Analysis of *in vitro* and *in silico* anti-hyperglycaemic action of bioflavonoids isolated from different citrus peels

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In this study, flavonoid-rich chloroform fraction (FRCF) of nine different citrus species was isolated by solvent fractionation technique and its effect on α-glucosidase, α-amylase, glucose uptake by yeast cells and glycosylation of haemoglobin was studied. FRCF of *Citrus maxima* was found to show higher activity in the *in vitro* assays and thus it was characterized by Electrospray Ionization Mass Spectrometry (ESI-MS) analysis. This led to the identification of icariin, hesperidin and diosmetin-6,8-di-C-glucoside. Their binding efficacy with targeted proteins such as glucokinase (GK), glycogen synthase kinases 3β (GSK 3β) and peroxisome proliferator-activated receptor-γ (PPAR-γ) was studied by *in silico* analysis using Schrödinger Maestro software. Icariin exhibited maximum docking energy with GSK 3β, relatively less for GK and null for PPAR-γ. Hesperidin and diosmetin-6, 8-di-C-glucoside showed good binding affinity with PPAR-γ compared to GSK 3β.

Keywords: Anti-hyperglycaemic activity, bioflavonoids, *Citrus maxima*, yeast cells.

INDIA has emerged as the diabetic capital of the world. The *Lancet* study has shown that there was a fourfold rise in the number of diabetics in the country – from 11.9 million in 1980 to 64.5 million in 2014 (ref. 1). Diabetes is characterized by high concentration of blood sugar which can cause serious complications in the organs like kidneys, eyes and in the cardiovascular system. There are several targets that are known to play a role in the development of diabetes. Some of these include aldose reductase, glucokinase, fructose 1,6-bisphosphatase, cytochrome P450, etc.[2] Glucokinase is a monomeric cytoplasmic enzyme found in the liver and pancreas, and is known to regulate the level of glucose in these organs. Irregularity in glucokinase often leads to elevated blood sugar levels[3]. The treatment of diabetes therefore mainly focuses on reducing fluctuations in blood sugar level and subsequent complications. Another target for diabetes is peroxisome proliferator-activated receptor-γ (PPAR-γ), which regulates glucose metabolism via gene expression[4]. α-Amylase and α-glucosidase are major enzymes found in the brush border cells that line the small intestine. They rapidly cleave the more complex carbohydrates such as starches, oligosaccharides and disaccharides into monosaccharide molecules before being absorbed in the duodenum and upper jejunum. This increases the rapid absorption of glucose and hence causes hyperglycaemic effect[5,6]. Anti-diabetic drugs work by competitively inhibiting these enzymes, slowing down the process of cleavage of carbohydrates and promoting slow absorption of glucose. The α-amylase and α-glucosidase inhibitors are currently used for treatment of diabetes. Acarbose is a commercially available enzyme inhibitor; biguanides and sulphonylureas are commonly used in the treatment of type-II diabetes. However, they have been reported to cause various side effects such that abdominal distention, flatulence and possibly diarrhoea[7]. Thus managing diabetes without any side effects is still a challenge, and in the search of alternative remedies, plant secondary metabolites are proving to be less toxic and free from side effects than synthetic ones[8].

Medicinal plants have potential efficacy against diabetes and phytochemicals play a major role in its management. Pharmacological and clinical trials of medicinal plants have shown anti-diabetic effects and repair of β-cells of the islets of Langerhans[9]. Flavonoids in plants, vegetables and fruits are key compounds for treating and managing diabetes. Flavonoids such as quercetin cause insulin secretion and are also considered as a strong inhibitor in sorbitol accumulation throughout the body[10]. The useful effect of flavonoids could be the result of increasing intercellular antioxidants levels, preventing the rupture of capillaries and boosting the immune system of the body, all of which are totally effective in diabetes improvement[11].

In previous studies, pure compounds and crude organic extracts of citrus fruits and peels have been screened for antidiabetic activity[12–17]. In the present study, peels of nine citrus species were extracted, fractionated into flavonoid fractions and assayed *in vitro* for anti-hyperglycaemic activity. The effective fraction of the species was explored for its active constituent. Availability of bioinformatics tools has facilitated the drug discovery process, and serves to identify drugs and targets based on their known structure and predicted properties. Identification

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of the interaction of targeted cell receptors for diabetes with the drug plays a major role in drug discovery. *In silico* techniques therefore serve as an excellent tool for the purpose of generation of preliminary information on drug likeliness and understanding the mode of action, thereby saving time and resources[18]. In this study, compounds identified in citrus peel were examined for their interaction with target proteins related to anti-hyperglycaemic activity using Schrödinger Maestro software.

