Evaluation of *Medicago sativa* L. sprouts as antihyperlipidemic and antihyperglycemic agent

Ahmed Seida[1,2], Hala El-Hefnawy[1]*, Dina Abou-Hussein[1,3], Fatma Alzahraa Mokhtar[1] and Ashraf Abdel-Naim[4,5]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Kasr El Aini, Cairo, Egypt
[2]Department of Pharmacognosy, Faculty of Pharmacy, October 6 University, Sixth of October, Egypt
[3]Department of Natural Products and Alternative Medicine, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia
[4]Department of Pharmacogology and Toxicology, Faculty of Pharmacy, Ain Shams University, Abbasia, Cairo, Egypt
[5]Department of Pharmacogology and Toxicology, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia

Abstract: *Medicago sativa* L. (Alfalfa) is traditionally used to treat diabetes. This study was designed to investigate the potential antihyperlipidemic and antihyperglycemic activity of *M. sativa* sprouts in streptozotocin (STZ) induced diabetes *via i.p*. injection of 55mg/kg of STZ. Experimental animals were divided into the following groups: GP1 (normal), GP2 (STZ-hyperlipidemic), GP3 (rouvastatin), GP4 (metformin), GP 5-9 (diabetic treated with methanolic, petroleum ether, chloroform, ethyl acetate and butanol extracts). The administration of the total methanolic extract (500 mg/kg), the petroleum ether (32.5mg) and butanol fractions (60mg) for 4 weeks significantly decreased ($p<0.05$) triglycerides (TG), total cholesterol (TC), low-density lipoproteins (LDL) and very low density lipoproteins (VLDL) in comparison to rouvastatin. Petroleum ether fraction proved to exhibit the best activity as antihyperlipidemic agent (12.23%). On the other hand, ethyl acetate fraction retained the best activity (*vs.* metformin) as antihyperglycemic agent. Histopathological evidences on liver, pancreas and spleen were in agreement with the abovementioned results. Purification, characterization, and identification of isolated compounds from the active fractions afforded 9 compounds: β-sitosterol and stigmasterol from the petroleum ether fraction; 10-hydroxy-coumestrol, apigenin, genistein, *p*-hydroxy-benzoic-acid, 7, 4′- dihydroxyflavone, quercetin-3-glucoside and sissotrin from the ethyl acetate fraction.

Keywords: *Medicago sativa* L. sprouts, streptozotocin, antihyperglycemic, antihyperlipidemic, sterols, flavonoids.

INTRODUCTION

Diabetes mellitus, recognized by chronic hyperglycemia, is a metabolic disorder with distinguished symptoms ranging from polythirst and polyuria in minor cases to ketoacidosis or a non-ketotic hyperosmolar state in severe forms. In absence of effective treatment, diabetes may lead to stupor, coma and death. Complications may develop in long term *viz:* retinopathy, neuropathy, foot ulcers, sexual dysfunction (Alberti and Zimmet, 1998; Marcovecchio *et al.,* 2005) and sometimes, especially in patients with uncontrolled diet and those who have elevated lipid profile, there is a risk of vascular and cardiac diseases (O'Keefe and Bell, 2007). It is considered as one of the five leading causes of death in the world (Wild *et al*., 2004). Patients are more encouraged to use natural antidiabetic products to overcome the side effects that may appear by the use of insulin and oral hypoglycemic agents (Robertson and Home, 1993; Micheal and Fowler, 2007).

Herbal medicine development is one of the important subjects of study in the 21stcentury to treat diabetes and hyperlipidemia (Kameswara *et al*., 1997). *Medicago sativa* L. (Alfalfa) grows wild as escape from cultivation in Nile Delta, Oases, Sinai and Mediterranean regions (Boulos, 1999). It is a traditional herb used to treat diabetes in folk medicine (Lust, 1986). Many studies on STZ-diabetes mice have confirmed the antihyperglycemic and antihyperlipidemic efficacy of the aerial parts of the plant (Gray and Flatt, 1997; Khaleel *et al.*, 2005; Baxi *et al*., 2010). The current study was designed to assess the potential of the methanolic extract of the sprouts and its fractions as antihyperglycemic and antihyperlipidemic, especially that they are common food for humans and major source of phytoestrogens known by their effects for the treatment and prevention of cancer, vascular and cardiac diseases (Knuckles *et al*., 1976; Franke *et al*., 1995).

MATERIALS AND METHODS

Plant material

Dry seeds were collected from fully mature plants in late June 2010, from Keram farms, Moderayat Al-Tahrer, Beheira, Egypt. The seeds (0.7-1cm) were sprouted as follows; 4.5kg of dry seeds were soaked in water for 18h, drained off and rinsed thoroughly every 12h for 5 days till the shoots reached a length of 7-12cm (Fratamico and Bagi, 2001). These are considered as typical sprouts and

**Corresponding author:* e-mail: hala.elhefnawy@pharma.cu.edu.eg

were further shade dried to yield 4.3 kg of material. A herbarium (# 0130416) is deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Extracts of M. sativa sprouts

The dry sprouts (4.3kg) were extracted with 70% methanol by percolation (10x12 L); the solvent was evaporated under vacuum using rotary evaporator at 45ºC. A weight of 500g of the dry methanolic extract was suspended in least amount of water and partitioned successively with petroleum ether, chloroform, ethyl acetate and *n*-butanol. Solvents were evaporated under reduced pressure and the fractions were put in a desiccator till constant weights.

Material and methods for biological study

A. Experimental animals

Mature male albino rats, Wistar strain, weighing about 90 ±10g were kept in constant environment (Animal house of NODCAR, Dokki, Giza), exposed to unlimited standard food (for normal groups) and water *ad libitum*. Animal protocol was approved by the ethical committee of Faculty of Pharmacy, Ain Shams University.

B. Drugs, reagents and kits

Streptozotocin (Sigma Chemical Co., St. Louis, MO, USA), Metformin (Cidophage®, Cid) as reference antihyperglycemic, Rouvastatin (Crestor®, Astra Zeneca) as reference antihyperlipidemic, Citrate buffer pH 4.5, 0.1 M (El-Nasr Chemical Company, Cairo, Egypt), Spinreact® reagent (Carretera Santa Cloma, Spain) kits for assessment of lipids and serum glucose level.

C. Induction of hyperlipidemia

All the experimental animals except the normal groups were fed high cholesterol diet (Sheyla *et al*., 2005) for 8 weeks to induce hyperlipidemia in addition to low dose STZ, the rats considered hypercholesterolemic after 3 days following STZ injection only when total cholesterol >120mg/dl and triglycerides >140 mg/dl (Srinivasan *et al.*, 2005).

D. Induction of hyperglycemia

After induction of hyperlipidemia for 8 weeks all the rats except those of normal group were fasted for 24h before the induction of hyperglycemia using streptozotocin (STZ) injection. This was performed by single *i.p.* injection of STZ (freshly prepared before injection in citrate buffer) at a dose of 55mg/kg bwt. The animals were considered diabetic only if the FBG ≥200mg/dl after 3 days (Wu and Huan, 2008), this was considered the zero time of the experiment.

E. Experimental design

Nine groups of rats (8 for each) were randomely divided as follows: GP1 (Control Group): normoglycemic, normolipidemic; GP2 (Diabetic Group): hyperglycemic and hypercholesterolemic; GP3 (Rouvastatin Group): diabetic orally administered daily with the standard hypolipidemic drug 10mg/kg rouvastatin; GP4 (Metformin Group): treated with standard antidiabetic drug metformin in a dose of 500mg/kg per day; GP 5-9 were treated daily with plant extracts: total methanolic extract (500mg/kg), petroleum ether fraction (32.5 mg/kg), chloroform fraction (10.5mg/kg), ethyl acetate fraction (11.0mg/kg) and butanol fraction (60mg/kg), respectively.

Tested samples and drugs were administered orally in the cited doses for 28 days and blood samples were collected in the fasting state (8, 12 h) from the retro orbital vein to measure the fasting blood glucose level (FBG) and lipid profile (TC, TG, LDL, HDL and VLDL), respectively. Blood samples were collected in test tubes, incubated at 37ºC for 10 minutes, centrifuged at 4000 rpm for 15 minutes, the serum was obtained and preserved frozen at -80ºC till subsequent analysis. Blood samples were taken at zero, 7, 14, 21, and 28 days for FBG and at zero, 14 and 28 days for lipid profile (Wu and Huan, 2008).

