Original Paper

Anti-Diabetic Potential of the Leaves of *Anisomeles malabarica* in Streptozotocin Induced Diabetic Rats

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Key Words

Experimental diabetes mellitus • Anti-diabetic activity • *Anisomeles malabarica* • Insulin sensitivity and HbA1c

Abstract

Background/Aims: Diabetes mellitus is a pandemic metabolic disorder that is affecting a majority of populations in recent years. There is a requirement for new drugs that are safer and cheaper due to the side effects associated with the available medications. **Methods:** We investigated the anti-diabetic activity of leaves of *Anisomeles malabarica* following bioactivity guided fractionation. The different solvent (hexane, ethyl acetate, methanol and water) extracts of *A. malabarica* leaves were used in acute treatment studies to evaluate and identify the active fraction. The ethyl acetate extract was subjected to further fractionation using silica gel column chromatography and the compounds were identified by LC-SRM/MS and GC-MS. Additional chronic treatment studies were carried out using this active fraction (AMAF) for 30 days in experimental diabetic rats. Fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), plasma insulin levels and glucose tolerance were measured along with insulin resistance/sensitivity indicators (HOMA-IR, HOMA- β and QUICKI) to assess the beneficial effects of *A. malabarica* in the management of diabetes mellitus. **Results:** Among the different solvent extracts tested, ethyl acetate extract showed maximum (66%) anti-hyperglycemic activity. The hexane and ethyl acetate (1:1) fraction that has maximum anti-diabetic activity was identified as active fraction of *A. malabarica* (AMAF). The FBG, HbA1c, plasma insulin levels and insulin sensitivity/resistance indicators such as glucose tolerance, HOMA-IR, HOMA- β and QUICKI were significantly improved to near normal in diabetic rats treated with AMAF. Further, we identified key flavonoids and fatty acids as the anti-diabetic active principles from the AMAF of *A. malabarica* leaves. **Conclusion:** The results of our study suggest that *Anisomeles malabarica* has potential anti-diabetic activity in STZ induced diabetic rats.

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Published by S. Karger AG, Basel

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Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia due to altered insulin secretion from β -cells of pancreas and/or altered hepatic and peripheral tissues' insulin sensitivity [1]. The prolonged chronic hyperglycemia leads to development of dyslipidemia followed by micro and macro vascular complications [2]. According to current international diabetes federation projections, an estimated 78 million people have diabetes in the South-East Asia and this number will likely to be doubled by 2040 [3, 4]. Management of diabetes starting with oral hypoglycemic agents and insulin, is not satisfactory and also have been reported to have several side effects that include hypoglycemia, hypersensitivity, gastrointestinal discomfort, nausea, liver and heart failure, and diarrhea [5]. Right now, there is every necessity to identify novel anti-diabetic therapeutics with lesser or no side effects. Due to their lower cost, increased tolerability and fewer side effects compared to conventional anti-hyperglycemic or anti-diabetic drugs, plant based drugs are receiving increased attention towards disease control/management [6, 7]. Plant parts and other natural components are considerable sources of medicine as evidenced by the discovery of metformin from *Galega officinalis*. To identify novel treatments and management strategies one should focus on conducting systematic scientific studies to identify and characterize the active principles and their efficacy in the management of diabetes mellitus.

Anisomeles malabarica is an aromatic, perennial herb belonging to the family Lamiaceae and is widely distributed throughout the southern and tropical regions of Asia [8]. The leaves of *A. malabarica* are used as traditional medicine in the treatment of gastrointestinal diseases, diabetes and hyperlipidemia [9]. The leaves are used in treatment of dyspepsia, catarrhal affections, intermittent fever, bowel disorder, boils, and tetanus from ancient period [10]. Recently another species of *Anisomeles* genus, *Anisomeles indica*, has been demonstrated to have anti-diabetic activity in streptozotocin (STZ) induced diabetic mice [11], but there are no scientific reports on the anti-diabetic activity of *A. malabarica*. Hence in the present study, systematic studies were conducted to evaluate the anti-diabetic activity of leaves of *A. malabarica* in STZ induced diabetic rats and also identify the active principle(s) following bioassay guided fractionation.

Materials and Methods

The flow chart of bioassay guided fractionation is outlined in Fig. 1.

Collection of plant material

Anisomeles malabarica leaves were collected from the Tirumala hills area (Andhra Pradesh) and identified by taxonomist. A voucher specimen (voucher number: 964) was deposited in the herbarium of Department of Botany, Sri Venkateswara University, Tirupati. The leaves were shade dried, mechanically ground and the obtained leaf powder was used for further studies.

Induction of diabetes mellitus

Animal studies were conducted as approved by Institute's Animal Ethics Committee of Sri Venkateswara University, Tirupati. Diabetes mellitus was induced in overnight-fasted rats by a single intraperitoneal injection of freshly prepared STZ (50 mg/kg, dissolved in 0.1 M cold citrate buffer, pH 4.5) as described by our group earlier [12, 13]. Diabetes was identified by polydipsia, polyuria and by measuring fasting blood glucose (FBG) levels. Rats were tested for successful induction of diabetes 3 days after STZ injection by determining FBG levels. Rats with blood glucose levels >250 mg/dL were used for the study.

