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ANTIOXIDANT AND HYPOGLYCEMIC EFFECTS OF WATERCRESS (*NASTURTIUM OFFICINALE*) EXTRACTS IN DIABETIC RATS

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Abstract

Background: Watercress is a semi-aquatic plant used in traditional medicine to treat various ailments, such as flu, cough, avitaminosis, and anorexia; it is also used as a diuretic and for hypoglycemia treatment in diabetes. In this study, we report the antioxidant and hypoglycemic activity of orally administered aqueous (WAQE), acetonic (WAE), and alcoholic (WOHE) watercress extracts. The effect of subchronic administration of watercress extracts on oxidative stress was also studied.
Materials and Methods: WAQE, WAE, and WOHE were obtained and administered orally. Alloxan (200 mg/kg) and streptozotocin (60 mg/kg) were applied to induce hyperglycemia in male Wistar rats. Phenolic and flavonoid content, as well as antioxidant activity of the extracts were measured. The acute and subchronic effects (8 weeks) of WAQE were evaluated. The activity of antioxidant enzymes levels of malondialdehyde, hepatic enzyme markers in the serum, and renal function markers, were assessed. Histopathological evaluation of the pancreas, kidney, and liver was performed using hematoxylin-eosin staining.
Results: Watercress extracts have high concentrations of phenols, polyphenols, and flavonoids, in addition to a very high antioxidant effect. The hypoglycemic effect of WAQE upon acute administration was 76.6% higher than that of insulin. When administered chronically, glucose levels were normalized on the third week up to the eighth week. Furthermore, the antioxidant enzymes and biochemical parameters improved.
Conclusion: WAQE administration to diabetic rats reduced oxidative stress damage and decreased glucose levels. This study supports the use of this plant for the treatment of diabetes.

Keywords: Antioxidant, Diabetes, Watercress (*Nasturtium officinale*), Oxidative stress, Pancreas.

Abbreviations: AE: antiradical efficiency, A/G: albumin/globulin, ALP: alkaline phosphatase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, ALX: alloxan, ANOVA: analysis of variance, AST: aspartate aminotransferase, BHT: butylated hydroxytoluene, CK: creatine kinase, DPPH: 1,1-diphenyl-2-picrylhydrazyl, EDTA: ethylenediaminetetraacetic acid, GAE: gallic acid equivalents, GPX: glutathione peroxidase, GR: glutathione reductase, MDA: malondialdehyde, PVPP: polyvinylpolypyrrolidone, QE: quercetin equivalents, ROS: reactive oxygen species, SD: standard deviation, SOD: superoxide dismutase, STZ: streptozotocin, TAS: total antioxidant state, TBARS: thiobarbituric acid reactive substances, TEP: 1,1,3,3-tetraethoxypropane, WAQE: watercress aqueous extract, WAE: watercress acetonic extract, WOHE: watercress alcoholic extract.

Introduction

The American Diabetes Association has classified diabetes mellitus as a metabolic disease characterized by hyperglycemia, which is caused by defects in insulin secretion and/or insulin action. Approximately 2.8% of the population

worldwide has diabetes mellitus, and it is estimated that by 2025, it will affect 5.4% of the population. In 2016, the Mexican National Health and Nutrition Survey showed that diabetes mellitus constitutes a public health problem, with 9.4 % of the population in Mexico estimated to have this disease, that significantly impacts the quality of life of patients ENSANUT (2016).

One of the main causes of diabetic complications and obesity is the increased production of reactive oxygen species (ROS) in the mitochondria, which undergo dynamic morphological changes in response to hyperglycemia. Oxidative stress plays a major role in the etiology of diabetes and can result in microvascular and macrovascular complications, cataracts, nephropathy, and neurological disorders. In addition, patients with diabetes have diminished antioxidant defenses and are at increased risk of tissue damage by free radicals (Evans et al. 2002; Chen and Gow-Yen, 2007).

When the ability to secrete insulin decreases, the resulting hyperglycemia can lead to the accumulation of free radicals through autoxidation and non-enzymatic glycosylation of proteins.Therefore, the generation of free radicals from cytokines resulting from autoimmune reactions, increases oxidative stress, causing damage to biomolecules such as lipids, carbohydrates, proteins, nucleic acids, and connective tissue macromolecules, thereby interfering with cell function (West, 2000).

Two studies have reported the hypoglycemic activity of watercress; the study conducted by Hoseini et al. (2009) showed that watercress had a significant and comparable effect to that of glibenclamide; the other study, conducted by Shahrokhi et al. (2009), showed a reduction in glucose levels following administration of aqueous extracts of watercress to diabetic rats for 4 and 8 weeks. The repeated administration of watercress extracts caused a more significant reduction in glucose levels compared to values reported for insulin. However, there are no reports on the effects of aqueous extracts from watercress herbs on oxidative stress in diabetic rats or its effects on the enzymes and biochemical reactions involved in this process. Information on the time taken for the extracts to induce hypoglycemic action is also lacking. We, therefore, studied these effects in this study.