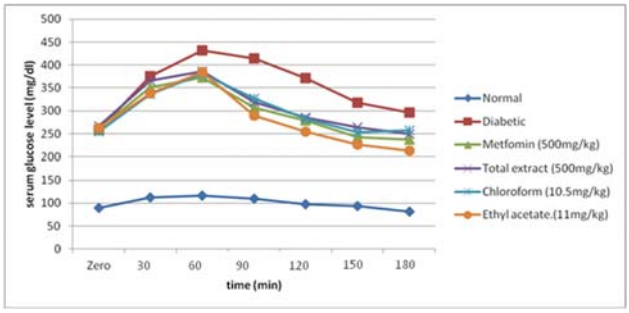

Fig. 1: The effect of total methanolic extract of *M. sativa* sprouts, chloroform and ethyl acetate fractions on serum glucose level over 180 min (OGTT). Values are expressed as mean ± S.E.M.

F. Measurement of lipid profile

1ml spinreact® reagent for each triglycerides (TG), total cholesterol (TC) and high density lipoproteins (HDL) was added to 10µl serum sample and measured by STAT-FAX 3300 spectrophotometer (Florida, USA) at wavelength 546nm. Very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol levels were calculated from the above measurements using Friedwald formula (Friedwald *et al.*, 1972).

VLDL = TG/5 LDL = TC- (HDL+ TG/5)

G. Measurement of fasting blood glucose (FBG)

1ml spinreact® reagent was added to 10 µl serum and measured spectrophotometrically at wavelength 540 nm.

H. oral glucose tolerance test (OGTT)

After the last FBG sampling (day 28), the identification of most promising fractions that lower glucose (total methanolic extract, chloroform and ethyl acetate

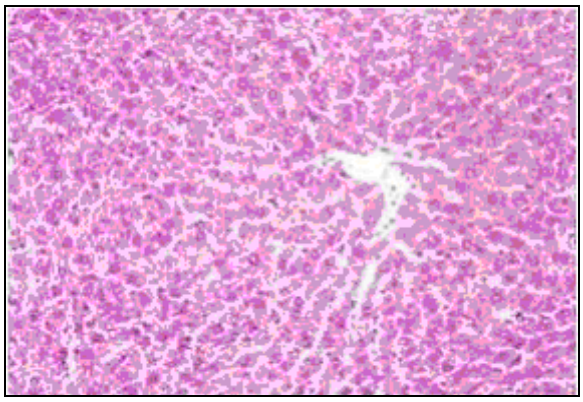

Fig. (2a): Liver of control (Normal) rats showing the normal histological structure of hepatic lobule.

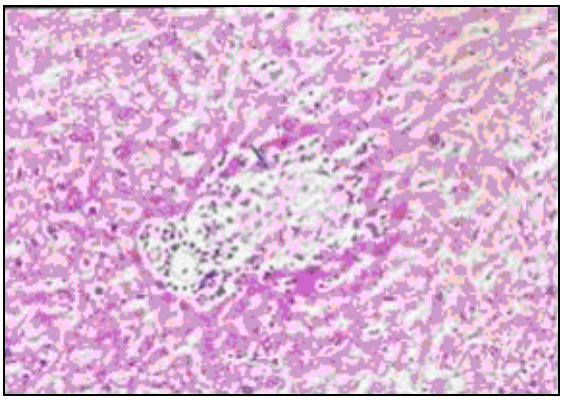

Fig. (2ab): Liver of diabetic rats showing vascular degeneration of hepatocytes and focal area of necrosis.

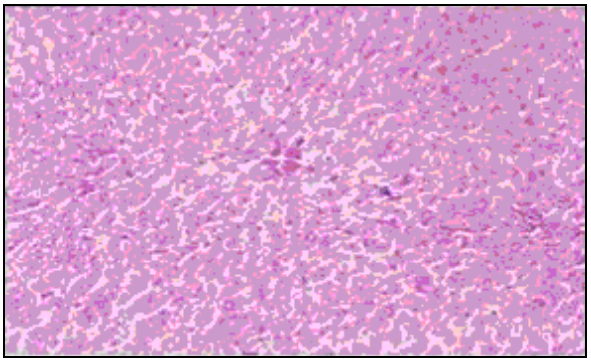

Fig. (2c): Liver of rats from total extract group showing kupffer cells activation.

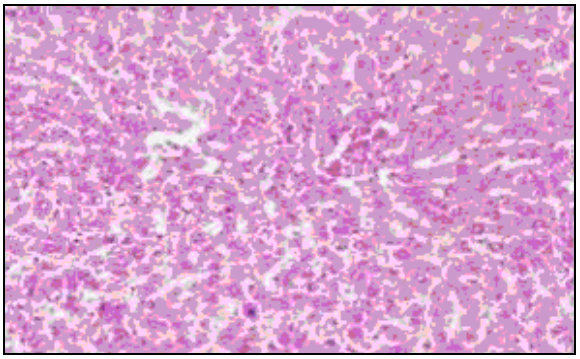

Fig. (2d): Liver of rats from petroleum ether group showing no histopathological changes.

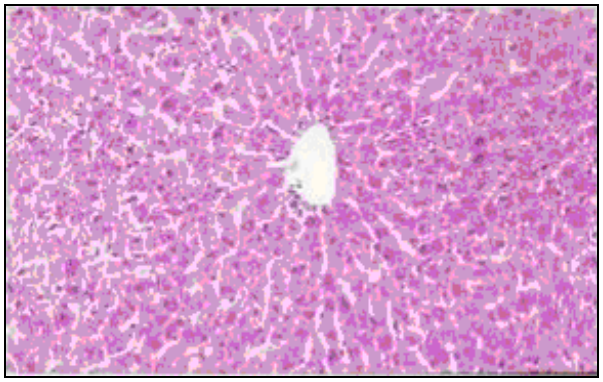

Fig. (2e): Liver of rats from chloroform group showing no histopathological changes.

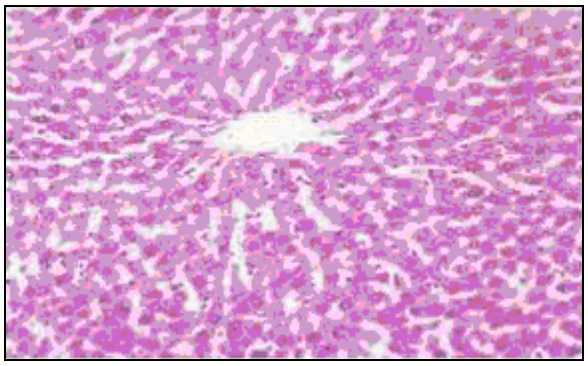

Fig. (2f): Liver of rats from ethyl acetate group showing no histopathological changes.

All tissues were stained with H&E stain (X=200)

Fig. 2: Effect of *M.* sativa sprouts total methanolic extract and fractions on liver tissue of rats

fractions) was performed. For these three groups at day 30 an (OGTT) is carried out by administrating a glucose oral load (10g/kg bwt) as 40% solution (w/v) in distilled H_2O for 15 h fasting rats, each group was dosed by its fraction 30 minutes before the oral glucose loading. Measurements were performed using Accu-check G glucometer (Mannheim, Germany) at (loading time) zero, 30, 60, 90, 120, 150 and 180 min after glucose loading (Srinivasan *et al.*, 2005).

I. Histopathological investigation

After OGTT all rats were sacrified by cervical decapitation. Organs (liver, spleen and pancreas) were removed and placed for 24h in Alfac fixing solution (85% ethanol, 10% formaldehyde and 5% glacial acetic acid). Histological cuts (5µm thickness) were prepared from the fixed organs, stained with hematoxylin and eosin (H and E) then photographed with the aid of electronic microscope (Slauson and Cooper, 2002).

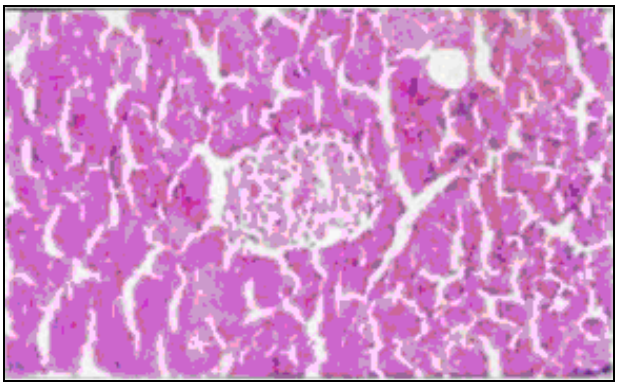

Fig. (3a): Pancreas of normal group showing the normal histological structure of pancreatic cells.

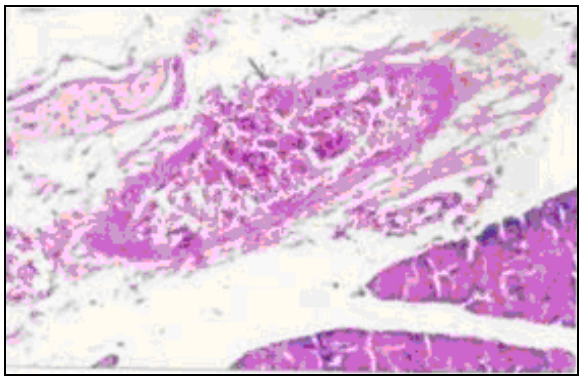

Fig. (3b): Pancreas of diabetic rats showing severe congestion of pancreatic blood vessels.

