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Original Article

Phytochemical Compounds of *Cichorium intybus* by Exploring its Antioxidant and Antidiabetic Activities

Dina Kanj[1], Karim Raafat[1,*], Abdalla El-Lakany[1], Safaa Baydoun[2], Maha Aboul-Ela[1]

ABSTRACT

Introduction: The current study aims to evaluate the therapeutic effect of *Cichorium intybus* n-hexane extract on diabetes mellitus and its antioxidant effect *in vivo* in alloxinated animals. Diabetic neuropathy improvement was also tested as well as insulin levels and histology of the pancreas were performed. **Methods:** The chromatographic standardization of *C. intybus* extract was performed using isocratic HPLC, which indicated the presence of numerous phyto-constituents. The hexane extract was studied for its effect on blood glucose levels and painful Diabetic neuropathy (DN) in diabetic mice. Hyperalgesia and mechanical-allodynia were evaluated using thermal stimuli, pain response to radiant energy experiments and a mechanical sensitivity test respectively. Subsequently, after eight weeks of being alloxinated, BGL, body weight, antioxidant activity, insulin levels and glycated hemoglobin were recorded to evaluate antidiabetic potential and the DN. **Results:** The administration of *Cichorium intybus* extract (50, 75 and 100 mg/kg) and a combination of *Cichorium intybus* extract and *Camellia sinensis* (50 + 200 mg/kg, respectively) have revealed an acute hypoglycemic effect ranging from 14.15% and 42.4%. The sub-chronic anti-diabetic effect ranged from 23.41% and 44.8%. They diminished hyperalgesia and tangible allodynia significantly ($p<0.05$), ($n=7$ per group). The powerful neuroprotective properties might serve as potential lead-compounds for further analysis. **Conclusion:** The histological study and the potent antioxidant effect showed that they could assist in the management of diabetes mellitus and DN by amelioration of insulin levels and regeneration of pancreatic beta cells.

Key words: *Cichorium intybus*, Phytochemical analysis, Antioxidant, Serum insulin, Anti-diabetic effects.

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INTRODUCTION

Type 1 Diabetes mellitus (T1DM) is identified by an impairment of the secretion of insulin or insulin shortage that leads to an imbalance of glucose metabolism. DM is considered as the prime of the five main causes of death in the world.[1] This is due to the life-style differences that result in lack of exercise and a rise in obesity.[2] The most frequent cause of death among these persons is due to myocardial infarction.[1]

Diabetic neuropathies (DN) are the predominant chronic complications of diabetes. This varied group of circumstances affects different areas of the nervous system and presents diverse clinical signs. The early identification and proper management of neuropathy in the patient with diabetes are essential for many reasons and may be treatable by specific methods. Some of the drugs' choices exist for symptomatic diabetic neuropathy. Identification of autonomic neuropathy and being under control may ameliorate symptoms, decrease sequelae, improve quality of life and help patients in danger for injuries to their insensate feet.

The diabetic state is postulated in the rise of oxidative stress. High levels of ROS have a role in the expansion of diabetic complications. The evolution of hyperglycemia can be attributed to an inequity between ROS, for instance, Hydroxyl radicals (HO), superoxide anions (O_2) and H_2O_2.[3-4]

Oxidation is a natural response that includes the transmission of an electron (ē) from (ē) effluent to a (ē) lacking object. The term oxidizer or oxidizing agent is an electron deficient molecule. The antioxidants are produced either endogenously or are received from exogenous sources.[5]

Catalases are ordinary enzymes, of the antioxidant nature, that generate the change of H_2O_2 to H_2O and O_2. Catalases exist everywhere in aerobic life, including almost all mammalian tissues. The predominant enzyme action resides in the liver and erythrocytes. Catalases, which mostly reside in peroxisomes in cells and mitochondria as both soluble and membrane-bound forms.[6]

Cichorium intybus (*C. intybus or* CI) took its name from the Latin term, "*Cichorium*" signifying field and "*intybus*" is partially the result of the Latin term, "to cut".[7] *C. intybus* L., being of the Asteraceae, is

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a bushy biennial herb that may be utilized in the food industry.[8-9] It is indigenous to Europe and Asia.[10]*C. intybus* can reach 170 cm in height and the roots are shaped as spindle.[11] The herb may germinate in a wild manner in its ordinary territory and/or river banks. Flowering periods last from June to October.[10] *C. intybus* L. or "Hendibeh" is eaten in Lebanon, the Middle East and in other countries.[9] *Camellia sinensis* (*C. sinensis*) is also commended for having hypoglycemic effects as it has proven to have some potential in preventing insulin resistance.[12]

A study conducted in 2007, by Pushparaj *et al.*[13] was performed to examine the activity of *C. intybus* ethanolic extract on hyperglycemia. This extract was used as a conventional drug for the management of DM. The result showed that the extract might improve DM. There is no study performed on the DN effect of the *C. intybus* hexane extracts.

Consequently, the study aims to explore not only the hypoglycemic effect of *C. intybus,* but also the possible antioxidant activity, improvement of DN, amelioration of insulin levels and enhancement of pancreatic beta cells.

MATERIALS AND METHODS

Plants materials

Total plant fragments of *Cichorium intybus* were assimilated from the RCED and identified by Prof. Nelly Arnold. The parts used are the roots which were dried and pulverized. The oil was extracted with hexane. No essential oil (EO) yield was obtained from the aerial parts.

Preparing the Cichorium intybus Extract

The extract was readied by adding 100ml of hexane. The mixture was put in the sonicator at a temperature of 56°C for 2h. The extract was filtered by Whatman filter paper.[14] The solvent was evaporated using the rotary evaporator to one-third of its starting volume. The collected final extract was kept in the freezer at -26°C for later use.

Animals for in vivo studies

The animals used for *in vivo* studies were Male Swiss-Webster mice, aging between 12 to 16 weeks and weighing between 25 to 32g provided by the Animal House, Faculty of Pharmacy, Beirut Arab University. The mice were kept in an adaptation environment prior to the experiment. The mice were reserved in distinct plexi-boxes at an ambient climate (22 ± 1°C) and a light/dark rotation of 12 h each. The mice were given standard pellets of proteins (20), fats (5) and multivitamins (1) to consume and had free access to water. The fasting of the mice was made 16 hrs prior to the experiment; the mice were prevented of the feeding pellets but they had free access to water. The mice that were subject to experimentation were treated with care (Table 1), conferring to the regulations of the Lebanese Ministry of Higher Education and the animal experiment legislation and with the approval code (2016A-0040-P-M-0144) of the Institutional Review Board (IRB) of Beirut Arab University.

Aqueous extract of Camellia sinensis

Five pills of 450 mg of *Camellia sinensis* (Green Tea powder Thompson) were soaked in 22.5 ml hot water for 15 min. The Filtrate was done by Whatman filter paper and kept in the fridge for later use.