Materials and methods

Plant materials and extraction procedure

Nine different citrus fruits *Citrus aurantifolia* (*Christm) Swingle* (*C. aurantifolia*) – greenish-yellow, *Citrus aurantium* Amara L. (*C. aurantium*) – orange, *Citrus hystrix* DC (*C. hystrix*) – green, *Citrus limetta* Risso (*C. limetta*) – greenish-yellow, *Citrus limonia* Osbeck (pro. sp) (*C. limonia*) – greenish-yellow, *Citrus maxima* Burm.f. Merr (*C. maxima*) – greenish-orange, *Citrus medica* L. (*C. medica*) – yellow, *Citrus reticulate* Blanco (*C. reticulata*) – orange, *Citrus sinensis* (L.) Osbeck (pro. sp) (*C. sinensis*) – orange were collected from Vellore and Chennai districts in Tamil Nadu, and from Andhra Pradesh in February 2014. The species were authenticated by Jayagen Biologics, Chennai. A voucher specimen (CS-01 to CS-09) was submitted to Anna University, BIT campus, Tiruchirappalli. The outer layers were peeled-off, cleaned, dried for about two weeks and ground in a ball mill. Next, 20 g of peel powder was extracted with methanol (200 ml) in a Soxhlet extractor for 36 h. The extract was evaporated in a rotary evaporator under vacuum. The methanolic extract was weighed and suspended in water and extracted successively with different solvents, viz. *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The presence of flavonoids was confirmed in the chloroform fraction by preliminary tests.

Preliminary phytochemical analysis

Qualitative phytochemical analysis was done for both methanolic extract and chloroform fraction to screen the phytoconstituents as described by standard methods[19].

Quantitative estimation of flavonoids

Flavonoids present in the chloroform fraction were quantitatively estimated using aluminum chloride colorimetric method [20]. Then 10 mg of flavonoid-rich chloroform fraction (FRCF) of each fruit was dissolved in 10 ml of methanol to obtain 1 mg/ml solution. Next 0.5 ml of each chloroform fraction was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The reaction mixture was incubated at room temperature for 30 min and measured at 415 nm. Quercetin at concentration of 5–100 µg/ml in methanol was used as standard. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line. Then, the content of flavonoids in extracts was expressed in terms of quercetin equivalent (mg/g of FRCF).

In vitro assays

Carbohydrates are normally converted into simple sugars by enzymes like α-amylase and α-glucosidase. In diabetic condition, these enzymes rapidly cleave the carbohydrate and release glucose leading to hyperglycaemic effect. The inhibitory effect of FRCF on these enzymes was assessed *in vitro* by determining a decrease in the amount of glucose liberated from substrate after incubation with the enzyme. Therefore, *in vitro* assays were performed to evaluate the enzyme inhibitory action of FRCF samples.

Determination of α-amylase inhibitory activity

α-Amylase is an enzyme that hydrolyses α-bonds of large, α-linked polysaccharides yielding glucose and maltose. *In vitro* assay was performed to evaluate the enzyme inhibitory action of FRCF samples of citrus species using 3,5-dinitrosalicylic acid (DNSA) method[21]. The FRCF samples were dissolved in minimum amount of 10% DMSO and phosphate buffer (pH 6.9) to give concentrations ranging from 20 to 1000 µg/ml. A volume of 200 µl of α-amylase solution (2 units/ml) was mixed with 200 µl of FRCF and incubated for 10 min at 30°C. Thereafter, 200 µl of starch solution (1% in water (w/v)) was added to each tube and incubated for 3 min. The reaction was terminated by the addition of 200 µl DNSA reagent and was boiled for 10 min in a water bath at 85–90°C. The mixture was cooled and diluted with 5 ml of distilled water. The absorbance was measured at 540 nm using a UV-vis spectrophotometer. The blank with 100% enzyme activity was prepared with 200 µl of buffer. A positive control sample was prepared using acarbose (2–100 µg/ml) and analysed as mentioned above. The α-amylase inhibitory activity was expressed as per cent inhibition and was calculated as given below. The concentration of acarbose and FRCF required to inhibit 50% of α-amylase activity under the conditions was defined as the IC_{50} value.

$$\%\ \alpha\text{-Amylase inhibition} = 100 \times \frac{\text{Abs 100\% control} - \text{Abs sample}}{\text{Abs 100\% control}},$$

defined as the absorbance of 100% control indicates only solvent with enzyme (only enzyme activity), a test sample (with enzyme) respectively.

Determination of α-glucosidase inhibitory activity

The enzyme yeast α-glucosidase was dissolved in 100 mM phosphate buffer pH 6.8 and ρ-nitrophenyl-α-D-glucopyranoside (Sigma-Aldrich, Switzerland) was used as the substrate. The reaction mixture consisting of 10 µl of sample at different concentrations (20–100 µg/ml) was premixed with 490 µl of 100 mM phosphate buffer (pH 6.8) and 250 µl of 5 mM *p*-nitrophenyl α-D-glucopyranoside. After preincubating at 37°C for 5 min, 250 µl α-glucosidase (0.15 unit/ml) was added and incubated at 37°C for 15 min. The reaction was terminated by the addition of 2000 µl Na_2CO_3 0.25 M. α-glycosidase activity was determined spectrophotometrically at 400 nm using a UV-vis spectrophotometer (Shimadzu 265, Jepang) by measuring the quantity of *p*-nitrophenol released from *p*-NPG[22]. The control samples were prepared without FRCF. Acarbose was used as the standard drug at a concentration of 100 µg/ml. The % inhibition was calculated as follows

$$\text{Inhibition (\%)} = \left(\frac{\text{Control} - \text{Extract}}{\text{Control}}\right) \times 100.$$

