STRUCTURE ELUCIDATION, PHYTOCHEMICAL INVESTIGATION AND PHARMACOLOGICAL SCREENING OF *SPINACIA OLERACEA* FOR ANTI-DIABETIC ACTIVITY

***[1]Dharna Singh Rajawat, [2]Brajesh Kumar Tiwari, [3]Neeraj Sharma**

**[1]Research Scholar, Department of Pharmaceutical Chemistry, Madhyanchal Professional University, Bhopal, MP, India*

[2,3]Professor, Department of Pharmaceutical Chemistry, Madhyanchal Professional University, Bhopal, MP, India

Corresponding author's details:Dharna Singh

dharna.ch91@gmail.com

ABSTRACT

The present study was based on structure elucidation, phytochemical investigation, and pharmacological screening of *Spinacia oleracea* for anti-diabetic activity. In this study, the methanolic extract of S. oleracea had shown the presence of numerous phytoconstituents. The isolation of compounds was performed on the methanolic extract of S. oleracea using column chromatography. In *S. oleracea*, erythritol was isolated kaempferol was isolated. The compounds were identified based on the spectral studies i.e., Infrared spectroscopy (IR), Nuclear Magnetic Resonance ($_1$H-NMR & $_{13}$C-NMR) and Mass Spectra and the structure was elucidated. The diabetic rats exhibited lower activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and reduced glutathione (GSH) content in pancreas and hepatic tissues as compared with normal rats. The activities of SOD, CAT, GPx and GSH were found to be increased in S. oleracea (200mg/kg) treated diabetic rats. The increased level of malondialdehyde, an index of lipid peroxidation in diabetic rats was also found to be reverted back to near-normal status in extract-treated groups. S. oleracea (200mg/kg) ameliorated the histopathological changes in the hepatic and pancreas tissues. Glibenclamide was used as a reference and showed similar antidiabetic effect. The structural changes are clearly oxidative in nature and are associated with development of vascular disease in diabetes. In results, it demonstrated statistically significant hypoglycemic and hypocholesteremia-like action when observed and compared with control group. It also exhibited potent anti-oxidant potential. In conclusion, it might be said that *Spinacia oleracea* is effective in the management of diabetes mellitus having numerous potential bioactive molecules. It could be used in the treatment of Type-II diabetes mellitus and to replenish the electrolytes and minerals in abundance.

Keywords: Herbal extract, antidiabetic, Column chromatography, blood glucose level, and histopathology.

DOI: 10.48047/ecb/2023.12.Si11.040

INTRODUCTION

Diabetes has been recognized as a major health problem worldwide for the twenty-first century. Developing countries of Asia and Africa are the most viable areas where the disease is feared to raise 2-3 folds [1]. In particular, the number of people with diabetes in India is around 40.9 million by 2004 and is expected to rise to 69.9 million by 2030 [2]. India leads the world with largest number of diabetic subjects thus earning the dubious distinction of being termed the ‘‘Diabetes Capital of the World” [3]. Diabetes mellitus was known to ancient Indian physicians as ‘madumeha’ [4]. Diabetes mellitus is a chronic and metabolic disease affecting glucose, fat, and protein metabolism. DM causes complications such as nephropathy, neuropathy, retinopathy, blindness, obesity, limb amputation and failure of various organs, in particular the blood vessels and nerves and increases mortality rate [5]. Chronic exposure to elevated levels of glucose and fasting blood glucose causes β-cell dysfunction and may induce β-cell apoptosis in type 2 diabetes. The deficit of β-cell mass seems to be caused mainly by increased β-cell apoptosis [6].

Spinacia oleracea is an edible flowering plant in the family of Chenopodiaceae, common name is spinach or in Hindi known as Palak. It is an annual plant, which grows to a height of up to 30 cm. Spinach may survive over winter in temperate regions. The leaves are alternate, simple, and ovate to triangular-based, very variable in size from about 2-30 cm long and 1-15 cm broad, with larger leaves at the base of the plant and small leaves higher on the flowering stem. The flowers are inconspicuous, yellow-green, 3-4 mm diameter, maturing into a small, hard, dry, lumpy fruit cluster 5-10 mm across containing several seeds [7].

Figure 1. *Spinacia oleracea* L. Plant

Native to South- west Asia; cultivated throughout India. Spinach has a high nutritional value and is extremely rich in antioxidants, especially when fresh, steamed, or quickly boiled. It is a rich source of vitamin A, vitamin C, vitamin E, vitamin K, magnesium, manganese, folate,

betaine, iron, calcium, vitamin B6, folic acid, copper, protein, phosphorus, zinc, niacin, selenium, and omega-3 fatty acids. Recently, opioid peptides called rubiscolins have also been found in spinach. It is a source of folic acid (Vitamin B9), and this vitamin was first purified from spinach. It contains ascorbic acid, dehydroascorbic acid, two spinach flavonoids; 5,3',4' -trihydroxy-3-methoxy-6:7-methylenedioxyflavone and spinacetin and carotenoid; β-caroteneand, lutein [8]. It also contains mainly three glycolipids: monogalactosyl diacylglycerol, digalactosyl diacylglycerol, and sulfoquinovosyl diacylglycerol, two antifungal peptides; designated alpha- and beta- basrubrins [9][10].

MATERIALS AND METHODS

Drugs and chemicals

Glibenclamide was gifted from Nicholas Piramal, Mumbai. All chemicals used were of analytical grade.

Equipments

Table 2. List of equipments with model names

Equipment name	Model
Digital Weighing Balance	Mettler
Digital pH meter	Equip-tronics
Incubator	Thermolab
Tissue Homogenizer	Remi-motors
Laboratory Centrifuge	Remi-motors
HPTLC	Camag
Electronic balance	Sartorius, CP2248
Hot air oven	Lab Tech
Water bath	Lab Tech
Soxhlet apparatus	ASGI
Rotatory Evaporator	Popular India
Melting Point apparatus	Lab. Hosp. Corporation
FTIR	Shimadzu 8400 S FT-IR
1H- NMR	Brucker multinuclear FT NMR
13C- NMR	Brucker
Mass spectrometer	TOF MS ES+ Mass spectrometer

Collection and authentication of the plant leaves

The leaves of Spinacia oleracea were collected from the outfield near Krishi College, Indore, India, during the month of July which shows a green color with a rough surface. The plant leaves were washed thoroughly in tap water, dried in shade, finely powdered and used for successive extraction methods. The plant was identified by Dr V. Chelladurai, Retd. Research Officer, Botany (Scientist C) at Central Council for Research in Ayurveda, Bhopal, India.

Extraction of plant

The successive extraction of plant materials was prepared in following steps [11].

Powdered drug

The correctly identified plant leaves is dried in shade at room temperature & after 4-4 days, it is formed in powder by mixer grinder. These should be extracted with different solvent in order to their increasing polarity to get the correct and dependable retention factor.

Defatting

Powdered drug 100gm was weighed and packed in Soxhlet. The drug was continuously extracted with petroleum ether for about 72 hours. Complete defatting was ensured by placing a drop form the thimble on a filter paper give any oily spot. The mare was dried in air to remove traces of petroleum ether.