Preparation of solvent extracts

Hexane, ethyl acetate, methanol and aqueous extracts were prepared by successive solvent extraction of leaf powder in soxhlet apparatus at 65°C-70°C. The filtrates obtained were distilled and concentrated under reduced pressure at low temperature (40°C to 45°C) in Buchi rotavapor R-200 and finally freeze dried. The extracts were stored at 4°C in airtight containers until used for further studies.

Fig. 1. Bioassay guided fractionation of leaves of *Anisomeles malabarica*. Values in the parenthesis indicate the percentage of fall in fasting blood glucose.

Experimental design

Effect of acute treatment with different solvent extracts of *A. malabarica* leaves in experimental diabetic rats:

The animals were divided into 7 groups with 6 rats in each group.

Group I: normal untreated rats

Group II: diabetic untreated rats

Group III: diabetic rats treated with hexane extract

Group IV: diabetic rats treated with ethyl acetate extract

Group V: diabetic rats treated with methanol extract

Group VI: diabetic rats treated with aqueous extract

Group VII: diabetic rats treated with glibenclamide (standard oral anti-diabetic drug)

After an overnight fast, either the extract (500 mg/kg b.w.) or the glibenclamide (20 mg/kg b.w.) suspended in water was administered to the corresponding groups of rats through oral gavage needle. Group-I and II rats were given water alone. Blood samples were collected from the tail veins of all the animals, every hour, from 0 to 6 hours after treatment for estimation of blood glucose.

Isolation of active fraction using Column Chromatography

Ten grams of ethyl acetate extract was subjected to column chromatography on silica gel (60-120 mesh size) using hexane and ethyl acetate gradient solvent system i.e. 100% n-hexane, different ratios of n-hexane : ethyl acetate (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9) and 100% ethyl acetate. The collected fractions were analyzed by thin layer chromatography (TLC) on silica gel. The fractions with similar TLC profiles were pooled into six different fractions viz. F1 to F6. The obtained fractions were distilled and concentrated under reduced pressure at low temperature (40°C to 45°C) in Buchi rotavapor R-200, freeze dried and were stored at 4°C until further studies.

Evaluation of anti-hyperglycemic activity of the fractions (F1-F6) in acute treatment studies

All six fractions (F1 to F6) were subjected to test for anti-diabetic activity in STZ induced diabetic rats. The animals were classified into nine groups ($n = 6$). Group I: normal control, Group II: diabetic control, Groups III, IV, V, VI, VII and VIII: diabetic rats received F1, F2, F3, F4, F5 and F6 fractions respectively, at a dose of 50 mg/kg b.w. Group IX: glibenclamide (20 mg/kg b.w.) treated diabetic rats. After an overnight fasting either the specific fraction or glibenclamide suspended in water were administered to the animals orally by a force feeding needle. Untreated control groups received water alone. Blood samples were collected from the tail veins of the animals at hourly intervals from 0 to 6 hours after the treatment for estimation of blood glucose. Based on its highest anti-hyperglycemic activity, the fraction F4 is further referred as active fraction (AMAF).

Effect of AMAF on oral glucose tolerance (OGT)

The oral glucose tolerance test was performed in overnight fasted rats as described earlier [12]. Five groups of diabetic rats (n = 6 in each group) were used for this study. Glucose solution (2 g/kg b.w) was administered orally to all groups of rats using a force feeding needle. Simultaneously, normal control (NC) and diabetic control (DC) rats received distilled water, normal treated rats (NT) and diabetic treated rats (DT) received 50 mg of AMAF/kg b.w. and, glibenclamide treated diabetic rats (DG) received glibenclamide (20 mg/kg b.w.) orally. Blood samples were collected every 30 minutes from the tail veins of rats from 0 to 3 hours of glucose and AMAF/glibenclamide/placebo administration.

Effect of chronic treatment with AMAF on fasting blood glucose and plasma insulin levels of normal and diabetic rats

The animals were divided in to 5 groups of 6 rats each.

Group I (NC): normal untreated rats

Group II (NT): normal rats treated with AMAF

Group III (DC): diabetic untreated rats

Group IV (DT): AMAF treated diabetic rats

Group V (DG): glibenclamide treated diabetic rats

Active fraction (AMAF, 50 mg/kg b.w) and glibenclamide (20 mg/kg b.w) were administered to the respective groups of rats once every day morning in fasting state for 30 days by gastric intubation. Fasting blood glucose levels were measured in all groups of animals at the beginning of the study and at the end of 8th, 15th, 22nd and 31st days. At the end of study, after overnight fasting, animals were sacrificed by asphyxiation followed by cervical dislocation. Then blood and tissues were collected, processed and immediately stored at -80°C until further analysis.

Biochemical analysis

Estimation of blood glucose was carried out using dextrostix with Accucheck glucometer (Glucose oxidase method) [12, 13]. HbA1c was estimated by the method of Eross et al [14]. Plasma insulin levels were measured according to the modified method of Herbert et al. [15] using RIA kit obtained from BARC, Mumbai, India. Homeostatic model assessment of insulin resistance (HOMA-IR), β -cell function (HOMA- β) and quantitative insulin sensitivity check index (QUICKI) were calculated before and after treatment based on fasting glucose and insulin values of experimental rats.

