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Antidiabetic potential of polyherbal formulation DB14201: Preclinical development, safety and efficacy studies

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ABSTRACT

Ethnopharmacological relevance: The poly-herbal formulation DB14201 is a new combination of ayurvedic ingredients for treatment of diabetes. The aim of present study was to investigate safety and *in vivo* efficacy of DB14201 extract. Further this work was aimed to develop, characterize and standardize DB14201 extract and develop it as a botanical drug.

Materials and methods: The polyherbal extract was standardized using four chemical markers. The LC-MS/MS method was developed for identification and quantification of mangiferin, berberine, kaempferol and curcumin. The extract was standardized for heavy metal content, aflotoxins, and microbial tests. The mechanism of action of DB14201 extract was explored through glucose uptake by adipocytes, TNF-α production and free fatty acid release, *in vitro*, was studied using murine adipocytes (3T3-L1). The effect of extract on insulin release was evaluated using murine pancreatic beta cell (β TC-6). The safety and *in vivo* efficacy of extract was studied using suitable animal model. Hematology and blood biochemistry parameters were also assessed.

Results: *In vitro* studies of DB14201 in murine adipocytes and murine pancreatic beta cells demonstrated the plausible mechanism of action of DB14201 could be through increase in glucose uptake and by stimulation of insulin release by RIN-5f cells. The microbial load, heavy metals were found to be within the AYUSH permissible limits and aflotoxins were absent. Preclinical efficacy studies in animal models proved the anti-diabetic potential of the extract. The preclinical acute dose toxicity study and 90-days repeated dose toxicity study of DB14201 extract in wistar rats by oral route indicated that the extract is safe up to 1000 mg/kg dose. Hematology and blood biochemistry parameters were within the normal range.

Conclusions: The data presented herein demonstrated anti-diabetic potential of developed DB14201 extract and this study will serve as the benchmark for the further research on this polyherbal formulation.

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Abbreviations: WHO, World Health Organization; US-FDA, US Food and Drug Administration; ADME, Absorption, Distribution, Metabolism, and Excretion; HPLC, High-performance liquid chromatography; EDTA, Ethylenediaminetetraacetic acid; MS, mass spectrometry; Pb, Lead; Cd, Cadmium; Hg, Mercury; As, Arsenic; TNF-α, Tumor necrosis factor alpha; DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal Bovine Serum; IBMX, Isobutylmethylxanthine; CO_2, Carbon dioxide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; DMSO, Dimethyl sulfoxide; SGOT, Serum glutamic oxaloacetic transaminase; SGPT, Serum glutamic-pyruvic transaminase; ELISA, Enzyme-linked immunosorbent assay; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; LOD, limit of detection; LOQ, limit of quantitation; PBS, Phosphate-buffered saline; 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose; IL-6, Interleukin 6; LPS, Lipopoly-saccharide; GTT, Glucose Tolerance Test; SOD, Superoxide dismutase; OECD, Organization for Economic Co-operation and Development; ICP-MS, Inductively coupled plasma mass spectrometry

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1. Introduction

Diabetes is characterized by altered carbohydrate, lipid and protein metabolism which not only causes hyperglycemia but also results in many complications such as hyperlipidemia, hyperinsulinemia, hypertension and atherosclerosis (Herman-Edelstein et al., 2014; Tag et al., 2012). The estimate of 2010 by WHO stated that 3.4 million people died from consequences of high fasting blood sugar level and more than 80% of deaths due to diabetes occur in low and middle income countries (Marles and Farnsworth, 1995; US-FDA, 2004; WHO/TRMJ/91.4, 1991). Treatment with most of the oral hypoglycemic agents is reported to have side effects related to pharmacokinetic properties, secondary failure rates, hypoglycemia, gastrointestinal disturbances, skin reactions, hematological disorders and rise in hepatic enzyme level. However, herbal medicines have been used effectively in treating diseases throughout the world because it is considered to be the less toxic and free from side effects in comparison to synthetic ones (Elvin-Lewis, 2001; Kumar et al., 2015). There are more than 1200 species of plants which possess antidiabetic activities and have been used to treat this disease. Furthermore, more than 200 plant-derived natural products are known to possess blood glucose lowering activity (He et al., 2005; Ji et al., 2009; Jung et al., 2006; Marles and Farnsworth, 1995).

For commercialization of botanical products, the responsibility of maintaining their quality falls to a large extent on the scientists and to a certain extent on the manufacturers. In this situation, the assurance of safety, quality and efficacy of medicinal herbs and botanical products has become an important issue. The National Center for Complementary and Alternative Medicine and WHO stress the importance of qualitative and quantitative methods for characterizing botanical samples, quantification of the biomarkers and/or chemical markers and the fingerprint profiles. The herbal drug preparation itself as a whole is regarded as the active substance. Hence, the reproducibility of the total configuration of herbal drug constituents is important. Different approaches can be used for chemical standardization such as pretreatment that involves drying and grinding; selection of a suitable method of extraction; analysis of compounds using suitable chromatographic or spectroscopic methods; the analysis of data based on bioactive or marker compounds; quality control; elucidation of the properties of ADME and metabonomics evaluation of medicinal plants. Also, there is a need to approach scientific proof and clinical validation with chemical standardization, biological assays, animal models and clinical trials for botanicals (Heng et al., 2013).

The poly-herbal formulation DB14201 is a new combination of ayurvedic ingredients which was used in southern India as a herbal drinking water supplement for diabetic patients from 2002 to 2007 (Krishnan, 2012). It is being developed for the treatment and prophylaxis of Diabetes Mellitus and related complications. DB14201 is a poly herbal extract of a combination of 16 herbs. All herbs present in DB14201 *viz. Ziziphus jujuba* (L.) Lam. (Abdel-Zaher et al., 2005; Bhatia and Mishra, 2010), *Strychnos potatorum* L. f. (Umamaheswari and Prince, 2007), *Aerva lanata* Juss (Vetrichelvan and Jegadeesan, 2002), *Coscinium fenestratum* (Goetgh.) Colebr (Shirwaikar et al., 2005), *Curcuma longa* L. (El-Moselhy et al., 2011), *Mangifera indica* L. (Sangeetha et al., 2010), *Emblica officinalis* Gaertn (Rao et al., 2005), *Terminalia chebula* (Gaertn) Retz. (Murali et al., 2007), *Syzygium cumini* (L) (Helmstädter, 2008), *Biophytum sensitivum* (L) DC (Puri, 2001), *Cyclea peltata* (Lam.) Hook. f. & Thoms. (Kirana and Srinivasan, 2010), *Salacia oblonga* Wall. ex Wight & Arn. (Krishnakumar et al., 1999), *Centella asiatica* (L.) Urb (Tenni et al., 1988), *Embelia tsjeriam-cottom* (Roem. & Schult.) A. DC. (Pandey and Ojha, 2011), *Vetiveria zizanioids* (L) (Lans, 2006) and *Cyperus rotundus* (L) (Raut and Gaikwad, 2006) have been studied for anti-diabetic as well as other pharmacodynamic activities by scientists and sufficient evidences about these activities exist in literature.

Considering its available history of safe use over this period and randomly reported efficacy by diabetic consumers, it was decided to select this herbal formulation for further investigations and develop it as a botanical drug for treatment of diabetes. Hence the present study was undertaken with an objective to evaluate *in vivo* safety and efficacy of the botanical extract and to standardize and characterize the extract. The mechanism of action of polyherbal formulation DB14201 was also explored using *in vitro* assays *viz.* glucose uptake, cytokine inhibition, free fatty acid release by adipocytes and *in vitro* insulin release by RIN-5f cells.

2. Materials and methods

2.1. Plant materials

The botanical raw materials *Ziziphus jujuba* (L.) Lam. (fruits with seeds), *Strychnos potatorum* L. f. (seeds), *Aerva lanata* Juss (whole plant with flower), *Coscinium fenestratum* (Goetgh.) Colebr (stem with bark), *Curcuma longa* L. (rhizomes), *Mangifera indica* L. (seed without cover), *Emblica officinalis* Gaertn (fruits), *Terminalia chebula* (Gaertn) Retz. (fruits), *Syzygium cumini* (L) (fruits with seeds), *Biophytum sensitivum* (L) DC (whole plant), *Cyclea peltata* (Lam.) Hook. f. & Thoms. (rhizomes), *Salacia oblonga* Wall. ex Wight & Arn. (roots), *Centella asiatica* (L.) Urb (whole plant), *Embelia tsjeriam-cottom* (Roem. & Schult.) A. DC. (seeds), *Vetiveria zizanioids* (L) (roots) and *Cyperus rotundus* (L) (rhizomes) were purchased from the local market of Coimbatore, India. The herbal materials was identified by T. Stanes & Company Ltd., Coimbatore 641018, India and Trivandrum Ayurveda college by a certified Pharmacognosist by comparing them to their herbarium specimen. These were identified and the voucher specimen/Accession numbers (TSPPTL/HD029/2010, TSPPTL/HD06/2010, P13/4016, TSPPTL/HD032/2010, TSPPTL/HD017/2010, AC no. 15239, TSPPTL/HD015/2010, AC no. 19189, TSPPTL/HD011/2010, P13/4012, P01/4013, CDR/4033, P13/4015, TSPPTL/HD014/2010, TSPPTL/HD026/2010, TSPPTL/HD023/2010) were deposited in the 'Herbal division' of T. Stanes & Company Ltd., Coimbatore – 641018, India and in Janaki Ammal Herbarium of CSIR- Indian Institute of Integrative Medicine, Jammu 180001, India. Each herbal material was washed separately, dried and powdered (sieve #65). The raw material was packed in airtight container and was analyzed for its quality before it was taken further for formulation development.