Evaluation of haemoglobin glycosylation

Preparation of haemoglobin: Blood was collected from a healthy human volunteer and transferred into a blood bottle containing an anticoagulant. Hemolysate was prepared based on the principle of hypotonic lysis. The red blood collected was washed thrice with 0.14 M NaCl solution and one volume of red blood cells suspension was lysed with two volumes of 0.01 M phosphate buffer (pH 7.4) and 0.5 volume of CCl_4. The haemolysate was then freed from the debris by centrifugation at 2300 rpm for 15 min at room temperature. The haemoglobin-rich fraction, i.e. the upper layer was separated and dispensed into sample bottle for storage and refrigerated until for use[23].

Effect of FRCF on haemoglobin glycosylation: To 1 ml of haemoglobin solution, 1 ml of glucose solution and 5 µl of gentamycin in 0.01 M phosphate buffer (pH 7.4) were mixed and incubated in the dark at room temperature in the presence of 30 µg/ml gallic acid and 100 µg/ml FRCF respectively. Haemoglobin concentrations were estimated over an incubation period of 72 h spectrophotometrically at 443 nm, as an index for measuring the degree of haemoglobin glycosylation[23].

Glucose uptake in yeast cells

Commercial baker's yeast in distilled water was subjected to repeated centrifugation (3000 rpm for 5 min) until clear supernatant fluids were obtained and a 10% v/v of the suspension was prepared in distilled water. The FRCF samples of nine citrus species with 100 µg/ml concentration (50 µl) were added to 1 ml of 10 mM glucose solution and incubated together for 10 min at 37°C. Reaction was started by adding 100 µl of yeast suspension followed by vortexing and further incubation at 37°C for 60 min. Then the tubes were centrifuged (2500 rpm for 5 min) and the amount of glucose was estimated in the supernatant[24]. Metronidazole was used as standard drug at 100 µg/ml concentration. The percentage increase in glucose uptake by yeast cells was calculated using the formula

$$\text{Increase in glucose uptake (\%)} = \frac{\text{Abs 540 (control)} - \text{Abs 540 (extract)}}{\text{Abs 540 (control)}} \times 100.$$

ESI-MS analysis of FRCF of Citrus maxima

FRCF of citrus peels which exhibit maximum enzyme inhibitory activity was subjected to Electrospray Ionization Mass Spectrometry (ESI-MS) analysis. Using on-line MassBank database, the flavonoid present in the chloroform fraction of the species was identified. In this analysis, 1 ml of 10 pg/µl of FRCF in 50 : 50 isopropanol/water was prepared and injected into the instrument. Mass spectral detection was done at ionization mode, ES+; scan range, 150–900 amu; scan rate, 1 scan/s and cone voltage, 20 eV. Peak identities were obtained by matching expected molecular weights, UV spectra and elution properties from a library of standards.

In silico studies

Flavonoids identified in the ESI-MS analysis were examined for their interaction with target proteins related to anti-hyperglycaemic activity using *in silico* analysis. The receptor–drug (ligand) interaction was studied using Schrödinger Maestro software, a GLIDE program which searches for favourable interactions. Co-crystallized structure of ligand from the Protein Data Bank was imported into Maestro. Multimeric complexes were simplified and metal ions, cofactors and ligand bond orders were adjusted. The final structure was refined. The combination of position and orientation of a ligand relative to the receptor, along with its conformation in flexible docking, is referred to as a ligand pose. The ligand poses were passed through an initial filter to test the spatial fit and complementarity of ligand–receptor interactions using a grid-based empirical ChemScore function. Poses that passed these initial screens entered the final stage to screen energy-minimized poses based on OPLS-AA algorithm[25]. A composite E-model score was then used to rank the

poses. The poses of all the ligands of interest were selected and entered into groups based on title or glide lignum. Sort button on the Project Table toolbar used to sort by glide score in ascending order. From the poses, the flavonoids which interacted proficiently with the receptors were identified.

Results and discussion

Percentage yield of the fraction

Table 1 shows the percentage yield of flavonoid fractions, i.e. chloroform fractions obtained from the nine citrus species. The yield of chloroform fractions from the methanolic extract was in the range 30–50%.

Preliminary phytochemical analysis

Table 2 shows results of preliminary phytochemical analysis of chloroform fractions of all the species. It was found that chloroform fractions of all citrus species were enriched only with flavones, flavonoids, phenols and glycosides, and hence referred as 'flavonoid-rich chloroform fractions'. These flavonoids in FRCF were quantitatively estimated using $AlCl_3$ assay.

Flavonoid estimation using aluminium chloride assay

Flavonoid concentration in FRCF of different citrus species was determined quantitatively using quercetin as standard (Figure 1). Concentration of flavonoids was found to be higher in *C. maxima*. Quantity of flavonoids in the citrus peels was in the following descending order: *C. maxima* > *C. reticulata* > *C. aurantifolia* > *C. limetta* > *C. medica* > *C. limonia* > *C. sinensis* > *C. aurantium* > *C. hystrix*. Total flavonoid content of methanolic extracts of different citrus species has been reported in the literature[26–28]. In the present study, total flavonoid content of the chloroform fraction of methanolic extract of nine citrus species has been reported, among which *C. maxima* was found to show highest flavonoid content.

