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Research Article

In silico and *in vitro* assessment on antidiabetic efficacy of secondary metabolites from *Syzygium cumini* (L.) Skeels

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Abstract

India ranks high for prevalence of diabetes and the treatment of diabetes without any side effects is still challenging. Though herbal remedies help reduce the side effect, proper standardization of phytochemical which prove as a bioactive compound, its proper dose and clinical trials are lacking. In our investigation, we studied the binding mechanism of the secondary metabolites of *Syzygium cumini*, their *in vitro* antidiabetic activity and the number of phytochemicals present. *In silico* study revealed that ellagic acid has a potential to modulate the carbohydrate metabolizing enzyme activity showing higher affinity for the enzymes with much lesser binding energy, -4.73 kcal/mol for alpha amylase, -4.87 kcal/mol for beta-glucosidase, -4.79 kcal/mol for glycogen synthase kinase, -4.18 kcal/mol for glucokinase and -4.49 kcal/mol for alpha-glucosidase. *In vitro*-Alpha amylase inhibitory activity assay showed that ethanol extract has the highest value of percent inhibition (73.33%) as compared to standard drug Acarbose (65.99%). Finally, TLC analysis cleared that ethanol extract contains five compounds one of which may be a bioactive compound, ellagic acid. Further purification and characterization of the ellagic acid is needed.

Keywords
Diabetes mellitus; *Syzygium cumini;* Docking studies; Alpha amylase; Thin Layer Chromatography

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Introduction

India is known as the "Diabetes capital of the world" with high prevalence of diabetes (more than 62 million) in the country. Every fifth diabetes patient occurs in the world is an Indian. It is estimated that, in India up to 79.4 million people will affect adversely with diabetes by 2030 (Joshi *et al.*, 2007; Kaveeshwar *et al.*, 2004). The two major types of diabetes *viz*. Type I diabetes (T1D), an autonomous disease cause due to insufficient or no production of insulin in the body and Type II diabetes (T2D) in which progressive insulin resistance is developed. Out of these, type I accounts only for 10-15% incidence while that of type II for 85-90%. Type II diabetes has a higher dominance worldwide and is a serious growing public health problem. Apart from the health consequences, diabetes is one of the economic burdens, including cost of treatment of the disease and its associated complications, increased mortality and morbidity cost and the cost required for informal care etc. (Hex *et al.*, 2012).

T2D is predominant in the individual of age 40 or above 40, hence also considered as a disease

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Table 1. Binding energies kcal/mol obtained during docking analysis of secondary metabolites found in *S. cumini* with their target enzymes

Sl. No.	Compounds	Alpha-amylase	Beta-glucosidase	Glycogen synthase kinase-3β	Glucokinase	Alpha-Glucosidase
1	Caffeic acid	-3.43	-3.63	-4.59	-3.74	-3.22
2	1-galloyl glucose	-1.19	-2.67	-1.52	-1.82	-2.15
3	Corilagin	-1.73	-2.09	-1.15	-1.2	-1.00
4	Ellagic acid	**-4.73**	**-4.87**	**-4.79**	**-4.18**	**-4.49**
5	Ferulic acid	-3.01	-3.34	-4.46	-2.51	-3.19
6	Gallic acid	-3.12	-2.63	-3.79	-3.33	-3.44
7	Guajacol	-3.54	-3.24	-3.00	-2.77	-3.21
8	Quercetin	-3.42	-4.69	-4.34	-3.68	-3.48
9	Veratrol	-3.16	-3.17	-2.98	-3.05	-3.09
10	Acarbose	-0.46	-2.72	-2.66	-1.87	-2.05

