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ORIGINAL ARTICLE

Antidiabetic, Toxicological, and Metabolomic Profiling of Aqueous Extract of *Cichorium intybus* Seeds

Kailash Chandra[1], Washim Khan[2], Sujata Jetley[1], Sayeed Ahmad[2], S. K. Jain[1]

[1]Departments of Biochemistry and Pathology, HIMSR, Jamia Hamdard, [2]Department of Pharmacognosy and Phytochemistry, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India

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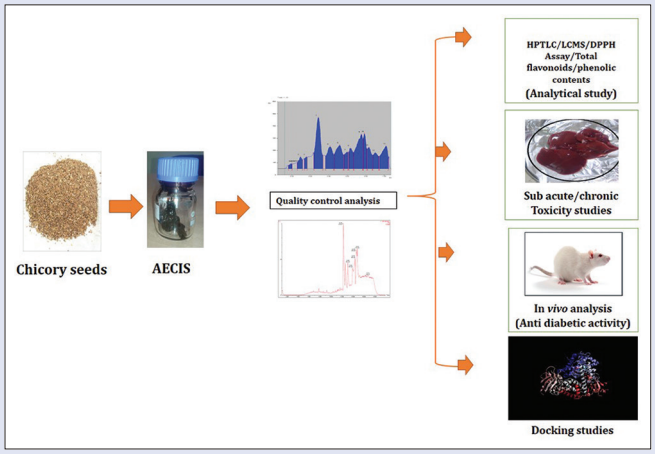
ABSTRACT

Background: *Cichorium intybus* has a wide range of therapeutic applications in Indian traditional systems of medicine, especially in metabolic disorders. **Objective:** To evaluate the toxicity profile and to investigate the antidiabetic, antihyperlipidemic, and antioxidative efficacy of *C. intybus* seeds in Wistar rats. **Materials and Methods:** The aqueous extract of seeds was prepared by decoction, and its quality control analysis was carried out by thin-layer chromatography and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) fingerprinting. Wistar rats were fed with high-fat diet for 5 weeks followed by a single dose of streptozotocin intraperitoneally to induce diabetes. The protective group of rats was given aqueous extract during and after the induction of type 2 diabetes mellitus. Further, repeated dose 28-day (subacute) and repeated dose 90-day (chronic) toxicity studies were conducted as per the OECD guidelines. **Results:** A total of 18 metabolites have been tentatively identified by UPLC-MS profiling in aqueous extract of *C. intybus* seeds. No significant changes in mortality and biochemical parameters have been observed during toxicity studies. Moreover, administration of the extract to a protective group of diabetic rats attenuated serum glucose and triglyceride levels by 52.7% and 65.3%, respectively, supported by similar results for parameters related to insulin resistance and oxidative stress. The beneficial effect of extract has also been confirmed through *in silico* screening. **Conclusion:** *C. intybus* can be used as a natural dietary supplement for the prevention and management of diabetes and can be explored to develop a potent phytopharmaceutical for diabetes.

Key words: Chicory, docking, homeostatic model assessment of insulin resistance, thin-layer chromatography, ultra-performance liquid chromatography-mass spectrometry

SUMMARY

- Quality control analysis of *Cichorium intybus* seeds
- Toxicological profiling (subacute and chronic toxicity) of aqueous extract has been carried out in rats and confirmed that aqueous extract of *C. intybus* seeds (AECIS) has no ill effect
- Oral administration of AECIS at the dose of 200 mg/kg body weight (BW) resulted in significant reduction in serum glucose and triglycerides levels and decreased the oxidative burden in high-fat-diet-induced diabetic rats
- Metabolites responsible for antidiabetic activity have been tentatively identified through ultra-performance liquid chromatography-mass spectrometry-based metabolomic analysis and found 18 active metabolites being present
- *In silico* screening was done for confirming the possible mechanism of action and found only three (11,13-dihydro-lactucin; 2′,6-dihydroxyflavone; and coumarin) revealed high fitness score (<−5.0).



Abbreviations used: AECIS: Aqueous extract of *Cichorium intybus* seeds; HOMA-IR: Homeostatic model assessment of insulin resistance; OGTT: Oral glucose tolerance test; TLC: Thin-layer chromatography; UPLC-MS: Ultra-performance liquid chromatography-mass spectrometry.