Preparing the Insulin stock and standard solutions

The stock insulin was prepared by adding 0.01M HCl at a concentration of 100 µg/mL. Using diluted stock insulin solution, the standards were prepared at (5, 10 and 25 µg/mL).[15]

Extraction procedure

A blank plasma at 0.2 mL was transferred to a 12 mL centrifuge with 1mL of phosphate buffer (pH 7.4) and swirled the solutions for a short time. One mL of CH_2Cl_2 was placed into the tube and swirled for 1 min. The tube was swirled for 3 mins at 2,000 x g. The organic phase was moved to a 5 mL tube. I added 0.15 mL of 0.05M HCl into this tube and swirled it for 1 min. The clear liquid was then moved to another tube and left to evaporate at ambient climate. I rebuilt the deposit by putting it over 1mL of 0.01M HCl. The solution was filtrated through a syringe filter vessel of 0.45 µm. A sample of 20 µL was analyzed by placing it into the automatic sample injector of HPLC system.[16]

Diabetes mellitus induction

DM is experimentally induced by giving alloxan monohydrate (Sigma-Aldrich, Germany) freshly prepared through intraperitoneal (IP) injection to mice. Alloxan was dissolved in saline solution (0.9%) and was delivered every other day (three times a week) at a dose of 180mg/kg of body weight (BW).

TLC analysis of Cichorium intybus extract

The stationary phase of silica gel was prepared. Using TLC spotting capillaries tubes 3 different spots were put. C1 = 2 drops of CI Ex, C2 = 5 drops of CI extract and C3 = 10 drops of CI Ex. 10% ethanolic H_2SO_4 reagent was prepared for the detection method, the plate was then put in the oven to dry at a temperature of 115°C for 15 min. The detection was in the UV light 365nm.[17]

HPLC analysis of Cichorium intybus extract

High performance liquid chromatography (HPLC) is a highly improved form of liquid chromatography. A faster technique where the liquid is forced through a column under very high pressures up to 400 atmospheres.

HPLCWaters717 plus Autosampler Multi λ fluorescence detector with Waters 2487 Dual λ Absorbance detector is the apparatus used. HPLC method was adapted from the method mentioned by Sakamoto, *et al.*[18]

HPLC analysis (Isocratic elution with MeOH/H_2O 9:1; λ = 265nm; flow 9.5mL/min; injection volume = 20 µl; and the runtime = 40 min). The MeOH/H_2O was prepared by adding 45ml MeOH to 5ml DDW. The sample stock (1) was prepared by weighing 1 mg of the *C. intybus* residue which was added to 1 ml of ACN, from stock (1) 200 µl was added to 800 µl ACN.

Determination of blood glucose levels

After three days of alloxinating the mice, the BGL of all the animals was measured, using ACCU-CHEK Performa™ Test Meter (Roche, USA) to confirm hyperglycemia. The blood samples were taken carefully from the animal's tail. A solution of 5% Glucose was administered as drinking water to the mice. The BGL were expressed as mg/dl. The mice with BGL higher than 200mg/dl were considered as hyperglycemic and were used in the experimental research.[16]

Management of diabetic neuropathy (DN)

Hot plate (HP) test

HP analgesia meter (Ugo Basile, Italy) was used for valuation of DN. Each mouse from the six groups treated with *C. intybus* extract alone and in association with *C. sinensis* extract at different doses was placed alone in the hot plate at a temperature of 50 ± 0.1°C. Response time to jumping or hind paw licking was observed utilizing a timer. To prevent tissue harm, 30 secs of shut-off time was selected.[19]

Tail flick (TF) test

TF apparatus (Hugo-Sachs-Bektronik, Germany) was used to assess DN. The tail flick test is a pain response test for animals. It is used to measure the usefulness of analgesics, by observing heat response. This meter measures a mouse's reaction time to radiant energy, from a light source. The energy of the light source can be adjusted. The light intensity was set to 8; a cut-off time of 10.00±0.50 sec was set in order to prevent tissue damage.[20]

Table 1: Study scheme.

A. Diabetic			
Group	**n =**	**Name**	**Dosage at 0h, 0.5h, 2h and 6 h**
I	7	NC	Saline (0.9% NaCl)
III	7	DC	Saline (0.9% NaCl)
IV	7	DC+CI 50	CI extract 50 µl
V	7	DC+CI 75	CI extract 75 µl
VI	7	DC+CI 100	CI extract 100 µl
VII	7	DC+CI 50+CS 200	CI extract 50 µl + C. sinensis extract 200 µ
B. Subchronic effect			
Group	**n =**	**Name**	**Dosage: 3 times a week (once a day) for 8 days**
VIII	7	NC	Saline (0.9% NaCl)
IX	7	DC	Saline (0.9% NaCl)
X	7	DC+CI 50	CI extract 50 µl
XI	7	DC+CI 75	CI extract 75 µl
XII	7	DC+CI 100	CI extract 100 µl
XIII	7	DC+CI50+CS 200	CI extract 50 µl + C. sinensis extract 200 µ
B. Diabetic neuropathy			
Group	**n =**	**Name**	**Dosage: 3 times a week (once a day) for 8 weeks**
XIV	7	NC	Saline (0.9% NaCl)
XV	7	DC	Saline (0.9% NaCl)
XVI	7	DC + CI 50	CI extract 50 µl
XVII	7	DC + CI 75	CI extract 75 µl
XVIII	7	DC + CI 100	CI extract 100 µl
XIX	7	DC+CI 50+CS 200	CI extract 50 µl + C. sinensis extract 200 µl

Von Frey filaments (VFF) test

The use of Von Frey filaments (OptiHair TM, MarstockNervtest™, Germany) leads to the assessment of a rodent's sensitivity to a mechanical stimulus (tactile allodynia). Fibers with rising calibration (0.5 - 45.3g ± 10%) were inserted at 90 degree angle through the mesh to poke the animal's hind paw and using an up-down method. A power just enough to slightly bow the fiber that was inserted for 5 sec. Affirmative reactions for the animal include abrupt withdrawing or licking or shaking the paw.[20]

Measuring glycated hemoglobin profile

In vitro analysis of the glycated hemoglobin (HbA1c) concentrations were measured in mice blood by pricking the tail, utilizing (Analyticon, Analyticon Biotechnologies AG, Am Munsterberg, Germany) test kit, of the different six groups, mentioned previously after 8 weeks of the onset of the drugs' administrations.

Effects of C. intybus and C. sinensis extracts on diabetic neuropathy

The potential effect of *C. intybus* extract alone and in association with *C. sinensis* extract on DN was studied. The six groups of mice were tested. After 4-weeks of DM induction and for 8 consecutive weeks at an interval of one week, DN achievement rate and their neurological results were verified by:

In vivo antioxidant effect of C. intybus alone and in association with C. sinensis extract

To prove that *C. intybus* and *C. sinensis* extracts have an antioxidant effect, serum catalase activity was studied. After 8-weeks of the drugs' administrations, 50 µl of blood samples were taken from the mice's tails from the six groups. The samples were swirled and the serum was collected. CAT was established by an adapted method described by Yasmineh, *et al.*[21] Serum was added to the phosphate buffer freshly prepared. The reaction took place as soon as H_2O_2 is added. The rate of the reaction was established by observing the reduction in the absorbance of H_2O_2 at 240nm for 5 min by a UV NIS spectrophotometer (SP-3000 plus, Optima, Japan).