The HOMA-IR and HOMA- β were calculated using the formula [16] given below

$HOMA-IR = [(fasting\ plasma\ insulin\ in\ \mu U/L) \times (fasting\ blood\ glucose\ in\ mg/dL)] / 405$

$HOMA-\beta = [(360 \times (fasting\ plasma\ insulin\ in\ \mu U/L)) / ((fasting\ blood\ glucose\ in\ mg/dL) - 63)]$

QUICKI of experimental rats was also calculated using the formula [17]:

$QUICKI = 1 / [\log (fasting\ plasma\ insulin\ in\ \mu U/L) + \log (fasting\ blood\ glucose\ in\ mg/dL)]$

Liquid chromatography-selected reaction monitoring/mass spectrometry (LC-SRM/MS) analysis of AMAF

11.2 mg of AMAF was extracted with 1 ml of methanol and sonicated for 2-3 minutes and centrifuged at 14, 000 rpm for 5 minutes. The supernatant was spiked with 25 ng of Umbelliferone, an internal standard (ISTD), and then dried under vacuum. The same procedure was followed to prepare different phenolic standards (quinic acid, gallic acid, caffeic acid, chlorogenic acid, epicatechin, leutolin, catechin, epigallocatechin-3-gallate, quercetin, naringenin, epicatechin and kaempferol) in 100 μ l of methanol and spiked with 10 μ l reference standard. The dried sample and standards were reconstituted in 100 μ l of 50% methanol followed by water bath sonication for 5 minutes and then centrifuged at 14, 000 rpm for 5 minutes. Ten microliters of this supernatant was finally injected into Ultra Performance Liquid Chromatographic separation (UHPLC) system (Agilent 1290I Infinity UHPLC). 10mM ammonium acetate in water with 0.1% formic acid was used as the mobile phase-A while acetonitrile with 0.1% formic acid was used as mobile phase-B. The total run time was 30 minutes and column used was shim-pack XR-ODS III, 2 μ , C18, 150 mm x 2 mm, with a flow rate of 200 μ l/min. The column oven temperature was set to 40°C. The UHPLC was coupled with a Thermo Fisher-TSQ vantage mass spectrometer and the selected reaction monitoring (SRM) experiment was performed on the mass spectrometer operated in both positive (4000V) as well as negative (2800V) ion mode with vaporizer temperature of 300°C. The MS parameters such as sheath gas and auxiliary gas values were set to 20 and 10 arbitrary units, respectively and the scan time was 0.05 seconds.

GC-MS analysis of AMAF

The AMAF was dissolved in analytical grade methanol and 2 µl of the fraction was subjected to GC-MS using JEOL GCMATE II GC-MS (Agilent Technologies 6890N Network GC system for gas chromatography), equipped with secondary electron multiplier. The column (HP5) used was fused silica 50 m x 0.25 mm I.D. Analysis conditions were 20 minutes at 100°C, 3 minutes at 235°C for column temperature, 240°C for injector temperature, helium as the carrier gas and split ratio was 5:4. One micro liter sample was evaporated in a splitless injector at 300°C with a run time of 30 minutes. The compounds were identified by gas chromatography coupled with mass spectrometry. The molecular weight and structure of the compounds of test materials were ascertained by interpretation on mass spectrum of GC-MS using the database of National Institute Standard and Technology (NIST).

Statistical analysis

Statistical analysis was conducted using student t-test and one way ANOVA test followed by Duncan's multiple range test (DMRT).

Results

Acute effect of different solvent extracts of *A. malabarica* leaves in experimental diabetic rats

The effects of hexane, ethyl acetate, methanol and aqueous extracts of *A. malabarica* leaves on the fasting blood glucose levels of diabetic rats are shown in Table 1. The percentage yield of hexane, ethyl acetate, methanol and aqueous extracts of *A. malabarica* leaves were 9.3%, 22.2%, 10.5% and 17.1% w/w respectively. The ethyl acetate extract revealed maximum anti-hyperglycemic activity (66.4% decrease in FBG after 6 hours of treatment), whereas glibenclamide has produced 33.3% reduction in the FBG levels in treated diabetic

Table 1. Effect of different solvent extracts of *A. malabarica* on fasting blood glucose levels in experimental diabetic rats. Values are given as mean ± S.D (n = 6). †P<0.0001 compared with 0h blood glucose levels of normal control group (group I). *P<0.001 compared with the 0h blood glucose levels in the respective group. **P<0.0001 compared with the 0h blood glucose levels in the respective group. Numbers in parenthesis indicate the percentage of fall in blood glucose. Group I and II: Normal and diabetic untreated rats respectively; Group III, IV, V, VI and VII: Diabetic rats treated with hexane, ethyl acetate, methanol, aqueous extracts and glibenclamide respectively

Groups	Fasting Blood Glucose (mg/dl)						
	0h	1h	2h	3h	4h	5h	6h
I	84.1±2.1	86.3±2.9	90.8±4.9	90.8±5.3	84.6±1.9	89.1±4.2	84.8±3.5
II	362.2±5.9†	364.4±5.3	367.6±6.7	378.1±7.3	381.9±6.3	386.4±5.7	392.2±6.3
III	368.4±33.9†	365.2±31.3	362.9±36.4	459.4±49.9	351.2±32.7	346.9±32.7	323.8±15.7* (12.2%)
IV	379.2±24.1†	365.8±28.9	350.8±54.9	309.0±54.1**	275.6±51.1**	190.1±26.3**	127.6±19.0** (66.4%)
V	319.5±38.2†	291.4±36.9	290.8±36.4*	266.0±41.3	239.4±46.3**	203.3±52.4**	161.6±62.9** (49.5%)
VI	386.3±33.9†	361.7±46.3	341.7±46.9*	304.9±53.6	293.4±53.6**	281.4±50.1**	253.6±46.8** (34.4%)
VII	369.4±10.1†	353.6±12.3	327.4±13.8	314.6±19.5*	284.3±10.7	244.8±11.8** (33.8%)	269.5±7.6** (27.1%)