Table 1. Percentage yield of chloroform fraction of nine different citrus species

Citrus species	Flavonoid-rich chloroform fraction percentage yield
Citrus aurantifolia	47.6 ± 0.98
Citrus aurantium	30.6 ± 0.87
Citrus hystrix	30.8 ± 1.45
Citrus limetta	32.8 ± 2.09
Citrus limonia	33.3 ± 1.67
Citrus maxima	36.8 ± 2.36
Citrus medica	44.0 ± 3.45
Citrus reticulata	35.5 ± 3.30
Citrus sinensis	42.0 ± 1.08

Value indicates mean ± SEM of three values.

Determination of α-amylase inhibitory activity

Table 3 shows the inhibitory effects of citrus peels and acarbose on α-amylase. The potential inhibition of all the species against α-amylase at 100 µg/ml concentration ranged from 32.88% to 76.58%. The α-amylase inhibition activities of *C. maxima* and *C. limetta* were 76.58% and 69.32% respectively, which were higher than the activity of standard drug acarbose (66.26%). The IC_{50} values of *C. maxima* and *C. limetta* were also higher than that of acarbose. There was no statistical difference between the standard and these two species, whereas a minimum level of significance ($P < 0.05$) was found with other citrus species. α-Amylase inhibitory activities of the citrus peels were in the following order: *C. maxima* > *C. limetta* > *C. hystrix* > *C. aurantifolia* > *C. limonia* > *C. reticulata* > *C. sinensis* > *C. aurantium* > *C. medica*. Maximum inhibitory activity of 76.58% was exhibited by FRCF sample of *C. maxima* and hence the flavonoids in *C. maxima* competitively bind with starch and prevent its degradation by α-amylase. Therefore, it has been suggested that inhibition activities against α-amylase could be part of the possible mechanisms of *C. maxima* variety in therapeutic/dietary management of diabetes, by retardation of starch hydrolysis in the gastrointestinal tract. α-Amylase inhibitors play a crucial role in blocking the release of glucose from dietary source of carbohydrates and extending glucose absorption leading to decreased postprandial plasma glucose levels and further minimize postprandial hyperglycaemia[29]. The exopolysaccharides (EPS) of *C. maxima* were reported to show good α-amylase inhibitory activity (86.59%)[30],

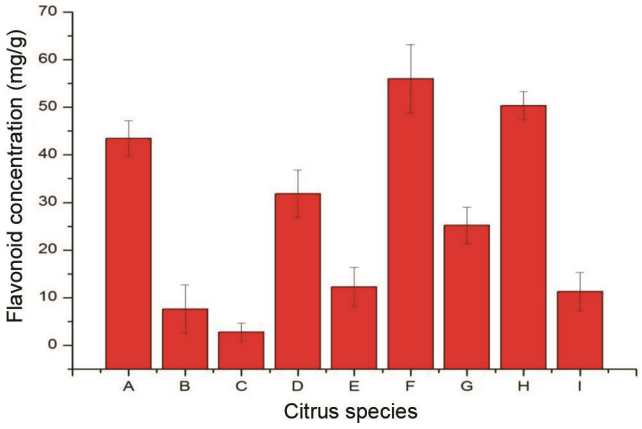

Figure 1. Total flavonoid content in nine citrus species. Values are expressed as mean ± SEM. (A) *C. aurantifolia*, (B) *C. aurantium*, (C) *C. hystrix*, (D) *C. limetta*, (E) *C. limonia*, (F) *C. maxima*, (G) *C. medica*, (H) *C. reticulata* and (I) *C. sinensis*.

Table 2. Preliminary phytochemical analysis results of FRCF of nine different citrus species

Citrus species	Alkaloids	Flavones	Saponins	Tannins	Flavonoids	Phenols	Glycosides	Amino acids
C. aurantifolia	–	+	–	–	+	+	+	–
C. aurantium	–	+	–	–	+	+	–	–
C. hystrix	–	+	–	–	+	+	–	–
C. limetta	–	+	–	–	+	+	+	–
C. limonia	–	+	–	–	+	+	+	–
C. maxima	–	+	–	–	+	+	+	–
C. medica	–	+	–	–	+	+	+	–
C. reticulata	–	+	–	–	+	–	–	–
C. sinensis	–	+	–	–	+	+	+	–

'+' Indicates presence and '–' indicates absence of a particular phytoconstituent.