Statistical Analysis

All data is presented as a sum of averages ± SEM. Statistical variances between the test group and control group were tested through a one-way analysis of variance (ANOVA) entailed by the Student-Newman-Keuls test utilizing the "OriginPro" statistic computer software. A variance in the mean values of $p < 0.05$ was accepted to be statistically significant.

RESULTS

Pharmacognostic description

C. intybus root consisted of the fleshy deep taproot, deep, branched, up to seventy-five cm long, with a milky-sap.

Chromatographic analysis of the oil extract of C. intybus

Constituents' identification contributes in more understanding of *C. intybus* importance. Thus, chromatographic analysis of the oil extract of *C. intybus* was done.

Thin layer chromatography

C. intybus hexane extract has undergone a TLC analysis. The chromatographic standardization, using TLC, indicated the presence of fructose, glucose and sucrose, ketose, nystose and the inulin type DP fructans: DP4, DP5 and DP6 (Figure S. 1).

Chromatographic standardization of Cichorium intybus

The chromatographic standardization, using isocratic HPLC, indicated that the most active compounds of *C. intybus* extract fraction major peaks were in the retention time range from 10 to 35 min. The six peaks found to represent a total percentage of 92.22%. The major compounds found in *C. intybus* extract are Chicoric acid at a high percentage (64.70%), Isochlorogenic acid A/B/C (8.90%), Dicaffeoylquinic acid (6.87%), Chlorogenic acid (4.81%), Esculetin (3.54%), Caffeic acid (3.40%) (Figure 1). The major compounds found in *C. intybus* extract are in the (Table 2).

Chromatographic standardization of Insulin

The chromatographic standardization of Insulin using an isocratic HPLC, (Isocratic elution with ACN/Na_2SO_4 (0.2M) 25:75; λ = 214nm; flow 1.2 mL/min; injection volume = 20 µl; and the runtime = 30 minutes), indicated that the active compounds of Insulin fractions major peaks were in the retention time range from 10 to 25 min[22] (Figure S. 2).

Acute antidiabetic effect of the C. intybus and C. sinensis extracts in alloxan-induced diabetic mice

The acute anti-diabetic result of numerous doses of *C. intybus* extract (50 µl, 75 µl and 100 µl) alone and in combination with *C. sinensis* at doses (50 µl + 200 µl, respectively) in induced diabetic mice is summarized in the (Table 3) CI 50 = *C. intybus* extract 50 µl, CI 75 = *C. intybus* extract 75 µl, CI 100 = *C. intybus* extract 100 µl, CI 50 + CS 200 = *C. intybus* extract 50 µl + *C. sinensis* extract 200 µl. The administration of *C. intybus* extract showed, at the 6 hours of the first day, a decrease in the percentage

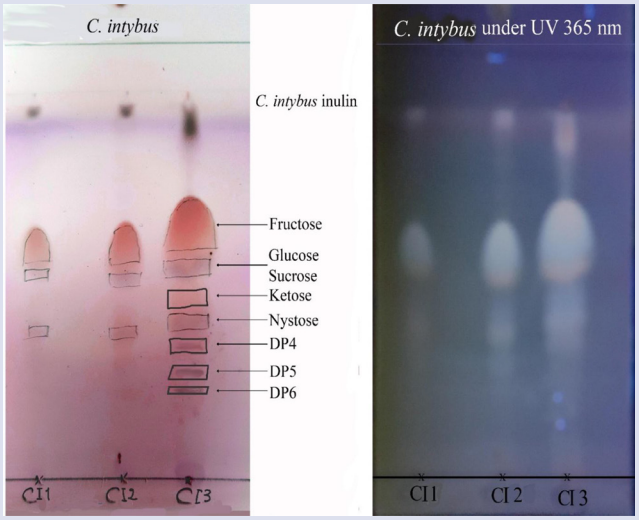

Figure S1: C 1 = 2 drops of CI extract, C 2 = 5 drops drops of CI extract and C 3 = 10 drops drops of CI extract. 10% ethanolic H_2SO_4 reagent was prepared for the detection method, the plate was then put in the oven to dry at a temperature of 115°C for 15 minutes. The detection was in the UV light 365 nm

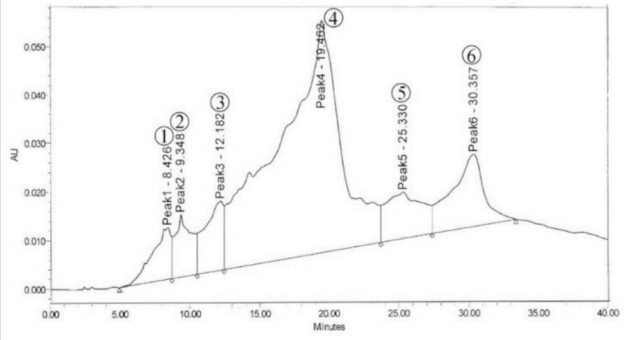

Figure 1: HPLC chromatogram of *C. intybus* using an isocratic HPLC (Isocratic elution with MeOH/H_2O 9:1; λ = 265 nm; flow 9.5 mL/min; injection volume = 20 µl; and the run time = 40 min).

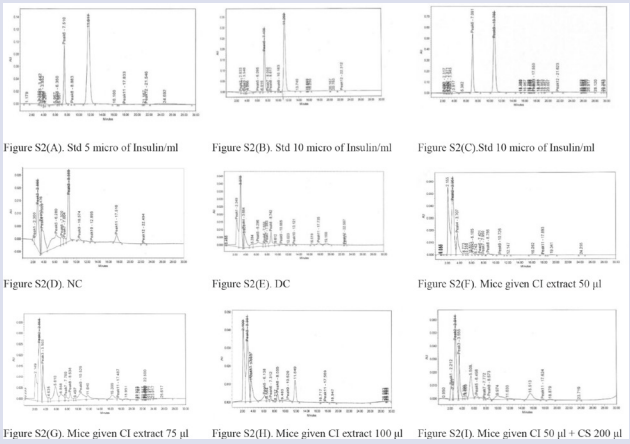

Figure S2. Represent Chromatographic standardization of Insulin using an isocratic HPLC (Isocratic elution with ACN/Na_2SO_4 (0.2M) 25:75; λ = 214 nm; flow 1.2 mL/min; injection volume = 20 µl; and the run time = 30 min).

Table 2: Identification of the major compounds found in *C. intybus* extract by HPLC.

Peak number	RT (min)	Identification	%
1	8.426	Caftaric acid	3.40
2	9.348	Aesculin	3.54
3	12.182	Chlorogenic acid	4.81
4	19.462	Chicoric acid	64.70
5	25.330	Dicaffeoylquinic acid	6.87
6	30.357	Isochlorogenic acid A/B/C	8.90

Table 3: Acute antidiabetic activity of *C. intybus* extract on BGL in alloxan-induced diabetic mice.