Correspondence:

Prof. S.K. Jain,
Department of Biochemistry,
HIMSR, Jamia Hamdard,
New Delhi - 110 062, India.
E-mail: skjain@jamiahamdard.ac.in
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INTRODUCTION

Diabetes mellitus is a major health problem globally. Its prevention and treatment are crucial for public health. Many individuals suffer from type 2 diabetes mellitus (T2DM) and the incidence is increasing rapidly, especially it now being diagnosed at much younger age. The International Diabetes Federation published the data on diabetes and showed that one out of 11 adults have diabetes and it is expected to be one out of 10 adults by 2040.[1] Although several drugs are available for maintaining blood glucose levels, many patients traditionally seek additional remedy from natural plant extracts. A number of medicinal plants are conventionally used for the management of diabetes mellitus; many of these offer other benefits also.[2,3]

Cichorium intybus commonly known as chicory belongs to family *Asteraceae* and is widely distributed in Asia.[4] It is best known for the use of its roots as coffee substitute or additives to coffee as it provides

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bitterness in taste without having any caffeine. Besides, its leaves, flowers, seeds, and roots are conventionally used as herbal medicine since ancient times. More than 50 active constituents have been identified from the *C. intybus* plant.[5] One of its most investigated and relatively well-recognized flavonoids is quercetin, present as free as well as in the form of its glycoside derivatives, especially rutin (quercetin 3-O-glucopyranoside).[6] Chlorogenic acid, a constituent of chicory, has antiobesity properties and improves lipid metabolism in mice.[7] A complete profiling of constituents in its biologically active extract through thin-layer chromatography (TLC) and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) can be used for quality control analysis.

Experimental studies with aqueous extract of *C. intybus* seeds (AECIS) in animal models showed hepatoprotective,[8] antioxidative, and antithrombotic[9] activities. Moreover, intraperitoneal administration of chicory seeds has been reported to have antidiabetic activity on rats.[10] Chicory may be useful as a natural dietary supplement for lowering the pace of diabetes progression. The alcoholic and aqueous extract of seeds revealed hepatoprotective effect in experimentally induced hepatic damage in rats. The biochemical investigations showed significant reduction of liver enzymes.[11] The aqueous extract of the seeds revealed antibacterial activity against *Staphylococcus aureus* but was inactive against *Escherichia coli*.[12] Moreover, the ethanolic extract of seeds showed *in vivo* and *in vitro* antimalarial activity against NK 65 strain of *Plasmodium berghei*.[13]

Although chicory has been used in traditional systems of medicine and seems to be safe for human use. However, a detailed scientific study to evaluate its medicinal properties and toxicological assessment has not been done. Therefore, the present study was designed to carry out detailed toxicity (subacute and chronic toxicity) and investigate its antihyperlipidemic, antioxidative, and antidiabetic efficacy of AECIS in Wistar rats as per its traditional claim for scientific validation.

MATERIALS AND METHODS

Plant material and extract preparation

C. intybus L. seeds (achenes) were collected from M/S Hamdard Laboratories, Ghaziabad, in August 2015 and authenticated by a taxonomist. A voucher specimen of the herbarium was deposited in the Bioactive Natural Product Laboratory (No.-KC/FP/BNPL/C1-01). The seeds were cleaned and shade dried for 4–5 days and crushed to make a coarse powder. The seeds were soaked in distilled water (1:10 w/v) for 12 h at room temperature and then kept in a boiling water bath until the volume reduced to its half. The suspension was filtered and evaporated to get the AECIS.

Quality control analysis of aqueous extract of *Cichorium intybus* seeds

Total phenolic and flavonoid contents

The total phenolic content was estimated using Folin–Ciocalteu colorimetric method[14] and reported as a percentage of gallic acid equivalents per 100 g of extract. Total flavonoid content was measured[15] and reported as a percentage of total rutin equivalents per 100 g of extract.

1,1-diphenyl-2-picryl-hydrazyl scavenging assay

The free radical scavenging activity of the extract was measured at 517 nm by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method.[16] The ability of the sample to scavenge DPPH radicals was calculated as follows:

DPPH scavenging capacity = (Absorbance$_{control}$ − Absorbance$_{sample}$) ×100/Absorbance$_{control}$

Thin-layer chromatography fingerprint quantification of rutin and quercetin in aqueous extract of Cichorium intybus seeds

AECIS was reconstituted in methanol at the desired concentration. The extract was hydrolyzed using 2 N HCl, and further, it was extracted with ethyl acetate.[17] The ethyl acetate fraction of the hydrolyzed extract was dried, reconstituted in methanol, and used for analysis. For TLC analysis, 5 µl of prepared samples were separately applied on silica gel 60 F254 precoated TLC plates, 5 cm × 10 cm (Merck, Germany) with the help of Camag Linomat-V (CAMAG, Switzerland) applicator. The flow rate of sample application was 150 nL/s and eluted the plate to a distance of 7.5 cm at 25°C in solvent system toluene:ethyl acetate: formic acid (3:4:1, v/v/v). Plates were developed in a glass tank presaturated with the mobile phase for 40 min at the room temperature. After the complete run, the plates were dried at room temperature. The densitometric scanning was carried out at 254 and 360 nm with a Camag TLC Scanner III. The sample application, scanning, and interpretation were done using the winCATS V123 software. Further, the TLC was used for the quantification of rutin and quercetin as described earlier.[18]

Metabolomic profiling of aqueous extract of Cichorium intybus seeds by ultra-performance liquid chromatography-mass spectrometry

