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Inhibitory effect of banana (*Musa* sp. var. Nanjangud rasa bale) flower extract and its constituents Umbelliferone and Lupeol on α-glucosidase, aldose reductase and glycation at multiple stages

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ABSTRACT

Postprandial hyperglycaemia is characterized as the earliest symptom of diabetes and its management attenuates several of the associated secondary complications. In this context, we investigated the role of ethanol extract of banana flower (EF) for its antihyperglycaemic effects. The EF showed a strong inhibition towards α-glucosidase and pancreatic amylase which play a vital role in clinical management of postprandial hyperglycaemia. The major active compounds present in EF were identified as Umbelliferone (C1) and Lupeol (C2) using various spectroscopic methods. C1 (IC_{50}: 7.08 ± 0.17 μg/ml) and C2 (IC_{50}: 7.18 ± 0.14 μg/ml) were found to inhibit α-glucosidase in a non-competitive mode of inhibition, with low K_i values. Further, *in vitro* glycation assays showed that EF and its compounds prevented each stage of protein glycation and formation of its intermediary compounds. EF, C1 and C2 also exhibited a potent inhibition on aldose reductase with IC_{50} values of 2.25 ± 0.29, 1.32 ± 0.22 & 1.53 ± 0.29 μg/ml respectively. Our results suggest that, the observed potential of EF in antihyperglycaemic activity *via* inhibition of α-glucosidase and in antidiabetogenic effect by inhibition of polyol pathway and protein glycation is more likely to be attributed to the presence of C1 and C2.

1. Introduction

Diabetes mellitus, a chronic metabolic condition is attributed to an impaired regulation of carbohydrate and lipid metabolism by inappropriate functioning of pancreatic hormone, insulin, which is characterized by increased blood glucose levels. According to the International Diabetes Federation (IDF), the number of diabetic patients is likely to reach 592 million by the year 2035 which will reflect on the cost for treating it which is expected to reach US $548 billion, annually (International Diabetes Federation, 2013). Rapid absorption of dietary carbohydrates aided by glycoside hydrolases (α-glucosidase, α-amylase) converts them into simpler monosaccharide units, which results in elevated blood glucose levels characterized as postprandial hyperglycaemia. This is distinguished as the earliest symptom of diabetes, and the use of glycoside inhibitors is widely accepted as an efficient method in restraining postprandial hyperglycaemia by inhibiting the release of free glucose units facilitating a smooth glucose profile (Ramith et al., 2014). Prolonged postprandial hyperglycaemia leads to diverse secondary complications all of which result in a series of diabetic complications *viz.*, neuropathy, retinopathy and nephropathy. High blood glucose levels cause a significant flux of glucose into the polyol pathway to convert them into sorbitol by the action of aldose reductase (Kador et al., 1985). Diabetic state leads to overproduction of sorbitol, which in turn leads to its accumulation in tissues *viz.*, nerves, kidney, retina and lens due to impaired membrane permeability or reduced metabolism by sorbitol dehydrogenase. Sorbitol accumulation is associated with various microvascular complications and cardiovascular diseases which can be efficiently averted by inhibition of the key enzyme aldose reductase (Peter et al., 1985). Other major concern in diabetes is glycation of proteins resulting in partial loss of its activity as a consequence of perpetual hyperglycaemia. A non-enzymatic nucleophilic addition reaction of the carbonyl residue of sugar with the free amino group of proteins forming a reversible Schiff base, which further forms a more stable Amadori product constitutes the primary step in protein glycation. The Amadori products subsequently undergo a series of reactions *via* the dicarbonyl intermediates resulting in less characterized compounds known as the advanced glycation end products (AGEs) which subsequently accumulate in the tissues and are responsible for the aetiology of diabetic micro- and macro-vascular complications (Nessar, 2005). Consequently, the identification of compounds that inhibit each stage of glycation as well as the formation of intermediary products in the pathway proves useful in

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generation of novel therapeutic interventions to delay and prevent these complications (Samuel and James, 2003). Also, the glycated proteins and AGEs by interfering in the signal transduction cascade induce the generation of reactive oxygen species (ROS) concomitantly resulting in oxidative stress, which constitutes key factors in the progression of several vascular complications in diabetes (Su-Yen and Mark, 2008). The paramount method of protection from ROS mediated damage is provided by the antioxidants which neutralize the generated free radicals. Collectively, a therapy that involves an integrated antioxidant and antiglycation properties with a potent α-glucosidase inhibition and lower α-amylase inhibition can prove optimal in control of diabetes and its complications (Prathapan et al., 2012).

In this regard, owing to the harmful effects of the long known synthetic antidiabetic drugs, recently there has been increasing focus on natural substances which have inhibitory potential on the development of hyperglycaemia and its associated complications. Banana fruit consumed worldwide is known for several beneficial properties. Banana flower, one of the secondary products of banana cultivation, has been consumed as vegetable in many countries (Joshi, 2000). It is a part of traditional medicine, used in treatment of various disorders like kidney stone and ulcers (Ivan, 2003). Its antihyperglycaemic effects have also been accounted and included in some of the Ayurvedic formulations in the treatment of diabetes (Bhaskar et al., 2011; Joshi, 2000). However, the mechanism underlying and the active principles responsible for the beneficial role are less exploited. Hence, the aim of the present study is to evaluate the potential of ethanol extract of banana flower (EF) in inhibiting α-glucosidase and α-amylase that are responsible for hyperglycaemia as well as inhibition of aldose reductase and protein glycation that are responsible for various diabetic complications. Glycation markers at each stage of glycation *viz.*, fructosamines (early stage), protein carbonyls (intermediate stage) and AGEs (late stage) were also evaluated. Besides, isolation of active compounds responsible for the beneficial properties was carried out. The findings could become a rationale to include EF as an effective therapeutic adjunct for management of hyperglycaemia and associated complications.

2. Materials and methods

2.1. Chemicals

Butylated hydroxyl anisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS), phenazine methosulfate (PMS), nitro blue tetrazolium (NBT), gallic acid, dinitro phenyl hydrazine (DNPH), L-cysteine, porcine pancreatic α-amylase (EC 3.2.1.1), α-glucosidase (EC 3.2.1.20) from *Saccharomyces cerevisiae*, *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG), β-NADPH and aminoguanidine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Thiobarbituric acid (TBA), trichloro acetic acid (TCA), sodium carbonate and nicotinamide adenine dinucleotide-reduced (NADH) were obtained from Sisco Research laboratories (Mumbai, India). Acarbose (Glucobay, 50 mg) was obtained from Bayer India (Thane, India). Reagents and solvents used for extraction and silica-gel for column chromatography were procured from Merck (Mumbai, India). All of the other reagents were of analytical grade.

2.2. Plant material

Immaculate inflorescences of *Musa* sp. cv. Nanjangud rasa bale were collected from banana cultivating farms of Nanjangud, Karnataka, India. The specimen was identified by the Department of Horticulture, Government of Karnataka, Mysore, India. Flowers were separated from the inflorescence followed by discarding the spathe. The isolated flowers were cleaned, cut into small pieces and dried at 40 °C in an oven. This was powdered using a homogenizer and further stored at 4 °C until use.

2.3. Extraction

The coarse powder was subjected to hot extraction using ethanol in a Soxhlet apparatus. Extraction was performed twice with 95% ethanol (500 ml) and filtered. The resulting filtrate was concentrated under vacuum using rotary evaporator (Rotavapor R-200, Buchi, Switzerland) and the yield of ethanol extract was recorded. Subsequently, ethanol extract of banana flower (EF) was subjected to preliminary phytochemical screening to identify the phytoconstituents present based on standard protocols (Harbone, 1973). The total phenol content (TPC) for the EF was estimated as per Shuxia et al. (2013).