Chloroform extraction

Defatted drug was subjected to extraction with chloroform in Soxhlet apparatus, the extraction was completed in 17-18 hrs. The extract was dried & stored in dark place.

Ethyl acetate extraction

Drug was subjected to extraction with ethyl acetate in Soxhlet apparatus, the extraction was completed in 17-18 cycles. The extract was dried & stored in dark place. 3. Ethanolic extraction: Drug was subjected to extraction with ethanol (90%) in Soxhlet apparatus, the extraction was completed in 24 cycles. The extract was dried & stored in dark place.

Methanolic extraction

Drug was subjected to extraction with methanol in Soxhlet apparatus, the extraction was completed in 24 cycles. The extract was dried & stored in dark place. 5. Aqueous extraction: The Drug was subjected to extraction with water by Hot Water Extraction. The % Yield of the Petroleum ether, Chloroform, Ethyl acetate, Ethanol, Methanol, & Aqueous extract of Spinacia oleracea was calculated by using the following formula-

$$\% \text{ Yield} = \frac{\text{Net weight of powder in gram after extraction} \times 100}{\text{Total weight of leaf powder in gram taken for extraction}}$$

Phytochemical Screening [12]

Detection of Carbohydrate

400 mg of extract was dissolved in 4 ml of distilled water and filtered. The filtrate was used to test the presence of carbohydrates.

Molisch's Test

To 1 ml of filtrate, 2 drops of Molisch's reagent was added in a test tube and 2 ml of concentrated sulphuric acid added carefully along the side of the test tube. Formation of violet ring at the junction indicates the presence of carbohydrate.

Molisch's reagent:

10 gm of alpha napthol was dissolve in 100 ml of 94% alcohol to prepare Molish's reagent.

Fehling's Test

To 1 ml of filtrate, 4 ml of Fehling's solution was added in a test tube and heated for 10 minutes in a water bath. Formation of red precipitate indicates the presence of reducing sugar.

Fehling's solution:

(a) 34.66 gm of copper sulphate was dissolved in distilled water and volume was made up to 400 ml.

(b) 173 gm of potassium sodium tartarate and 40 gm of sodium hydroxide were dissolved in distilled water and made up to 400 ml.
(a) and (b) solutions were mixed in equal volume to give Fehling's solution.

Detection of Glycosides

The 0.4 gm of extract was hydrolyzed with 20 ml of dilute hydrochloric acid (0.1 N) and filtered. The filtrate was used to test the presence glycosides.

Modified Borntrager's Test

To 01 ml of filtrate, 02 ml of 1% ferric chloride solution was added in a test tube and heated for 10 minutes in boiling water bath. The mixture was cooled and shaken with equal volume of benzene. The benzene layer was separated and treated with half of its volume of ammonia solution. Formation of rose pink or cherry colour in the ammonia layer indicates the presence of glycoside.

Killer Killiani Test

Small portion from the respective extracts was shaken with 1 ml glacial acetic acid containing a trace of ferric chloride. 1 ml of conc. sulphuric acid (H2SO4) was added carefully by the sides of the test tube. A blue colour in the acetic acid layer and red colour at the junction of the two liquids indicate the presence of glycosides.

Detection of Alkaloids

The 0.4 gm of extract was dissolved in 10 ml of dilute hydrochloric acid (0.1 N) and filtered. The filtrate was used to test the presence of alkaloids.

Mayer's Test

Filtrates were treated with Mayer's reagent; formation of yellow cream coloured precipitate indicates the presence of alkaloids.

Mayer's reagent:

(a) Dissolve 1.36 gm of mercuric chloride in 60 ml of distilled water.
(b) Dissolve 4 gm of potassium iodide in 20 ml distilled water.
(c) Mix (a) and (b) and adjust the volume to 100 ml with distilled water.

Dragendroff's Test

Filtrates were treated with Dragendroff's reagent; formation of red colored precipitate indicates the presence of alkaloids.

Dragendroff's reagent:

(a) Dissolve 8 gm of bismuth nitrate in 20 ml of nitric acid.
(b) Dissolve 27.2 gm of potassium iodide in 40 ml distilled water.
(c) Mix (a) and (b) and adjust the volume to 100 ml with distilled water.

Hager's Test

Filtrates were treated with Hager's reagent; formation of yellow coloured precipitate indicates the presence of alkaloids.

Hager's reagent:

Saturated solution of picric acid in distilled water.

Detection of phytosterols and triterpenoids

The 0.4 gm of extract was treated with 10 ml chloroform and filtered. The filtrate was used to test the presence of phytosterols and triterpenoids.

Salkowaski Test

To the test extract solution added few drops of conc. H_2SO_4 shaken and allowed to stand, lower layer turns reddish brown or golden yellow indicating the presence of triterpenes.

Detection of Protein and amino acid

The 100 mg of each extract was taken in 10 ml of water and filtered. The filtrate was used to test the presence of protein and amino acids.

Millon's Test:

2 ml of filtrate was treated with 2 ml of Million's reagent in a test tube and heated in a water bath for 4 minutes, cooled and added few drops of Sodium Nitrate solution. Formation of white precipitate, which turns to red upon heating, indicates the presence of proteins and amino acid.

Millon's reagent:

Dissolve 1 gm mercury in 9 ml of fuming nitric acid. Keeping the mixture well cooled during the reaction. When the reaction is completed, add equal volume of distilled water.

Ninhydrin Test:

To 2 ml of filtrate, 0.24% Ninhydrin reagent was added in a test tube and boiled for 2 minutes. Formation of blue color indicates the presence of amino acids.

Ninhydrin reagent:

0.24% solution in n- butanol.

Biuret test

2 ml of filtrate was treated with 2 ml of 10% sodium hydroxide solution in a test tube and heated for 10 minutes. A drop of 7% copper sulphate solution was added in the above mixture. Formation of purplish violet colour indicates the presence of proteins.

Detection of Fixed oils and Fats test

One drop of each extract was placed on filter paper and solvent was allowed to evaporate. An oily stain on filter paper indicates the presence of foxed oil.

Detection of Phenolics and Tannins

The 100 mg of each extract was boiled with 1 ml of distilled water and filtered. The filtrate was used for following tests.

Ferric chloride test

To 2 ml of filtrate, 2 ml of 1% ferric chloride solution was added in a test tube. Formation of bluish black colour indicates the presence of phenolic nucleus.

Lead Acetate Test

To 2 ml of filtrate, few drops lead acetate solution was added in a test tube. Formation of yellow precipitate indicates the presence of tannins.

Detection of Flavonoids Alkaline test

To 100 mg of extract, few drops of sodium hydroxide solution were added in a test tube. Formation of intense yellow colour that becomes colourless on addition of few drops of dilute acid (HCl) indicates the presence of Flavonoids.

Detection of Saponin Foam Test

Extracts were diluted with distilled water to 20 ml and Shaken in a graduated cylinder for 14 minutes. Formation of one cm layer of foam indicates the presence of Saponin.

Detection of Mucilage

The 10 ml of the aqueous extract was tested for mucilage; the extract was added with 24 ml of 94% alcohol with constant stirring. The so formed precipitate was centrifuged and washed

with alcohol, the dissolved in water (10 ml) and reprecipitated. After washing the precipitate was collected & dried in desiccators. On addition of a drop of water and allowed to stand for some time, it swelled to give a viscous mass which gave indication for presence of mucilage [13].