of middle-aged and elderly (Singh, 2011; Ozougwu *et al.,* 2013). It is a multifactorial disease involving various genetic as well as environmental risk factors responsible for the disease. It is a hereditary disease can cause due to sedentary lifestyle, alcohol drinking, smoking, aging, obesity, etc. (Kohei *et al.*, 2010; Mehta *et al.*, 2009). If untreated or not diagnosed at early stages, it leads to various complications that finally results in morbidity and mortality. Infections, hypertension, limb amputations, dyslipidemia, renal failure, blindness are some of the complications arise due to the T2D. Treatment for the T2D includes proper diet and exercise, administration of hypoglycemic agents. Pharmacological treatments are also effective in reducing the risk (DiStefano *et al*., 2010; Bastaki 2005). Herbal remedies are found to be effective in order to minimize the side effects arising from the oral hypoglycemic agents. Bioactive potential present in the plants is due to the presence of various phytochemicals such as alkaloids, phenolics, flavonoids, tannins, terpenoids etc. (Rao *et al*., 2010). Today 21,000 medicinal plants are listed by the World Health Organization (WHO) among which 2500 species are of Indian origin. Proper standardization of the phytochemicals present, biological activity profile as well as their proper doses and clinical trials is of greatest interest so as to achieve the target of phyto-pharmaceutical market (Kamboj, 2000; Peixoto *et al*., 2013).

Syzygium cumini or *Eugenia jambolana* is originated from India and is very often cultivated. It is also known as Indian Blackberry or Jamun. Apart from India it is also found in the Philippines, Thailand (Sharma *et al.,* 2006), South Asia, Pakistan, Sri Lanka, Nepal, Bangladesh, Indonesia, Burma etc. (Ayyanar *et al*., 2012). It is a rich source of phytochemicals such as alkaloid like jambosine (Ayyanar *et al.,* 2012), ellagic acid, terpenoids, kaempferol, myricetin, quercetin, isoquercitin (Afify *et al.,* 2011), gallic acid, jamboline as well as some anthocyanins like malvidine glucoside, petunidin, cyaniding etc. (Raza *et al.*, 2015). Out 13.5 million tones of worldwide production of jamun India rates highest with 15.4% of total production (Raza *et al.,* 2015). Due to the presence of a variety of secondary metabolites, the plant shows diverse biological activities. Hydroalcoholic leaf extract shows effective antibacterial activity against various pathogens, including multidrug resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Oliveira *et al*., 2007). Seed kernels extract in combination with the Acarbose found to be very effective in preventing the development of diabetes induced ulcerogenic stimuli (Jonnalagadda *et al.,* 2013). Apart from this *Syzygium cumini* posses other pharmacological activities like anti-hyperglycemic activity, cardioprotective activity, anti-inflammatory activity, antioxidant activity etc. (Chagas *et al*., 2015). ɣ-sitosterol and Kaempferol 7-Omethylether isolated from an ethanol extract of *S. cumini* fruit possesses antioxidant and anticancer activities (Sharafeldin *et al*,. 2015). Anthocyanin isolated from the fruit and pulp has shown anticancer activity against the early stage of HCT-116 human colon cancer cells. It induces apoptosis as well as inhibits self-renewal property of colon cancer stem cells (colon CSCs) (Charepalli *et al.,* 2016). Not only fruit and seed but other parts of the *S. cumini* have proven to be a medicinally important properties. Aqueous extract of leaves and bark possess antiviral activity against avian influenza (H5N1) virus (Sood *et al.,* 2012).