Table 2. Effect of acute treatment with fractions obtained by column chromatography of ethyl acetate extract of *A. malabarica* leaves in experimental diabetic rats. Values are given as mean $\pm$ S.D (n = 6). †P<0.0001 compared with 0h blood glucose levels of normal control group (group I). *P<0.001 compared with the 0h blood glucose levels in the respective group. **P<0.0001 compared with the 0h blood glucose levels in the respective group. Numbers in parenthesis indicate the percentage of fall in blood glucose. Group I and II: normal and diabetic control rats respectively, Groups III to VIII: diabetic rats received F1, F2, F3, F4, F5 and F6 fractions respectively, at a dose of 50 mg/kg b.w. Group IX: glibenclamide (20 mg/kg b.w) treated diabetic rats

Groups	Fasting Blood Glucose (mg/dL)						
	0h	1h	2h	3h	4h	5h	6h
I	82.4 $\pm$ 37.3	86.1 $\pm$ 61.7	83.4 $\pm$ 29.5	87.3 $\pm$ 44.4	85.8 $\pm$ 19.2	89.6 $\pm$ 28.4	87.2 $\pm$ 11.3
II	392.6 $\pm$ 25.2†	390.7 $\pm$ 32.1	362.6 $\pm$ 9.4	367.8 $\pm$ 22.3	382.6 $\pm$ 59.6	391.3 $\pm$ 38.6	408.6 $\pm$ 41.2
III	321.9 $\pm$ 14.2†	329.5 $\pm$ 38.4	340.3 $\pm$ 54.3	352.2 $\pm$ 42.7	355.4 $\pm$ 55.2	364.9 $\pm$ 27.4	373.1 $\pm$ 50.3
IV	346.3 $\pm$ 46.6†	321.0 $\pm$ 46.9	325.5 $\pm$ 26.1	328.4 $\pm$ 37.5	322.8 $\pm$ 29.7	314.6 $\pm$ 33.2	315.9 $\pm$ 25.9
V	357.8 $\pm$ 49.1†	355.5 $\pm$ 60.0	336.0 $\pm$ 76.7	331.0 $\pm$ 81.7	331.4 $\pm$ 88.8	326.8 $\pm$ 98.5	324.6 $\pm$ 95.4
VI	413.6 $\pm$ 12.4†	294.2 $\pm$ 41.2*	254.7 $\pm$ 16.9*	209.3 $\pm$ 23.9*	182.6 $\pm$ 34.5	148.1 $\pm$ 40.4*	104.2 $\pm$ 11.6** (74.8%)
VII	403.5 $\pm$ 42.1†	334.1 $\pm$ 38.7*	298 $\pm$ 36.6	267 $\pm$ 49.8*	241.9 $\pm$ 54.6	216.0 $\pm$ 46.8	190.1 $\pm$ 34.6** (52.8%)
VIII	309.6 $\pm$ 44.6†	286.7 $\pm$ 41.6*	266 $\pm$ 29.7	249 $\pm$ 32.6	234.5 $\pm$ 32.5*	215.3 $\pm$ 31.5	203.6 $\pm$ 37.3* (34.3%)
IX	377.8 $\pm$ 33.5†	357.3 $\pm$ 26.6	343 $\pm$ 45.2	298 $\pm$ 15.3*	267.2 $\pm$ 38.9	244.1 $\pm$ 61.5	239.7 $\pm$ 36.2*

rats. Hexane, methanol and aqueous extracts showed maximum anti-hyperglycemic activity of 12.2%, 49.5% and 34.4% respectively in STZ diabetic rats.

Anti-hyperglycemic activity of the different fractions (F1-F6) of the ethyl acetate extract

Effect of the six fractions isolated from ethyl acetate extract of *A. malabarica* on FBG levels are given in Table 2. Among these fractions, fraction F4 has decreased the FBG levels significantly from 413.6 $\pm$ 12.4 mg/dL to 104.2 $\pm$ 11.6 mg/dL in diabetic rats after six hours of treatment. And the anti-hyperglycemic effect of fraction F4 (74.8%) is significantly higher than that of the standard oral hypoglycemic agent glibenclamide and also those of other fractions.

Effect of AMAF on oral glucose tolerance (OGT) in normal and diabetic rats

The effects of AMAF on OGT in normal and STZ induced diabetic rats are shown in Fig. 2. The oral administration of 50 mg AMAF/kg b.w has significantly improved the glucose tolerance of the diabetic rats within 180 minutes of oral glucose load and it is similar to that of glibenclamide. While in the diabetic control rats, blood glucose levels remained elevated beyond 180 minutes of oral glucose load. No significant difference in the glucose tolerance was observed between the normal control and normal treated groups.

