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Antihyperlipidaemic effect of *Aegle marmelos* fruit extract in streptozotocin-induced diabetes in rats

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Abstract: The present study determines the effect of an aqueous extract of *Aegle marmelos* fruits on serum and tissue lipids in experimental diabetes. Albino Wistar rats were rendered diabetic by intraperitoneal administration of streptozotocin (45 mg kg^{-1}). Serum and tissue lipids such as total cholesterol, triglycerides, free fatty acids and phospholipids were elevated in diabetic rats. Oral administration of *A marmelos* fruit extract at doses of 125 and 250 mg kg^{-1} to diabetic rats twice daily for 1 month led to a significant lowering of these lipids in diabetic rats. The effect exerted by the fruit extract at a dose of 250 mg kg^{-1} was greater than that of the dose of 125 mg kg^{-1} or of glibenclamide (300 μg kg^{-1}). The results of this study demonstrate that an aqueous *A marmelos* fruit extract exhibits an antihyperlipidaemic effect in streptozotocin-induced diabetic rats.

Keywords: *Aegle marmelos*; antihyperlipidaemic; lipids; insulin; streptozotocin-induced diabetes

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action or both. Chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels.[1] Type 2 diabetes mellitus is the most common form of diabetes mellitus. Currently available therapeutic options for type 2 DM, such as dietary modification, oral hypoglycaemics and insulin, have limitations of their own.[2] Therefore the search for more effective and safer antihyperglycaemic and antihyperlipidaemic agents has become an area of active research. In recent years the popularity of complementary medicine has increased. Dietary measures and traditional plant therapies as prescribed by Ayurvedic and other indigenous systems of medicine are commonly used in India.[3] The World Health Organization (WHO) has also recommended the evaluation of the effectiveness of plants traditionally used for the treatment of various conditions where we lack safe modern drugs.[4]

Aegle marmelos Correa, commonly known as bael, is indigenous to India and is grown throughout the subcontinent as well as in Myanmar, Pakistan and Bangladesh. The bael tree is one of the most useful medicinal plants of India. All parts of this tree (stem, bark, root, leaves and fruits) have medicinal virtues and have a long tradition as herbal medicines.[5] Bael leaves and fruits are widely used in folk medicines for the treatment of diabetes mellitus.[6] The unripe bael fruit is mostly used for curing diarrhoea and dysentery.[7] Bael fruit taken in the form of a beverage helps to heal ulcers. A decoction of the small and unripe fruits is prescribed in cases of haemorrhoids.[8] Preliminary studies indicate antidiabetic and hypocholesterolaemic effects of leaves of *A marmelos* (AM).[9] It has also been demonstrated that an alcoholic extract of AM fruits lowers blood glucose levels in non-diabetic rabbits.[8] We have already reported the hypoglycaemic and antioxidant properties of an aqueous extract of AM fruits in streptozotocin-induced diabetic rats.[10–12] The following constituents are reported to be present in *A marmelos* fruits: aegelin, alloimperatorin, imperatorin, marmelosin, psoralen, scoparone, scopoletin, tannic acid, umbelliferone, xanthotoxol and β-sitosterol.[7]

As there is no scientific literature available on the efficacy of AM fruits on serum and tissue lipids in streptozotocin-induced diabetic rats, we undertook the present study to evaluate the antihyperlipidaemic effect of AM fruits and to compare it with that of an oral hypoglycaemic drug, glibenclamide.

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MATERIALS AND METHODS

Plant extract

Aqueous *A marmelos* fruit extract was obtained from Chemiloids (Vijayawada, Andhra Pradesh, India). The herb/product ratio was 8:1. The extract was suspended in distilled water prior to use.

Drugs and chemicals

Streptozotocin (STZ) was purchased from Sigma Chemical Co (St Louis, MO, USA). All other chemicals used were of analytical grade.