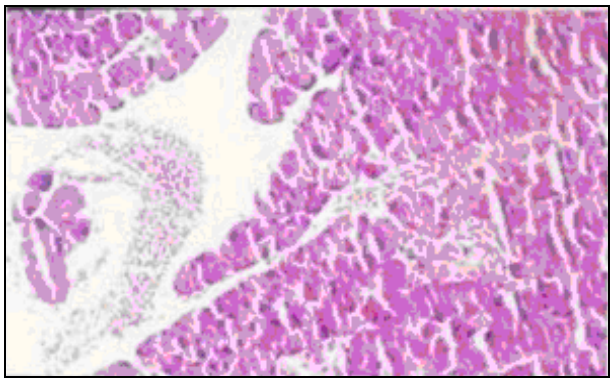

Fig. (3c): pancreas of rats from total extract group showing pancreatic hemorrhage.

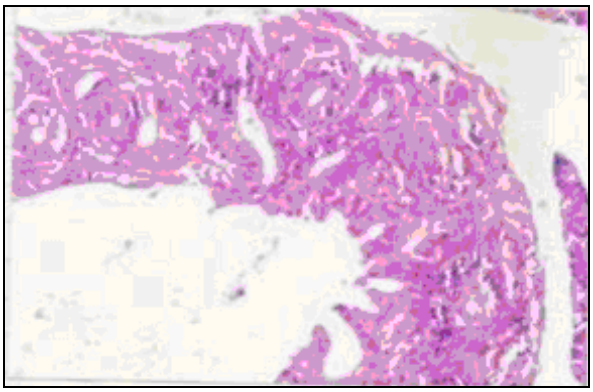

Fig. (3d): pancreas of rats from petroleum ether group showing hyperplasia of epithelial lining of pancreatic duct

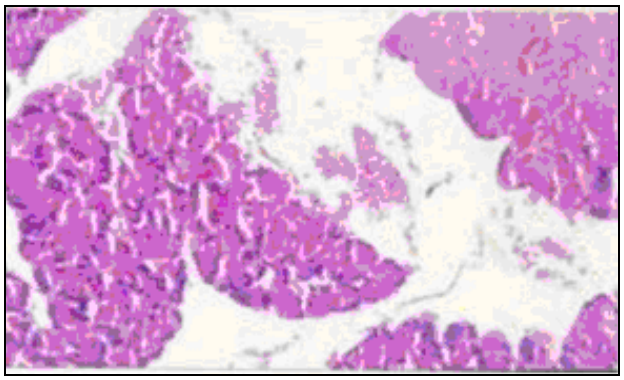

Fig. (3e): Pancreas of rats from chloroform group showing sever pancreatic hemorrhage

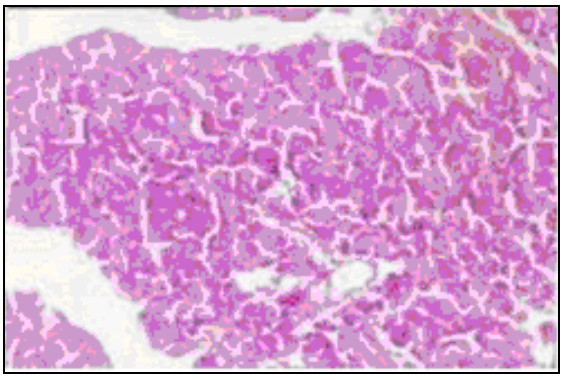

Fig. (3f): Pancreas of rats from ethyl acetate group showing no histopathological changes

All tissues were stained with H&E stain (X=200)

Fig. 3: Effect of *M.* sativa sprouts total methanolic extract and fractions on pancreatic tissue of rats

J. Statistical analysis

SPSS® statistical package version 17 was used to analyze data. One way ANOVA followed by Tukey HSD test at $p<0.05$ were applied to check the significant difference between groups (Winer, 1971).

Material and methods for phytochemical study

A. General

Melting points of the isolated compounds were inaccurate and measured on a digital apparatus (Electothermal IA 9000 series). Mass spectrometer (Finningan, USA), SSQ7000 was used for EI-MS. ^{1}H (500 MHz) and ^{13}C

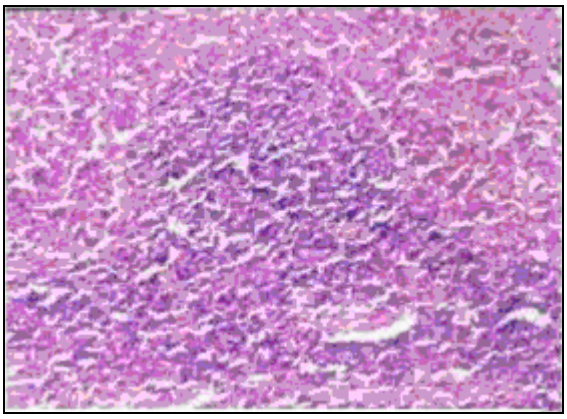

Fig. (4a): Spleen of Normal group showing normal histological spleen cells

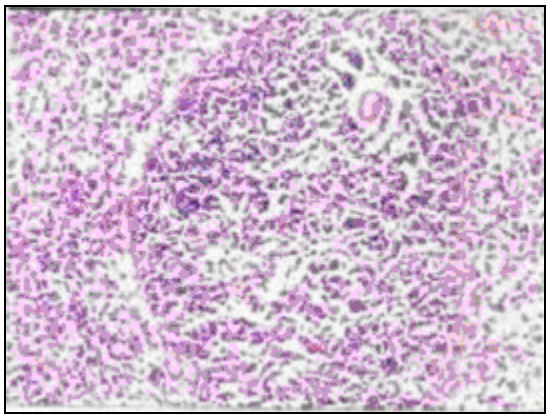

Fig. (4b): Spleen of Diabetic rats showing lymphocytic necrosis and depletion

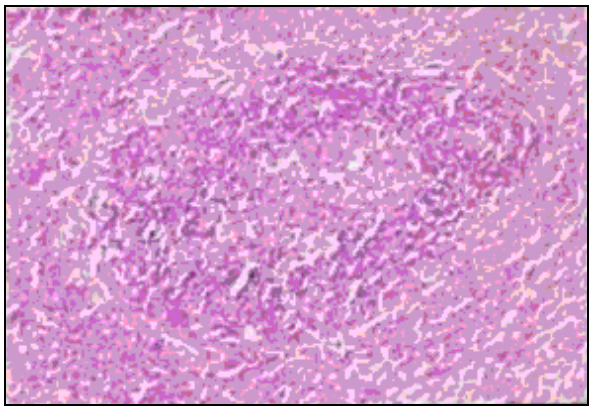

Fig. (4c): Spleen of total extract group showing no histopathological changes

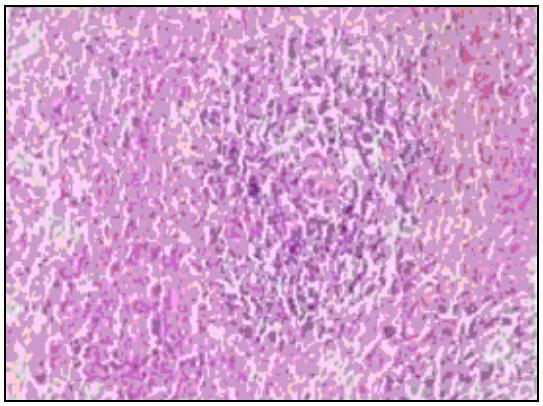

Fig. (4d): Spleen of ethyl acetate group showing no histopathological changes

All tissues were stained with H&E stain (X=200)

Fig. 4: Effect of *M.* sativa sprouts total methanolic extract and fractions on spleen tissue of rats

(150 MHz) NMR spectra were traced on Joel Ex-270 (Japan) apparatus at 25ºC in DMSO-*d6*, with TMS internal standard and chemical shifts in δ values. Beckman Du-7 and Shimadzu-265 spectrophotometer was used for recording the UV absorption spectra. Silica gel 60 (70-230 mesh, Merck), Sephadex LH-20 (Sigma) and Polyamide S6 (Merck) were used for column chromatography. Thin layer chromatography were carried out on pre-coated silica gel 60 F_{254} plates (0.25mm thickness, Merck) and visualization was accomplished by UV (254/365nm) for localization of spots and by *p*-anisaldehyde reagent.