2.2. Chemicals and reference compounds

Reference marker compounds oleanolic acid, kaempferol, berberine hydrochloride, curcumin, gallic acid, isoorientin, berbamine dihydrochloride, mangiferin, asiaticoside, embelin, khusenic acid and oleic acid were purchased from Natural Remedies Pvt. Ltd., Chromadex, Sigma-aldrich and Sami labs. Acetonitrile (Merck), methanol (Merck), trifluoroacetic acid (Sigma-Aldrich), ethanol (Merck), potassium dihydrogen orthophosphate (Sisco Research Laboratories), orthophosphoric acid (Spectro chem), formic acid (Fluka), α-glucosidase and α-amylase (Sigma-Aldrich) were used for the study. The reference standards, reagents and chemicals used for efficacy, safety and toxicity studies were; dextrose 5% (Baxter), formaldehyde (Merck), EDTA (Sigma), potassium dihydrogen phosphate (Sigma), disodium hydrogen phosphate (Qualigens), potassium chloride (Merck), sodium chloride (Merck), streptozotocin (Sigma), glibenclamide (Brand name: Daonil, Aventis Pharma Ltd.), biphasic isophane insulin injection I.P. (Brand name: Human Mixtard®, Torrent Pharmaceuticals Ltd.), metformin (Brand name: Glyciphage®, Franco Indian

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Table 1
The % content of marker compounds in polyherbal DB14201 extract.

Marker compound (Medicinal herb)	Retention time (min)/ Molecular mass $[M+H]^+$	% Content of marker compound in DB 14201 extract			Mean ± SD	Regression equation	R^2	Linear range (ng/mL)	LOQ (ng/mL) (S/N)	LOD (ng/mL) (S/N)
		Batch 1	Batch 2	Batch 3						
Mangiferin	11.5/423.2	0.115	0.113	0.118	0.115 ± 0.003	y = 22.298 × x − 233.518	0.996	195.3–12500	48	12
Berberine	14.2/336.2	0.003	0.0024	0.004	0.003 ± 0.001	y = 104.357 × x + 273.91	0.999	12.20–1562.50	12.2	3.0
Kaempferol	15.6/287	0.001	0.0009	0.001	0.001 ± 0.000	y = 31.637 × x + 250.73	0.994	12.20–1562.50	15	5.0
Curcumin	18.4/369.2	0.0025	0.0021	0.0034	0.003 ± 0.001	y = 49.481 × x + 809.44	0.992	12.20–1562.50	12.2	3.0

Pharmaceutical Pvt. Ltd.), sodium citrate (Sigma), citric acid (Merck), carboxy methyl cellulose (Sigma), Potassium dihydrogen orthophosphate (Glaxo), Trichloroacetic acid (Spectrochem), sodium azide (Sigma), Dipotassium hydrogen phosphate (E. Merck), sodium phosphate (monobasic) (Sigma), sodium phosphate (dibasic) (Sigma), glacial acetic acid (E. Merck), sodium lauryl sulfate (Sigma), nitro blue tetrazolium solution (Sigma), potassium phosphate (Sigma), hydrochloric acid (Qualigens), creatinine kit (Bayer), D-glucose (Sigma-Aldrich), ethanol (Merck), ether (SRL Ranbaxy), glucose kit (Bayer), picric acid (Qualigens), phenol red (Glaxo), rat insulin ELISA kit (Mercodia), SGOT (AST) kit (Bayer), SGPT (ALT) kit (Bayer), triglycerides kit (Bayer), total cholesterol kit (Bayer), urea kit (Bayer).

2.3. Apparatus

Agilent 6410B triple quad LC-MS/MS, HPLC (Shimadzu LC-2010 C_{HT}), columns (YMC ODS-A, 3 μm, 150 × 4.6 mm; YMC ODS-A, 5 μm, 250 × 4.6 mm; Inertsil ODS-3, 3 μm, 150 × 4.6 mm; BDS Hypersil C_{18}, 5 μm, 100 × 4.6 mm), Chromolith® high resolution RP-18 endcapped, 50 × 4.6 mm, LC-MS (Waters), Oasis HLB 1cc cartridge (Waters), sonicator and micropipettes were used for the study.

2.4. Characterization and specification development of DB14201

2.4.1. Extraction, isolation and qualitative chemical analysis of markers from botanical raw materials (individual herbs)

Markers oleanolic acid, kaempferol, curcumin, gallic acid, isoorientin, berberine hydrochloride, berbamine dihydrochloride, embelin, mangiferin, asiaticoside, khusenic acid and oleic acid were selected from individual herb (Fig. S1). These markers were extracted from respective herb using the optimized protocols. Table S1 depicts the detailed protocol and experimental conditions for extraction of selected marker from individual herb. Isolated markers were characterized by comparison of their HPLC profile and MS data of reference standards. The HPLC conditions for analysis of markers from individual herbs are listed in Table S2.

2.4.2. Composition and method of extraction of DB14201

DB14201 is herbal mixture of 16 herbs. The raw material consisted of all herbs, which are mentioned in Section 2.1, in equal quantity (by weight) except *Embelia tsjeriam-cottom* (Roem. & Schult.) A. DC. and *Centella asiatica* (L.) Urb, which were exactly half quantity by weight of the rest herbs.

Each batch of the raw herb premix was extracted in 3 stages. During first stage of extraction, raw material was mixed with 1000 L of water and it was kept for 3 h at 80–90 °C. It was concentrated and dried using rotary vacuum dryer at 70 °C. The obtained solid was pulverized and sieved through #40 mesh. The 2nd and 3rd stage of extraction was carried out using 800 L of water. The resulting mixture was kept for 2 h at 70–80 °C. The resulting solution was concentrated and dried using rotary vacuum dryer at 70 °C. The obtained solid was pulverized and sieved through #40 mesh. DB14201 extract was prepared by mixing 1 part of solid obtained from first extraction and 0.5 part of solid obtained from both, 2nd and 3rd extraction in equal proportion.

2.4.3. LC-MS/MS method for simultaneous estimation of four markers in polyherbal extract

Mangiferin, berberine, curcumin and kaempferol were selected to find out batch-to-batch % content of markers in DB14201. Stock solution of mangiferin, berberine and curcumin was prepared in ACN: water mixture (4:1). Stock solution of kaempferol was prepared in methanol. Simultaneous estimation of markers in DB14201 was performed on Chromolith® high resolution RP-18 endcapped; 50 × 4.6 mm column maintained at 30 °C. The mobile phase containing buffer (0.2% formic acid in water) and acetonitrile at the flow rate of 0.6 mL/min was used. The volume of injection was 5 μL, the total run time of 23 min. The method of estimation was (SIM) LC-MS. The Instrument parameters during LC-ESI-MS/MS analysis and gradient details are given in Table S3-a and b. For simultaneous estimation of markers in DB14201, 10 mg of extract was transferred in a vial containing 1 mL of acetonitrile and the mixture was sonicated. The supernatant was injected in the LC-MS/MS system. The chromatogram of mixture of reference markers and markers extracted from DB14201 were compared. The marker compounds in three different batches of DB14201 were quantified and the data shown in Table 1. The developed method was validated for LOD (limit of detection), LOQ (limit of quantitation) and linearity. The data is shown in Table 1.

2.5. Standardization and quality control of extract

Standardization and quality control of extract was carried out in terms of chemistry, manufacturing and control information. The extract was evaluated for following parameters *viz*. acid insoluble ash, loss on drying at 105 °C, pH of 1% w/w solution, total ash, water soluble extractive, 50% alcohol soluble extractive.

Heavy metals (Pb, Cd, Hg and As) and aflotoxins content (B1, B2, G1, G2) was also determined. For determination of heavy metals, approximately 0.25–5.0 sample was digested with 5–8 mL of nitric acid (concentrated) and the vessel was placed in the microwave oven. After completing digestion the vessel was removed from microwave and solution was transferred in 50 mL centrifuge tube and volume made upto 50 mL with elemental water. 100 μL of 10 ppm internal standard was added to the sample in volume 50 mL (*e.g* 20 ppb). This solution was injected directly into ICP-MS (Inductively coupled plasma mass spectrometry) for determination of metals (Method-990.08/993.14, 2005).

Analysis of aflotoxins was performed as follows: To 50 g of extract, 200 mL of methanol and 50 mL of 0.1 N HCl was added.

