Effects profiles of complete aqueous extract and hexane and aqueous fractions of Phalaris canariensis L. seeds on fructose-induced metabolic syndrome in rats

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Abstract
Phalaris canariensis L. (P. canariensis), commonly known as canary seed, is used in the traditional medicine of Mexico for the treatment of hypertension, diabetes, and obesity. Recent preclinical studies with different P. canariensis seed extracts support such uses. The main interest of this study was to describe the protective effect of the seeds on fructose-induced metabolic syndrome (MS) in rats and to assess the benefits gained from use of a complete aqueous extract. Our results indicate that the antihypertensive component(s) of P. canariensis seeds is (are) found in the aqueous fraction and that the substances responsible for the effects on obesity are found in both the aqueous and the hexane fractions. We conclude that both fractions are effective in attenuating manifestations of MS, and that administration of the complete aqueous extract of the P. canariensis seed is necessary for effective control when the P. canariensis seeds are used as an alternative herbal medicine.

Keywords: Phalaris canariensis, metabolic syndrome, seeds extract, rats

1. Introduction
Metabolic syndrome (MS) is characterized by high waist circumference (WC), atherogenic dyslipidemia, elevated blood pressure, insulin resistance, high fasting glucose, increased risk for cardiovascular disease (CVD), and diabetes mellitus type 2 [1]. MS is diagnosed if a person has at least three of the following metabolic risk factors: a large waistline, high blood pressure, high fasting blood glucose, high serum triglycerides (TGs), and a low HDL-C level [2]. In Mexico, the prevalence of MS has increased alarmingly; for example, in obese children and adolescents treated at a hospital clinic, the prevalence of MS was 36.7%; it was higher in females (41.7 vs. 33.3%) [3]. Lifestyle (diet and physical activity) interventions have been indicated as most important for treating this multifactorial syndrome and further trials are needed on interventions affecting three or more factors [4]. Since medicinal plants contain numerous active ingredients, it is possible to treat SM with a single plant. The ability of herbs and spices to reduce the symptoms associated with metabolic syndrome is promising. Based on cell cultures along with animal and human studies, it appears that several commonly used spices have the potential to regulate glucose, lower LDL and total cholesterol, and exert antioxidant and anti-inflammatory effects [5]. In recent years, increasing scientific evidence has emerged regarding the role of P. canariensis seeds as a promising therapeutic agent in the treatment of metabolic disorders. Canary seed (Phalaris canariensis) is used as food for birds but this cereal has recently been shown to have promising nutraceutical potential for humans [6]. In Mexican traditional medicine, it is used for the treatment of hypertension, diabetes, and obesity [7-9]. In laboratory experiments, the hexane extract of Phalaris canariensis seeds has been shown to reduce serum glucose and inhibit insulin resistance, lipid abnormalities, and oxidative stress in streptozotocin-induced mildly diabetic and severely diabetic mice [9]. However, no change in plasma lipids was observed in rats with induced hyperlipidemia treated with the hydroalcoholic extract of canary grass seeds [10]. In a chronic inflammation model (cotton pellet-induced granuloma), the chloroform extract of P. canariensis exhibited an anti-inflammatory effect through mechanisms involving reduced neutrophil influx and decreased production of inflammatory cytokines [11]. The aqueous extract of P. canariensis showed a hypotensive effect in anesthetized Wistar rats, without renal alterations [12], in addition to a vasodilator effect by nitric oxide induction of venous inflow [13]. In adult normotensive Wistar rats and spontaneously hypertensive rats (SHRs) and in prehypertensive young SHRs (SHRYs, 3 weeks old) treated with P. canariensis, an antihypertensive effect was observed, without induction of any significant risk of
nephrototoxicity [14]. Due to the fact that much of the aforementioned work on P. canariensis was carried out with either polar or non-polar extracts, the aim of the present study was to systematically establish the in vivo effects of the complete aqueous extract of P. canariensis seeds on fructose-induced metabolic syndrome in rats in addition to the activity against the same MS of two fractions containing substances of different solubility.