The 5 mg/mL solution of AECIS in methanol was filtered through 0.2 µM polytetrafluoroethylene membranes and used for UPLC-MS analysis. UPLC was performed on a Water's ACQUITY UPLC™ system (Waters Corp., MA, USA) equipped with a binary solvent delivery system, an autosampler, a column manager, and a tunable MS detector (Waters, Manchester, UK). The system was operated by the Empower software (Waters, USA). Data acquisition was done in positive mode. Chromatography was performed using acetonitrile and water as the mobile phases on the monolithic capillary silica-based C18 column (ACQUITY UPLC® BEH C18 1.7 µm, 2.1 mm × 100 mm), with the precolumn split ratio 1:5 and flow rate 10 µl/min at ambient temperature. Separation was obtained by stepwise gradients from 95% to 5% acetonitrile. The flow rate of the nebulizer gas was set to 500 L/h; for cone gas, it was set to 50 L/h, and the source temperature was fixed at 100°C. The capillary voltages and cone voltage were set at 3.0 and 40 KV, respectively. For collision, argon was employed at a pressure of 5.3×10^{-5} Torr. The accurate mass and composition of the precursor ions and of the fragment ions were calculated using Mass Lynx V 4.1 software (Waters Corp., MA, USA) incorporated in the instrument. Separated metabolites present in AECIS were tentatively identified based on their m/z ratio and literature reports.

Toxicity study

Experimental animals

Wistar rats (120–180 g) obtained from the Central Animal Facility, Jamia Hamdard, were used for the study. The animals were acclimatized for 7 days to laboratory conditions before experimentation. The protocol was approved by the Institutional Animal Ethical Committee, Jamia Hamdard, New Delhi, India (Approval No.-1132), and the study was performed as per the animal ethical guidelines.

Repeated dose 28-day oral toxicity study

A 28-day repeated oral toxicity study was performed according to the OECD guideline.[19] The experimental rats were divided into four groups with 10 animals (5 in either sex) in each group. Group 1 served as control and received normal saline as vehicle and Groups 2, 3, and 4 received AECIS at 50, 200, and 600 mg/kg body weight (BW)/day, respectively, in distilled water for 28 days.

Repeated dose 90-day oral toxicity (chronic) study

After getting no significant toxicity in repeated dose 28-day oral toxicity study, we investigated repeated dose 90-day oral toxicity study to rule out any unwanted effect due to chronic toxicity of the extract. Both sexes of rats were divided into four groups (Groups 5–8) with 10 animals (5 in either sex) in each group. Group 5 served as control and received normal saline as vehicle and Group 6 received 600 mg/kg BW/day of AECIS (maximum dose used in repeated 28-day toxicity study) in distilled water for 90 days. Group 7 served as satellite control (received vehicle) and Group 8 served as treatment satellite group which received AECIS at 600 mg/kg BW/day orally for 90 days. The satellite groups (Groups 7 and 8) were scheduled for follow-up observations for the next 14 days (i.e., day 91–104) without the vehicle or the AECIS administration during this period, respectively.

The animals were observed for mortality and morbidity twice a day, till the completion of treatment. Clinical observations were made once daily to detect signs of toxicity, at the same time on each day (1 h after vehicle or AECIS administration). BW of the animals was recorded on the alternate days throughout the study period. The food and water intake were measured to calculate daily consumption.

Induction of experimental type 2 diabetes mellitus

After acclimatization, the rats were fed with high-fat diet (HFD), which was composed of 60.3% fats, 18.4% proteins, and 21.3% carbohydrates, for 5 weeks. After 5 weeks of dietary manipulation, the HFD was replaced with standard rodent pelleted diet and animal received a single intraperitoneal injection of a low dose of streptozotocin (STZ) (35 mg/kg BW), dissolved in citrate buffer (pH 4.5). Fasting glucose, serum insulin, and triglyceride (TG) levels were measured after 3 days of STZ injection. Rats with glucose level >250 mg/dL and significantly higher level of homeostatic model assessment of insulin resistance (HOMA-IR) score and TG levels (as compared to controls) were considered as the diabetic and included in the study.[20]

Further, animals were divided into five groups of five rats each and given following treatment for 4 weeks. Group 1A served as untreated normal control group and received normal saline orally once a day along with standard pellet diet. Group 2A rats (protective group) received AECIS (200 mg/kg BW) during (5 weeks) and after the induction of T2DM (4 weeks) for total 9 weeks of study. Group 3A rats (diabetic control) received normal saline. Group 4A diabetic rats were given extract orally at the dose of 200 mg/kg BW/day (AECIS treatment). The dose of AECIS (200 mg/kg BW/day) was equivalent to pharmacopeia dose and calculated on the basis of extract value.[21] Moreover, standard group (5A) of rats received metformin orally at the dose of 42 mg/kg BW/day.

At the end of the stipulated treatment period (28 days), the overnight-fasted (water allowed) animals were anesthetized, and blood samples were collected in the respective blood collection tubes for hematological and biochemical analysis. Biochemical parameters (glucose, total cholesterol, TGs, and high-density lipoprotein [HDL]) were analyzed by automated biochemistry analyzer (Dimension Xpand plus, Siemens, India). The HOMA-IR values were calculated by fasting plasma glucose and insulin.[22] The oxidative markers (malondialdehyde [MDA], reduced glutathione [GSH], and catalase) were measured by Spectrophotometer (Labomed, USA) using standard protocols.