Materials and Methods

Plants

Leaves from watercress plants were acquired from a local market in Morelia, Michoacan, Mexico. Plant identification and voucher specimen (No. 026808) referencing were done at the Biology Faculty Herbarium at the Michoacan University of Saint Nicholas of Hidalgo (UMSNH). The leaves were disinfected with a chlorine solution (5%) and rinsed with distilled water, prior to using them to prepare the different extracts.

Preparation of the aqueous extract (WAQE)

To prepare the 900 mg/kg dose, the disinfected watercress leaves were homogenized using distilled water. The homogenate was subsequently filtered and centrifuged (1800 g; 15 min; 4°C). The supernatant containing the aqueous extract was collected.

Preparation of the acetone extract (WAE) and the alcoholic extract (WOHE)

The disinfected leaves (1 kg) were air-dried and extracted five times with acetone (1 kg/1 L each), followed by five extractions using alcohol. The solvent extracts were evaporated to dryness using simple distillation to obtain WAE and WOHE.

Bromatological analysis

WAQE, WAE, and WOHE were analyzed in the Laboratory of Food and Nutrition Analysis at the Veterinary Faculty (FMVZ-UMSNH). The parameters measured included moisture, dry material, ether extract, crude fiber, crude protein, ashes (minerals) and extracts without nitrogen (carbohydrates).

Animals

Ethical approval for this study was obtained from the Institutional Animal Care Committee Experimental procedures were performed in accordance with the Mexican Official Norm (NOM-062-ZOO-1999) and the Guide for Care and Use of Laboratory Animals (2011). Adequate measures were taken to minimize the animal´s pain or discomfort. Healthy male Wistar albino rats (body weight: 150-250 g) were used to perform the experiments. The animals were housed in standard environmental conditions (temperature: 25 ± 7°C) with a relative humidity of 66-65% under a 12 h light to dark cycle at the Postgraduate Division of the Faculty of Medicine and Biological Sciences at UMSNH. Rats were fed a standard diet and provided free access to water before and during the experiments.

The rats were randomized into various groups and allowed to acclimatize for 7 days under standard environmental conditions before initiating the experiments. Animals described as fasting were deprived of food and water for 12 h.

Induction of diabetes

Diabetes was induced in two rat groups. One group of rats that were fasted 12 h received a single intraperitoneal injection of 200 mg/kg of alloxan monohydrate (ALX; Sigma-Aldrich St. Louis, MO, USA) dissolved in sterile saline Dunn et al. (1943). The second group was administered 60mg/kg of streptozotocin (STZ; Sigma-Aldrich). The rats were allowed to stabilize for 2 days in order to confirm the development of diabetes mellitus. Blood samples were drawn from the tail vein to measure blood glucose concentrations. Rats with blood glucose values ≥180 mg/dL were considered hyperglycemic and were included in the study.

Experimental design

The rats were randomly divided into five groups with six rats in each group. Solutions or aqueous extracts were administered using an animal feeding cannula (animal feeding needles 16 G X3", 3 mm curved; Cadence Science, Inc., Cranston, RI, USA). Insulin was administrated via subcutaneous (SC) injection.

In the acute experiments, the following groups were included:
Group I Untreated healthy rats received saline solution (SS);
Group II Diabetic rats (alloxan) received SS;
Group III Diabetic rats (alloxan) received insulin (4 IU/kg);
Group IV Diabetic rats received WAQE (900 mg/kg);
Group V Diabetic rats received either WAE or WOHE (4 mg/kg).

In the sub-chronic experiments, the following groups were included:
Group I Untreated healthy rats, received SS;
Group II Diabetic rats received insulin (4 IU/kg);
Group IV Diabetic (alloxan) rats received WAQE (900 mg/kg);
Group IV Diabetic (streptozotocin) rats received (WAQE (900 mg/kg).

Acute hypoglycemic effects
Effect of watercress extracts in oral glucose tolerance test

The rats from all test groups received an oral dose of glucose (1.5 g/kg) 30 min after administration of either saline, neutral protamine Hagedorn (NPH) insulin (4 IU/kg), WAQE (900 mg/kg), and WAE (4 mg/kg) or WOHE (4 mg/kg). Blood samples were collected from the tail vein just prior to administration of the extract/drug, at the following times: 0, 15, 30, 60, 120, 180, and 240 min. Blood glucose concentration was measured using a blood glucose meter system (ACCU-CHEK Performa Nano System Roche, D.C., Mexico). The acute effect was evaluated over a 4 h period. Results were expressed as the glucose variation in percentage vs. time intervals.

Effect of watercress extracts on fasting glucose test.

The test was performed as described above. The animals fasted for 12 h; the test groups were administered either saline, NPH insulin (4 IU/kg), WAQE (900 mg/kg), and WAE (4 mg/kg) or WOHE (4 mg/kg) orally. Blood samples were subsequently collected from the tail vein after 0, 60, 120, 180, 240, 300, 360, and 420 min. The hypoglycemic effect was evaluated as described above.

Sub-chronic administration

The subchronic treatment consisted of 8 weeks of daily administration of WAQE (900 mg/kg) using an oral feeding cannula (animal feeding needles 16 G X3", 3 mm curved; Cadence Science Inc.) to ALX and STZ treated rats, which comprised the diabetes-induced groups. The control group was administered subcutaneous insulin glargine (4 IU/kg) daily. Glucose levels and weights were measured weekly for up to 8 weeks (n=6 in each group).