Isolation of Active compounds from fractions of Spinacia oleracea

Thin Layer Chromatography

On the precoated TLC plate, test samples (after dissolving in respective solvents) were applied in the form of spots with the help of fine capillary. Spots were marked on the top of the plate for their identification. Rectangular glass chambers were used for chromatography. To avoid insufficient chamber saturation and undesirable edge effect, a smooth sheet of filter paper was placed in TLC chamber and was allowed to be in the developing solvent. Plate was sprayed with anisaldehyde-sulphuric acid and heated at 1150C for 5 minutes. Solvent n-hexane: ethyl acetate (72:28) was used as solvent system. After development of plates, they were air-dried and number of spots, color and Rf values were recorded [14].

TLC plate-: Precoated TLC plate silica gel G

Bioactive extract-: dichlomethane extract

Solvent system-: n-hexane:ethyl acetate (72:28)

Spraying agents-: Anisaldehyde sulphuric acid

Preparation of the column

The slurry of adsorbent (silica gel; 60-120 mesh) was prepared by mixing the adsorbent in the n-hexane and used as stationary phase. It was then poured into glass column (90cm 41 x 3cm) and allowed to settle. The air entrapped was removed by stirring with glass rod. This method of column filling is called as wet filling method. A small amount of sand was kept at the top of the column. Excess of solvent was run off until the level of mobile phase fell to one cm just above the top of the sand layer [15].

Preparation of sample and loading

Dichloromethane extract (10 g) was dissolved in a minimum volume of dichloromethane and adsorbed on silica gel (60-120 mesh), dried and applied on the column to separate possible phytoconstituents.

Selection of mobile phase for separation of phytoconstituents

Active dichloromethane extract (10 g) of Spinacia Oleracea was subjected to chromatographic separation by loading out on a glass column. The solvent system was used as n-hexane: ethyl acetate. The gradient elusion was followed for the isolation. Initially n-hexane was used as pure solvent and then column was eluted by increasing quantity (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40 and finally 50:50) of ethyl acetate. All the fractions were monitored simultaneously on a TLC plate using n-hexane: ethyl acetate (72:28) as solvent system. The fractions showing same color and Rf on TLC were pooled together and finally 4 fractions (F1-F4) were obtained. All the fractions (F1-F4) were subjected to their highest Immunomodulatory activity by determine their phagocytic response.

Spectral analysis and Structure elucidation

It was tried to purify compounds, which were obtained by employing Column chromatography and TLC and by re-crystallizing them in different solvents. The compounds were weighted and there melting point determined. The identification of a molecule was done through the interpretation of the data obtained from spectroscopic analysis [16].

FT-IR

Infrared (IR) spectra were recorded on a Shimadzu (Japan) 8400 S FT-IR spectrophotometer model using potassium bromide pellets (υmax in cm-1).

NMR

1HNMR spectra were recorded on Brucker multinuclear FT NMR spectrometer model AV-400, 400 MHz using deuterated-chloroform or deuterated dimethylsulfoxide-containing tetramethylsilane (Me4Si) as internal standard (chemical shifts in δ, ppm). The spin multiplicities are indicated by symbols, s (singlet), d (doublet), t (triplet), m (multiplet), and q (quartet). The MASS spectrum was recorded on TOF MS ES+ Mass spectrometer. The purity of compounds was established by thin layer chromatography (TLC). Precoated silica gel aluminium plate 60F-244 (20 cm X 20 cm with 240 μm thickness was used for TLC. Iodine was used to develop the TLC plates [17].

Mass spectroscopy

Mass is mainly used to determine m/z ratio. This ratio is dependent upon fragmentation pattern of compounds. In this technique, sample under investigation was bombarded by the help of electron proton and compound showed various types of fragmentation pattern. In fragmented pattern we find out base peak that showed m/z ratio [18].

Preparation of animals

Wistar Albino rats of either sex (140 to 200 g) were purchased from DRDE, Gwalior. They were maintained under standard laboratory conditions at 24 ± 2°C, relative humidity (40 ± 14%) and normal photoperiod (12-hour light-dark cycle) were used for the experiment. Commercial pellet diet MFD, by Amrut trade corporation, Gwalior were given to the experimental animals throughout the study. The Experimental Protocol was duly approved by IAEC having NoIPS/COP/IAEC/02 according to CPCSEA guidelines.

Selection of Dose (OECD guideline 2001)

Acute oral toxicity test was carried out according to the OECD guideline No. 423. Wistar Albino Rats were kept for overnight fasting prior to drug administration. A total of three animals were used, which received a single oral dose in 2000 mg/kg, body weight of different extracts. The animals were observed for a period of 24 hr for the changes in behavior, hypersensitivity reactions etc. Mortality, if any, was determined over a period of 2 weeks. Hence in our studies we selected 1/10 and 1/4th dose i.e., 200 and 400 mg/kg dose [19].

Group design

In order to assess the anti-diabetic activity, the animals were divided in five groups of six animals in each group.

Group 1: Normal control, 0.9% NaCl-treated animals

Group 2: Diabetic control, STZ -treated rats (40 mg/kg body weight)

Group 3: Treated with methanolic extract of Spinacia oleracea (200 mg/kg body weight)

Group 4: Treated with methanolic extract of Spinacia oleracea (400 mg/kg body weight)

Group 5: Standard drug, Glibenclamide- treated rats (4 mg/kg body weight)
The test drug and reference drug were administered orally at two dose level for a period of 21 days from starting day of diabetes.

Streptozotocin (STZ) induced diabetes in rats

After fasting 18 hours, the rats were injected intraperitoneal injection through tail vein with a single dose of 40 mg/kg Streptozocin (Sigma, St. Louis, Mo, USA), freshly dissolved in citrate buffer (pH 4.4). After injection, the rats had free access to food and water and were given 4% glucose solution to drink overnight to counter hypoglycemic shock. Diabetes in rats was observed by moderate Polydipsia and marked Polyuria. The diabetes was confirmed by estimating the blood glucose level after 3 days by glucometer based on glucose oxidation method. Rats having blood glucose level more than 240 mg/dl were selected for further study [20].

Blood collection and biochemical estimations in serum & Pancreas

On 22nd day, fasting blood samples were collected from the tail vein of all the groups of rats. Whole blood was collected for estimation of blood glucose by using the glucometer (Easy Gluco, Morepen Laboratories Ltd. New Delhi), glycosylated hemoglobin (HbA1C) (Murray et.al. 2003) and glutathione levels (Goldstein et al.1982). Serum was separated for estimation of specific serum marker enzymes, namely, lactate dehydrogenase (LDH) (karunanayke et al.1990) and creatine kinase (CK) (Murray et al. 2000). Streptozocin-induced oxidative stress in diabetes is also a predictor of cardiac damage. Since LDH and CK are specific cardiac marker enzymes, increased serum LDH and CK levels were considered as marker of oxidative stress-induced cardiac damage [21].