Groups/ Day	Mean of BGL ± S.E.M (mg/dl)			
	0 hr	0.5 hr	2 hr	6 hr
NC	137.50 ± 1.50	131.00 ± 1.00	111.00 ± 1.00	133.00 ± 3.00
DC[a]	198.50± 1.50	160.00 ±2.00	173.50 ± 4.50	208.50 ± 5.50***
CI 50[b]	183.00 ± 22.00	148.00 ± 1.00	157.50 ± 1.50	155.00 ± 21.00*
CI 75[b]	180.00 ± 13.00	132.00 ± 5.00	119.50 ± 1.50	143.00 ± 12.00**
CI 100[b]	209.00 ± 41.00	121.50 ±1.50	103.00 ± 3.00	127.00 ± 17.00**
CI 50 + CS 200[b]	199.50 ± 16.500	160.00 ± 5.00	133.50 ± 1.50	128.50 ± 11.50**

"*" means $p<0.05$, "**" means $p<0.01$, "***" means $p<0.001$. [a] Compared to NC, [b] Compared to DC. n= 7 mice/group.

of BGL when compared to DC. The percentage of BGL decrease for the dose CI extract 50 µl was 14.15%, for the dose of CI extract 75 µl was 19.69%, for the dose of CI extract 100 µl was 42.4% and finally for the dose of CI extract 50 µl + CS 200 µl was 35.19% when compared to DC. (Table 3).

Subchronic effect of the C. intybus and C. sinensis extracts in alloxan-induced diabetic mice

The Sub-chronic anti-diabetic activity, of several dosages of *C. intybus* extract (50 µl, 75 µl and 100 µl) alone and in combination with *C. sinensis* at doses (50 µl + 200 µl, respectively) in hyperglycemic mice, is summarized in the (Table 4). CI 50 = *C. intybus* extract 50 µl, CI 75 = *C. intybus* extract 75 µl, CI 100 = *C. intybus* extract 100 µl, CI 50 + CS 200 = *C. intybus* extract 50 µl + *C. sinensis* extract 200 µl. The administration of *C. intybus* extract showed, at the 8th day, a decrease in the percentage of BGL when compared to DC. The percentage of BGL decrease for the dose CI extract 50 µl was 23.41%, for the dose of CI extract 75 µl was 25.39%, for the dose of CI extract 100 µl was 44.8% and finally for the dose of CI extract 50 µl + CS 200 µl was 35.19% when compared to DC.

During the 8 days of the drugs administrations of the hyperglycemic mice, with *C. intybus* extract (50 µl, 75 µl and 100 µl) alone and in combination with *C. sinensis* at doses (50 µl + 200 µl, respectively), the weights of the mice were also observed for changes (Table 5). The administration of *C. intybus* extract showed, at the 8th day, an increase in the percentage of BW when compared to DC. The percentage of BW increase for the

Table 4: Subchronic effect of *C. intybus* extract on BGL of alloxinated mice.

Groups/ Day	Mean BGL± S.E.M (mg/dl)			
	1st day	3rd day	5th day	8th day
NC	128.50 ± 8.50	134.00 ± 7.00	135.00 ± 5.00	132.00 ± 1.00
DC[a]	207.00 ± 7.00	200.00 ± 3.00	165.50 ± 3.50	210.00 ± 18.00**
CI 50[b]	180.50 ± 4.50	137.50 ± 3.50	155.50 ± 9.50	143.00 ± 14.00*
CI 75[b]	144.50 ± 18.50	152.50 ± 15.50	120.00 ± 8.00	135.00 ± 9.00*
CI 100[b]	133.50 ± 76.50	142.50 ± 5.50	149.00 ± 18.00	123 ± 15.00*
CI 50 + CS 200[b]	146.50 ± 38.50	151.50 ± 0.50	150.50 ± 21.50	121.00 ± 10.00*

"*" means $p<0.05$, "**" means $p<0.01$. [a] Compared to NC, [b] Compared to DC. n= 7 mice/group.

Table 5: Subchronic effect of *C. intybus* extract on BW in alloxinated animals.

Groups	Mean BW ± S.E.M (g)			
	1st day	3rd day	5th day	8th day
NC	30.00 ± 0.05	27.70 ± 0.20	29.05 ± 0.55	30.05 ± 0.05
DC[a]	30.05 ± 0.15	29.25 ± 0.25	29.75 ± 0.75	28.45 ± 0.45**
CI 50[b]	29.15 ± 0.05	27.77 ± 1.23	28.50 ± 0.40	29.65 ± 0.05*
CI 75[b]	28.30 ± 0.70	29.55 ± 0.05	29.70 ± 0.30	30.35 ± 0.05*
CI 100[b]	27.20 ± 0.30	29.30 ± 0.10	31.00 ± 0.50	31.50 ± 0.50**
CI 50 + CS 200[b]	27.75 ± 0.75	30.90 ± 0.40	31.90 ± 0.60	31.90 ± 0.60**

"*" means $p<0.05$, "**" means $p<0.01$. [a] Compared to NC, [b] Compared to DC. n= 7 mice/group.

dose CI extract 50 µl was 6%, for the dose of CI extract 75 µl was 8.5%, for the dose of CI extract 100 µl was 14.2% and finally for the dose of CI extract 50 µl + CS 200 µl was 15% when compared to DC.

Measurement of Glycated Hemoglobin profile

After 8 weeks of starting the drugs' administrations, HbA1c was measured for hyperglycemic mice. Four different concentration of *C. intybus* extract (50 µl, 75 µl and 100 µl) and a combination of *C. intybus* extract with *C. sinensis* extract (50 µl + 200 µl) were used. For each group, HbA1c was measured to indicate the long-term hyperglycemia in mice.

The Figure 2 Shows that the NC of HbA1c has a normal result (4.5% ± 0.23), whereas in DC HbA1c was (8.5% ± 0.43). The percentage of the result for the dose CI extract 50 µl was (7.3% ± 0.37), for the dose of CI extract 75 µl was (6.9% ± 0.35), for the dose of CI extract 100 µl was (6.27% ± 0.32) and finally for the dose of CI extract 50 µl + CS 200 µl was (6.5% ± 0.33). These doses were effective and maintained the HbA1C within the "good control" range and the highest effects were with the extract of CI at the dose 100 µl and the combination of the extract of CI and CS at doses of 50µl + 200µl, respectively, when compared to DC.

Measurement of Insulin profile

After 8 weeks of starting the drugs' administrations, Insulin was measured for alloxan-induced diabetic mice. Four different concentration of *C. intybus* extract (50 µl, 75 µland 100 µl) and a combination of *C. intybus* extract with *C. sinensis* extract (50 µl + 200 µl) were delivered.