The animals were sacrificed 24 h after the last treatment with an intraperitoneal injection of a high dose of sodium pentobarbital (150 mg/kg), following the specifications of the Mexican Official Norm (NOM–062-ZOO-1999). Tissue samples of the pancreas, kidney, and liver were obtained, rinsed using a sterile saline solution, and frozen at -70°C. A portion of the tissues was fixed using formaldehyde (10%) for further histological processing. Other portions were used to analyze the antioxidant activity of the enzymatic extracts.

Blood samples for biochemical and hematological analyses were divided into two samples; one was added to an empty tube (without anticoagulant), where the serum was separated by centrifuging at 2000 g for 15 min; the other one was added to a tube with ethylenediaminetetraacetic acid (EDTA).

Blood biochemistry

An automated analytical chemical analyzer ES-218 (KontroLab, Guidonia, Italy) was used for spectrophotometric analysis of the serum samples. The following biochemical parameters were analyzed: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, globulin, albumin/globulin (A/G) ratio, urea, uric acid, creatinine, creatine kinase (CK), total protein, glucose, cholesterol, triglycerides, calcium, and phosphorous.

Enzyme measurements

The antioxidant enzymes glutathione peroxidase (GP_X), glutathione reductase (GR), superoxide dismutase (SOD), and the total antioxidant state (TAS) were quantified with RANDOX (Randox Laboratories, Crumlin, UK) using the blood of hyperglycemic rats treated for 8 weeks with WAQE. The enzyme catalase was measured in erythrocytes and tissues (pancreas, liver, and kidney) using the method described by Aebi (1984).

Measurement of malondialdehyde (MDA) concentration in plasma

MDA was used as an indicator of lipid peroxidation. It was measured in the plasma of the experimental animals following treatment using the method described by Rezaeizadeh et al. (2011). A standard curve was prepared with absorbance values for different concentrations of 1,1,3,3-tetraethoxypropane (TEP; 0.1-5 μM/L).

Measurement of MDA concentration in tissue

MDA level was measured in the pancreas, liver, and kidney of the rats after treatment, using the method described by Rezaeizadeh et al. (2011). A standard curve was prepared with different concentrations of TEP (2.5-50 μM/L). The values obtained were expressed in concentrations of thiobarbituric acid reactive substances (TBARS; nmol/g) of the tissues.

Total polyphenol content assay

The polyphenolic content was analyzed using the Folin and Ciocalteau (1927) method. The polyphenol content was detected through measurement of tungsten and molybdenum salts using the spectrophotometer Jenway 6305 UV/Vis (Keison International, United Kingdom) at 760 nm. To avoid interferences, a portion of the samples was treated with polyvinylpolypyrrolidone (PVPP) linked to polyphenols in an acid medium. A standard curve was developed using phloroglucinol as a reference. The absorbance of unknown samples was determined by interpolation. The differences between the absorbance of the treated and untreated samples reflect the polyphenol content, reported as mg phloroglucinol/g extract.

Total phenol content assay

Using a modification of the Folin-Ciocalteau method (Kim et al. (2003), the extracts were analyzed for their phenolic content. Using a Gallic acid standard curve (50, 100, 150, 250 and 500 mg/L), concentrations were obtained, and the total phenolic content was measured in milligrams of Gallic acid equivalents per gram of extract (mg GAE/g extract).

Total flavonoid content assay

The flavonoid content of the extracts was determined by the colorimetric Dowd´s method (Ebrahimzadeh et al. 2010; Ramamoorthy et al. 2007). A standard curve was prepared using quercetin at the following concentrations: 3.125, 6.25, 12.5, 25, 50, 80, and 100 mg/L. The absorbance of the unknown samples in the standard curve was determined by interpolation. Total flavonoid content was estimated in milligrams of quercetin equivalents per gram of extract (mg QE/g extract).

Antioxidant analysis

The free radical scavenging activity of the extracts was measured *in vitro* using 1,1-diphenyl-2-picrylhydrazyl (DPPH). The percentage of the remaining DPPH (% $DPPH_{REM}$) was obtained using the following formula: DPPH final/DPPH initial*100. A graphic comparison of the standard concentration or samples vs. $DPPH_{REM}$ was conducted to obtain the amount of antioxidants necessary to decrease the initial DPPH concentration by 50% (EC_{50}). The kinetic behavior was measured using DPPH, and the sample was analyzed. The time needed to reach steady state concentration corresponding to EC_{50} (T_{EC50}) was calculated, and the antiradical efficiency (AE) as a parameter to characterize the antioxidant compounds was measured, where $AE=1/(EC_{50}*T_{EC50})$ Brand-Williams et al. (1995), Sánchez-Moreno et al. (1998).