Table 3. α-Glucosidase and α-amylase inhibitory activity of FRCF of citrus species

	α-Glucosidase inhibition		α-Amylase inhibition	
Citrus species	Percentage inhibition at 100 µg/ml	IC 50 (±SEM, µg/ml)	Percentage inhibition at 100 µg/ml	IC 50 (±SEM, µg/ml)
C. aurantifolia	–	–	48.3 ± 2.35*	128.26 ± 5.68*
C. aurantium	43.82 ± 4.45	114.22 ± 5.34	53.5 ± 1.13*	90.34 ± 6.72*
C. hystrix	14.15 ± 2.02*	365.24 ± 7.88	60.97 ± 3.78*	88.44 ± 7.89*
C. limetta	–	–	69.32 ± 2.86	52.32 ± 4.56
C. limonia	–	–	37.44 ± 1.22*	187.45 ± 8.34*
C. maxima	57.14 ± 3.34*	92.56 ± 5.78	76.58 ± 4.56	45.34 ± 5.34
C. medica	–	–	32.88 ± 0.98*	166.58 ± 6.67*
C. reticulata	43.22 ± 2.76	118.90 ± 4.87	35.53 ± 1.56*	155.86 ± 4.88*
C. sinensis	–	–	38.89 ± 2.35*	176.58 ± 9.64*
Acarbose	28.57 ± 2.33	143.22 ± 5.21	66.26 ± 2.57	75.88 ± 3.45

Values are expressed as mean ± SEM. *Represents significant difference @ $P < 0.05$ level when compared with standard.

which was very high compared to the present results. The bound phenolic extracts from *C. maxima* exhibited significantly higher α-amylase inhibitory activity than free phenolics[31]. The present study explores the potency of total flavonoids present in *C. maxima* as α-amylase inhibitors.

Determination of α-glucosidase inhibitory activity

From the assay it was found that FRCF of *C. maxima* possesses effective α-glucosidase inhibitory action of 57.14% (Table 3) by competitively binding α-glucosidase than other species. The inhibitory activity of citrus species was in the descending order of *C. maxima* > *C. aurantium* > *C. reticulata* > *C. hystrix*. It was found that only four citrus species showed α-glucosidase inhibition whereas the remaining five species were found to be inactive. In addition, the inhibitory percentage of certain citrus peels (*C. maxima* > *C. aurantium* > *C. reticulata*) exceeded the activity of standard drug acarbose (28.57%). It was observed from the literature that citrus peel, pulp and seed extracts were found to possess α-glucosidase inhibitory property[32,33]. However, the present study indicates that the flavonoids from citrus peels are effective α-glucosidase inhibitors.

α-Amylase and α-glucosidase inhibitory potential of citrus peels would delay the degradation of starch and oligosaccharides, which would in turn cause a decrease in the absorption of glucose and consequently inhibit the increase in postprandial blood glucose[33]. Among the nine citrus species tested for α-amylase and α-glucosidase inhibitory properties, *C. maxima* exhibited the best activity and the dual inhibitory potential against these target enzymes might be due to the presence of specific flavonoids present in *C. maxima.*

Effect of citrus species on glycated haemoglobin and glucose uptake in yeast cells

The effect of FRCF of nine different citrus species was studied on inhibition in glycosylated haemoglobin and increase in glucose uptake by yeast cells. Table 4 shows the results. In general, increased concentration of glucose in the blood leads to its binding to haemoglobin which may result in the formation of reactive oxygen species, this is known as glycation. In the present study, FRCF of nine different citrus species was studied *in vitro* to inhibit haemoglobin glycation. Results of the study revealed that among the nine species, FRCF of *C. maxima* inhibited haemoglobin glycation (47.36%) and also helped increase

Table 4. Effect of nine citrus species on glycosylation of haemoglobin and glucose uptake by yeast cells

Sample	Glycosylated haemoglobin (% inhibition after 72 h)	Increase in glucose uptake (%)
C. aurantifolia	7.28 ± 1.22*	55.31 ± 3.44*
C. aurantium	16.92 ± 0.98*	21.27 ± 2.97*
C. hystrix	26.72 ± 2.33*	29.78 ± 3.22*
C. limetta	22.67 ± 1.97*	48.93 ± 1.96*
C. limonia	4.85 ± 0.86*	10.63 ± 0.95*
C. maxima	47.36 ± 1.13	65.95 ± 1.22
C. medica	27.53 ± 2.08*	40.42 ± 0.88*
C. reticulata	1.21 ± 0.11*	38.29 ± 1.27*
C. sinensis	29.55 ± 0.76*	48.93 ± 2.32*
Standard	52.45 ± 4.23	62.97 ± 3.33

Values indicate mean ± SEM; $n = 3$. *$P < 0.05$ considered significant.

