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Exploiting the therapeutic potential of secondary metabolites from *Salvadora persica* for diabetes using *in silico* and *in vitro* approach

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ABSTRACT

Diabetes is the fifth leading cause of death in most developed countries. In spite of this, the drugs available in the market for treatment of diabetes are more expensive with side effect. Therefore, there is a high demand of cost-effective novel natural antidiabetic drug without any side effect. In our investigation, we studied *in silico* the mechanism of secondary metabolites isolated from *S. persica* which modify the enzyme action, their *in vitro* inhibitory activity of alpha amylase and the phyto-constituent analysis those are responsible for its activity. *In silico* study revealed that kaempferol from *S. persica* is a most effective metabolite which can bind, interact and modulate the activity of all enzymes with much higher binding affinity and lesser binding energy such as -4.58 kcal/mol for alpha amylase, -4.76 kcal/mol for beta glucosidase, -4.97 kcal/mol for glycogen synthase kinase, -4.3kcal/mol for glucokinase and -3.85 kcal/mol for alpha glucosidase. Aqueous extract of *S. persica* has a high ability with 72.39% inhibition value to inhibit the activity of alpha amylase and 376µg /ml IC_{50} value in comparison with standard drug which show 65.99% inhibition value. Total 15 compounds have been detected in TLC analysis in different solvent system and different extracts. Kaempferol might be one of them, which attribute to the anti-diabetic property. Further purification and characterization of kaempferol is required, which may prove a probable anti-diabetic drug

KEYWORDS Diabetes mellitus, Molecular docking, Alpha amylase, Kaempferol, *S. persica*

INTRODUCTION

Diabetes is a metabolic disease which disturbs not only glucose homeostasis but also carbohydrates and lipid metabolism. It is a life threatening disease in the today's era and considered as a 5th leading cause of death in most developed countries (Perera *et al.*, 2014). This can cause badly short-term and long-term consequences, including peripheral and vascular disease, ischemic heart diseases, end stage renal disease, neuropathy, lower extremity amputation and blindness. It also increases the risk of stroke and heart failure (Hazarika *et al.*, 2012; Nguyen *et al., 2012*). Diabetes mellitus is characterized by high blood glucose level either due to failure of insulin secretion by β pancreatic cell or cellular resistance of insulin uptake (Channabasava *et al.*,2014).Treatment of the type I diabetes is limited to supplementation of insulin while treatment of type II diabetes can be done by using various oral glucose lowering drugs (Funke *et al.*, 2006). Though various glucose metabolizing drugs with different mechanism of action are available in market, finding a 'novel natural antidiabetic drug' with high potential of preventive action, cost effective and low side effect is still challenging (Castellano *et al.*,2013).A drug is a molecule which when bind and interact with the active site region and thereby alters (either activate or inhibit) the action of key molecule of the pathway that is specific for a disease condition to stop the functioning however, not affecting a function of other important analog molecule. Drug designing is to design such drug candidate either artificially or from natural products like plant secondary metabolite (Ramanathan *et al.*,2010).Prior to *in vitro* approach, *in silico* docking studies supporting structure based virtual screening is necessary which screens the interaction between chemical compounds with their biological target molecules and help identifying the desired activity from a huge database of chemical compounds and reduce the time as well as cost of identifying chemical

hits. That means work flow of drug designing involves from *in silico* to *in vitro* approach and lastly if drug found suitable then *in vivo* and its clinical trials (Hyeon *et al.*,2015)

Salvadora persica L.also known as a 'toothbrush tree' is wildly used as an oral hygiene tool all over the world especially in a Muslim community including African and Arab countries (Nordin *et al.*,2012; Haque *et al.,* 2015). It is also known as a 'miswak', a word originated from Arab meaning tooth-cleaning stick (Halawany *et al.*,2012).It help preventing the tooth decay. *S. persica* belongs to family Salvadoraceae. It is an evergreen shrub approximately 4-6 m in height. The name *Salvadora* was given in honor of the apothecary of Barcelona, Juan Salvadory Bosca while the term *perscia* derived from word Persia. *L*.is the standard author abbreviation indicating Carl Linnaeus (Ahmad *et al.*,2013).Plant contains different biologically active compounds such as alkaloids, flavonoids, tannins, saponins, pyrrolidine, glycosides(Ibrahim *et al.*,2015) minerals like calcium, phosphorous and fluorides (Abdallah *et al.*,2015) essential oil such as 1,8-cineole (eucalyptol), α-caryophellene, β-pinene, and 9-epi-(E)-caryophellene(Akhtar *et al.*,2011) as well as salvadoricine, an indole alkaloid also found in leaves of the *S. persica*(Gupta *et al.*,2015).