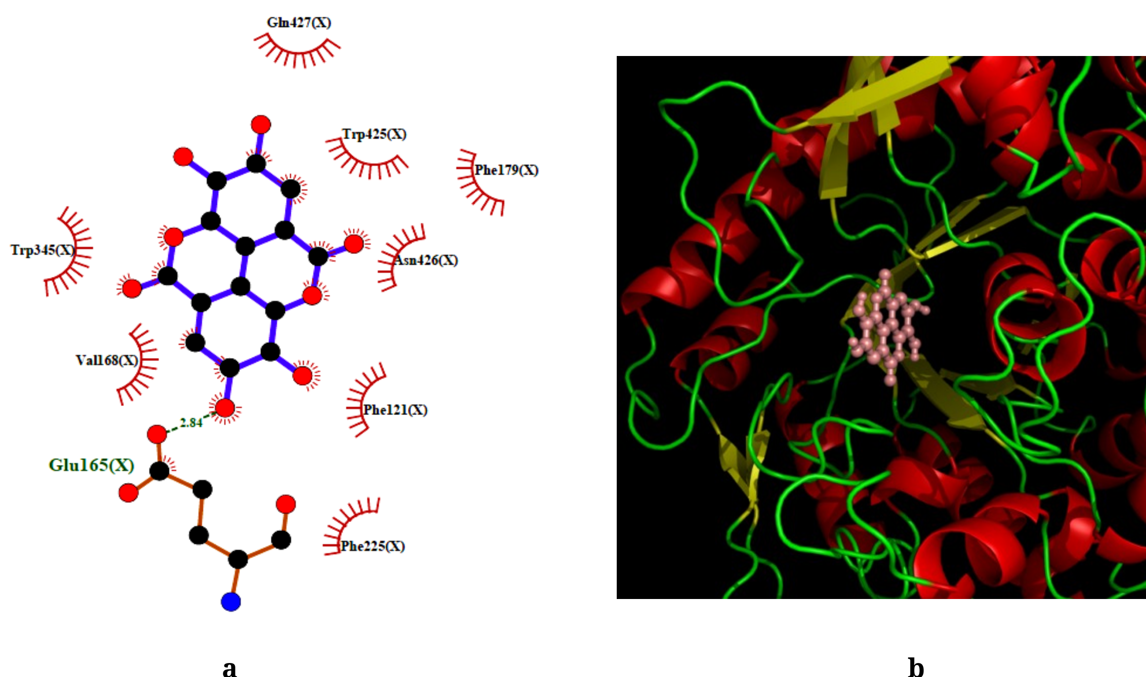

Fig 1. (a) Hydrophobic and hydrophilic interactions of ellagic acid with beta glucosidase residues. Brown colored half circle indicates the hydrophobic reactions of ellagic acid with the enzymes. Green dotted lines indicate the hydrogen bond along with their bond length. (b) Best binding mode of ellagic acid with beta glucosidase enzyme.

Herbal medicines to cure diabetes mellitus are always preferred because of its less or no side effect. Considering this view, we have designed the study to check the antidiabetic potential of the plant *S. cumini* using *in silico* and *in vitro* approach and the possible bioactive compound was also studied.

Materials and Methods

In silico approach

Molecular docking

The structure of the target proteins in PDB format was retrieved from a crystallographic database (www.rcsb.org/pdb/). PDB file of different enzyme structures *viz.* Human pancreatic alpha amylase (PDB ID: 1HNY), Human cytosolic beta glucosidase (PDB ID: 2JFE), Human glycogen synthase kinase-3β (PDB ID: 4ACD), Human Glucokinase (PDB ID: 1V4T), Sugar beet alpha-glucosidase (PDB ID: 3W37), were downloaded.

According to the literature (European Agency for the Evaluation of Medicinal Products) nine major secondary metabolites are found in the seed part of the *S. cumini* as shown in the Table 1. The structure of all nine compounds was mined from the PubChem database in their SDF format and considered as a ligand. These ligands then converted into Pdbqt format using Open Babel, which is a suitable file format for docking in PyRx.

All the ligands were then subjected to energy minimization followed by docking analysis using Autodock module available in the PyRx Version 0.8 software (http://pyrx.sourceforge.net/). Total 10 conformers of each ligand molecule were analyzed to predict the most favorable interaction. Finally docking interaction of the docked compounds with its protein was analyzed using LigPlot software (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

In vitro approach

Plant material and extract preparation

S. cumini seeds were collected from a region of Ahmednagar (MS), India and authenticated by the Department of Botany, Padmashri Vikhe Patil College, Pravaranagar (Loni), Tal. Rahata, District Ahmednagar, (MS), India. Seeds were ground into fine powder and were successively extracted by maceration in petroleum ether, chloroform, ethanol and aqueous (increasing order of their polarity). Solvent containing extract was evaporated to obtain dry crude extracts. Percent yield of the extract in each solvent was calculated (Okoro *et al.*, 2014).