B. Reagents

Shift reagents for UV spectral analysis of flavonoids were prepared from analytical grade chemicals (Mabry *et al*., 1996) and *p*-anisaldehyde spray reagent was used for TLC analysis (Stahl, 1969).

C. Reference material

In GLC determination of unsaponifiable and saponifiable matters, reference materials of hydrocarbons and sterols (Merck) and those of fatty acid methyl esters (Nu-Check Prop) were supplied by The Central Laboratory, Faculty of Agriculture, Cairo University. Authentic sterols for TLC, were previously isolated, identified and available in the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

D. Preparation and gas-liquid chromatography analysis of unsaponifiable and saponifiable matters

M. sativa sprouts (500g) were extracted with petroleum ether till exhaustion. The solvent was evaporated under reduced pressure at a temperature, not exceeding 40°C. 2g of the residue was treated with $CHCl_3$/MeOH mixture (2:1 v/v) to extract the lipids. To remove non-lipids, each extract was washed thrice with MeOH/H_2O mixture (1:1 v/v) and the chloroform layers were filtered over anhydrous sodium sulfate then distilled under vacuum at 40°C to get rid of the solvent (AOAC, 1990). The lipid sample under investigation was saponified overnight with ethanolic KOH (20%) at room temperature and the liberated fatty acids methylated with diazomethane (Vogel *et al*., 1996). Each of the unsaponifiable matter and fatty acid methyl esters was subjected to GLC analysis.

Table 1: Effect of total methanolic extract and fractions of *M. sativa* sprouts on serum lipids (TG, TC, LDL-C, HDL-C and VLDL-C) in STZ-diabetic hyperlipidemic rats (n=8)

Parameter	Days	Normal	Diabetic hyperlipidemic	Rouvastatin (10mg/kg)	Total methanolic extract (500mg/kg)	Pet Ether (32.5mg/kg)	Chloroform (10.5mg/kg)	Ethyl acetate (11mg/kg)	Butanol (60mg/kg)
TG (mg/dl)	0	93.50^{b}±2.72	233.43^{a}±6.28	227.56^{a}±8.34	229.1^{a}±10.27	231.36^{a}±8.15	230.01^{a}±7.54	222.16^{a}±11.41	228.17^{a}±6.4
	14	95.00^{b}±3.02	237.78^{a}±9.41	201.68^{a}±11.02	197.52^{a}±7.14	174.02^{ab}±9.55	225.15^{a}±10.75	207.28^{a}±7.57	202.03^{a}±8.04
	28	99.25^{b}±5.01	240.70^{a}±7.16	169.06^{b}±9.28	146.01^{b}±5.67	151.63^{b}±8.69	213.67^{a}±8.65	185.92^{a}±6.27	163.25^{a}±5.31
TC (mg/dl)	0	72.63^{b}±2.37	142.28^{a}±6.92	143.44^{a}±5.42	141.42^{a}±6.53	135.83^{a}±7.34	144.5^{a}±8.93	139.66^{a}±8.55	140.83^{a}±11.37
	14	74.80^{b}±3.92	157.6^{a}±7.1	107.0^{b}±6.4	116.8^{a}±16.1	111.5^{b}±5.06	140.91^{a}±10.24	137.9^{a}±6.77	125.87^{b}±4.09
	28	73.05^{b}±4.06	153.4^{a}±11.47	95.30^{b}±7.96	101.80^{b}±5.82	97.80^{b}±4.09	121.67^{a}±8.93	120.53^{a}±10.07	102.80^{b}±7.72
LDL - C (mg/dl)	0	12.63^{b}±0.73	56.38^{a}±4.34	59.11^{a}±2.76	55.58^{a}±3.46	50.21^{a}±4.84	61.33^{a}±5.39	56.51^{a}±4.63	56.47^{a}±9.53
	14	15.55^{b}±2.2	73.21^{a}±3.81	27.99^{b}±3.49	34.19^{a}±13.19	34.39^{a}±3.09	57.7^{a}±6.45	57.18^{a}±3.91	48.14^{a}±1.86
	28	11.45^{b}±2.21	66.36^{a}±9.24	22.41^{b}±5.9	28.58^{a}±3.61	23.35^{b}±1.44	41.31^{a}±6.58	43.52^{a}±6.98	31.11^{b}±4.23
HDL - C (mg/dl)	0	41.25±1.1	39.13±1.32	38.78±0.99	40±1.02	39.31±0.87	37.17±2.03	38.16±1.64	38.5±0.56
	14	40.25±1.1	36.83±1.49	38.67±0.71	43.1±1.02	42.30±1.06	38.18±1.64	39.26±1.35	37.25±0.62
	28	41.75±0.85	38.9±1.88	39.07±1.2	43.94±0.87	44.12±0.91	37.63±1.62	39.83±1.79	39.07±2.41
VLDL - C (mg/dl)	0	18.7^{b}±0.54	46.68^{a}±1.26	45.51^{a}±1.6	45.82^{a}±2.05	46.27^{a}±1.63	46.02^{a}±1.51	44.43^{a}±2.28	45.63^{a}±1.28
	14	19^{b}±0.62	47.56^{a}±1.88	40.34^{a}±2.2	39.5^{a}±1.43	34.8^{a}±1.91	45.05^{a}±2.15	41.46^{a}±1.51	40.41^{a}±1.61
	28	19.85^{b}±1.0	48.14^{a}±1.43	33.81^{a}±1.86	29.2^{b}±1.34	30.33^{b}±1.74	42.73^{a}±1.73	37.18^{a}±1.25	32.65^{a}±1.06

Values are expressed as mean ± S.E.M. ^{a} significantly different from normal group at p<0.05, ^{b} significantly different from the diabetic group at p<0.05, ^{ab} significantly different from both groups.

Table 2: Effect of total methanolic extract and fractions of *M. sativa*. sprouts on FBG in STZ-diabetic rats at 7 days intervals (n = 8).

Treatment group	Zero day	7 day	14 day	21 day	28 day
	Serum FBG (mg/dl)				
Normal	84.75^{b}±2.49	85.24^{b}±3.88	85.3^{b}±5.53	87.11^{b}±4.81	82.56^{b}±3.04
Diabetic	267.16^{a}±18.33	317.66 ^{a}±26.12	308.62 ^{a}±23.78	312.50 ^{a}±22.6	314.10 ^{a}±37.63
Metformin (500mg/kg)	269.40 ^{a}±24.42	203.43 ^{a}±16.51	187.94 ^{a}±19.81	152.96 ^{ab}±17.73	129.08^{b}±21.05
Total methanolic extract (500mg/kg)	271.14 ^{a}±16.1	266.19 ^{a}±26.44	246.41 ^{a}±20.74	220.72 ^{a} ±17.99	184.84^{ab}±24.93
Pet. Ether (32.5mg/kg)	265.57 ^{a}±27.15	261.36 ^{a}±17.98	255.22 ^{a}±18.95	249.44 ^{a}±23.45	232.63 ^{a}±28.54
Chloroform (10.5mg/kg)	265.00 ^{a}±19.8	244.71 ^{a}±21.00	236.47 ^{a}±23.16	207.32 ^{a}±16.03	174.16^{ab}±20.08
Ethyl acetate (11mg/kg)	270.65 ^{a}±22.7	212.33 ^{a}±9.72	200.52 ^{a}±13.78	143.53^{ab}±31.82	117.27^{b}±16.77
Butanol (60mg/kg)	237.51 ^{a}±30.11	246.29 ^{a}±30.01	251.65 ^{a}±25.12	242.25 ^{a}±29.88	227.52^{a}±24.59

Values are expressed as mean ±S.E.M at p <0.05. ^{a} significantly different from normal group at p<0.05, ^{b} significantly different from diabetic group at p<0.05.

Table 3: Effect of total methanolic extract and fractions of *M. sativa*. sprouts on serum glucose levels in STZ-diabetic rats after a bolus injection of 10 g/kg glucose during oral glucose tolerance test (n = 8)

Time (minutes)	Zero	30	60	90	120	150	180
Serum glucose level mg/dl							
Normal	89.5[b]±10.6	113.0[b]±10.1	116.2[b]±17.0	109.2[b]±11.0	97.2[b]±7.9	93.5[b]±6.4	81.5[b]±7.6
Diabetic	259.0[a]±31.7	375.0[a]±29.8	431.1[a]±16.6	414.7[a]±20.6	371.9[a]±24.1	317.7[a]±18.7	296.0[a]±26.2
Metformin (500mg/kg)	261.0[a]±19.4	351.2[a]±18.0	372.8[a]±22.7	306.8[a]±17.2	278.6[a]±21.8	242.4[a]±20.1	237.2[a]±17.8
Total methanolic extract (500mg/kg)	267.2[a]±24.4	366.0[a]±26.1	386.2[a]±10.9	319.0[a]±25.3	285.0[a]±18.6	264.0[a]±16.3	249.4[a]±27.5
Chloroform (10.5mg/kg)	254.0[a]±25.6	337.0[a]±16.7	380.5[a]±18.4	327.7[a]±22.8	283.5[a]±21.3	252.7[a]±27.8	257.5[a]±20.7
Ethyl acetate (11mg/kg)	262.3[a]±17.9	338.2[a]±21.2	384.5[a]±23.6	289.6[a]±14.9	255.3[a]±12.2	226.0[a]±14.82	212.7[b]±17.4

Values are expressed as mean ± S.E.M. [a] significantly different from normal group at $p<0.05$., [b] significantly different from diabetic group at $p<0.05$.