Histopathological studies

After fixing the tissue in 10% formaldehyde, the tissues were dehydrated, and paraffin blocks were created. Sectioning was subsequently done at 5 μm thickness. Routine histopathology was performed using the hematoxylin-eosin stain. The pancreas was also stained with Gomori's trichromic stain. The cuts made to the pancreatic islets (7 μm) were identified by the stains and counted using an optical microscope (Leica DM1000, Leica Microsystems Inc., Buffalo Grove IL, USA). The program LAS Core was used to visualize the images 100X in 5 slides. More than 20 islets were obtained from the pancreas. The different sizes observed on the islets were also recorded (Rupnik, 2009).

Statistical analysis

The data are expressed as the mean ± SD and analyzed using ANOVA tests. Tukey's tests were used to determine differences between the pairs of means for which ANOVA indicated significance ($p<0.001$). In pancreas, results were expressed as the mean of the number of beta cells ± standard deviation. The statistical difference was determined by comparing the hyperglycemic animals to the control using one-way ANOVA.

Results

Bromatological analyses (Table 1) showed that WAQE had the highest concentration of dry material. The quantities of crude fiber, protein, and ashes were similar among the different extracts. The extracts without nitrogen (carbohydrates) were higher in WAQE. The ethereal extract or fat was greater in WAE, as shown in Table 1.

Table 1: Bromatological Analysis

g%	**WAQE**	**WAE**	**WOHE**
Moisture	98.39	99.53	99.5
Dry material	1.61	0.47	0.5
Ethereal Extract	0.27	20.35	9.6
Crude fiber	12.72	15.42	12.06
Crude protein	47.91	33.51	43.14
Ashes (minerals)	13.67	21.46	23.64
Extract without nitrogen (carbohydrates)	25.44	9.26	11.56

WAQE: watercress aqueous extract, WAE: watercress acetonic extract, WOHE: watercress alcoholic extract.

Phenols, polyphenols, and flavonoids

Table 2 shows the concentration of polyphenols, phenols, and flavonoids. All watercress extracts had high concentrations of secondary compounds.

Table 2: Total phenols, polyphenols, and flavonoid content in watercress extracts

Extract	Phenols (mg GA/g)	Polyphenols (mg phloroglucinol/mL)	Flavonoids (mg QE/g)
WAQE	61.47±8.47a	568.5±50.13 a	773±64.38 a
WAE	112±9.45 b	812.75±6.70 b	1400±207 b
WOHE	552.5±39.12 c	1680.25±127.37 c	5067±116.83 c

WAQE: watercress aqueous extract, WAE: watercress acetone extracts, WOHE: watercress alcoholic extracts GA: Gallic acid, QE: Quercetin. Data represent the mean ± SD. Each experiment was performed in triplicate and repeated three times. Different lowercase letters in the same column indicate significant difference at $p \leq 0.001$.

Antioxidant capacity *in vitro*

In Table 3 the EC_{50}, TEC_{50}, and AE are summarized. Ascorbic acid and Gallic acid used as controls demonstrated similar AE values to those reported by Sanchez- Moreno (1998).

Table 3: Antioxidant analysis of Watercress extracts

	EC50 (g Antioxidant/kg DPPH) [1]	TEC50 (min) [1,2]	EA x 10^{-3}
Ascorbic Acid	78.14±4.5 a	1.375 ± 0.25 a	9.30
Gallic Acid	31.64±0.18 b	14 ± 1.12 b	2.25
WAQE	20.2±0.14 c	9.8 ± 4.98 c	5.05
WAE	31.55±0.43 b	1.3 ± 0.02 a	24.37
WOHE	25.37±0.035[c]	30 ± 0.0 d	0.0013

DPPH: 1,1-diphenyl-2-picrylhydrazyl, GA: Gallic acid, QE: Quercetin, WAQE: watercress aqueous extract, WAE: watercress acetone extracts, WOHE: watercress alcoholic extracts. 1 Each value is the mean ± standard deviation (n= 6). 2.- TEC50: Time needed to reach the steady state of EC concentration, 3.- Antiradical Efficiency AE=1/(EC50*TEC50). Different lowercase letters in the same column indicate significant difference at a: $p \leq 0.001$, b, c: $p \leq 0.05$, d: $p \leq 0.001$.

Hypoglycemic activity

The glucose tolerance test showed that WAQE (900 mg/kg) had 76.6% hypoglycemic activity compared to 55.8% for insulin ($p<0.05$). After 120 min, WAE decreased glucose levels to 8.8% while WOHE had no effect at 4 mg/kg, though a higher dose of 8 mg/kg was tested and had comparable results (data not shown). The diabetic rats receiving saline solution showed an increase in glucose level of 57%. In healthy rats, there were no variations in glucose levels.
When comparing groups, there was a significant difference between the following: insulin and saline solutions ($p<0.001$); WAQE and saline solution ($p<0.0001$); healthy + WAQE and WAQE ($p<0.001$); and WAQE and WAE treatments ($p<0.001$). There were no significant differences between the hypoglycemic effects of insulin and WAQE. (Figure1A).

Fasting glucose test

When using WAQE (900 mg/kg), a glucose blood reduction of 25.9% was observed compared to pretreatment levels. WAE and WOHE (4 mg/kg) showed a hypoglycemic effect of 26.4% and 36.4%, respectively. Insulin decreased blood glucose by 77.5%, and an increase of 7% was observed in rats administered the saline solution. There was a significant difference in glucose levels ($p<0.001$) between rats administered insulin, and those administered the watercress extracts or saline solution. The extracts analyzed showed significant differences at 240 min vs. saline solution. In the case of insulin, the reduction was observed at 60 min. (Figure 1B).

