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Isolation and *in silico* evaluation of antidiabetic molecules of *Cynodon dactylon* (L.)

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ABSTRACT

Cynodon dactylon is a potential source of metabolites such as flavanoids, alkaloids, glycosides and β-sitosterol and has been traditionally employed to treat urinary tract and other microbial infections and dysentery. The present work attempts to evaluate the activity of *C. dactylon* extracts for glycemic control. Aqueous extracts of *C. dactylon* analyzed by HPLC–ESI MS have identified the presence of apigenin, luteolin, 6-*C*-pentosyl-8-*C*-hexosyl apigenin and 6-*C*-hexosyl-8-*C*-pentosyl luteolin. Evaluation of hypoglycemic activity through an extensive *in silico* docking approach with PPARγ (*Peroxisome Proliferator-Activated Receptor)*, GLUT-4 (glucose transporter-4) and SGLT2 (sodium glucose co-transporter-2) revealed that luteolin, apigenin, 6-*C*-pentosyl-8-*C*-hexosyl apigenin, 6-*C*-hexosyl-8-*C*-pentosyl luteolin interact with SGLT2. Interactions of these molecules with Gln 295 and Asp 294 residues of SGLT2 have been shown to compare well with that of the phase III drug, dapagliflozin. These residues have been proven to be responsible for sugar sensing and transport. This work establishes *C. dactylon* extract as a potential SGLT2 inhibitor for diabetic neuropathy thus enabling a possibility of this plant extract as a new alternative to existing diabetic approaches.

1. Introduction

Type 2 diabetes mellitus (T2DM) is an emerging epidemic estimated to increase to 69% and 29% in adult population of developing and developed countries, respectively [1]. Recent survey on T2DM population shows that there are over 60 million diabetic patients in India (7.8% of the adult population) exceeding the predicted worldwide figures for 2030 [2]. T2DM is a progressive disease caused by peripheral insulin resistance, visceral obesity, impaired glucose utilization in adipose tissues and muscle and steady deterioration of beta cell function which causes β-cell failure [3]. Inadequate control of blood sugar levels necessitates use of multiple oral hypoglycemic agents and insulin therapy in diabetic patients with a history of more than 10 years of diabetes [4]. Drugs in the armour of diabetologists focus on the control of HbA_{1C} levels towards preventing complications such as diabetic neuropathy and nephropathy. However, prolonged use of multiple drugs for diabetic control, results in side effects, such as increased body weight and hypoglycemia. Reducing post-prandial glucose is an important mechanism for treating diabetic patients and there is a need for new and risk free drugs to treat hyperglycemia wherein the new targets address these issues through inhibition of glucose transport in the small intestine, and reduction of glucose reabsorption from the kidney [5,6]. Fructose-1,6-bisphosphatase, GLP-1, GLUT-4, amylin, incretin and protein kinase C are other targets evaluated towards reducing the post-meal hyperglycemia and associated complications [7–15].

Competitive inhibition of glucose uptake in the intestine and kidney is a preferred strategy to treat T2DM since this facilitates glucose reduction without triggering insulin secretion. Inhibitors for sodium glucose co-transporter-2 (SGLT2) have progressed to late stages of clinical development. Phlorizin (Fig. 1a) is a sodium glucose co-transporter inhibitor isolated from root bark of apple (*Pyrus communis*) and is present in many plants like cherry, pear and other trees of the *Rosaceae* family [16]. Phlorizin acts by inhibiting SGLT2 thereby reducing glucose reabsorption from kidney. Taking lead from phlorizin, which is a natural O-glycoside, several *C*-aryl glycosides have been developed as SGLT-2 inhibitors. Recently, dapagliflozin (Fig. 1b), a molecule jointly developed by Bristol-Myers Squibb in partnership with Astra Zeneca has been withheld by the United States Food & Drug Administration [17]. *Cynodon dactylon* has been reported to contain *C*-glycosides [18] and since the *C*-glycosides have demonstrated SGLT-2 inhibition, identification of structures of compounds present in this plant and

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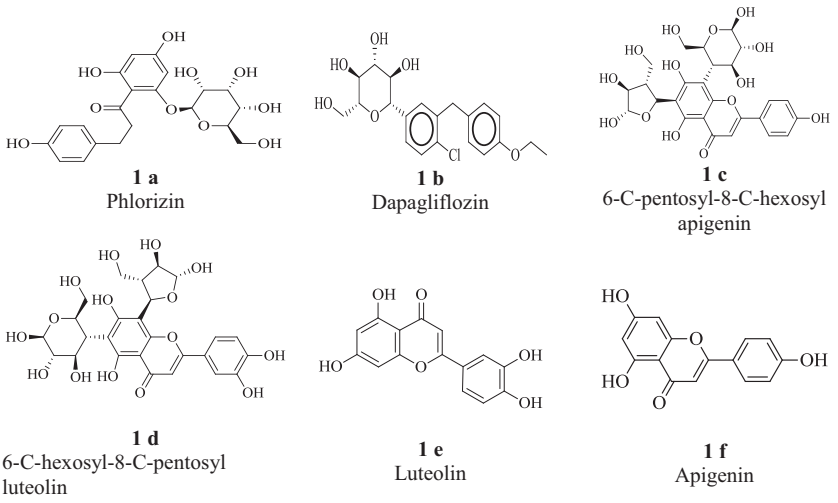

Fig. 1. Structures of known SGLT-2 inhibitors phlorizin (a), dapagliflozin (b) disaccharide oapigenin (c), disaccharide of luteolin (d), luteolin (e) and apigenin (f).

evaluation of *in silico* docking study with SGLT-2 was undertaken. Although aqueous extracts of *C. dactylon* have been shown to possess anti-diabetic activity, their mechanism of action has not been established [19,20].

In the current work, an eco friendly extraction procedure has been adopted and the constituents identified using HPLC–MS/MS techniques. Extensive *in silico* docking procedures have also been carried out to examine the interactions of the plant components with SGLT2 and other diabetic targets. An aqueous extraction procedure is done as the drug is usually administered in the form of a juice.

2. Materials and methods

2.1. Plant materials and chemicals

The whole plant of *C. dactylon* (L.) pers. was collected from Tirumalaisamudram village in Thanjavur, India. The species was identified using the Flora of Presidency of Madras and authenticated with specimens (RHT: 6643) deposited at Rapinat Herbarium, Tiruchirappalli, India. HPLC grade methanol from Merck Specialties Pvt. Ltd and MilliQ water were used in the entire study.

2.2. Extraction procedures

2.2.1. Successive extraction

The plants were cleaned and dried in room temperature for 10 days and coarsely powdered. 100 g of plant material was successively extracted with n-hexane, chloroform, and ethanol by cold percolation method. Extraction using n-hexane and chloroform yielded 0.55 g and 1.68 g of the concentrate, respectively, while 8.82 g of the concentrate was obtained through the ethanol extraction procedure.