Animals and treatment

The 30 female albino Wistar rats used in this study, weighing 160–190 g each, were obtained from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital (Annamalai University, Tamil Nadu, India). They were housed in polypropylene cages ($47 \times 34 \times 20\ cm^3$) lined with husk, which was renewed every 24 h, under a 12/12 h light/dark cycle at around 22 °C and had free access to tap water and food. The rats were fed a standard pellet diet (Kamadhenu Agencies, Bangalore, India) consisting of 22.02% crude protein, 4.25% crude oil, 3.02% crude fibre, 7.5% ash, 1.38% sand silica, 0.8% calcium, 0.6% phosphorus, 2.46% glucose, 1.8% vitamins and 56.17% carbohydrates. It provided a metabolisable energy of 3600 kcal.

Diabetes was induced in the rats by a single intraperitoneal injection of freshly prepared STZ (45 mg kg^{-1} body weight)[13] in citrate buffer (0.1 M, pH 4.5) in 1 ml kg^{-1}. Two days after STZ administration the blood glucose level of each rat was determined. Rats with a blood glucose range of 250–300 mg dl^{-1} were considered diabetic and included in the study.

Experimental design

A total of 30 rats (six non-diabetic, 24 STZ-treated diabetic) were used in our study. The animals were divided into five groups of six rats each: group 1, non-diabetic untreated rats; group 2, STZ-treated diabetic rats; groups 3 and 4, STZ-treated diabetic rats administered with an aqueous extract of AM fruits (AMFEt) at 125 and 250 mg kg^{-1} body weight respectively using an intragastric tube twice daily for 1 month;[11] group 5, STZ-treated diabetic rats administered with glibenclamide (300 μg kg^{-1} body weight) in distilled water using an intragastric tube twice daily for 1 month.[11]

After the last treatment, all animals were sacrificed by cervical decapitation after an overnight fast. Blood was collected in tubes with or without respective anticoagulants. Blood samples collected in potassium oxalate/sodium fluoride-containing tubes were used for the estimation of glucose. Plasma and serum were separated and used for the determination of insulin and lipids respectively. Tissues such as liver, kidney and heart were removed immediately and stored in ice-chilled normal saline for various biochemical estimations.

Estimation of blood glucose

Blood glucose levels were determined by the method of Sasaki *et al*.[14] A 0.1 ml aliquot of freshly drawn blood was immediately mixed with 1.9 ml of trichloroacetic acid solution to precipitate proteins and then centrifuged. A 1 ml aliquot of the supernatant was mixed with 4 ml of *o*-toluidine reagent and kept in a boiling water bath for 15 min, and the green colour developed was read at 620 nm.

Assay of plasma insulin

The plasma insulin assay was performed by the ELISA method using a Boehringer Mannheim kit (Boehringer Es 300 analyser, Mannheim, Germany). A 0.1 ml aliquot of plasma was injected into the plastic tube coated with monoclonal anti-insulin antibodies. Phosphate buffer (40 mM, pH 7.0) and anti-insulin peroxidase conjugate were added to form anti-insulin antibody/peroxidase conjugate. Substrate (phosphate/citrate 100 mM, pH 4.4)/chromogen (diammonium 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonate)) solution was then added to produce the indicator reaction. After the development of colour the absorbance was read at 420 nm.

Extraction of lipids

Lipids were extracted by the method of Folch *et al*.[15] The tissues were homogenised, plasma was added with cold chloroform/methanol (2:1 v/v) and the contents were extracted after 24 h. The extraction was repeated four times. The combined filtrate was washed with 0.7% potassium chloride and the aqueous layer was discarded. The organic layer was made up to a known volume with chloroform and used for various estimations.

Estimation of cholesterol

The cholesterol content was estimated by the method of Zlatkis *et al*.[16] A 0.1 ml aliquot of lipid extract was evaporated to dryness and 5 ml of ferric chloride/acetic acid reagent was added. Then 3 ml of concentrated sulphuric acid was added and the absorbance was read after 20 min at 560 nm.

Estimation of triglycerides

Triglycerides were estimated by the method of Foster and Dunn.[17] A 0.1 ml aliquot of lipid extract was evaporated to dryness, then 0.1 ml of methanol was added followed by 4 ml of isopropanol and 0.4 mg of alumina. All the tubes were shaken well for 15 min and centrifuged, then 2 ml of the supernatant fluid was transferred to labelled tubes. The tubes were placed in a water bath at 65 °C for 15 min for saponification, after adding 0.6 ml of saponification reagent followed by 0.5 ml of acetyl acetone reagent. After mixing, the tubes

were kept in a water bath at 65 °C for 1 h, the contents were cooled and the absorbance was read at 420 nm.

