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CHRONIC HYPOGLYCEMIC AND HYPOLIPIDEMIC EFFECT OF *Equisetum myriochaetum* AERIAL PARTS ON STREPTOZOTOCIN-INDUCED DIABETIC RATS.

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Abstract

Type 2 diabetes mellitus is a chronic multifactorial metabolic disorder that affects 463 million people and caused 4.2 million deaths in 2019. Although there are many approved treatments,, the number of diabetic patients is increasing. One of the reasons can be the lack of treatment adherence due to different causes, being the unwanted side effects one of these. Medicinal plants can fulfill the need for new treatments especially because these treatments are better accepted, have none or mild side effects, are widely available and, have a lower cost.

In this study, we demonstrated the chronic hypoglycemic and hypolipidemic effect of the aqueous extract of the aerial parts of *E. myriochaetum* on streptozotocin -induced diabetic rats by administrating a daily oral dose of 7 mg/kg (BW) of the aqueous extract for 33 days and testing its effect against glibenclamide-treated diabetic rats and diabetic rats with no treatment. We also demonstrated the absence of side effects determined by the observation of clinical non-invasive parameters.

The hypolipidemic effect of the extract, not previously reported, is an additional benefit for the treatment of diabetic patients due to the association of this disease with dyslipidemia. The hypolipidemic effect may be one of the possible mechanisms of action contributing to the hypoglycemic effect of *E. myriochaetum*, not excluding any other mechanism.

The long term hypoglycemic and hypolipidemic effect associated with the absence of side effects, toxicity, and genotoxicity, previously reported, give support for the safe extensive use of this decoction for the treatment of diabetes.

Keywords: *Equisetum myriochaetum, hypoglycemic effect, hypolipidemic effect, STZ induced diabetes*

Introduction

Type 2 diabetes mellitus is a chronic multifactorial metabolic disorder characterized by hyperglycemia due to defective insulin secretion, inadequate insulin action, or both that result from the interaction of genetic and environmental factors (1). It is a health problem due to the increase in its prevalence, chronicity, long term disabling complications, and high social and economic costs.

According to the last International Diabetes Federation (IDF) report, in 2019, 463 million people were diabetic, a number that will increase to 578 million in 2030 and to 700 million in 2045, which represents an increase of 51%. In 2019 diabetes complications were responsible for the dead of 4.2 million patients, and from those, 46.2% were patients with ages from 20 to 79 years (2).

The long term and elevated glucose levels, characteristic condition of the diabetic patients, is associated with damage, dysfunction, and failure of different organs and adverse effects on carbohydrate, lipid, and protein metabolism that lead to micro- and macro-vascular complications. Microvascular complications are responsible for the increased risk of developing retinopathy, peripheral neuropathy and nephropathy, and end-stage renal disease. Macrovascular complications, including coronary heart disease, stroke, and peripheral vascular disease, are the principal causes of mortality in diabetic patients (3,4).

Besides the recommended control with diet and exercise, that has not great compliance, diabetes is being treated with different pharmaceutical products or with a combination of them, including insulin, that have different mechanisms of action, and correct the glucose levels acting on one or more of the pathophysiological alterations that characterize the disease. Still, the problem is that all of them have different unwanted side effects, including hypoglycemia (5).

The increasing global number of diabetic patients demonstrate that even though the high availability of different medications, the real situation is that most patients do not take the medications as indicated for various reason that includes important side effects, not yielding the wanted results and the high cost.
These patients progress to the long-term complications characteristic of the disease and finally die with a high social and economic cost.

Thus, all the efforts made to find new or alternative treatments for this disease are of great importance, mainly it must be considered that the treatments must be better accepted by patients, have none or mild side effects, and that adherence to treatment can be ensured, characteristics that can be fulfilled by the hypoglycemic plants.

Herbal extracts contain several active constituents that can act through different mechanisms and on multiple pathways so that considerable benefits can be obtained with its administration (6-8).

To demonstrate the sustained hypoglycemic effect of the herbal extracts, identify active compounds, their mechanisms of action, confirm the absence of toxicity, and the absence or the presence of mild side effects, extensive research must be performed.

