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Supercritical carbon dioxide extracts of small cardamom and yellow mustard seeds have fasting hypoglycaemic effects: diabetic rat, predictive iHOMA2 models and molecular docking study

Kaninika Paul[1], Soumi Chakraborty[1], Priyanka Mallick[2], Paramita Bhattacharjee[1]*, Tapan Kumar Pal[3], Nilendra Chatterjee[4] and Saikat Chakrabarti[2]

[1]*Department of Food Technology and Biochemical Engineering, Jadavpur University, Kolkata 700032, India*
[2]*Division of Structural Biology and Bioinformatics, CSIR-Indian Institute of Chemical Biology, Kolkata 700032, India*
[3]*Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India*
[4]*TAAB Biostudy, Jadavpur, Kolkata 700032, India*

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Abstract

In the present investigation, the supercritical carbon dioxide (SC-CO_2) extracts of small cardamom (SC) and yellow mustard (YM) seeds have been investigated for their efficacies in combating type 2 diabetes in streptozotocin-induced Wistar albino rats. Fasting blood glucose (FBG) levels in the rats were monitored on days 8, 15 and 21. On day 15, FBG level reduced appreciably by 31·49 % in rats treated with SC seed extract and by 32·28 % in rats treated with YM seed extract, comparable to metformin (30·70 %) and BGR-34 (a commercial polyherbal drug) (31·81 %) administered rats. Either extract exhibited desirable effects on hepatic glucose-6-phosphatase, glucose-6-phosphate dehydrogenase (G6PD) and catalase activities in controlling diabetes. A molecular docking exercise was conducted to identify specific compounds in the extracts which possessed augmenting effect on G6PD. The results revealed that all the bioactive compounds in the extracts have binding affinities with the enzyme and contributed to the antidiabetic efficacies of the extracts as G6PD augmenters. The effects of the extracts on insulin sensitivity and glucose uptake were investigated using non-invasive modelling by iHOMA2 software. This *in vitro* approach indicated that extract administration resulted in increased both insulin sensitivity of the liver and glucose uptake in the gut. The findings of the present study attest these SC-CO_2 extracts of the spices as safe alternatives of metformin and BGR-34 in combating type 2 diabetes and could be safely subjected to clinical studies. These extracts could also be employed in designing proactive food supplements in mitigating the metabolic disorder.

Key words: Hypoglycaemic activity: Supercritical carbon dioxide extraction: Small cardamom seeds: Yellow mustard seeds: iHOMA2

Herbs and spices are well recognised for their medicinal benefits since Ayurvedic ages. Small cardamom (SC), the 'Queen of spices' (*Elettaria cardamomum*) and yellow mustard (YM) (*Brassica campestris*), commonly known as field mustard, are two of the widely used spices in India. These spices are reportedly known to possess strong antioxidant, anti-inflammatory and antimicrobial properties[1,2]. Authors have successfully extracted bioactive components, that is, 1,8-cineole from SC seeds and melatonin from YM seeds employing supercritical carbon dioxide (SC-CO_2) extraction[3,4]. The SC-CO_2 extracts thus obtained have revealed appreciable antioxidant properties along with *in vitro* α-amylase inhibitory activities[3,4]. Moreover, *in vivo* hypocholesterolaemic efficacies of the extracts have been established in Wistar albino rats in terms of their total cholesterol and lipoprotein levels[5].

Reportedly, both 1,8-cineole and melatonin in their synthetic forms have low LD_{50} (lethal dose, 50 %) values and exhibited adverse effects in rat models as well as in clinical trials[6–10]. The aforementioned findings of the extracts and reported toxicities of synthetic 1,8-cineole and melatonin prompted us to investigate the efficacies of the natural SC-CO_2 extracts as alternate preventive therapeutics for diabetes (type 2) *in vivo,* in Wistar albino rats. To the best of our knowledge, there is no literature report on cardamom and mustard seed extracts as antidiabetic agents.

Prevention of type 2 diabetes occurs through a myriad of pathways chiefly, regulation of blood glucose level, enhancing insulin secretion, facilitating insulin sensitivity by increasing peripheral glucose utilisation and regulation of antioxidant activity, to name a few[11]. In type 2 diabetes, augmentation of

Abbreviations: b.w, body weight; FBG, fasting blood glucose; GOLD, Genetic Optimization for Ligand Docking; G6Pase, glucose-6-phosphatase; G6PD, glucose-6-phosphate dehydrogenase; SC, small cardamom; SC-CO_2, supercritical carbon dioxide; YM, yellow mustard.

* **Corresponding author:** Paramita Bhattacharjee, fax +91 33 2414 6822, email paramita.bhattacharjee@jadavpuruniversity.in

glucose-6-phosphatase (G6Pase) activity and reduction of glucose-6-phosphate dehydrogenase (G6PD) activity result in increase in fasting blood glucose (FBG) level[12,13]. Therefore, the present study also investigates the effect of SC-CO_2 extracts on these aforesaid enzymes to ascertain whether usage of these extracts lowers FBG level in hyperglycaemic animals through desirable up- and down-regulations of the enzymes.

According to Yu *et al.*[14], hyperglycaemia leads to production of reactive oxygen species and simultaneously attenuation of free radical scavenging compounds. Antioxidant enzymes such as catalase appear to be essential for cellular defence against reactive oxygen species[15]. Since both these extracts exhibited strong antioxidant activities (in terms of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity), it is envisaged that these spice extracts would exhibit secondary effects by scavenging reactive oxygen species production in type 2 diabetes. Therefore, the effects of the aforesaid extracts on hepatic catalase activity have also been analysed.

The present study forays into *in vivo* investigation of hypoglycaemic efficacies of SC-CO_2 extracts (SC and YM seeds) in streptozotocin (STZ)-induced hyperglycaemic Wistar albino rats in terms of FBG level, serum lipid profile, plasma insulin level along with their effects on G6Pase, G6PD and catalase activities for 3 weeks. An *in vitro* approach was adopted to explore the effects of the extracts on insulin sensitivity as well as on glucose uptake using the non-invasive method of iHOMA2[16]. Additionally, *in silico* molecular docking studies have been conducted by docking the major extract constituents with G6PD. This exercise allowed us to identify which chemical compounds among the major extracted constituents augmented the activity of the aforesaid enzyme. The findings of *in silico* and *in vivo* studies were then corroborated to eliminate ambiguity in conferring these spice extracts as novel biotherapeutics. The investigative schemes of the present study have been illustrated in Fig. 1. The uniqueness of the present study lies in investigating the antidiabetic roles of 1,8-cineole-rich and melatonin-rich extracts of globally consumed spices *viz.* SC and mustard, respectively. The outcome of the present study can safely be extrapolated to redress type 2 diabetes in human. These extracts would serve as antidiabetic supplements or could be ingredients for new spice-based therapeutic foods or drugs.