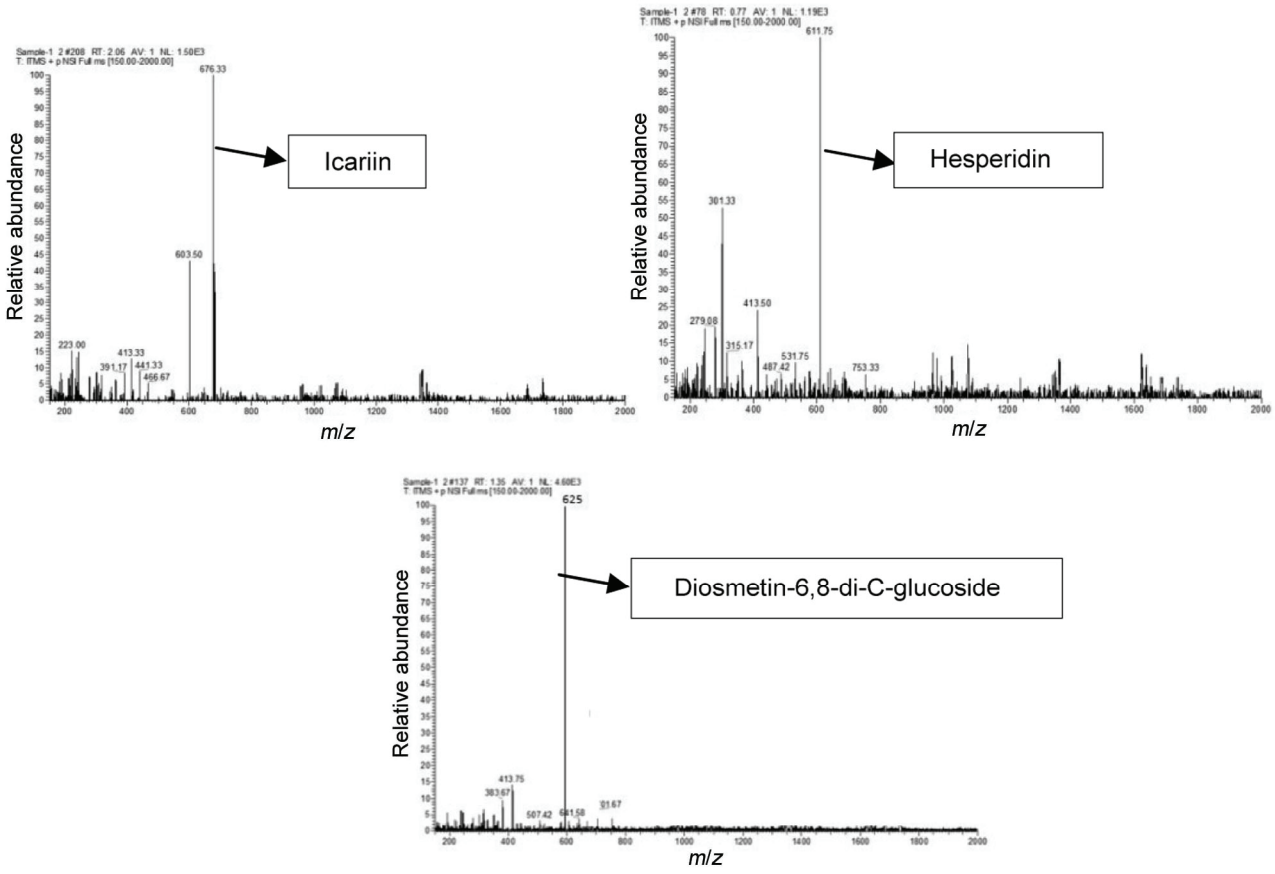

Figure 2. ESI–MS peaks showing the presence of icariin, hesperidin, diosmetin 6,8-di-C-glucoside.

glucose uptake by yeast cells (65.95%) in a marked manner when compared to the other eight citrus species. Except *C. maxima*, all other citrus species showed a minimum level of significance ($P < 0.05$) with the standard, indicating that the effect of *C. maxima* is equivalent to the standard. *C. sinensis* peel extract at 250 and 500 mg/kg body weight administration was able to reduce the glycosylation of haemoglobin in streptozotocin-induced diabetic rats[12]. Other citrus species were not studied for their effects on glycosylation of haemoglobin. *In vitro* antidiabetic activity screening indicated that among the nine citrus species, *C. maxima* exhibited leading activity in all the four models, which may be due to its higher flavonoid content.

ESI-MS analysis

From the above *in vitro* antidiabetic assay reports, interpretations were made which showed that *C. maxima* possessed maximum activity. Hence it was used for ESI-MS