Biochemical estimation in pancreatic tissue

After blood collection, all the animals were sacrificed and pancreas was dissected out. Tissue was washed with ice cold saline, weighed and minced; 10% homogenate was prepared in 0.14M ice-cold KCl for TBARS (thiobarbituric acid-reactive substances), a marker for lipid per oxidation (Chen et al.2001) and protein estimation; in 0.02M EDTA for glutathione estimation (Goldstein et. al.1982) and in phosphate buffer (pH 7.4) for superoxide dismutase (SOD) (Koenig et al.1976) and catalase estimations (Koenig et al.1976) using a Teflon tissue homogenizer. Decrease in levels of endogenous antioxidants with rise in TBARS levels was considered as oxidative stress [22].

Statistical analysis

The values are expressed in mean ± SEM. The results were analyzed by using one way analysis of variance (ANOVA) followed by Dunnet's "t" test to determine the statistical significance. $p < 0.04$ was chosen as the level of significance. Statistical analysis was performed using Graph Pad Prism Software 4.0 version

RESULTS AND DISCUSSION

The following table demonstrates the presence or absence of phytochemical moieties in the extract-

Table 1. Qualitative phytochemical analysis of methanolic extract of *S. oleracea*

Type of Constituent	Methanolic extract
Alkaloids	-
Tannins	+ + +
Flavonoids	+
Saponins	+
Steroids	+ + +
Triterpenoids	+ + +
Proteins	+ +
Carbohydrates	+
Glycosides	+ +

The below mentioned tables 2 and 3 show the electrolytes that plant consists and HPTLC finger print analysis-

Table 2. Estimation of Inorganic Constituents in *S. oleracea*

Elements	Quantity of elements (mg/g) in dried powder
Zn	1.660
Mn	3.876
Cu	0.687
Cr	0.123
Pb	0.31
As	<0.001
Co	26
Na	<0.01
K	<0.01

Table 3. HPTLC finger print analysis of methanolic extract of *S. oleracea*

Extract	**Solvent system**	**No. of peaks**	**Rf Value**	**Percent Area (%)**
Methanolic extract	Toluene: Ethyl Acetate: Formic acid 5:4:1	15	0.06, 0.11, 0.20, 0.31, 0.44, 0.53, 0.56, 0.61, 0.65, 0.70, 0.75, 0.82, 0.86, 0.95, 0.99	1.14, 1.80, 0.39, 3.86, 7.05, 3.37, 2.84, 5.60, 4.87, 6.18, 8.00, 12.91,8.36, 28.85, 4.79

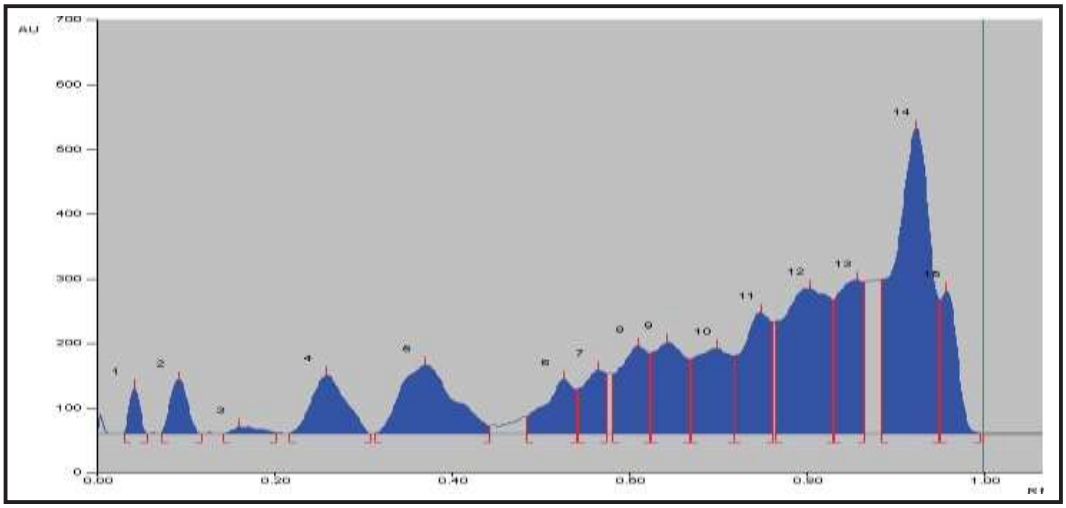

Figure 2. HPTLC Chromatogram of methanolic extract of *S. oleracea*

Table 4. Column Chromatography of S. oleracea Extract and examination of Elutes

Eluent	Solvent Ratio	Fractions	Compound
Methanol	100%	1-11	Greenish color residue
Methanol: EtoAc	98:2	12-26	Waxy residue
Methanol: EtoAc	95:5	27-32	Yellow residue
Methanol: EtoAc	9:1	33-46	Reddish brown residue
Methanol: EtoAc	8.5:1.5	47-57	SHM-1
Methanol: EtoAc	3:1	58-65	Mixture
Methanol: EtoAc	1:1	66-73	Intangible mass
EtoAc	100%	74-83	Waxy residue