2.2.2. Aqueous extract of dry powder

100 g of shade dried and coarsely powdered *C. dactylon* whole plant was mixed with 1 l of distilled water and boiled for 1 h. The mixture was then filtered and the filtrate was concentrated in vacuo. The concentrate was analyzed by HPLC–MS/MS and compounds were identified from the mass spectral data. A portion of the crude extract was subjected to hydrolysis by adding sulphuric acid (1:50) followed by heating for 20 min. The mixture was cooled and filtered. The precipitate was washed with water till filtrate attained neutral pH and was then dried to obtain hydrolysed compounds. This was further extracted with ethanol and the filtrate concentrated in vacuo and analyzed using HPLC–MS/MS.

2.2.3. Aqueous extract of C. dactylon fresh whole plant

100 g of fresh *C. dactylon* whole plant was mixed with 1 l of distilled water and the above procedure repeated to obtain aqueous extract as well as the hydrolysed product.

2.2.4. Methanol extract

1 kg of dried coarsely powdered plant material was defatted with n-hexane and extracted using methanol (3 × 5 l) by cold percolation method. The concentrated filtrate was partitioned between ethylacetate and n-butanol and the dissolved portion from these two solvents were separated and concentrated in vacuo to obtain 26.82 and 18.28 g of extracts, respectively.

The ethyl acetate fraction was further subjected to column chromatography over celite column. Gradient elution was done in the following order, $CHCl_3$:MeOH (1:0; 8:2; 6:4; 5:5; 4:6; 2:8 and 0:1), solvent was removed by evaporation in vacuo. The residue was further fractionated through column chromatography over silica gel by gradient elution of $CHCl_3$ 100%, $CHCl_3$:MeOH (8:2; 6:4; 5:5; 4:6; 2:8) and 100% MeOH. The solvent was evaporated in vacuo and the concentrate subjected to HPLC–MS/MS analysis.

2.3. Qualitative analysis of components using electro spray ionization mass spectrometry (ESI-MS/MS)

In order to realize a qualitative analysis on the compounds present in *C. dactylon*, the aqueous extract was analyzed by LC/ESI/MS using Bruker UHPLC 3000 chromatograph coupled to a quadrupole-ToF mass selective detector (microToF QII, Bruker, Germany). Chromatographic separation of the extract was done using RP C18 column (100 mm × 3.9 mm with internal diameter of 5 μm, Acclaim®, Sunnyvale, CA, USA). Gradient elution was performed by using a mobile phase A represented by acetonitrile and mobile phase B represented by MilliQ water acidified with acetic acid (2%) with the following gradient profile: initially 95% B to 80% B over 5 min; to 70% B over 5 min; to 65% B over 5 min; to 40% B over 5 min; to 0% B over 5 min; to 95% B over 2.5 min and continuing at 95% B until completion of the run. Flow rate was set to 200 μL/min and uv absorbance was detected at 335 nm. Mass spectra in negative ion mode were generated. The ligand molecules were identified from HPLC–MS/MS technique and the structures of the same were obtained from PubChem small molecular database [21].

2.4. 2.4 Molecular docking

The ligands considered for the study were obtained from literature and from mass spectrometric observations reported in this work. Apigenin, luteolin, orientin, iso orientin and truxillic acid were the ligands whose interactions with receptors PPARγ, GLUT-4 and SGLT-2 ligands were explored. Docking and interaction studies were done using the Schrödinger suite [22]. LigPrep [23] module was used to prepare high quality, all atom 3D structures of the ligands. The atomic coordinates of the receptors were retrieved from the PDB and UniProt and the protein structures were prepared and optimized by exhaustive sampling.

Since GLUT-4 and SGLT2 do not have experimental crystal structures, these structures were modelled using MODELLER 9v3 before they were docked to the respective ligands. The details of the templates, their sequence identity and the rmsd of the modelled structures with respect to the template are specified below. Single template modelling was considered for SGLT2 while GLUT-4 was modelled using multiple templates. All these structures were validated and energy minimized after modelling before the docking exercise was initiated.

Modelling of SGLT2

Template PDB code: 2XQ2 (The mechanism of sodium and substrate release from the binding pocket of vSGLT. Watanabe et al. Nature 2010)

Resolution: 2.73 Å

Organism: Vibrio parahaemolyticus

Sequence identity: 33%

rmsd = 0.26 Å

Modelling of GLUT4 (multi template modelling)

Template: 2EAY (203–300) and 1YQT (343–504)

Resolution: 1.90 Å; 2EAY: 1.95 Å

Sequence identity: 1YQT: 33% 2EAY 30%

rmsd: 0.41 Å

PPARγ

PDB code: 2XKW

Organism: Homo sapiens

Resolution = 2.02 Å

PPAR pan agonist

PDB code: 3ET3

Resolution: 1.95 Å

Organism: Homo sapiens

Metal ions and cofactors were adjusted and a restrained energy minimization of the protein was done. Possible active sites for the receptors, which are not co-crystallized with a ligand, were generated. For receptors such as PPARγ, the available crystal structure has been solved with a ligand (ET1, in the case of PPARγ agonist and indeglitazar in the case of PPAR pan agonist). In these cases, the ligand in the crystal structure was removed and its binding site residues were considered for docking the new ligand. In the modelled structure of GLUT-4 and SGLT2, sitemap was generated and the highest ranking site chosen as the best probable binding site and the grid was centred on this site. Extra-precision (XP) docking of the ligands with the three receptors was carried out using Glide [24] and the residues involved in hydrogen bonding and hydrophobic interactions of the resultant complexes were analyzed. The binding site residues of the docked complex thus identified were visualized using Chimera [25]. Ligand activity was studied using a pharmacophore model generated using PHASE [26]. The three diabetic related targets PPARγ, GLUT-4 and SGLT-2 were chosen for the study because of the following reasons:

SGLT2: Since it is known that *C*-glycosides act as inhibitors of SGLT-2 [27]. *C*,*C*-disaccharides were identified from *C. dactylon* extract and hence we wished to evaluate their *in silico* binding with the target.

GLUT-4 and PPARγ: PPARγ and GLUT-4 are important targets in diabetes since they are involved in insulin resistance and sensitization. Further to this, there are several reports of luteolin and apigenin (from other plant extracts) acting through PPARγ [28]. Therefore, we wanted to include them for comparison with the disaccharides reported for the first time from *C. dactylon.* This is the reason for evaluating PPARγ and GLUT-4 as targets in the current investigation.

Concerning other diabetic targets, like GPR-40 and DPP-4, we have not considered them because the ligands for these receptors are different than the *C*,*C*-diglycosides investigated from *C. dactylon.* For GPR-40, the natural ligand is linoleic acid, a linera free fatty acid and potency of agonists was shown to depend on the chain length of the saturated fatty acids whose chain length should be either 15 or 16. During our previous programme on GPR-40 agonists, we realized that either very rigid molecules or compounds lacking propionic acid moiety were ineffective to elicit GPR-40 agonist activity [29] and further that they did not answer GPR-40 assay although presumably acting through GPCRs since the compounds lacking propionic acid moiety have shown *in vivo* glucose reduction. This is the reason why we have not considered GPR-40 as a target for the *C*,*C*-disaccharides identified from *C. dactylon* extract because these disaccharides lack any COOH group for interaction with GPR-40 and also because these disaccharides are bulky in nature. Regarding DPP-4 inhibitors, it has been established through Structure-activity relationship studies that a characteristic electrophilic group is a pharmacophoric requirement wherein an interaction of the polar group with the hydroxyl group of the residue serine located in the active binding site. Prominent groups are nitrile or boronic acid or diphenylphosponate group which imparts selectivity towards DPP-4 against DPP-2, DPP-8, DPP-9, a factor in turn attributed to the presence of this polar group [30]. Since the flavanoids disaccharides evaluated from the present study do not possess any of these polar groups, it was thought that they might not have any influence on DPP-4 inhibition and so DPP-4 was not chosen as a diabetes target for the docking study.