Table 4: Histopathological investigation of liver, pancreas and spleen

Microscopic study in comparison							
Microscopic features	LIVER GROUPS						
	Control group	Diabetic group	Total methanolic extract group	Pet. Ether group	Chloroform group	Ethyl acetate group	Butanol group
Lobular architecture	Maintained	Maintained	Maintained	Maintained	Maintained	Maintained	Maintained
Vacular degeneration	-	+++	-	-	-	-	+
Necrosis	-	+++	-	-	-	-	-
Kupffer cell activation	-	-	+	-	-	-	-
Microscopic features	PANCREAS GROUPS						
	Control group	Diabetic group	Total methanolic extract group	Pet. Ether group	Chloroform group	Ethyl acetate group	Butanol group
Congestion of blood vessels	-	+++	-	-	+	_	_
Pancreatic hemorrhage	-	+++	+	-	+++	-	_
Necrosis	-	+++	-	++	-	+	-
Epithelial hyperplasia	-	+	-	+	-	-	+++
Microscopic features	SPLEEN GROUPS						
	Control group	Diabetic group	Total methanolic extract group	Pet. Ether group	Chloroform group	Ethyl acetate group	Butanol group
Lymphocytic necrosis	-	+	-	-	+	_	_
Depletion	-	+	-	-	-	-	_

Symbol +++,++,+ and – indicates the presence of severe degree, moderate degree, mild degree and no changes respectively, with respect to microscopic features of liver, pancreas and spleen of control, diabetic, total methanolic extract, petroleum ether, chloroform, ethyl acetate and butanol groups

Table 5: Results of GLC analysis of the fatty acids methyl esters of saponifiable fraction of petroleum ether fraction of the sprouts of *M. sativa*.

Peak No.	Identified compounds	RRT*	Relative%
1.	Decanoic (Capric) acid C10:0	0.486	1.340
2.	Dodecanoic (Lauric) acid C12:0	0.550	1.420
3.	Tetradecanoic (Myristic) acid C14:0	0.724	0.670
4.	Hexadecanoic (Palmitic) acid C16:0	0.941	12.751
5.	Hexadecanoic (Palmitoleic) acid C16:1	1.000	27.580
6.	Octadecanoic (Stearic) acid C18:0	1.390	3.792
7.	Octadecenoic (Oleic) acid C18:1	1.084	8.640
8.	Octadecadienoic (Linoleic) acid C18:2	1.660	26.850
9.	Octadecadienoic (Linoelaidic) acid C18:2	1.890	12.071
10.	Octadecatrienoic (Lionleic) acid 18:3	2.310	0.53
11.	Eicosanoic (Arachidic) acid 20:0	2.600	1.591
12.	Eicosatetrienoic (Archidonic) acid 20:4	3.250	0.650
13.	Eicosapentaenoic acid (EPA)	3.720	1.000
14.	Docosahexaenoic acid (DHA)	4.545	1.1200
Percentage of identified saturated fatty acids		21.571	
Percentage of identified unsaturated fatty acids		78.429	

RRT*: Relative Retention time to Palmitoleic acid with R_t=5.97mim

Table 6: Results of GLC analysis of the unsaponifiable fraction of the petroleum ether fraction of the sprouts of *M. sativa*.

Peak No.	Identified compounds	RRT**	Relative%
1.	Tetradecane	0.492	1.690
2.	Pentadecane	0.506	0.726
3.	Hexadecane	0.530	1.010
4.	Heptadecane	0.549	0.322
5.	Octadecane	0.555	1.613
6.	Nonadecane	0.601	0.848
7.	Eicosane	0.607	10.010
8.	Heneicosane	0.637	0.906
9.	Docosane	0.679	5.277
10.	Tricosane	0.736	1.487
11.	Tetracosane	0.753	13.254
12.	Hexacosane	0.804	8.290
13.	Heptacosane	0.878	1.539
14.	Octacosane	0.935	3.864
15.	Nonacosane	0.949	0.941
16.	Stigmasterol	1.000	18.526
17.	β-Sitosterol	1.009	9.851
18.	Triacontane	1.017	5.203
19.	Dotriacontane	1.023	14.54
Percentage of total hydrocarbons		71.62	
Percentage of total sterols		28.38	

RRT **: Relative retention time to stigmasterol with R_t=44.2min

GLC analysis of unsaponifiable matter was performed on Pye UNICAM Gas Liquid Chromatography, series 304 set with Flame Ionization Detector (FID). For separation, a coiled glass column (UNICAM Pro GC, 2.8mx4mm) was used with diatomite packing (100-120mesh) and 3% OV-17 coating. The injector port temperature was set at 280^{o}C (splitless mode) and the detector cell at 300^{o}C. The flow rate of the carrier, N_2, was 30mL/min, that of hydrogen was 33mL/min, while air flow rates was 330 mL/min. The column temperature was 70^{o}C and increased to 270^{o}C by the rate 10^{o}C/min then set isothermally for 25 min, with a total run time of 50min.

Table 7: ^{1}H (500 MHz), ^{13}C (150 MHz) Spectral data for compounds 4,5,7-9 at 25ºC, recorded in DMSO-d_6

	Compound 4		Compound 5		Compound 7		Compound 8		Compound 9	
Position	δ of ^{1}H (*J*, Hz)	δ of ^{13}C (*J*, Hz)	δ of ^{1}H (*J*, Hz)	δ of ^{13}C (*J*, Hz)	δ of ^{1}H (*J*, Hz)	δ of ^{13}C (*J*, Hz)	δ of ^{1}H (*J*, Hz)	δ of ^{13}C (*J*, Hz)	δ of ^{1}H (*J*, Hz)	δ of ^{13}C (*J*, Hz)
2	-	161.29	8.33(s)	154.27	-	163.56	-	158.21	8.11 (s)	154.59
3	6.74 (s)	105.58	-	122.94	6.64(s)	104.69	-	134.41	-	123.42
4	-	180.46	-	180.10	-	177.51	-	178.25	-	180.56
5	-	164.58	-	162.02	7.81 (d,8.7)	127.01	-	161.60	-	162.46
6	6.19 (d,2.0)	104.77	6.21(d,1.9)	99.05	6.89(dd,2.1,8.7)	115.74	6.16(d,2.3)	99.73	6.26 (d,2.2)	97.38
7	-	160.16	-	164.37	-	163.41	-	165.98	-	164.97
8	6.44 (d,2.0)	99.27	6.38(d,1.9)	93.37	6.92 (d,2.2)	103.00	6.36(d,2.3)	93.91	6.1(d,2.2)	94.25
9	-	160.69	-	159.19	-	158.02	-	158.01	-	158.08
10	-	109.41	-	104.45	-	116.13	-	104.29	-	104.90
1`	-	123.16	-	121.95	-	122.15	-	122.08	-	122.44
2`	7.88 (d,8.8)	129.53	7.46(d,8.4)	130.18	7.85 (d,8.7)	127.01	7.54(d,6.1)	114.33	7.46 (d,8.4)	130.64
3`	6.89 (d,8.8)	117.12	6.96(d,8.4)	113.69	6.91 (d,8.7)	128.73	-	144.97	6.98 (d,8.4)	114.19
4`	-	162.61	-	157.61	-	161.18	-	149.36	-	159.63
5`	6.88 (d,8.8)	117.12	6.96(d,8.4)	113.69	6.91(d,8.7)	115.74	6.81 (d,9.1)	116.41	6.98 (d,8.4)	114.19
6`	7.88 (d,8.8)	129.5	7.46(d,8.4)	130.18	7.85(d,8.7)	128.72	7.54 (d,6.1)	121.89	7.46 (d,8.4)	130.64
1``							5.42 (d,6.8)	103.26	5.08 (d,7.1)	99.57
2``							3.05-3.52 (m)	74.48	3.6-3.83(m)	75.35
3``							3.05-3.52 (m)	76.97	3.6-3.83(m)	77.19
4``							3.05-3.52 (m)	69.51	3.6-3.83(m)	70.79
5``							3.05-3.52 (m)	77.20	3.6-3.83(m)	77.24
6``							3.05-3.52 (m)	61.74	3.6-3.83(m)	71.07
4`-OCH_3									3.31	55.65