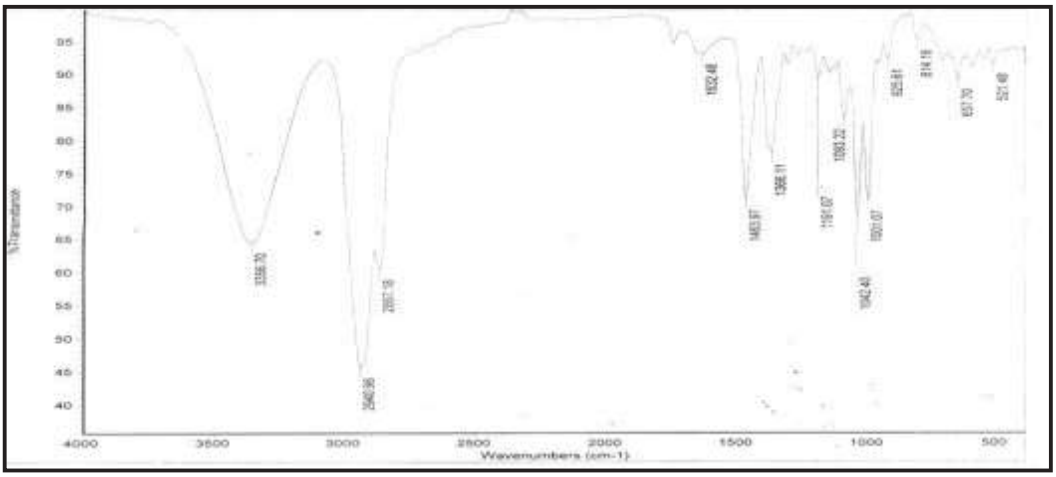

Figure 3. IR spectra of compound MESH-1

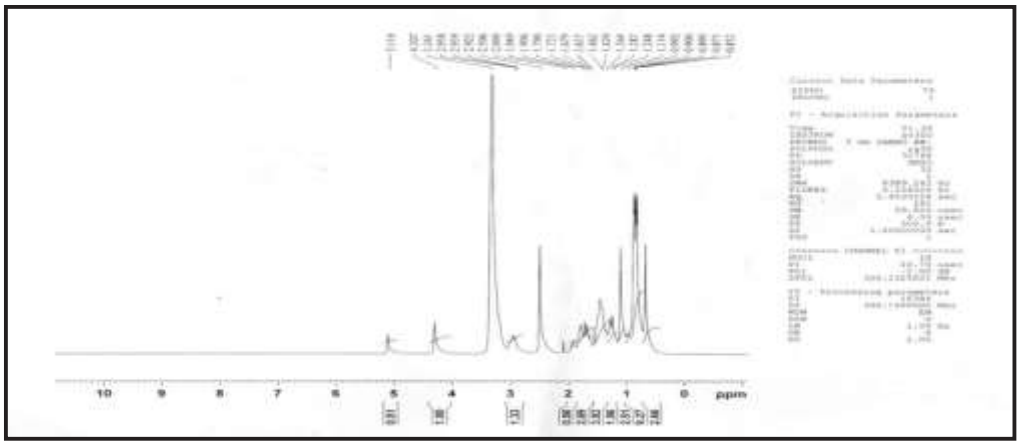

Figure 4. ^{1}H spectra of compound MESH-1

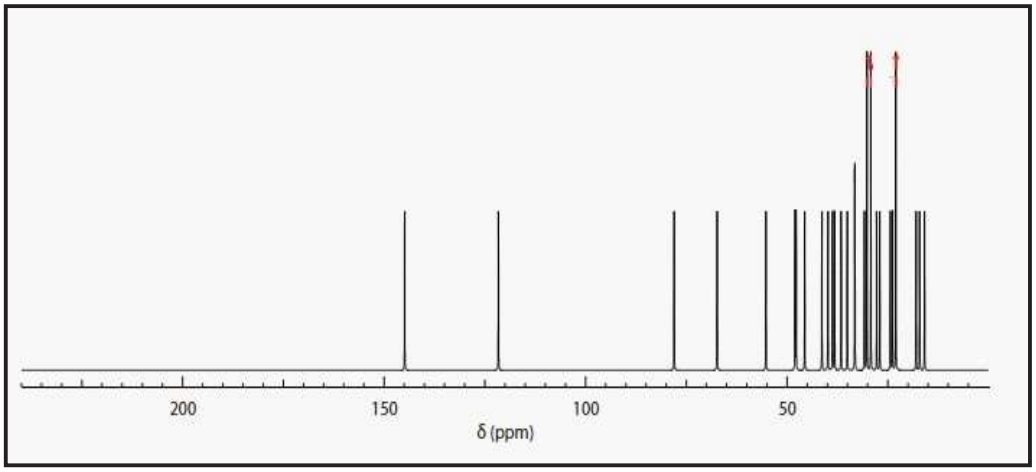

Figure 5. ^{13}C spectra of compound MESH-1

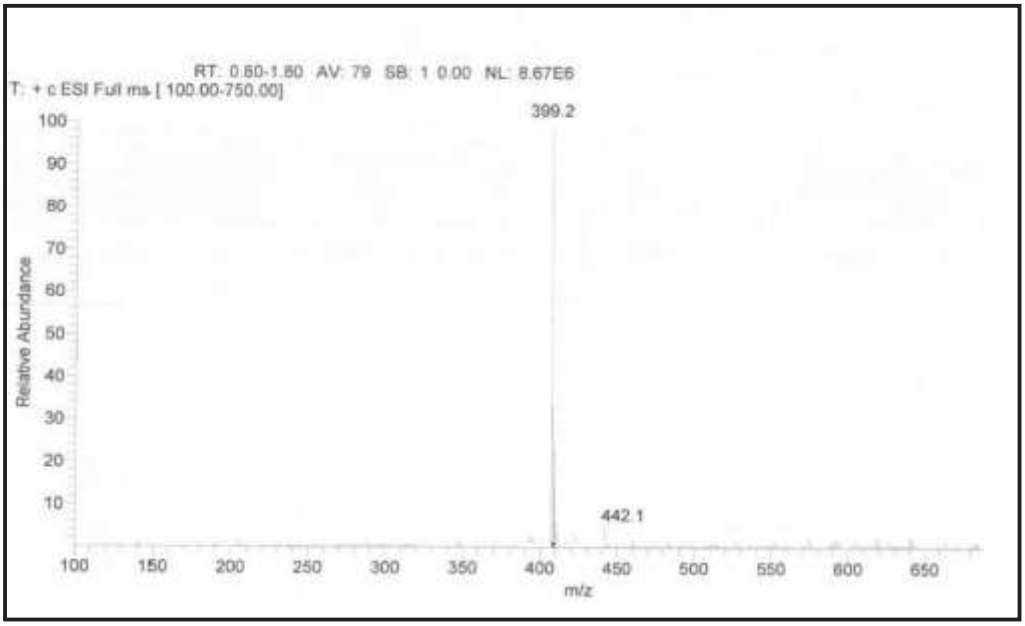

Figure 6. Mass spectra of compound MESH-1

Table 5. Invitro antioxidant activity of MESH by DPPH inhibition Assay

Conc (µg/ml)	Percentage Inhibition	
	Vitamin C	MESH
10	34.8±0.98	11.14±0.84
20	55.9±0.92	19.6±1.2
40	63.4±0.85	32.8±1.10
60	76.7±0.92	49.6±1.18
80	83.2±1.02	64.9±1.02
IC50 (µg/ml)	19.77	60.91

Data are presented as the mean ± SEM (n = 3)

Table: 6. Effect of MESH and High fructose diet on blood glucose level

Blood Glucose level (mg/dl)				
Treatment	**0 day**	**7th day**	**14th day**	**21st day**
Control 0.5% CMC (1ml/kg; p.o)	85.12± 1.87	86.12 ± 2.12	86.54±1.92	87.12±1.24
Fructose Diet	271.76 ± 4.90	280.45±3.87	288.78±4.32	296.56±4.87
Fructose Diet + MESH (200mg/kg, b.wt; p.o)	276.56±3.65 NS	170.78±2.98b*	158.87±3.12 b*	153.87±2.87b*
Fructose Diet + MESH (400mg/kg, b.wt; p.o)	274.64 ± 3.82	150.65±3.72	135.82±2.12	115.32±1.76
Fructose Diet + Glibinclamide (600 µg/kg, b.wt; p.o)	272.24 ± 4.65	104.25±2.34	98.98±1.65	89.21±0.87

The values are expressed ad Mean ± SEM, n=6.Comparisons is made between [a] Fructose diet vs control; [b] Fructose diet vs Fructose diet + MESH (200mg/kg) and [c] Fructose diet vs Fructose diet + MESH (400mg/kg); [d] Fructose diet vs Fructose diet + Glibinclamide. * Statistically significant, $p<0.05$; NS- Non-Significant

Table: 7. Effect of MESH and High fructose diet on hepatic enzymic antioxidants level

Treatment	SOD	CAT	GPx
Control 0.5% CMC (1ml/kg; p.o)	2.66±0.01	15.21±0.07	1.71±0.05
Fructose Diet	0.45±0.02 [a*]	2.76±0.04 [a*]	0.36±0.02 [a*]
Fructose Diet + MESH (200mg/kg, b.wt; p.o)	0.96±0.02 [b*]	10.64±0.16 [b*]	0.72±0.04 [b*]
Fructose Diet + MESH (400mg/kg, b.wt; p.o)	1.82±0.08 [c*]	12.87±0.14 [c*]	1.54 ±0.08
Fructose Diet + Glibenclamide (600 µg/kg, b.wt; p.o)	2.13±0.02 [d*]	13.50±0.24 [d*]	1.70±0.09 [b*]

Units: SOD: U/mg of Protein; CAT: U/mg of Protein; GPx: nmol/min/mg protein. The values are expressed ad Mean ± SEM, n=6.Comparisons is made between a Fructose diet vs control; b Fructose diet vs Fructose diet + MESH (200mg/kg) and c Fructose diet vs Fructose diet + MESH (400mg/kg); d Fructose diet vs Fructose diet + Glibenclamide. * Statistically significant, $p<0.05$; NS-Non-Significant.