Materials and methods

Materials

Authenticated Alleppey green cardamom (*Eletteria cardamomum*) seeds (of export quality) were procured from Spices Board, Cochin, Kerala, India and authenticated B_9 variety of YM seeds (*Brassica campestris*) were provided by the Faculty Center for Integrated Rural Development and Management, Narendrapur, South 24 Parganas, West Bengal, India. Food-grade CO_2 was purchased from BOC India Ltd. SPE-ED matrix for SFE vessel packing was procured from M/s Applied

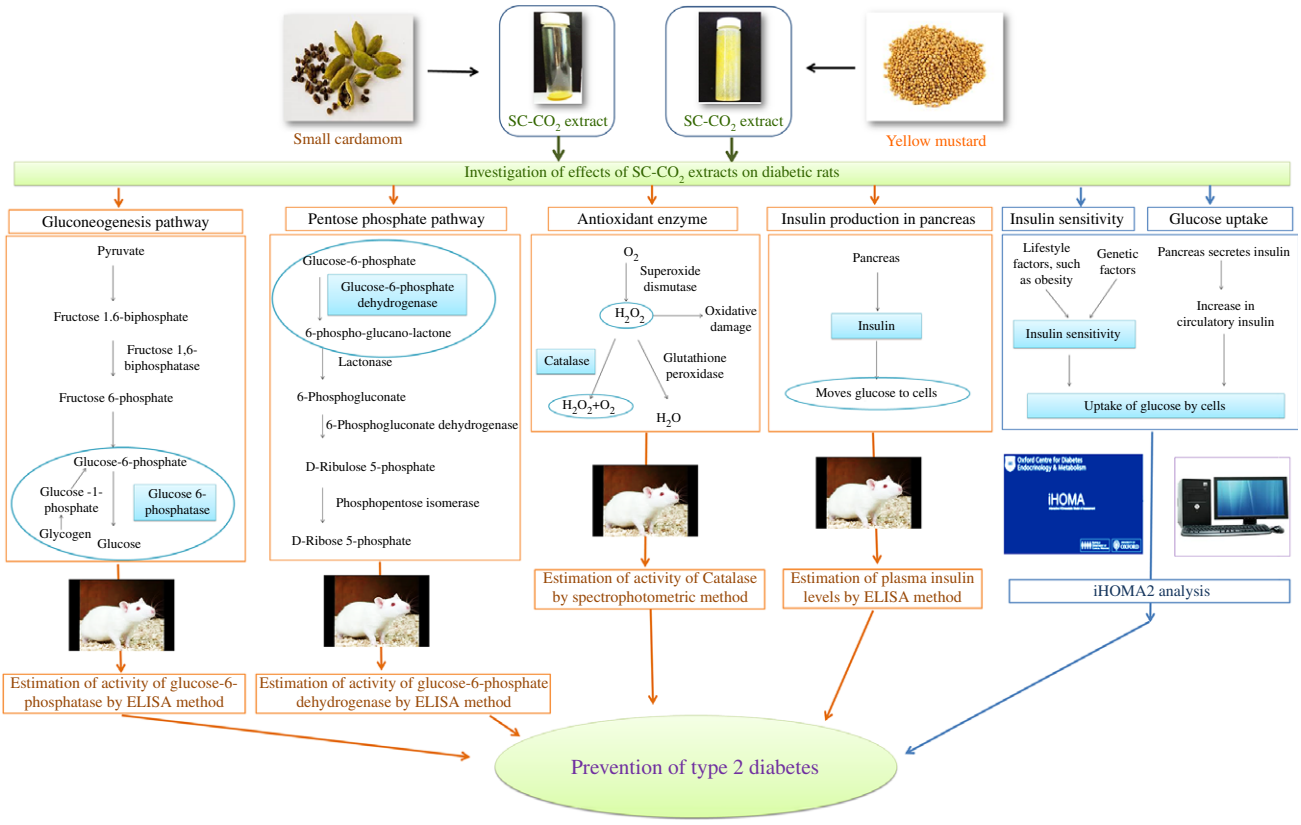


Fig. 1. Schematic representation of investigative pathways for supercritical carbon dioxide (SC-CO_2) extracts. ▭, *In vivo* method; ▭, *in vitro* method.

Separations. ELISA kits for G6pase and G6PD were procured from Elabscience. ELISA kit for insulin estimation was purchased from Bioassay Technology Laboratory.

Supercritical carbon dioxide extraction of bioactive components: 1,8-cineole from small cardamom seeds and melatonin from yellow mustard seeds

A laboratory scale 'SCF Green Technology SPE-ED SFE 2' model of M/s Applied Separations was used for SC-CO_2 extractions of melatonin and 1,8-cineole from YM and SC, respectively (online Supplementary Fig. S1). The extraction pressure (100, 200 and 300 bar), temperature (40, 50 and 60°C) and extraction time (30, 60 and 90 min) were optimised using a 3^3 full factorial design on the basis of the best combination of yield of 1,8-cineole and phytochemical properties, *viz.* antioxidant, antidiabetic and hypocholesterolaemic in the extracts. The optimised extraction conditions for 1,8-cineole from SC seeds were a sample size of 25 g of SC seeds ($d_p = 0{\cdot}5$ mm), an extraction pressure of 200 bar, an extraction temperature of 50°C, an extraction time of 90 min and a flow rate of 2 litres/min of CO_2[3].

A 3^2 full factorial design was employed for optimisation of extraction parameters (pressure (200, 300 and 400 bar), temperature (40, 50 and 60°C)) of SC-CO_2 extraction from YM seeds on the basis of the best combination of yield of melatonin and phytochemical properties, *viz.* antioxidant, antidiabetic and hypocholesterolaemic in the extracts. The optimised extraction conditions were 300 bar, 50°C, 120 min, a flow rate of 2 litres/min of CO_2 and 30 g of YM seeds ($d_p = 0{\cdot}5$ mm). The extracts were stored in N_2-flushed amber-coloured screw-capped glass vials at controlled temperature (YM extract at –20°C, SC seed extract at 4°C) until further study. These extracts being free of solvents could be directly employed *in vivo* in animal model studies. The extracts of SC and YM seeds were designated as SC_E and YM_E, respectively.

Identification and quantification of bioactive components: 1,8-cineole in SC_E and melatonin in YM_E

The presence of 1,8-cineole in SC_E and melatonin in YM_E has been detected employing GC-MS and LC-MS analyses, respectively.

GC-MS and LC-MS analyses of SC_E and YM_E

The extract having the maximum content of 1,8-cineole (obtained at 200 bar, 50°C, 90 min) was analysed by GC-MS in accordance with the method reported by Ghosh *et al.*[1], while YM_E was subjected to LC-electrospray ionisation (ESI)-MS analysis in accordance with the method reported by Chakraborty *et al.*[5]. Identification of the components of the extracts was based on comparison of the mass spectra of compounds in the National Institute of Standards and Technology (NIST)[17] compound library and those published in literature[18,19].

Quantification of 1,8-cineole in SC_E and melatonin in YM_E

High-performance TLC of SC_E was conducted to estimate the 1,8 cineole content therein in accordance with the method described by Ghosh *et al.*[1]. Melatonin in YM_E was quantified by HPLC in accordance with the method reported by Chakraborty & Bhattacharjee[4]. Both extracts were diluted in food-grade 10 % dimethyl sulfoxide (DMSO) immediately before oral administration to rats.

Experimental animal acclimatisation and welfare-related assessments

Male Wister albino rats (130–150 g) aged 2 months were procured from M/s Rita Ghosh Private Ltd. The study was conducted in the laboratory of Department of Physiology, Nutrition and Microbiology, Raja N.L. Khan Women's College, Midnapore, West Bengal, India, in the months of January to March 2017. The experiments were conducted as per approved guidelines of Institutional Animal Ethical Committee guidelines and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, registration no. 190/PO/Re/S/2016/CPCSEA.).

The animals were maintained and acclimatised under standard environmental conditions (17–23°C, 60 (SD 5) % relative humidity) for 3 weeks prior to the study. Acclimatised rats were selected randomly and three rats per polyacrylic cage (30 × 23 × 10 cm) were housed inside a temperature and humidity-controlled room with 12 h dark –12 h light – (180 to 200 lux at day time) cycles. They were provided with standard rat feed (a mixture of wheat flour, Bengal gram flour, milk powder, salt and distilled water, on weight basis) and distilled water *ad libitum* throughout the experimental period. Animal care and personal hygiene of the researchers were maintained according to the Guide to the Care and Use of Experimental Animals[20]. Food intake and body weight (b.w.) were measured regularly during the experimental period.