3. Results

3.1. HPLC–MS/MS analysis of the aqueous extracts of C. dactylon

To identify the constituents of the aqueous extracts of *C. dactylon*, a LC–MS/MS analysis was performed. Mass spectrometric data identified 6-*C*-pentosyl-8-*C*-hexosyl apigenin (Fig. 1c), 6-*C*-hexosyl-8-*C*-pentosyl luteolin (Fig. 1d) and luteolin (Fig. 1e). Though literature has documented the presence of apigenin (Fig. 1f) in the extracts of *C. dactylon* [31,32], our aqueous extracts did not identify apigenin as a constituent molecular entity. The constituent compounds of *C. dactylon* analyzed using HPLC–MS-MS are shown in Table 1.The presence of flavonoid disaccharides was confirmed by comparing the mass spectrometric indices of the same compounds isolated from other plant sources. The fragmentation patterns obtained were compared with those reported in literature [33] for these compounds and a pattern matching arrived at. A similar HPLC chromatogram pattern was observed for the extracts from dry powder (Fig. 2) as well as the fresh grass (Fig. 3) of *C. dactylon.* LC–MS spectra for luteolin, 6-*C*-hexosyl-8-*C*-pentosyl luteolin and 6-*C*-pentosyl-8-*C*-hexosyl apigenin are shown in Figs. 4–6. Though apigenin was not observed in the above two extracts, it could be identified from the hydrolysed fraction from the extracts (Fig. 7) albeit in very small concentration indicating the resistance of *C*,*C*-diglycosides towards hydrolysis. Based on the similarity of compounds isolated by different extraction methods (Table 2), it is decided to use the eco-friendly aqueous extraction method which provided the *C*,*C*-disaccharides. These are the only

Table 1
LC–MS-MS identification of constituents of *Cynodon dactylon* aqueous extract.

Compound	Retention time (min)	$[M-H]^-$ m/z	Fragments in MS (HPLC–ESI-MS^n)
6-*C*-pentosyl-8-*C*-hexosyl apigenin (3)	12.65	563.1	473, 443, 413, 425, 353, 383
6-*C*-hexosyl-8-*C*-pentosyl luteolin (4)	11.30	579.1	489, 441, 429, 399, 369
Luteolin (5)	19.70	285.0	201, 175, 133, 121
Apigenin (6)	22.70	269.0	117

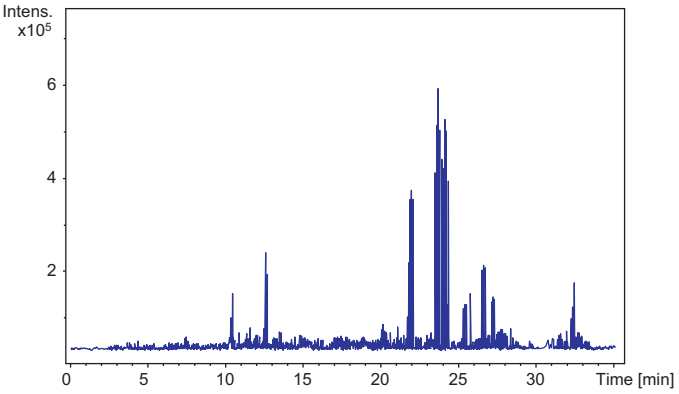

Fig. 2. HPLC chromatogram of dried *C. dactylon* aqueous extract.

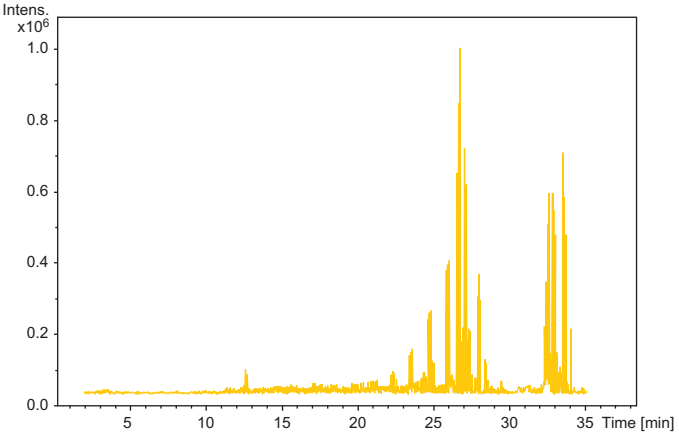

Fig. 3. HPLC chromatogram for hydrolysed aqueous extract of dried *C. dactylon*.

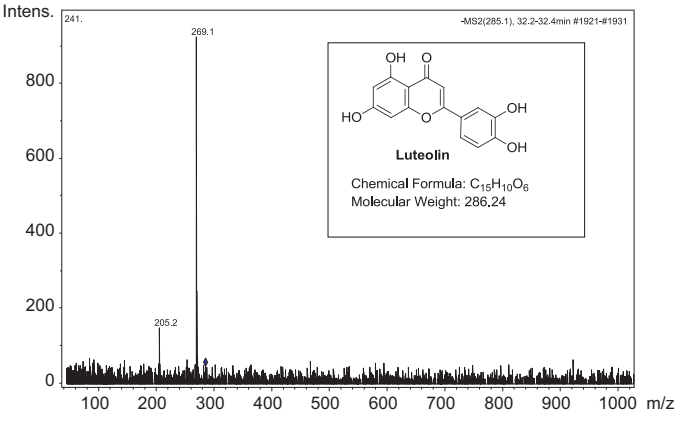

Fig. 4. LC–MS spectrum of luteolin.

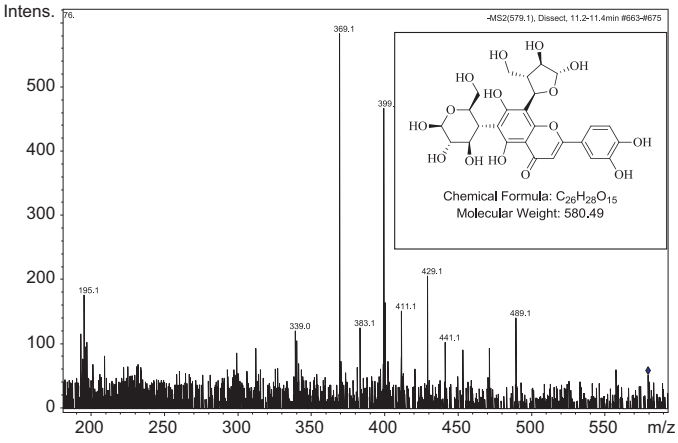

Fig. 5. LC–MS spectrum of 6-*C*-hexosyl-8-*C*-pentosyl luteolin.