2. Materials and methods

2.1 Chemicals
A commercial formulation of crystalline fructose powder (Archer Daniels Midland Company, USA) was used. Seeds of P. canariensis were purchased at the Sonora market in Mexico. Histological sections of the seed ruled out the presence of spicules in the glumes (palea and lemma), indicating that the material used was P. canariensis [15]. Kits for determination of serum TGs, HDL-C, and glucose were supplied by Polymer Technology Systems®. All chemicals used in the study were high purity analytical grade obtained from Sigma-Aldrich and Merck.

2.2 Preparation of plant extracts
A total of 100 g of P. canariensis seeds was macerated with double distilled water at room temperature (20-22 °C) for 8 h. The macerate was liquefied and filtered through a medium mesh plastic strainer. The filtrate was dried at room temperature with constant ventilation for 6 h. The dried residue was pulverized to a particle size capable of passing through a 60-micron mesh, producing the complete aqueous extract (yield=35%). A part of this crude extract (10 g) was partitioned with hexane. Two parts were obtained from this process: the hexane-insoluble part (96%) and the soluble part (3.58%). The latter extract was filtered and concentrated by a rotary vacuum evaporator and kept in a vacuum desiccator for complete hexane removal.

2.3 Animals
The study was conducted in 90-day-old male Wistar rats. All animals were studied at the vivarium of the Department of Pharmacology at the UNAM School of Medicine by trained personnel, housed in wire cages (3 per cage), maintained at 21–23 °C and 60% humidity, subjected to a 12-h light/12-h dark cycle, and given pellet food (Purina 5001 Rodent Laboratory Chow) and tap water ad libitum for three weeks so that they might adapt to the laboratory environment prior to the experiments. The experiments reported in this study were conducted in accordance with our Federal Regulations for Animal Experimentation and Care (NOM-062-ZOO-1999 and NOM-087-SEMARNAT-SSA1-2002) and official international guidelines (Guide for the Care and Use of Laboratory Animals. NIH Publication 85-23, revised in 1985).

2.4 Induction of metabolic syndrome
In order to determine the concentration and time of fructose intake needed to induce MS, in a first series of experiments, forty animals were randomly divided into the following four groups (10 rats per group): three groups were fed with experimental high-fructose diets consisting of 70%, 80%, or 90% normal rat chow pellets enriched, respectively, with 10%, 20%, or 30% fructose, plus free access to 10% (10MS group), 20% (20MS group), and 30% (30MS group) fructose w/v in drinking water for 12 weeks for induction of MS. Control group rats received Purina chow without added fructose and normal drinking water.

2.5 Metabolic syndrome treatment
In a second series of experiments, twenty-four rats with MS induced after three months of ingesting 10% fructose in the food and drinking water were randomly divided into the following four groups (6 rats per group): MS + SS control group: rats received an oral dose of 0.2 mL/100g/day of saline solution (SS); MS + Pc group: rats received an oral dose of 310 mg/kg/day of complete aqueous extract of P. canariensis seeds; MS + sib group: rats received an oral dose of 5 mg/kg/day of sibutramine. All treatments lasted 8 weeks, during which the rats were fed the same diet that induced MS. Non-MS group: rats received Purina chow without added fructose and normal drinking water. In a third series of experiments, thirty-six rats with MS induced after three months of ingesting 10% fructose in the food and drinking water were randomly divided into the following 6 groups (6 rats per group): MS + SS control group: rats received an oral dose of 0.2 mL/100g/day of SS; MS + aqueous group: rats received an oral dose of 310 mg/kg/day of aqueous fraction (hexane-insoluble part) of P. canariensis seeds; MS + Tween group: rats received an oral dose of 0.2 mL/100g/day for SS with 1% of Tween 80. MS + hex group: rats received an oral dose of 310 mg/kg/day of hexane fraction (hexane-soluble part). All treatments lasted 8 weeks, during which the rats were fed the same diet that induced MS. Non-MS group: rats received Purina chow without added fructose and normal drinking water.