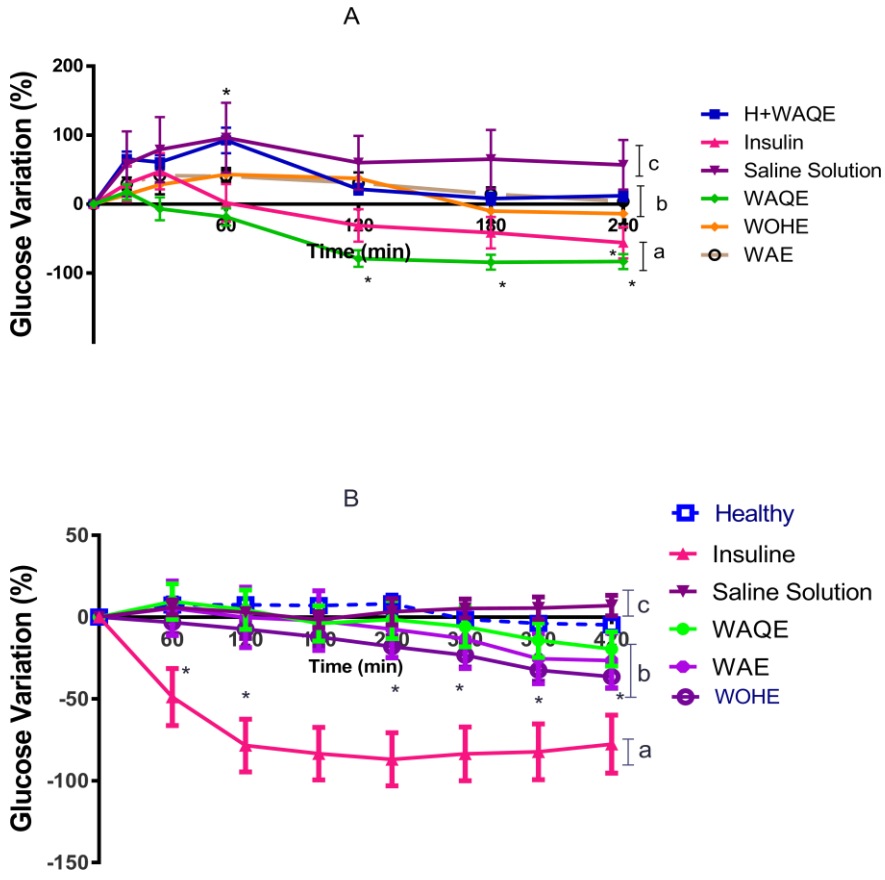

Figure 1: (A) Hypoglycemic effect of WAQE, WAE, and WOHE using the glucose tolerance curve. (B) Fasting glucose test with WAQE, WAE, and WOHE.

The graph represents glucose variation vs. time (min). WAQE exhibits the highest glucose variation. Points presented as mean ± SD (n=6). *p<0.001 intragroup differences vs. time. Letters represent significant differences of p<0.001 between groups. H: healthy, WAQE: watercress aqueous extract, WAE: watercress acetone extracts, WOHE: watercress alcoholic extracts.

Sub-chronic administration

The results of weight and circulating glucose in the groups treated sub-chronically are presented in Figure 2A, 2B. Weight continuously increased in all groups.