2.4. α-Amylase inhibition assay

The α-amylase (EC 3.2.1.1, categorised as type-VI B porcine pancreatic α-amylase) inhibition was assayed according to the procedure described by Worthington (1993) with slight modifications. The α-amylase activity was determined, using soluble starch (1%) as a substrate in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl). The enzyme (0.5 mg/ml) dissolved in 500 μl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) and 500 μl (0–1000 μg/ml) of EF (dissolved in DMSO) were pre-incubated for 10 min at 25 °C. The residual α-amylase activity was evaluated by adding 1% starch solution (500 μl) and incubation at 25 °C for 15 min. The reaction was stopped by adding 1000 μl of DNS reagent and kept in a boiling water bath (85 °C) for 5 min. The resulting mixture was diluted by 10 ml distilled water and absorbance (A) was measured at 540 nm in a spectrophotometer (Shimadzu UV-1800). Subsequently, the absorbance of blank (without enzyme) was subtracted from each test sample (EF) and the results were compared with the control (without analyte). Acarbose was used as a positive control. The α-amylase inhibitory activity was expressed in percent inhibition.

$$\text{Inhibition}\ (\%) = \left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}} \times 100$$

IC_{50} values were determined from a curve relating the % inhibition of each sample to the concentration of sample.

2.5. Yeast α-glucosidase inhibition assay

The yeast α-glucosidase (EC 3.2.1.20, categorised as type-1 α-glucosidase) inhibition was assayed using the substrate *p*NPG according to the modified method described by Worawalai et al. (2012). In short, 700 μl phosphate buffer (50 mM, pH 6.8) and 100 μl EF with varying concentrations dissolved in dimethyl sulfoxide (DMSO) were mixed prior to the addition of 100 μl of yeast α-glucosidase (0.4 U/ml). One unit of α-glucosidase is defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol formed from *p*NPG per minute under the assay conditions specified. The mixture was pre-incubated for 10 min at 37 °C. After incubation, 100 μl of 0.5 mM *p*NPG solution in 50 mM phosphate buffer (pH 6.8) was added and the reaction was maintained at 37 °C for 20 min. The reaction was terminated by adding 250 μl 0.1 M Na_2CO_3. Enzyme activity was determined by measuring the absorbance of the liberated *p*-nitrophenol from *p*NPG at 405 nm using micro-plate reader (Spectramax 340, Molecular Devices, Sunnyvale, USA). The absorbance was compared with the control, containing buffer instead of test sample. Acarbose was used as a positive control. The results were expressed as percent α-glucosidase inhibition obtained using the formula given below:

$$\text{Inhibition}\ (\%) = \left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}} \times 100.$$

Each experiment was performed in triplicates, along with appropriate blanks. The concentration required to inhibit 50% of the α-

glucosidase activity under the specified assay conditions was described as the IC_{50}.

2.6. Aldose reductase (AR) inhibition assay

Human recombinant AR activity was measured according to the procedure of Nishimura et al. (1991) with slight modifications. In brief, reaction mixture consisted of 870 μl sodium phosphate buffer (100 mM, pH 6.2), 50 μl NADPH (3 mM), 10 μl AR enzyme (0.2 U/ml) and 20 μl of EF (dissolved in DMSO) with diverse concentration, in a final volume of 1000 μl. The enzyme reaction was initiated by the addition of 50 μl substrate (DL-glyceraldehyde, 5 mM) and incubated for 3 min at 25 °C. Enzyme activity was determined by measuring the absorbance (decrease in NADPH absorption) at 340 nm over a 10 min period. The inhibitory activity was evaluated by comparing the absorbance change of test to that of the control. All the tests were performed in triplicates and the IC_{50} was calculated as described in α-amylase inhibition assay.

2.7. Evaluation of antiglycation potential

2.7.1. In vitro bovine serum albumin (BSA) glycation

The formation of albumin glycation was assayed using BSA/fructose system according to the modified method described by McPherson et al. (1988). The assay was performed by adding 1 ml BSA (10 mg/ml) in 4 ml assay system containing 1 ml fructose (300 mM), 1 ml phosphate buffer (100 mM, pH 7.4 with 0.02% sodium azide) along with EF (test) or aminoguanidine (positive control) at different concentrations. Reaction mixture was incubated for 21 days at 37 °C in sterile conditions. Prior to incubation, filtration of reaction mixture through 0.22 μm membrane filters was performed under aseptic conditions to sustain sterility. Subsequently, samples free of microbial contamination were subjected to dialysis against phosphate buffer (100 mM, pH 7.4) and kept frozen at −80 °C until analysis. The control (EF replaced by carrier solvent) was sustained under analogous state and all the incubations were performed in triplicates.

2.7.2. Analysis of AGE formation

The fluorescence intensities of the resulting AGE formed (fluorescent products) from albumin glycation and control were determined using spectral scanning multimode reader (Thermo Scientific, Varioskan Flash) at excitation and emission wavelengths of 370 and 440 nm (with slit = 10 nm) respectively. The fluorescence of control and test (EF) was blanked against BSA and apposite treatment controls. The corrected fluorescence readings (F) of the control (F_c) and the test (F_T) were acquired to resolve the percent inhibition of AGE formation and calculated as follows:

$$\text{Inhibition}\ (\%) = (F_c - F_T)/F_c \times 100.$$

2.7.3. Analysis of fructosamine

The formation of fructosamine (an Amadori product) in the glycated BSA and the control was assessed using NBT assay described by Johnson et al. (1983) with slight modifications. In brief, 200 μl glycated samples and control were mixed with 0.8 ml of carbonate buffer (100 mM, pH 10.4) and 0.5 mM NBT and incubated at 37 °C. Absorbance was measured at 530 nm in a spectrophotometer at 10 and 15 min time intervals. The concentration of fructosamine was determined using 1-deoxy-1-morpholino-fructose (1-DMF) as standard. The inhibitory activity against fructosamine was calculated by comparing the absorbance change of test samples (A_T) to that of the control (A_c), where the test samples were replaced by carrier solvent and calculated by the equation below:

$$\text{Inhibition}\ (\%) = (A_c - A_T)/A_c \times 100.$$

2.7.4. Analysis of protein carbonyl content

The carbonyl group, a marker for protein oxidative damage was assayed using glycated BSA according to the method described by Koji et al. (1998) with slight modifications. In brief, 400 μl of glycated protein samples and control were mixed with 600 μl of DNPH (0.1% in 2 M HCl) and incubated for 60 min at 37 °C. The proteins in the reaction mixture were precipitated with 500 μl TCA (20% w/v). Consequently, the precipitate was cleansed thrice with ethanol:ethyl acetate (1:1 v/v) following centrifugation. All the preparations were performed at 4 °C. The protein sample was then solubilised with 2 ml of 8 M guanidine chloride and absorbance was recorded at 365 nm. The results were expressed as nmol carbonyl/mg protein based on molar extinction coefficient (365 nm = 21 mM per cm) of DNPH. Percent inhibition of protein carbonyl content by test samples was calculated using the equation as described for fructosamine.

2.7.5. Analysis of protein thiol content

The free thiols in the glycated samples and the control were estimated by Ellman's method (Ellman, 1959). The concentration of free thiols was determined using L-cysteine as standard and expressed as nmol/mg protein. Results were expressed as percent protection to protein thiols by test samples in comparison with the control.