2.6 Prevention of metabolic syndrome
In a fourth series of experiments, twenty-four rats were randomly divided into the following 4 groups (6 rats per group): fructose + SS control group: rats received an oral dose of 0.2 mL/100g/day of SS; fructose + Pc group: rats received an oral dose of 310 mg/kg/day of complete aqueous extract of P. canariensis seeds; fructose + sib group: rats received an oral dose of 5 mg/kg/day of sibutramine. All treatments lasted 12 weeks, during which the rats received Purina chow and drinking water with 10% fructose each. Non-MS group: rats received Purina chow without added fructose and normal drinking water.

2.7 Determination of metabolic and clinic alterations
Systolic blood pressure (SBP), Lee index (LI), waist circumference (WC), body weight (BW), TGs, HDL-C, and glucose were measured every month and for two to three consecutive months. LI and WC were determined in rats previously anesthetized with sodium pentobarbital (30 mg/kg).

2.8 Measurement of blood pressure
SBP measurements were taken monthly; the rats were maintained at 32 °C in an LE 5650/6 heater and scanner unit (Letica Scientific instruments). A pulse transducer and pressure cuff (LE 5160/R) were placed around the rat tail and connected to an LE 5007 automatic blood pressure computer. SBP was recorded using the standard tail cuff method three times for each. The mean of three consecutive readings was used.

2.9 Obesity indicators
The body weight of each rat was measured once per week and the total amount of food consumed was recorded three times each week. In previously anesthetized rats, WC was measured 2 cm below the rib rim and rat length was measured from the tip of the snout to the anus. Subsequently, IL was calculated according to the following formula [16]: IL = (√p/L)na, where
\(3\sqrt{ }\) = cubic root, \(p\) = body weight (g), \(L_{na}\) = naso-anal length (cm).

2.10 Biochemical tests
TGs, HDL-C, and glucose were determined using metabolic panel reagent strips (REF 2400) and a CardioChek PA analyzer (Polymer Technology Systems®). Every morning, one hour after the food was removed, 40 µL of blood was taken from the capillaries of the caudal vein.

2.11 Statistical analysis
Experiments to induce metabolic syndrome were carried out ten times, and the other experiments were carried out six times. The results are reported as mean values ± the standard error of the median. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA). For multiple comparisons, a one-way ANOVA followed by Dunnett’s test \((p<0.05)\) was performed in order to detect statistical differences.

3. Results
3.1 Induction of metabolic syndrome
The MS indicators for the first series of experiments are shown in Figure 1. In all groups (10MS, 20MS, and 30MS), additional fructose intake significantly increased TGs and three clinical indicators: weight/length ratio (LI), WC, and SBP \((p<0.05, p<0.005)\). This MS profile was very consistent at 60 and 90 days.

[Image: Fig 1: Effect of administration of 10% (10MS), 20% (20MS), and 30% (30MS) fructose on TG, HDL-C, glucose, LI, WC, and SBP levels. Fructose was added to the food and drinking water in the same percentage. These indicators were measured at time zero \((0, n=40)\) and after the three groups were formed (10 rats per group) at 30, 60, and 90 days of fructose consumption. In the control group \((-\)), fructose was not added to food or water. Each bar represents the mean ± SEM. * = \(p<0.05\), ** = \(p<0.005\) significance level compared to the corresponding control group. Time zero represents the mean of all animals studied.

A significant increase in TG levels was observed in all groups after the 30 days of fructose consumption. Inconsistent differences were observed in HDL-C (1MS) and glucose (3MS) levels between the control group and the fructose-fed groups. Based on this first series of experiments, feeding adult rats with a purified high-fructose (10%) diet plus 10% in drinking water for 90 days was judged to be an appropriate protocol for the rest of the experiments reported in this work.