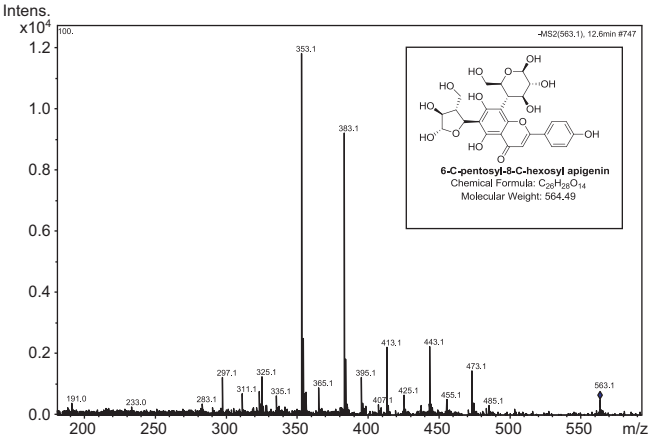

Fig. 6. LC–MS spectrum of 6-*C*-pentosyl-8-*C*-hexosyl apigenin.

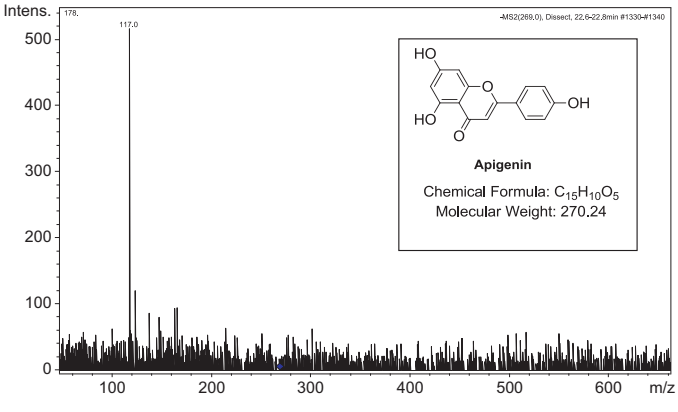

Fig. 7. MS/MS spectrum of apigenin from hydrolysed aqueous extract of dried *C. dactylon*.

Table 2
Compounds identified through different extraction methods.

S. no.	Extract details	Compounds identified
1	Ethanol	6-*C*-pentosyl-8-*C*-hexosyl apigenin 6-*C*-hexosyl-8-*C*-pentosyl luteolin Luteolin
2	Ethyl Acetate	6-*C*-pentosyl-8-*C*-hexosyl apigenin 6-*C*-hexosyl-8-*C*-pentosyl luteolin
3	Ethyl acetate (butanol)	6-*C*-pentosyl-8-*C*-hexosyl apigenin Luteolin
4	Dried water extract	6-*C*-pentosyl-8-*C*-hexosyl apigenin 6-*C*-hexosyl-8-*C*-pentosyl luteolin Luteolin
5	Dried water extract (hydrolyzed)	6-*C*-pentosyl-8-*C*-hexosyl apigenin 6-*C*-hexosyl-8-*C*-pentosyl luteolin Luteolin Apigenin
6	Fresh water extract	6-*C*-pentosyl-8-*C*-hexosyl apigenin 6-*C*-hexosyl-8-*C*-pentosyl luteolin Luteolin
7	Fresh water extract (hydrolyzed)	6-*C*-pentosyl-8-*C*-hexosyl apigenin 6-*C*-hexosyl-8-*C*-pentosyl luteolin (weak) Luteolin Apigenin

four compounds that could be identified from the aqueous extracts of *C. dactylon*, although beta-sitosterol, linolenic acid, docosanoic acid ethyl ester, hexadecanoic acid ethyl ester, eicosanoic acid ethyl ester and 2,6-dimethoxy phenol were identified through GC–MS analysis of organic (hexane) extracts. Since we are interested in aqueous extract only, data for the compounds identified from aqueous extract have been shown and discussed in the paper.

3.2. Analysis of receptor–ligand interactions in C. dactylon

To evaluate the anti-diabetic effect of flavanoids and its related compounds in *C. dactylon*, molecular docking was carried out for the targets PPAR gamma (PPARγ), glucose transporter type 4 (GLUT-4) and sodium glucose co-transporters (SGLT2) which are involved in the anti-diabetic pathway. The disaccharides of luteolin and apigenin identified by LC–MS–MS were also subjected to docking with the receptors mentioned above.

C-aryl glycosides such as dapagliflozin have proved to be effective anti-diabetic compounds presumably due to the resistance of the *C*-glycosyl flavones to undergo hydrolysis by beta glucosidases in the intestine compared to their O-glycoside counterparts [34]. Also since the disaccharides 6-*C*-pentosyl-8-*C*-hexosyl apigenin and 6-*C*-hexosyl-8-*C*-pentosyl luteolin have not been evaluated for diabetes targets, especially for SGLT-2, the dissacharides were docked to the aforementioned receptors to determine their binding affinities. The same receptors were also subjected to docking with test compounds like dapaglifozin, sergiflozin, fascentin, rosiglitazone and genistin that were proven to show inhibitory effects, so as to ascertain if the glide scores are comparable. The glide scores for all the docking analysis are summarized in Table 3.

The reference compound for PPARγ is Rosiglitazone and that for GLUT-4 is genistin.

In the present evaluation, using PPAR pan agonist cocrystal structure (3ET3), where the ligand was removed and docked with the aglycones and rosiglitazone (a PPARγ agonist), we obtained high docking scores for the aglycones compared with rosiglitazone, presumably due to the selection structure of PPAR pan agonist instead of PPARγ agonist. After running the docking experiment with PPAR pan agonist, the dock scores obtained are, indeglitazar: −9.99, apigenin: −6.86 and luteolin: −7.48. However, when the structure of PPARγ (2XKW) was used for docking with reference ligand rosiglitazone, the scores are, rosiglitazone: −5.81, apigenin: −4.04, luteolin: −6.1. The above docking results are in agreement with the published experimental activity data for the PPAR activity of aglycones. EC_{50} of rosiglitazone for PPARα is 15,000 nM and for PPARγ is 245 nM.

Similarly there are several folds of lower EC_{50} observed for PPARγ activation by PPAR α ligands. PPARα selective ligand [35] for example, RO4899100 was found to be 100-fold more potent for PPAR than for PPARγ.

However the present study identifies several key interactions with other residues. Hence it is possible that due to the inability of the aglycones to form a U-shaped conformation to fit into the LBS of PPARγ, they might be making interactions with other residues. Further, it is well established that aglycones such as luteolin were found to act as a partial agonist albeit through interaction with proinflammatory signalling. Visualization of crystal structures of PPARγ (2XKW) and PPAR pan agonist cocrystal structure (3ET3), reveals the striking difference in the binding mode of the two types of ligands (Fig. 12). The observation that the EC_{50} values luteolin was 30 times less potent than rosiglitazone and apigenin was nearly 1000 times less potent than rosiglitazone [36] has been explained due to the rigid flavanoids failing to not adapt a U-shaped configuration in the LBP unlike the TZDs. This indicates that the aglycones could be considered as weak or partial PPARγ agonists and hence they may not qualify as better ligands than rosiglitazone as far as PPARγ activation is concerned.