Table 8: UV absorption shifts of compounds 4,5,7-9

	UV: λ_{max} (nm)					
Compound	MeOH	NaOMe	NaOAc	NaOAc/Boric acid	$ALCL_3$	$ALCL_3$/HCL
4	267, 336	280, 318, 385	276, 299, 370	268, 305 (*sh*), 338	277, 302, 345,380	275, 300, 340, 380
5	261, 329 (*sh*)	276, 327 (*sh*)	272, 326	263, 336 (*sh*)	273,307(*sh*), 373	273, 310 (*sh*), 373
7	253, 313 (*sh*), 328	251, 262 (*sh*), 329, 386	262, 309, 320 (*sh*), 369	256(*sh*), 314 (*sh*), 329	231(*sh*), 255(*sh*), 312, 327, 382	246 (*sh*), 254 (*sh*), 309, 328, 396
8	257, 295 (*sh*), 358	277, 325, 370, 405	272, 323 (*sh*),407	262, 332 (*sh*), 380	275,340, 430	268, 300, 360, 397
9	261, 330	275, 328	271, 327	262, 332	272,310, 370	273, 318, 340, 370

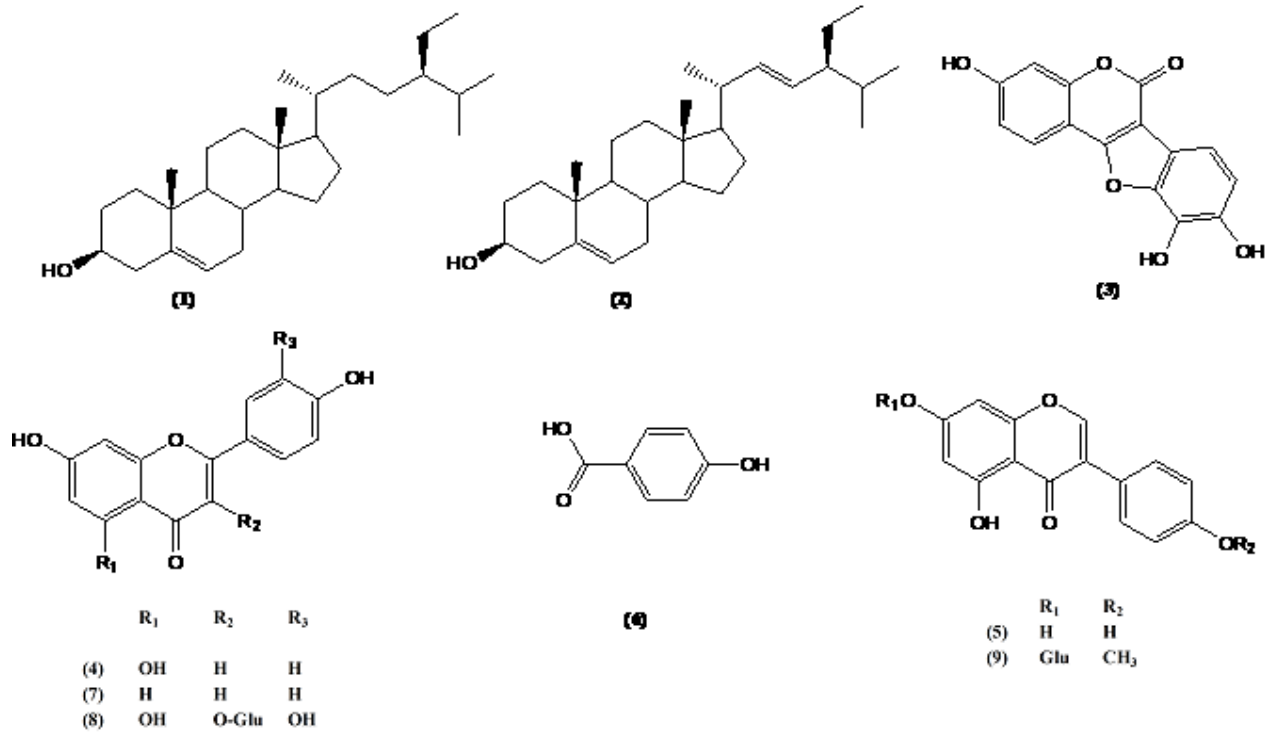

Fig. 5: Structures of compounds 1-9 isolated from *M.sativa* sprouts

GLC analysis of fatty acid methyl esters was carried out as for the unsaponifiable matter except that a coiled glass column (UNICAM Pro GC, 1.5 m x 4mm) with diatomite packing (100-120 mesh) and 10% polyethylene glycol adipate (PEGA) coating was used. The column temperature program was set from 70°C to 190°C by the rate 8°C/min then maintained isothermal for 25min, with a total run time of 50 min.

Identification of the hydrocarbons, sterols and fatty acid methyl esters (FAME) was carried out by comparing the retention time of the peaks with those of the pure corresponding authentics. The quantitative estimation of each peak was done by peak area measurement using a computing integrator.

Isolation of the main constituents from the bioactive fractions

A. Column chromatographic fractionation of petroleum ether fraction

The petroleum ether fraction (30g) was chromatographed on silica gel (800g) column (140cm x 8cm). Fractions (250ml, each) were collected by gradient elution of *n*-hexane/ethyl acetate mixtures, then similar fractions were pooled by TLC monitoring to afford eleven fractions. Fraction 4 (940mg) eluted with 25% ethyl acetate/*n*-hexane and fraction 5 (420mg) eluted with 30% ethyl acetate/*n*-hexane, were separately purified on silica gel column eluted by *n*-hexane/ethyl acetate mixtures in increasing polarities and gave compound 1 (73mg) and compound 2 (280mg), respectively.

B. Column chromatographic fractionation of ethyl acetate fraction

The ethyl acetate fraction (9g) was loaded on the top of polyamide (250g) column (120cm x5cm); gradient elution was carried out with 0-100% MeOH/H_2O mixtures, and thirteen fractions were obtained by TLC monitoring. Fraction 5 (190mg) eluted with 40% MeOH/H_2O and fraction 8 (275mg) eluted with 70% MeOH/H_2O, were separately repurified on sephadex LH-20 column using 80% MeOH/H_2O for elution; fraction 5 gave compound 3 (7mg), while fraction 8 afforded compound 4 (8mg) and compound 5 (11mg). Similarly, fraction 9 (220mg) eluted with 75% MeOH/H_2O from the polyamide column, fraction 11 (195mg) eluted with 90% MeOH/H_2O and fraction 12 (380mg) eluted with 100% MeOH, when subjected separately to sephadex column eluted with 50% MeOH/H_2O afforded the isolation of four compounds: compound 6 (6mg) from fraction 9, compound 7 (12mg) from fraction 11, compound 8 (10mg) and compound 9 (21mg) from fraction 12.

RESULTS

Yield of extractives

The percentage yield of methanol extractive was 21%. Successive fractionation of 500g methanolic extract yielded petroleum ether fraction (32.5g, 6.5%), chloroform fraction (10.5g, 2.2%), ethyl acetate fraction (11g, 2.3%), *n*-butanol fraction (60g, 12%) and remaining aqueous fraction (385g, 77%).

Effect on serum lipid profile of STZ induced diabetic rats

The lipid profile was evaluated by estimating triglycerides (TG), total cholesterol (TC), LDL-cholesterol (LDL), HDL-cholesterol (HDL), VLDL-cholesterol (VLDL). The STZ diabetic hyperlipidemic animals showed a significant increase in the above mentioned parameters levels and small increase in HDL levels compared to control group. After treatment with methanolic extract, petroleum ether, chloroform, ethyl acetate and butanol fractions of sprouts of *M. sativa*, a significant treatment-duration related decrease in the TG, TC, LDL and VLDL levels were observed compared to the untreated diabetic rats in a level similar to those of reference hypolipidemic drug rouvastatin. The total methanolic extract of *M. sativa* decreased the lipid profile in different degrees TG, TC, LDL, VLDL (36.28%, 28.0%, 47.6%, 36.28%) followed by petroleum ether fraction (34.46%, 27.98%, 52.2%, 34.46%), also a significant elevation in HDL levels by petroleum ether fraction and total methanolic extract (12.23%, 9.85%) was observed. From these results petroleum ether fraction was the most effective fraction of *M. sativa* sprouts as antihyperlipidemic as illustrated in table (1).