In México, the aqueous extract of *Equisetum myriochaetum* (cola de caballo) has been used for a long time by traditional healers to treat kidney diseases and type 2 diabetes (9) and the reported main constituents of the water and butanolic extracts are kaempferol-3-O-sophoroside, kaempferol-3,7-di-O-b glucoside, caffeoyl-methylate-4- b-glucopyranoside and kaempferol-3-O-sophoroside-4´-O-b-glucoside (10,11) and pinocembrin, chrysin, b -D-glycosyl-sitosterol, b -D-glucose and fatty acids (12).

In addition to its traditional use, and in order to demonstrate the effectiveness and security of the administration of *Equisetum myriochaetum*, two acute controlled studies have been performed.

In streptozotocin-induced diabetic rats, the hypoglycemic effect was demonstrated with the oral administration of single doses of water (7 and 13 mg/kg BW) and butanolic (8 and 16 mg/kg BW) extracts (10).

The hypoglycemic effect of a single dose of the water extract (0.33g/kg BW) was also demonstrated in recently diagnosed type 2 diabetic patients (13).

Considering the results of these studies, the aim of this work was to demonstrate the chronic hypoglycemic effect of the aqueous extract of the aerial parts of *Equisetum myriochaetum* in streptozotocin-induced diabetic rats, to identify a possible mechanism of action and confirm the absence of side effects.

The study was approved by the Research Committee of the Health of the Research Coordination of the Mexican Institute of Social Security. It was conducted following the internationally accepted principles for laboratory animal use and care, as found in the US guidelines, NIH publication #85-23, revised in 1985, and the Mexican guidelines NOM-062-ZOO-1999.

Methods

Plant Material

Samples of *Equisetum myriochaetum* Schlecht. & Cham (Equisetaceae), (cola de caballo) were collected in Xochipala, Guerrero, México. They were identified and a voucher specimen IMSSM 11266 was deposited at the IMSS Herbarium in México City.

Preparation of the extract

The dried powdered material of the aerial parts of the plant (700 g) was extracted with water by refluxing for 4 hrs.; the extract was lyophilized and stored at 4°C. For the study, the lyophilized material was resuspended in a physiological NaCl solution (0.9%).

Experimental animals

Thirty-five female Sprague-Dawley rats (weighing 200-250g) obtained from the Bioterium of the National Medical Center were housed in an air-conditioned room at 25°C with 55% humidity, under a 12 hrs. light/dark cycle, with free access to food (standard laboratory pellet diet) and water for one week prior to the inclusion in the study, they were maintained in these conditions during the entire study.

Induction of experimental diabetes

Diabetes was induced by a single intraperitoneal injection of 50 mg/kg (BW) of buffered streptozotocin (STZ) (Sigma No. 242-646-8) (acetate buffer 0.1M, pH 4.5) to the overnight fasted experimental rats; overnight fasted control rats were injected with the acetate buffer.

Rats were considered diabetic with glucose levels ≥250 mg/dl 48hrs after the STZ injection.
The diabetic rats were assigned to 3 groups, each with seven rats. For 33 days, Group 1 (DE) received at 8:00 am, a daily oral dose of 7 mg/kg (BW) of the aqueous extract of *E. myriochaetum* in 0.25 ml of physiological NaCl solution (0.9%) (vehicle). Group 2 (DG) received at 8:00 am a daily oral dose of 3 mg/kg (BW) of the hypoglycemic agent glibenclamide in the same vehicle, and Group 3 (DC) received a daily oral dose of 0.25 ml of the vehicle.

Non-diabetic rats were assigned to 2 groups, each with seven rats. For 33 days, Group 4 (CE) received at 8:00 am a daily oral dose of 7 mg/kg (BW) of the aqueous extract of *E. myriochaetum* in 0.25 ml of physiological NaCl solution (0.9%) (vehicle) and Group 5 (NDC) received at 8:00 am a daily dose of 0.25 ml of the vehicle.

Determination of the bodyweight

Every three days at 8:00 am. the body weight was determined.