2.8. Antioxidant assays

In this study, three principle methods, DPPH free radical, ABTS cation radical and superoxide anion radical scavenging activities were employed and measured as per Nurhanani et al. (2008). Radical scavenging potential was expressed as EC_{50} values. An EC_{50} value represents 50% of free, cation and anion radicals scavenged by the test samples. All the samples were analysed in triplicates. BHA was used as positive control.

2.9. Assay of lipid peroxidation inhibition

The antioxidative action of EF and its constituents was determined in a liposome model (Lipid peroxidation inhibition in biological systems) as described by Meghashri et al. (2010). Results were expressed as EC_{50} values as described in antioxidant assays.

2.10. Reducing power assay

Reducing power of iron ion was determined according to the method reported by Yen et al. (2002) with slight modifications. In brief, 0.75 ml EF of varying concentrations was mixed with equal volume of 0.2 M phosphate buffer (pH 6.6) and 0.75 ml 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min and subsequently mixed with 0.75 ml TCA (10%) and centrifuged at 3000 rpm for 10 min. The supernatant was mixed with equal volume of distilled water and 0.1% ferric chloride (0.5 ml). The absorbance of the resulting solution was measured at 700 nm. Ascorbic acid was used as reference standard and phosphate buffer as blank solution. An increase in the absorbance of the reaction mixture was taken as a measure of reducing power.

2.11. Metal ion chelating activity

The ferrous ion chelating activity was measured by the ferrous ion–ferrozine complex at 562 nm. The ability of the test samples to chelate ferrous ion was carried out as described by Meghashri et al. (2010).

2.12. Isolation and identification of bioactive components in EF

The crude EF (25 g each) was subjected to silica-gel (100–200 mesh) column (length 100 cm and 3 cm diameter) chromatography (elution rate of 2 ml/min flow with a total elution of 600 ml). It was eluted with diverse solvents (100 ml each) ranging from non-polar to polar

series *viz.*, ether:chloroform (1:1), ethyl acetate, acetone, ethanol, methanol and water. The fractions were collected and spotted over pre-coated silica gel F254 plates (20 × 20 cm, Merck, Germany). The optimum resolution was attained in the combination of solvent system n-butanol, water and acetic acid (5:4:1 v/v). To visualize the spot, TLC plate was exposed to iodine fumes. The fractions (Fr) with similar retention factor (R_f) in TLC pattern were pooled together. Hence, methanol (Fr. I-16–20) and ethanol (Fr. I-21–25) fractions with same R_f were pooled, yet again subjected to silica-gel column chromatography and eluted stepwise with linear gradients of methanol:ethanol (90:10; 80:20; 70:30; 60:40; 50:50; 20:80; 100:0 v/v). A single spot on TLC with n-butanol, water and acetic acid (5:4:1 v/v) mobile phase led to the isolation of Umbelliferone (96 mg) and Lupeol (90 mg) from Fr. II (36–40) and Fr. II (46–50) fractions respectively. The amount of compounds (1) and (2) were found to be 0.38% and 0.36%, respectively.

2.12.1. Nuclear magnetic spectroscopy (NMR) analysis

The NMR spectra were recorded on a Bruker DRX-400 spectrometer (Bruker Biospin Co., Karlsruhe, Germany) with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz. The isolated compounds were prepared using deuterated solvent with tetramethylsilane (TMS) as an internal standard in 5 mm NMR tubes. Data were measured in $CDCl_3$ with chemical shifts according to the TMS signal and were expressed in parts per million (δ).

2.12.2. High-performance liquid chromatography mass spectrometer

The mass spectrum was obtained using LCMS2010A (Shimadzu, Japan) having probes APCI & ESI. The separation was performed on a C-18 column (Agilent Technologies, Palo Alto, CA, USA) using a mobile phase consisting of methanol/water (90:10). Flow rate was maintained at 0.2 ml/min and the elutes were monitored at 254 nm. The mass spectrum (MS) was obtained in ESI mode at CDL temperature at 250 °C, block temperature at 200 °C, detector voltage at 1.6 kV and with nitrogen nebulizer gas flow at 1.5 l/min.

2.12.3. Other analyses

The IR spectra were recorded using KBr discs on a NICOLET 380 FT IR spectrometer (Thermo Fisher Scientific, France) in the range of 400 to 4000 nm. The ultraviolet (UV) spectra of the compounds in methanol were recorded on a Shimadzu UV-1800 spectrophotometer. The melting points were determined on an electrically heated VMP-III melting point apparatus and were uncorrected. Further elemental analysis of the compounds was performed on a Perkin Elmer 2400 elemental analyzer.

2.13. Kinetics of α-glucosidase inhibition

The enzyme kinetics on inhibition of α-glucosidase activity by the isolated compounds were studied using diverse concentrations of substrate against IC_{20}, IC_{40} and IC_{60} inhibitory concentrations of the isolated compounds. The type of inhibition, K_m and V_{max} were determined by double reciprocal plot (Lineweaver and Burk, 1934) of the substrate concentration and the velocity (1/V *versus* 1/[pNPG]). Further, the inhibitory constant (K_i) was determined by Dixon plot (Dixon, 1953).

2.14. Statistical analysis

The experiments were done in triplicates. Results were expressed as mean ± SE. Statistical comparisons between the treatment groups and control were performed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test using SPSS Software (version 21.0, Chicago, USA). The results were considered statistically significant if the 'p' values were 0.05 or less. GraphPad PRISM software (version 4.03) was used for calculating IC_{50} values.

3. Results

In this study, EF exhibited an effective inhibition on α-glucosidase at a more significant level than the drug acarbose, with a higher free radical scavenging activity in all the assays tested. In addition, EF was effective in inhibiting the polyol pathway by inhibition of the key enzyme, aldose reductase and prevented the formation of advanced glycation end products remarkably better than the positive control. These findings suggested the presence of potential inhibitory compounds in the extract. Hence an attempt was made to identify the potential bioactive principles in EF responsible for its antiglycaemic activity.

3.1. Isolation and identification of Umbelliferone and Lupeol from EF

The preliminary phytochemical investigation of EF showed the presence of triterpenoids, coumarins and phenolic compounds. To characterize the bioactive components responsible for inhibition of α-glucosidase, EF was subjected to column chromatography. Repeated chromatographic separations through silica gel column chromatography lead to the identification of Umbelliferone and Lupeol (Fig. 1). The structural elucidation of the compounds was determined on various physicochemical and spectroscopic methods (UV, IR, ^{1}H NMR, ^{13}C NMR and MS). The elucidation of their structures is as follows.

Umbelliferone (C1): obtained from Fr. II (36–40) as light yellow semi-solid. m.p. 225 °C. UV (MeOH): λ max 324 nm. IR (KBr) 3610–3590 cm^{-1} (O–H). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.6 (1H, bs, OH), 6.46 (1H, d, H-3), 6.61 (1H, s, H-8), 6.66 (1H, d, H-6), 7.44 (1H, d, H-5), 7.8 (1H, d, H-4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.6 (C-2), 116.2 (C-3), 145.3 (C-4), 128.4 (C-5), 112.6 (C-6), 157.5 (C-7), 108.6 (C-8), 152.2 (C-9), 120.2 (C-10). EIMS for $C_9H_6O_3$ m/z: 162 [M + 1]. Analytical calculated data for ($C_9H_6O_3$): C, 66.67; H, 3.73%. Found C, 66.69; H, 3.75%. The identity of the compound was deciphered on the basis of the above results as well in comparison with the NMR and MS data in the literatures (Rajbir et al., 2010).