The Figure 3 shows that the NC of Insulin is in the normal range (9.61% ± 0.48), whereas in DC it was (0.87% ± 0.13) and for the dose CI extract 50 µl was (1.87% ± 0.17), for the dose of CI extract 75 µl was (3.22% ± 0.16), for the dose of CI extract 100 µl was (9.06% ± 0.3) and finally for

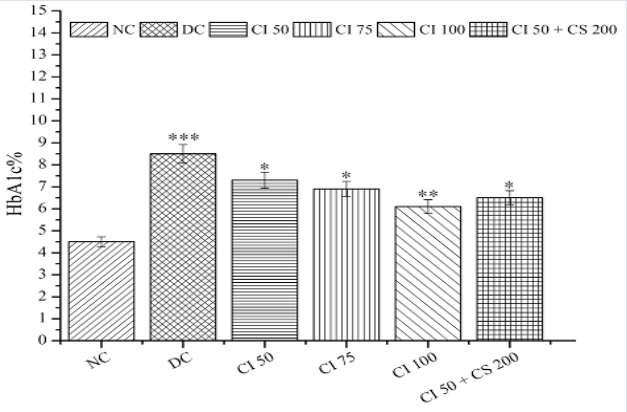

Figure 2: Measurement of glycated hemoglobin profile in alloxinated animals of *C. intybus* and C. sinensis extracts. NC = Normal control, DC = Diabetic control, CI 50 = *C. intybus extract* 50 µl, CI 75 = *C. intybus extract* 75 µl, CI 100 = *C. intybus* extract 100 µl, CI 50 + CS 200 = *C. intybus* extract 50 µl + *C. sinensis* extract 200 µl. Data are expressed in mean ± S.E.M. (standard error of the mean). "*" means $p<0.05$, "**" means $p<0.01$, "***" means $p<0.001$. n= 7 mice/group.

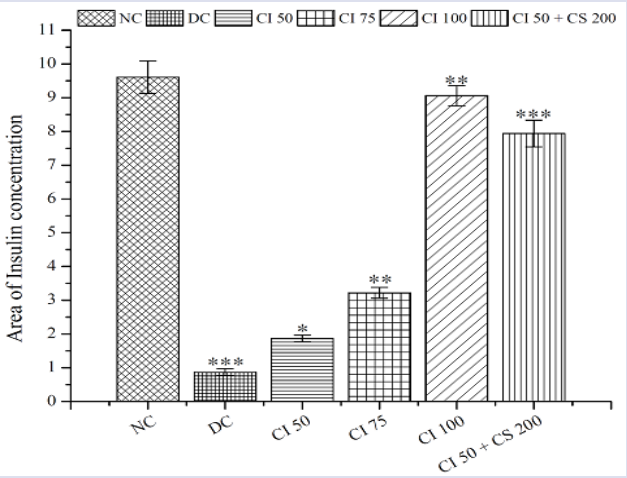

Figure 3: Measurement of Insulin profile in alloxinated animals of *C. intybus* and *C. sinensis* extracts. NC = Normal control, DC = Diabetic control, CI 50 = *C. intybus* extract 50 µl, CI 75 = *C. intybus* extract 75 µl, CI 100 = *C. intybus* extract 100 µl, CI 50 + CS 200 = *C. intybus* extract 50 µl+ *C. sinensis* extract 200 µl. Data are expressed in mean ± S.E.M (standard error of the mean). "**" means $p<0.01$, "***" means $p<0.001$. n= 7 mice/group.

the dose of CI extract 50 µl + CS 200 µl was (7.94% ± 0.39). The rise of insulin rate was a dose-dependent. The highest Insulin rates were with the CI extract 100 µl and the combination CI and CS extract at doses of 50µl + 200µl, respectively, when compared to DC.

Analysis of antioxidant activity

CAT serum's levels of each mice group were measured, eight weeks after the onset of the drugs' administration for alloxan-induced diabetic mice, to assess the antioxidant activity of *C. intybus*. The results, summarized in Figure 4, show that the diabetic animals were monitored by changes

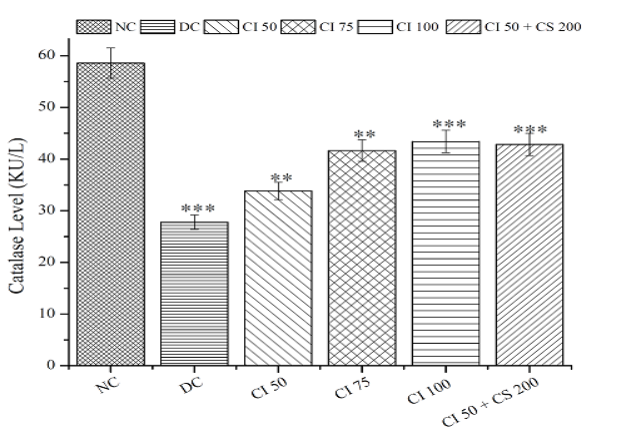

Figure 4: *In vivo* analysis of the antioxidant effect of *C. intybus* and *C. sinensis* extracts. NC = Normal control, DC = Diabetic control, CI 50 = *C. intybus* extract 50 µl, CI 75 = *C. intybus* extract 75 µl, CI 100 = *C. intybus* extract 100 µl, CI 50 + CS 200 = *C. intybus* extract 50 µl + *C. sinensis* extract 200 µl. Data are expressed in mean ± S.E.M (standard error of the mean). "**" means $p<0.01$, "***" means $p<0.001$. n= 7 mice/group.

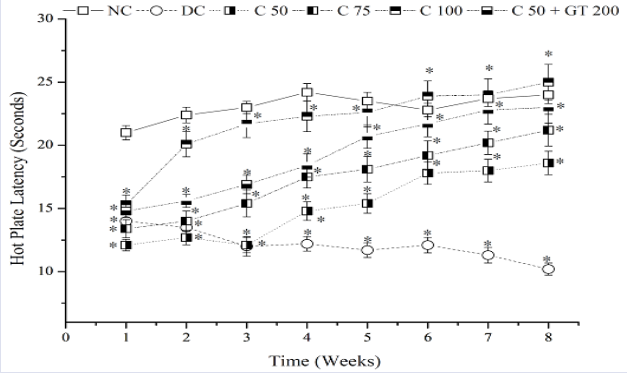

Figure 5: Effect of *C. intybus* on the hot plate latency in alloxan-induced diabetic mice. NC = Normal control, DC = Diabetic control, CI 50 = *C. intybus* extract 50 µl, CI 75 = *C. intybus* extract 75 µl, CI 100 = *C. intybus* extract 100 µl, CI 50 + CS 200 = *C. intybus* extract 50 µl + *C. sinensis* extract 200 µl. Data are expressed in mean ± S.E.M (standard error of the mean). "*" means $p<0.05$. n= 7 mice/group.

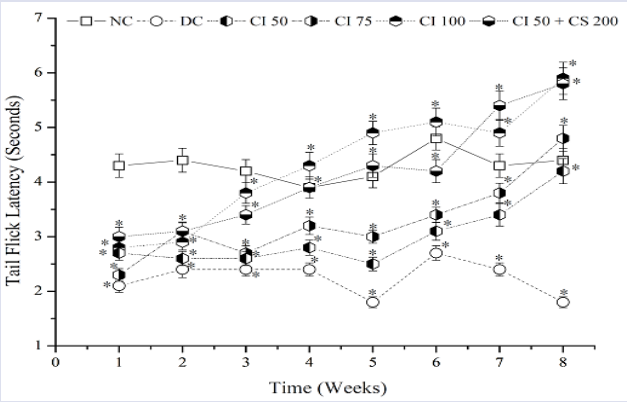

Figure 6: Effect of *C. intybus* on the tail flick latency in alloxan-induced diabetic mice. NC = Normal control, DC = Diabetic control, CI 50 = *C. intybus* extract 50 µl, CI 75 = *C. intybus* extract 75 µl, CI 100 = *C. intybus* extract 100 µl, CI 50 + CS 200 = *C. intybus* extract 50 µl + *C. sinensis* extract 200 µl. Data are expressed in mean ± S.E.M (standard error of the mean). "*" means $p<0.05$. n= 7 mice/group.

in serum's CAT levels. The delivering of *C. intybus* extract was at doses (50 µl, 75 µl and 100 µl) and an association of *C. intybus* extract with *C. sinensis* at doses (50 µl + 200 µl, respectively) resulted in an important increase in serum CAT activity. The percentage of CAT serum's levels increase for the dose CI extract 50 µl was 30.11 %, for the dose of CI extract 75 µl was 53.34 %, for the dose of CI extract 100 µl was 66.96 % and finally for the dose of CI extract 50 µl + CS 200 µl was 64.76 % when compared to DC.