In vitro alpha amylase inhibitory activity assay

The assay was performed according to simple modification of Narkhede *et al.*, 2011 method. A volume of 250 µl of the test samples and different concentrations of standard drug *i.e.* 100-1000 µg/ml were mixed with 250 µl of 0.20mM phosphate buffer (pH 6.9) and alpha amylase (0.5 mg/ml in 0.02M phosphate buffer, pH-6.9 with 0.006M Sodium Chloride), pre-incubated the mixture at 25°C for 10 min. 250 µl of a 1% starch

Table 2. Alpha amylase inhibition by *S. cumini* using different solvent extracts. Tests were carried out in triplicate manner and values are expressed as the mean ± SD. The IC_{50} value is the concentration of inhibitor which inhibits 50% of its activity under the assayed conditions. (SCPE-*S. cumini* petroleum ether extract, SCCE- *S. cumini* chloroform extract, SCEE- *S. cumini* ethanol extract, SCAE- *S. cumini* aqueous extract)

Sr. No.	Concentration (µg/ml)	% Inhibition				
		Acarbose	SCPE	SCCE	SCEE	SCAE
1	100	42.15 ± 0.68	15.90 ± 3.09	21.88 ±0.31	35.12 ±0.19	33.69±5.77
2	200	57.88 ± 2.42	17.03 ± 2.63	23.40 ±2.35	40.80 ±5.36	34.35±5.62
3	400	62.24 ± 0.53	19.02 ± 0.57	25.24 ±2.19	56.62 ±5.41	36.00±4.98
4	600	63.76 ± 0.96	19.64 ± 0.16	25.89 ±2.26	61.15 ±5.96	36.22±4.74
5	800	64.66 ± 1.26	20.98 ± 0.81	28.37 ±0.63	69.62 ±0.09	38.61±4.68
6	1000	65.99 ± 1.76	23.41 ± 2.10	29.85 ±0.82	73.33 ±0.16	42.90±2.49
IC_{50} values (µg /ml)		52	4497	3365	374	1932

solution (in 0.02 M sodium phosphate buffer, pH 6.9) was added to each tube containing reaction mixture and again incubated at 25°C for 10 min. The reaction was then terminated with 500 µl of DNSA (3, 5 dinitrosalicylic acid, a chromogen) and product formed were analyzed by boiling the mixture in boiling water bath for 5 min. Tubes were cooled at room temperature and diluted the solution with 5 ml of distilled water. Intensity was measured at 540nm. Control represent 100% enzyme activity, prepared using the same procedure replacing the extract with distilled water (Narkhede *et al.*, 2011).

The α-amylase inhibitory activity was calculated by using following formula and IC_{50} value was determined graphically:

$$\% \text{ Inhibition} = [(Abs_{control} - Abs_{extracts})/Abs_{control}] \times 100$$

Chromatographic separation

The presence of the bioactive compounds was determined by Thin Layer Chromatography. Chromatoplates were prepared on microscope slide by mixing silica gel and distilled water in 1:2 ratios at a uniform thickness of 0.5mm. Plates were then dried and were activated in an oven at 110^{0}C for 1hr. 10 µl of the extract was spotted on a chromatoplate, dried and allowed to develop a chromatogram with the help of an appropriate solvent shown in Table 3. Developed chromatogram was observed by using iodine vapor (Singh *et al.*, 2005). Rf value was calculated using following formula:

Rf = Distance travelled by solute (cm) /Distance travelled by solvent (cm)

Result and Discussion

In silico - molecular docking analysis

Molecular docking analysis of the secondary metabolites found in the *S. cumini* with the major enzymes those incorporate into the diabetes mellitus were performed to gain insight into the potential antidiabetic activity of these compounds. The binding mode and the favorable docking interaction of the selected secondary metabolites were analyzed based on the binding energies obtained by the docking results (Ganugapati *et al.*, 2012). Docking reveals the proper orientation of ligand–protein complex as well as various potential binding sites (Mobley *et al.*, 2009). The docking result with their binding energies is shown in the Table 1 while the Figure 1(b) and Figure 3 indicate the most favorable binding mode of the compounds. Out of nine secondary metabolites studied Ellagic acid shown higher affinity for the enzymes with much lesser binding energy , -4.73 kcal/mol for alpha amylase, -4.87 kcal/mol for beta-glucosidase, -4.79 kcal/mol for glycogen synthase kinase, -4.18 kcal/mol for glucokinase and -4.49 kcal/mol for alpha-glucosidase (Table 1). More or less ellagic acid have shown an anti-diabetic potential against all enzymes studied but comparing in between them, beta-glucosidase shows highest binding affinity. Considering this the further interaction of the enzyme is studied in order to understand the proper orientation and interaction. There are two active site residues of beta-glucosidase enzyme that plays a crucial role in the enzyme catalyzed reaction *viz*. Tyr315 and Glu440 (Badieyan *et al.*, 2012). Favorable binding of the ellagic acid to the beta-glucosidase reveals the 'novel' binding site residues that can play important role in binding and interactions of drugs with the enzyme. Some of these binding site residues such as Val68, Phe121, Phe179, Phe225, Trp345, Trp425, Asn426, Gln427 interact with hydrophobic interactions while others like Glu 165 interact with hydrogen bonding (Figure 1 (a)). Hydrogen bond distance of the Glu 165 residue is 2.84A^{0}. Considering the hydrogen bonding interaction of the other enzymes, alpha amylase forms total two