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The resulting mixture was shaken at high speed for 5 min and filtered through Whatman filter paper 1. To 50 mL of the filtrate, 50 mL of 10% NaCl solution and hexane was added and the mixture was shaken for 30 s. Hexane layer was discarded and aqueous layer was again partitioned using 25 mL dichloromethane. The lower layer of dichloromethane was collected and anhydrous sodium sulfate was added to remove water, if any. The partitioning was performed twice with dichloromethane as mentioned above. The collected elute was concentrated on steam bath and loaded on to silica gel column for separation of aflotoxins. Aflotoxins were eluted with 100 mL of mixture of dichloromethane and acetone (9:1). Elute was evaporated on steam bath upto 6 mL and was divided into 3 parts for further analysis. After transferring, 2 mL of elute into vial, it was evaporated to dryness using nitrogen. Derivatization of sample was carried out by adding 200 μL of hexane and 50 μL TFA. To this solution, 2 mL of ACN- water mixture (1:9) was added and vortexed for 30 s 25 μL of lower aqueous layer was injected into LC system. For this, Shimadzu LC-10ATVP with Autosampler SIL-10 ADVP or equivalent; fluorescence detector Shimadzu RF-10 AXL Ex 360 nm, Em 450 nm fitted with PC Windows 2000 was used. LC column- 25 cm × 4.6 mm id, 5 μm RP-18 or equivalent with 20 cm × 1 cm id was used. The LC elution solvent was H_2O-CH_3CN-CH_3OH (700:200:200). Individual aflatoxin concentration (B1, B2, G1 and G2) was calculated from standard calibration curve (Section-49.2.17, 2005). Microbial testing of extract was also performed to find out total aerobic bacterial count; total yeast, mold count; *Enterobatecriace* count; *Escherichia coli, Salmonella spp.*; *Staphylococcus aureus and Pseudomonas aeruginosa.*

2.6. Evaluation of anti-diabetic potential of DB14201 using murine adipocytes (3T3-L1) and murine pancreatic beta cells (β TC-6) in vitro

The mechanistic profiling of DB14201 by evaluation of effect on four key parameters *viz.* glucose uptake (Choi et al., 2009), TNF-α production (Martineau et al., 2006), free fatty acids release by adipocytes (Dave et al., 2012) and effect of DB14201 on release of insulin by pancreatic-beta cells (Berg et al., 2004) was studied.

Differentiation of 3T3-L1 pre-adipocytes into adipocytes was induced by treatment of confluent monolayer of cells with Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 10 μg/mL Insulin, 0.5 mM Isobutylmethylxanthine (IBMX) and 0.25 μM Dexamethasone. After 2 days, the cell culture medium was changed with DMEM containing 10 μg/mL insulin and 10% FBS. The medium was replaced with DMEM containing 10 μg/mL insulin and 10% FBS after every two days till 6 days (Harkins et al., 2004; Martineau et al., 2006; Tripp et al., 2012).

Non-cytotoxic concentrations of DB14201 was determined on differentiated 3T3-L1 adipocytes (Boden et al., 2005; Kang et al., 2013; Youl et al., 2010). The cells were trypsinized and a single cell suspension was obtained. The cell suspension was counted on a hemocytometer. The cells were seeded at the density of 10×10^3 cells/well in 96 well plate. The seeded plate was incubated overnight at 37 °C in a 5% CO_2 incubator. Subsequent to incubation, 3T3-L1 preadipocytes were differentiated to mature adipocytes as mentioned above. 3T3-L1 adipocytes were treated with different concentrations of DB14201 ranging from 0.1 to 2000 μg/mL in triplicates. Subsequently, treated cells were incubated for a time period of 48 h at 37 °C in a 5% CO_2 incubator. The untreated cells were used as controls. After treatment of 3T3-L1 adipocytes with DB14201 at concentrations ranging from 0.1 μg/mL to 2000 μg/mL for a time period of 48 h at 37 °C in a 5% CO_2 incubator, the cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) based assay. 20 μL of 5 mg/mL of MTT was added to all the wells and incubated for 3 h at 37 °C in a 5% CO_2 incubator. Subsequent to incubations, the supernatant was aspirated without disturbing the formazan complex and 150 μL of DMSO was added to all wells to dissolve the formazan crystals. The optical density of each well was recorded at 540 nm on a multiplate Biotek reader.

Non-cytotoxic concentrations of DB14201 was determined on RIN-5F cells as per following protocol (Hassan et al., 2010). The cells were trypsinised and a single cell suspension was obtained. The cell suspension was counted on a hemocytometer. The cells were seeded at the density of 5×10^3 cells/well in 96 well plate. The seeded plate was incubated overnight at 37 °C in a 5% CO_2 incubator. RIN-5F cells were treated with different concentrations of DB14201 ranging from 0.1 μg/mL to 500 μg/mL in duplicates. Subsequently, treated cells were incubated for a time period of 6 h at 37 °C in a 5% CO_2 incubator. The untreated cells were used as controls. After treatment of RIN-5F cells with DB14201 at concentrations ranging from 0.1 to 500 μg/mL for a time period of 6 h at 37 °C in a 5% CO_2 incubator, the viability of the cells were determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) based assay. 20 μL of 5 mg/mL of MTT was added to all the wells and incubated for 3 h at 37 °C in a 5% CO_2 incubator. Subsequent to incubations, the supernatant was aspirated without disturbing the formazan complex and 150 μL of DMSO was added to all wells to dissolve the formazan crystals. The optical density of each well was read at 540 nm on a multiplate Biotek reader.

2.6.1. Effect on glucose uptake by adipocytes by fluorescence based assay

3T3-L1 pre-adipocytes were plated in 96 well plate at a density of 1×10^3 cells per well. 3T3-L1 pre-adipocytes were incubated overnight at 37 °C in 5% CO_2 incubator. Subsequent to incubation, 3T3-L1 pre-adipocytes were differentiated to mature adipocytes as per protocol mentioned in above Section. 3T3-L1 adipocytes were treated with predetermined non-cytotoxic concentrations of DB14201 ranging from 0.1 to 600 μg/mL and Insulin (175 nM). The treated cells were incubated for 24 h at 37 °C in 5% CO_2 incubator. Following the incubation period, 3T3-L1 adipocytes were washed twice with PBS (pH 7.4) and were incubated for 30 min at 37 °C in 1 × PBS (pH 7.4). Subsequent to incubation, adipocytes were washed twice with PBS (pH 7.4) at 37 °C. 300 μM of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) prepared in PBS (pH 7.4) was added to all the wells and incubated for 10 min at 37 °C. Cells were lysed by freeze thaw in PBS (pH 7.4). Fluorescence of 2-NBDG were measured in cell lysates using multiplate Biotek reader at an emission wavelength of 528 nm and excitation wavelength of 485 nm.

2.6.2. Effect on cytokine release (IL-6)

The cells were trypsinized and a single cell suspension was obtained. The cell suspension was counted on a hemocytometer. The cells were seeded at the density of 0.1×10^6 cells/well in 6 well plate. The seeded plate was incubated overnight at 37 °C in a 5% CO_2 incubator. Cells were serum starved with 0.1% FBS containing DMEM for 24 h at 37 °C in a 5% CO_2 incubator. Subsequent to incubation, 3T3-L1 preadipocytes were treated with different concentrations of DB14201 from 1 to 500 μg/mL in duplicates for 24 h. Cells were stimulated with LPS (100 ng/mL) for 8 h at 37 °C in a 5% CO_2 incubator. After completion of incubation time, supernatants were collected and analyzed for IL-6 release using mouse IL-6 immunoassay kit, Cat no. M6000B, Quantikine. Percent inhibition of IL-6 release in response to different concentrations of DB14201 was calculated as compared to LPS alone (Hoareau et al., 2010).

2.6.3. Effect on adipolysis (Glycerol release)

The cells were trypsinized and a single cell suspension was obtained. The cell suspension was counted on a hemocytometer.

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The cells were seeded at the density of 0.1×10^6 cells/well in 6 well plate. The seeded plate was incubated overnight at 37 °C in a 5% CO_2 incubator. Subsequent to incubation, 3T3-L1 preadipocytes were differentiated to mature adipocytes as mentioned above. Cells were serum starved with DMEM containing 1% FBS and incubated for 24 h at 37 °C in a 5% CO_2 incubator. 3T3-L1 adipocytes were treated with different concentrations of DB14201 ranging from 0.1 to 100 μg/mL in duplicates and incubated at 37 °C in a 5% CO_2 incubator for 24 h. After 24 h, cells were stimulated with TNF-α (10 ng/mL)+ Glucose (25 mM) and incubated at 37 °C in a 5% CO_2 incubator for another 24 h. After 24 h, supernatants were collected and glycerol released into culture medium was analyzed using Adipolysis assay kit, Cat No. 10009381, Cayman (Lee and Fried, 2012).

2.6.4. Effect on insulin release

The RIN-5 F cells were trypsinized and a single cell suspension was obtained. The cell suspension was counted on a hemocytometer. The cells were seeded at the density of 10×10^3 cells/well in 96 well plate. The seeded plate was incubated overnight at 37 °C in a 5% CO_2 incubator. After overnight incubation, cells were washed with KRB buffer ×5 times and incubated in KRB buffer for 40 min at 37 °C in a 5% CO_2 incubator. RIN-5 F cells were treated with different concentrations of DB14201 ranging from 0.1 to 100 μg/mL in duplicates and incubated at 37 °C in a 5% CO_2 incubator for 6 h. After 6 h, supernatants were collected and analyzed for insulin release using ELISA assay kit, High sensitive mouse insulin immunoassay kit, Cat No. EHSMISY, Symansis. Data for insulin release were analyzed using 4-parameter curve fit analysis on graph pad prism version 4 (Sakoda et al., 2000).