Management of diabetic neuropathy

Diabetic patients with peripheral neuropathy possess an important indicator which is the decrease in the peripheral nerve conduction. On the 8th week of the onset of the management of in alloxinated animals, we examined the effect of *C. intybus* extract alone at doses (50 µl, 75 µl and 100 µl) and an association of *C. intybus* extract with *C. sinensis* extract at doses (50 µl + 200 µl, respectively) on peripheral function by evaluating the thermal latency with HP test and TF and sensitivity to a mechanical stimulus by VFF. The results in the Figure 5 show a noticeable improvement in the thermal latency when compared to DC group which showed a temporary hyperalgesic response in the HP test. The percentage of improvement in the thermal latency (Hot Plate Latency) for the dose CI extract 50 µl was 25.25%, for the dose of CI extract 75 µl was 43.29%, for the dose of CI extract 100 µl was 80.30% and finally for the dose of CI extract 50 µl + CS 200 µl was 57.73% when compared to DC.

The results in the Figure 6 show a noticeable improvement in the thermal latency (TF Latency) when compared to vehicle-treated groups which showed a temporary hyperalgesic response in the TF test. The percentage of improvement in the thermal latency (Tail Flick Latency) for the dose CI extract 50 µl was 32.77%, for the dose of CI extract 75 µl was 46.11%, for the dose of CI extract 100 µl was 92.22% and finally for the dose of CI extract 50 µl + CS 200 µl was 83.88% when compared to DC.

The sensitivity to a mechanical stimulus using VFF was improved markedly on the week eight after the onset of the drug administration of alloxinated animals Figure 7. The improvement in the tactile allodynia for the dose CI extract 50 µl was 11.58 folds, for the dose of CI extract 75 µl was 14.16 folds, for the dose of CI extract 100 µl was 23.48 folds and finally for the dose of CI extract 50 µl + CS 200 µl was 18.90 folds when compared to DC.

Histology results of mice's pancreas (diabetic mice)

The histological sections have to be stained with H and E stain (Hematoxylin and eosin stain). Staining of the cells' components gives them a bright color, together with a counterstain that stains the rest of the cell a different color. The resulting colors are Nuclei, violet; muscle, pink; cytoplasm, pink; basophilic, blue; acidophilic, pink.

The following images are the results of the histology of NC, DC and the treated mice. The Figure 8 shows: In A. is the result of the pancreas' histology for NC: β cells are 73.46%. In B. pancreas' histology for DC: we see a decrease in β cells to 23.80%. In C. pancreas' histology for the one treated by (CI extract 50 µl): we see an increase in β cells to 30.61%. In D. pancreas' histology for the one treated by (CI extract 75 µl): we see an increase in β cells to 40.81%. In E. pancreas' histology for the one treated

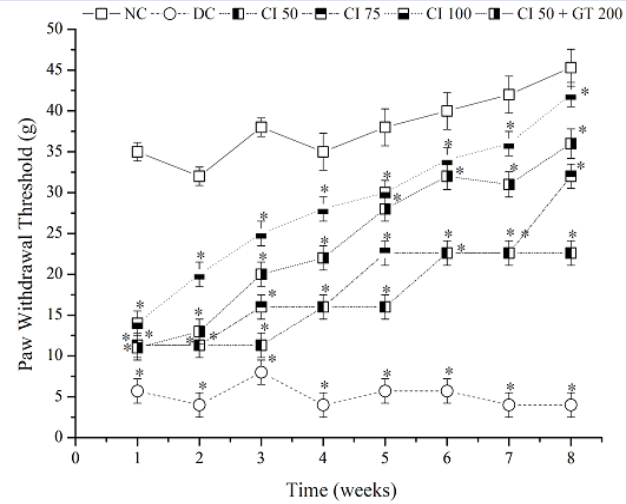

Figure 7: Effect of *C. intybus* on sensitivity to a mechanical stimulus in neuropathic model in alloxinated animals. Paw withdrawal thresholds to Von Frey Filaments were determined on hind paw up to 8 weeks after IP injection of *C. intybus*. NC = Normal control, DC = Diabetic control, CI 50 = *C. intybus* extract 50 µl, CI 75 = *C. intybus* extract 75 µl, CI 100 = *C. intybus* extract 100 µl, CI 50 + CS 200 = *C. intybus* extract 50 µl + *C. sinensis* extract 200 µl. Data are expressed in mean ± S.E.M (standard error of the mean). "*" means $p<0.05$. n= 7 mice/group.

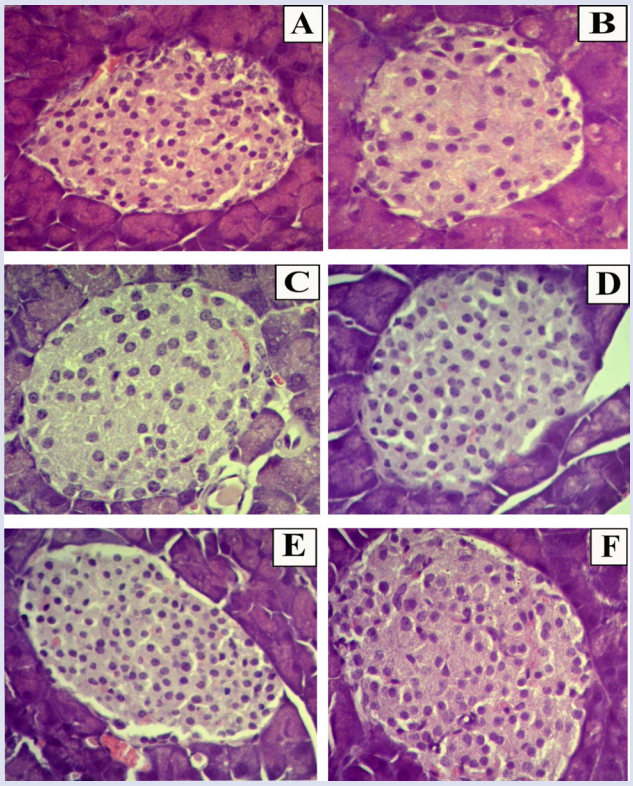

Figure 8: Figures 8 A- 8 F: A. Histology of the pancreas in Normal Control: β cells are 73.46%. B. Histology of the pancreas in Diabetic Control: β cells are 23.80%. C. Histology of the mouse's pancreas treated by (CI extract 50 µl): β cells are 30.61%. D. Histology of the mouse's pancreas treated by (CI extract 75 µl): β cells are 40.81%. E. Histology of the mouse's pancreas treated by (CI extract 100 µl): β cells are 69.04%. F. Histology of the mouse's pancreas treated by (CI extract 50 µl + CS extract 200 µl): β cells are 65.30%.

by (CI extract 100 µl): we see an increase in β cells to 69.04%. In F. pancreas' histology for the one treated by (CI extract 50 µl + CS extract 200 µl): we see an increase in β cells to 65.30%.