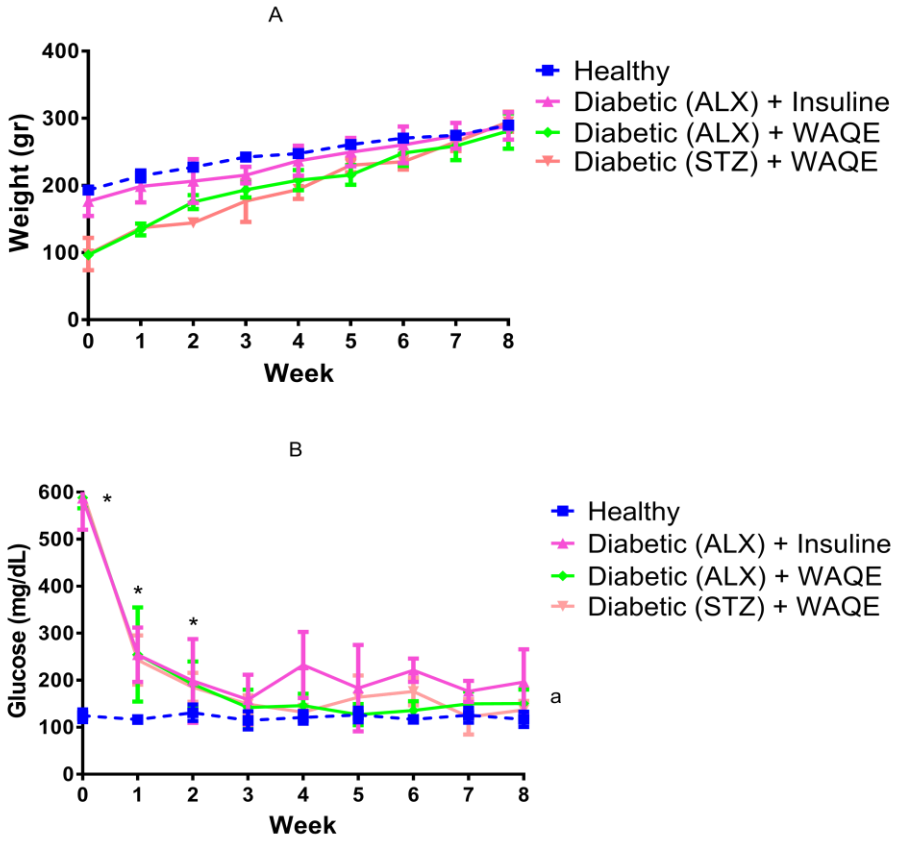

Figure 2: (A) Weight during sub-chronic administration (8weeks). (B) Glucose concentrations during subchronic administration (8 weeks). ALX: alloxan, STZ: streptozotocin, WAQE: watercress aqueous extract. *p<0.05 compared to healthy rats. Letters represent no significant differences between groups.

Oxidative stress *in vivo*

Values of antioxidant enzymes for healthy, diabetic + insulin, and diabetic + WAQE-treated rats are presented in Table 4.

Table 4: Antioxidant enzyme concentrations

Erythrocyte Catalase (µmol H_2O_2/mL/min).	H+SS	D+I	Diabetic (ALX)+ WAQE	Diabetic (STZ)+ WAQE
	119.26 ± 32.21 **a**	256.88±1.25 b	100.91±18.027 a	181±14.21 a
Catalase in Tissues (U /mg protein)				
Pancreas	68.80± 29.6 a	82.56 ±26.48a	91.74 ±5.29a	45.87±11b
Liver	134.5 ±35.2 a	61.16±13.32 b	128.44 ±19.09 a	73.39±21.18 b
Kidney	103.9 ±8.1 a	39.75±21.40 b	143.73±18.6 a	88.68±49.21 b
Superoxide dismutase	258+12.55 a	43.44+5.14b	86.67 + 34c	113.28+26.47c
Glutathione reductase	67.68±0.62 a	13.85±5.99 b	8.09±2.02 b	22.54±10.50 b,c
Glutathione peroxidase	40143 ±1583 a	31872 ± 5570b	46469±576.8c,d	43982.7±670 a,d
Total antioxidant status (TAS)	1.44 ±0.5 a	0.85±0.1 b	1.88±0.3 a	0.99±0.342 a
Malondialdehyde				
Serum	4.6±0.7 a	5.47±0.417 c	2.5±0.1 b	2.35±0.19 b
Pancreas	63.61±15.15 a	73.74±7.87 a	67.63±0.032 a	43.62±0.003 b
Liver	35.71±10.28 a	55.89±11.73 b	32.15±0.005 a	38.87±0.04 a
Kidney	46.057±0.05 a	60.84±10.18 b	44.08±3.7 a	25.86±5.82 c

Groups: H+SS: healthy + saline solution, D+I: diabetic + insulin. **Units**: [1]superoxide dismutase: % inhibition, [2]glutathione reductase: U/g hemoglobin, [3]glutathione peroxidase: U/L whole blood, [4]catalase: CAT/mg of protein, [5]total antioxidant status: mmol/L of plasma, [6]malondialdehyde in serum: µmol/L, [7]malondialdehyde in tissues: µmol/mg protein. Letters represent statistical differences between groups at p<0.05.

Biochemical parameters

After subchronic administration for 8 weeks, biochemical serum analysis was performed. Results are shown in Table 5.

Table 5: Biochemical parameters of animals that received 8-week treatment

	HEALTHY	D+I	ALX+WAQE	STZ + WAQE
Glucose[1]	7.76±3.7	9.2±1.6	11.3±3.5	12.8±4.2
Urea[1]	6.4±1.7	15.6±2.1*	17.8±4.8±	22.3±6.1*
Creatinine[2]	46.3±10.9	128.5±65.5*	79.3±6.4	79.6±13.2
Proteins[3]	30.4±1.9	71.5±0.1*	52.5±9.3*	49.6±4.9*
Albumin[3]	15±8.9	40.4±8.1*	22.1±5.9	24.9±1.5
Globulin[3]	15.4±10.1	31±8	30.3±3.6	24.7±6
Albumin/Globulin	0.69±0.3	1.49±0.6*	0.7±0.1	1.2±0.4
Cholesterol[1]	1.8±0.5	2.7±0.2*	1.9±0.2	2.0±0.4
Calcium[1]	1.3±0.8	3.4±1.2*	1.9±0.3	1.9±0.03
Phosphorum[1]	3±0.8	3.6±1.1	3.6±2.2	4.5±1.5
ALT[4]	108±3.7	128.5±6.5*	114.2±14.2	113±4.8
AST[4]	192.5±19.6	226±84.8	176.6±60.3	172.5±84.8
ALP[4]	432.3±120.9	492±196	424±41.8	377.9±21.9

[1]mmol/L, [2]µmol/L, [3]g/L, [4]U/L. (n=6). *p<0.05 vs. healthy.
ALP: alkaline phosphatase, ALT: alanine aminotransferase, ALX: alloxan, AST: aspartate aminotransferase, STZ: streptozotocin, WAQE: watercress aqueous extract.