Determination of the biochemical parameters

Every three days at 8:00 am blood samples (drop) were taken from the tail vein (according to Guideline 9 (3/10/99) IACUC, 1999) to determine glucose levels of the overnight fasted rats with the Accu-chek glucometer (Roche).

On day 34, all overnight fasted rats were sacrificed by craneo-cervical dislocation; whole blood was obtained and centrifugated at 3,000 rpm for 15 min. in a Beckman GS-15R centrifuge. The serum determination of cholesterol (mg/dl), triglycerides

(mg/dl), was performed with the Vitros Ektachem DT 60II Analyzer (Johnson Medical).

Clinical observations

Every day at the same time in the morning (8:00 am.) and in the evening (16:00 and 22:00 pm.), clinical observations were performed by the same person to determine the physical condition, behavior, general and motor activity, equilibrium, presence of tremors, seizures, lethargy, sleep, and changes in the circadian cycle, feeding, water drinking, feces aspect, fur aspect, secretion in the nose and eyes.

Statistical analysis

The analysis was performed with a non-parametric test, the Wilcoxon sign-rank for two correlated samples, and the Kruskal Wallis test for more than two groups. p-values <0.05 were considered statistically significant.

Results

Bodyweight

The bodyweight of the diabetic groups had a significant reduction (p<0.0001) when compared with the initial weight. When analyzed by percentage of weight loss, the DE group had less weight loss (Table 1). No weight loss was observed in the CE group or in the NDC group.

Blood glucose levels

Before diabetes was induced, the mean glucose level was 97.45 ± 8.79 mg/dl. no significant differences in the glucose levels between the five groups could be observed (p = 0.261). On day 1 of the study, 48 hours after the STZ injection, the mean glucose level of the diabetic groups (DC, DE, DG) was 343.89 ± 87.66 mg/dl, with no significant difference (p=0.816).

On day 7, a significant hypoglycemic effect of the water extract of *E. myriochaetum* was observed in the DE group (p=0.007). On day 34, the glucose level of the DE group was 274 ± 49 mg/dl, and when compared with the glucose level of the DC group that was 472 ± 55 mg/dl. (p<0.0001),a reduction of 41.95% was achieved.

A significant effect of glibenclamide on the glucose levels of the DG group, compared with the DC group, was first detected on day 10 (p=0.017) and was maintained until day 34. On day 34, the mean glucose levels of the DG group were 361 ± 149 mg/dl, which represents a reduction of 23.52%.

There was no significant difference in the glucose levels of the non diabetic group treated with *E. myriochaetum* (CE), and in the levels of the non-diabetic control group with no treatment (NDC) Fig. 1

Serum triglycerides levels

On day 34, the serum triglycerides level of the DE group was 138.9 ± 24.1 mg/dl, 58.2% lower than that of the DC group 331.5 ± 85.3 mg/dl (p = 0.001). The triglyceride levels of the DG group were not significantly different from the DC group. The triglycerides levels in groups CE and NDC were not significantly different (Figure 2).

Serum cholesterol levels

On day 34, the serum cholesterol levels of the DE group (69.7 ± 12.9 mg/dl) were significantly different from the levels of the DC group (104.5 ± 29.8 mg/dl) (p = 0.014), representing a reduction of 33.3%.

No significant reduction of the cholesterol levels was observed in the DG group.

Cholesterol levels were not significantly different in the group of the non-diabetic rats treated with *E. myriochaetum* (CE) or in the untreated group (NDC) (Figure 3)

Clinical observations

No changes in the general behavior of the diabetic treated groups (DE and DG) or the CE and NDC groups were observed. DC group showed slightly delayed reactions.

The physical condition of the rats of the DC group deteriorated progressively; the deterioration of the diabetic treated groups, DE and DG, was significantly less, especially in the DE group. The characteristic changes in the fur aspect of diabetic rats were significantly less in the diabetic groups with treatment, mainly in the DE group, which had less piloerection and fewer areas with coloration

changes. No changes in motor activity or equilibrium were observed. No tremors, seizures, lethargy, sleep alterations, or modifications in the circadian cycle were observed.
Diabetic groups consumed more food and water than the NDC and CE groups. Only the feces of two rats of the DC group had a loose aspect. No presence of secretion in the eyes or nose was observed. At the end of the study, an improvement of the physical condition of the DE group was clearly demonstrated, not achieved by the DG group or by the DC group.