3.2. Treatment of metabolic syndrome
The profile effects due to the oral administration of SS (2 mL/100 g/day), sibutramine (5 mg/kg/day), or complete aqueous extract of *P. canariensis* seeds (310 mg/kg/day) in rats with MS induced by a diet supplemented with 10% fructose for 90 days are summarized in Figure 2. The results of this second series of experiments show that the oral administration of complete aqueous extract of *P. canariensis* seeds (MS + Pc group) at a dose of 310 mg/kg/day to SM rats for 60 days significantly \((p<0.05, p<0.005)\) reduced the high recorded values of LI, WC, TGs, and SBP, while no effects were observed for HDL-C or glucose compared to the control group (MS + SS control group).
Fig 2: Effect of administration of saline solution (MS + SS group), sibutramine (MS + sib group), or complete aqueous extract of *P. canariensis* seeds (MS + *Pc* group), in rats with MS induced with 10% fructose added in food and drinking water. TG, HDL-C, glucose, LI, WC, and SBP levels were measured at time zero (n=18) and after the three groups were formed (6 per group) at 30 and 60 days of fructose consumption plus co-administration of treatments. In the non-MS group ( ), rats received Purina chow without added fructose and normal drinking water. Each bar represents the mean ± SEM. * = p<0.05, ** = p<0.005 significance compared to the corresponding control group (MS + SS group). a Time zero represents the mean of all animals studied.

The daily administration of sibutramine at a dose of 5 mg/kg/day to the positive control group (MS + sib group) for 60 days significantly reduced the same indicators of MS that the complete aqueous extract of *P. canariensis* seeds reduced. In addition, sibutramine produced a significant reduction in blood glucose level (p<0.05).

The profile effects due to the oral administration of 0.2 mL/100 g/day of SS with 1% Tween 80 (MS + Tween group), 310 mg/kg/day of hexane fraction of *P. canariensis* seeds (MS + hex group), 0.2 mL/100 g/day of SS (MS + SS group), or 310 mg/kg/day of aqueous fraction of *P. canariensis* seeds (MS + aqueous group) in rats with MS induced by a diet supplemented with 10% fructose for 90 days are summarized in Figures 3 and 4. In both figures, time zero represents the mean of all animals studied, and the means of the records obtained from the non-MS group are the same.

Fig 3: Effect of administration of Tween (MS + Tween control group) or hexane fraction of *P. canariensis* seeds (MS + hex group), in rats with MS induced with 10% fructose added in food and drinking water. TG, HDL-C, glucose, LI, WC, and SBP levels were measured at time zero (n=12) and after the three groups were formed (6 per group) at 30 and 60 days of fructose consumption plus Tween or hexane fraction. In the non-MS group ( ), rats received Purina chow without added fructose and normal drinking water. Each bar represents the mean ± SEM. * = p<0.05 significance compared to the corresponding control group (MS + Tween group). a Time zero represents the mean of all animals studied.
This third series of experiments revealed that the oral administration of hexane fraction of *P. canariensis* seeds (MS + hex group) at a dose of 310 mg/kg/day in SM rats for 60 days significantly reduced (*p*<0.05) the high recorded values of WC, while no effects were observed in the other SM indicators when compared to the MS + Tween control group (Figure 3). In contrast, daily administration of or aqueous fraction of *P. canariensis* seeds at a similar dose of 310 mg/kg/day to SM rats for 30 and 60 days significantly reduced SBP (*p*<0.005, *p*<0.05). The results also demonstrate a significant control of TGs up to 60 days of treatment with responses that were lower than those observed in the MS + *Pc* group.