Lupeol (C2): obtained from Fr. II (46–50) as creamish yellow semi-solid. m.p. 214 °C. UV (MeOH): λ max 200 nm. IR (KBr) 3610–3590 cm^{-1} (O–H). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.58, 4.62 (2H, s, H-29a, 29b), 3.19 (1H, dd, J = 4.79, 11.00 Hz, H-3), 0.74, 0.77, 0.86, 0.95, 0.96, 1.05, 1.26 (each 3H, s, Me × 7). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 151.3 (C-20), 109.4 (C-29), 79.5 (C-3), 55.4 (C-5), 50.7 (C-9), 48.4 (C-18), 48.5 (C-19), 43.4 (C-17), 43.3 (C-14), 41.2 (C-8), 40.3 (C-22), 39.2 (C-13), 38.6 (C-4), 38.7 (C-1), 37.5 (C-10), 35.6 (C-16), 34.4 (C-7), 30.2 (C-21), 28.3 (C-23), 27.6 (C-15), 27.7 (C-12), 25.1 (C-2), 21.3 (C-11), 19.4 (C-30), 18.2 (C-6), 18.3 (C-28), 16.4 (C-25), 16.5 (C-26), 15.6 (C-24), 14.6 (C-27). EIMS for $C_{30}H_{50}O$ m/z: 427 [M + 1]. Analytical calculated data for ($C_{31}H_{52}O$): C, 84.44; H, 11.81%. Found C, 84.47; H, 11.85%. On the basis of the above results as well in comparison with the NMR and MS data in the literature (Aynilian et al., 1972), compound (2) was identified as Lupeol.

3.2. Inhibitory effects on yeast α-glucosidase and α-amylase

In vitro α-glucosidase inhibitory studies recognized that EF and its active compounds had effective inhibitory potential. The IC_{50} values were found to be 7.79, 7.08 and 7.18 μg/ml for EF, C1 and C2 respectively. Acarbose, investigated under same conditions had IC_{50} value of 9.68 μg/ml, therefore, EF, C1 and C2 showed higher inhibitory effect in this assay. In terms of IC_{50} values, it is evident that C1 and C2 possessed a strong inhibition on yeast α-glucosidase and were significantly higher ($p < 0.05$) than acarbose and EF (Table 1). The inhibition ascended in the order: C1 > C2 > EF > Acarbose.

Furthermore, similar studies were conducted to evaluate whether EF and its constituents also inhibited α-amylase, another key carbohydrate hydrolyzing enzyme. The 50% inhibition of α-amylase by EF and its active compounds is detailed in Table 1. Results showed that C1 (IC_{50}:

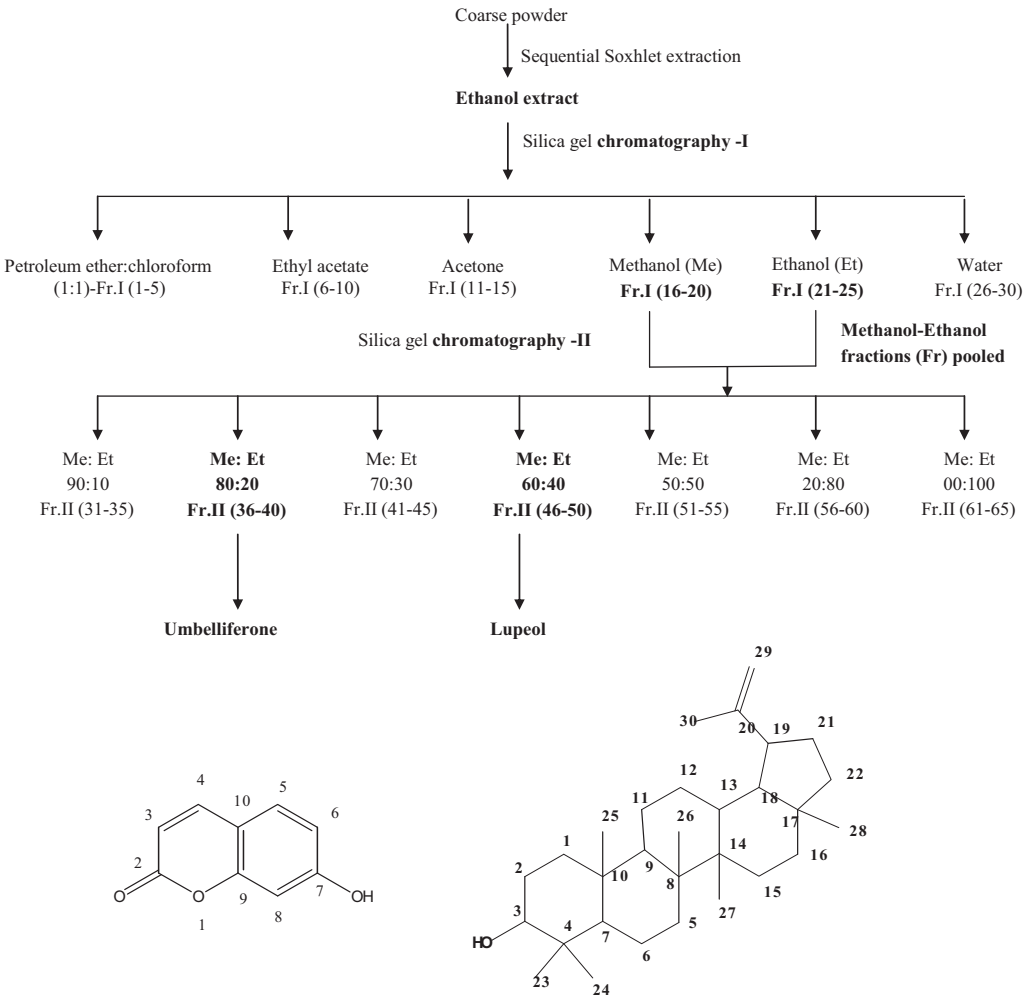

Fig. 1. Separation scheme of active compounds from ethanol extract of banana flower and structures of isolated compounds.

32.26 μg/ml) possessed the highest inhibitory activity as compared to C2 (IC_{50}: 32.90 μg/ml), whereas EF (IC_{50}: 36.05 μg/ml) had the lowest inhibitory effect. The α-amylase inhibitory effect (based on IC_{50} values) of EF, C1 and C2 was comparatively lower ($p < 0.05$) than the therapeutic drug acarbose (IC_{50}: 29.71 μg/ml).

3.3. Kinetic analysis of α-glucosidase inhibition by C1 and C2

C1 and C2 were selected for further kinetic inhibition experiments against yeast α-glucosidase since it wielded a remarkable inhibitory activity. For kinetic studies, enzyme was incubated with designated concentrations of substrate *p*NPG in the absence (control) or presence of IC_{20}, IC_{40} and IC_{60} inhibitory concentrations of C1 and C2. Mode of inhibition, [K_m] and [V_{max}] values were determined by graphical means using Lineweaver–Burk plots. Lineweaver–Burk plots revealed that the intersecting point for different concentrations of C1 (Fig. 2A) and C2 (Fig. 2B) arises from the same x-intercept as uninhibited enzyme other than diverse slopes and y-intercepts. With the increasing concentrations of C1 and C2, both the slope and the vertical axis intercept increased, whereas, the horizontal axis intercept ($-1/K_m$) remained the same. The kinetic results established that C1 and C2 retarded the maximum velocity (V_{max}) of the reaction (with increasing concentrations) catalysed by α-glucosidase without much change in K_m values (Table 2). These results indicated that the mechanism of α-glucosidase inhibition was reversible, corresponding to the classical pattern of non-competitive inhibition. The inhibitory constant (K_i), determined from Dixon plots for α-glucosidase was 1.61 and 2.87 μg of C1 and C2 respectively.