Oral glucose tolerance test

Further, another set of rats (3 groups, named normal control, diabetic control, and AECIS-treated diabetic rats) consisting of six rats each was subjected to oral glucose tolerance test (OGTT). AECIS-treated diabetic rats were treated with AECIS at the dose of 200 mg/kg BW/day for 10 days. At day 11, blood glucose levels were measured after an overnight fast, and the glucose solution (2 g/kg BW) and AECIS (200 mg/kg BW) were orally administered. Normal and diabetic control rats have received glucose solution (2 g/kg BW) along with normal saline. Blood glucose was estimated at every 30 min from the tail vein using glucometer (Glu Check, Aspen Diagnostic).

Histopathology

Necropsy was done in all animals on day 29 in 28-day repeated oral toxicity study and on day 91 in repeated dose 90-day oral toxicity (chronic) study, except for the satellite groups, where it was done on day 104. After blood collection, all the animals were euthanized for gross pathological examinations of major internal organs such as liver, kidney, and spleen.

Statistical analysis

All the experiments were performed in triplicate, and data were expressed as mean with standard error of mean (SEM) Results were analyzed by two-way analysis of variance, and the level of significant differences among different groups was determined by Bonferroni posttest test using GraphPad Prism, ver. 5.0 software (GraphPad Software, Inc., California, USA). At 95% confidence interval, $P < 0.05$ considered as statistical significant.

In silico screening of identified metabolites

The possible mode of interaction of the potent metabolites present in AECIS was carried out by docking analysis using MOPAC 6 software package. Different proteins that may interact with metabolites of interest, such as GLUT2, mitochondrial K+ ATPase, AMP kinase[23] phosphatidylinositol 3 kinase (PI3K), GLUT 4,[24] dipeptidyl peptidase 4 (DPP4),[25] and sulfonylurea receptor (SUR),[26] were selected for docking analysis. Metabolites such as 11,13-dihydro-lactucin, 2',6-dihydroxyflavone, cichoric acid, phosphatidylethanolamine, and coumarin have been previously studied for their antidiabetic potential.[27-30]

In docking analysis, solvation parameters, Kollman United atom type charges, and essential hydrogen atoms were added with the aid of AutoDock tools.[31] AutoGrid program was used for the generation of affinity (grid) maps of × Å grid points and 0.375 Å spacing. The van der Waals and the electrostatic terms were calculated by AutoDock parameter set and distance-dependent dielectric functions, respectively. Simultaneously, docking was executed using the Lamarckian genetic algorithm and the Solis and Wets local search method.[32] Initial position, orientation, and torsions of the ligand molecules were selected randomly. During docking, all rotatable torsions were released. A translational step of 0.2 Å and quaternion and torsion steps of 5 were applied during the search. The structure of molecules in mol format was drawn in ChemDraw Ultra 7.0.1 (CambridgeSoft Corporation, USA) and converted to input ligand format (PDB) for docking by Open Babel version 2.3.2 (Journal of Chemo Informatics).

RESULTS AND DISCUSSION

Quality control analysis of aqueous extract of *Cichorium intybus* seeds

Phytochemicals

The total yield of water-soluble material in the extract (AECIS) was 26.3% w/w. The total flavonoids and phenolic contents of the extract were 8.3% and 34.88% w/w, respectively, as calculated using the standard curve equation y = 0.0216 × −0.0325, R^2 = 0.9885 and y = 0.0241 × −0.1118, R^2 = 0.9839 of rutin for flavonoid and quercetin for phenolics. The DPPH assay of extract assessed the dose-dependent free radical scavenging

capacity. A maximum of 73% ± 3% inhibition has been recorded at 50 µg/mL dose (IC_{50} = 14.15 µg/mL). The scavenging activity of AECIS may be due to the presence of high levels of flavonoids and phenolics.[33]

Thin-layer chromatography densitometry fingerprint profile

TLC fingerprinting of AECIS was carried out by scanning at 254 and 360 nm. Scanning at 254 nm resulted in the identification of 12 peaks with R_f ranging from 0.1 to 0.90. However, at 360 nm, the same number of peaks with R_f ranging from 0.11 to 0.99 was observed. However, the pattern of metabolites in terms of R_f was different at two wavelengths [Figure 1 and Table 1]. Two common markers (rutin and quercetin) were identified and quantified. The content of rutin and quercetin was 3.5% and 5.4% w/w of total extract, respectively.