Administration of supercritical carbon dioxide extracts to hyperglycaemic Wistar albino rats

Diabetes was induced in each rat by injecting STZ intraperitoneally at a standard dose of 60 mg/kg of the b.w. on day 1 in the morning at 09.00 hours, in accordance with the method reported by Paul *et al.*[21]. The FBG levels were determined in STZ-injected rats after 72 h of injection. Rats with FBG levels between 120 and 130 mg/dl were considered for the study. The highest dose of the SC-CO_2 extracts for oral administration was selected to be 550 mg/kg b.w., much lower than the LD_{50} of the extracts, that is, 5000 mg/kg b.w.[5]. Groups C and HC were treated as normal control and negative control, respectively, and were given normal food and water *ad libitum*. Groups M and B served as positive controls and were treated with metformin (50 mg/kg b.w.) and a polyherbal drug (BGR 34 (600 mg/kg b.w.) developed by the Council of Scientific & Industrial Research (CSIR)), respectively. Groups E_1–E_{12} were orally administered (commonly used method for administering drugs into the gastrointestinal tract) with SC-CO_2 extracts (SC and YM seeds) at regular doses of 55, 175, 550 mg/kg b.w. (Table 1) at 10.00 hours. from day 4 onwards for 21 d in accordance with Organisation for Economic Co-operation and Development (OECD) guidelines[22]. The total number of rats used in the present study was ninety-six where each group had six rats. Blood was collected from the retro-orbital plexus of animals on days 8, 15 and 21 of the experimental period, and liver and kidney were collected after

British Journal of Nutrition

Table 1. Groups of streptozotocin-injected animals treated with metformin, supercritical carbon dioxide (SC-CO_2) extracts of yellow mustard (YM) and small cardamom (SC)

Groups	Details of groups	Treatment given
C	Control	Normal diet and 10 % DMSO solution
HC	Diabetic control	Injected with streptozotocin (50 mg/kg b.w.) intraperitoneally
M	Metformin	Oral administration of metformin at the dose of 50 mg/kg b.w. for 21 d
B	BGR-34	Oral administration of BGR-34 at the dose of 600 mg/kg b.w.
E1	YM-550	Oral administration of SC-CO_2 extract of yellow mustard seeds at the dose of 550 mg/kg b.w. for 21 d
E2	YM-175	Oral administration of SC-CO_2 extract of yellow mustard seeds at the dose of 175 mg/kg b.w. for 21 d
E3	YM-55	Oral administration of SC-CO_2 extract of yellow mustard seeds at the dose of 55 mg/kg b.w. for 21 d
E4	SC-550	Oral administration of SC-CO_2 extract of small cardamom seeds at the dose of 550 mg/kg b.w. for 21 d
E5	SC-175	Oral administration of SC-CO_2 extract of small cardamom seeds at the dose of 175 mg/kg b.w. for 21 d
E6	SC-55	Oral administration of SC-CO_2 extract of small cardamom seeds at the dose of 55 mg/kg b.w. for 21 d
E7	YM-550+M	Oral administration of SC-CO_2 extract of yellow mustard seeds at the dose of 550 mg/kg b.w. for 21 d along with metformin (50 mg/kg b.w.)
E8	YM-175+M	Oral administration of SC-CO_2 extract of yellow mustard seeds at the dose of 175 mg/kg b.w. for 21 d along with metformin (50 mg/kg b.w.)
E9	YM-55+M	Oral administration of SC-CO_2 extract of yellow mustard seeds at the dose of 55 mg/kg b.w. for 21 d along with metformin (50 mg/kg b.w.)
E10	SC-550+M	Oral administration of SC-CO_2 extract of small cardamom seeds at the dose of 550 mg/kg b.w. for 21 d along with metformin (50 mg/kg b.w.)
E11	SC-175+M	Oral administration of SC-CO_2 extract of small cardamom seeds at the dose of 175 mg/kg b.w. for 21 d along with metformin (50 mg/kg b.w.)
E12	SC-55+M	Oral administration of SC-CO_2 extract of small cardamom seeds at the dose of 55 mg/kg b.w. for 21 d along with metformin (50 mg/kg b.w.)

DMSO, dimethyl sulfoxide; b.w., body weight.

killing animals on day 21. The serum and plasma were prepared in accordance with standard protocols and the samples were stored at −20°C for further study[23]. Details of *in vivo* experiments are schematically represented in online Supplementary Fig. S2.

In vivo *evaluation of effects of SC_E and YM_E on hyperglycaemic Wistar albino rats*

Estimation of fasting blood glucose level, serum lipid profile and plasma insulin level. The FBG levels of rats (all groups) were periodically monitored during the treatment on days 8, 15 and 21. The blood samples of all rats were estimated for FBG levels and total cholesterol, TAG, HDL and LDL were estimated in the serum samples by semi-autoanalyser (Merck), according to standard protocols[24–27]. Plasma insulin levels were evaluated using ELISA kit with assay sensitivity of 0·05 mIU/l[21].

Monitoring stability in fasting blood glucose level after discontinuation of oral administration of SC_E and YM_E. The optimised dose and duration of administration of SC_E and YM_E (obtained from above experiment) were found to be 550 mg/kg b.w. and 15 d, respectively (details of optimisation have been described later). Since there were negligible changes in FBG levels of the extract-administered rats on days 15 and 21, the FBG levels of rats were monitored on days 15, 17, 19 and 21 (by withdrawing blood from the retro-orbital plexus of the rats) after discontinuation of extract administration on day 15 to ascertain whether the FBG levels remained unaltered up to day 21. Concomitantly, the wellness (physical and/or psychological) parameters of the rats were also monitored.

Estimation of glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and catalase activity. The rat liver homogenates were prepared in ice-cold 50 mM TRIS–HCl buffer (pH 7·5) according to the method reported by Ahmad & Ahmad[28] and the protein content therein was estimated using the method described by Bradford[29]. Both G6Pase and G6PD activities in rat livers were assayed using ELISA kits. The hepatic catalase activity was estimated according to the method elaborated by Sinha[30].

Histopathological studies and determination of liver and kidney markers. To confirm whether there are any morphological changes in livers and kidneys of the experimental rats, the said organs were examined under light microscopy (M/s Olympus). The levels of different liver (serum glutamic pyruvic transaminase (SGPT) and serum glutamic-oxaloacetic transaminase (SGOT)) and kidney markers (BUN, serum urea and creatinine) were determined to ascertain toxic effects of the extracts on the said organs, if any[31].

Estimation of human equivalent dose. There are four different methods of extrapolation of dose from animals to human, namely: dose by factor, similar drug, pharmacokinetically guided and comparative approaches[32]. In the present study, the 'dose by factor' approach has been used to estimate human equivalent dose values of SC_E and YM_E from their respective optimised animal doses.

Molecular docking studies of extract components with glucose-6-phosphate dehydrogenase. The binding affinities of major components of SC_E and YM_E with G6PD were computed by molecular docking to identify the chemical compounds in the extracts which redressed type 2 diabetes. The major components of the aforesaid extracts include limonene and linalool, besides α-terpinyl acetate, α-terpineol and 1,8-cineole in SC_E; and melatonin, tocopherol and ascorbic acid in YM_E. Therefore, the aforesaid components were docked at the known

British Journal of Nutrition

substrate binding site of G6PD receptor using the GOLD (Genetic Optimization for Ligand Docking) package[33].