Table 8. Effect of MESH and High fructose diet on hepatic MDA and GSH level

Treatment	MDA	GSH
Control 0.5% CMC (1ml/kg; p.o)	1.22±0.06	4.82±0.23
Fructose Diet	5.85±0.32[a*]	1.42±0.16 [a*]
Fructose Diet + MESH (200mg/kg, b.wt; p.o)	3.76±0.25[b*]	2.13±0.04 [b*]
Fructose Diet + MESH (400mg/kg, b.wt; p.o)	2.20±0.11 [c*]	4.12±0.18 [c*]
Fructose Diet + Glibenclamide (600 µg/kg, b.wt; p.o)	1.97±0.12 [d*]	4.58±0.21 [c*]

Units: MDA: µmoles/ mg of tissue; GSH: µmoles/ mg of tissue. The values are expressed ad Mean ± SEM, n=6.Comparisons is made between [a] Fructose diet vs control; [b] Fructose diet vs Fructose diet + MESH (200mg/kg) and [c] Fructose diet vs Fructose diet + MESH (400mg/kg); [d] Fructose diet vs Fructose diet + Glibenclamide. * Statistically significant, $p<0.05$; NS-Non- Significant

Table 9. Effect of MESH and High fructose diet on pancreatic enzymic antioxidants level

Treatment	SOD	CAT	GPx
Control 0.5% CMC (1ml/kg; p.o)	4.05±0.01	17.90±0.54	2.82±0.08
Fructose Diet	1.53±0.05 [a*]	7.81±0.29 [a*]	0.85±0.05 [a*]
Fructose Diet + MESH (200mg/kg, b.wt; p.o)	2.34±0.05 [b*]	12.76±0.43 [b*]	1.65±0.04 [b*]
Fructose Diet + MESH (400mg/kg, b.wt; p.o)	3.21±0.02 [c*]	14.36±0.56 [c*]	2.12±0.05 [c*]
Fructose Diet + Glibenclamide (600 µg/kg, b.wt; p.o)	3.96±0.03 [d*]	15.17±0.58 [d*]	2.64±0.02 [d*]

Units: SOD: U/mg of Protein; CAT: U/mg of Protein; GPx: nmol/min/mg protein. The values are expressed ad Mean ± SEM, n=6.Comparisons is made between a Fructose diet vs control; b Fructose diet vs Fructose diet + MESH (200mg/kg) and c Fructose diet vs Fructose diet + MESH (400mg/kg); d Fructose diet vs Fructose diet + Glibenclamide. * Statistically significant, $p<0.05$; NS-Non-significant.

Table 10. Effect of MESH and High fructose diet on pancreatic MDA and GSH level

Treatment	MDA	GSH
Control 0.5% CMC (1ml/kg; p.o)	3.24±0.08	2.93±0.07
Fructose Diet	6.78±0.24[a*]	0.67±0.01 [a*]
Fructose Diet + MESH (200mg/kg, b.wt; p.o)	5.06±0.18 [b*]	1.12±0.03 [b*]
Fructose Diet + MESH (400mg/kg, b.wt; p.o)	4.12±0.18[c*]	1.98±0.03 [c*]
Fructose Diet + Glibenclamide (600 µg/kg, b.wt; p.o)	3.64±0.09 [d*]	2.46±0.06 [d*]

Units: MDA: µmoles/ mg of tissue; GSH: µmoles/ mg of tissue. The values are expressed ad Mean ± SEM, n=6.Comparisons is made between [a] Fructose diet vs control; [b] Fructose diet vs Fructose diet + MESH (200mg/kg) and [c] Fructose diet vs Fructose diet + MESH (400mg/kg); [d] Fructose diet vs Fructose diet + Glibinclamide. * Statistically significant, $p<0.05$; NS-Non-significant.

Table 11. Effect of MESH and High fructose diet serum on lipid markers.

Groups	TC (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	TG (mg/dl)
Control 0.5% CMC (1ml/kg; p.o)	119.65±4.56	45.97±4.83	74.83±2.84	93.96±3.27
Fructose Diet	264.60±7.87 a*	20.99±3.64 a*	191.72±10.25 a*	218.50±12.33 a*
Fructose Diet + MESH (200mg/kg, b.wt; p.o)	180.87±6.32 b*	32.18±3.12 b*	134.76±5.42 b*	145.82±5.34 b*
Fructose Diet + MESH (400mg/kg, b.wt; p.o)	144.29±6.87c*	40.39±2.63 c*	80.61±2.12 c*	112.13±4.12 c*

The values are expressed ad Mean ± SEM, n=6.Comparisons is made between [a] Fructose diet vs control; [b] Fructose diet vs Fructose diet + MESH (200mg/kg) and [c] Fructose diet vs Fructose diet + MESH (400mg/kg); * Statistically significant, $p<0.05$; NS-Non-Significant.