Discussion

The high incidence of diabetes and the high impact on health, quality, and expectancy of life of diabetic patients demand adequate and global treatments to reduce de glucose levels and prevent the development of complications related to the disease. There are many approved treatments, therapeutic algorithms, and clinical practice recommendations to achieve the glycemic control and prevent the progression of the disease, but to achieve effective glycemic control, the treatments must be individualized and indicated according to each patient's condition, considering other comorbidities (14). Although the IDF report shows that the number of diabetic patients is increasing, and so are the deaths due to the complications (2).

Medicinal plants with antidiabetic properties have been used for a long time in the traditional medicine of many countries and are actually used in developing and developed countries. Its acceptance is increasing due to the belief that natural products are effective, have no side effects, are widely available, and have a low cost.

More than 1200 species of plants have been used to treat diabetes (15, 16), but not all of them have scientific studies that support its effectiveness and the absence of side effects.

Preclinical studies with diabetic animal models have been used to test the hypoglycemic effect and the absence of side effects. The STZ-induction of diabetes is a well-demonstrated mechanism to induce diabetes in rats (17). STZ is the agent of choice for reproducible induction of a diabetic metabolic state in experimental animals (18), and it is equally effective for the induction of experimental diabetes administrated to fasted or fed animals (19). STZ has a half life of 5-15 minutes (20) is excreted (70-80%) mainly in urine and (8-9%) in the feces in a 6 hours period, has a considerable metabolic transformation and a rapid renal clearance (21, 22).
Glibenclamide is a sulfonylurea that has been used for a long time in the treatment of diabetes acting by promoting the insulin secretion through the inhibition of ATP-sensitive K^+ channels in the pancreatic cells and has the additional effect acting against the reactive oxygen species (ROS) that produce oxidative stress responsible for the increased risk of cardiovascular diseases (23,24). Glibenclamide has been used in different studies with STZ diabetic rats as a control drug, in doses that vary from 0.5 to 50 mg/kg (25-27); in our study, we used the 3mg/kg dose as in a previous study (10).

In this chronic study performed in rats with severe hyperglycemia, we demonstrated the significant reduction of the glucose levels of the diabetic rats treated for 33 days with 7 mg/kg (BW) of the aerial parts of the *Equisetum myriochaetum* aqueous extract ($p<0.0001$), that represents a reduction of 41.95%, that was not achieved with the glibenclamide treatment. An interesting and important observation was that the extract did not reduce the glucose levels of the rats of the CE group, and the glucose levels of this group were comparable to the glucose levels of the NDC group ($p=0.517$).

We also demonstrated that in the DE group, the extract produced a significant reduction in the cholesterol (33%) and triglycerides levels (58.2%) ($p<0.001$), a reduction that was not achieved in the glibenclamide treated group (DG). No significant changes were found in the non-diabetic rats treated with the extract (CE) when compared with the non-diabetic rats with no treatment (NDC).

It has been established in trends in plant research for antidiabetic treatment over the past 20 years, that the antidiabetic acting mechanisms of plant extracts can be categorized into six groups: alteration of glucose metabolism, hypolipidemic

effect, pancreatic effect, antioxidative effect, diabetes complication treatment, and insulin-like effect (28, 29).

Different studies have demonstrated that the hypoglycemic effect of the aerial parts of *E. myriochaetum* is not due to stimulation in the secretion of insulin as was demonstrated in STZ diabetic rats (10) and in recently diagnosed diabetic patients (13) nor due to an inhibition of intestinal a glucosidase as was demonstrated in diabetic rats and confirmed with an *in vitro* assay (30).
Recently it was demonstrated with the n-STZ model that *E. myriochaetum* has an inhibitory effect on the hepatic glucose output and has no inhibitory effect on the G6Pase system, considering that its mechanism of action can also be due the inhibition of other gluconeogenic enzymes (31).