From the three-dimensional (3D) structures, the respective aforesaid active ingredients of SC_E and YM_E were retrieved from the PubChem database, and solvent accessible surface area was generated using the Chimera program[34,35]. Top ten energetically preferred conformers of all the active ingredient molecules were generated using FROG2 (FRee On line druG conformation generation) software[36]. Molecular docking of these cardamom extract components was performed using the structure of G6PD receptor collected from the protein data bank (PDB)[37] (PDB ID: 5UKW). Ingredient molecules were docked at the known substrate binding site of G6PD receptor using the GOLD package Cambridge Crystallographic Data Centre (CCDC) (Gold Suite 5.2.2) with two scoring systems, that is, GOLD Scoring and ChemPLP Scoring. GOLD software optimises the fitness score of many possible docking solutions using a genetic algorithm. Following parameters were used in the docking cycles: population size (10), selection pressure (1·100 000), number of operations (100 000), number of islands (5), niche size (2), crossover weight (95), mutate weight (95) and migrate weight (10). Docking solutions from various conformers were clustered based on their root mean square deviation representing structural similarity. Subclusters with minimum of three solutions with root mean square deviation < 2 Å with each other were considered for further analysis. Top three scoring solutions (pose) from the largest cluster were selected for further comparison. For comparison purpose, rescoring of glucose-6-phosphate-bound structure of G6PD receptor structure was carried out using the GOLD program. Ligand–protein interactions were identified by the Schrödinger suite[38] (Maestro, Schrödinger)

In vitro *evaluation of effects of SC_E and YM_E on insulin sensitivity, β-cell function and glucose uptake using iHOMA2*

The model of iHOMA2 can be used to predict the effects of therapeutic agents on *β*-cell function (%*β*), insulin sensitivity (%S) and glucose uptake by a non-invasive method[16,21]. In the present study, %S and %*β* were calculated from experimental glucose and insulin values using iHOMA2 in analytical mode. There was an increase in %S and %*β* (discussed later) in the extract administered rats compared with that of diabetic rats. Six possible sites of action were hypothesised and modelled (Table 2) using iHOMA2 (predictive mode) on the basis of insulin sensitivity (liver and periphery) and glucose uptake (brain and gut). Since insulin facilitates cellular glucose uptake, the hypotheses were modelled by assuming %S to be directly proportional to glucose uptake in brain and gut[39]. Since the hypotheses are based on %S and glucose uptake, increase in %*β* was kept constant in all hypotheses. The predicted values of insulin were compared with the experimental values using Bland–Altman plots to assess the agreement between the two methods of insulin estimation, that is, by ELISA kit assay and by predictive mode of iHOMA2 software. The predicted values of glucose and insulin were further analysed using *F* tests for model fit.

Table 2. Hypotheses modelled in iHOMA2

	Hypotheses
1	Increases *β*-cell function and insulin sensitivity change is equally partitioned between liver and periphery
2	Increases *β*-cell function and insulin resistance change is only sited at the liver
3	Increases *β*-cell function and insulin resistance change is only sited at the periphery
4	Increases *β*-cell function and glucose uptake change is equally partitioned between brain and gut
5	Increases *β*-cell function and glucose uptake is only sited at the gut
6	Increases *β*-cell function and glucose uptake is only sited at the brain

Statistical analyses

The experimental results are expressed as means of the experimental data obtained from six rats in each group. Significant differences between mean values were determined by Duncan's multiple range test to determine significant differences among means of different parameters of of rat serum and plasma. A value of $P \leq 0{\cdot}05$ was considered significant to establish differences in all tests. All statistical tests were performed by STATISTICA 8.0 software (Statsoft).

Results

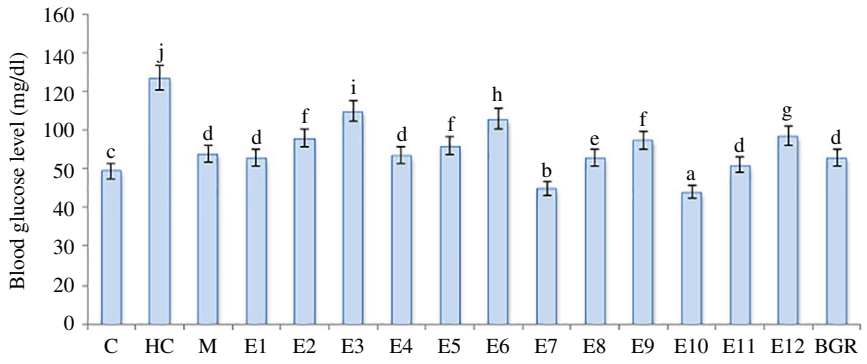
1,8-cineole content in SC_E and melatonin in YM_E

GC-MS analysis revealed the presence of 1,8-cineole (along with *α*-terpinyl acetate, limonene, *α*-terpineol and linalool) in SC_E, while LC-MS analysis of YM_E revealed presence of melatonin (limonene, linalool, tocopherol and ascorbic acid) in the extract. The amount of 1,8-cineole present in SC_E was 46·7 mg/100 mg extract, as determined by high-performance TLC analysis of SC_E and amount of melatonin present in YM_E was 427·26 ng/100 mg extract, as determined by HPLC analysis.

Effects of SC_E and YM_E on fasting blood glucose levels

The FBG levels in rats fed with SC_E and YM_E remained unchanged until day 8. However on day 15, there was 31·49 (SD 2·94) % and 32·28 (SD 2·29) % reduction in FBG level in rats administered with SC_E and YM_E, respectively, at an amount of 550 mg/kg b.w. It was also observed that there was an insignificant ($P = 0{\cdot}102$) decrease in FBG levels in animals administered with 550 mg/kg b.w. of extracts on day 21 (87·34 mg/dl for SC_E and 86·10 mg/dl for YM_E) compared with those on day 15, indicating that administration of the aforesaid dose for 15 d (87·11 mg/dl for SC_E and 86·93 mg/dl for YM_E) could achieve normal blood glucose levels in diabetic rats. The reduction in FBG levels in animals administered with extracts at a dose of 175 mg/kg b.w. was lower than that with 550 mg/kg b.w. on day 21. Administration of extracts at 55 mg/kg b.w. did not result in any reduction in FBG level in rats (Fig. 2) on day 21.

However, when 175 mg/kg b.w. of extracts were co-administered with metformin, the reduction in FBG level was similar to that in rats fed with 550 mg/kg b.w. extract only. The lowest dose of extracts, that is, 55 mg/kg b.w. also resulted in reduction in FBG when co-administered with metformin (Fig. 2). FBG level of the rats co-administered with 550 mg/kg b.w. of either extract and



Group details	
C	Control
HC	Diabetic control
M	Metformin
B	BGR-34
E1	YM-550
E2	YM-175
E3	YM-55
E4	SC-550
E5	SC-175
E6	SC-55
E7	YM-550+M
E8	YM-175+M
E9	YM-55+M
E10	SC-550+M
E11	SC-175+M
E12	SC-55+M

Fig. 2. Fasting blood glucose levels of Wistar albino rats on day 15. [a–j] Unlike letters indicate significant difference ($P < 0{\cdot}05$). YM, yellow mustard; SC, small cardamom. Data reported in mg/dl can be expressed in mmol/l by multiplying the data by 0·055.

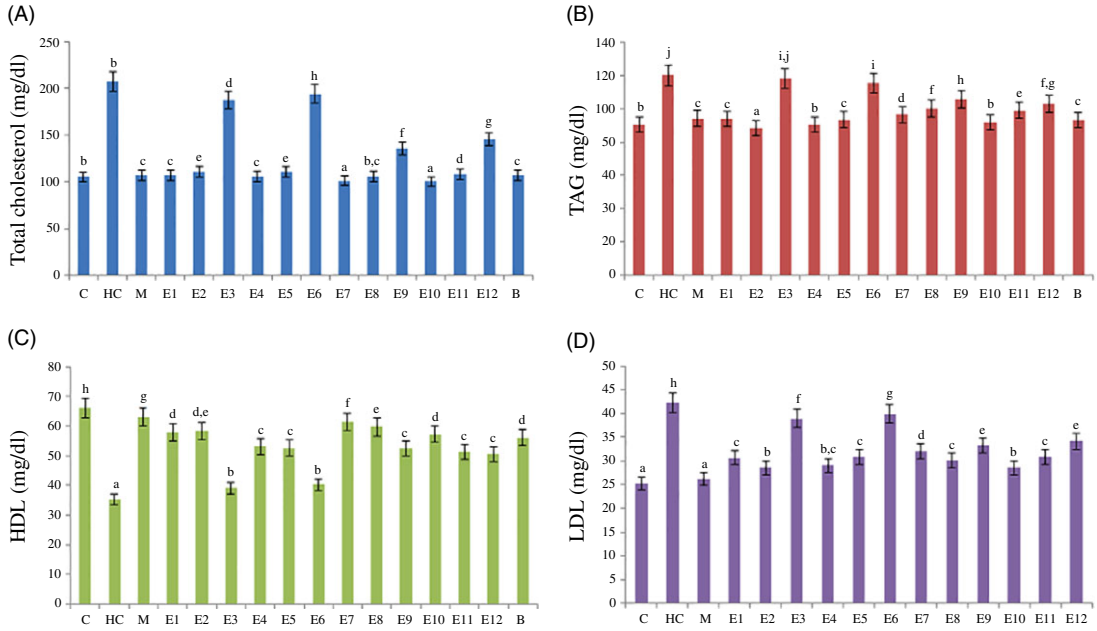
metformin was below 65 mg/dl (below 70 mg/dl indicates hypoglycaemia)[40] indicating hyperglycaemia in the animals. Administration of BGR-34 for 21 d also improved the FBG level (86·61 mg/dl) in rats, without inducing hypoglycaemia. No adverse effects in the animals were found during the experimental period.