Estimation of free fatty acids

Free fatty acids were estimated by the method of Falholt *et al*.[18] A 0.1 ml aliquot of lipid extract was evaporated to dryness, then 1 ml of phosphate buffer (pH 6.4), 6 ml of extraction solvent (chloroform/heptane/methanol 5:5:1 v/v/v) and 2.5 ml of copper reagent were added. All the tubes were shaken vigorously, then 200 mg of activated silicic acid was added. After 30 min the tubes were centrifuged and 3 ml of the upper layer was transferred to another tube containing 0.5 ml of diphenyl carbazide and mixed carefully. The absorbance was read at 550 nm immediately.

Estimation of phospholipids

The phospholipid content was estimated by the method of Zilversmit and Davis.[19] A 0.1 ml aliquot of lipid extract was digested with 1 ml of concentrated sulphuric acid and 1 ml of concentrated nitric acid to give a colourless solution. To this, 1 ml of 2.5% ammonium molybdate and 0.1 ml of amino-2-naphthol-4-sulphonic acid (ANSA) were added. The volume was then made up to 5 ml with distilled water and the absorbance was read at 660 nm.

Statistical analysis

All the grouped data were statistically evaluated and the obtained data were analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). The values are expressed as mean ± SD for six rats in each group. P values <0.05 were considered as significant.

RESULTS

Table 1 shows the changes in blood glucose levels, plasma insulin and body weight in non-diabetic and diabetic animals. As a result of STZ treatment, diabetic rats showed a significant ($P < 0.05$) increase in blood glucose and a significant decrease in plasma insulin and body weight as compared with normal rats. Administration of AMFEt orally at doses of 125 and 250 mg kg^{-1} body weight twice daily for 1 month significantly ($P < 0.05$) lowered the levels of blood glucose, increased insulin levels and improved the body weight in STZ-induced diabetic rats.

Table 2 presents the changes in the levels of serum lipids in non-diabetic and diabetic rats. STZ administration caused a significant ($P < 0.05$) increase in serum total cholesterol, triglycerides (TGs), free fatty acids (FFAs) and phospholipids (PLs) in rats as compared with non-diabetic rats. Oral administration of AMFEt at doses of 125 and 250 mg kg^{-1} twice daily for 1 month caused a significant ($P < 0.05$) reduction in these values in diabetic rats.

Tables 3–5 show the concentrations of total cholesterol, triglycerides, free fatty acids and phospholipids in hepatic, renal and cardiac tissues of non-diabetic and diabetic animals. There was a significant ($P < 0.05$) increase in the concentrations of total cholesterol, FFAs, TGs and PLs in those tissues in diabetic animals as compared with non-diabetic rats. Oral administration of aqueous AMFEt at doses of 125 and 250 mg kg^{-1} twice daily for 1 month to STZ-treated rats resulted in a significant ($P < 0.05$) decrease in these parameters.

Table 1. Effect of *Aegle marmelos* fruit extract on blood glucose levels, plasma insulin and body weight in STZ-treated diabetic rats

	Blood glucose (mg dl^{-1})		Plasma insulin (μU ml^{-1})	Body weight (g)	
Group	Initial	Final	Final	Initial	Final
Non-diabetic	81.6 ± 4.8	85.8 ± 4.4a	19.5 ± 0.7a	172.0 ± 6.4	183.8 ± 6.0a
STZ-treated	286.2 ± 13.2	336.8 ± 22.5b	10.7 ± 1.4b	186.4 ± 8.0	160.9 ± 7.1b
STZ-treated + AMFEt 125 mg	270.6 ± 16.5	162.2 ± 10.9c	15.2 ± 1.1c	184.2 ± 6.3	192.7 ± 6.0a
STZ-treated + AMFEt 250 mg	280.4 ± 19.2	96.2 ± 5.3a	17.9 ± 1.2d	180.0 ± 8.6	190.0 ± 8.2a
STZ-treated + glibenclamide (300 μg)	285.4 ± 18.4	130.0 ± 8.0d	15.6 ± 0.9c	182.5 ± 7.2	187.0 ± 7.5a

Each value is mean ± SD for six rats in each group. Values within a column not sharing a common letter differ significantly at $P < 0.05$ (DMRT).