In addition to this reported mechanism of action, the results of this study demonstrated that the significant hypolipidemic effect of the aqueous extract of *E. myriochaetum* could be another mechanism involved in the hypoglycemic effect by contributing to the control of hyperlipidemia, a related metabolic alteration of diabetes (32,33).

The reduction of the cholesterol and triglycerides levels are findings, not previously reported for *E. myriochaetum*, that represents an additional benefit for the treatment of diabetic patients due to the association of this disease with dyslipidemia, related to the coronary heart disease.

It has been demonstrated that besides the high glucose levels, hyperlipidemia and oxidative stress play an important role in the pathogenesis of diabetes (34-37).

Numerous studies have shown the importance of controlling individual risk factors for cardiovascular diseases in type 2 diabetic patients and that the benefits are greater if multiple risk factors are controlled simultaneously (38).

It well established that intensive glycemic control lowers the incidence and progression of diabetes, but several randomized studies and two meta-analyses have demonstrated that the intensive glycemic control alone is not enough to prevent macrovascular complications and that a global approach is needed in order to avoid cardiovascular events, an approach that includes among others the control of lipid levels (39,40).

Taking epidemiological data together, both moderate and severe hypertriglyceridemia is associated with a substantially increased long term total mortality and cardiovascular disease (CVD) risk. Triglycerides may also stimulate atherogenesis by other mechanisms, which include the production of proinflammatory cytokines, fibrinogen, and coagulation factors and impairment of fibrinolysis (41). This independent association with long term all-cause mortality supports the idea that serum triglycerides play a role in type 2 diabetic patients' mortality risk. (42, 43).

The association of high levels of serum cholesterol and diabetes has a significant contribution to CVD mortality, and the reduction of total cholesterol is translated into 39% reduction in the incidence and the recurrent antiplatelet events in diabetic patients. The reduction of the cholesterol levels exerts a protective effect on the incident and recurrent nonfatal myocardial infarction (MI) and incident nonfatal stroke (44, 45). It has also been demonstrated that the alterations of plasma and islets cholesterol can contribute to islet dysfunction and loss of insulin secretion (46).

Genetic, epidemiological and clinical studies have also demonstrated that elevated serum cholesterol is positively correlated with the incidence of coronary heart disease and to lower serum cholesterol concentration and improve blood flow changes can prevent the occurrence and development of coronary heart disease (CHD) and other cardio-cerebrovascular diseases (47). Dislipidemia is also associated with the development and progression of diabetic nephropathy (48).

Hyperlipidemia treatment involves dietary control, exercise, and pharmaceutical therapy; however, the lipid-lowering drugs, mainly statins, fibrates, nicotinic acid and bile acid sequestrants or a combination of them, have different adverse effects, have interactions with other drugs and have

specific contraindications and most of them have high costs (49).
For this reason, plant extracts could be one solution because they have, and fewer or no side effects than pharmaceutical drugs (29).

As previously mentioned, the absence of toxicity and side effects must be tested to consider the security of the administration of the herbal treatments. In relation to the toxicity of the aqueous extract of the aerial parts of *E. myriochaetum*, it is important to mention that no acute toxicity of the aqueous extract was found in Drosophila or human lymphocytes in culture in a wide range of concentrations. The genotoxic assays, somatic mutations, and recombination test (SMART) and the *in vitro* cytokinesis-block micronucleus assay indicated a lack of genotoxicity (50).

The long term administration of the *Equisetum* extract did not induce alterations in the clinical parameters analyzed in this study, and improvement in the general condition and a less weight loss in the DE group could be observed, an improvement that can be explained by the significant reduction in the glucose and lipid levels that were not achieved by glibenclamide.

It has already been established that flavonoids are effective antioxidants that can protect against several chronic diseases, including type 2 diabetes (51). *in vivo* and *in vitro* studies support that antioxidants can improve insulin sensitivity and ameliorate diabetic symptoms and that plants are potential sources of natural antioxidants (52). Additionally, it has been demonstrated that kaempferol, one of the main constituents of the *Equisetum myriochetum* extract has a hyperglycemic effect and a significant inhibitory effect on NO production (53), can promote hypoglycemia through increase glucose uptake and glycogen synthesis and can potentially act at multiple targets to ameliorate hyperglycemia, including its action as partial agonists of PPARgamma (52). Kaempferol can also reduce hyperglycemia by inhibiting the hepatic gluconeogenesis and improving muscle glucose metabolism (54).