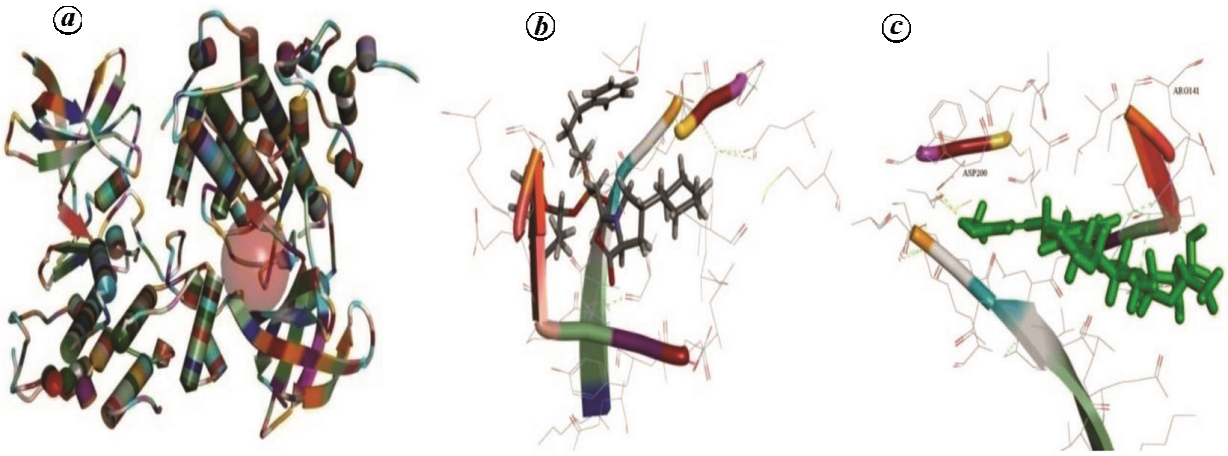

Figure 3. *In silico* analysis of glycogen synthase kinases 3β with citrus flavonoids. ***a***, Interaction site of glycogen synthase kinases 3β. ***b***, Interaction with standard drug I5. ***c***, Interaction of glycogen synthase kinases 3β with citrus flavonoids.

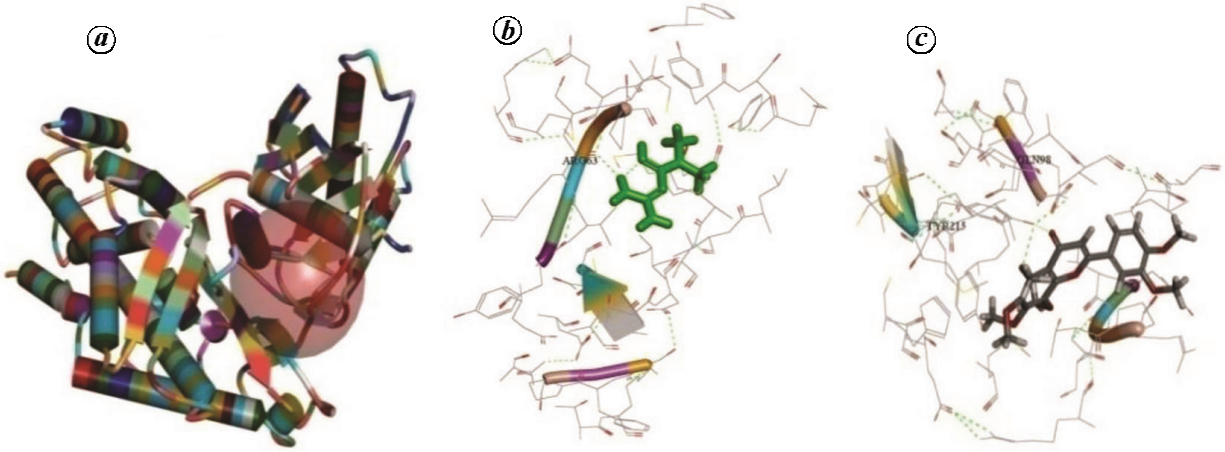

Figure 4. *In silico* analysis of glucokinase with citrus flavonoids. ***a***, Interaction site of glucokinase. ***b***, Interaction with standard drug metformin. ***c***, Interaction of glucokinase with citrus flavonoids.

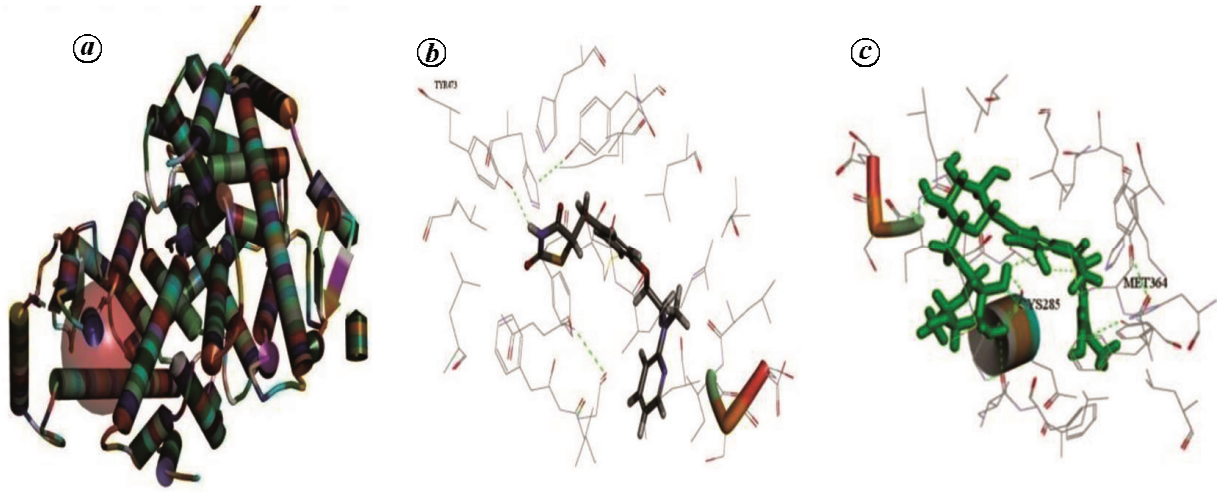

Figure 5. *In silico* analysis of PPAR-γ with citrus flavonoids. ***a***, Interaction site of PPAR-γ. ***b***, Interaction with standard drug rosaglitazone. ***c***, Interaction of PPAR-γ with citrus flavonoids.