Effect of chronic treatment with AMAF on fasting blood glucose and plasma insulin levels of normal and diabetic rats

Effects of AMAF on FBG and plasma insulin levels are given in Table 3. During the treatment period and at the end of the treatment we observed continuous rise in the blood glucose levels in untreated diabetic rats. AMAF treated diabetic rats (50 mg/kg b.w.) have consistently shown significant reduction in the blood glucose levels over the period of four weeks. As anticipated, similar effects were observed in diabetic rats treated with glibenclamide. Diabetic untreated group showed significantly decreased plasma insulin levels (6.6 $\pm$ 0.9 μ U/l) compared to those of normal rats (12.7 $\pm$ 1.6 μ U/l), whereas treatment with AMAF significantly

increased the plasma insulin levels ($9.6 \pm 0.6 \mu\text{U/l}$) in the diabetic treated group. Elevated plasma insulin levels were also observed in glibenclamide treated diabetic rats ($8.4 \pm 1.1 \mu\text{U/l}$) indicating the presence of remnant pancreatic β -cells in STZ diabetic rats. There were no significant changes in plasma insulin levels of normal treated rats after the treatment.

Effect of AMAF on insulin sensitivity (HOMA-IR, HOMA- β and QUICKI scores) in normal and diabetic rats

The effect of AMAF on insulin resistance and insulin sensitivity using three mathematical indices, HOMA-IR, HOMA- β and QUICKI scores, in experimental rats before and after treatment are given in

Table 4. HOMA-IR values were increased (6.15 ± 1.11) in diabetic rats compared to those in normal rats (2.54 ± 0.39) indicating increased insulin resistance in the diabetic rats. HOMA- β and QUICKI values were decreased in diabetic rats suggesting the lower insulin sensitivity in these animals when compared to normal rats. After 30 days of AMAF or glibenclamide treatment HOMA-IR, HOMA- β and QUICKI values were significantly improved to near normal levels in diabetic treated groups. HOMA-IR, HOMA- β and QUICKI scores of normal treated rats did not show any changes compared to normal control rats.

Fig. 2. Effect of active fraction of *Anisomeles malabarica* (AMAF) on oral glucose tolerance in normal and diabetic rats. $n = 6$; NC: normal control rats, NT: normal rats treated with AMAF, DC: diabetic control rats, DT: diabetic rats treated with AMAF and DG: diabetic rats treated with glibenclamide. Values represented in mean $\pm$ standard deviation. $\dagger P < 0.0001$ compared with 0h blood glucose levels of normal control group (NC). $* P < 0.001$ compared with the 0h blood glucose levels in the respective group. $** P < 0.0001$ compared with the 0h blood glucose levels in the respective group.

Table 3. Effects of active fraction (AMAF) of *A. malabarica* on fasting blood glucose and plasma insulin levels in normal and diabetic rats. BT: before treatment, AT: After treatment, NC: normal control rats, NT: normal rats treated with AMAF, DC: diabetic control rats, DT: diabetic rats treated with AMAF and DG: diabetic rats treated with glibenclamide. Values are calculated as mean $\pm$ S.D ($n=6$). Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT)

Groups	Blood glucose (mg/dL) at different days during the experimental period					Plasma insulin($\mu\text{U/l}$)	
	BT	8 th day	15 th day	22 nd day	31 st day	BT	AT
NC	84.4 $\pm$ 3.2 ^a	81.6 $\pm$ 3.6 ^a	83.4 $\pm$ 2.9 ^a	83.2 $\pm$ 4.4 ^a	82.6 $\pm$ 3.9 ^a	12.7 $\pm$ 1.6 ^a	12.5 $\pm$ 0.8 ^a
NT	84.6 $\pm$ 6.7 ^a	87.6 $\pm$ 5.6 ^a	82.83 $\pm$ 4.3 ^a	85.8 $\pm$ 2.9 ^a	85.6 $\pm$ 3.1 ^a	11.8 $\pm$ 1.7 ^a	12.1 $\pm$ 1.2 ^a
DC	368.5 $\pm$ 63.0 ^b	371.3 $\pm$ 69.6 ^d	386.1 $\pm$ 77.1 ^c	368.5 $\pm$ 78.3 ^c	349.6 $\pm$ 80.7 ^c	6.6 $\pm$ 0.9 ^c	6.1 $\pm$ 0.6 ^c
DT	369.3 $\pm$ 35.5 ^b	281.8 $\pm$ 12.3 ^b	219.8 $\pm$ 14.3 ^b	130.1 $\pm$ 44.4 ^a	117.8 $\pm$ 21.9 ^a	6.5 $\pm$ 0.8 ^c	9.6 $\pm$ 0.6 ^b
DG	361.5 $\pm$ 25.6 ^b	285.4 $\pm$ 11.6 ^b	273.3 $\pm$ 12.9 ^b	169.1 $\pm$ 21.5 ^b	159.8 $\pm$ 25.4 ^b	6.9 $\pm$ 0.5 ^c	8.4 $\pm$ 1.1 ^b

Table 4. Effect of active fraction (AMAF) of *A. malabarica* on insulin sensitivity in normal and experimental diabetic rats. NC: normal control rats, NT: normal rats treated with AMAF, DC: diabetic control rats, DT: diabetic rats treated with AMAF and DG: diabetic rats treated with glibenclamide. Values are calculated as mean $\pm$ S.D from six rats in each group. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT)