2.7. Efficacy studies of DB14201

For *in vivo* studies, all protocols were approved by animal ethical committee of Dabur Research Foundation, Ghaziabad – 201010, Uttar Pradesh, India. The animal ethical committee permission number is IAEC/DO/99-2.

2.7.1. Anti-diabetic potential of DB14201 in streptozotocin (STZ) induced type I diabetes mellitus in rats (Junod et al., 1969)

The study was performed to evaluate anti-hyperglycemic effect of DB14201 administered by oral route in STZ induced Type I diabetic rats. Type I diabetes can be induced in rodents by a single STZ injection (Wu and Yan, 2015). Hyperglycemia was induced in these rats by intravenous injection *via* tail vein with 45 mg/kg STZ in normal saline (pH 4.5) on day–14. STZ causes Type I diabetes by the rapid destruction of pancreatic β-cells and thus an absence of insulin release. Hyperglycemia causes oxidative damage by the generation of reactive oxygen species (Mohamed et al., 1999) and results in the development of diabetic complications (Baynes and Thorpe, 1999; Donnini et al., 1996).

Male Wistar rats, 8–10 weeks of age and weighing 180–230 g were used for the study. After 12 days of acclimatization, STZ was administered by intravenous bolus injection to 45 male rats fasted overnight for 12 h. A stabilization period of 14 days was followed by Glucose Tolerance Test (GTT), which was used as a screening test for selection of diabetic animals. 24 rats with fasting glucose >130 mg/dl during the GTT profile were considered as diabetic animals and randomized into 3 subsequent groups. Additionally, 8 untreated Wistar rats were considered as non- diabetic controls (Huang et al., 2000).

In summary, Group I (n=8), the non-diabetic control group, did not receive STZ and was given sterile deionized water in equivalent volumes to the treatment dose. The second group (n=8), the hyperglycemic vehicle control group, received STZ and sterile deionized water in equivalent volumes to the treatment dose. The third and fourth groups (n=8) were hyperglycemic rats receiving DB14201 at two dose levels of 250 mg/kg and 500 mg/kg respectively. Observations comprised of mortality, general cage side and clinical observations, body weight, food and water consumption and weekly blood glucose estimation and other biochemical parameters on day 1 and day 15. Statistical analysis was done using Graph pad PRISM version-4 software. The statistical result shows significant ($P < 0.01$) anti-hyperglycemic activity of DB14201 at a dose of 500 mg/kg with reference to the hyperglycemic control animals.

2.7.2. Effect of DB14201 in STZ induced diabetic nephropathy in C57BL/6 mice

The objective of this study was to evaluate the role of DB14201 on STZ induced diabetic nephropathy in C57BL/6 mice. The extract was tested on C57BL/6 mice as it is commonly reported animal model for evaluation of STZ induced diabetic nephropathy (Breyer et al., 2005). 45 male C57BL/6 mice were fasted for 6 h. Hyperglycemia was induced in these mice by intra-peritoneal injection of STZ at 55 mg/kg using 29 G insulin needle. To complete the induction of diabetes, STZ was injected in same manner for five consecutive days. The body weight of all animals was recorded bi-weekly. One week after STZ induction, the fasting (six hours) basal glucose level of all the animals was measured and allocated to different group accordingly. Non-diabetic control animals (n=5) were grouped separately. After 2 week of STZ induction, the dosing was started, G1 served as non diabetic control (negative control) and G2 served as diabetic control (positive control) and both received 10 mL/kg of milli Q water. G3 and G4 were treated with test item at the dose of 250 mg/kg and 500 mg/kg respectively. All the groups were treated orally for 28 days using disposable syringes tipped with an oral gavage needle. All animals were observed daily for mortality, morbidity and clinical signs of toxicity. Body weight was recorded daily throughout the dosing period. Blood and urine was collected for biochemical estimations on 1st, 14th and 28th day of dosing period. After completion of dosing period, animals were humanely sacrificed and observed for gross pathological changes. Kidney of each animal were isolated and histopathological examination was performed (Whitton and Hems, 1975).

2.7.3. Effect of DB14201 in STZ induced diabetic atherosclerosis in C57BL/6 mice

The study was conducted to evaluate the test item on STZ induced diabetic atherosclerosis in C57BL/6 mice (Wu and Huan, 2007). 40 female C57BL/6 mice were fasted for 6 h and hyperglycemia was induced in these mice by intra-peritoneal injection of STZ at 40 mg/kg using 29 G insulin needle. To complete the induction of diabetes, STZ was injected in same manner for five consecutive days. One week after final STZ injection, the fasting basal glucose level (6 h fasting) of all the animals were measured. Animals having elevated blood glucose level were selected, randomized and grouped. Non-diabetic control animals were randomized and grouped separately in two groups of five animals each. Atherogenic diet was supplemented to all animals except group 1. After one week of atherogenic feeding and two weeks of STZ injection, dosing was initiated. G1 served as non-diabetic control fed with normal diet and G2 served as non-diabetic control with atherogenic diet. Both the groups received 10 mL/kg of milli-Q water. G3 served as a diabetic control fed with atherogenic diet and received 10 mL/kg of milli-Q water. G4 and G5 were treated with test item at the dose of 250 mg/kg and 500 mg/kg respectively and both were subjected to atherogenic diet. All groups were treated orally for 28 days using disposable syringes tipped with an oral gavage needle. All animals were observed daily for mortality, clinical signs of toxicity. Body weights were recorded daily throughout the dosing period. Water and feed consumption

were recorded daily. Blood was collected for biochemical estimation on 1st, 14th and 28th day of dosing period. After completion of dosing period animals were humanely sacrificed and observed for gross pathology. Aorta was isolated and analyzed for histopathological changes.

2.7.4. Effect of DB14201 in combination with standard drug metformin in STZ induced diabetes mellitus in wistar rats

95 Female wistar rats (Andrade-Cetto et al., 2005; Rees and Alcolado, 2005) were fasted overnight for 12–16 h. Hyperglycemia was induced in these rats by intra-peritoneal injection of STZ 55 mg/kg in citrate buffer (pH 4.5). One week post STZ inductions, fasting blood glucose level of all STZ injected animals (95 animals) were estimated. Out of 95 diabetes induced animals, 56 animals were randomized and allocated to different groups accordingly. Non-diabetic control animals (n=5) were grouped separately. Number of animals in diabetic group was decided depending upon glucose level after STZ induction. G1 served as non diabetic control (negative control) and G2 served as diabetic control (vehicle control) and both groups received 10 mL/kg of milli-Q water. G3 and G4 were treated with 250 mg/kg and 500 mg/kg of DB14201 (test item) respectively. G5 and G6 were administered with optimal dose (500 mg/kg) and suboptimal dose (250 mg/kg) of metformin respectively as previously reported (Alhaider et al., 2011; Quaile et al., 2010; Tanaka et al., 1999; Vianna et al., 2011). G7 was treated with combination of 250 mg/kg of metformin and 250 mg/kg of DB14201. G8 was administered with combination of 250 mg/kg of metformin and 500 mg/kg of DB14201. In case of G7 and G8, test item was administered 2 h post metformin treatment. All groups were treated orally for 21 days using disposable syringes tipped with an oral gavage needle. Fasting blood glucose level was estimated once in three days. All animals were observed daily for mortality and clinical signs of toxicity. Feed and water consumption for each group was recorded daily throughout the dosing period. Body weight of each rat was recorded daily throughout the dosing period. On 22nd day, plasma was collected and stored at −80 °C for HbA1c estimation.

2.7.5. Effect of DB14201 in combination with standard drug glibenclamide in STZ induced diabetes mellitus in wistar rats (Andrade-Cetto et al., 2005)

95 Female wistar rats were fasted overnight for 12–16 h. Hyperglycemia was induced in these rats by intra-peritoneal injection of STZ 55 mg/kg in citrate buffer (pH 4.5). One week post STZ inductions, fasting blood glucose level of all STZ injected animals (95 animals) were estimated. Out of 95 diabetes induced animals, 56 animals were randomized and allocated to different groups depending upon the glucose levels. Non-diabetic control animals (n=5) were grouped separately. Number of animals in diabetic group (n=8) was decided depending upon STZ induction.

G1 served as non diabetic control (negative control) and G2 served as diabetic control (vehicle control) and received 10 mL/kg of milli Q water. G3 and G4 were treated with 250 mg/kg and 500 mg/kg of DB14201 respectively. G5 and G6 were administered with optimal dose (5 mg/kg) and suboptimal dose (1 mg/kg) of glibenclamide respectively (Peungvicha et al., 1998). G7 was treated with combination of 1 mg/kg of glibenclamide and 250 mg/kg of DB14201. G8 was administered with combination of 1 mg/kg of glibenclamide and 500 mg/kg of DB14201. In case of G7 and G8, test item was administered 2 h post glibenclamide treatment. The groups were treated orally for 21 days using disposable syringes tipped with an oral gavage needle. Fasting blood glucose level was estimated once in three days. All animals were observed daily for mortality and clinical signs of toxicity. Feed and water consumption for each group was recorded daily throughout dosing period. Body weight of each animal was recorded daily throughout dosing period. On the 22nd day, blood was collected and stored at −20 °C for HbA1c estimation.