The aglycones, luteolin and apigenin were shown to interact with a number of targets and since the *C*,*C*-disaccharides show good interaction with SGLT-2 alone and not with other targets evaluated in the present work, we anticipate these disaccharides to be selective towards SGLT-2 in potentially offering anti-diabetic activity. Further, flavanoids such as quercetin, apigenin, luteolin and their O-glycosides have been shown to elicit α-glucosidase inhibitory activity [37] competitive inhibition [38] of sodium dependent glucose transporter-1 and also aldose reductase inhibition [39].

In the case of interactions essential for activation of GLUT-4, genistin makes H-bonded interactions with Gln275 through A ring OH group. In addition to this, ATP binding pocket observed in the loop between TM8 and TM9 is surrounded mainly by the residues R169, H353, R467 and other residues required for glucose transport while residues E345, E409 and R416 facilitate conformational change of the transporter. The two OH groups present in genistin A ring is involved in interaction with ATP binding site. However, in the case of apigenin the interactions observed are mainly H-bonded interaction of B ring OH group with Gln270, while luteolin makes H-bonded interaction through the OH group of A ring with Gln270. Although H-bonded interactions through Gln residues are important in glucose recognition, the extent to which these observed interactions could influence glucose translocation depends on the interaction with ATP binding domain and the synergy. These flavanoids do not make such interactions with ATP binding site and hence the docking scores observed for luteolin and apigenin for GLUT-4 could be taken as a guiding factor only and further study would provide support for this.

The docked complexes of apigenin, luteolin and truxillic acid with PPARγ and GLUT-4 are shown in Fig. 8. The docking outputs of Rosiglitazone, an inhibitor of PPARγ and genistin, an inhibitor of GLUT-4 are also shown in the same figure. It can be observed that ligands orientin and luteolin showed higher scores with all the receptors when compared to the other ligands (Table 3). The docking scores of dapagliflozin and sergliflozin, known to be the best inhibitors of SGLT-2 were found to be comparable to that of luteolin and orientin when docked with SGLT-2. Furthermore, genistin and fascentin, clinically proven inhibitors of GLUT-4 scored high in docking and were closer to the quantified docking outputs of truxilic acid and apigenin with GLUT-4. Rosiglitazone scored comparably well with that of truxillic acid and luteolin docked to PPARγ.

Table 3
Docking score for compounds present in *Cynodon dactylon* with diabetes targets.

Ligands	Receptors (diabetes targets)			
	SGLT-2	GLUT-4	PPAR pan agonist	PPARγ agonist
Truxillic acid	−9.18	−5.17	−0.85	−0.85
Orientin	−11.01	−9.12	−14.8	−14.8
Apigenin[a]	−8.93	−4.23	−6.86	−4.04
Luteolin[a]	−7.73	−6.00	−7.48	−6.1
Iso-orientin	−10.70	−6.09		
Dapagliflozin (test)	−6.44			
Sergliflozin (test)				
Rosiglitazone (test)			−7.80	−5.81
Genistin (test)		−4.69		
Indeglitazar (test)			−9.99	
Fascentin (test)		−5.19		
Apigenin disaccharide[a]	−11.62			
Luteolin disaccharide[a]	−10.09			

[a] Compounds identified by LC–MS/MS of *Cynodon dactylon*.

Furthermore, it was observed that the disaccharide forms of apigenin and luteolin had a higher glide score when compared to their monosaccharide counterparts. This emphasises the higher affinity and significance of the new compounds obtained from this study.

When the ligands apigenin, luteolin and orientin were docked to the GLUT-4 receptor, an interaction with Glu 270 of GLUT-4 was observed in all the docked receptor ligand complexes. However truxillic acid showed an interaction with Gln 277 of GLUT-4, similar

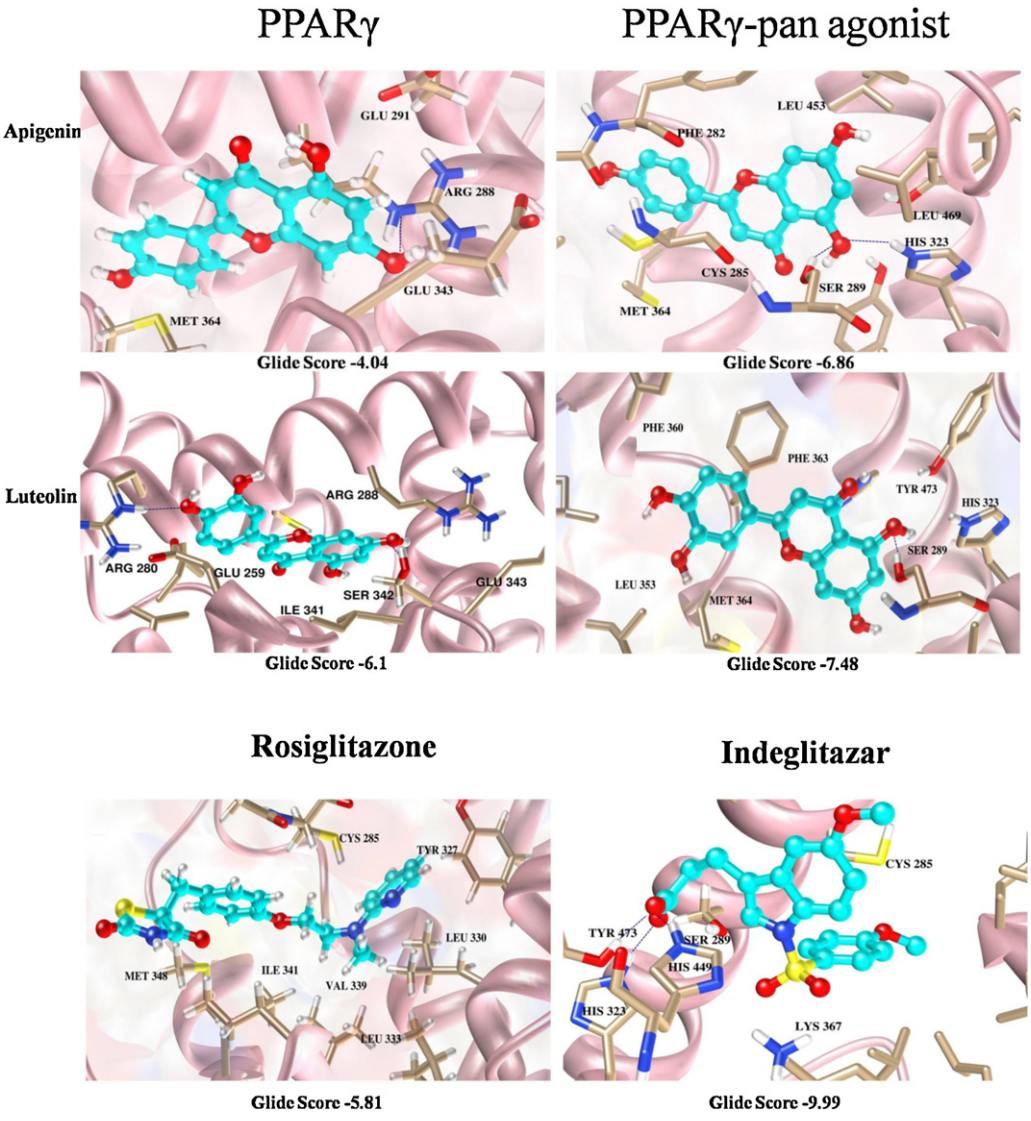

Fig. 8. Docked complexes of apigenin, luteolin in comparison with indeglitazar-PPAR pan agonist and rosiglitazone-PPARγ agonist.