On discontinuation of oral administration of either extract (SC_E and YM_E) after 15 d of regular administration, the FBG levels did not change significantly ($P = 0{\cdot}200$) up to day 21 (online Supplementary Fig. S3). Therefore, it is evident that FBG levels in animals were stable up to day 21 even after discontinuation of extract administration on day 15.

Histological changes and effects on liver and kidney markers

The histological sections of livers and kidneys of the diabetic control rats and the rats treated with SC_E and YM_E (550 mg/kg b.w.) are shown in Fig. 4. Histological structures showed morphological variations in livers of diabetic rats. The kidneys of diabetic rats had severe disorganisation of glomerulus and dilation of renal tubules. The rats treated with SC-CO_2 extracts, however, revealed normal organised structures of both liver and kidney.

The levels of serum BUN, urea and creatinine (liver markers), serum SGPT and SGOT (kidney markers) levels of the rats are presented in Table 3. No alternations in serum urea and creatinine levels were observed in the rats treated with metformin, BGR-34, SC_E and YM_E, during the experimental period of 21 d; however, the levels changed significantly ($P = 0{\cdot}000$) in the HC group of rats. Considering the animal dose to be 550 mg/kg b.w. at which no adverse effect in the animals was found, human equivalent dose was calculated to be 89 mg/kg b.w. for SC_E and YM_E.



Group details	
C	Control
HC	Diabetic control
M	Metformin
B	BGR-34
E1	YM-550
E2	YM-175
E3	YM-55
E4	SC-550
E5	SC-175
E6	SC-55
E7	YM-550+M
E8	YM-175+M
E9	YM-55+M
E10	SC-550+M
E11	SC-175+M
E12	SC-55+M

Fig. 3. Levels of (A) total cholesterol, (B) TAG, (C) HDL and (D) LDL in Wistar albino rats on day 15. [a–j] Unlike letters indicate significant difference ($P < 0{\cdot}05$). YM, yellow mustard; SC, small cardamom.

British Journal of Nutrition

Table 3. Effect of metformin, BGR-34, supercritical carbon dioxide extracts of yellow mustard and small cardamom seeds on kidney and liver markers in diabetic rats
(Mean values and standard deviations of six rats in each group)

	BUN (mg/dl)		Creatinine (mg/dl)		Urea (mg/dl)		SGOT (IU/l)		SGPT (IU/l)	
Groups	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C	16·87[a]	0·90	0·61[a]	0·02	35·29[a]	1·30	41·70[a]	1·01	42·20[a]	1·11
HC	26·05[b]	0·81	0·99[b]	0·03	69·33[b]	2·01	88·77[b]	1·89	53·91[b]	1·90
M	17·21[a]	0·58	0·72[c]	0·03	34·06[a]	1·08	43·05[a]	4·31	38·12[a]	5·01
B	19·22[a]	0·79	0·77[c]	0·05	40·55[c]	1·42	44·82[a]	4·19	39·59[a]	3·99
E1	20·08[a]	0·62	0·87[c]	0·06	51·07[d]	1·05	48·09[a]	4·18	43·05[a]	6·07
E2	20·99[a]	0·79	0·91[b]	0·04	60·69[d]	0·98	47·99[a]	3·13	42·26[a]	1·88
E3	19·00[a]	0·85	0·85[c]	0·04	40·23[c]	1·59	44·14[a]	4·56	40·05[a]	4·56
E4	21·52[a]	0·92	0·81[c]	0·03	51·44[d]	2·13	46·08[a]	6·32	42·85[a]	6·32
E5	20·77[a]	0·69	0·83[c]	0·05	63·04[d]	1·34	48·99[a]	2·18	42·77[a]	2·18
E6	19·11[a]	0·75	0·80[c]	0·03	65·01[c]	1·52	47·39[a]	4·03	39·99[a]	2·56
E7	21·02[a]	0·90	0·85[c]	0·06	55·02[d]	1·15	45·73[a]	3·33	40·15[a]	5·17
E8	20·19[a]	0·69	0·89[b]	0·04	57·69[d]	0·90	45·14[a]	4·56	43·06[a]	1·98
E9	21·33[a]	0·80	0·83[c]	0·06	58·23[d]	1·39	46·58[a]	6·32	44·05[a]	3·56
E10	20·52[a]	0·92	0·79[c]	0·07	50·24[d]	2·03	43·92[a]	2·17	41·98[a]	6·40
E11	19·87[a]	0·79	0·82[c]	0·07	53·04[d]	1·24	44·09[a]	4·18	42·79[a]	1·67
E12	19·39[a]	0·67	0·85[c]	0·06	60·55[d]	1·22	47·93[a]	3·41	41·95[a]	3·29

BUN, blood urea N; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.
[a,b,c,d] Values with unlike superscript letters were significantly different ($P < 0·05$) between groups by Duncan's multiple range test.