2.7.6. Effect of DB14201 in combination with standard drug insulin in STZ induced diabetes mellitus in wistar rats

95 Female wistar rats were fasted overnight for 12–16 h. Hyperglycemia was induced in these rats by intra-peritoneal injection of STZ 55 mg/kg in citrate buffer (pH 4.5). One week post STZ inductions, fasting blood glucose level of all STZ injected animals (95 animals) were estimated. Out of 95 diabetes induced animals, 63 animals were randomized and allocated to different groups accordingly. Non-diabetic control animals (n=5) were grouped separately. Number of animals in diabetic group was decided depending upon STZ induction (Andrade-Cetto et al., 2005; Haritha et al., 2013).

G1 served as non diabetic control (negative control) and G2 served as diabetic control (vehicle control) and both the groups received 10 mL/kg of milli Q water. G3 and G4 were treated with 250 mg/kg and 500 mg/kg of DB14201 respectively. G5 and G6 were administered with optimal dose (5 μg/kg, i.p.) and suboptimal dose (1 μg/kg, i.p.) of Insulin respectively. G7 was treated with combination of 1 μg/kg, i.p. of insulin and 250 mg/kg of DB14201. G8 was administered with combination of 1 μg/kg, i.p. of insulin and 500 mg/kg of DB14201. In case of G7 and G8, test item was administered 2 h post insulin treatment. Test item was administered orally using disposable syringe tipped with an oral gavage needle and insulin was administered intraperitoneally for 21 days. Fasting blood glucose level was estimated once in three days. All animals were observed daily for mortality and clinical signs of toxicity. Feed and water consumption for each group was recorded daily throughout dosing period. Body weight of each animal was recorded daily throughout dosing period. On the 22nd day, blood was collected and stored at −20 °C for HbA1c estimation.

2.7.7. Effect of DB14201 on prevention of development of STZ induced diabetes mellitus in wistar rats

This study was conducted to evaluate the potential of DB14201 to prevent the development of Streptozotocin induced Diabetes Mellitus in Wistar rats 35 female Wistar rats were randomized and allocated to different groups on the basis of body weight (Junod et al., 1969; Wang et al., 2012). G1 served as non diabetic control (Negative Control) and G2 served as Diabetic control (Vehicle Control) and received 10 mL/kg of Milli Q water. G3 and G4 were treated with 500 mg/kg and 250 mg/kg of DB14201 respectively. All the groups were treated orally for 21 days using disposable syringes tipped with an oral gavage needle. At the end of dosing period, hyperglycemia was induced in G2, G3 and G4 rats by intraperitoneal injection of streptozotocin (STZ) 55 mg/kg in citrate buffer (pH 4.5). Fasting blood glucose level was estimated before induction of diabetes and on 2nd and 7th days post induction. All animals were observed daily for mortality and clinical signs of toxicity throughout the experimental period. Body weight of each animal was recorded daily throughout the experimental period. On the 8th day post STZ injection, animals were killed by cervical dislocation. Their pancreatic tissues were quickly removed. Tissues were washed in normal saline and visible clots were removed to minimize blood contamination. Part of the pancreatic tissue was evaluated for histological changes. With second part of tissue, homogenates was prepared and stored at −70 °C until the determination of biochemical parameters and enzyme activity.

2.7.8. Effect of DB14201 on uncontrolled diabetes

100 Male Wistar rats were fasted overnight for 12–16 h (Lenzen, 2008). Hyperglycemia was induced in these rats by intraperitoneal injection of streptozotocin (STZ) 65 mg/kg in citrate

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buffer (pH 4.5). One week post STZ induction, fasting blood glucose level of all STZ injected animals was estimated. Out of 100 diabetes induced animals, 70 were randomized and allocated to different group accordingly (Herman-Edelstein et al., 2014; Mohamed et al., 1999). Non- Diabetic control animals (n=5) were grouped separately. G1 served as non diabetic control (Negative Control) and G2 served as Diabetic control (Vehicle Control) and both received 10 mL/kg of Milli Q water. G3 and G4 were treated with 250 mg/kg and 500 mg/kg of DB14201 respectively. G5 and G6 were administered with optimal dose (500 mg/kg) and Sub optimal dose (250 mg/kg) of Metformin respectively. G7 was treated with combination of 250 mg/kg of Metformin and 250 mg/kg of DB14201. G8 was administered with combination of 250 mg/kg of Metformin and 500 mg/kg of DB14201. Body weight of each rat was recorded daily throughout the dosing period. For G7 and G8, test item was administered 2 h post Metformin treatment. All groups were treated orally for 21 days using disposable syringes tipped with an oral gavage needle. Fasting blood glucose level was estimated once in three days. All animals were observed daily for mortality and clinical signs of toxicity. Blood glucose level, body weight, toxic signs and survival rate of animal was recorded throughout the dosing period.

2.8. Safety studies

2.8.1. Acute oral toxicity study of DB14201 in wistar rats and swiss albino mice

The study was performed as per OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects- Test No. 420: Acute oral toxicity- Fixed dose procedure, 2002, DOI: 10.1787/9789264070943-en. In the present study, both the sexes of Wistar rats and Swiss albino mice were treated orally with DB14201 at 2000 mg/kg, single dose to determine the acute toxicity (LD_{50}). 5 g of the extract was dissolved in 20 mL of Milli-Q water in a 250 mL conical flask and boiled till the volume reduced to half. The mixture was cooled to room temperature and filtered through a sieve and over a filter paper. A final concentration of 500 mg/mL of the formulation was prepared by adjusting the volume. The control animals were administered 5% dextrose. All animals were observed for treatment related signs and symptoms for 14 days post treatment (Ghosh and Suryawanshi, 2001).

2.8.2. 90-day repeated dose toxicity study of DB14201 in wistar rats by oral route

The study was performed as per the guidelines of Schedule Y: Drug and Cosmetics (IInd Amendment) Rules, 2005 and OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects–Test No. 408: Repeated dose 90-day oral toxicity study in rodents, 1998, DOI: 10.1787/9789264070707-en. The study plan was approved as per the Standard Operating Procedures and regulations of the committee for the purpose of control and supervision of experiments on animals (CPCSEA, Dabur Research Foundation Registration No.64/1999/CPCSEA).

A 90 day repeated dose oral toxicity study was designed and conducted to determine the toxicity profile of DB14201 when administered daily for 90 days in Wistar rats *via* the intended clinical route which is oral. Male and female Wistar rats, 6-8 weeks of age were used for the study. There were 15 animals/sex/group except the reversal group which comprised of 10 animals/sex. The animals were acclimatized for six days. DB14201 was administered at dose levels of 1000 mg/kg, 500 mg/kg and 250 mg/kg body weight. The reversal group was dosed at 1000 mg/kg. Control animals were administered 5% dextrose. Administration was once daily for 90 days, orally. All animals were observed for 90 days except for reversal animals which were further observed for 15 and 29 days following which they were sacrificed. Observations comprised of mortality, clinical signs, body weight and food consumption. Prior to termination, blood and urine were collected from all animals. Hematology and blood biochemistry parameters were assessed. The gross observations and organ weights were recorded for each animal. Histopathology was done for representative animals of high dose and control (Felig et al., 1970).

3. Results and discussion

DB14201 is a polyherbal formulation which is being used in humans for treatment of diabetes. The present study was undertaken in order to develop this herbal formulation as a botanical product.

3.1. Standardization, characterization, analytical method development and quality control of DB14201

In the present work, one of our objectives was to carry out quantitative estimation of multiple markers in DB14201 and to establish HPLC method for standardization of this formulation with 4 markers. Twelve markers oleanolic acid, kaempferol, curcumin, gallic acid, isoorientin, berberine hydrochloride, berbamine dihydrochloride, embelin, mangiferin, asiaticoside, khusenic acid and oleic acid were selected for quantification from all sixteen herbs on the basis of their antidiabetic activity reported in the literature (Fig. S1). The %content of these markers in DB14201 was determined using RP-HPLC analysis. Individual herb was analyzed qualitatively for the presence of selected marker component. The retention time and molecular mass of marker compound extracted from respective individual herb matched with their reference standards, which confirmed their identity. The %content of each marker compound in DB14201 is depicted in Table S1. The markers (%w/w) observed in DB14201 were 0.16 ± 0.015 oleanolic acid, $0.0006 \pm .0004$ kaempferol, 0.0026 ± 0.002 curcumin, 1.33 ± 0.258 gallic acid, 0.0005 ± 0.0002 isoorientine, 0.0001 ± 0.00002 berbamine and 0.0003 ± 0.0002 embelin. These markers showed excellent batch-to-batch consistency; on the other hand markers *viz.* berberine hydrochloride (0.0085 ± 0.0025), mangiferin (0.015 ± 0.0184), asiaticoside ($0.0116 \pm .0071$), khusenic acid (0.0001 ± 0.00005) and oleic acid (0.075 ± 0.0280) showed slight variation in the % content. It is prerequisite to standardize botanical extract/ formulation using minimum four markers (either analytical and/or biomarkers) as per phytopharmaceutical guideline (Anonymous, 2013). Therefore, mangiferin, berberine, kaempferol and curcumin were selected as analytical markers for standardization of formulation by LC-MS/MS analysis. Standardization of polyherbal formulation with multiple markers selected from each individual herb minimizes batch to batch variation, assures safety, efficacy, quality and acceptability of the polyherbal formulations (Gadre et al., 2001).