Table 1
Inhibitory activities of ethanol extract of flower (EF) and its isolated compounds on α-amylase, α-glucosidase and aldose reductase enzymes.

Enzymes	IC_{50}[x,y] (μg/ml)			
	EF	Umbelliferone	Lupeol	Acarbose/*quercetin
α-Amylase	36.05 ± 0.48[c]	32.26 ± 1.80[b]	32.90 ± 1.18[b]	29.71 ± 1.51[a]
α-Glucosidase	7.79 ± 0.11[b]	7.08 ± 0.17[a]	7.18 ± 0.14[a]	9.68 ± 0.48[c]
Aldose reductase	2.25 ± 0.29[b]	1.32 ± 0.22[a]	1.53 ± 0.29[a]	*3.42 ± 0.14[c]

[x] Values are expressed as mean ± SE. Means in the same row with distinct superscripts are significantly different ($p \leq 0.05$) as separated by Duncan's multiple range test.
[y] The IC_{50} value is defined as the inhibitor concentration to inhibit 50% of enzyme activity under assay conditions.
* Quercetin was used as positive control in aldose reductase assay.

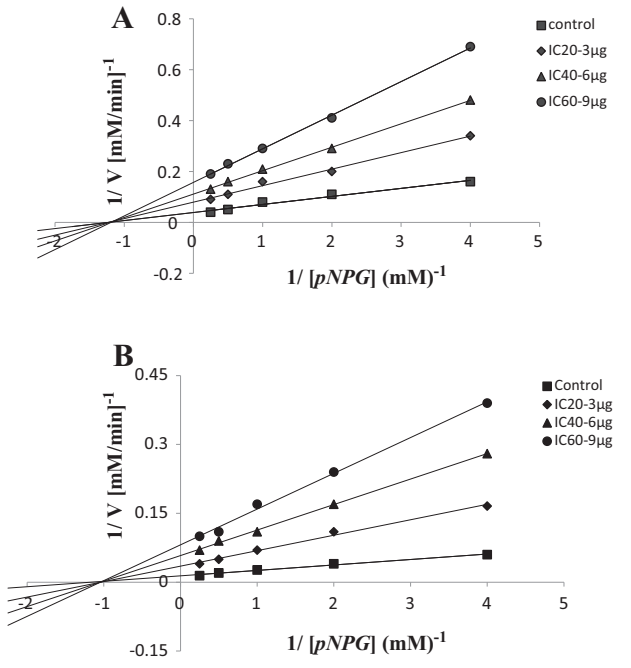

Fig. 2. Lineweaver–Burk plot of substrate dependent enzyme kinetics on inhibition of α-glucosidase activity by Umbelliferone (A) and Lupeol (B). α-Glucosidase was incubated with diverse concentrations of *p*-nitrophenyl-α-D-glucopyranoside (pNPG, 0.25–4 mM) in the absence or presence of Umbelliferone and Lupeol at IC_{20}, IC_{40} and IC_{60} inhibiting concentrations.

3.4. Aldose reductase inhibitory potential

The inhibitory effect of EF and its constituents on aldose reductase was found to be 2-fold higher than quercetin, the phenolic inhibitor. EF inhibited aldose reductase with an IC_{50} of 2.25 μg/ml (Table 1). C2 (IC_{50}: 1.53 μg/ml) isolated from EF was found to be a potent inhibitor, but nonetheless the inhibition was slightly lower than C1 (IC_{50}: 1.32 μg/ml). On the whole, EF and its constituents were stronger inhibitors ($p < 0.05$) than quercetin.

3.5. Antioxidant ability and TPC

The free radical scavenging ability of EF and its constituents was measured by employing a battery of *in vitro* assays *viz.*, DPPH, ABTS and Superoxide while BHA was used as a positive control. Results expressed as EC_{50} values (μg of tests per ml) summarized in Table 3, indicated that the isolated compounds C1 and C2 were comparatively higher ($p < 0.05$) than EF in radical scavenging activities. In all the assays used in this study, EF, C1 and C2 were 2-fold effective than BHA, and activities ascended in the order C1 > C2 > EF > BHA. In general, C1 isolated from EF exhibited a higher free radical scavenging activity in three antioxidant assays while C2 was relatively lower (based on EC_{50} values) but statistically similar ($p < 0.05$) to C1. The results revealed that C1 and C2 possess strong antioxidant ability and are significantly higher ($p < 0.05$) than the positive control.

The ethanol extract of banana was found to have high phenolic content (227 mg GAE/g) with an extraction yield of 125.08 g/kg. The results implied that the content of total phenols was concentrated in the EF and the compounds isolated from these active extract showed high TPC in the same order: C1 (36.52) > C2 (28.28 mg GAE/g respectively) of antioxidant assays.

3.6. Reducing power

The increase in the absorbance at 700 nm of the reaction mixture caused by the EF, C1 and C2 was descriptive of their increased reducing power. As shown in Fig. 3E it is evident that EF and its constituents exhibited increased reducing power with the increased concentration as ascorbic acid. The reducing power decreased in the order of ascorbic acid > C1 > C2 > EF, in the increasing concentrations (0.2–1 mg/ml).

3.7. Lipid peroxidation inhibitory activity

The lipid peroxidation inhibitory activity of EF and its constituents is shown in Table 3. The ethanolic extract (EC_{50}: 36.43 μg/ml) displayed 1.3 fold higher inhibitory activity compared to the standard antioxidant, BHA (EC_{50}: 46.72 μg/ml). The EC_{50} value for C2 was found to be 27.97 μg/ml and C1 showed an EC_{50} value of 23.81 μg/ml, which is more active than C2. All the compounds and EF exhibited a higher inhibitory effect, with the order: C1 > C2 > EF > BHA.

3.8. Metal chelating activity

Activity of test samples on chelating the ferrous ions is shown in Table 3. EF showed 50% chelating ability at the concentration of 52.53 μg/ml. The EC_{50} value of BHA was 67.58 μg/ml. Both the compounds C1 (33.89 μg/ml) and C2 (39.20 μg/ml) exhibited higher metal chelating activity than BHA. The results illustrated that EF and its constituents possess ion chelating effects and are significantly higher ($p < 0.05$) than BHA.

3.9. Antiglycation potential at various levels

The inhibitory effects of diverse concentrations of EF, C1, C2 and aminoguanidine (25, 50 and 100 μg/ml) on early glycation product (fructosamine), intermediate (protein carbonyls) and late stage glycation moieties (AGEs) after 3 weeks of incubation are shown in Fig. 3A–C. Incubation with EF and its constituents inhibited albumin glycation at various stages in a dose dependent fashion. At the end of the study period, it was evident that both C1 and C2 exhibited higher inhibitory activities (in all the levels) compared to EF with diverse concentrations. At a concentration of 100 μg/ml, the formation of

Table 2
Enzyme kinetics of α-glucosidase by Umbelliferone (1) and Lupeol (2).