S. persica possess various biological activities such as antioxidant activity (Mohamed *et al.*,2013), antifungal activity (Noumi *et al.*,2010), antibacterial activity (Al-Ayed *et al.*,2016), antimicrobial activity (Al-Bayati *et al.*,2008), anti-inflammatory activity (Ibrahim *et al.*,2011), analgesic activity (Sulaiman *et al.*,1996), anti-hyperlipidemic and anti-tumor (Iyer *et al.*,2012), anti-biofilm activity (Al-Sohaibani *et al.*,2012) etc. Miswak is not only well known for its use as mouth wash and toothpastes but also for its use as endodontic irrigation solution in root canal irrigation. It can effectively remove the daily bacterial plaque accumulated on the surface of teeth. Roots of the S. persica are used as a bio-preservative in the process of food preservation (Niazi *et al.,* 2016). Roots of *S. persica* also act as a bioreductant and help reduce graphene oxide (GRO) (Khan *et al.,* 2015). All the biological activities of *Salvadora persica* have found without side effects. Hence it is expected that in the herbal management of diabetes mellitus it would prove as a cost effective drug without any side effect.

Considering these characteristics we investigated the secondary metabolites of *S. persica* for its anti-diabetic potential applying both *in silico* and *in vitro* approach.

MATERIALS AND METHODS

In-silico Approach (Molecular Docking)

Selected proteins for docking study were nothing but the key regulatory enzymes those are potential target in the development of lead compound for the treatment of diabetes mellitus such as human pancreatic alpha amylase[PDB ID 1HNY] (Oboh *et al.*,2012), human cytosolic beta glucosidase [PDB ID: 1JFE] (Pandey *et al.*,2013),Human glycogen synthase kinase-3β [PDB ID: 4ACD] (Cohen *et al.*,2004), Human Glucokinase [PDB ID: 1V4T] (Matschinsky *et al.*,2010), Sugar beet alpha-glucosidase [PDB ID: 3W37] (van de Laar, 2008). All structures were retrieved from the protein data bank (PDB) (http://www.rcsb.org/pdb/).

As per literature survey total 14 secondary metabolites (Table1) were collected those are reported to be isolated from S. persica [Halawany *et al.*,2012; Tambe *et al.*,2010; Arora *et al.*,2011]. All the compounds were downloaded in SDF file format from PubChem database and converted to .pdbqt file, required ligand file format using Open Babel software.

Energy minimization of all fourteen ligands along with standard Acarbose were carried out and then subjected to docking analysis using Auto dock module available in PyRx Version 0.8 software (http://pyrx.sourceforge.net/) (Balajee *et al.*,2011). For each ligand molecule total 10 conformers were studied for their all possible interaction with target proteins. Finally, these interactions were studied using a freeware LigPlot (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

In vitro approach

Plant Material and Extract Preparation

Slavadora persica leaves were obtained from a local area of India and authenticated by the, Department of Botany, Padmashri Vikhe Patil College, Pravaranagar (Loni), Tal.Rahata, District Ahmednagar, (MS), India. The leaves were washed, dried and ground into fine powder and then subjected to successive extraction by maceration in petroleum ether, chloroform, ethanol and aqueous (increasing order

of their polarity). The extract was concentrated by evaporation. Percent yield of the extract in each solvent was calculated (data not given) (Agrawal *et al.*, 2012).

***In-vitro* Alpha amylase inhibitory activity assay**

The assay was done according to Narkhede *et al*. 2011 with simple modification.

Different concentrations of standards were prepared from 100-1000µg/ml. Both sample and standards were taken in 250 µl of volume and 250µl of 0.20 mM phosphate buffer (pH 6.9) containing α-amylase (0.5mg/ml in 0.02M phosphate buffer, pH-6.9 with 0.006M Sodium Chloride) solution was added to this mixture. The mixture was pre-incubated at 25°C for 10 min and 250 µl of a 1% starch solution (in 0.02 M sodium phosphate buffer, pH 6.9) was added to each tube after incubation. Again the mixture was incubated at 25°C for 10 min and the reaction was terminated with 500 ml of DNSA (3, 5 dinitrosalicylic acid, a chromogen). The test tubes were kept in a boiling water bath for 5 min and allowed to cool at room temperature. The reaction solution was then diluted with 5 ml distilled water. Absorbance of the reaction mixture was recorded at 540 nm. Control indicates 100% enzyme activity and 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

S. persica plant extracts were subjected to Thin Layer Chromatography to assess the bioactive compound present. Chromatoplates were prepared by applying the slurry (silica gel and distilled water in 1:2 ratios) on a microscope slide at a uniform thickness of 0.5mm and allowed to dry. The plates were then activated in an oven at 110^0C, 1hr. 10 µl of extract was taken and spotted on a chromatoplate, dried and allowed to develop a chromatogram using an appropriate solvent shown in Table 3. Developed chromatogram was analyzed by exposure of the plates to 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