Ultra-performance liquid chromatography-mass spectrometry profiling of aqueous extract of Cichorium intybus seeds

The UPLC-MS chromatogram [Figure 2] shows complete separation of metabolites, which were tentatively identified from literature and database survey. The UPLC-MS fingerprinting analysis resulted in the tentative identification of 18 metabolites [Table 2]. Cichoroside (R_t = 14.81), glucose-6-phosphate (R_t = 16.72), 11,13-dihydro-lactucin (R_t = 12.25), and 2',6 dihydroxyflavone (R_t = 13.92) are the major metabolites found in AECIS.

Toxicity analysis

There were no treatment-related toxicity signs, and no mortality was observed in both sexes of rats treated at 50, 200, and 600 mg/kg BW/day orally for 28 days in repeated dose 28-day oral toxicity study. The animals gained BW during the study period. The total BW gain in both the sexes of Group 2, 3, and 4 rats showed the insignificant difference in compared to the Group 1 (control). The difference of average feed consumption and water intake in treated groups (Groups 2, 3, and 4) and control rats (Group 1) was found insignificant in both the sexes. There was no significant difference in hematological and biochemical parameters, such as hemoglobin (Hb), creatinine, total protein, albumin, alanine transaminase, aspartate transaminase, and total cholesterol observed between control and treated groups. The subacute toxicity study (28 days) revealed no weight loss, feed and water intake were relatively constant and no significant difference in the biochemical parameters, even at a very high dose of AECIS (600 mg/kg BW/day). These data suggested that there was no subacute toxicity associated with AECIS administration. To ensure the AECIS has any toxic effect on long-term use, chronic toxicity study for repeated dose 90 days was carried out. In chronic oral toxicity study, no toxicity sign and mortality were observed in test group as well as satellite group, treated at 600 mg/kg BW/day for 90 days. The difference in total BW gain, average feed consumption, and water intake in test and satellite groups of rats was found insignificant as compared to the control group. Further, no weight loss occurred in experimental rats throughout the period of chronic toxicity study. The differences in biochemical parameters such as alanine transaminase, aspartate transaminase, creatinine, total Protein, albumin, total cholesterol, and Hb in both sexes of control and treated group (group 6) of rats were insignificant [Table 3]. The results of subacute and chronic toxicity experiments taken together confirmed that AECIS has no ill effect.

Histopathological observations

The gross examination of liver and kidney of rats fed with 600 mg/kg BW/day of AECIS in subacute and chronic toxicity studies shows no abnormalities as compared to control rats [Supplementary Figure 1].

There were no signs of toxicity with respect to histopathological analysis, biochemical parameters, and physiological examination in AECIS-treated satellite group of rats (Group 8) as compared to the satellite control group. It can be concluded that AECIS will not

Table 1: Data pertaining to thin-layer chromatography densitometry fingerprint of aqueous extract of *Cichorium intybus* seeds, recorded at 254 and 360 nm

Detection wavelength (nm)/number of spots	R_f (area %)
254 (12)	0.1 (3.25), 0.14 (2.07), 0.29 (18.4), 0.36 (9.43), 0.42 (11.46), 0.52 (4.12), 0.62 (5.89), 0.64 (7.21), 0.77 (15.3), 0.79 (5.97), 0.88 (5.76), 0.92 (10.04)
360 (12)	0.11 (27.6), 0.21 (5.17), 0.23 (5.81), 0.28 (5.66), 0.32 (10.26), 0.46 (1.86), 0.49 (1.8), 0.53 (2.18), 0.58 (5.42), 0.69 (27.1), 0.75 (5.48), 0.90 (1.64)

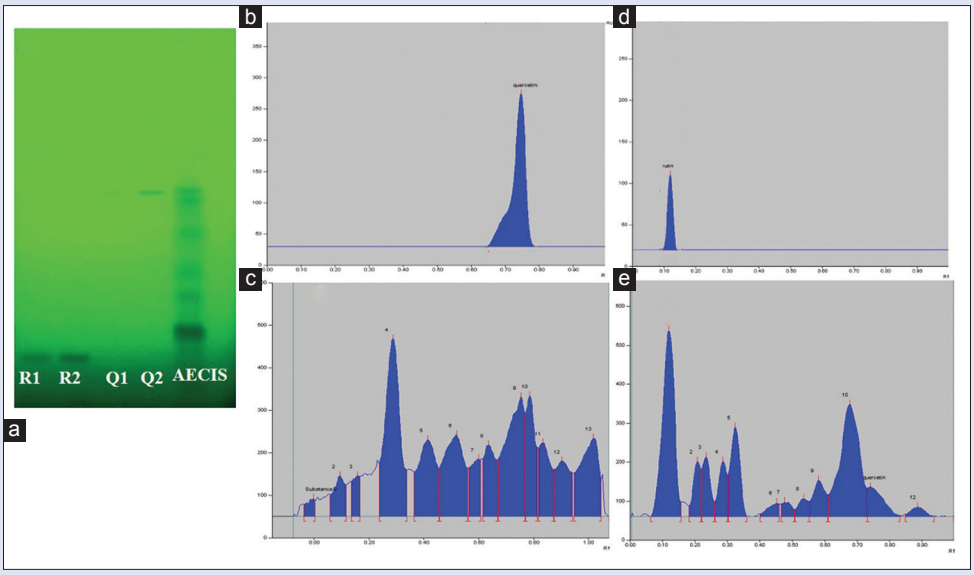

Figure 1: (a) Developed thin-layer chromatography plate at visualized at 254 nm. (b) Chromatogram standard Q2 (c) at 254 nm, (d) chromatogram standard R2 and (e) at 360 nm, (R1-rutin [0.5 mg/mL], R2-rutin [1 mg/mL], Q1-quercetin [0.5 mg/mL], Q2-quercetin [1 mg/mL], AECIS-aqueous extract of *Cichorium intybus* seeds)