Effect on fasting blood glucose level of STZ induced diabetic rats

STZ injection of a dose of 55mg/kg caused a significant elevation of FBG level of the experimental rats > 200mg/dl compared to control group. Administration of *M. sativa* sprouts total methanolic extract (500mg/kg) and fractions to diabetic rats for a period of 28days ameliorated STZ-induced alterations of FBG levels in comparison to untreated diabetic rats in different degrees. Total methanolic extract of *M. sativa* decreased the FBG level after 28 days by 31%, the most active fraction as antihyperglycemic was that of ethyl acetate (56%) followed by chloroform fraction (34.5%); the effect was comparable to standard antihyperglycemic drug metformin, as illustrated in table (2).

Effect on blood glucose level on OGTT of STZ induced diabetic rats

Blood glucose level of STZ-induced diabetic rats reached its peak after 60 minutes of administrating a glucose bolus of 10 g/kg and started to decline gradually to reach its starting level at 150 minutes in all treated groups. In case of ethyl acetate fraction, the glucose level reached a level less than the starting level at 150, 180 minutes. No groups showed extreme elevations like diabetic group so they have a good hypoglycemic effect in different degrees as illustrated in table (3) and fig. (1).

Hisopathological investigation

Liver of normal rats (fig. 2) revealed the normal histological structure of hepatic lobule (2a). Meanwhile, liver of diabetic rat showed vacular degeneration of hepatocytes and focal area of hepatic necrosis (2b). Examined liver of rats treated with total methanolic extract showed no changes except Kupffer cells activation (2c). In diabetic rats treated with petroleum ether (2d), chloroform (2e) and ethyl acetate (2f), liver sections maintained lobular structure with no histopathological changes.

Sections of pancreas of normal rats (fig. 3) showed pancreatic lobules separated by connective tissue septa. Most of the lobules show small, round, light staining islets of Langerhans. The center of the islet cells consists of aggregates of small beta-cells (3a). Sections of diabetic group show severe congestion of pancreatic blood vessels, pancreatic haemmorrhage and necrosis of β cells of islets of Langerhan's (3b). Sections from pancreas of total methanolic extract group showed no histopathological changes, also seen are few degenerated β cells (3c). Sections of petroleum ether group showed hyperplasia of epithelial lining pancreatic ducts and vacuolation of some β cells of islets of Langerhan's (3d). Sections of chloroform group revealed vaculations of β cells of islets of Langerhan's and severe pancreatic hemorrhage (3e). Sections of ethyl acetate group showed normal pancreatic tissue (3f), while those of butanol group revealed hyperplasia of epithelial lining pancreatic duct.

Microscopically, spleen of normal group (fig. 4) revealed the normal histological pattern (4a), conversely, spleen of diabetic rats revealed lymphocytic necrosis and depletion (4b). Spleens sections of total methanolic extract group (4c) and ethyl acetate group (4d) showed normal histopthological pattern. Results of histopathological investigation were summarized in table (4).

GLC analysis of lipoidal content

Saponification of 2g lipoidal matter yielded 680mg of fatty acid fraction (34%) and 915mg of unsaponifiable fraction (45.75%). Results of GLC analysis of the fatty acids methyl esters of saponifiable fraction revealed the presence of 12 saturated and unsaturated fatty acids in addition to polyunsaturated fatty acids (Omega 3 fatty acids); Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) in ratios (1.0%, 1.1%), respectively as illustrated in table (5). GLC analysis of the unsaponifiable fraction resulted in the identification of 17 hydrocarbons and two plant sterols; stigmasterol and β-sitosterol in concentrations 18.5% and 9.8%, respectively as illustrated in table (6).

Compounds isolated from the bioactive fractions

β-sitosterol $C_{29}H_{48}O$ (1)

White powder, mp 160-162°C, EI-MS *m/z* 412.

Stigmasterol $C_{29}H_{50}O$ (2)

White powder, mp 160-162°C, EI-MS *m/z* 414. Both sterols were isolated before from *M. sativa* (Shingo *et al*., 1987)

10-hydroxy coumestrol $C_{15}H_8O_6$ (3)
White powder, mp 241-242°C. ^{1}H NMR (500 MHz, DMSO-d_6), δ 7.87 (1H, d, *J*=8.4 Hz, H-1), 7.79 (1H, d, *J* =8.8 Hz, H-7), 6.89 (2H, m, H-2, H-8), 6.21(1H, d, *J*= 2.1 Hz, H-4). It was previously isolated from aerial parts of *M. sativa*. (Hong *et al*., 2011).

Apigenin $C_{15}H_{10}O_5$ (4)
Yellow powder, mp 345-348°C, previously isolated from the aerial parts of *M. sativa* (Seguin *et al.*, 2004).

Genistein $C_{15}H_{10}O_5$ (5)
Yellow powder, mp 299-301°C, previously isolated from the aerial parts of *M. sativa* (Martin *et al*., 2006).

p-hydroxy benzoic acid $C_6H_7O_3$ (6)
White powder, mp 217-218°C, EI-MS *m/z* 138.^{1}H NMR (500 MHz, DMSO-d_6), δ 6.71 (2H, d, *J* = 8.6 Hz, H-2, H-6), δ 7.79 (2H, d, *J* = 8.6 Hz, H-3, H-5). It was previously isolated from the shoots of *M. sativa* (Newbya *et al.*, 1980).

7, 4` dihydroxy flavone $C_{15}H_{10}O_4$ (7)
Yellow powder, mp 324-326°C, previously isolated from the aerial parts of *M. sativa* (Bickoff *et al.*, 1966) and from the seed coats of both *M. arborea* and *M. strasseri* (Monirul *et al*., 2012).

Quercetin-3-β-D-glucopyranoside $C_{21}H_{20}O_{12}$ (8)
Yellow powder, mp 225-226 °C, previously isolated from the seed coats of both *M. arborea* and *M. strasseri* (Felix *et al*., 1992).

Sissotrin $C_{22}H_{22}O_{10}$ (9)
Yellowish white powder, mp 214-217°C, was previously isolated from the aerial parts of *M. littoralis* (Bertoli *et al*.**,** 2010).

Data of compounds 4,5,7-9 were dependent on ^{1}H and ^{13}C NMR results as illustrated in table (7), UV shift reagent results in table (8). Structures of compounds 1-9 were illustrated in fig. (5).

DISCUSSION

The methanolic extract, petroleum ether, chloroform, ethyl acetate and butanol fractions of sprouts of *M. sativa*, showed a significant treatment-duration related decrease in the TG, TC, LDL and VLDL levels compared to the untreated diabetic rats in a level similar to those of reference hypolipidemic drug rouvastatin. The high content of omega -3 fatty acids (2.1%) and phytosterols (28.3%) in petroleum ether fraction may be the cause of the hypolipidemic action of petroleum ether fraction and the total extract of *M. sativa* sprouts; as reported before, stigmasterol reduces plasma cholesterol levels and inhibits hepatic synthesis and intestinal absorption in the rat (Wallace, 2004; Batta *et al*., 2006). β-sitosterol has a reducing effect on hypercholesterolemia (Batta *et al*., 2006) and omega -3 fatty acids have a reducing effect on elevated cholesterolemia in NIDDM patients (Goh *et al.*,1997).

Total methanolic extract of *M. sativa*, ethyl acetate and chloroform fractions decreased the FBG level after 28 days. The most active fraction was the ethyl acetate fraction with activity comparable to standard drug metformin.

Oral glucose tolerance test (OGTT) was performed in diabetic rats after receiving glucose orally (10 g/ kg) clearly indicated that total methanolic extract and ethyl acetate fraction showed a gradual reduction in blood glucose levels reaching the starting level at 150 minutes. The reports of histopathological study concluded the protecting and regenerating effect of extracts of *M. sativa* sprouts against toxic actions of STZ.

The isolated compounds from ethyl acetate were mostly flavones and isoflavone derivatives, which may be the responsible compounds for the hypoglycemic action especially in type 2 diabetes mellitus as reported in previous literature of other plants (Ammar and El-Okbi, 1988; Adebajo *et al*., 2009). Additionally triterpene saponins reported as constituents of the plant possess antihypercholestremic activity, which contributes to the activity of the total methanolic extract of the plant (Mazahery *et al*., 2011; Malinow *et al*., 1980).

CONCLUSION

The study findings revealed that the methanolic extract of *M. sativa* sprouts exerted antihyperlipidemic and improved antihyperglycemic effect in STZ diabetic hyperlipidemic rats. Phytochemical investigation of the bioactive fractions led to the isolation of nine compounds: β-sitosterol and stigmasterol from the petroleum ether fraction; 10-hydroxy-coumestrol, apigenin, genistein, *p*-hydroxy-benzoic-acid, 7, 4′- dihydroxyflavone, quercetin-3-glucoside and sissotrin from the ethyl acetate fraction.

REFERENCES

Adebajo AC, Iwalewa EO, Obuotor EM, Ibikunle GF, Omisore NO, Adewunmi CO, Obaparusi OO, Klaesf M, Adetogun GE, Schmidt TJ and Verspohl EJ (2009). Pharmacological properties of the extract and some isolated compounds of *Clausenalansium* stem bark: Anti-trichomonal, antidiabetic, anti-inflammatory, hepatoprotective and antioxidant effects. *J. Ethnopharmacol*., **122**: 10-19.