Groups	Before Treatment			After Treatment		
	HOMA-IR	HOMA- β %	QUICKI	HOMA-IR	HOMA- β %	QUICKI
NC	2.54 ^b $\pm$ 0.39	211.68 ^b $\pm$ 22.80	0.332 ^b $\pm$ 0.007	2.53 ^b $\pm$ 0.10	245.57 ^b $\pm$ 56.59	0.332 ^b $\pm$ 0.002
NT	2.45 ^b $\pm$ 0.36	228.61 ^b $\pm$ 10.72	0.334 ^b $\pm$ 0.007	2.53 ^b $\pm$ 0.30	217.68 ^b $\pm$ 32.83	0.331 ^b $\pm$ 0.005
DC	6.15 ^a $\pm$ 1.11	8.18 ^a $\pm$ 2.11	0.295 ^a $\pm$ 0.008	5.61 ^a $\pm$ 0.65	7.42 ^a $\pm$ 1.72	0.298 ^a $\pm$ 0.004
DT	5.95 ^a $\pm$ 0.61	7.72 ^a $\pm$ 1.27	0.295 ^a $\pm$ 0.003	2.82 ^b $\pm$ 0.36	68.38 ^d $\pm$ 20.81	0.326 ^b $\pm$ 0.004
DG	6.18 ^a $\pm$ 0.63	8.40 ^a $\pm$ 0.92	0.294 ^a $\pm$ 0.004	3.30 ^c $\pm$ 0.51	33.69 ^c $\pm$ 9.91	0.320 ^c $\pm$ 0.006

Effect of AMAF on total hemoglobin and glycosylated hemoglobin levels of normal and diabetic rats

Total hemoglobin (Hb) and glycosylated hemoglobin (HbA1c) levels of the experimental groups of rats are shown in Fig. 3. HbA1c of diabetic control rats were significantly higher than those of normal rats. However, the treatment with AMAF significantly decreased the levels of HbA1c (Fig. 3A) in diabetic rats. Glibenclamide treatment has also resulted in improvement in the HbA1c levels of diabetic rats but the improvement is significantly lower than that with AMAF. A reduction in the total hemoglobin levels were observed in diabetic untreated rats when compared to normal rats. In case of diabetic treated rats either with AMAF or glibenclamide the total hemoglobin levels were significantly increased (Fig. 3B). No significant changes in HbA1c or Hb levels were observed in normal treated rats.

LC-SRM/MS and GC-MS analysis of AMAF

Phenolic/flavonoid compounds of AMAF were identified and quantified using LC-SRM/MS by comparing with different standard phenolic compounds. AMAF contained significant levels of leutolin (28 ng/mg), caffeic acid (0.85 ng/mg) and quinic acid (0.52 ng/mg). On the other hand chlorogenic acid and kaempferol were identified but found below quantitation levels in AMAF. The chromatogram of the phenolics/flavonoids is shown in Fig. 4A and their molecular weights, chemical formulae and their retention times are given in Table 5. Twelve fatty acids were detected and identified in AMAF using GC-MS analysis (Table 6). The representative mass spectrogram of AMAF is presented in Fig. 4B. These compounds were detected and identified by their fragmentation pattern and in conjunction with the NIST library.

Fig. 3. Effect of active fraction of *Anisomeles malabarica* (AMAF) on the levels of HbA1c (A) and total hemoglobin (B) in normal and diabetic rats. $n = 6$, NC: normal control rats, NT: normal rats treated with AMAF, DC: diabetic control rats, DT: diabetic rats treated with AMAF and DG: diabetic rats treated with glibenclamide. Values are presented in mean $\pm$ standard deviation. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

Fig. 4. A) LC-SRM/MS phenolics/flavonoid chromatograms of AMAF; B) GC-MS chromatogram of AMAF.

Table 5. Quantification of phenolics/flavonoids in AMAF by LC-SRM/MS analysis. BLQ: Below quantification level

S.No	Compounds	Retention Time (minutes)	Molecular Weight (g/mol)	Molecular formula	Concentration (ng/mg)
1	Quinic acid	1.82	192.17	C ₇ H ₁₂ O ₆	0.52
2	Chlorogenic acid	10.16	354.31	C ₁₆ H ₁₈ O ₉	BLQ
3	Caffeic acid	11.11	180.16	C ₉ H ₈ O ₄	0.85
4	Luteolin	17.01	286.24	C ₁₅ H ₁₀ O ₆	28.40
5	Kaempferol	19.09	286.23	C ₁₅ H ₁₀ O ₆	BLQ

Table 6. The list of compounds present in the AMAF with their retention times, molecular weights and molecular formulas