Chromatographic identification, characterization and quantification showed that all markers selected were present in the DB14201 extract but gallic acid and oleanolic acid were present in higher quantity compared to the others. The reason might be that gallic acid is present in four herbs *viz. Mangifera indica, Emblica officinalis, Terminalia chebula* and *Syzygium cumini* while oleanolic acid is present in the two herbs *viz. Zizyphus jujube* and *Strychnos potatorum.* It might be possible that antidiabetic activity of DB14201 extract is due to gallic acid and oleanolic acid. The antidiabetic action of gallic acid may be due to the enhancement of insulin receptor sensitivity by this compound (Hsu and Yen, 2007; Raid, 2010; Rice-Evans et al., 1996) while oleanolic acid has strong *α-glucosidase* inhibition activity (Wenyi et al., 2012). The oral administration of kaempferol showed significant hypoglycemic effect

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in normal and diabetic rats (De-Sousa et al., 2004). Curcumin and its derivatives exhibited cardioprotective, antidiabetic, antioxidant and nematicidal activities (Nishiyama et al., 2005; Preetha et al., 2008). All these accumulating evidences suggest that both pancreatic and extra pancreatic mechanisms might be involved in antidiabetic or antihyperglycemic action of DB14201 extract (Muruganandan et al., 2005).

Further, DB14201 extract was standardized using four markers by LC-MS/MS method so as to confirm identity, quality and quantity of their markers. All these three batches of DB14201 extract showed consistent chromatogram of marker compounds *viz.* mangiferin, berberine, kaempferol and curcumin which were similar to the chromatogram obtained with the standard marker compounds as shown in Fig. 1. The retention time for mangiferin, berberine, kaempferol and curcumin was found to be 11.5, 14.1, 15.6 and 18.4 min respectively in reference standard and in DB14201 extract. The developed LC-MS/MS method validated for linearity, LOQ and LOD. The results are depicted in Table 1.

The extract was also evaluated for different quality control parameters as listed in Table 2. The heavy metals (Pb, Cd, Hg and As) and aflotoxins content (B1, B2, G1, G2) was found within the acceptable limit as per AYUSH and US-FDA guidelines. The microbial load was within the acceptable limit as per British Pharmacopoeia 2001 and WHO/Pharma/92.

3.2. Evaluation of anti-diabetic potential of DB14201 in vitro

The plausible mechanism of action of DB14201 extract was evaluated based on key parameters *viz.* glucose uptake, TNF-α production and free fatty acids release by adipocytes and effect of insulin release by pancreatic-beta cells. DB14201 was found to be non cytotoxic at concentrations from 0.1 to 600 μg/mL for 48 h on 3T3-L1 adipocytes and 0.1–100 μg/mL for 6 h on RIN-5F cells. The criteria for evaluation of non-cytotoxic concentrations are cell viability considered as ≥ 70% as compared to control cells. Hence this concentration range was found to be non-cytotoxic and safe to use for the further evaluation of anti-diabetic potential of DB14201. The developed extract also demonstrated 68–133% increase in glucose uptake in 3T3-L1 adipocytes at concentrations from 0.1 to 600 μg/mL as compared to untreated control cells after 24 h. A maximum of 133% increase in glucose uptake was recorded at 1 μg/mL concentration. The effect of DB14201 on cytokine release in 3T3-L1 preadipocytes was also studied. 3T3-L1 preadipocytes after stimulation with LPS (100 ng/mL) led to an increase in IL-6 release from 9.87 pg/mL (untreated control cells) to 28.76 pg/mL. Whereas, 3T3-L1 preadipocytes which are pretreated with DB14201 for 24 h at concentrations ranging from 1–500 μg/mL demonstrated considerable inhibition in IL-6 release of 6.89–16.41 pg/mL or 42.94–76.04% inhibition as compared to untreated control cells. The effect of DB14201 on adipolysis demonstrated that co-treatment of TNF-α (10 ng/mL) and glucose (25 mM) led to an increase in glycerol release from 2.90 μg/mL (untreated control cells) to 19.84 μg/mL. Pretreatment with DB14201 for 24 h at concentrations from 1 to 25 μg/mL of stimulated 3T3-L1 adipocytes led to inhibition of glycerol release from 19.84 to 1.37 μg/mL – 12.39 μg/mL or 37.57–93.08% as compared to untreated control cells. Hence, DB14201 (0.1–25 μg/mL) demonstrated considerable inhibition of glycerol release against TNF-α and glucose co-stimulated levels. DB14201 (0.1–100 μg/mL) demonstrated 3.66–458.40% increase in insulin release in RIN-5F cells after 6 h of treatment. A maximum of 458.40% increase in insulin release was recorded at concentration of 100 μg/mL.

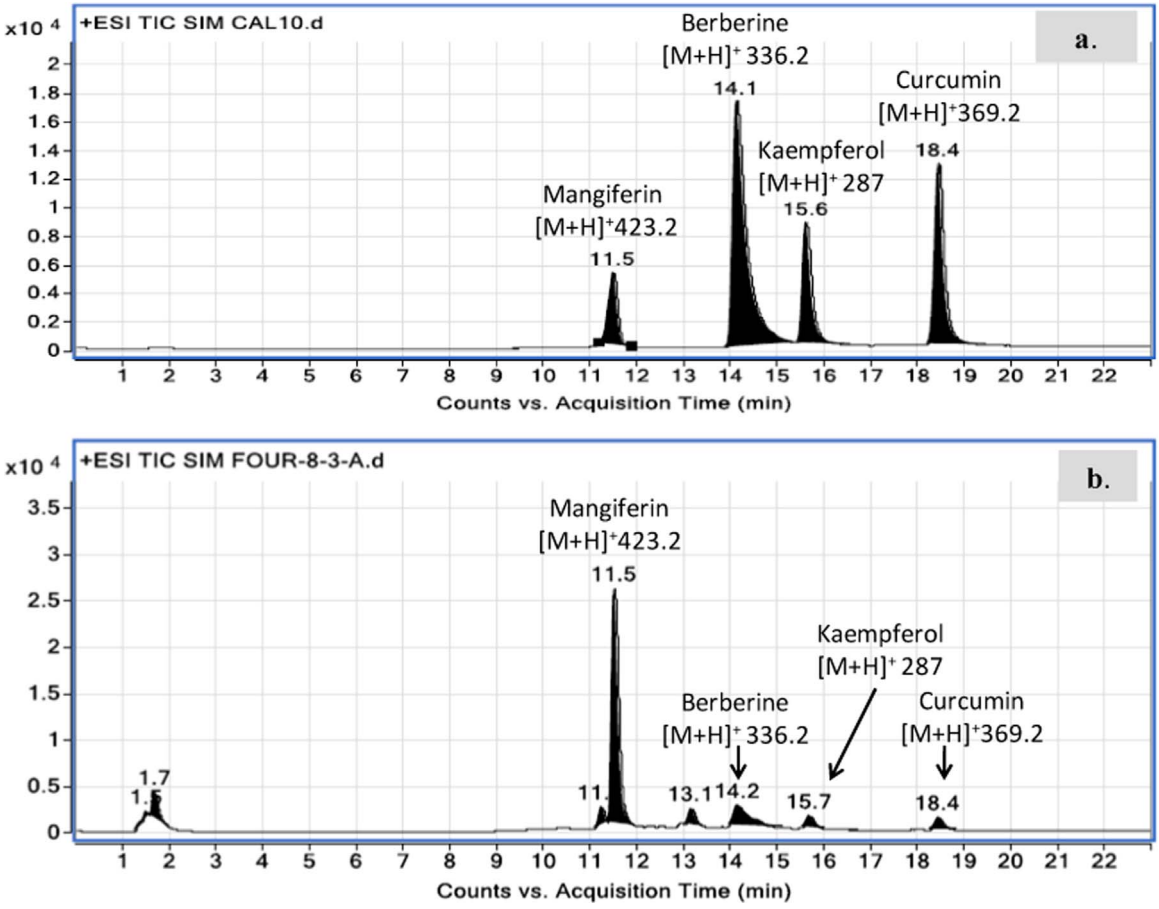

Fig. 1. (a). LC-MS/MS chromatogram of reference marker compounds, (b). LC-MS/MS chromatogram of marker compounds in DB14201 extract.

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Table 2
Quality control parameters of DB14201 extract.