cause delayed onset of toxicity. The overall observations confirm that AECIS is a nontoxic herbal drug and can safely be taken for extended periods.

Antidiabetic, antihyperlipidemic, and antioxidative activities of aqueous extract of Cichorium intybus seeds

The beneficial effect of AECIS in the protective group was more pronounced. The average blood glucose level and average HOMA-IR score in diabetic control rats were 400.3 ± 7.23 mg/dL and 18.35 ± 1.61 (mean ± SEM), respectively. The diabetic control rats (Group 3A) showed a significant increase in plasma glucose (73.9%) and HOMA-IR values as compared to normal rats. The Group 2A rats treated with AECIS-200 mg/kg BW/day for total 9 weeks showed a decrease of 52.7% in blood glucose level as compared to untreated diabetic rats. Further, in Group 4A rats, where AECIS was given for 4 weeks after induction of diabetes mellitus, blood glucose level was similar to the standard group of rats. However, after giving the treatment, the HOMA-IR values showed a decrease of 56.9%, 48.7%, and 63.0% in the Groups 2A, 4A, and 5A, respectively, as compared to untreated diabetic rats. The average TG level in diabetic rats was 336 ± 23.9 mg/dL (mean ± SEM). The diabetic rats treated with AECIS revealed the significant lowering of plasma TG levels in Groups 2A, 4A, and 5A. Moreover, good cholesterol and HDL level in blood are significantly increased in treatment and standard group of rats. The difference of oxidative markers (plasma MDA, GSH, and catalase) in test and control groups were statistically significant. The data show that AECIS treatment reduced the oxidative burden in diabetic rats [Figure 3]. The AECIS is rich in phenolic compounds and flavonoids that may be responsible for the antioxidative activity of the extract. The antioxidative activity of AECIS may be due to the presence of metabolites such as kaempferol, quercetin, and cichoric acid.[34]

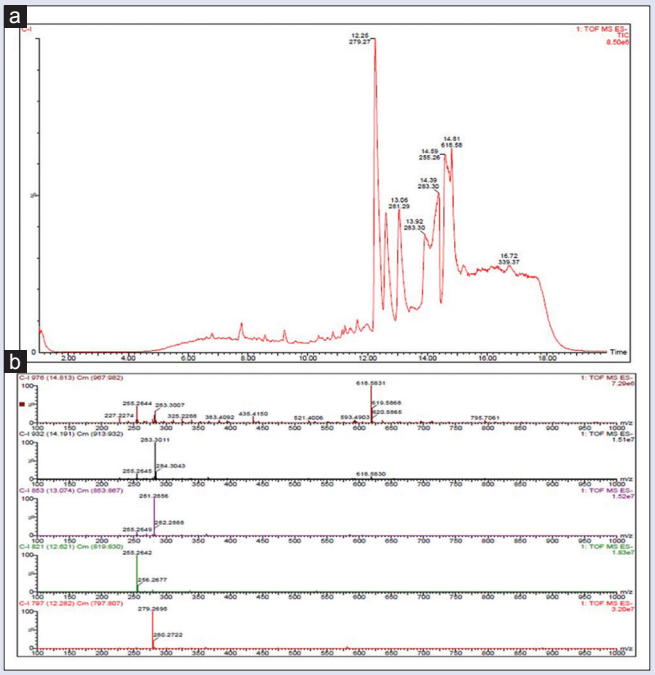

Figure 2: (a) Ultra-performance liquid chromatography-mass spectrometry chromatogram and (b) mass spectrum of separated metabolites of aqueous extract of *Cichorium intybus* seeds

OGTT was performed in normal, diabetic, and AECIS-treated group rats [Figure 4]. The AECIS (200 mg/kg/BW)-treated rats showed significant lowering in blood glucose than the diabetic control rats at 2 h after a load of glucose solution. However, these values did not reach the same level as in normal control group animals. The overall observations revealed that AECIS decreases the pace of diabetes in HFD-induced T2DM Wistar rats. These observations are similar to the standard drug.[10,35]