Molecular docking analysis

The main objective of this work was to test the bioactive compound from the natural products which can get docked with the targeted regulatory enzymes used for the treatment of diabetes. Total 14 secondary metabolites of *S. persica* were obtained from literature. Docking analysis was based on the binding energy and was tabulated in table 1. The lesser binding energy of all five docked enzymes varies in between -4.97kcal/mol to -3.85 kcal/mol (Table 1).The more negative the docking/binding energy is, the more favorable the interaction and the more potential the drug is. Therefore, molecular docking studies are significant in finding the stable complex within the active binding site (Singh *et al.*,2015). Our study revealed that, out of 14 secondary metabolites selected, kaempferol showed the more favorable interaction and hence high binding affinity for all target proteins with the lesser binding energy of -4.58 kcal/mol for alpha amylase, -4.76 kcal/mol for beta glucosidase, -4.97 kcal/mol for glycogen synthase kinase, -4.3kcal/mol for glucokinase and -3.85 kcal/mol for alpha glucosidase (Table 1). Best binding poses of all theses enzymes are shown in Figure1b and Figure 2. The kaempferol is a member of flavonol and is considered as a dietary flavonoid as it constitutes a significant part of the human diet (Chen *et al.*, 2013). It found to have more favorable interaction with all key regulatory enzyme but among them glycogen synthase kinase -3β showed higher affinity than others (Table 1). Docking analysis also gives an idea about non-covalent interactions between the ligand-protein complex like hydrophobic interaction and hydrogen bonding (Gholivand *et al.*, 2013). Non-covalent interactions are the chemical interaction between ligand and target proteins without a significant share of the electrons between them. It allows the molecule to recognize themselves, approach towards each other and pack together (Saleh *et al.*,2012).Our drug molecule interacted well with targets by non-covalent interactions (Figure 1a and Figure 3). Firstly, glycogen synthase kinase -3β, the enzyme which binds with more affinity with kaempferol interact non-covalently with formation of three hydrogen bonds, first at A chain of Lys292 with a bond distance of $2.79A^0$ while other, two hydrogen bonds are formed at B chain of Lys205 with bond distance $2.89A^0$ and $2.99A^0$ (Figure1a). Residues involved in the hydrophobic interaction between active binding site of glycogen synthase kinase -3β and kaempferol are Phe93, Arg96, Arg180,

Asn213, Val214, Glu290, Phe29 (Figure1a). Hydrogen bonding between alpha amylase and kaempferol includes in between A chain of Leu69 and His185 with bond distance 3.26 A^0 and 3.04 A^0 respectively (Figure 3A). Amino acid residue involved in hydrogen bond formation of kaempferol and beta-glucosidase are Asp50, Val51 and Arg432 with bond distance 3.02 A^0, 2.84 A^0 and 3.30 A^0 respectively (Figure 3B). Glucokinase form only one hydrogen bond with kaempferol at Lys458 on A chain and bond distance is 3.13 A^0 (Figure 3C).

Finally, considering the last enzyme in the docking analysis, alpha glucosidase involve in the interaction by forming a hydrophobic interaction with kaempferol but no formation of hydrogen bonds is found. Total eight amino acid residues involved in hydrogen bond formation, including Arg647, Thr648, Gly651, Leu652, Leu726, Phe727, Asn728, Tyr729 (Figure 3D). In brief, it depicts that kaempferol can also act as a drug that can alter the activity of all five enzymes studied potentially.

Table 1: Binding energies of revealed during docking analysis of the compounds with their target key enzymes

Sr. No.	Compounds	Alpha-amylase	Beta-glucosidase	Glycogen synthase kinase-3β	Glucokinase	Alpha – glucosidase
1	Trimethylamine	-2.82	-3.67	-2.25	-3.18	-3.71
2	Quercetin Glucoside	-1.67	-1.8	-2.71	-1.67	-1.66
3	Kaempferol-3-0-rutinoside	-1.5	-0.38	-1.49	-0.99	-0.84
4	Liriodendrin	-0.17	1.01	-0.1	-0.15	0.68
5	Qercetin	-3.79	-4.17	-4.29	-3.27	-3.13
6	Mauritianin	0.28	-0.43	-0.45	0.83	1.08
7	**Kaempferol**	**-4.58**	**-4.76**	**-4.97**	**-4.3**	**-3.85**
8	Rutin	-2.17	-1.24	-1.24	-0.87	-1.05
9	Astragalin	-2.89	-2.58	-3.22	-1.11	-1.26
10	Syringin	-1.69	-2.69	-2.36	-0.88	-1.12
11	Quercetrin	-3.32	-2.7	-3.22	-2.34	-3.37
12	Narcissin	-1.3	-0.37	-0.96	-0.57	-0.3
13	Sisterol-3-0-glucopyranoside	-3.56	-2.19	-3.01	-3.6	0.12
14	Glucotropaeolin	-1.65	-1.33	-1.96	-1.87	-1.18
15	Acarbose	-0.46	-2.72	-2.66	-1.87	-2.05