DISCUSSION

The evaluation of the effect of DM, one of the oldest metabolic disorder known, is characterized as an endocrine disorder leading to an impairment of insulin secretion or insulin shortage that leads to an imbalance of glucose metabolism. Several factors or causes complicate the management of DM.[22] According to statistics done by the International Diabetes Federation, the total adult population per 1,000 of Lebanese between the ages of 20 and 79, is 3,801 with the prevalence of diabetes in adults being 12.2%. The Number of deceases in grownups due to hyperglycemia is 5,723.80. The budget of the person with hyperglycemia is $ 870.2.[23]

The common side effect of insulin injection is hypoglycemia. Symptoms comprise of headaches, hunger, faintness, sweating, tremors, irritability, trouble with concentration, shortness of breath, rapid heartbeats, collapsing, or seizures (severe hypoglycemia can be fatal).[24] Also, there are possible side effects of diabetes medications. The Sulfonyl ureas' side effects are a drop in blood sugar, an irritated stomach, allergies and an increase in body weight. The Biguanides/Metformin's side effects are weight loss, alcohol sickness, kidney problems, an irritated stomach, exhaustion or faintness, metal taste. The Alpha-glucosidase inhibitors' side effects are gas, bloating and diarrhea. The Thiazolidinediones' side effects are weight gain, the risk of liver disease, anemia risk, inflammation of legs or ankles. The Meglitinides's side effects are weight gain, low blood sugar.[24]

All these side effects sited previously made the discovery of hypoglycemic agents with minimal or no adverse effects, the challenge. For a long time, only few plants, among many, have demonstrated that they are useful in the management of DM and have hypoglycemic activity. The use of complementary and alternative medications was admitted to be used by 30% of the patients.[25]

C. intybus hexane extract TLC revealed the presence of fructose, glucose and sucrose, ketose, nystose and the inulin type DP fructans: DP4, DP5 and DP6.

The chromatographic standardization of *C. intybus* extract, using isocratic HPLC, indicated that the abundant composites are available in the extract. The analysis of the sample revealed the presence of six peaks, which represent a total percentage of 92.22%. The major compounds found in *C. intybus* extract are Chicoric acid at a high percentage (64.70%), Isochlorogenic acid A/B/C (8.90%), Dicaffeoylquinic acid (6.87%), Chlorogenic acid (4.81%), Esculetin (3.54%), Caffeic acid (3.40%).

According to Pushparaj *et al.*[13] had conducted a study to investigate the hypoglycemic effect of a *C. intybus* ethanolic extract. The extract is extensively prepared in India as a conventional drug for the management of DM. Hypoglycemic effects of *C. intybus* extracts were perceived in an oral glucose tolerance test (OGTT) that encourages the conventional faith that *C. intybus* might improve hyperglycemia. In a study conducted by haidari *et al.*[26] results clearly showed that the oral administration of *Camellia sinensis* extract at a quantity of 200 mg/Kg improved the BGL. The BGL reached the normal levels in rats. The researchers suggested that EGCG (epigallocatechin gallate), one of the catechins in *Camellia sinensis*, enhances the oral glucose tolerance in severely diabetic mice.[13]

C. intybus extract was tested alone using the following doses, CI 50 = *C. intybus* extract 50 µl, CI 75 = C. *intybus* extract 75 µl, CI 100 = *C. intybus* extract 100 µl and in combination with *C. sinensis* CI 50 + CS 200 = *C. intybus* extract 50 µl + *C. sinensis* extract 200 µl. The dose of the *C. intybus* extract and *C. sinensis* extract was adjusted after many trials on different doses that most of them led to mice's dying from hypoglycemia. The latter dose was found to be the most appropriate.

DM was induced in the mouse by alloxan according to Raafat and Samy[27-29] and Raafat and El-Lakany.[27] The results showed successful induction of DM based on the rise in blood glucose to (208.50 ± 5.50 mg/dl) and HbA1c levels (8.50 ± 0.43 mg/dl) in mice alloxinated animals coupled with the failure of the mice to gain weight. The results are explained in the following paragraphs.[19]

The acute effect of *C. intybus* extract on BGL of alloxinated animals showed an important hypoglycemic effect and dose dependent effects on diabetic mice (Table 3). The hypoglycemic effect was more promising over longer periods. After 6h post administration of the drug at doses (50 µl, 75 µl, 100 µl) and (50 µl + 200 µl) the decrease of blood glucose levels (BGL) was 14.15% significant at (p<0.05), 19.69%, 42.4% and 35.19% and they are all significant at (p<0.01), respectively; furthermore, the doses (100 µl) and (50 µl + 200 µl) were the most effective doses in lowering mice blood glucose levels (BGL).

The Subchronic effect of *C. intybus* extract on BGL of alloxan-induced diabetic mice showed a more important hypoglycemic effect (Table 4). The results showed dose dependent effects of *C. intybus* alone and in combination with *C. sinensis*. After 8 days of the starting of drug administration, at the doses (50 µl, 75 µl, 100 µl) and (50 µl + 200 µl) the decrease of blood glucose levels (BGL) was 23.41%, 25.39%, 44.8% and 39.35%, respectively. All the dosages were statistically significant at (p<0.05); furthermore the doses (100 µl) and (50 µl + 200 µl) were the most effective doses in lowering mice blood glucose levels (BGL).

The sub-chronic effect of *C. intybus* extract on BW in alloxinated animals showed on the 8th day an increase in the percentage of body weight when compared to DC. The results showed dose dependent effects of *C. intybus* alone and in combination with *C. sinensis* on *DM*. The percentage of body weight increase for the doses (50 µl, 75 µl, 100 µl) and (50 µl + 200 µl) was 6%, 8.5% (significant, at (p<0.05), 14.2% and 15% (significant at (p<0.01), respectively, when compared to DC (28.45 ± 0.45) and NC weight (30.05 ± 0.05). All these weights elevations reflect the amelioration of hyperglycemia as demonstrated previously with other DM phytotherapies.[28]

The glycated hemoglobin (HbA1c) was set as a principal of diagnosing criteria for DM (≥ 6.5%) and pre-diabetes (5.7–6.4%) by the 2010 American Diabetes Association (ADA) principles of maintenance for hyperglycemic, founded mainly on the judgment of an international expert committee.[29] HbA1c, measured 8 weeks after dose administration to diabetic mice, showed dose dependent effects of *C. intybus* alone and in combination with *C. sinensis*. For each group, HbA1c was measured to indicate the long-term hyperglycemia in mice. For the doses (50 µl, 75 µl, 100 µl) and (50 µl + 200 µl), HbA1c was (7.3% ± 0.37), (6.9% ± 0.35), (6.27% ± 0.32) and (6.5% ± 0.33) when compared to DC group (8.5% ± 0.43). Although the four different doses have shown significant, at (p<0.05), HbAlc reductions, the highest reduction was observed with CI extract 100 µl, with an HbAlc value of (6.27% ± 0.32). These doses were effective and maintained the HbA1c within the "good control" range. These results could be an evidence of the good glycemic control that CI extract alone and in combination with CS extract possess.