In silico screening

Considering results obtained from *in vivo* and analytical study, it was pertinent to think that *in silico* analysis would predict metabolites responsible for antidiabetic activity through ligand-receptor interaction based on binding energies or fitness score.[36] The docking of tested metabolites with targeted proteins was therefore performed, and corresponding fitness scores were determined [Supplementary Table 1]. High fitness scored metabolites were subjected to the elucidation of their interaction surface and total intermolecular energy with targeted molecules and proteins separately.[37] Five ligands (11,13-dihydro-lactucin, 2',6-dihydroxyflavone, cichoric acid, phosphatidylethanolamine, and coumarin) were selected for this study. However, only three (11,13-dihydro-lactucin; 2',6-dihydroxyflavone; and coumarin) revealed high fitness score (<−5.0). The estimated free energy of ligand–protein interaction was found <−5.0 for GLUT2, mitochondrial

Table 2: Metabolites tentatively identified through ultra-performance liquid chromatography-mass spectrometry in aqueous extract of *Cichorium intybus* seeds

Mass	Compound name	Percentage	Molecular formula	Database
227.2	2'-Deoxycytidine	5.66	C9H13N3O4	BML80258
255.2	N-alpha-(tert-Butoxycarbonyl)-L-histidine	9.10	C11H17N3O4	BML81771
256.2	2',6-Dihydroxyflavanone	5.25	C15H12O4	BML80228
278.3	11,13-dihydro-lactucin	11.50	C14H21N3O3	PubChem CID 9970764
280.2	Linoleic acid	3.60	C18H32O2	MT000114
281.2	Coumarin	9.25	C18H19NO2	BML80992
282.28	Oleic acid	2.01	C18H34O2	MT000029
283.09	Guanosine	5.25	C10H13N5O5	KO008966
283.3	5,7-Dimethoxyflavanone	8.3	C17H16O4	BML80455
284.3	3 (4'-Chlorophenyl)-4,6-dimethylcoumarin	1.01	C17H13ClO2	BML80307
339.9	Alpha-D-Glucose-1,6-diphosphate	4.12	C6H14O12P2	PR100628
383.4	1-Myristoyl-2-hydroxy-sn-glycero-3-phosphate	2.94	C17H35O7P	PR100784
435.4	Quercetin-3-Arabinoside	8.45	C20H18O11	PR101030
519.5	Phosphatidylcholine lyso 18:2	0.25	C26H50NO7P	UT001085
521.3	Phosphatidylcholine lyso 18:1	0.95	C26H52NO7P	UT001084
594.1	Kaempferol-3-Glucoside-2"-p-coumaroyl	5.44	C30H26O13	PR101018
618.5	Unhydrolyse cichoroside	9.56		
795.6	Phosphatidylethanolamine 20:0-20:4	3.2	C45H82NO8P	UT001152

Table 3: Biochemical and physiological changes in repeated 28 days and 90 days oral toxicity study

Treatment	Sex	TBW	AFI	AWI	Hb	Creatinine	ALT	AST	TP	Albumin	T. Chol
28 days oral toxicity study (percentage change)											
AECIS - 50 mg/kg BW	Male (*n*=5)	3.9±0.4	5.0±0.6	9.1±1.1	0.6±0.2	5.0±0.2	3.2±0.3	6.1±0.5	1.4±0.02	6.6±0.08	7.9±0.9
	Female (*n*=5)	1.3±0.1	2.0±0.2	7.2±0.5	1.9±0.1	0.5±0.02	5.1±0.9	1.6±0.4	1.4±0.03	0.5±0.01	0.2±0.01
AECIS - 200 mg/kg BW	Male (*n*=5)	4.9±0.5	8.0±0.8	8.7±1.0	4.1±0.2	0.8±0.04	6.9±0.4	7.0±0.9	2.8±0.04	6.6±0.8	7.0±0.8
	Female (*n*=5)	2.6±0.4	6.3±0.6	9.0±0.9	5.3±0.5	7.3±0.8	7.1±0.9	0.5±0.02	0.5±0.01	6.6±0.9	9.6±1.2
AECIS - 600 mg/kg BW	Male (*n*=5)	7.4±0.9	8.1±0.7	9.0±0.8	1.3±0.04	0.9±0.01	5.1±0.3	8.8±0.8	0.9±0.02	0.8±0.07	8.8±1.1
	Female (*n*=5)	6.7±0.7	7.4±0.5	6.9±0.6	4.6±0.1	7.5±0.5	3.8±0.02	3.3±0.02	4.3±0.03	0.8±0.08	4.4±0.4
90 days oral toxicity study (percentage change)											
AECIS - 600 mg/kg BW	Male (*n*=5)	8.0±0.9	6.8±0.9	7.4±0.5	5.8±0.8	0.5±0.02	8.3±0.9	4.8±0.2	1.4±0.02	0.5±0.01	2.9±0.1
	Female (*n*=5)	8.9±0.8	4.0±0.3	5.3±0.6	2.1±0.4	0.8±0.02	2.9±0.4	1.0±0.05	1.4±0.03	0.8±0.02	7.1±0.9
Satellite group	Male (*n*=5)	8.0±0.9	8.1±0.8	5.6±0.4	0.6±0.01	7.1±0.9	6.9±0.8	7.5±0.8	5.8±0.07	6.6±0.9	0.5±0.01
AECIS - 600 mg/kg BW (follow-up observations)	Female (*n*=5)	6.5±0.7	3.7±0.1	4.2±0.3	0.7±0.01	6.3±0.5	5.0±0.9	2.0±0.02	2.7±0.03	0.9±0.06	0.9±0.02