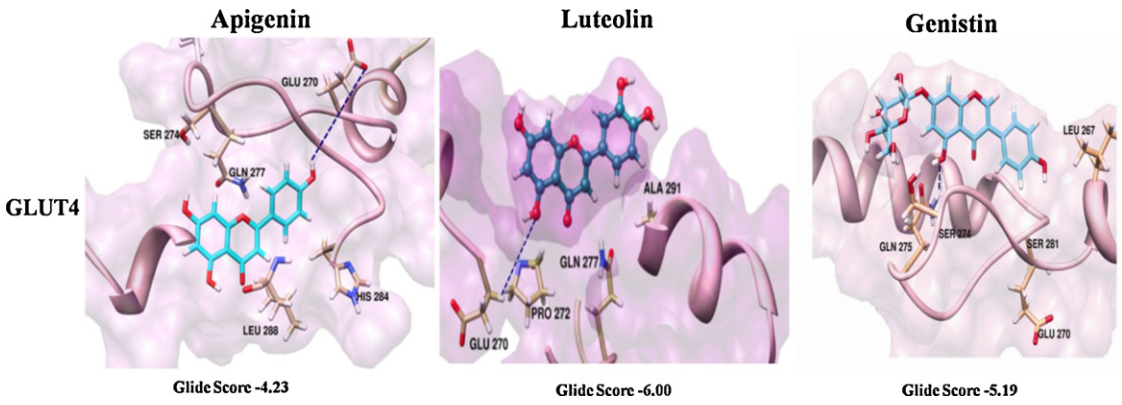

Fig. 9. Interaction observed for apigenin and luteolin with GLUT-4 in comparison with test compound genistin.

to the interaction observed in the test compound genistin (Fig. 9). Orientin which interacts with both Glu 270 and Gln 277 of GLUT-4 showed a higher glide score of −9.12 when compared to the other ligands (Fig. 8). Rosiglitazone a test compound for PPARγ showed one hydrophobic interaction with Phe 282 and orientin which also exhibited the same interaction has a higher glide score of −14.8. Dapagliflozin, the test compound for SGLT-2 interacts with Asp 294 and Gln 295. A similar interaction is also seen in the disaccharides forms of luteolin and apigenin.

3.3. Development of pharmacophore models for SGLT-2 ligands

A pharmacophore model conveys the characteristics of 3D chemical structures that are critical for binding. In this case, a common pharmacophore model was developed using a set of pharmacophore features to generate sites for all the compounds. The initial pharmacophore model was developed with five ligands namely, orientin, apigenin, luteolin, iso-orientin and truxilic acid. Common pharmacophore feature for this set was found to be AAAR comprising three acceptors and an aromatic ring. In order to confirm the obtained feature AAAR, the pharmacophore model for the test compounds dapagliflozin, rosiglitazone and fascentin was generated. The pharmacophore model of disaccharide forms of apigenin and luteolin when superimposed with their aglycones showed identical features at the same sites. This shows the importance of three acceptor groups and one aromatic ring for the activity of these ligands against the receptors. The generated pharmacophore model is shown in Fig. 10.

4. Discussion

C. dactylon juice has been traditionally and extensively used as a tonic and as powder for several ailments. *C. dactylon* has been employed [40,41] in the treatment of cancer, anti-inflammatory, diuretic, antidiabetic, hypolipidemic, and hepatoprotective effects and also to prevent vomiting and burning sensation, hallucinations and fever. *C. dactylon* aqueous extracts in streptozotocin induced diabetic rats have been shown to result in hypoglycaemia and weight-gain [19,20]. Several reports that exist on the extraction and identification of *C. dactylon* constituent compounds [18] point to *C*-glycosides, apigenin, luteolin, beta sitosterol, citronellol, phytol, linoleniec acid, docosanoic acid, syringol, hexadecanoic acid, eicosanoic acid and truxillic acid. Biological activity and properties of the extracts of *C. dactylon* in diabetes, cancer, CNS activity and kidney stone formation have been reported. However, the active compounds and their mechanism of action have not been ascertained through the studies reported so far. Ethanol extract [42] has been reported to contain flavonoids, alkaloids and saponin, while flavonoid and saponin were present in aqueous extract.

Identification of natural product inhibitors of SGLT-2 could serve as alternate to the *C*-aryl glycosides developed for inhibiting renal glucose reabsorption. Flavanoid glycosides are present in many natural products and those reported by various investigators include apigenin and luteolin and their *C*-glucosides, orientin, vitexin, iso-orientin and iso-vitexin. Though flavanoids and their glycosides have been reported to exhibit glucose reduction in diabetic patients, their mechanism of action has been ascribed to alpha glucosidase inhibition, intestinal glucose transporter GLUT-2.

In the present work, *C*-pentosyl-*C*-hexosyl disaccahrides of apigenin and luteloin having *C*-glycosidic linkage were isolated from aqueous extract of *C. dactylon* for the first time. Structural assignments for the *C*-pentosyl-*C*-hexosyl disaccahrides of apigenin and luteloin have been made on the basis of published LC–MS–MS data for the same compounds isolated from other plants. Apigenin-6-*C*-hexoside-8-*C*-pentoside has been identified from alcoholic extract of *Artemesia argenta* L Her [43]. 6-*C*-pentosyl-*C*-hexosyl apigenin and 6-*C*-hexosyl-*C*-pentosyl luteloin have been isolated from *Capsicum* [44,45] and from *Cymbopogan citratus* leaves [32]. Pharmacophore analysis [46,47] of SGLT-2 inhibition revealed that there exists flexible sugar binding to glucose site, providing opportunity for variations in flavones part as well as sugar part. Inhibition of SGLT-2 by flavanoid glycosides has led to the development of novel *C*-aryl glycosides and several novel analogues. During extraction and characterization of active compounds from *C. dactylon*, it was observed that the novel 6-*C*-pentosyl-8-*C*-hexosyl apigenin, 6-*C*-hexosyl-8-*C*-pentosyl luteolin were present in aqueous extracts as well as in organic solvent extraction. In view of the need for natural remedy for blood sugar control, isolation of flavanoid *C*-glycosides from *C. dactylon* and their use in diabetes treatment gains importance. Further, traditional use of *C. dactylon* juice in treating burning sensation prompted investigation of the plant for inhibiting glucose reabsorption from kidney, since the first SGLT-2 inhibitor phlorizin, a *C*-aryl glycosides work on this mechanism. Therefore, the *C*,*C*-disaccharides isolated for the first time from *C. dactylon*, were considered as potential inhibitors of SGLT-2. Previously, novel synthetic agonists for GPR40, a transmembrane GPCR has been developed and shown to have glucose stimulated insulin secretion [48].

