1.95 [c]	0.006
AMPK	1± 0 [a]	5.3± 1.61 [b]	17. 36± 3.27 [c]	0.0002

[A, b, c] in the same row means that there were significant differences between the collected samples at $p < 0.05$.

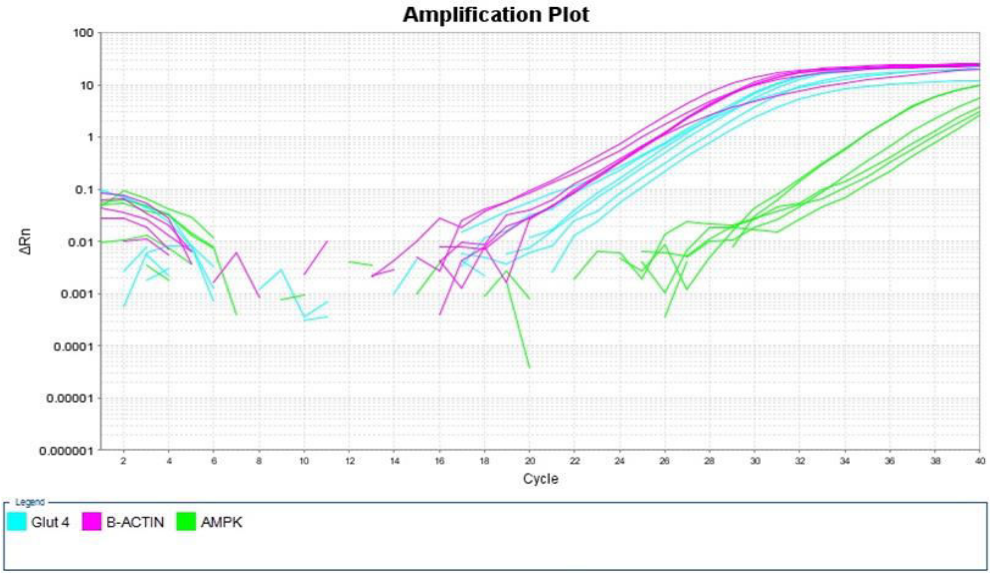


Fig.4. Amplification plot of a run of 9 samples showing GLUT4 and AMPK expression to β-actin in experimental rats of group 1, 2 and 4 (at 8 weeks).

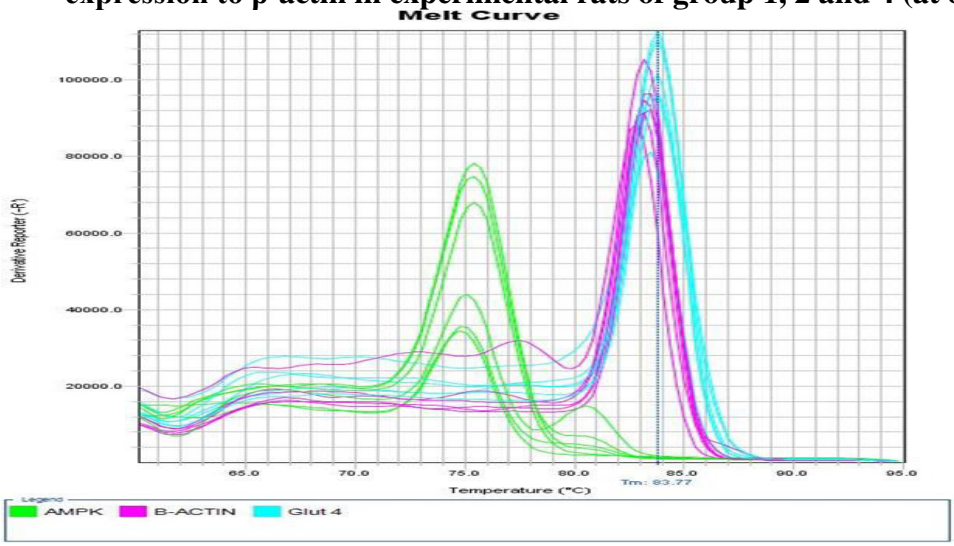


Fig.5.Melting curve of a run of 9 samples showing GLUT4 and AMPK expression to β-actin in experimental rats of group 1, 2 and 4 (at 8 weeks).