Table 2. Effect of *Aegle marmelos* fruit extract on serum total cholesterol, triglycerides, free fatty acids and phospholipids in STZ-treated diabetic rats (mg dl^{-1})

Group	Total cholesterol	Triglycerides	Free fatty acids	Phospholipids
Non-diabetic	79.3 ± 3.6a	11.4 ± 0.5a	50.3 ± 2.6a	116.5 ± 3.8a
STZ-treated	203.7 ± 12.3b	22.3 ± 1.2b	126.9 ± 9.2b	204.1 ± 16.6b
STZ-treated + AMFEt 125 mg	157.1 ± 8.9c	18.6 ± 0.9c	92.47 ± 7.4c	151.3 ± 1.4c
STZ-treated + AMFEt 250 mg	92.2 ± 4.0d	12.8 ± 0.4d	61.6 ± 3.8d	120.3 ± 4.3a
STZ-treated + glibenclamide (300 μg)	108.6 ± 5.7e	16.0 ± 1.0e	70.8 ± 5.1e	138.1 ± 4.7d

Each value is mean ± SD for six rats in each group. Values within a column not sharing a common letter differ significantly at $P < 0.05$ (DMRT).

Table 3. Effect of *Aegle marmelos* fruit extract on total cholesterol, triglycerides, free fatty acids and phospholipids in hepatic tissue of STZ-treated diabetic rats (mg g^{-1} wet tissue)

Group	Total cholesterol	Triglycerides	Free fatty acids	Phospholipids
Non-diabetic	7.2 ± 0.37a	4.8 ± 0.22a	8.8 ± 0.52a	22.3 ± 1.43a
STZ-treated	12.4 ± 0.81b	10.5 ± 0.74b	19.3 ± 1.56b	48.6 ± 2.34b
STZ-treated + AMFEt 125 mg	9.4 ± 0.69c	8.0 ± 0.46c	14.7 ± 1.09c	41.4 ± 2.02c
STZ-treated + AMFEt 250 mg	8.0 ± 0.34d	6.1 ± 0.32d	10.9 ± 0.81d	26.7 ± 1.27d
STZ-treated + glibenclamide (300 μg)	8.3 ± 0.40d	7.5 ± 0.40c	13.2 ± 1.14e	31.1 ± 2.82e

Each value is mean ± SD for six rats in each group. Values within a column not sharing a common letter differ significantly at $P < 0.05$ (DMRT).

Table 4. Effect of *Aegle marmelos* fruit extract on total cholesterol, triglycerides, free fatty acids and phospholipids in renal tissue of STZ-treated diabetic rats (mg g^{-1} wet tissue)

Group	Total cholesterol	Triglycerides	Free fatty acids	Phospholipids
Non-diabetic	3.6 ± 0.17a	2.1 ± 0.12a	18.6 ± 1.47a	15.8 ± 0.66a
STZ-treated	8.4 ± 0.62b	6.2 ± 0.51b	28.2 ± 2.12b	33.8 ± 2.14b
STZ-treated + AMFEt 125 mg	6.2 ± 0.36c	4.8 ± 0.28c	22.4 ± 1.93c	26.6 ± 1.48c
STZ-treated + AMFEt 250 mg	4.1 ± 0.21d	3.1 ± 0.17d	20.2 ± 1.26ac	17.9 ± 0.82d
STZ-treated + glibenclamide (300 μg)	4.9 ± 0.24e	4.2 ± 0.20e	20.7 ± 1.05ac	22.3 ± 1.13e

Each value is mean ± SD for six rats in each group. Values within a column not sharing a common letter differ significantly at $P < 0.05$ (DMRT).