Compounds	Treatment	Mode of inhibition[x]	K_m (mM)	V_{max} (10^3(μM/min)$^{-1}$)	K_i (μg)[y,z]
1	Control	Non-competitive	0.85	76.92	1.61 ± 0.13
	IC_{20} 3 μg		0.94	28.57	
	IC_{40} 6 μg		0.96	17.54	
	IC_{60} 9 μg		0.95	12.35	
2	Control	Non-competitive	0.80	25.64	2.87 ± 0.16
	IC_{20} 3 μg		0.81	12.66	
	IC_{40} 6 μg		0.82	9.01	
	IC_{60}9 μg		0.83	6.37	

[x] Inhibition mode was determined from Lineweaver and Burk plot.
[y] K_i = dissociation constant.
[z] Values are expressed as mean ± SE.

Table 3
Yield, antioxidant activity and total phenolic contents (TPC) of ethanol extract of flower (EF) and its isolated compounds.

Sample	Yield (g/kg)	TPC (mg GAE/g)	EC_{50}[x,y] (μg/ml)				
			Radical scavenging activities			Lipid peroxidation	Metal chelation
			DPPH	ABTS	Superoxide		
EF	125.08 ± 1.84[b]	227.08 ± 1.92[c]	28.85 ± 3.42[b]	27.17 ± 1.81[b]	53.39 ± 4.06[b]	36.43 ± 0.83[c]	52.53 ± 0.22[c]
Umbelliferone	3.85 ± 0.14[b]	36.52 ± 2.28[b]	21.70 ± 0.37[a]	21.99 ± 0.28[a]	47.39 ± 1.68[a]	23.81 ± 1.60[a]	33.89 ± 1.34[a]
Lupeol	3.63 ± 0.09[a]	28.28 ± 1.27[a]	24.14 ± 1.82[a]	22.05 ± 0.80[a]	48.79 ± 0.61[a]	27.97 ± 1.16[b]	39.20 ± 1.64[b]
BHA	–	–	40.43 ± 0.81[c]	32.31 ± 1.22[c]	73.04 ± 0.93[c]	46.72 ± 0.42[d]	67.58 ± 1.05[d]

[x] Values are expressed as mean ± SE. Means in the same column with distinct superscripts are significantly different ($p \leq 0.05$) as separated by Duncan's multiple range test.
[y] The EC_{50} value is defined as the effective concentration of the test samples to show 50% of antioxidant activity under assay conditions.

fructosamines was found to be inhibited by EF and its constituents with the range of 85 to 90% (Fig. 3A), while the protein carbonyl compounds was found to be inhibited at 80–86% (Fig. 3B) and in the presence of C1 it was maximally diminished. Fluorescence studies on AGEs as illustrated in Fig. 3C implied that EF, C1 and C2 can inhibit them in the range of 71–82%. It is worth mentioning that EF, C1 and C2 exhibited higher inhibition compared to a known inhibitor aminoguanidine at diverse concentrations on 21 day incubation.

Subsequently, Table 4 gives the amount of fructosamine and protein carbonyl content in the various incubated test samples (EF, C1 and C2) with aminoguanidine at 1, 2 and 3 weeks. The results showed that the amount of fructosamine increased in the samples incubated with fructose and BSA (BSA/fructose) on all the 3 weeks in comparison to EF, C1, C2 and aminoguanidine samples at a concentration of 100 μg/ml. C1 exhibited the maximum inhibition in the formation of fructosamine followed by C2 and EF. Aminoguanidine also showed inhibition but was significantly lower ($p < 0.05$) than EF and its constituents. Similarly the addition of fructose to BSA increased the amount of protein carbonyl formation compared to BSA alone. It was found that suppression of protein carbonyl formation by EF and its constituents was significantly higher ($p < 0.05$) than aminoguanidine, when compared to BSA incubated with fructose.

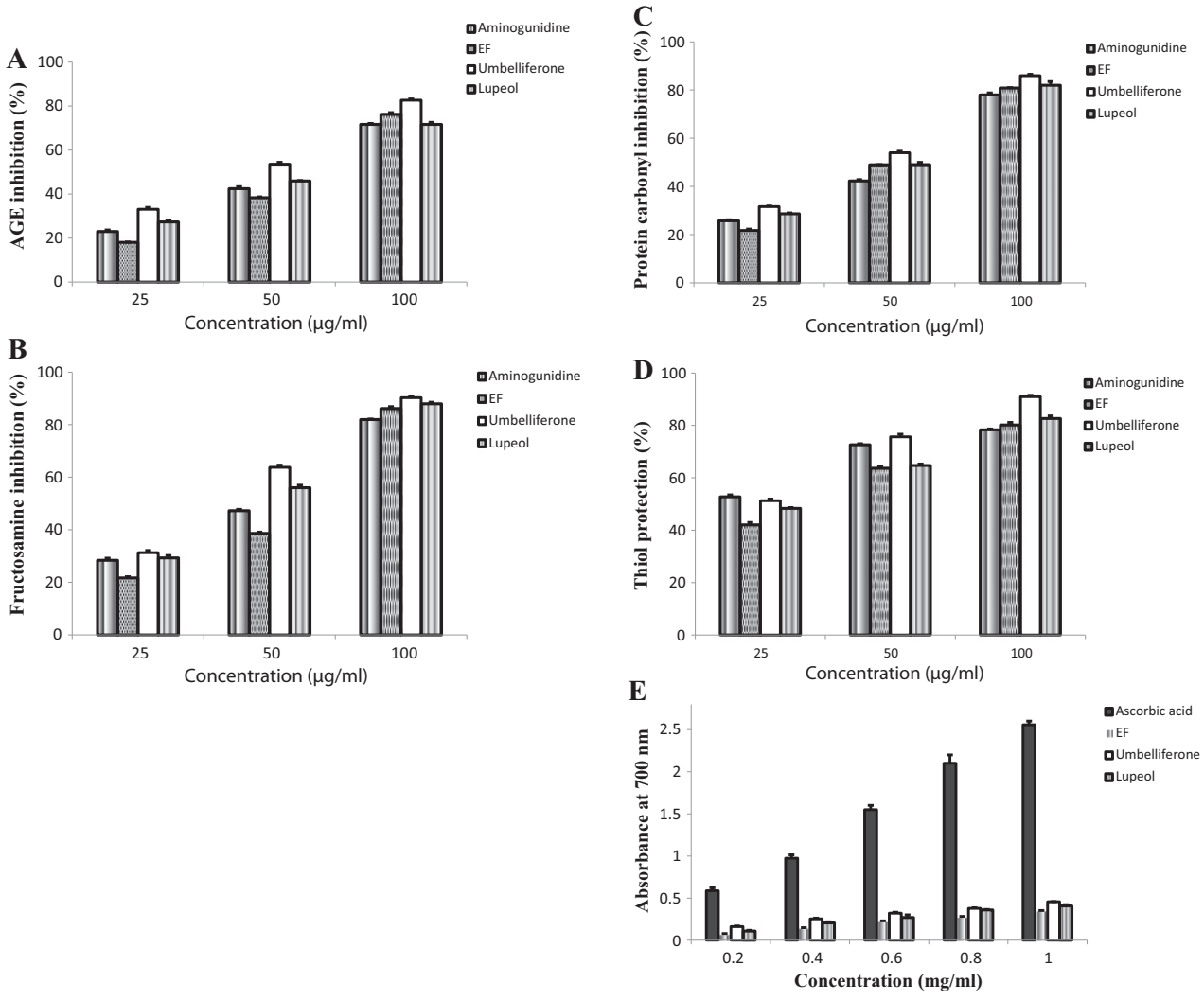

Fig. 3. Inhibitory effects of ethanol extract of flower (EF) and its isolated compounds on (A) AGE formation; (B) Fructosamine; (C) Protein carbonyls; and (D) Protein thiol protection at different concentrations. (E) Reducing power of EF and its isolated compounds. Values are mean ± SE (n = 3).