The data represented in (**Table.4, Figs.6&7**) showed that expression of GLUT4 and AMPK genes significantly increased ($p < 0.05$) at 8 weeks in both treated groups compared to the controls.

Table 4.Effects of watercress aqueous extract on gene expression (RQ) of GLUT4 and AMPK in experimental rats (at 8 weeks)

Experimental periods	Groups				P value
	Group 1	Group 2	Group 3	Group 4	
GLUT 4	1± 0 [a]	6.5± 1.12 [b]	12.37±1.55[c]	17.86± 1.43[d]	0.000
AMPK	1± 0 [a]	5± 1.05 [b]	18.77± 2.34[c]	20.92± 1.89 [d]	0.000

A, b, c, d in the same row means that there were significant differences between the collected samples at $p < 0.05$.

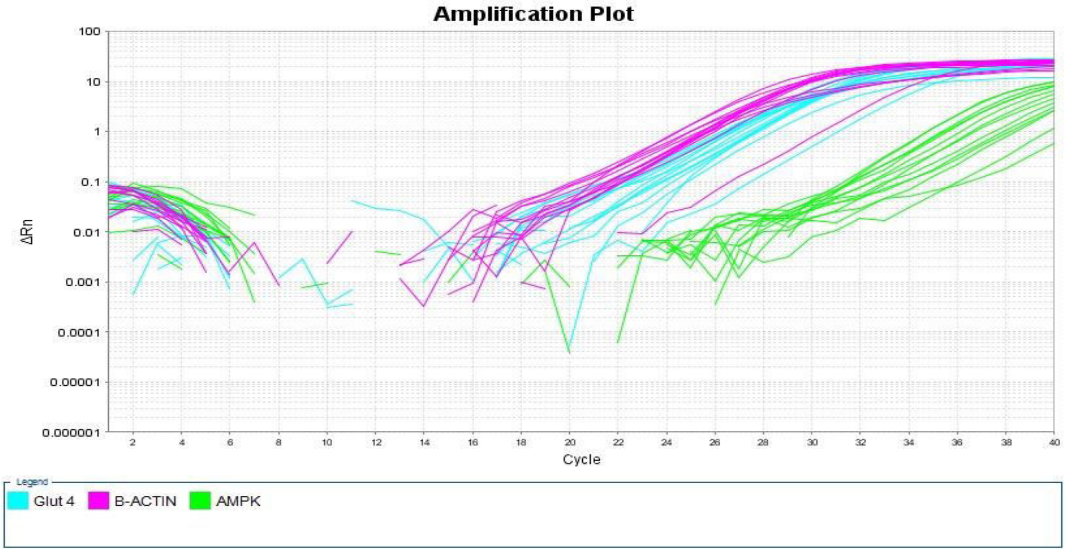


Fig.6. Amplification plot of a run of 12 samples showing GLUT4 and AMPK expression to β-actin (at 8 weeks).