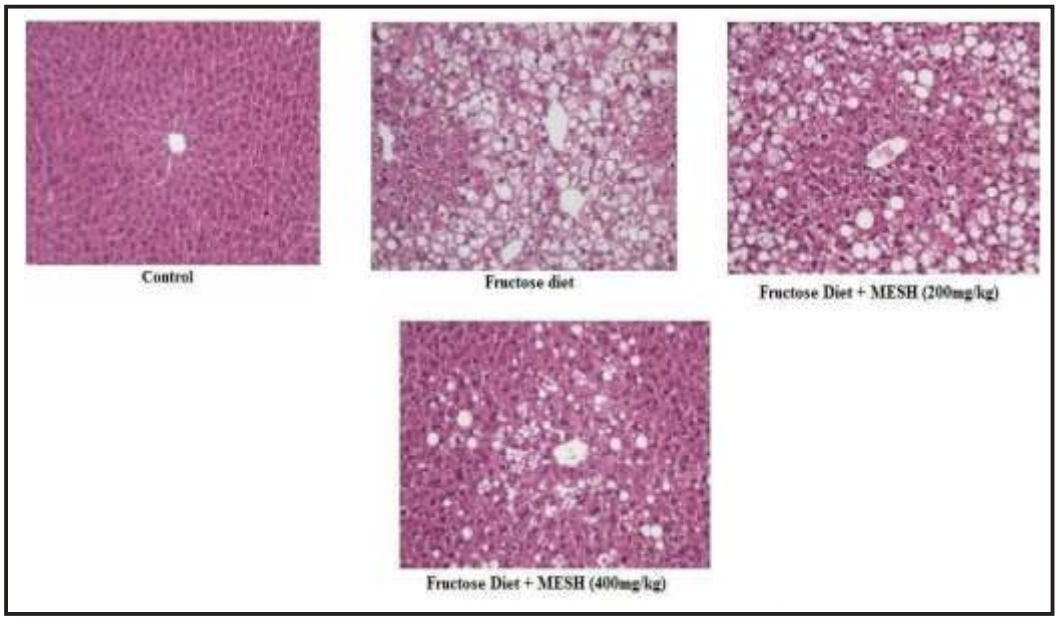

Figure 7. Effect of MESH and High fructose diet on liver histopathology

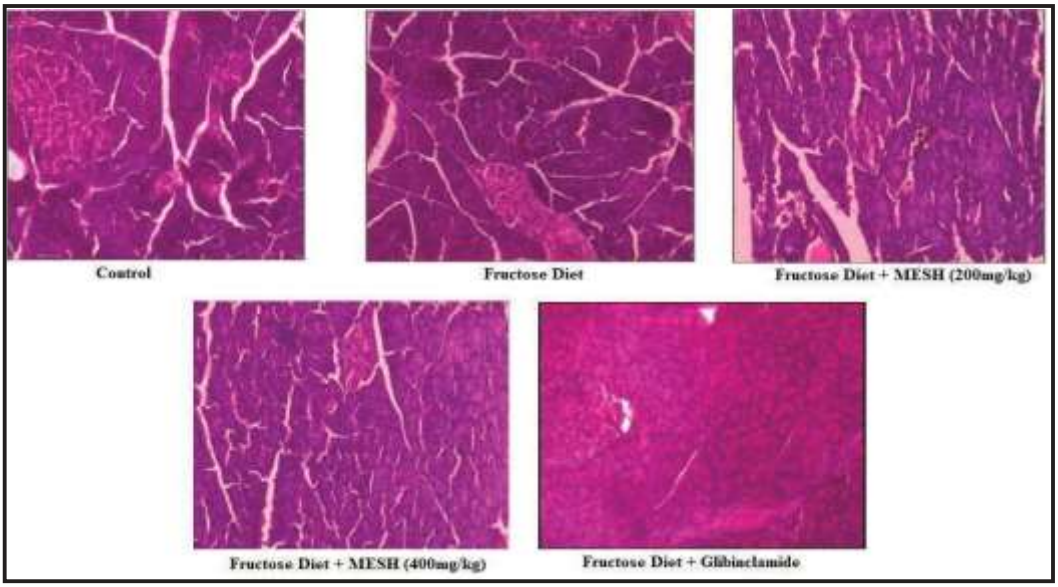

Figure 8. Effect of MESH and High fructose diet on pancreas histopathology

The treatment of diabetes with medicines of plant origin that proved much safer than synthetic drugs is an integral part of many cultures throughout the world and has gained importance in recent years. India has a rich history of using various potent herbs and herbal components for treating various diseases including diabetes [23]. Several phytomolecules including flavonoids, alkaloids, glycosides, saponins, glycolipids, dietary fibers, polysaccharides, peptidoglycans, carbohydrates, amino acids and others obtained from various plant sources have been reported as potent hypoglycemic agent [24].

Administration of MESH and MESA extracts to food induced diabetic rats displayed significant reduction in the blood glucose levels to near normal. Albeit, the exact mechanism of action of the extract is unknown, the reduction in blood glucose level could be due to increased pancreatic insulin secretion from existing β-cell of the pancreas [25].

The body deals with oxidative stress by employing a series of low molecular weight antioxidants that neutralize the reactive oxygen species before they can produce oxidative changes in tissues [26].

β- cell is the most abundant cell type in the endocrine pancreas; β- cell number is the most important factor that determines islet area. Pancreas of diabetic rats showed ruptured islet and decreased β- cell. The area of islet was reduced markedly in diabetic rats which were restored in MESH, MESA and glibenclamide treated diabetic rats. The restoration of damages in pancrease may be improved control over the toxic activity of free radical by MESH and MESA. Diabetic rats showed shrunken islets and fatty infiltration may be due to high fructose diet induced highly reactive free radicals can deplete GSH which also destroy hepatic and renal cell [27]. The activity of antioxidant enzymes such as superoxides dismutase (SOD), catalase and glutathione peroxidase which is low in islet cells when compared to other tissues

under diabetic conditions. Glycation mediated reactive oxygen species leads to apoptosis of β- cell and reduced insulin gene transcription [28]. The treatment of diabetic rats with MESH and MESA prevents the glycation process by its antihyperglycemic activity and thus it protects the β-cell of the pancreas from hyperglycemia-induced injuries. Rats are commonly used animal models for studying the adverse effects of obesity [29]. Humans and rodents have a similar tendency to gain weight when exposed to a long-term high calorie diet intake. Hence, diet induced obesity model of rat possess excellent utility in predicting weight loss in man. Hyperlipidemia is responsible for the onset and progression of atherosclerosis [30]. a major risk factor in the development of coronary heart diseases (CHDs) such as ischemic heart disease, myocardial infarction and stroke [31]. In clinical practice, effective and intensive lipid-lowering is important in order to reduce and prevent CHDs. MESH and MESA significantly reduced TC, TG and LDL-c concentrations. These reductions in TC, TG and LDL levels suggest the ameliorative potential of MESH and MESA in hyperlipidemia. The elevation of TC concentration in this study was achieved by the indirect stimulation of HMG CoA reductase following induction of hyperlipidemia [32]. Hence the possible TC lowering effects of MESH and MESA could be attributed to decreased activity of hepatic HMG CoA reductase and/or stimulation of cholesterol-7-alpha-hydroxylase, which converts cholesterol into bile acids. It could also be due to the presence of saponins, a phytochemical which forms insoluble complexes with cholesterol or their bile salt precursor, thus making them unavailable for absorption. The results obtained in this work conform to earlier report by that phytochemical possesses anti hyperlipidaemic activity. Additionally, obesity is the most important risk factor for complex and chronic liver disorders. These liver disorders begin as steatosis and may progress to steatohepatitis, cirrhosis, liver failure and hepatocellular carcinoma. Hypercholesterolemia, elevated low-density lipoprotein (LDL), and triglycerides all are associated with obesity. Obesity is also linked to low levels of high-density lipoprotein (HDL).

In results, it demonstrated statistically significant hypoglycemic and hypocholesteremia-like action when observed and compared with control group. It also exhibited potent anti-oxidant potential.

CONCLUSION

In this study, high fructose diet (HFD) induced rat model of obesity was used to check the antihyperlipidemic potential of MESH and MESA. The mechanism of HFD induced obesity is still unclear, but long-term exposure to a HFD can increase body weight and adiposity in human and animals. Lipid abnormalities are very common in obese and are considered a major risk factor for development of atherosclerosis. Hence it is very important to prevent and control early stages of hyperlipidemia, completely with drug therapies.

In conclusion, it might be said that *Spinacia oleracea* is effective in the management of diabetes mellitus having numerous potential bioactive molecules. It could be used in the treatment of Type-II diabetes mellitus and to replenish the electrolytes and minerals in abundance.