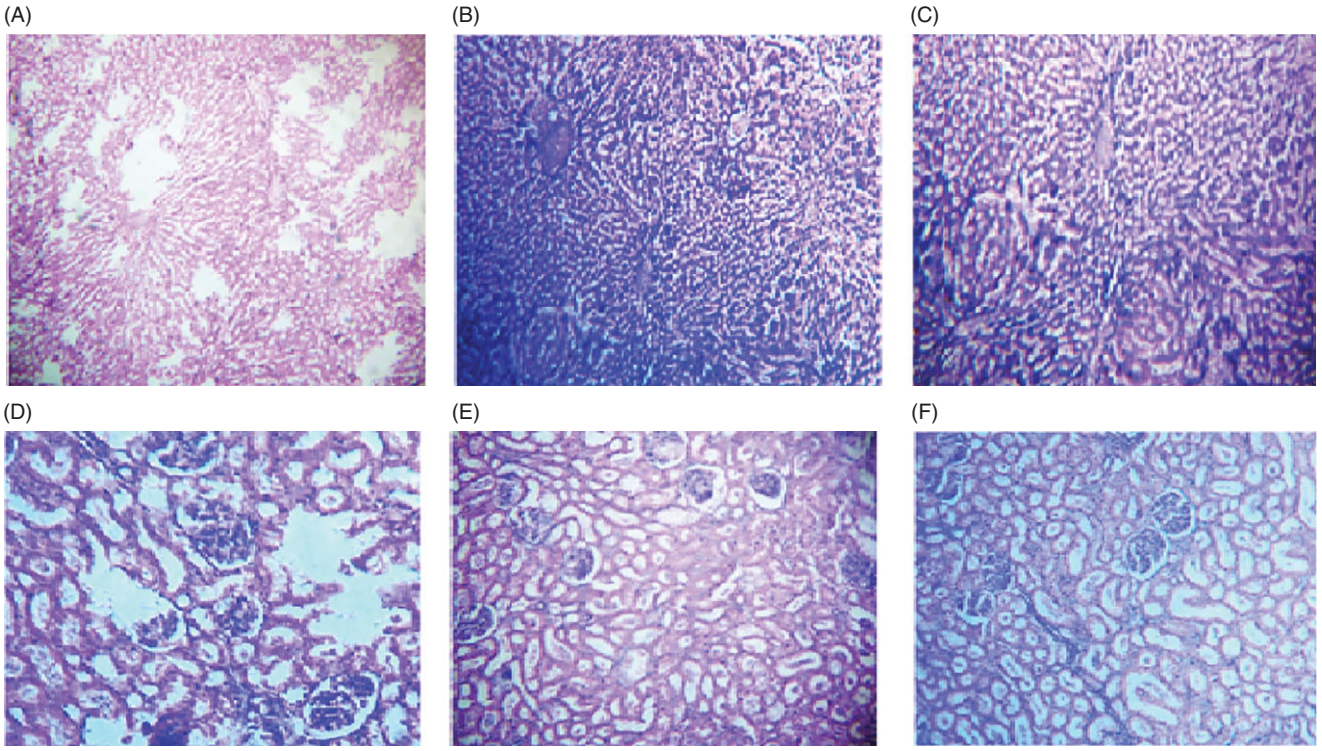


Fig. 4. (A) Histology of liver of diabetic control group of rats showing disorganised structure of liver. (B) Histology of liver of group of rats treated with 550 mg/kg body weight (b.w.) of supercritical carbon dioxide (SC-CO_2) extract of small cardamom (SC) seeds showing well-organised lobular structure. (C) Histology of liver of group of rats treated with 550 mg/kg b.w. of SC-CO_2 extract of yellow mustard (YM) seeds showing organised structure of lobules. (D) Histology of kidney of diabetic control group of rats showing severe disorganisation in the renal tubules. (E) Histology of kidney of group of rats treated with 550 mg/kg b.w. of SC-CO_2 extract of SC seeds showing normal renal tubules with intact well-organised cellular boundary. (F) Histology of liver of group of rats treated with 550 mg/kg b.w. of SC-CO_2 extract of YM seeds showing normal organisation in the renal tubules.

Effects of SC_E and YM_E on serum lipid profile

As diabetes is a metabolic disorder, it is characterised not only by increased FBG levels in rats but also by altered level of lipid profile. In the present study, total cholesterol, TAG and LDL were significantly ($P = 0·002$) elevated, while HDL was significantly ($P = 0·007$) lowered in diabetic rats. However, the lipid profile of the animals administered with the SC-CO_2 extracts were improved as compared with that of the diabetic animals (Fig. 3).

British Journal of Nutrition

British Journal of Nutrition

Effects of SC_E and YM_E on glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and catalase activities

The activities of G6PD significantly decreased, whereas the activities of G6Pase significantly increased in the STZ-injected diabetic rats with respect to the normal control group. After the administration of the SC_E and YM_E to the diabetic rats, significant recoveries were noted in the activities of the aforesaid enzymes. The decrease in hepatic G6Pase activity was most prominent amounting to decreases of 25·41 % and 23·50 % in the groups treated with 550 mg/kg b.w. per d of extracts of SC and YM seeds, respectively (Fig. 5(A)). There was no significant difference ($P > 0·05$) in activity of G6Pase when the rats were treated with either SC_E or YM_E at 550 mg/kg b.w. However, G6Pase activities of the animals varied significantly ($P < 0·05$) among the groups of rats administered with SC_E and YM_E at doses of 175 and 55 mg/kg b.w, respectively.

The diabetic rats showed reduction in hepatic G6PD activity as compared with that of normal control rats after day 21. In STZ-induced diabetic rats, the reduction of G6PD activity in liver obstructs glucose utilisation through pentose phosphate pathway. There were 36·71 % and 34·40 % increases in the activities of G6PD in the groups treated with SC-CO_2 extracts of SC and YM seeds (550 mg/kg b.w.), respectively (Fig. 5(B)). There was no significant difference ($P > 0·05$) in the activity of G6PD in the rats treated with SC_E and YM_E at 550, 175 and 55 mg/kg b.w. It was observed that the administration of metformin and BGR-34 to the rats resulted in reduction in G6Pase (30·95 % and 25·76, respectively) and increases in G6PD (28·76 % and 27·99 %, respectively) activities in rats (Fig. 5(B)).

The catalase activities of the rats are presented in Fig. 5(C). The animals treated with 550 mg/kg b.w. of SC_E and YM_E, the activities of catalase (21·09 (SD 1·01) and 20·98 (SD 0·99) IU/mg of protein for SC and YM seeds, respectively) were similar to that of the control group (22·12 (SD 1·17) IU/mg of protein) on day 21. Although activity of catalase was similar ($P > 0·05$) in the rats treated with either extract at doses of 550 and 175 mg/kg b.w., there was significant difference ($P < 0·05$) in activity of catalase in the rats administered with SC_E and YM_E at the lowest dose, that is, 55 mg/kg b.w.

Augmentative effects of extract components on glucose-6-phosphate dehydrogenase in terms of docking scores

Fig. 5 provides the docking scores of active ingredients of SC_E (1,8-cineole, α-terpinyl acetate, terpineol, limonene and linalool) and YM_E (melatonin, ascorbic acid and tocopherol) with respect to the bound substrate (glucose-6-phosphate) of G6PD receptor protein. Ingredient molecules were docked at the known ligand binding site of G6PD receptor using GOLD package (CCDC). Average docking scores of top three scoring solutions (pose) from the largest cluster of all active ingredient molecules were calculated and compared against GOLD-generated rescores derived from the original substrate bound to G6PD. Online Supplementary Fig. S4 provides the corresponding docking poses with the respective ligands. For SC_E, high docking scores were achieved by linalool and limonene, whereas for YM_E, docking score was appreciably high for tocopherol apart from that of melatonin.

Effects of supercritical carbon dioxide extracts of small cardamom and yellow mustard seeds on serum insulin levels and model assessment in iHOMA2

The insulin content in the rats along with the respective calculated %S and %B values are presented in Table 4. The plasma