Table 4
Effects of ethanol extract of flower (EF) and its isolated compounds on fructosamine, protein carbonyl and thiol group content in BSA/fructose system after incubation for 3 weeks.

	Week	BSA	BSA/fructose	EF	Umbelliferone	Lupeol	Aminoguanidine
A.	Fructosamine (mmol/mg protein)						
	1	–	37.30 ± 0.51^{e}	26.20 ± 0.34^{c}	22.22 ± 0.38^{a}	25.04 ± 0.06^{b}	30.12 ± 0.20^{d}
	2	–	45.43 ± 0.49^{e}	27.40 ± 0.45^{c}	22.52 ± 0.47^{a}	25.70 ± 0.51^{b}	32.12 ± 0.20^{d}
	3	–	51.30 ± 0.10^{e}	29.49 ± 0.43^{c}	23.62 ± 0.11^{a}	27.07 ± 0.06^{b}	33.18 ± 0.18^{d}
B.	Protein carbonyl content (nmol/mg protein)						
	1	0.47 ± 0.01^{a}	0.92 ± 0.07^{f}	0.61 ± 0.27^{d}	0.56 ± 0.63^{b}	0.58 ± 0.19^{c}	0.72 ± 0.09^{e}
	2	0.48 ± 0.05^{a}	2.54 ± 0.23^{f}	0.66 ± 0.30^{d}	0.58 ± 0.44^{b}	0.61 ± 0.11^{c}	0.69 ± 0.56^{e}
	3	0.48 ± 0.39^{a}	4.42 ± 0.31^{f}	0.69 ± 0.11^{d}	0.61 ± 0.05^{b}	0.65 ± 0.10^{c}	0.75 ± 0.02^{e}
C.	Thiols group (nmol/mg protein)						
	1	1.89 ± 0.03^{d}	1.12 ± 0.08^{c}	0.85 ± 0.02^{b}	0.74 ± 0.02^{a}	0.77 ± 0.01^{a}	0.86 ± 0.03^{b}
	2	1.88 ± 0.02^{d}	1.10 ± 0.07^{c}	0.88 ± 0.03^{b}	0.77 ± 0.01^{a}	0.80 ± 0.04^{a}	0.92 ± 0.04^{b}
	3	1.88 ± 0.12^{d}	1.14 ± 0.03^{c}	0.93 ± 0.02^{b}	0.79 ± 0.01^{a}	0.82 ± 0.05^{a}	0.95 ± 0.05^{b}

Values are expressed as mean ± SE. Means in the same row with distinct superscripts are significantly different ($p \leq 0.05$) as separated by Duncan's multiple range test.

In addition, the potential of EF and its constituents on thiol group modification was investigated in the present experimental conditions. Treatment with EF and its constituents provided strong shielding from denaturation *i.e.* significant improvement in the thiol levels. Table 4 depicts the content of obtainable free thiol groups and oxidative modification of BSA on EF and its constituents. As depicted in Fig. 3D, both the compounds and EF illustrated a remarkable protection against thiol oxidation in a dose dependent fashion. At a concentration of 100 μg/ml, results showed that thiols shielding values ranged from 80–91% in the presence of EF, C1 and C2. For comparison, aminoguanidine at 100 μg/ml showed 78% thiol group protection under similar assay conditions.

4. Discussion

Diabetes mellitus is a chronic metabolic complication with multifactorial effects. Characterized by a hyperglycaemic state, diabetes is closely associated to several other complications of the kidney, eye and heart collectively induced by the excessive oxidative stress, augmented formation of AGE and enhanced activation of aldose reductase-associated polyol pathway (Michael, 2001). Successful management of hyperglycaemia is a prerequisite to organize the frequency, advancement, and seriousness of diabetic complications. Conversely, use of a single therapeutic procedure has unfortunately not been successful in negating all the complications caused by the high level of glucose in blood. Hence, auxiliary adjunct rehabilitations, such as α-glucosidase inhibitors, AR inhibitors, antiglycation agents and antioxidants could be viable remedial choice to constrict the toxic impacts of glucose. Numerous studies have demonstrated the adequacy of the unrefined plant concentrate and also its bioactive mixes in bringing down blood glucose levels (Geetanjali et al., 2010). Banana flower, being an incidental or secondary product of the banana cultivation, is known to have hypoglycaemic impacts and was used to treat diabetes in conventional medicine (Joshi, 2000). Recent work in this regard, provided a stronger evidence of its antioxidant potential and hypoglycaemic effects (Dhanabal et al., 2005; Pari and Maheswari, 1999) on alloxan induced diabetic rats. However, a detailed study on the mechanism underlying this role as well as the active principles corresponding to this biological effect is deficient and so in this study, we have focused on the antidiabetic prospective of EF against multiple targets involved in the pathogenesis of diabetes and its complications.

Mammalian α-glucosidases present on the brush border of the epithelial cells of the small intestine and α-amylase secreted by the pancreatic cells are the key enzymes in the hydrolysis of starch to free glucose units, consequence of which is the postprandial hyperglycaemia. Use of the enzyme inhibitors over the past few decades, has assisted in delaying the disease progression by reduction of hyperglycaemic effect (Casirola and Ferraris, 2006). In the present investigation, the banana flower extracted with ethanol exhibited a notable inhibition against α-glucosidase enzyme in comparison with the therapeutic drug acarbose. Further, the repeated silica gel column chromatography of EF led to the isolation of yellow semi-solid and creamish yellow semi-solid compounds which were identified analytically pure by thin layer chromatography. Various bioanalytical techniques (UV, IR, ^{1}H NMR, ^{13}C NMR and MS) determined that these two compounds were Umbelliferone and Lupeol, belonging to the coumarin and triterpenoids family respectively. For bioevaluation studies, Umbelliferone and Lupeol isolated from banana flower were tested for their ability to operate an antihyperglycaemic activity by targeting the key enzymes of the carbohydrate metabolism. Umbelliferone is known to be a potent antirheumatic, antipyretic and analgesic compound (Molnar and Garai, 2005), while, Lupeol is known for its antiinflammatory and anticancerous effects (Mohammad, 2009). Also, both the compounds have been reported to encompass significant biological activities suggesting its effectiveness in treatment of various metabolic disorders (Margareth and Miranda, 2009; Wal et al., 2011; Aoife and Richard, 2004; Revathi and Manju, 2013). However, its role with regard to antihyperglycaemia has not been reported to date. In our study, Umbelliferone and Lupeol isolated from EF were proved to be two α-glucosidase inhibitors, of which Umbelliferone was the most potent one. Further, in order to identify the mechanism underlying this inhibition, a Lineweaver–Burk plot was prepared from the kinetics data which indicated a reversible, non-competitive type of inhibition, with low K_i values. The results illustrated that these active principles bind to a site other than the active site of the enzyme thereby retarding the enzyme substrate reaction, without competing with the substrate for the active site of the enzyme. A similar finding on non-competitive inhibition was shown by Genistein, a soy isoflavone (Lee and Lee, 2001). In contrast, EF and its constituents were assessed for its inhibition on another key carbohydrate hydrolyzing enzyme α-amylase. The inhibitory effect on α-amylase was relatively low than that of acarbose. In general, the results showed that extract and its active compounds had a strong inhibitory activity on α-glucosidase and mild inhibition over α-amylase. Yet, our results are in agreement with prior findings that plant-based inhibitors have mild inhibition on α-amylase and a strong inhibition over α-glucosidase (Kwon et al., 2010; Ramith et al., 2014).