BW: Body weight; TBW: Total body weight gain; AFI: Average feed intake/day; AWI: Average water intake/day; Hb: Hemoglobin; ALT: Alanine transaminase; AST: Aspartate transaminase; TP: Total protein; T. Chol: Total cholesterol, percentage change of all the parameters were calculated as compared to the control; AECIS: Aqueous extract of *Cichorium intybus* seeds

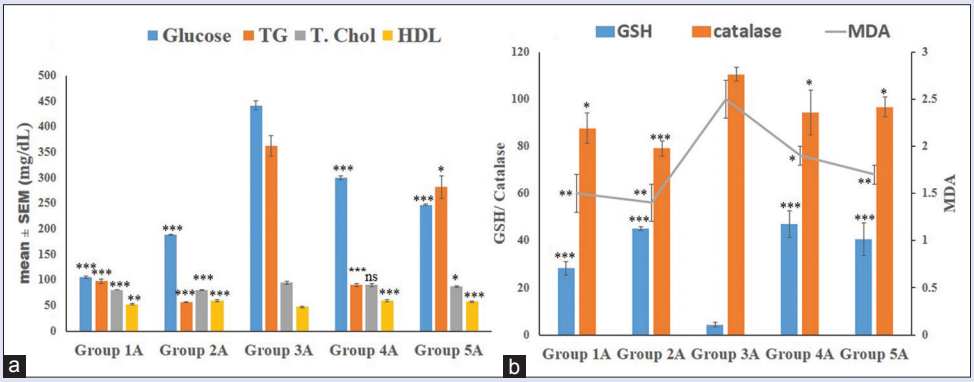

Figure 3: Effect of aqueous extract of *Cichorium intybus* seeds on biochemical (a) and oxidative (b) parameters. Data are expressed as mean ± standard error of mean ($n = 5$), ns $P > 0.05$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; *denoting that data were compared with toxic control; (Group 1A, 2A, 3A, 4A, and 5A-normal control, protective, toxic control, aqueous extract of *Cichorium intybus* seeds-treated, and standard drug groups)

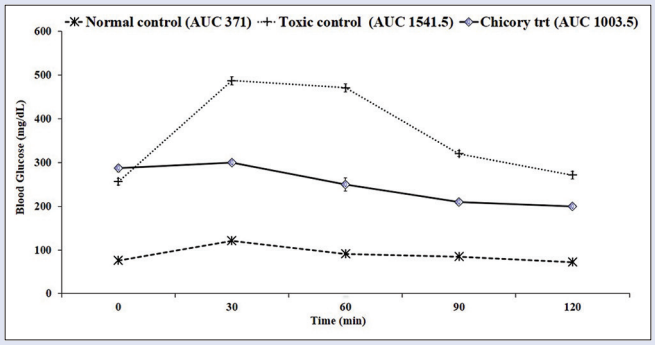

Figure 4: Effects of aqueous extract of *Cichorium intybus* seeds on glucose tolerance test. Rats were administered with 2 g/kg glucose solution ($n = 6$) (control: saline)

K+ ATPase, AMP kinase, PI3K, GLUT 4, DPP4, and SUR and this interaction indicating that affinity of these proteins toward targeted metabolites might be changed after oral administration of AECIS. Interaction of protein and ligand has been shown in Supplementary Figures 2-4. The docking studies present probable mechanism of action of antidiabetic activity of AECIS. Further, a molecular-based study is required for confirmation of the above-proposed mechanism.

A few studies have been carried out to see the beneficial effect of AECIS on lifestyle diseases; however, this is the first report where a comprehensive study has been carried out to see short- and long-term toxicity of the AECIS and to see its beneficial effect on T2DM and hyperlipidemia.

CONCLUSION

It may be stated that *C. intybus* may be considered as a potential source of natural antioxidants, phenolics, and flavonoids with antidiabetic, antioxidative, and antihyperlipidemic activities. Based on 28-day and 90-day repeated dose toxicity study, it is to conclude that AECIS is a nontoxic supplement. The present study substantiates the safety of AECIS, which was found to be in line with the long history of its use in the traditional systems of medicine. Oral administration of AECIS resulted in significant reduction in serum glucose and TG levels and decreased the oxidative burden in HFD-induced diabetic rats. Further, it has significant antidiabetic, antioxidative, and antihyperlipidemic effects, which have also been confirmed by *in silico* analysis. Further, it is proved that *C. intybus* seeds are beneficial to healthy as well as those with diabetes mellitus and can be administered as a dietary supplement in pharmacopeial dose as well as same can be explored for the development of phytopharmaceuticals for metabolic disorders.

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Conflicts of interest

There are no conflicts of interest.

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