### 3.3 Prevention of metabolic syndrome

The effects observed in TG, HDL-C, and glucose levels, as well as in LI and WC due to the oral administration of fructose plus co-administration of SS (2 mL/100 g/day), sibutramine (5 mg/kg/day), or complete aqueous extract of *P. canariensis* seeds (310 mg/kg/day) for 90 days in normal rats are summarized in Figure 5.
This fourth and final series of experiments showed that a dose of 310 mg/kg/day of complete aqueous extract of *P. canariensis* seeds significantly prevented an increase in LI (*p*<0.005) and WC at 90 days, while no effects were observed in the other SM indicators compared to the fructose + SS control group (Figure 4). The oral administration of a daily dose of sibutramine (5 mg/kg/day) in rats that consumed 10% in food and drinking water also prevented an increase in LI at 90 days and significantly (*p*<0.005) reduced blood glucose at 60 days.

4. Discussion

Intake for 60 and 90 days of 10%, 20%, or 30% fructose added to the food and drinking water of the Wistar rats resulted in increased TGs, LI, and SBP to a similar degree at all three concentrations of fructose employed. These results are consistent with numerous studies in which intake of fructose was shown to cause metabolic changes in animal models, mainly with regard to weight gain, hyperlipidemia [17], and hypertension [18]. They also agree with a study conducted in Sprague-Dawley rats in which 10% fructose in drinking water produced hypertensive and hyperlipidemic effects similar to those obtained when the rats consumed 60% fructose added to the diet [19]. The decision to use 10% fructose to induce SM and administer the *P. canariensis* treatments was based on the fact that the animals that received this concentration had the best nutritional balance. There is evidence that the amount of food that rats eat depends more on the balance of kilocalorie (kcal) intake than it does on the smell, taste, and color of the food, which are important factors in the excessive intake of calories in humans [20]. Our results, not yet published, show that animals fed diets supplemented with fructose (10, 20, and 30%), from the first month modulate and compensate their kcal intake by eating less food than the control group. This behavior compensates for increased calories due to the addition of fructose to the food and drinking water, and it is consistent with Steve M. *et al.*, who observed a gradual decrease in food intake in rats that had *ad libitum* access to high-carbohydrate diets for long periods of time [21]. However, in the groups treated with 20% and 30% fructose, the lower food intake significantly decreased the intake of proteins and fats compared to the group treated with 10% fructose.

The results of this study reveal that the daily oral administration of 310 mg/kg aqueous extract of *P. canariensis* seed is more efficacious in the treatment of MS induced by 10% fructose than the administration of the hexane and water fractions obtained from this extract. The significant decrease in TGs and SBP observed in the MS + *Pc* group coincides with the findings of a study presented at the 34th Congress of the Spanish Society of Pharmacology by another research group, which also noted that the *P. canariensis* seed decreases glucose and protects against oxidative damage in rats with MS [22]. The decreases in LI and WC observed in this study’s MS + *Pc* group strengthen the highly significant evidence that a complete aqueous extract of canary seed is effective; from 30 to 60 days of treatment, it reduces the increase found in the three indicators (TGs, LI, and SBP) that allowed for the characterization of the fructose-induced MS.