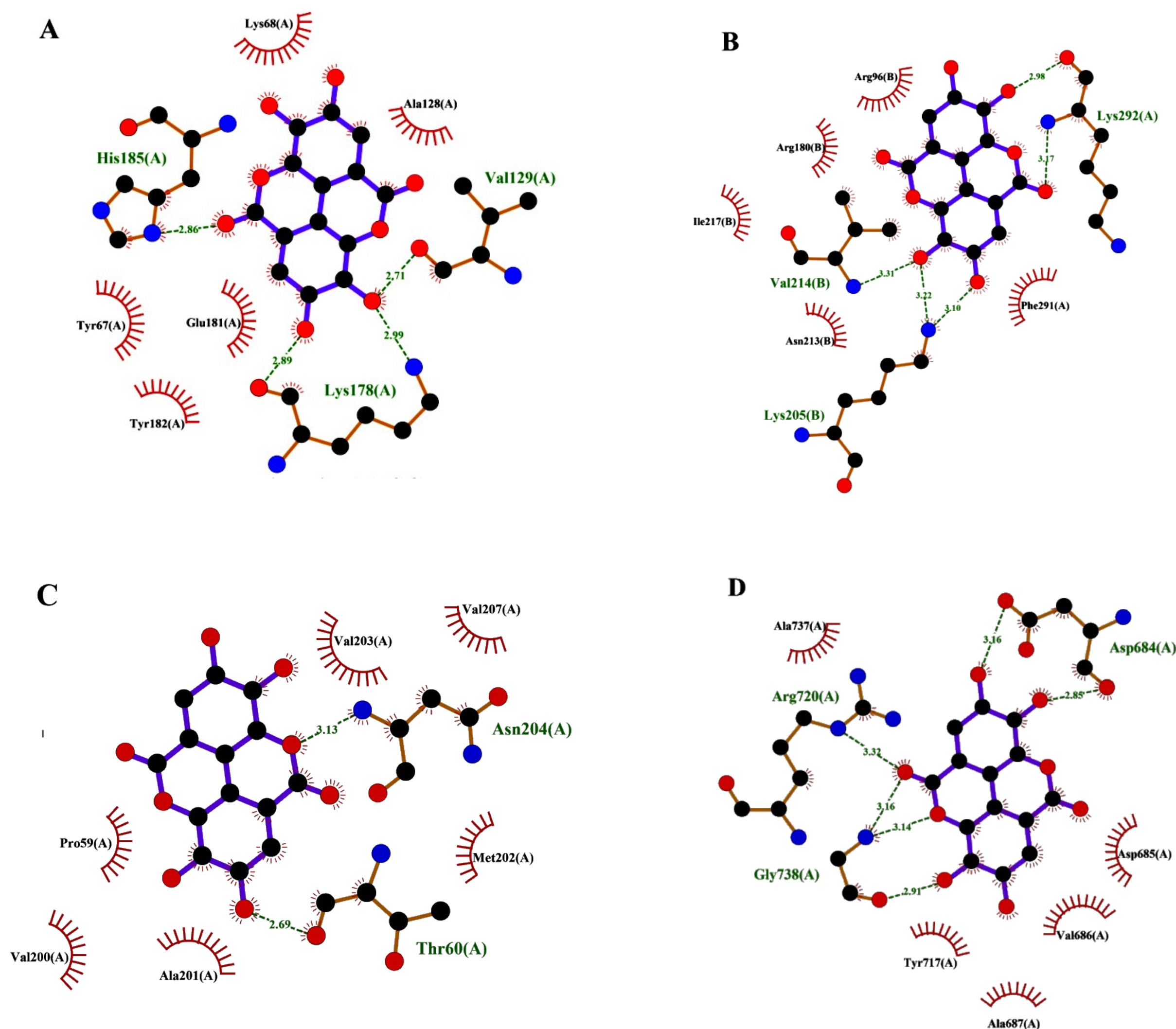

Fig 2. Hydrophobic interactions and hydrogen bonds of ellagic acid with amino acid residues of A. Alpha amylase, B. Glykogen synthase kinase, C.Glucokinase D. Alpha glucosidase. Brown colored half circle indicates the hydrophobic reactions of ellagic acid with the enzymes. Green dotted lines indicate the hydrogen bond along with their bond length.

hydrogen bonds Lys 178 and His 185 with the bond distance 2.99 A^0 and 2.86A^0 respectively (Figure 2(A)), Glycogen synthase kinase forms total four hydrogen bonds out of which two are from B chain of Lys 205 with bond distance 3.10 A^0 and 3.22 A^0 while other two are from Val 214 (chain B) and Lys 292 (chain A) with bond distance 3.31 A^0 and 3.17 A^0 respectively (Figure 2(B)). Glucokinase forms only one hydrogen bond with Asn 204 of A chain and the bond distance is 3.13 A^0 (Figure 2(C)). Finally alpha glucosidase forms three hydrogen bonds from A chain residue. Arg720 forms a hydrogen bond with bond distance 3.32 A^0 while, Gly738 with bond distance 3.14 A^0 and 3.16 A^0 (Figure 2 (D).

Out of five enzymes studied *in silico*, which showed favorable interactions with all secondary metabolites from *S. cumini*, alpha amylase inhibition is a potent weapon against the diabetes as it reduces the breakdown of complex carbohydrates, thereby lowering the levels of postprandial hyperglycemia (Alagesan *et al.*, 2012a). Therefore, we have screened the different solvent plant extracts for their significant alpha amylase inhibitory activity which can give an idea about the extract which shows the highest potential containing a bioactive substance in it specifically above said compound *i.e.* ellagic acid.

*In vitro - a*lpha amylase inhibitory activity analysis

Decreasing post- prandial blood glucose level is one of the therapeutic approaches used to treat the Diabetes mellitus (Khacheba *et al.*, 2014). This can be achieved by using some starch or carbohydrate blockers that delay the process of carbohydrate hydrolysis and absorption and thereby decreasing the postprandial increase of blood glucose after, a starch or mixed carbohydrate diet (Sales *et al.*, 2011; McEwan *et*