Histological analysis

The pancreatic analysis of the number of β-cells is presented in Table 6.

Table 6: Number of β-cells in the different groups after 8 weeks of treatment

Group	β-cells	±SE
Healthy	123*	34
Insulin	72	15
Alloxan	135*	7
Streptozotocin	110*	4

SE: Standard error, *p<0.05 vs. the insulin group.

Discussion

The bromatological content shown in Table 1 is consistent with the report on the nutritional content of watercress extracts Muñoz de Chávez (2002). The watercress extracts contained very high concentrations of phenols, polyphenols, and flavonoids particularly the WOHE (Table 2).

According to the Antiradical efficiency (AE) classification Sanchez-Moreno (1998), our results show that WAQE had high AE, WAE had very high AE, and WOHE had low AE (Table 3). Our data are consistent with those of a previous report on the relevant antioxidant properties of watercress Özen (2009), and also establish the AE which has not been reported previously.

The hypoglycemic activity of the watercress extracts following acute administration was measured using two main parameters in diabetic studies. Fasting glucose measurement is used in clinics to diagnose diabetes mellitus. It evaluates the hepatic production of glucose (gluconeogenesis) from glycogen, lactate, or amino acids during fasting periods. Using medicinal plants and animal models, this test provides the means to measure the hypoglycemic activity of an extract by evaluating its effect on hepatic glucose metabolism, specifically in gluconeogenesis Andrade-Cetto (2012). Similarly, the glucose tolerance test is used in clinics to diagnose patients with diabetes mellitus or insulin resistance. With medicinal plants, this test also allows for the evaluation of glucose absorption, hepatic metabolism (gluconeogenesis), and peripheral tissues, as well as the evaluation of pancreatic production of insulin Attanayake et al. (2013).

The hypoglycemic action of watercress extracts (Figures 1A and 1B) is due to the presence of secondary plant metabolites present in the plant, such as flavonoids, quercetin, rutin, flavonol, kaempferol, and glucosinolate (gluconasturtiin, most highly hydroxylated). These compounds were shown to participate in the peripheral utilization of glucose, by inhibiting the action of glucose transporters in the intestine. They also increase the secretion of insulin and activate adenosine monophosphate (activated protein kinase) in white adipose tissues and skeletal muscles. This significantly enhances the expression of GLUT4, which in turn increases the uptake of glucose by these compounds

(Jadhav et al. 2012, Oyenihi et al. 2014). The higher hypoglycemic activity of WOHE is consistent with the presence of a higher concentration of hydroxylated compounds (Table 2). In the case of WAQE, the greater hypoglycemic effect was due to synergistic effects of all the hypoglycemic compounds present in the extract.

The observed increases in weight were gradual and constant (Figure 2A), suggesting that the components of the extract do not affect the absorption or utilization of nutrients by the animal obtained from feeding. The acute and subchronic toxicological evaluation of watercress in our lab showed watercress to be non-toxic and completely safe to consume.

Weight maintenance in hyperglycemic rats is also associated with adequate glycemic control since, in hyperglycemia, muscle mass in both smooth and skeletal muscles decrease due to proteolysis; which this is approximately 30% reduction compared to healthy controls. These parameters are also kept under control when insulin is administered Smith et al. (1989).

The hypoglycemic activity observed with subchronic administration of watercress (Figure 2B), is consistent with a previous finding from by Shahrokhi et al. (2009), where the watercress used was air-dried, powdered, and extracted using the percolation method with ethanol (80%). Doses of 25 and 75 mg/kg were administered orally to diabetic rats. A decrease of 96% was observed with the 75 mg/kg dose, which was significantly higher than the 49% decrease found in the insulin group ($p<0.001$). The authors also measured the glucose levels at week 4 and 8 and observed the most optimal results at week 8. A report by Hoseini et al. (2009), where ethyl acetate, methanol, and aqueous extracts were tested, showed positive results with methanol extracts only at 800 and 1000 mg/kg after 1 week and 2 months, respectively.

We used the aqueous extract, without previous extraction. From the dose standardization, the best results were observed at 900 mg/kg (data not shown). In the reports mentioned above Shahrokhi et al. (2009), Hoseini et al. (2009), glucose levels were not evaluated weekly. Our results showed a significant decrease at the beginning of the first week, and these glucose levels normalized beginning on the third week up to the eighth week. The extracts did not affect liver glucose metabolism (i.e., enzymes that control gluconeogenesis, increase accumulation of glucose in the liver, or reduce glycogen breakdown) since no glucose variations were observed in healthy animals.