Presence of excessive free glucose units carries certain amount of secondary complications and they are also to be addressed while designing an optimum antidiabetic therapy. Incidentally, polyol pathway which is a component of the intermediary metabolism is affected during the presence of excessive glucose levels. Studies suggest that about 3% of glucose is metabolised through this pathway during normoglycemic conditions, whereas, during hyperglycaemic state, utilization of glucose is enhanced to more than 30% (Gonzalez et al., 1984). It is through this pathway that glucose is converted into sorbitol by the catalysis of aldose reductase (AR), the rate limiting enzyme of this pathway. Despite the studies suggesting the role of AR in detoxification of aldehydes which is a by-product of lipid peroxidation, its relevance in carbohydrate metabolism is unknown. However, inhibition of this enzyme renders a

protective role against cataract development in case of diabetic patients (Anthony et al., 1970). This suggests the importance in targeting the inhibition of AR during diabetes. In our studies, EF and its constituents were seen to inhibit aldose reductase activity far better than quercetin, which was used as the positive control in this assay signifying its potential use in this pathway.

During prolonged hyperglycaemia, the increased blood glucose levels accelerate the glycation process of various proteins rendering them inactive, known as Maillard reaction. Though the role of glucose in the Maillard reaction is well established, more recently, role of fructose is also identified to result in protein glycation attributing to its increased intake in the diet. Fructose either can glycate the protein residues in a non-enzymatic manner or might activate the polyol pathway which enhances the formation of fructose units from free glucose. These can in turn participate in protein glycation at a much higher rate than glucose (Casper et al., 2004). The process of glycation is initiated by the nucleophilic addition reaction between the free amino group of proteins and the carbonyl group of reducing sugars to yield a reversible Schiff base formation. This progressively turns into a more irreversible Amadori product like fructosamine, which is characterized as the early stage of glycation (Ivo, 1994). Reduction of fructosamine is a potential target for short term control of diabetes and in turn in delay of vascular complications (Shield et al., 1994). In our study, exposure of BSA to a high fructose level significantly elevated the fructosamine concentrations in comparison to the non-glycated BSA. Further, treatment with EF and its constituents demonstrated a considerable decrease in fructosamine. The second stage in glycation involves the degradation of fructosamines to a range of carbonyl compounds (namely glyoxal, methylglyoxal and deoxyglucosones) which marks the intermediate stage of glycation (Singh et al., 2001). Further, these compounds induce protein oxidation by formation of carbonyl proteins which leads to a loss of protein thiols which is an efficient indicator of protein oxidation (Michael, 2001). In the present investigation, the BSA exposed to a high fructose showed significant formation of protein carbonyl contents and a converse reduction in the protein thiols in comparison with the non glycated BSA. EF and its components when treated to such a system significantly augmented the formation of protein carbonyl as well as prevented the loss of protein thiol groups in a concentration dependent manner. Summing up, these results suggest the protective ability of the EF and its components in loss of protein thiol groups and a noteworthy inhibition to the early (formation of fructosamine) and the intermediary (protein carbonyl formation) stages of protein glycation process.

The final stage is described by the formation of insoluble, fluorescent advanced glycation end products (AGEs) which coupled with the glycated proteins play a pivotal role in pathogenesis of vascular complications of diabetes (Singh et al., 2001). Accumulation of AGE within the cells leads to impaired protein functions while its presence in the extracellular matrix results in abnormal cross-linking (Nessar, 2005). It also generates reactive oxygen species (ROS) by activation of several signalling cascades by binding to the receptors of AGE on the cell surfaces. Association of ROS in oxidative stress and vascular inflammation are among the several complications of postprandial hyperglycaemia (Samuel and James, 2003). Effect of EF and its constituents on the AGE formation was studied on the basis of its fluorescent ability which exhibited an antiglycation role throughout the 3 weeks of the experimental period. These results were superior to the inhibitory and protective ability exhibited by the synthetic glycation inhibitor aminoguanidine.

The role of widely accepted AGE inhibitor, aminoguanidine is to reduce the formation of carbonyl intermediates. Some of the other inhibitors also play a role acting either as metal chelating agents or as antioxidants. However, the long term implications of these molecules extend a hepatotoxic effect and thus safer alternatives are in need. Also, these molecules confiscate all the free radicals *in vivo* which are not desirable. To prevent these drawbacks, it is advantageous to stimulate the antioxidant defences in order to avert protein glycation (Paul, 2003; Samuel and James, 2003). In the present study, various *in vitro* assays were performed *viz*., DPPH, ABTS and superoxide for assessing the radical scavenging ability. Our results clearly established that EF and its constituents exhibited a higher free radical scavenging activity in all the assays used in the study, presenting a protective role against free radical mediated damage. The studies also revealed a high concentration of total phenolic content in EF which is well correlated to radical scavenging activity of the extract. It is recognized that free radicals have a vital role in autoxidation of unsaturated lipids in food stuffs (Kaur and Perkins, 1991). A stronger inhibitory potential against lipid oxidation suggests that EF and its constituents might intercept the free radical chain of oxidations and contribute hydrogen atom from the phenolic hydroxyl groups themselves, thereby forming stable free radicals which do not initiate or propagate further oxidation of lipids.

Although the above assays are based on different principles, it appears necessary to determine the reducing power of the extract and its compounds, since it serves as a major indicator of its potential antioxidant activity (Meghashri et al., 2010). The observed dose dependent increase in the absorbance at 700 nm of the reaction mixture caused by the reductones (EF and its constituents) was indicative of their increased reducing power. It is self-evident that potent reductones may also affect ions, particularly Cu^{2+} and Fe^{2+}. In this regard, iron the vital mineral for normal physiology, if present in excess, undergoes reduction and might form highly reactive hydroxyl radicals contributing to oxidative stress. An effective way to revert this would be identification of a chelating agent which would seize the pro-oxidant potential from these metal ions (Susanne and Erich, 1999). In food system, ferrous ions possess maximum pro-oxidant potential and hence in our study we examined the chelating ability of EF and its constituents towards the chelation of Fe^{2+}. The results projected a substantial chelating capacity of the compounds suggesting its interference in the formation of ferrozine complex from the ferrous ions.

5. Conclusion

In conclusion, EF extends a remarkable antihyperglycaemic effect *via* inhibition of carbohydrate hydrolysing enzymes as well as ameliorates the diabetes induced complications by inhibiting AR and AGE related pathways. The beneficial effects of EF in are in agreement with the positive effects of the isolated Umbelliferone and Lupeol, suggesting them as potential antidiabetic agents. Further, our results are intricately linked to the antioxidant activities of EF and its constituents which suggest its association with the antidiabetic properties. Also, identification of significant amount of Umbelliferone (0.38%) and Lupeol (0.36%) in EF provided an insight for a strong chemical basis to the alleged beneficial role of EF in antidiabetic properties. However, *in vivo* studies to provide a stronger basis for these evidences are essential before it can be established and incorporated in therapeutic procedures.

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Conflict of interest

The authors declare that there is no conflict of interest.

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