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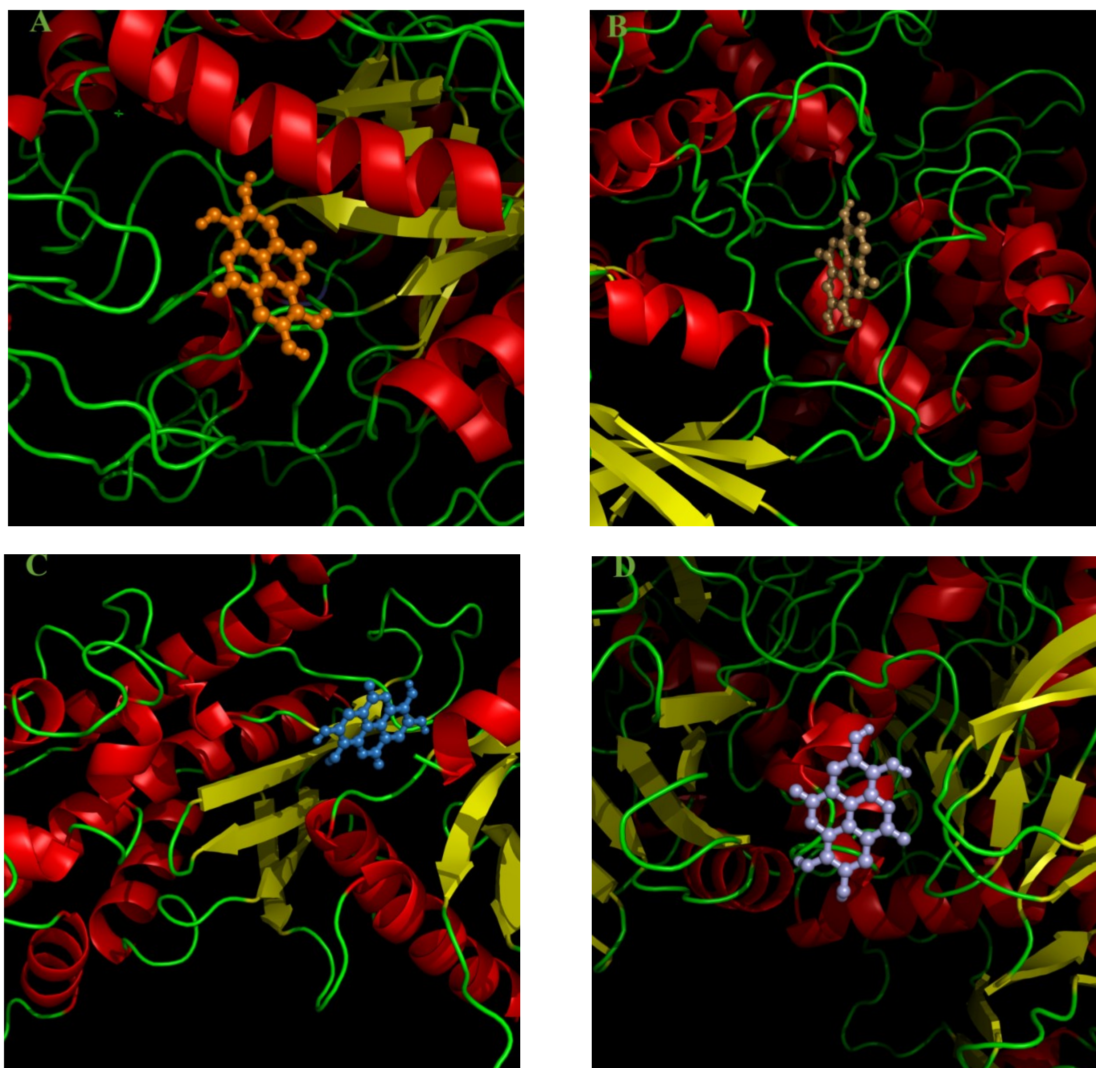

Fig 3. Best binding mode of ellagic acid with A. Alpha amylase, B. Glykogen synthase kinase, C.Glucokinase D. Alpha glucosidase.

al., 2010). In order to confirm antidiabetic effect of all nine secondary metabolites from *S. cumini* by the inhibition of alpha amylase, we studied *in vitro* the effect of these phytochemicals on the activity of alpha amylase at varying concentration of plant extracts, to identify the minimum concentration (IC_{50}) with the inhibitory ability on the enzyme. The results obtained were compared with the standard drug Acarbose as a control. Standard drug showed the highest percent inhibition 65.99 ± 1.76 at the concentration of 1mg/ml comparing with other plant extracts at the same concentration, SCPE showed the highest inhibition at 23.41 ± 2.10, SCCE showed highest percent inhibition at 29.85 ±0.82, SCEE showed highest percent inhibition at 73.33 ±0.16 while SCWE showed the highest percent inhibition at 42.90±2.49 (Table 2).

Among all extracts, ethanol extract of the *S. cumini* has highest value of percent inhibition. The peak is higher than that of the standard showing its potential in inhibiting the activity of an enzyme (Graph 1) and minimum inhibitory concentration (IC_{50}) as 374(μg /ml). The alpha amylase inhibitory activity of the plant extract is due to the presence of secondary metabolites present in it (Uddin *et al.*, 2014). Seed and leaf extract of *S. cumini* also reported to have a α-Glucosidase inhibitory activity and this activity is due to the presence of secondary metabolites apigenin 7-O-glucoside and dihydro-3,3',4',5,7 –pentahydroxyflavone glycoside respectively (Alagesan *et al.*, 2012b).