Table 5. Interaction of glycogen synthase kinases 3β, glucokinase and PPAR-γ with citrus flavonoids

Protein name	Docking energy (kcal/mol)	Docking interaction energy	Vander walls energy
Glycogen synthase kinases 3β			
I5	–16.858	–45.608	–8.64
Hesperidin	–0.1763	–50.887	–3.42
Icariin	–28.824	–39.993	–2.92
Diosmetin 6,8-di-C-glucoside	–6.720	–42.383	–4.86
Glucokinase			
Metformin	–21.270	–25.781	2.88
Icariin	–3.57	–12.78	–1.56
PPAR-γ			
Rosaglitazone	–44.555	–51.099	–8.64
Hesperidin	–17.7517	–22.844	–3.42
Diosmetin 6,8-di-C-glucoside	–14.56	–26.733	–2.97

analysis for detection of specific compounds in the sample responsible for maximum activity. The ESI-MS peaks in Figure 2 indicate the presence of various compounds in the FRCF sample of *C. maxima.* The MS report was interpreted using MassBank on-line database for identification of flavonoids and it identified the presence of icariin, hesperidin and diosmetin-6,8-di-C-glucoside. Icariin was a new moiety in the study which was identified from the results, having the base peak at 676.33 *m*/*z*. The activity of *C. maxima* may be due to the presence of these compounds. Hence, *in silico* docking studies were carried out for the phytoconstituents determined in the present study.

In silico analysis

Flavonoids identified using ESI-MS were analysed for interaction with target proteins like GSK 3β, GK and PPAR-γ. Schrödinger Maestro software was used for registering ligand–receptor interactions of glycogen synthase kinases 3β, glucokinase and PPAR-γ with hesperidin, icariin and diosmetin 6,8-di-C-glucoside, and compared with the standard drugs like metformin, rosaglitazone and I5 for identification of the molecule with effective binding energy. Figures 3–5 show the three-dimensional structure of these receptor–ligand interactions and Table 5 shows their corresponding docking energies. From the results it was found that icariin showed good interaction with glycogen synthase kinase 3β compared to the other two flavonoids with maximum docking energy. The higher negative interaction energy of about –28.824 kcal/mol of the inhibitor molecule icariin substantiates its inhibitory potential against the receptor GSK 3β. Hesperidin and diosmetin 6,8-di-C-glucoside exhibited maximum interaction with PPAR-γ, with docking energies of –17.7517 and –14.56 respectively. Hence, the mechanism of antidiabetic activity for hesperidin and diosmetin 6,8-di-C-glucoside may be the PPAR-γ pathway. Icariin displayed greater interaction energy with GSK-3β compared to standard drug I5, but the energies of hesperidin and diosmetin 6,8-di-C-glucoside for PPAR-γ were comparatively lesser than that of the standard drug rosaglitazone.

To evaluate the interaction between target proteins and flavonoids, knowledge about their 3D structure is essential. A series of 3D protein structures and their binding sites with ligands have been derived using various structural bioinformatics tools[34,35]. Binding energy plays a key role in the binding interaction between target receptor and ligand of any drug design process[36]. Maestro provides flexible energy. Docking analysis using the Autodock tool V 4.2 and ADT v1.5.4 programs showed that citrus flavonoids dock well with various targets related to diabetes mellitus[37]. Another study on induced-fit docking suggested that flavonoids induce significant conformational changes in the PPAR-γ receptor upon binding and hence can be used as leads/templates for development of novel ligands[38].

It has been reported in the literature that icariin[39] and hesperidin[40] possess antidiabetic activity in animal models. The results of the present study further indicate its possible interaction with target proteins which may be useful in exploring its mechanism of action in *in vivo* and other *in vitro* models in future. However, icariin is reported to have poor bioavailability in a study showing the absolute availability as 12.02% and the first-pass effect of liver as 45.17% (ref. 41). Similarly, the bioavailability of hesperidin[42] and disometin[43] was found to be low due to first-pass metabolism. As these flavonoids possess effective target binding, suitable delivery system to avoid first-pass metabolism can help develop these molecules as effective antihyperglycaemic agents. Specifically, the citrus flavonoid icariin or its analogue molecules could be further developed as an effective lead molecule for the therapeutic treatment of diabetes mellitus.

Conclusion

Flavonoid fraction from the peels of *C. maxima* was studied for *in vitro* anti-hyperglycaemic activity and its interaction with target proteins related to such activity. *In vitro* studies revealed that FRCF of *C. maxima* exhibited maximum inhibition of α-amylase and α-glucosidase enzymes. ESI-MS analysis showed the identification of flavonoids such as icariin, hesperidin and diosmetin-6,8-di-C-glucoside in FRCF of *C. maxima*. *In silico* studies showed that the icariin possesses good GLIDE score against the receptor glycogen synthase kinase 3β, comparable with that of other interactions. The above results demonstrate that citrus flavonoids might be developed as effective agents for blood glucose regulation by performing detailed *in vivo* studies for exploring the mechanism of action.

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