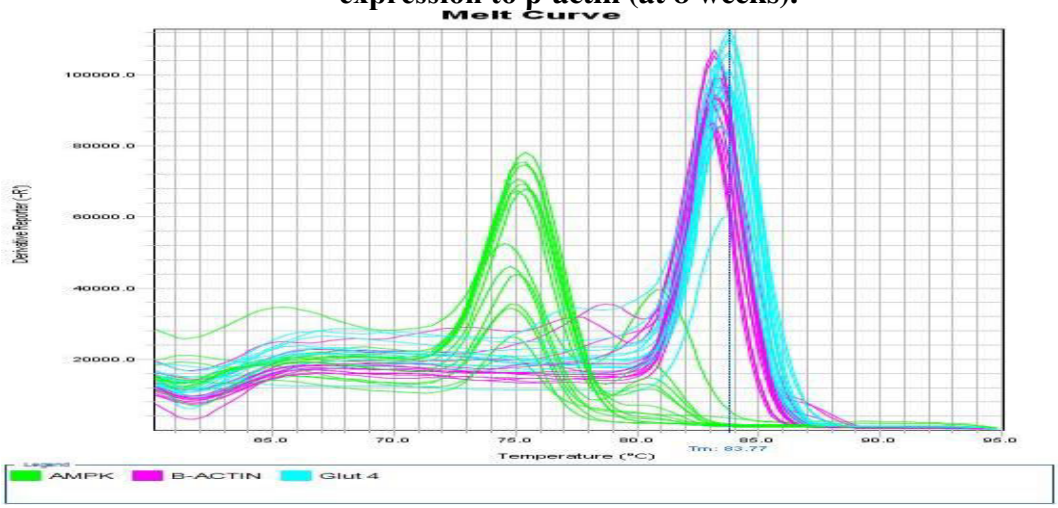


Fig.7. Melting curve of a run of 12 samples showing GLUT4 and AMPK expression to β-actin (at 8 weeks).

Table (5): Effects of watercress aqueous extract on fasting blood glucose levels (mg/dl) in experimental rats:

Experimental periods	Groups				P value
	Group 1	Group 2	Group 3	Group 4	
2 weeks	100.33 ± 11.68 [a]	445 ± 16.7 [c]	353 ± 54.02 [b]	377.3 ± 38.07 [b]	0.001
4 weeks	101.7 ± 14.01 [a]	439.3 ± 43.12 [c]	354 ± 9.63 [b]	314.6 ± 45.36 [b]	0.001
6 weeks	102.6 ± 6.65 [a]	475 ± 40.28 [d]	317 ± 15.71 [c]	215 ± 7.21 [b]	0.001
8 weeks	102.67 ± 6.03 [a]	486 ± 18.68 [d]	266.3 ± 17.09 [c]	179.7 ± 11.5 [b]	0.000

Values are expressed as means ± SD for 60 rats.[A, b, c, d] in the same row means that there were significant differences between the collected samples at $p < 0.05$.

Table 6. Effects of watercress aqueous extract on insulin hormone (µIU/ml) in experimental rats:

Experimental periods	Groups				P value
	Group 1	Group 2	Group 3	Group 4	
2 weeks	1.52 ± 0.02 [b]	1.04 ± 0.04 [a]	1.14 ± 0.18 [a]	1.11 ± 0.16 [a]	0.003
4 weeks	1.4 ± 0.03 [b]	1.06 ± 0.19 [a]	1.24 ± 0.19 [ab]	1.25 ± 0.09 [ab]	0.015
6 weeks	1.71 ± 0.03 [c]	0.84 ± 0.13 [a]	1.26 ± 0.05 [b]	1.41 ± 0.14 [bc]	0.001
8 weeks	1.61 ± 0.05 [c]	1.1 ± 0.03 [a]	1.3 ± 0.12 [b]	1.52 ± 0.08 [c]	0.001

Values are expressed as means ± SD for 60 rats.[A, b, c] in the same row means that there were significant differences between the collected samples at $p < 0.05$.

Table 7. Effects of watercress aqueous extract on HbA1c (%) in experimental rats (at 8 weeks):

Experimental periods	Groups				P value
	Group 1	Group 2	Group 3	Group 4	
8 weeks	4.19 ± 0.49 [a]	11.05 ± 1.55 [d]	9.42 ± 0.81 [c]	7.38 ± 0.76 [b]	0.001

Values are expressed as means ± SD for 60 rats.[A, b, c, d] in the same row means that there were significant differences between the collected samples at $p < 0.05$.