TLC profiling

TLC profiling is a crucial technique to identify active compound from a plant extracts (Simões-Pires *et al.*, 2009). With very minute analyte and short time span it provides effective separation of the active compound revealing the number of compounds present in that plant (Kamalakar *et al.*, 2014). Table 3 shows the result of TLC analysis. Different solvent system dissolves different secondary metabolites, therefore the solvent system for each extract is selected in

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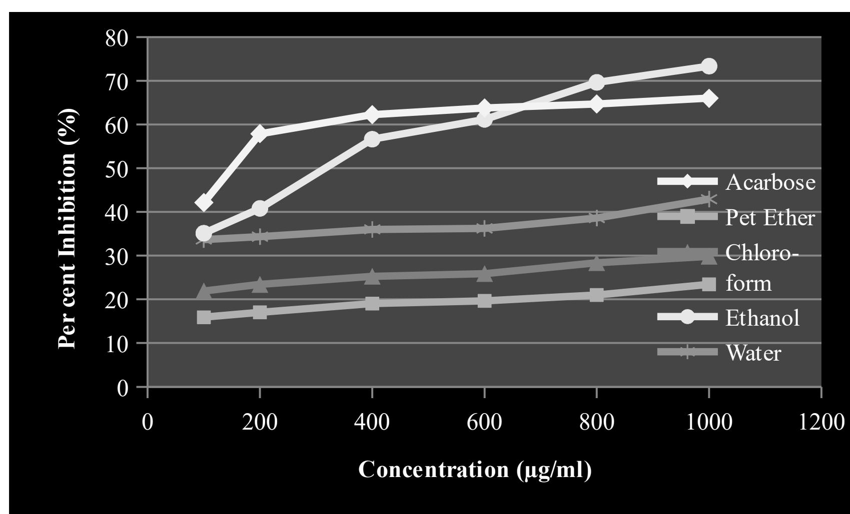

Graph 1. The enzyme inhibitory activity of different extracts of *S. cumini* seeds on α-amylase

Table 3. TLC result of different extracts of *S. cumini* visualized by iodine chamber

Sr No	Extract	Solvent system used	Rf value
1	Petroleum ether	Chloroform: ethyl acetate (3:1)	0.96, 0.58
2	Chloroform	Chloroform: ethyl acetate (4:6)	0.83, 0.63
3	Ethanol	Ethyl acetate :Methanol: Water (5:1.1:1)	0.93, 0.75, 0.46,0.39, 0.26
4	Aqueous	Toluene: Ethyl acetate (4:1)	0.85, 0.17

such a way that it might give a better separation of a particular compound. In our investigation, it is clear that in solvent system chloroform: ethyl acetate (3:1), petroleum ether extract shows two spots with Rf values 0.96, 0.58. In solvent system chloroform: ethyl acetate (4:6), chloroform extract shows two spots with Rf values 0.83, 0.63. In solvent system ethyl acetate: methanol: water (5:1.1:1), ethanol extract shows total five spots with Rf values 0.93, 0.75, 0.46, 0.39, 0.26. Finally, in solvent system toluene: ethyl acetate (4:1), aqueous extract shows two spots with Rf values 0.85, 0.17. Comparing the result, it is clear that ethanol extract contains more compounds concluding that might contain a biologically active compound which shows potent antidiabetic activity.

Conclusion

It is clear from the overall investigation that all the secondary metabolites present in the *S. cumini* shows antidiabetic potential. *In silico* analysis has contributed in finding that ellagic acid present in the seed has a great capacity to modulate the enzyme action, hence proves a biologically active compound. *In vitro* alpha amylase inhibition assay gives an idea about the extract and a required concentration (IC_{50}) that has greater capability to inhibit an enzyme action and thereby reducing the glucose level. Finally, in TLC cleared that ethanol extract contains various compounds, one of which possessing potent antidiabetic activity. But still further investigation is required to confirm the bioactive property of ellagic acid by purifying it from the extract and subjecting to the *in vitro* alpha amylase inhibitory analysis.

Competing Interest

The authors declare that they have no competing interests.

Authors' contributions

All authors have contributed significantly. They have performed the laboratory works and prepared the manuscript.

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