Alberti KG and Zimmet PZ (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. *Diabetic. Med.*, **15**: 539-553.

Ammar NM and El-Okbi S (1988). Effect of four flavonoids on blood glucose of rats. *Arch. Pharm. Res*., **11**(2): 166-168.

AOAC (1990). Official Methods of Analysis of the Association of Official Analytical Chemist. Washington, D.C, 15th Ed, pp. 951.

Batta A K, Xu G, Honda A, Miyazaki T and Salen G (2006). Stigmasterol reduces plasma cholesterol levels and inhibits hepatic synthesis and intestinal absorption in the rat. *J. Metbolism*., **55**(3): 292-299.

Baxi DB, Singh PK, Doshi AA, Arya S, Mukherjee R and Ramachadran AV (2010). *Medicago sativa* leaf extract supplementation corrects diabetes induced dyslipidemia, oxidative stress and hepatic renal functions and exerts antihyperglycemic action as effective as metformin. *Ann. Bio. Res*., **1**(3): 107-119.

Bertoli A, Daniela C, Fabio G and Luisa P (2010). Flavonoids isolated from *Medicago littoralis* Rhode (Fabaceae); their ecological and chemosystematic significance. *J. Caryologia*., **63**(1): 106-114.

Bickoff EM, Spencer RR, Lundin RE and Knuckle BE (1966). 3- Methoxycoumestrol from alfalfa: Isolation and characterization. *J. Agric. Food. Chem*., **14**(3): 444-446.

Boulos L (1999). *Flora of Egypt*. Al Hadara Publishing, Cairo, Egypt, Vol I, pp.249-276.

Felix P, Jose LC, Aldo EG and Itziar A (1992). Flavonoids in seed coats of *Medicago arborea* and *M. strasseri* (Leguminosae). *J. Bas. Microb.,* **32**(4): 241-248.

Franke AA, Custer LJ, Cerna CM and Narala KN (1995). Rapid HPLC analysis of dietary phytoestrogens from Legumes and from human urine. *Proc. Sec. Exp. Biol. Med.*, **208**: 18-26.

Fratamico PM and Bagi LK (2001). Comparison of an immunochromatographic method and the TaqMan® E. coli O157:H7 assay for detection of *Escherichia coli* O157:H7 in alfalfa sprout spent irrigation water and in sprouts after blanching. *J. Ind. Microb. Biotech*., **27**(2): 129-134.

Friedwald J, Levy YR and Friedrickson SD (1972). Estimation of concentration of low-density lipoprotein cholesterol in plasma without use of preparative ultracentrifuge. *J. Clin. Chem*., **18**: 499-502.

Goh YK, Jumpsen JA, Ryan EA and Clandinin MT (1997). Effect of omega 3 fatty acid on plasma lipids, cholesterol and lipoprotein fatty acid content in NIDDM patients. *Diabetologia.,* **40**(1): 45-52.

Gray, AM and Flatt PR (1997). Pancreatic and extra-pancreatic effects of traditional anti-diabetic plant, *Medicago sativa* (lucerne). *Br. J. Nutr*., **78**: 325-334.

Hong YH, Wang SC, Hsu C, Lin BF, Kuo YH and Huang CJ (2011). Phytoestrogenic compounds in alfalfa sprout (*Medicago sativa*) beyond coumestrol. *J. Agric. Food Chem.,* **59**(1): 131-137.

Kameswara R, Giri MM, Kesavalu MM and Apparao CH (1997). Herbal Medicine in The Management by Indigenous Resources. Interprint, New Delhi, India, pp. 375-377.

Khaleel EA, Gad ZM, El-Maraghy AS, Hifnawy SM and Abdel-Sattar E (2005). Study of hypocholesterolemic and antiatherosclerotic properties of *Medicago sativa* L. cultivated in Egypt. *J. Food and Drug Anal*., **13**(3): 212-218.

Knuckles BE, Defermery D and Koehler GO (1976). Coumestrol content of fractions obtained during wet processing of alfalfa. *J. Agric. Food. Chem*., **24**: 1177-1180.

Lust J (1986). The Herb Book. Bantam Books, London.

Mabry TJ, Markham KR and Thomas MB (1996). The systematic Identification of Flavonoids. 2nd Ed., Springer Verlag, New York, pp.41-164.

Malinow MR, Mclaughin P, Stafford S, Livingston AL and Kohler GO (1980). Alfalfa saponins and alfalfa seeds. Dietary effects on cholesterol- fed rabbits. *Atherosclerosis,* **37**(3): 433-438.

Marcovecchio M, Mohn A and Chiarelli F (2005). Type 2 diabetes mellitus in children and adolescents. *J. Endocrinol. Invest*, **28**: 853-863.

Martin ML, Castilhob C, Silveira I and Abrev MJ (2006). Liquid chromatographic validation of a quantitation method for phytoestrogens, biochanin-A, coumestrol, daidzein, formononetin, and genistein in lucerne. *J. Liq. Chrom. Rel. Tech.,* **29**(19): 2875-2884.

Mazahery LH, Yazdy S, Bagheri AR and Bagheri M (2011). Alfalfa (Medicago sativa) shoot saponins: Identification and bioactivity by the assessment of aphid feeding. *Br. J. Nutr.,* **105**(1): 62-70.

Michael J and Fowler MD (2007). Diabetes treatment, Part 2: Oral agents for glycemic management. *J. Clin. Diabetes*, **25**(4): 131-134.

Monirul I, Al-Amin MD, Mahboob AS, Shakila A, Mohammad MH, Nasim S and Sarwaruddin AM (2012). Isolation of Quercetin-3-O-beta-d-glucopyranoside from the leaves of azadirachta indica and antimicrobial and cytotoxic screening of the crude extracts. *J. Sci.,* **60**(1): 11-14.

Newbya VK, Sablon RM, Syngea RL, Casteele KV and Van Sumere KF (1980). Free and bound phenolic *acids of lucerne (Medicago sativa cv. europe). Phytochemistry,* **19**(4): 651-657.

O'Keefe JH and Bell DS (2007). Postprandial hyperglycemia/hyperlipidemia (postprandial dysmetabolism) is a cardiovascular risk factor. *Am. J. Cardiol*., **100**(5): 899-904.

Robertson DA and Home PD (1993). Problem and pitfalls of sulphonylurea therapy in older patients. *J. Drugs Aging*., **3**(6):510-524.

Seguin P, Zheng W and Souleimanov A (2004). Alfalfa phytoestrogen content: Impact of plant maturity and herbage components**.** *J. Agric. Crop Sci*., **190**(3): 211-217.

Sheyla LM, Heberth P, Maria LP, Rinaldo CS, Eduardo L, Deoclécio AC and Marcelo ES (2005). Dietary models

for inducing hypercholesterolemia in rats. *Braz. Archiv. Bio. Tech.*, **48**(2): 203-209.

Shingo H, Tacagishi H and Koushi H (1987). Variation in the content and composition of sterols in alfalfa seedlings treated with compactin (ML-236B) and mevalonic acid. *J. P. Cell. Physio*., **28**(4): 709-714.

Slauson BJ and Cooper DO (2002). Pathology the study of disease, in Mechanisms of Disease A Textbook of Comparative General Pathology, Mosby, St. Louis, Mo, USA, pp. 1-15.

Srinivasan K, Viswanad, B, Lydia A, Kaul CL and Ramarao P (2005). Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: A model for type 2 diabetes and pharmacological screening. *Pharmacol. Res*., **52**(4): 313-320.

Stahl E. (1969). Thin Layer Chromatography. 2nd Ed., Springer Verlag, Berlin, Heidelberg, New York, pp.322.

Vogel AL, Tatchell AR, Furnis BS, Hannaford AJ and Smith PW (1996). Vogel's Textbook of Practical Organic Chemistry, 5th Ed. Prentice Hall, UK, pp.87-130.

Wallace HY (2004). Plasma LDL cholesterol lowering by plant phytosterols in a hamster model**.** *J. Trends. F. Sci. Tech*., **15**: 528-531.

Wild S, Sireee R, Rog G, King H and Green A (2004)**.** Global prevalence of diabetic: Estimate of the year 2000 and projection for 2030. *J. Diabetes Care*, **27**: 1047-1053.

Winer BJ (1971). Statistical principles in experimental design. 2nd Ed. McGraw-Hill, New York, pp.1-907.

Wu KK and Huan Y (2008). UNIT 5.47 Streptozotocin-Induced Diabetic Models in Mice and Rats. *Curr. Protoc. Pharmacol.*, **40**(5): 47-48.