Diabetes is known to be associated with an increase in oxidative stress and a higher production of ROS such as hydroxyl radicals (HO), superoxide anions (O_2) and H_2O_2, which play a role in the development of diabetic complications and other disease states. A protective mechanism against ROS accumulation includes antioxidant enzymes, which when not controlled can lead to clinical manifestations West (2000). In diabetes, the overproduction of ROS cannot be adequately balanced by antioxidant enzymes. In this context, we evaluated the protective effect of WAQE subchronically administered for 8 weeks (Table 4).

The diabetic models used in this study, induced using ALX have been shown to generate oxidative stress. Pancreatic β-cell toxicity and the resultant diabetogenicity are due to redox cycling and the generation of toxic ROS. The impairment in antioxidant enzyme function induced via STZ may contribute to the observed experimental diabetes Szkudelski (2001).

Low concentrations of hydrogen peroxide act as a cellular messenger in insulin signaling, whereas at high concentrations, it is toxic. Catalase activity in erythrocytes was reportedly higher in diabetic rats treated with insulin, which is consistent with previous studies Qujeq and Rezvani (2007), where diabetic animals treated with watercress had catalase concentrations similar to healthy controls.

The reduction in catalase in the pancreas is due to the nitro-oxidative stress induced by STZ Szkudelski (2001). In the liver and kidneys, catalase activity was significantly lower in the diabetic + insulin (D+I) group, indicating susceptibility to oxidative damage. The protective effect of WAQE was evident from the tissue catalase values observed in other groups.

The activity of SOD, GPx, GR, and TAS were significantly lower in the D+I group than the control group, and in the watercress groups were not significantly different from that of healthy rats.

The plasma and tissue (liver and kidney) lipid peroxidation (measured by MDA) increased in diabetic rats treated with insulin, which is consistent with previous reports Ebuehi et al. (2009) and studies in humans (Clapés et al., 2001). However, lipid peroxidation in the diabetic groups treated with WAQE was similar to that found in healthy normal animals.

The phenolic, polyphenols, and flavonoids content, as well as the radical scavenging activity reported in Table 2, confirms the high antioxidant capacity and thus, protective effects against oxidative stress, of watercress extracts. This is consistent with recent reports (Zeb, 2015). The presence of rutin in watercress extracts can neutralize free radicals and inhibit lipoperoxidation, which in turn prevents oxidative stress induced by ALX and STZ, ultimately providing cytoprotection. Also, these compounds significantly decrease ROS and increase endogenous antioxidant enzymes in the kidneys of diabetic rats, thereby preventing or controlling the development of nephropathy Oyenihi et al. (2014).

The parameters measured in blood biochemistry (Table 5) helps to monitor the general health of individuals in the clinical setting. The present study examined whether watercress extracts are safe and whether they can be used in other species, including humans.

Diabetic hyperglycemia induces the elevation of plasma urea and creatinine, considered key markers of renal dysfunction. As shown in Table 4, D+I animals showed significantly higher levels of urea, creatinine, albumin and albumin/globulin, and cholesterol, all of which were significantly reduced following WAQE administration. Conversely,

total protein levels did not change in the experimental groups, indicating appropriate nutritional status, as well as adequate hepatic and renal functioning in these rats.

ALT and AST levels can be used to evaluate hepatic function. These enzymes were elevated in the diabetic animals, and cholesterol was found to be raised in the D+I group. Enzyme levels in the diabetic groups that received WAQE were similar to those observed in the healthy control groups. Our results are consistent with previous reports on watercress, where no significant changes in total serum cholesterol, high-density lipoprotein, and low-density lipoprotein levels were found in treated diabetic groups compared to untreated diabetic groups Qeini et al. (2010). Protection of liver tissue and reduction in cholesterol are likely due to the presence of flavonoids, which have been reported to be hepatoprotective agents. Scalbert (2005).

Drugs used to induce hyperglycemia, such as ALX and STZ, interact with β-pancreatic cells. ALX produces a reduction in β-cells, and STZ induces marked necrotic changes in the pancreas with a reduction of β-cells and hyperplasia of the nucleus (Dunn et al. 1943, Smirnov et al. 2012). In our study (Table 6), larger areas of islets and more significant number of β-cells were found in the groups that received WAQE. Liver and kidney histological analysis showed no apparent damage (data not shown).

These results are consistent with the pancreas-, liver-, and kidney-protecting effects of medicinal plants (Vessal et al. 2003, Chakravarthy et al. 1982) and quercetin Adewole et al. (2007). Our results differed from those reported by Qeini et al. (2008), where no beneficial effects of watercress treatment were found. The mechanism by which medicinal plants with flavonoids, quercetin, and kaempferol operate may be via the increase of hexokinase glucokinase activity and number of pancreatic islets Vessal et al. (2003). Watercress may function under the same mechanisms, though this remains to be examined.

Conclusion

Our analysis showed that WAQE orally administered to diabetic rats had beneficial effects with regard to reducing and maintaining normal glucose levels from the first week up to the eighth week of subchronic administration. This treatment provided additional protection against oxidative stress, which is commonly detected in diabetic patients but is not modified by insulin. This was evident given that antioxidant enzyme levels in treated rats were similar to control groups. These results suggest that watercress extracts possess antioxidant activity, provide protection to liver enzymes and β-cells, and reduce cholesterol.

Conflict of Interest: All authors declare no conflict of interests.

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