Discussion

Worldwide, there are numerous traditional plant-based treatments for diabetes mellitus. Diabetes management that has no adverse effects is still a challenge for the healthcare system. The demand for natural products with fewer side effects and antidiabetic activity increased as a result. Numerous herbs and plant products have been demonstrated to have hypoglycemic action, according to a review of the literature. Flavonoids, which are found in watercress, are recognized to be bioactive antidiabetic agents **(Jadhav and Puchchakayala, 2012).**

This work was carried out to evaluate the gene expression of Glucose

transporter 4 (GLUT4) and 5' AMP-activated protein kinase (AMPK) of streptozotocin-induced diabetic rats received watercress aqueous extract daily orally for 8 consecutive weeks

The study's findings suggest that GLUT4 and AMPK gene expression can be positively impacted by the aqueous extract of watercress. In our study we have observed that the expression of GLUT4 gene non significantly up regulated ($p < 0.05$) in group 3 but significantly up regulated ($p < 0.05$) in group 4 compared to the controls while expression of AMPK gene significantly up regulated ($p < 0.05$) in groups 3 and 4 compared to the controls at 4 weeks as in tables (2 and 3) and expression of both genes significantly up regulated ($p < 0.05$) at 8 weeks in both treated groups compared to the controls.

The hypoglycemic effect of watercress extract is attributed to the plant's metabolites, which include flavonoids, rutin, quercetin, flavonol, kaempferol, and glucosinolate (mostly highly hydroxylated gluconasturtiin). According to reports, these substances work by inhibiting the intestine's glucose transporters from carrying glucose to the peripheral tissues. Additionally, they cause skeletal muscles and white adipose tissues to release more insulin and activate adenosine monophosphate (activated protein kinase), or ATP. This greatly raises GLUT4 expression, which in turn causes these substances to absorb more glucose **(Jadhav and Puchchakayala, 2012 and Oyenihi et al. 2014).** The synergies of all the hypoglycemic compounds in the watercress aqueous extract were what caused the greater hypoglycemic effect **(Fenton-Navarro et al., 2018).**

These findings are consistent with **Bähr et al. (2012),** who discovered that pancreatic GLUT4 expression is downregulated in insulin-deficient type 1 diabetic rats. GLUT4 expression in the skeletal muscle of type 1 diabetic animals treated with alloxan or streptozotocin has also been demonstrated in several studies. Therefore, since insulin treatment also increased the GLUT4 mRNA expression levels in αTC1.9 cells, it is not possible to attribute the increased GLUT4 mRNA expression in the pancreas of type 1 diabetic animals to an effect in β-cells. However, it may be an effect in α-cells.

According to **Vannucci et al. (1998),** the cerebellum's GLUT4 protein expression levels seem to be influenced by the amount of circulating insulin and are lowered in streptozotocin-diabetic rats that have low insulin levels. Reductions in insulin and GLUT4 levels in the cerebellum are also associated with exercise training. These findings suggest that there may be acute variations in the glucose uptake of these GLUT4-expressing cells, coupled with a chronic insulin-sensitive regulation of GLUT4 in the rodent brain.

Miller and his coworker (2013) noticed that the downregulation of gluconeogenic genes expression resulted from the activation of AMP-activated protein kinase (AMPK). Furthermore, an increase in AMP concentration may suppress adenylate cyclase activity, which is a crucial mediator of glucagon action, and consequently suppress gluconeogenesis. The ability of AMPK to enhance skeletal muscle's absorption of glucose is a significant effect. This occurs both acutely via translocation of GLUT4 from intracellular storage vesicles to the plasma membrane, and in the longer term by up regulation of GLUT4 expression **(McGee et al., 2008).**

Kim and Park (2016) observed that AMPK controls the synchronization of anabolic processes and its

activation in different tissues can aid in achieving metabolic homeostasis, leading to improvements in lipid and glucose profiles in insulin-resistant animal models, as well as exhibiting anti-tumor activity and mitochondrial biogenesis.

Conclusion

Through up regulating the expression of the GLUT4 and AMPK genes, our analysis demonstrated that giving watercress aqueous extract orally to diabetic rats had positive effects on lowering and maintaining normal glucose levels. This suggests that watercress aqueous extract can be used as an alternative diabetes treatment.

Abbreviations: STZ: Streptozotocin, GLUT4: Glucose transporter 4, AMPK: 5' AMP-activated protein kinase, PCR: Polymerase chain reaction, RQ: Relative quantity of DNA

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