It is observed that the ligands orientin and luteolin from *C. dactylon* extracts showed higher affinity to all the receptors when compared to the other ligands. In addition to this, docking scores of dapagliflozin and sergliflozin which are known to be the best inhibitors of SGLT-2 [49], had scores comparable to that of luteolin and orientin same. Furthermore, the clinically proven inhibitors of GLUT-4, genistin and fascentin had the docking scores close to

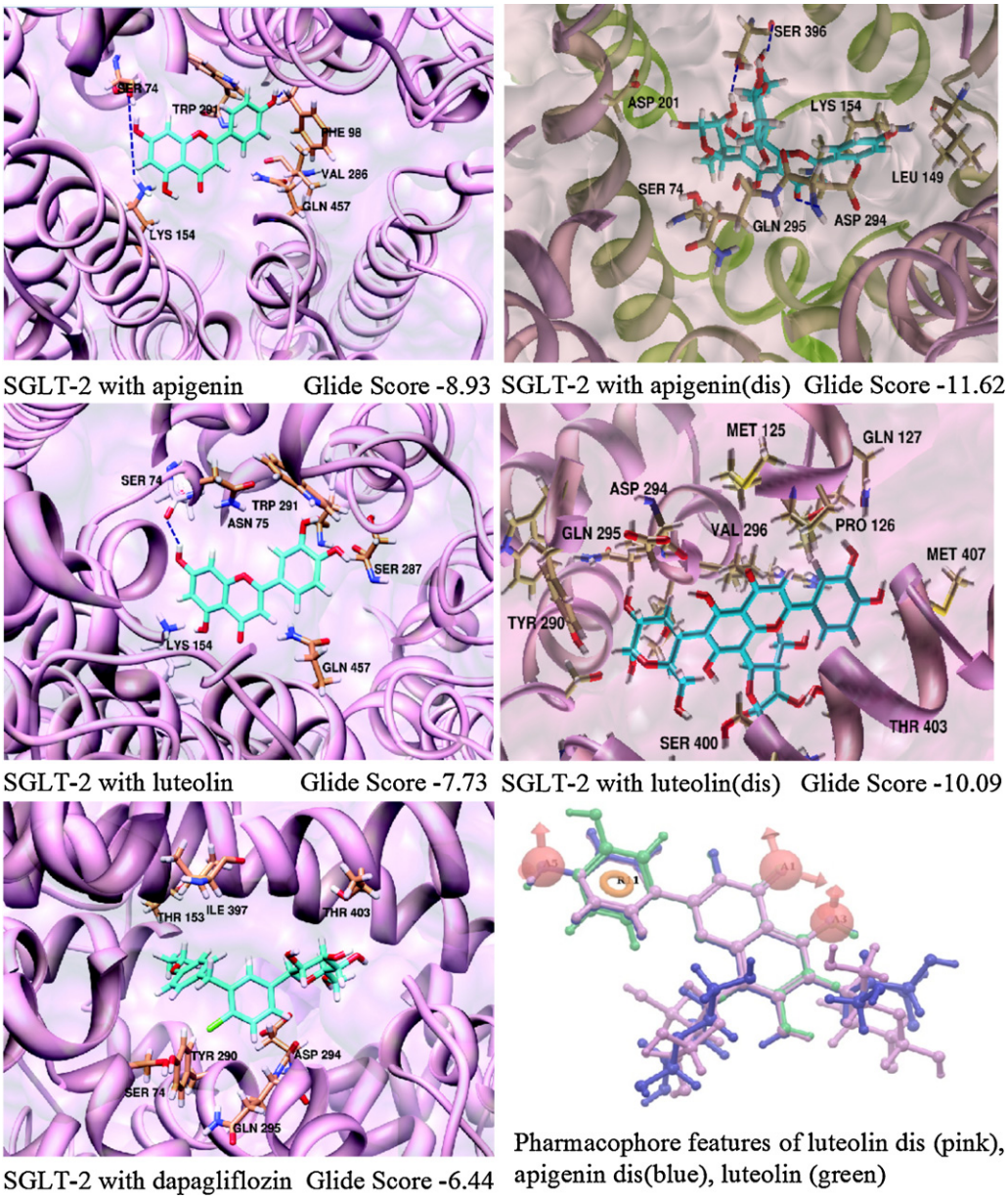

Fig. 10. Docked complexes of SGLT-2 ligands from *Cynodon dactylon*, dapagliflozin and pharmacophore model.

that of truxillic acid and apigenin with GLUT-4. Rosiglitazone, an inhibitor of PPARγ, had a comparable score with that of truxillic acid and luteolin.

It is also known that the facilitated glucose uptake mediated by insulin sensitive GLUT-4 receptors in skeletal muscle, adipose and intestine is inhibited by aglycones and the inhibition of glucose uptake by dietary polyphenols is considered to be mediated through this mechanism. However, active transport of glucose, influenced by Na^+ gradient in kidney is inhibited by glycosides while Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells [50].

The differences in activity with oral and intravenously administered phlorizin have been studied and the poor activity in the oral route is ascribed to the facile cleavage of O-glycosidic linkage leading to phloretin [51]. New phlorizin derivatives have been tested and found to possess hypoglycemic activity [52–58].

The pharmacokinetics of *C*-glycosyl flavones of Hawthorn leaf flavonoids in rat after single dose oral administration [59] has been studied and found that more than 60% of the parent glycosides remain intact after 24 h indicating that *C*-glycosides are resistant to hydrolysis. The role of *C*-6 alkyl residues of 6-deoxy-glucose imparting higher affinity towards SGLT-2 compared to SGLT1 could be construed as support for hydrophobic interaction [60] of *C*,*C*-disaccharides with SGLT2 Characteristics of renal Na^+-D-glucose co transport in the skate (*Raja erinacea*) and shark (*Squalus acanthias*). Phlorizin has also been reported to have interaction with Gln 457 and Thr 460 similar to the hydrogen bond interactions shown by D-glucose and further ligand-induced conformational changes were ascribed to this effect [61]. Significance of the interaction of flavanoid ring A [62] with Phe 602 and Phe 609 has been confirmed by single-molecule recognition atomic force microscopy and tryptophan scanning.