S.No	Compound name	Retention Time	Molecular weight	Molecular formula
1	Eicosanoic acid/Arachidic Acid	14.73	312.5	C ₂₀ H ₄₀ O ₂
2	2-Methyl-1-hexadecanol	14.78	256.4	C ₁₇ H ₃₆ O
3	Methyl 13-methylpentadecanoate	16.20	270.4	C ₁₇ H ₃₄ O ₂
4	Palmitic anhydride/Hexadecanoic anhydride	17.00	494.8	C ₃₂ H ₆₂ O ₃
5	Oleic acid	17.93	282.4	C ₁₈ H ₃₄ O ₂
6	Methyl 11-(3-pentyl-2-oxiranyl) undecanoate	18.13	312.4	C ₁₉ H ₃₆ O ₃
7	6-octadecenoic acid	18.60	282.4	C ₁₈ H ₃₄ O ₂
8	Hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester	19.35	286.4	C ₁₇ H ₃₄ O ₃
9	9-Hexadecenoic acid/palmitoleic acid	19.87	254.4	C ₁₆ H ₃₀ O ₂
10	10,13-octadecadiynoic acid, methyl ester	20.28	294.4	C ₁₉ H ₃₄ O ₂
11	4-hexyl-1-(7-methoxycarbonylheptyl) [4.4.0]deca-2,5,7-triene	21.48	372.5	C ₂₅ H ₄₀ O ₂
12	3,8,8-Trimethoxy-3-piperidyl-2,2- binaphthalene-1,1',4,4'-tetrone	22.35	487.5	C ₂₈ H ₂₅ NO ₇

Discussion

In recent years, plant-based therapies are getting more attention for the management of diabetes mellitus particularly in the developing countries [6, 18]. Herbs from Lamiaceae family are used for food preservation, culinary flavors and treatment of common illnesses as traditional medicine due to the presence of various phytochemicals and antioxidants [19]. Phenolics are an important class of phytochemicals with significant antioxidant properties which play a potential role in managing chronic diseases including diabetes mellitus [20]. *Anisomeles malabarica* (AM) is an aromatic herb belonging to Lamiaceae family, which is a great source of phenolics and natural antioxidants. Our initial acute treatment studies using different solvent extracts of leaves of *A. malabarica* revealed the maximum anti-hyperglycemic effect of ethyl acetate extract compared to the other solvent (hexane, methanol and aqueous) extracts. Other studies also demonstrated the ethyl acetate soluble active principles in lowering blood glucose levels [21, 22]. An advantage of using ethyl acetate for extraction is, it excludes the glycosides and hyperglycemic components that often interfere with anti-hyperglycemic or hypoglycemic effects of herbal products [23]. Our comparative studies with a known oral hypoglycemic drug, glibenclamide, indicated that the efficacy of the ethyl acetate extract is higher than that of glibenclamide. Additionally the improved glycemic control by oral hypoglycemic agents indicates the presence of remnant pancreatic β -cells in STZ induced diabetic rats or mice [24]. Further bioactivity guided fractionation of the ethyl acetate extract led to the identification of active fraction (AMAF) with higher anti-hyperglycemic activity than that of the solvent extracts. No hypoglycemia was observed during the treatment period with either crude solvent extracts or the active fraction. Further, the efficacy of AMAF at a dosage of 50 mg/kg b.w in reducing the blood glucose levels is much higher than that of the glibenclamide. In general active principle/s enriched fractions, like AMAF in the current study, demonstrate increased anti-hyperglycemic activity at lower doses when compared to plant crude extracts. This may not only be due to anticipated enrichment of the active principle(s) but also because of elimination of potential hyperglycemic compounds.

Oral glucose tolerance study indicated that the active fraction significantly improved the glucose tolerance in diabetic treated rats and it is higher in magnitude compared to that of glibenclamide. Elevated blood glucose stimulates insulin secretion from β -cells of pancreas thereby increasing peripheral glucose consumption and controls the glucose homeostasis through different mechanisms [25]. This is deranged in diabetic condition leading to glucose

intolerance. In our studies AMAF treated diabetic rats' blood glucose levels were brought down to near normal within 3 hours compared to the diabetic control group, indicating the beneficial role of AMAF in glucose utilization. It is well established that the effect of glibenclamide on glucose tolerance has been attributed to the enhanced activity of beta cells of the pancreas resulting in increased secretion of insulin. So the mechanism behind this beneficial effect of AMAF on glucose tolerance potentially may be due to stimulating remnant pancreatic β -cells, regeneration of β -cells or an insulin-like activity.

Decreased levels of total hemoglobin in diabetic rats, in the current study, is not very surprising as anemia was reported commonly in diabetics [26]. Diabetic untreated rats showed significantly increased levels of HbA1c indicating poor glycemic control. In chronic hyperglycemia increased glycosylation of number of proteins including hemoglobin were reported [27]. Administration of AMAF to diabetic rats for 30 days normalized the total hemoglobin and HbA1c levels. It could be due to improved glycemic control after the treatment with the active fraction of *A. malabarica*.

Further to understand the mechanism of action of the AMAF, we measured the insulin levels. The decreased plasma insulin levels of diabetic rats were significantly elevated after the treatment with the active fraction indicating potential β -cell regenerative and/or stimulatory (secretagogue) activity of the AMAF. Previously numerous phytochemical constituents were reported to improve glycemic control by stimulating remnant β -cells, increasing the mass of β -cells and/or enhancing the insulin action [28, 29]. Additionally fasting or postprandial hyperglycemia is a common pathogenesis of diabetes mellitus which is induced by partial pancreatic β -cell destruction and/or insulin resistance [30]. The quantitative assessment of insulin resistance (HOMA-IR) and insulin production/ β -cell function (HOMA- β) are key factors to assess control of diabetes and glucose homeostasis [31]. HOMA-IR is an important measure to evaluate insulin resistance that indicates micro-vascular and macro-vascular complication risk [32]. In our studies, insulin sensitivity (QUIKI) and β -cell function (HOMA- β) indices were improved significantly in diabetic rats treated with AMAF when compared to diabetic control rats. Improvement of β -cell function by the AMAF could be due to regeneration of β -cells or enhanced β -cell function compared to diabetic untreated control rats. HOMA-IR indices of diabetic control rats were significantly elevated when compared to normal control rats. After the oral administration of AMAF to diabetic rats HOMA-IR was brought down to near normal indicating that the AMAF could decrease the insulin resistance through improved tissue sensitivity to insulin or insulin secretagogue activity or both.