FUNDING

Nil.

CONFLICT OF INTEREST

‘None conflict of interest’ was confirmed by the authors.

REFERENCES

1. Akhtar MS, Ali MR (1985) Study of hypoglycaemic activity of Cuminum nigrum seeds in normal and alloxan diabetic rabbits. Planta Med 51(2):81-85.
2. Akhtar MS, Khan QM, Khaliq T (1984) Effects of Euphorbia prostrata and Fumaria parviflora in normoglycaemic and alloxan-treated hyperglycaemic rabbits. Planta Med 50(2):138-142.
3. Alain Puppo and Berry Halliwell (1988) Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron: is hemoglobin a biological fenton reagent? Biochem J 249:185-190.
4. Al-awadi FM, Khattar MA, Gumaa A (1985) On the mechanism of the hypoglycaemic effect of a plant extract. Diabetologia 28:432-434.
5. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC (1974) Enzymatic determination of total serum cholesterol. Clin Chem 20(4):470-5.
6. Ambavade SD, Mhetre NA, Tate VD, Bodhankar SL (2006) Pharmacological evaluation of the extracts of Sphaeranthus indicus flowers on anxiolytic activity in mice. Indian J Pharmacol 38:254–9.
7. Ames BN (1983) Dietary carcinogens and anticarcinogens Oxygen radicals and degenerative diseases Science 221(4617):1256-64.
8. Amos A, McCarty D, Zimmet P (1997) The rising global burden of diabetes and its complications: estimates and projections to the year 2010. Diabet Med 14:S1-S85.
9. Anja J. Gerrits, Eelo Gitz, Cornelis A. Koekman, Frank L. Visseren, Timon W. van Haeften, Jan Willem N. Akkerman (2012) Induction of insulin resistance by the adipokines resistin, leptin, plasminogen activator inhibitor-1 and retinol binding protein 4 in human megakaryocytes Haematologica 97(8):1149–1157.
10. Ali M, K. Nahar, M. Sintaha et al (2011) An evaluation of antihyperglycemic and antinociceptive effects of methanol extract of Heritiera fomes Buch- Ham. (Sterculiaceae) barks in Swiss albino mice," Advances in Natural and Applied Sciences 5(2):116–121.
11. Haffner SM, Miettinen H, Gaskill SP, Stern MP (1996) Decreased insulin action and insulin secretion predict the development of impaired glucose tolerance. Diabetologia 39:1201–1207
12. Harman D (1992) Role of free radicals in aging and disease. Ann N Y Acad Sci. 673:126–41.
13. Helen Vlassara (1992) Receptor mediated interactions of advanced glycosylation end products with cellular components within diabetic tissues. Diabetes 41(2):52-56.
14. Lee Hi Bahl, Hunjoo Ha and George L. King (2003) Reactive oxygen species and diabetic nephropathy. J. Am Soc. Nephrol 14:S209-S210.
15. Hossain MA, Panthi S, Asadujjaman M, Khan SR, Ferdous F, Sadhu SK (2013) Phytochemical and pharmacological assessment of the ethanol leaves extract of Heritiera fomes Buch. Ham. (Family- Sterculiaceae). J Porphyr Phthalocyanines 2:95–101.
16. Hundal RS, Krssak M, Dufour S, Laurent D, Lebon V, Chandramouli V, Inzucchi SE Schumann WC, Petersen KF, Landau BR, Shulman GI (2000) Mechanism by which metformin reduces glucose production in type 2 diabetes. Diabetes 49:2063–2069.

17. Hunter SJ, Garvey WT (1998) Insulin action and insulin resistance: diseases involving defects in insulin receptors, signal transduction, and the glucose transport effector system. Am J Med 105:331–45.
18. Ilyas M, Parveen M, Amin KMY (1998) Neriifolione, A triterpene from Euphorbia neriifolia. Phytochem. 48(3):561-563.
19. Inzucchi SE, Petersen KF, Shulman GI. (2002) Non-alcoholic fatty liver disease. N Engl J Med 347(10):768-9.
20. Ivorra MD, Paya M, Villar A (1989) A review of natural products and plants as potential antidiabetic drugs. J Ethnopharmacol 27:243-275.
21. Patra J K and H. Thatoi (2013) Anticancer activity and chromatography characterization of methanol extract of Heritiera fomes Buch. Ham., a mangrove plant from Bhitarkanika, India, Oriental Pharmacy and Experimental Medicine 13(2):133–142.
22. Jain A, Basal E (2003). Inhibition of Propionibacterium acnes-induced mediators of inflammation by Indian herbs. Phytomedicine 10(1):34-8.
23. Jakus V (2000) The role of free radical, oxidative stress and antioxidants systems in diabetic vascular disease. Bratisel Lek Listy 101(10):541-551.
24. Jennifer R. Pfaffly (2001) Diabetic complications, hyperglycemia and free radicals. Free radicals and radiation biology program 1-18.
25. Jha RK, Garud N, Nema RK (2009) Excision and incision wound healing activity of flower head alcoholic extract of Sphaeranthus indicus Linn.in albino rats. Global J Pharmacol 3:32-7.
26. John W. Bayness and Suzanne R. Thorpe (1999) Perspectives in diabetes, role of oxidative stress in diabetic complications: a new perspective on an old paradigm. Diabetes 48:1-9.
27. Joseph L Evans, Beatty A Maddux, Ira D (2003) Goldfine, Antioxidants in diabetic complications and insulin resistance, In diabetes: From research to diagnosis and treatment (I Raz, J Skyler, E Shafrir) chapter 29; 479-496.
28. Joseph L. Evans, Ira D. Goldfine, Betty A. Maddux and Gerold M. Grodsky (2003) Are oxidative stress activated signaling pathways mediators of insulin resistance and beta cell dysfunction? Diabetes 52:1-8.
29. Joseph L. Evans, Ira D. Goldfine, Betty A. Maddux and Gerold M. Grodsky (2002) Oxidative stress and stress activated signaling pathways: A unifying hypothesis of type 2 diabetes. Endocrine Reviews 23(5):599-622.
30. Kahn CR (1994) Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes. Diabetes 43:1066.
31. Kakkar R, Kalra J, Mantha SV, Prasad K (1995) Lipid peroxidation and activity of antioxidant enzymes in diabetic rats. Mol Cell Biochem 151:113-119,
32. Kalpesh Gaur, Rana AC, Cauhan LS, Sharma CS, Nema RK, Kori ML, Yashwant (2009) Investigation of Immunomodulatory potential of Euphorbia neriifolia Linn Against Betamethasone Induced Immunosuppression. International Journal of Pharmacy and Phyto Research 1(1):8-11.
33. Kaneto H, Fujii J, Myint T, Miyazawa N, Islam KN, Kawasaki Y, Suzuki K, Nakamura M, Tatsumi H, Yamasaki Y, Taniguchi N (1996) Reducing sugars trigger

oxidative modification and apoptosis in pancreatic beta-cells by provoking oxidative stress through the glycation reaction. Biochem J 320(3):855- 63.

34. Kawano J, Arora R (2009) The role of adiponectin in obesity, diabetes, and cardiovascular disease. J Cardiometab Syndr 4(1):44-9.