From the docking scores it can be seen that the *C*,*C*-disaccharides have affinity to SGLT-2. These disaccharides fit into a pharmacophore similar to the one adapted by the phase 3 drug dapagliflozin and it shows interaction with the residues Gln 295 and Asp 294 of SGLT-2 which are important for sugar binding. Mutation studies using SGLT revealed that Gln 457 amino group contributes

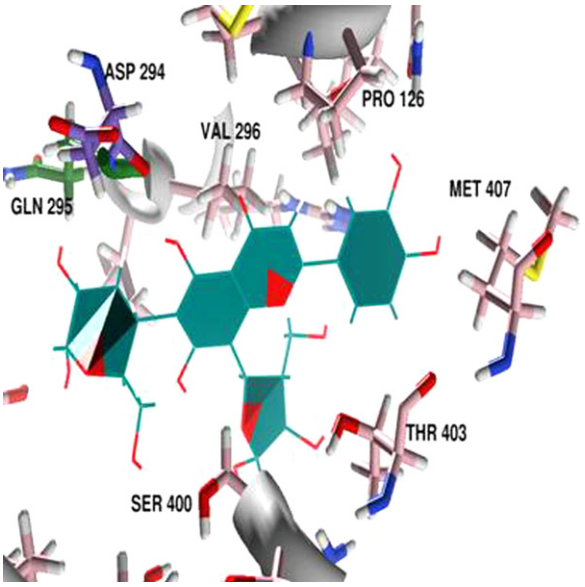

Fig. 11. Interaction observed between luteolin disaccharide with Asp 294 and other residues of SGLT-2.

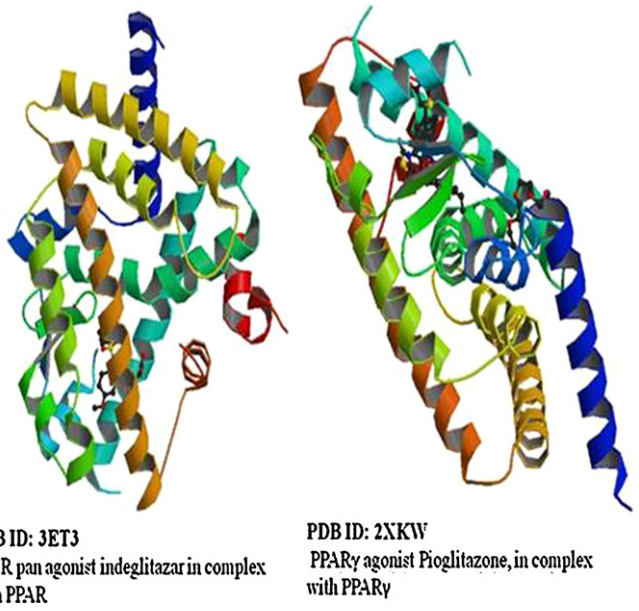

Fig. 12. PDB structures for cocrystal structures of PPAR pan agonist and PPARγ agonist.

to both sugar recognition and translocation through strong H-bond donor interaction with O-1 and with O-5 of the hexose, respectively [63]. Though this residue is not visible in the ligand interaction diagram, we observe a favourable interaction between Gln 295 with the *C*,*C*-disaccharides and dapagliflozin as well and this residue in place of Gln 457 might act as sugar sensor. Similarly, contribution of Asp 454 towards favouring glucose transport and suppression, by negative and positive charges, respectively, has been identified through mutation studies [64,65]. Similar to the above, the dissacharides shows interaction with Gln 295, Asp 294 of SGLT2 a pattern seen in dapagliflozin.

Truxillic acid and luteolin shows interaction with Gln 457 whereas orientin interacts with Gln 295. This indicates that the sugar unit present in orientin makes it interact with Gln 295 similar to the disaccharides of *C. dactylon*, while the other two compounds because of the absence of sugar unit in their structure are unable to make such interaction. All these three compounds have interaction with Asp 294.

In order to check the relative interaction of these compounds with other diabetes targets, we have evaluated their docking with PPAR gamma, GLUT-4 in addition to the docking with SGLT-2. The results reveal that the interaction with Gln 277 of GLUT-4 receptor is prominent in the docking of apigenin, isoorientin, luteolin, orientin and truxillic acid.

Truxillic acid is seen to interact with all the targets evaluated and its poor selectivity could be attributed to the size and dicarboxylic acid functionality present in it. Due to the absence of sugar units, the flavanoids, apigenin, luteolin behave differently in comparison to their glycoside counterparts.

The structural docking reported in this study points to a conserved interaction with Asp 294 and Gln 295 residues in SGLT-2 similar to the interactions observed in the phase 3 drug dapagliflozin indicating that these *C*,*C*-disaccharides present in *C. dactylon* could be useful as anti-diabetic compounds. Hydrogen bonded interaction at 3.94 Å is observed between Asp 294 and CH_2OH of the hexose attached at 6 position of luteolin disaccharide. However, weaker interactions are observed for apigenin disaccharide with these two residues of SGLT-2 (Fig. 11). Further, it is noted that either luteolin or apigenin, as aglycones do not interact with these residues of SGLT-2. The observation that the disaccahrides alone are able to make key interaction with Asp 294 and Gln 295 shows that the bulkiness of the disaccharides are forcing them to the binding pocket in order to make these key interactions required for glucose sensing and transport as required of SGLT-2 inhibition in managing diabetes. Pharmacokinetic study of oral administration of *C*-glycosyl flavones have established that more than 60% of the parent glycosides remain intact after 24 h indicating that *C*-glycosides are resistant to hydrolysis, it can be presumed that resistance of *C*,*C*-disaccharides derived from common plant, to hydrolysis in intestine would be an advantage of these compounds towards eliciting antidiabetic activity similar to the *C*-glycoside SGLT-2 inhibitors (Fig. 12).

5. Conclusions

Diabetic nephropathy is a complication observed in diabetic patients and these patients have symptoms of burning sensation in legs and other parts of the body. Since *C. dactylon* plant juice has been used for burning sensation, anti-diabetic property, especially for the glucose uptake for flavanoids and flavanoid *C*-glycosides from this plant were evaluated. The targets considered for evaluation are alpha glucosidase, PPARγ, GLUT-4 and SGLT2. The main focus is given to understand whether the flavanoids and or flavanoid glycosides present in *C. dactylon* could inhibit SGLT2 thereby preventing glucose reabsorption from kidney. Hence if these compounds act as inhibitors of SGLT2, that would help diabetic patients with a natural remedy. The flavanoid aglycone, *C*-glycosides and flavanoid *C*-pentosyl-*C*-hexosyl disaccharides are reported for the first time from *C. dactylon*. Further these disaccharides have been isolated using a simple and eco-friendly extraction method using water as solvent. This method provides better solvent systems compared to other solvents used. SGLT2 inhibition by these disaccharides evaluated by *in silico* techniques indicates that these *C*,*C*-disaccharides occupy position in the binding pocket and luteolin disaccharide shows a better interaction with SGLT2. Thus the present investigation has identified *C*,*C*-disaccharides for the first time in *C. dactylon* and shown to interact with diabetic target SGLT-2. Use of aqueous extract of common and popular plant *C. dactylon* might provide a way to natural remedy for diabetes overcoming the toxicity issues associated with synthetic drugs. Further *in vitro* and *in vivo* studies using the aqueous extracts is required to ascertain

their efficacy in reducing hyperglycemia. Because of the plieotropic activity manifested by aglycones with various diabetes targets, we intend to do a separation of the disaccharides separately and evaluate their interaction with the diabetes targets described in the paper. It is thought that we will get confirmatory results of the affinity of the compounds with the receptors when tested in pure form compared with mixture of components *in vitro* or *in vivo* experiments. This will form a basis for a future work which has been planned.

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