Sr. No.	Parameter	Batch 1	Batch 2	Batch 3	
Other parameters					
1.	Acid insoluble ash	3.43	1.55	1.86	
2.	Loss on drying at 105 °C	8.79	6.72	5.92	
3.	pH of 1% w/w solution	4.007	4.007	4.007	
4.	Total ash	13.052	14.3692	14.2582	
5.	Water soluble extractive	77.011	79.9494	82.236	
6.	50% alcohol soluble extractive	64.448	69.46	68.96	
Sr. No.	**Parameter**	**Batch 1**	**Batch 2**	**Batch 3**	**Limits**
Microbial testing					
1.	Total aerobic bacterial count	2.8×10^4	2.0×10^4	1.6×10^4	$< 10^3$
2.	Total yeast, mold count	6.0×10^4	30	Nil	$< 10^3$
3.	*Enterobatecriace* count	> 10 but < 10	< 10	< 10	$< 10^3$
4.	*Escherichia coli*	Absent	Absent	Absent	10/g
5.	*Salmonella spp.*	Absent	Absent	Absent	Absent
6.	*Staphylococcus aureus*	Absent	Absent	Absent	Absent
7.	*Pseudomonas aeruginosa*	Absent	Absent	Absent	Absent

Parameter	Batch 1	Batch 2	Batch 3	Limits (ppm) As per AYUSH	Limits (ppm) As per USFDA/USP
Heavy metals					
Pb	0.85	1.09	1.05	10	3–10 mg/kg
Cd	0.1	0.1	0.14	0.3	–
Hg	BDL	BDL	BDL	1.0	3.0 mg/kg
AS	0.01	BDL	BDL	3.0	3.0 mg/kg

Parameter	Batch 1	Batch 2	Batch 3
Aflatoxins content			
Aflatoxins (B1, B2, G1, G2)	BDL	BDL	BDL

BDL, Below detection limit.

3.3. Efficacy studies of DB14201

Anti-diabetic potential of DB14201 in streptozotocin (STZ) induced type I Diabetes mellitus in rats. The anti-hyperglycemic effect of DB14201 administered by oral route in STZ induced Type I diabetic rats was studied. After 14-days of continuous treatment with DB14201 in 500 mg/kg treatment group, the blood glucose levels decreased by 85.24% showing a significant ($P < 0.01$) anti-hyperglycemic potential of extract with reference to the hyperglycemic control animals. While a decline in blood glucose levels of animals treated with 250 mg/kg of the test item was observed, this was not found to be statistically significant. By the 15th day of treatment, there was a slight elevation in the serum insulin levels of both the treated groups as compared to the hyperglycemic control animals, however this was found to be statistically insignificant. The DB14201 treatment of hyperglycemic animals did not show any alterations in serum tri-glycerides and total cholesterol levels and these values were found comparable to that of the normal control animals on day 1 and 15. Urea, Creatinine and SGPT levels in serum were elevated in STZ induced diabetic rats as compared to the normal control animals on day 1 and 15 (Felig et al., 1970; Ghosh and Suryawanshi, 2001). These parameters did not decline significantly even after the 14-day treatment with the extract. The serum SGOT levels remained the same in normal control group as well as the STZ treated groups on both days 1 and 15. Treatment with DB14201 did not show any lowering effect. Glycogen synthesis in the rat liver is impaired in diabetes (Huang et al., 2000). In this study, a decrease was observed in the hepatic glycogen content of diabetic animals in consistence with the earlier studies (Whitton and Hems, 1975). In this study, the hepatic glycogen content of the 500 mg/kg treated animals showed a mild increase when compared to that seen in the hyperglycemic control group. The difference was not statistically significant indicating the inability of the test item to restore the hepatic glycogen content. Treatment with DB14201 seemed to offer protection against the hyperglycemic clinical symptoms *viz.* polyuria and polydipsia. DB14201 treated animals had a smooth and even fur coat as opposed to coarse fur coat in hyperglycemic control group. However there was no protection against polyphagia and diarrhea throughout the study period. The characteristic loss of body weight caused due to hyperglycemia showed no significant change upon treatment with DB14201. In conclusion, the study demonstrated the ability of DB14201 extract to effectively bring down blood sugar levels in hyperglycemic rats after 14-day continuous oral administration and this activity is seen best at a dose of 500 mg/kg/day body weight.

Effect of DB14201 in STZ induced diabetic nephropathy in C57BL/6 mice. The results indicated dose dependant promising anti-diabetic activity of DB14201 at the tested dose level and treatment period in multiple low dose of STZ induced diabetes model. Considerable inhibition in elevation of glycated hemoglobin indicates the regression of diabetic severity. Biochemical data at the end of treatment period suggests the utility of DB14201 treatment in diabetes induced complications. Two month of diabetic stabilization in experimental model leads to further biochemical changes that lead to several complications. Four week treatment of DB14201 at high dose (500 mg/kg) evidence of remarkable inhibition of circulating glucose level and urinary microalbumin strongly exhibits the significance of DB14201 in diabetes induced nephropathy. There was no change in glomerular number and nodular lesions and tubules, blood vessels and interstitium were found to be within normal limit in all assigned groups. The glomerular (G) loops have thin basement membranes normal complement of cells and the mesangial matrix is sparse. Tubular (T) basement membranes are within normal limits and tubular epithelium does not show any damage (Fig. 2).

Effect of DB14201 in STZ induced diabetic atherosclerosis in C57BL/6 mice. The results obtained from the STZ induced diabetes followed by atherogenic diet induced atherosclerosis animal model demonstrated that, 28 days oral administration of 500 mg/kg of DB14201 has potential role in reducing the levels of biomarkers that leads to the formation of atherosclerosis.

Effect of DB14201 in combination with standard drug metformin in STZ induced diabetes mellitus in wistar rats. Findings of this study demonstrated the anti-diabetic activity of DB14201 in combination with suboptimal dose of metformin as depicted in Fig. 3a. The combination of DB14201 250 mg/kg and 500 mg/kg with suboptimal dose of metformin *i.e.* 250 mg/kg showed additive anti-diabetic activity as compared to individual treatments. Interestingly, 21 days treatment of high dose of DB14201 with suboptimal dose of metformin demonstrated greater anti-diabetic activity as compared to optimal dose of metformin.

Effect of DB14201 in combination with standard drug glibenclamide in STZ induced diabetes mellitus in wistar rats. Findings of this study demonstrated the role of DB14201 in the potentiation of anti-diabetic effect of suboptimal dose of glibenclamide *i.e.* 1 mg/kg. Result indicated the anti-diabetic effect of combination treatment of high dose of DB14201 and sub optimal dose of glibenclamide was comparable to anti-diabetic effect of optimal dose

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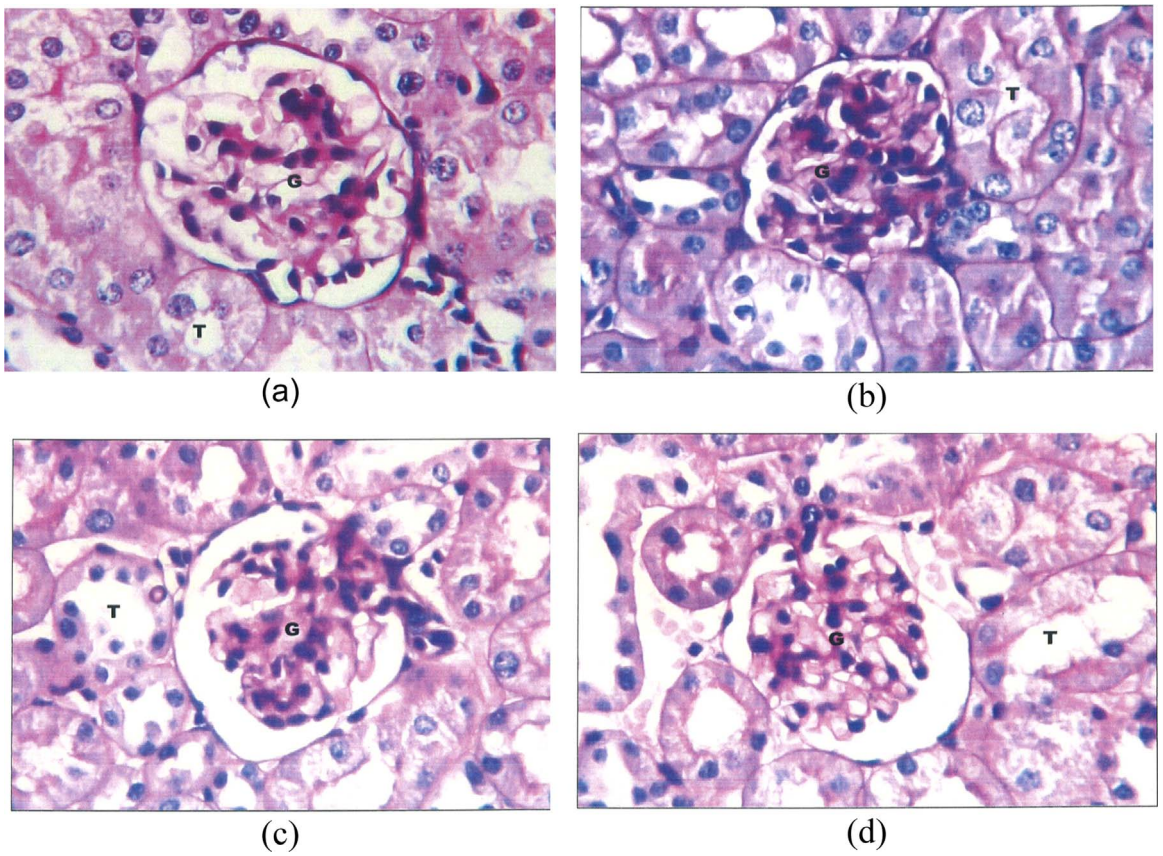

Fig. 2. High power photomicrograph of kidney showing normal renal parenchyma. a) Group 1; b) Group 2; c) Group 3; d) Group 4.

of glibenclamide (Fig. 3b). Hence it can be concluded that, the combination of sub optimal dose (1 mg/kg) of glibenclamide with DB14201 500 mg/kg shows superior results as compared to individual treatment of glibenclamide (1 mg/kg) and DB14201 500 mg/kg.