In the present study, we used LC-SRM/MS to verify the presence of phenolic compounds in AMAF. Our studies revealed the presence of phenolic compounds such as quinic acid, caffeic acid, leutolin, chlorogenic acid and kaempferol. Some of the beneficial roles of AMAF in STZ induced diabetic animals can be attributed to the phenolic compounds reported here. These compounds were shown to significantly regulate various insulin and diabetes associated pathologies in diabetics. Quinic acid is the polyphenol-rich compound, which has been reported to have significant anti-hyperglycaemic activity with the combination of quercetin [33]. Jung et al., demonstrated the potential anti-hyperglycemic and antioxidant activity of caffeic acid in db/db mice [34]. The natural flavonoids, luteolin, that we identified in AMAF is present in many vegetables and fruits. Luteolin ameliorates insulin resistance and inhibits alpha-amylase and alpha-glucosidase enzyme activities [35, 36]. Similarly, kaempferol is another typical dietary flavonoid that was shown to have many beneficial activities including anti-diabetic, antioxidant, and anti-inflammatory through promoting functions of pancreatic β -cell [37]. It was also shown to have positive effect on lipid metabolism, stimulate insulin-dependent glucose uptake and improve insulin resistance [38]. Chlorogenic acid, one of the most abundant polyphenol compounds in the human diet was also identified by us in AMAF. Previously chlorogenic acid was shown to regulate glucose and lipid metabolisms [39], restore carbohydrate metabolizing enzymes [40] and it was reported to possess anti-diabetic activity [41]. These phenolic compounds, partly or in whole, are responsible for the anti-diabetic activity of *A. malabarica* that we demonstrated in this study.

GC-MS studies have shown the presence of fatty acids, hydrocarbons and unsaturated fatty acids in the AMAF. Unsaturated fatty acids in the active fractions of *Joso* leaf extracts were shown to contribute for the anti-diabetic activity by rising sensitivity of PPAR-receptors and increasing the release of insulin from β -cells of pancreas [42]. Previous reports have been supporting the beneficial effects of omega-3- fatty acids, unsaturated fatty acids and polyunsaturated fatty acids on progression of diabetes, blood glucose concentration, insulin levels and antioxidant parameters in tissues of diabetic humans and animals [43-46]. Recently, Yang et al. [47] showed the beneficial effect of palmitoleic acid in the reduction of body weight increase, hyperglycemia, hypertriglyceridemia, insulin resistance and hepatic lipid accumulation. In this study, we report for the first time the presence of palmitic anhydride (hexadecanoic anhydride), 9-hexadecenoic acid (palmitoleic acid), hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester and methyl 13-methylpentadecanoate in the active fraction of *A. malabarica*. Oleic acid is also present in the active fraction of *A. malabarica*. Oleic acid rich diet improves insulin production, increase insulin-stimulated glucose transport and reduce insulin resistance [48, 49].

The results of our studies indicate that the leaves of *A. malabarica* contain more than one active principle such as the flavonoids (luteolin and kaempferol), phenolic compounds (quinic acid, chlorogenic acid and caffeic acid) and unsaturated fatty acids with anti-hyperglycemic activity. Further studies are underway to characterize the active principles of anti-diabetic activity and understand the molecular mechanisms how these active principles elicit beneficial effect in the treatment of diabetes mellitus.

Conclusion

In conclusion, from the results of the present study it is clear that hexane: ethyl acetate (1:1) fraction (AMAF) of ethyl acetate extract of the leaves of *Anisomeles malabarica* play potential role in the management of diabetes mellitus.

Abbreviations

STZ (streptozotocin); AMAF (active fraction of *Anisomeles malabarica*); LC-SRM/MS (Liquid Chromatography-Selected Reaction Monitoring/Mass Spectrometry); GC-MS (Gas Chromatography-Mass Spectrometry); FBG (fasting blood glucose); OGT (oral glucose tolerance); HbA1c (glycosylated hemoglobin A1c; Hb, total hemoglobin); HOMA-IR (homeostasis model assessment for insulin resistance); HOMA- β (homeostasis model assessment for β -cell function); QUICKI (quantitative insulin sensitivity check index).

Acknowledgements

Peddanna Kotha received Junior Research Fellowship from Department of Science and Technology-Promotion of University Scientific Excellence (DST-PURSE), Sri Venkateswara University, Tirupati; Authors acknowledge the support of Indian Institute of Technology (IIT-Madras), Chennai for GC-MS studies; Padma Ramakrishnan, C-CAMP/NCBS MS-Facility, Bangalore and Dr. EGTV Kumar, SVIMS, Tirupati for insulin studies.

Disclosure Statement

The authors declare that there is no conflict of interest.

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