Medicinal plants contain different compounds and the extract effect of the whole plant cannot be the same by administering isolated or purified constituents. The composition of the extracts is more efficient due that the compounds are additive and synergistic in their bioactivity.
The combined action of biologically active compounds is responsible for the potential benefits of the treatment (55). So, more than one mechanism may be involved in the *E. myriochaetum* hypoglycemic effect.

All of this information contributes to considering that *Equisetum myriochaetum* can have a reliable therapeutic efficacy and that more than one mechanism can be responsible for the hyperglycemic effect. Further controlled studies must be performed to determine all of the possible mechanisms of action.

The results of this study clearly demonstrated that the aqueous extract of the aerial parts of *E. myriochaetum* has a sustained long-term hypoglycemic and hypolipidemic effect on STZ-induced diabetic rats, that improves the general condition, including a less loss of weight, of the treated diabetic rats and exerts no side effects. The hypolipidemic effect added to the already demonstrated hypoglycemic effect is an additional benefit for the treatment of diabetic patients due to the association of this disease with dyslipidemia and the CVD and CHD so that it can be considered as an antidiabetic plant. These findings, added to the absence of toxicity and genotoxicity, give support for the safe extensive use of this decoction that has been used for many years by traditional healers to treat diabetes, and whose use has increased among diabetic patients of México.

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Table 1. Percentage of weight loss of the diabetic rats.

Group	% of weight loss
DE	33.42
DG	37.42
DC	39.15

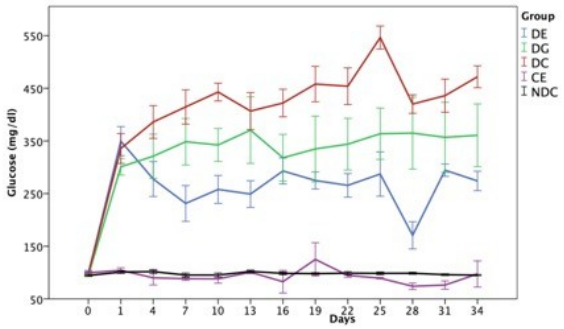

Figure 1. 33-day time-course variation in blood glucose (mg/dl). Data are expressed as mean ± SD

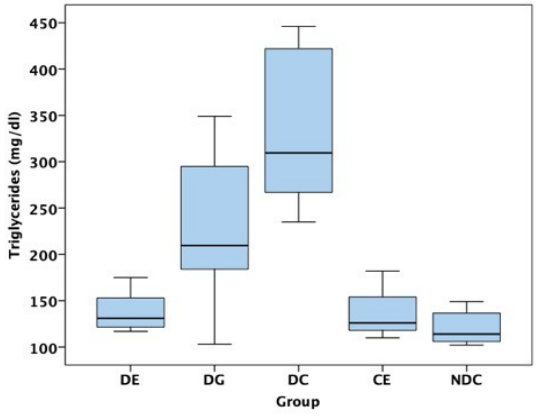

Figure 2. Serum triglycerides levels (mg/dl). Data are expressed as mean ± SD.

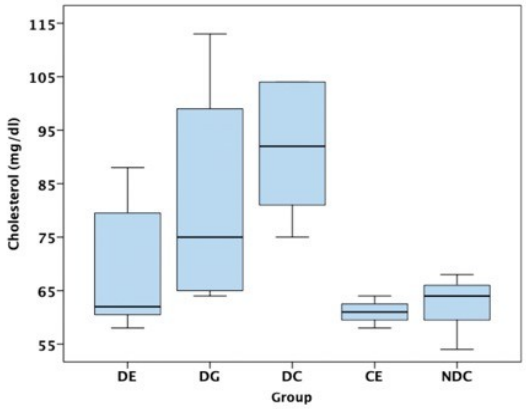

Figure 3. Serum levels of cholesterol (mg/dl). Data are expressed as mean ± SD.