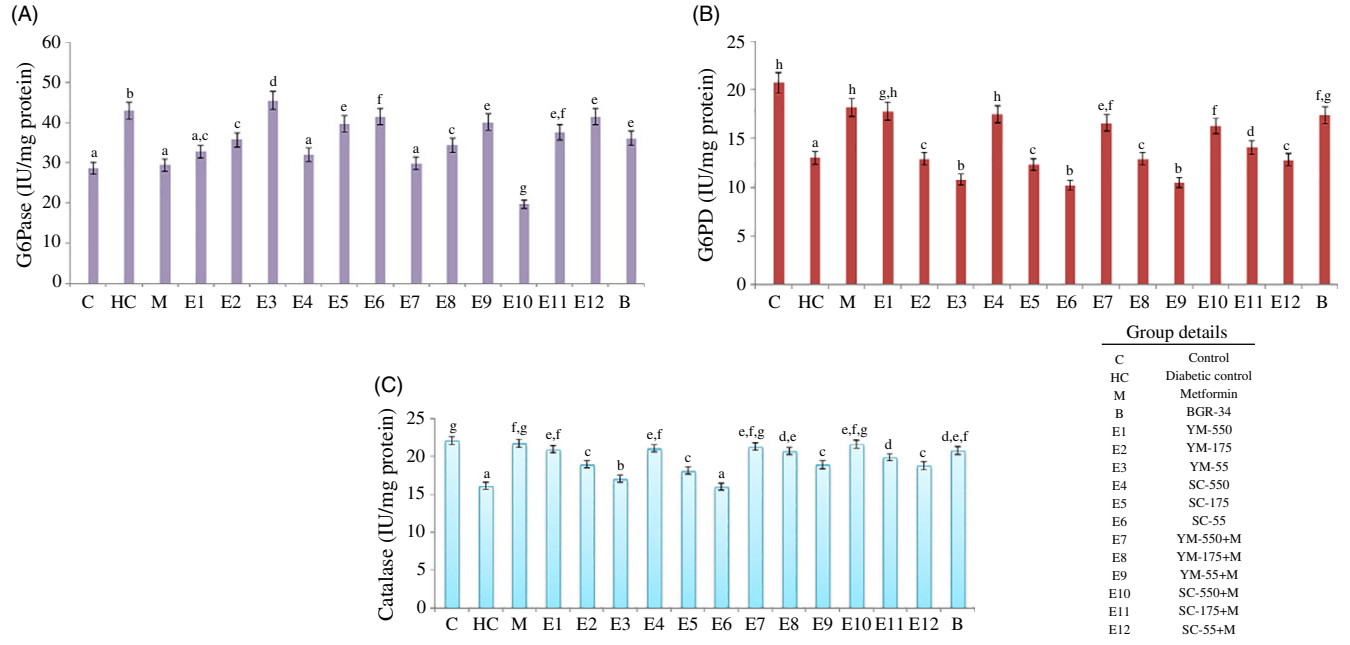


Fig. 5. (A) Activity of glucose-6-phosphatase (G6Pase) in Wistar albino rats on day 21. (B) Activity of glucose-6-phosphate dehydrogenase (G6PD) in Wistar albino rats on day 21. (C) Activity of catalase in Wistar albino rats on day 21. $^{a–g}$ Unlike letters indicate significant difference ($P < 0·05$ level). YM, yellow mustard; SC, small cardamom.

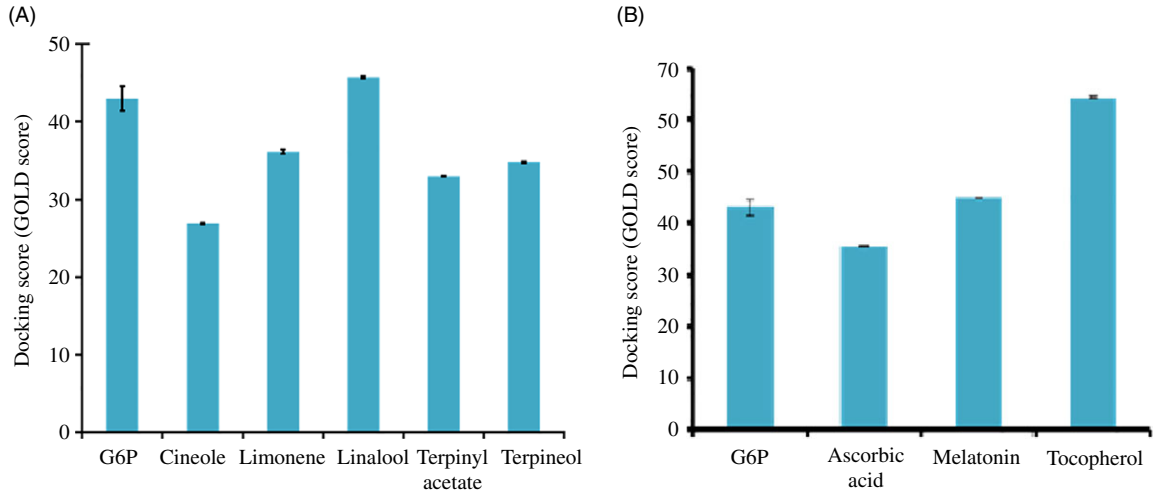


Fig. 6. (A) Average molecular docking score represented as Genetic Optimization for Ligand Docking (GOLD) score is plotted for each active ingredient of the cardamom extract when docked at the substrate binding site. (B) Average molecular docking scores represented as GOLD score for each active ingredient of the mustard extract when docked at the substrate binding site. G6P, glucose-6-phosphate.

insulin value was least in the diabetic rats, whereas the values increased in the metformin and extract-treated rats. From the experimental values of blood glucose and plasma insulin levels in the rats, it was found that increases of %S and %β for YM seed extract-treated rats were 33·08 % and 48·35 %, respectively, whereas the same for SC seed extract-treated rats were 35·68 % and 46·29 %, respectively. The Bland–Altman plots showed good fit between the experimental and predictive values of insulin in rats indicating agreement between the methods. The predictive results from iHOMA2 and the *F*-values are represented in Table 5. The *F* values indicated that the models based on the second hypothesis (insulin sensitivity increased in liver) and fifth hypotheses (glucose uptake in gut) fitted well to the experimental data (glucose and insulin) of extract-administered rats (Table 5). Similar findings were also observed in the analyses conducted with data obtained from rats treated with metformin and BGR-34. In a study conducted on antidiabetic potency of nanoliposomes of SC-CO_2 extract of SC seeds, Paul *et al.*[20] have reported that iHOMA2 results indicated that nanoliposomes had either increased insulin sensitivity in liver or increased glucose uptake in brain of Wistar albino rats.

Table 4. Plasma insulin level, insulin sensitivity and β-cell function of rats (Means and standard deviations of six rats)

	Insulin (mIU/l)			
Group	Mean	SD	% S	% β
C	12·55	0·98	76·8	133·7
HC	13·74	0·87	53·8	82·3
M	7·38	0·41	75·6	125·2
B	11·29	0·59	70·9	108·4
E1	10·99	0·69	71·6	122·1
E2	12·53	0·88	63·1	133·5
E3	11·10	0·91	51·2	114·1
E4	10·77	0·85	73·0	120·4
E5	13·13	0·78	60·2	134·6
E6	15·58	0·94	50·6	145·4
E7	12·22	0·76	63·5	115·4
E8	13·89	0·77	57·1	142·8
E9	15·97	0·82	48·6	129·8
E10	10·80	0·66	72·6	117·6
E11	13·72	0·78	55·9	110·5
E12	19·04	0·63	42·4	186·6

%S, insulin sensitivity; %B, β-cell function.

Discussion

Administration of either SC-CO_2 extract in the diabetic animals resulted in achieving normal FBG levels in rats on day 15 which remained unaltered till day 21, while co-administration of either SC_E or YM_E at a dose of 550 mg/kg b.w. with metformin resulted in hypoglycaemia in rats on the same day. Therefore, oral administration of either extract alone at a dose of 550 mg/kg b.w. is effective in restoring normal FBG levels in rats on day 15. Although either extract was found to be most effective in reducing blood glucose levels at the highest dose, that is, 550 mg/kg b.w., the concentrations of 1,8-cineole and melatonin in the respective extracts were different (discussed above). Thus, SC_E having 46·70 mg 1,8-cineole/100 mg extract and YM_E having 427·26 ng melatonin/100 mg extract successfully reduced FBG levels in diabetic rats. The reduction in FBG levels in rats administered with either extract at a dose of 550 mg/kg b.w. was similar to those administered with metformin and BGR-34.