An isocratic HPLC of insulin was conducted to study the percentage of insulin in the nine groups of mice. The insulin was measured, 8 weeks after dose administration to diabetic mice. The level of insulin was dose dependent effects of *C. intybus* alone and in combination with *C. sinensis*. The administration of the doses (50 µl, 75 µl, 100 µl) and (50 µl + 200 µl) showed an elevation of (1.87% ± 0.17) (significant at (p<0.01), (3.22% ± 0.16), (9.06% ± 0.3) and finally (7.94% ± 0.39) (significant at (p<0.001), respectively. The highest "Insulin" rates were with the doses 100 µl and (50 µl + 200 µl), respectively, when compared to DC (0.87% ± 0.13).

The diabetic state is postulated in the rise of oxidative stress. *In vivo*, a disproportion in antioxidant enzyme, Catalase (CAT), has been related to the increase of DM complications. The higher the oxidative stress, the lower the CAT and the greater the incidence of DM. Rising CAT causes oxidative stress to decrease, contributing to the control of diabetes. β-cells have the strong sensitivity to oxidative stress, leading to the death and to the development, of type 1 diabetes. This effect was decreased by antioxidants. Antioxidants have shown promise effects to prevent and to be helpful for treating diabetes and its complications.[30]

CAT serum's levels, of each mice group, were measured 8 weeks after dose administration to diabetic mice. The results show dose dependent effects of *C. intybus* alone and in combination with *C. sinensis*. The administration of the doses (50 µl, 75 µl and 100 µl) and (50 µl + 200 µl), showed an increase in CAT's levels (30.11%) (Significant, at (p<0.01, 53.34%, 66.96% and 64.76% (Significant, at (p<0.001), respectively, when compared to DC. Different doses of *C. intybus* alone and in combination with *C. sinensis* showed equi-potent antioxidant activity, which may indicate that the antioxidant effect of CI extract alone and in combination with CS extract over longer periodare potent even at a low doses.

Diabetic patients with peripheral neuropathy possess an important indicator, a decrease in the peripheral nerve conduction. Eight weeks after the dose administration to diabetic mice, the effect of *C. intybus* extract was examined alone at doses (50 µl, 75 µl and 100 µl) and with *C. sinensis* extract at doses (50 µl + 200 µl, respectively). The test was done on peripheral function by evaluating the thermal latency with HP test and TF and sensitivity to a mechanical stimulus by VFF. The results of the administration of the previously cited doses have eased hypersensitivity to pain situations by comparison to the DC in mice. The tests demonstrated that the different doses administered were very active against heat hypersensitivity and TA in animal models of diabetic neuropathy. In addition, the anti-nociceptive effect was dose-dependent alleviations with the maximum reduction observed with the highest dose (100 µl) and with the association of doses (50 µl + 200 µl), respectively. Furthermore, the most active dose against tactile allodynia producing 23.48 folds and 18.90 folds improvement was following the administration of the doses (100µl) and (50 µl + 200 µl) when compared to DC.

The histology of mice pancreas showed an increase in the percentage of the pancreatic β-cells in all treated groups of diabetic mice (Figure 8). The sub-chronic effect of the doses (50 µl, 75 µl and 100 µl) and (50 µl + 200 µl) showed an increase of 30.61%, 40.81%, 69.04% and 65.30%, respectively, when compared to DC (23.80%) and reaching approximately the normal levels (NC = 73.46%). The percentage of the pancreatic β-cells was dose dependent effects; furthermore, the most effective doses were (100 µl) and (50 µl + 200 µl).[30-32]

CONCLUSION

In the Arab world, *Cichorium intybus* is one of our traditional plants. Many studies have been conducted to investigate its therapeutic effects to prove the traditional uses. Our study investigated the n-hexane extract of *Cichorium intybus* roots, which revealed a promising anti-diabetic, diabetic neuropathy and antioxidant effect. This is the first study of the n-hexane extract of *Cichorium intybus* roots in Lebanon. Furthermore, the histological study and the potent antioxidant effect supported this postulation by amelioration of insulin levels and enhancement of pancreatic beta cells.

Cichorium intybus extract was extracted using hexane solvent and a TLC and HPLC analysis were performed on the hexane extracted oil of *Cichorium intybus* roots. The results indicated the presence of fructose, glucose and sucrose, ketose, nystose and the inulin type DP fructans: DP4, DP5 and DP6. HPLC indicated that the most active compounds of the hexane-extracted oil of *C. intybus* are Chicoric acid at a high percentage (64.70%), Isochlorogenic acid A/B/C (8.90%), Dicaffeoylquinic acid (6.87%), Chlorogenic acid (4.81%), Esculetin (3.54%), Caffeic acid (3.40%).

All the demonstrated data revealed that the combination of both; the hexane-extract of *Cichorium intybus* and the aqueous extract of *Camellia sinensis* could assist in the management of many diseases including *Diabetes Mellitus* and diabetic neuropathy. Furthermore, the histological study and the potent antioxidant effect supported this postulation by amelioration of insulin levels and regeneration of pancreatic beta-cells.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ACN: Acetonitrile; **BGL:** Blood Glucose Levels; **CI:** *Cichorium intybus*; **CAT:** Serum catalase; **DC:** Diabetic Control; **DDW:** Double distilled water; **DM:** Diabetes mellitus; **DMSO:** Dimethyl sulfoxide; **DN:** Diabetic Neuropathy; **NC:** Normal control; **S.E.M.:** Standard error of the mean.

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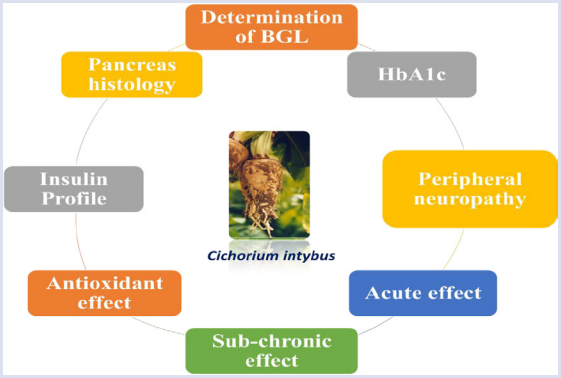
SUPPLEMENTARY MATERIALS

Figure S1 and Figure S2

GRAPHICAL ABSTRACT



SUMMARY

- All the demonstrated data revealed that the combination of both the hexane extract of *Cichorium intybus* and the aqueous extract of *Camellia sinensis* can assist in the management of diabetes mellitus and diabetic neuropathy. Furthermore, the histological study and the potent antioxidant effect supported this postulation by amelioration of insulin levels and the regeneration of pancreatic β-cells.

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