Effect of DB14201 in combination with standard drug insulin in STZ induced diabetes mellitus in wistar rats. Findings of this study demonstrated the role of DB14201 in the potentiation of anti-diabetic effect of suboptimal dose of insulin. Low dose of DB14201 in combination offered superior results (Fig. 3c). The combination of suboptimal dose of insulin *i.e.* 1 μg/kg with DB14201 500 mg/kg shows better results as compared to individual treatment of both insulin (1 μg/kg) and DB14201 (500 mg/kg). In addition to this the combination of suboptimal dose of insulin *i.e.* 1 μg/kg with DB14201 250 mg/kg showed enhanced result as compared to individual treatment of both insulin (1 μg/kg) and DB14201 (250 mg/kg). Interestingly, result indicated the anti-diabetic effect of combination treatment of low dose of DB14201 and sub optimal dose of insulin was comparable to anti-diabetic effect of optimal dose of insulin (5 μg/kg).

Effect of DB14201 on prevention of development of STZ induced diabetes mellitus in wistar rats. Findings of this study demonstrated that DB14201 treatment have prominent role in the prevention of STZ induced diabetes mellitus. Result indicated the prophylactic as well as therapeutic potential of DB14201 in this experimental model. DB14201 at the dose of 500 mg/kg was found to be more promising (Fig. 3d).

Effect of DB14201 on uncontrolled diabetes. Amongst all the groups treated with DB14201, G7 *i.e.* Combination of Metformin 250 mg/kg and DB14201 250 mg/kg showed maximum efficacy in lowering the blood glucose level as shown in Fig. 3e. However in the same group mortality rate was high. G3 *i.e.* DB14201-250 mg/kg showed maximum survival rate at the end of the study. The present study demonstrated that DB14201 250 mg/kg offers maximum protection from mortality in case of uncontrolled diabetes.

3.4. Safety studies of DB14201

Acute oral toxicity study of DB14201 in wistar rats and swiss albino mice. The test substance did not cause toxicity and all animals survived the study period of 14 days. The LD_{50} of DB14201 in both the sexes of rats and mice was found to be greater than 2000 mg/kg of the body weight.

90-day repeated dose toxicity study of DB14201 in wistar rats by oral route. All the animals from the control and the treated dose groups survived throughout the dosing period of 90 days and the reversal period except few incidental mortality in high dose and high dose reversal groups. These mortalities are considered incidental since these animals did not show any characteristic clinical signs, body weight loss, reduced feed consumption and also no abnormality in vital organs on necropsy. Male and female animals from all the treated animals including the reversal group exhibited comparable body weight gain with respect to the controls throughout the dosing period of 90 days and the reversal period. Food consumption of control and treated animals were found comparable throughout the dosing period of 90 days and was normal during the reversal period. Hematology, biochemistry and urine analysis parameters on day 90 and reversal did not show any treatment related changes. Gross pathological examination did not reveal any abnormality and the relative organ weights were comparable to control. Histopathological examination was carried out on 5 representative animals of both the sexes in control and high dose animals and did not reveal any test item related changes. Thus the 90 day oral No Observed Adverse Effect Level (NOAEL) of DB14201 in Wistar rats (male & female) may be considered as 1000 mg/kg b.w.

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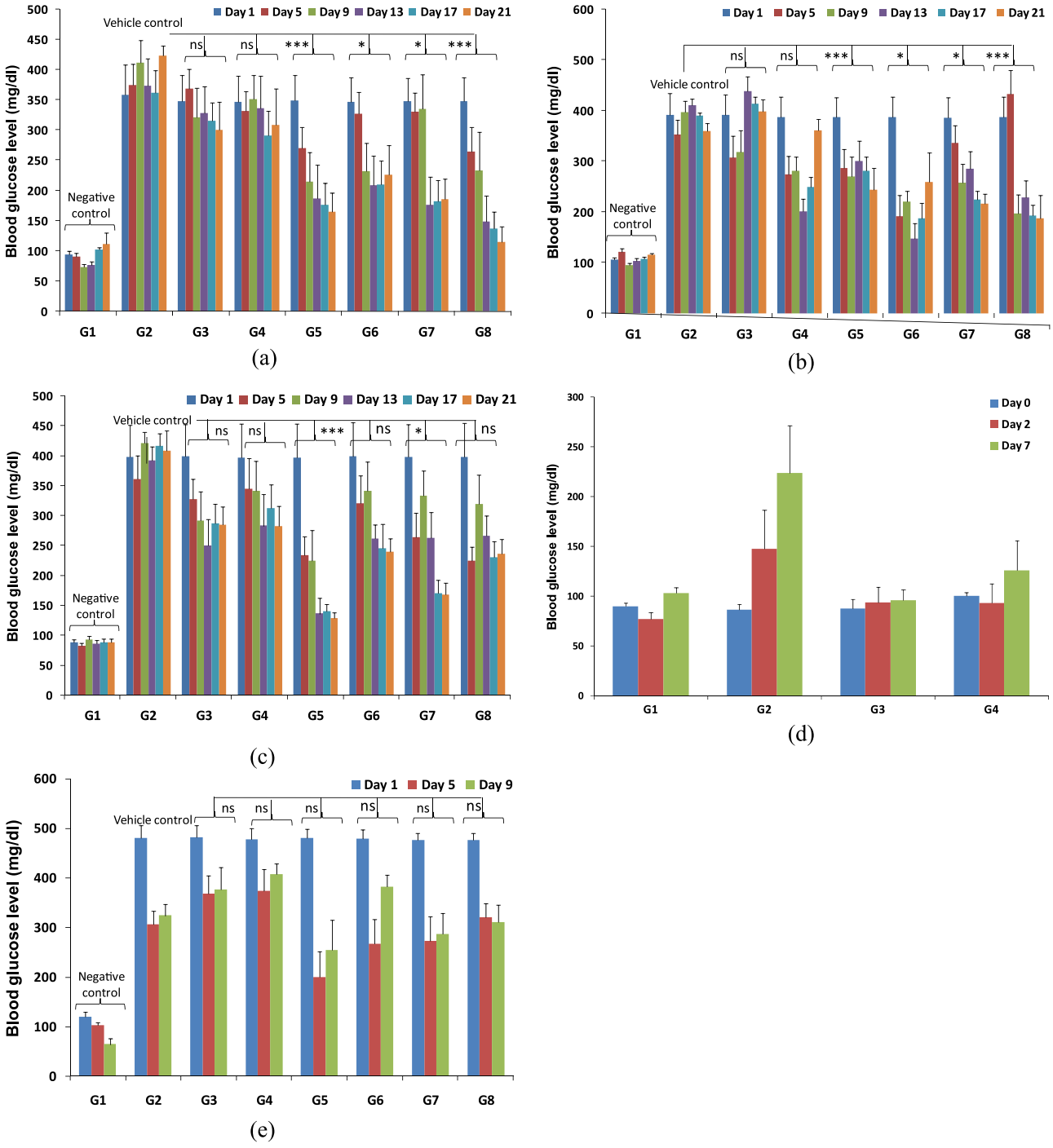

Fig. 3. Effect of DB14201 on blood glucose level a) in combination of standard drug metformin; b) in combination of standard drug glibenclamide; c) in combination of standard drug insulin; d) prevention of development of STZ induced diabetes mellitus in wistar rats; and e) in uncontrolled diabetes.

4. Conclusion

In summary, standardization, characterization and quality control of antidiabetic polyherbal DB14201 extract was carried out. Developed extract was found to be efficacious *in vivo* and was found to be safe as per OECD Guidelines for the Testing of Chemicals. The possible mechanism of action of DB14201 was also studied *in vitro*. The extract DB14201 demonstrated considerable increase in glucose uptake, inhibition of TNF-α and glucose co-stimulated glycerol release in 3T3-L1 adipocytes and inhibition of LPS stimulated IL-6 release in 3T3-L1 preadipocytes. It also showed increased insulin release in pancreatic beta cells. The data generated will serve as the benchmark for the further research on this developed polyherbal DB14201 formulation.

Conflict of interest

G. Geetha Krishnan is the inventor of the formulation DB14201 in the US patent application. He is also a shareholder in Innoveda Biological Solutions (P) Ltd, which developed the polyherbal formulation DB14201. The approved US patent on DB14201, is owned by Innoveda Biological Solutions (P) Ltd.

No other declaration of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2016.07.062.

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