Decrease in G6Pase activity extract-administered rats indicated repression of this key gluconeogenic enzyme (G6Pase) and was responsible for reduced FBG levels in the rats. Natural extracts of *Cinnamomum zeylanicum* and *Octomeles sumatrana* are also reportedly known to have inhibitory effects on G6Pase activities in rat models[41,42]. Moreover, increased levels of G6PD in rats treated with SC_E and YM_E were indicative of proper glucose utilisation through pentose phosphate pathway in the liver. Studies conducted by Kumar *et al.*[43] have also reported on restoration of G6PD activity in rats when administered with fenugreek seed extract. The effects of the extracts on hepatic G6Pase and G6PD activities possibly contributed to the maintenance of normal FBG levels in diabetic rats. It was

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Table 5. iHOMA2 results

Hypotheses	Predicted glucose (mmol/l)	Experimental glucose (mmol/l)	Predicted insulin (pmol/l)	Experimental insulin (pmol/l)	*F*
$SC\text{-}CO_2$ extract of SC seeds (550 mg/kg b.w.)					
Increase in insulin sensitivity is equally partitioned between liver and periphery	3·8	5·03	59·3	74·81	142·62
Increase in insulin sensitivity is only sited at the liver	3·9	5·03	62·9	74·81	93·98
Increase in insulin sensitivity is only sited at the periphery	3·7	5·03	55·7	74·81	125·88
Glucose uptake is equally partitioned between brain and gut	3·7	5·03	53·9	74·81	155·40
Glucose uptake is only sited at the gut	3·9	5·03	65·3	74·81	42·03
Glucose uptake is only sited at the brain	3·4	5·03	42·6	74·81	186·12
$SC\text{-}CO_2$ extract of YM seeds (550 mg/kg b.w.)					
Increase in insulin sensitivity is equally partitioned between liver and periphery	3·8	5·02	61·9	76·3	356·73
Increase in insulin sensitivity and the change is only sited at the liver	5·2	5·02	72·4	76·3	270·07
Increase in insulin sensitivity is only sited at the periphery	3·7	5·02	58·3	76·3	491·00
Glucose uptake is equally partitioned between brain and gut	3·7	5·02	57·3	76·3	546·09
Glucose uptake is only sited at the gut	3·9	5·02	68·0	76·3	208·24
Glucose uptake is only sited at the brain	3·5	5·02	46·7	76·3	832·15
Metformin					
Increase in insulin sensitivity is equally partitioned between liver and periphery	4·1	5·22	60·8	75·83	1182·20
Increase in insulin sensitivity is only sited at the liver	4·2	5·22	64·0	75·83	388·92
Increase in insulin sensitivity is only sited at the periphery	4·0	5·22	57·4	75·83	540·82
Glucose uptake is equally partitioned between brain and gut	3·9	5·22	56·7	75·83	337·33
Glucose uptake is only sited at the gut	4·3	5·22	61·0	75·83	284·78
Glucose uptake is only sited at the brain	4·0	5·22	55·2	75·83	333·36
BGR-34					
Increase in insulin sensitivity is equally partitioned between liver and periphery	4·3	5·32	53·6	71·23	387·65
Increase in insulin sensitivity is only sited at the liver	4·4	5·32	57·0	71·23	261·92
Increase in insulin sensitivity is only sited at the periphery	4·2	5·32	50·1	71·23	281·87
Glucose uptake is equally partitioned between brain and gut	4·4	5·32	61·3	71·23	390·73
Glucose uptake is only sited at the gut	4·7	5·32	64·4	71·23	273·85
Glucose uptake is only sited at the brain	4·1	5·32	59·8	71·23	579·62

$SC\text{-}CO_2$, supercritical carbon dioxide; SC, small cardamom; b.w., body weight; YM, yellow mustard.

evident from the results that the efficacies of the extracts in terms of activities of these hepatic enzymes were also comparable to those of metformin and BGR-34, affirming the suitability of the extracts as promising alternatives to metformin and BGR-34 in redressing type 2 diabetes.

The reduced activity of catalase observed in diabetic rats indicated accumulation of H_2O_2 leading to oxidative stress[44]. However, in rats treated with 550 mg/kg b.w. of SC_E and YM_E, the activities of catalase on day 21 indicated the efficiencies of the extracts in restoring catalase activity, owing to the presence of antioxidants namely 1,8-cineole and melatonin in SC_E and YM_E, respectively. Administration of either extract at 550 mg/kg b.w. successfully down-regulated G6Pase activity[12] and up-regulated G6PD[13] and catalase[14,15] activities in the animals owing to highest content of respective bioactive component in the extracts.

Additionally, improved lipid profiles of the animals administered with the $SC\text{-}CO_2$ extracts confirmed hypolipidaemic effect of the extracts. This finding was in agreement with that reported by Chakraborty *et al.*[5], where administration of $SC\text{-}CO_2$ extracts of SC and YM seeds resulted in improved lipid profile of hypercholesterolaemic Wistar albino rats. Histological sections of livers and kidneys of extract-administered rats attested to the fact that administration of extracts had no toxic effects on liver and kidney of animals. Therefore, these 'green extracts' as per WHO[45] guidelines could be termed as 'herbal preparations'.

Molecular docking results revealed that linalool achieved the highest docking scores in the G6PD-bound site, suggesting its strong augmenting action on G6PD. SC_E therefore exhibited its augmenting effect on hepatic G6PD activity not only due to 1,8-cineole alone but also due to the presence of linalool therein. Docking exercise at the known substrate binding site of G6PD suggests that some of the active ingredients of the cardamom extract (e.g. linalool) achieved docking scores comparable to the known substrate of the protein. However, the sizes and surface areas of few active molecules are considerably smaller than the substrate glucose-6-phosphate. Hence, they do not occupy

the full substrate binding pocket, instead binds to subpockets. In case of YM_E, tocopherol achieved appreciably high docking scores which implied that augmenting effect of the extract in the enzyme activity was due to the presence of both melatonin and tocopherol. In fact, the docking scores of the constituents of either extract revealed that each component had affected the enzyme activity desirably. Thus, it was the consortium of antioxidants present in either extract which augmented G6PD activity and not the respective bioactive principle alone. Chakraborty *et al.*[5] have reported synergistic presence of antioxidants in SC-CO_2 extracts of YM and SC seeds which contributed to their strong hypocholesterolaemic activities. In the present study too, it can be concluded from the molecular docking results that prominent antidiabetic activities of SC_E and YM_E were owing to the presence of consortia of bioactives therein and not due to 1,8-cineole or melatonin alone.

The results from iHOMA2 imply that the SC-CO_2 extracts either increase hepatic insulin sensitivity or glucose uptake in the gut or both. Therefore, increase in plasma insulin levels of the rat treated with either SC_E or YM_E was owing to either insulin sensitivity increased in liver or glucose uptake in gut or both in extract-administered rats. Similar findings were observed in the analyses conducted with data obtained from rats treated with metformin and BGR-34. The increase in hepatic insulin sensitivity (predicted by iHOMA2) in metformin-administered rats in our study is in agreement with the findings of Tiikkainen *et al.*[46]. Further *in vivo* studies related to the above mechanisms are suggested to substantiate the findings of iHOMA2.

In the present investigation, *in vivo* antidiabetic and *in silico* molecular docking results affirmed that oral administrations of SC_E and YM_E effectively inhibited hepatic G6Pase activities and restored FBG levels in the rats. Hence, these biotherapeutic molecules are therefore not invalid metabolic panaceas but authentic natural leads for the design of therapeutic foods and natural product-based drugs. Since both SC_E and YM_E are non-toxic and safe for consumption, either extract can be further employed in clinical trials at a human equivalent dose of 89 mg/kg b.w.

Conclusions

In the present study, it can be reasonably concluded that oral administration of SC and YM seed extracts in rats would exhibit dual benefits *viz.* inhibition of G6Pase activity which contributed to the maintenance of normal FBG levels in diabetic rats, and scavenging of reactive oxygen species leading to reduction of diabetes-induced oxidative stress. Additionally, the *in silico* molecular docking study revealed the consortia of bioactives that augmented G6PD activity in the rats. Thus, it can be reasonably concluded that the consortia of bioactives in YM_E and SC_E have contributed to their antidiabetic efficacies. The predicted results on effects on insulin sensitivity and glucose uptake obtained from the iHOMA2 model affirmed antidiabetic potencies of the above extracts. The findings from the present study are strongly suggestive of use of these extracts as safe alternative biotherapeutics in combating type 2 diabetes. These newly discovered biotherapeutic agents derived from spices consumed worldwide could be safely subjected to clinical studies with the aim to establish these as novel, natural antidiabetic supplements.

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There are no conflicts to declare.

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