Table 5. Effect of *Aegle marmelos* fruit extract on total cholesterol, triglycerides, free fatty acids and phospholipids in cardiac tissue of STZ-treated diabetic rats (mg g^{-1} wet tissue)

Group	Total cholesterol	Triglycerides	Free fatty acids	Phospholipids
Non-diabetic	2.5 ± 0.12a	3.0 ± 0.18a	3.6 ± 0.20a	1.6 ± 0.04a
STZ-treated	3.7 ± 0.22b	5.3 ± 0.38b	4.8 ± 0.31b	2.5 ± 0.16b
STZ-treated + AMFEt 125 mg	2.9 ± 0.18c	4.2 ± 0.26c	4.0 ± 0.30c	2.0 ± 0.11c
STZ-treated + AMFEt 250 mg	2.6 ± 0.12a	3.1 ± 0.16a	3.7 ± 0.18a	1.7 ± 0.08ad
STZ-treated + glibenclamide (300 μg)	2.8 ± 0.15c	3.5 ± 0.19d	3.8 ± 0.14a	1.8 ± 0.06d

Each value is mean ± SD for six rats in each group. Values within a column not sharing a common letter differ significantly at $P < 0.05$ (DMRT).

For all the parameters studied, AMFEt at doses of 125 and 250 mg kg^{-1} showed a significant effect when compared with non-treated diabetic rats. The effect at a dose of 250 mg kg^{-1} was more pronounced than that of the dose of 125 mg kg^{-1} or of glibenclamide (300 μg kg^{-1}).

DISCUSSION

Diabetes mellitus is associated with profound alterations in blood glucose, plasma lipids and lipoprotein profile and therefore with an increased risk of coronary heart disease.[20,21] The elevated blood glucose levels in STZ-treated rats were lowered by administering the aqueous fruit extract. The antihyperglycaemic action of the extract is due to the potentiation of insulin from existing β-cells of the islets of Langerhans. This is evidenced by the significant increase in the levels of plasma insulin in STZ-treated rats.

Lowering of serum lipid levels through dietary or drug therapy seems to lower the risk of vascular disease and related complications.[22] The rise in serum lipids observed in this study in STZ-induced diabetic rats indicates an increased mobilisation of free fatty acids from the peripheral depots. In AMFEt-treated diabetic rats the serum lipids were reduced markedly. The reduction in serum lipids may be due to decreased fat mobilisation and synthesis.

We have observed an increase in the concentrations of lipids in different tissues of STZ-induced diabetic rats. The increase in cholesterol levels in hepatic tissue may be due to an increase in the transport of chylomicron cholesterol to the liver.[23] The observed increase in the concentration of free fatty acids in the liver of diabetic rats may be due to an increased transport of fatty acids as a result of excessive mobilisation of fatty acids.[23] Higher levels of phospholipids might be caused by an increase in hepatic synthesis.[24] Under normal conditions, insulin activates lipoprotein lipase and hydrolyses triglycerides.[25,26] Hence increased levels of triglycerides in diabetic rats may be due to decreased lipoprotein lipase activity.

During diabetes the heart is under stress and therefore may preferentially utilise glucose for its energy production, resulting in a rise in the concentration of fatty acids in the myocardium.[27] The raised level of fatty acids in the diabetic heart may also be responsible for increased levels of triacylglycerol.[23]

The increased level of cholesterol observed in the diabetic kidney might have been due to the decreased levels of HDL cholesterol. Accumulation of fatty acids

in diabetes may result in elevated levels of their metabolites such as acyl carnitine and long-chain acyl-CoA, which interfere with Na^+/K^+ ATPase activity. This defect may be implicated in diabetic nephropathy. Thus the renal complications associated with diabetes mellitus may be partly due to abnormalities in lipid metabolism.[27] Oral administration of AMFEt lowered tissue lipids in diabetic rats.

The results of our study clearly indicate the lipid-lowering activity of AM fruits in STZ-induced diabetic rats. Diabetes-induced hyperlipidaemia is attributable to excess mobilisation of fat from the adipose tissue due to the under-utilisation of glucose.[28] The regression of the diabetic state on oral administration of AMFEt increases the utilisation of glucose (increased insulin secretion), thereby depressing the mobilisation of fat. The antihyperlipidaemic effect exerted by AMFEt may also be due to the various phyto constituents present in it. The mechanism of action is still unknown and further investigations are under way in our lab to reach a definite conclusion.

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