The 310 mg/kg dose of aqueous extract that we utilized was estimated based on information provided by folk healers from the Sonora region in Mexico, who prepare a homemade formulation called *leche de alpiste* (canary seed milk) for a person with approximately 70 kg body weight. This dose, also used in the hexane and water fractions, allowed for the dispersion of each treatment in its respective vehicle and for a relatively uncomplicated daily intraoral administration. Field studies indicate that the *P. canariensis* seed has anti-diabetic [8, 23-25], diuretic [7, 26, 27], hypcholesterolemic [17,23] and antihypertensive [17,18] properties. However, with regard to its use in the treatment of obesity, there is a lack of ethnobotanical studies that would allow for the appropriate characterization of such use. Nevertheless, experimental studies found that 400 mg/kg/day for 30 days of a *P. canariensis* seed extract obtained with hexane produced a significant reduction in body weight, blood glucose, serum cholesterol, TGs, lipoproteins, and increased HDL-Cin high-fat-diet-induced obese and streptozotocin-induced diabetic mice, probably as a result of serum insulin decreasing oxidative stress [28] or through the inhibition of enzymes related to obesity and diabetes mellitus [9]. In contrast, our results show that in Wistar rats, the treatment of the MS + hex group with a hexane extract significantly decreased only WC without modifying the LI or TGs. One possible explanation for this difference lies in the effects reported for the type of unsaturated fatty acids contained in *P. canariensis* seeds (the total amount of crude fat, 8.7%, contains 55% linoleic, 29% oleic, 11% palmitic, and 2.5% linolenic acid) [15], which are extracted by hexane. It has been reported that in rats with diet-induced obesity, the aforementioned fatty acids produce a redistribution of lipids from the abdomen to the rest of the body. Linoleic acid and alpha-linolenic acid, unlike oleic acid, do not increase body adiposity; oleic acid reduces total plasma cholesterol but does not affect TG or non-esterified fatty acid levels [29]. The effect that seems to prevail in our results is the redistribution of abdominal fat, which decreased WC without modifying body weight and TG levels. This finding coincides with data observed in humans in which consumption of unseparated fatty acids does not appear to produce major effects on body weight or serum lipids [30]. In contrast, 60 days of treatment with the aqueous extract in the MS + aqueous group resulted in a significant decrease in SBP and TG levels. The hypotensive and antihypertensive effects has been reported in numerous studies that evaluated aqueous extracts of *P. canariensis* seeds, which, in relation to the high protein content (18.7%) and high concentrations of amino acids such as cystine, tryptophan, phenylalanine and leucine (3.3, 2.8, 6.7 and 7.0g/100 g of protein respectively) [15], explained the hypotensive effect of the seed through the contribution to vessel relaxation and reduction of blood pressure made by the metabolism of tryptophan to kynurenine effected by the indoleamine 2, 3-dioxygenase (IDO) pathway [32], and it was also explained by encrypted peptides in the seed’s proteins that exhibit inhibitory activity against angiotensin-converting enzyme, a target in the treatment of systemic hypertension [6, 13].

The decrease in TGs due to the components of the seed that are soluble in an aqueous medium may be associated with the presence of phytate (18.2 mg/g) [33] because it has been observed that at physiological dosages (0.1–0.5% of diet), phytate inhibits rise in hepatic total lipids and triglycerides, resulting from administration of sucrose. The mechanism of this hypolipidaemic effect in the liver appears to be related to the inhibition of hepatic enzymes involved in lipogenesis [34]. As shown in Figures 4 and 5, the sum of the effects produced by the administration of the hexane and the aqueous fractions does not reproduce all of the effects nor the same improvement in MS observed with the administration of the complete aqueous extract. It is clear that the presence of various compounds of different polarity in the complete aqueous extract increases the efficacy and probably the potency of the active substances. However, the activity of
each compound has yet to be identified, so that their possible pharmacodynamic and pharmacokinetic interactions may be explored, thus substantiating the superiority shown by the complete extract over the fractions in the treatment of experimental MS. It is important to mention that during the conduct of the experiments no toxic effects were observed with any of the *P. canariensis* seed treatments, which is consistent with an extensive toxicological study that concluded that glabrous canary seed groats would be generally recognized as safe for consumption in their intended use in food [35]. In contrast, sibutramine used in this study as positive control has been withdrawn from the world market because it increases the risk of cardiovascular adverse events [36].

5. Conclusion
This study shows that an intraorally administered complete aqueous extract of *P. canariensis* seed is superior to the fractions obtained from this extract against some indicators of MS such as hypertriglyceridemia and increased WC and LI, observed in Wistar rats as a result of the addition of fructose in food and drinking water. This study provides evidence for and supports the general idea that the complete mixture of substances contained in the natural form of some plants improves effectiveness in the treatment of clinical entities that, as in MS, have multiple physiopathological manifestations. The preventive and palliative effects of the complete aqueous extract of *P. canariensis* seed in addition to the large concentrations of protein, essential amino acids, and unsaturated fatty acids that it contains make this plant a good prospect for studies in humans in order to substantiate its clinical utility.

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