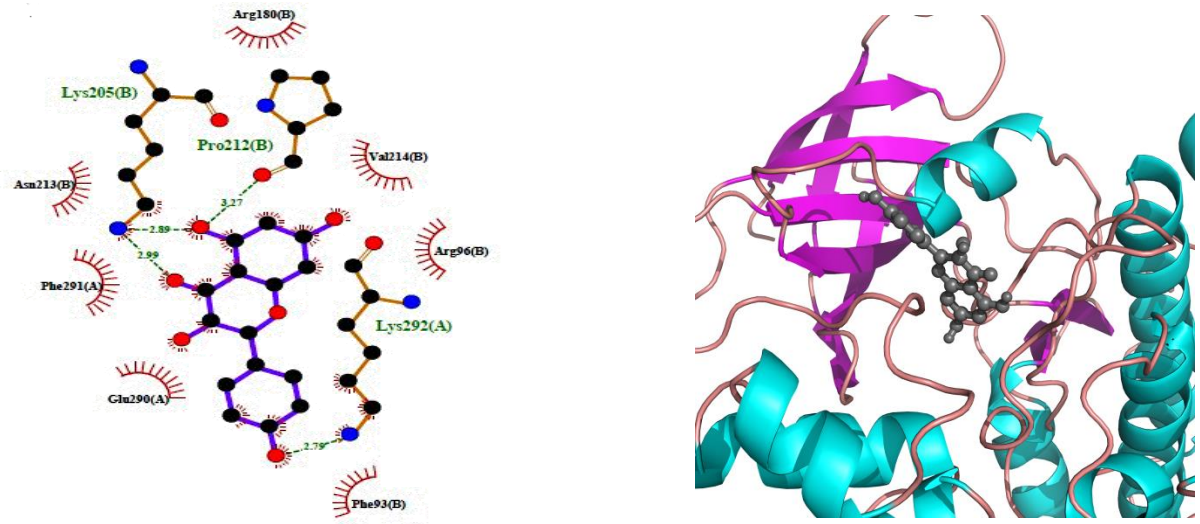
***In vitro* alpha amylase inhibitory activity assay**

Elevated postprandial blood glucose level, is the level of glucose measured two hours after a meal and is an important early stage indicator of failure of glucose regulation. Postprandial hyperglycemia contributes to the development of insulin resistance (Kellogg *et al.*,2014).Hydrolysis of starch is the main source of glucose that help elevate the blood glucose level, hence inhibition of the carbolytic enzymes is the targeted therapy for management of diabetes (Tamil *et al.*,2011). Alpha amylase inhibitors also known as starch blockers prevent the starch from digestion and enhance the digestion time of carbohydrates and reduce the glucose absorption rate (Oboh *et al.*, 2013). Alpha amylase inhibitors are used as an oral antidiabetic drug (Jhong *et al.*2015).Aiming to find the more potent inhibitor we have investigated the different extracts of *S. persica* for its potential to alpha amylase inhibition. Table 2 shows the result obtained in comparison with standard drug Acarbose as a control. It is clear from the data that at 1mg/ml concentration standard drug Acarbose showed 65.99 % inhibition value. SPPE showed activity with 46.73% alpha amylase inhibition, SPCE showed activity with 39.56% and SPEE showed activity with 41.35% alpha amylase inhibition. SPWE showed 72.39% alpha amylase inhibitory activity with IC_{50} value 376µg /ml. Comparing with the standard drug our plant extract showed appreciable alpha amylase inhibitory effect at the concentration of 1mg/ml. This may be due to the presence of secondary metabolites (listed Table 1) present (Sumathy *et al.*, 2013). Graph 1 shows the highest pick of amylase inhibitory activity for aqueous extract. It can be predicted that from the above *in silico* study kaempferol acts as a bioactive substance.

Thin Layer Chromatography Analysis

Thin layer chromatography is an important tool for analysis of a small amount of bioactive compound from a natural mixture. It is an effective mean of purification of secondary metabolites (Jóźwiak *et al.*, 2007).The objective of the TLC was to find out the principle compounds present in the mixture of different solvent extracts. Different solvent used for better resolution for different extract were shown in the Table 3. All four extract gave impressive results indicating the number of phytochemicals present in the extracts. Rf value

gives information about the polarity of the compounds. Chromatogram observed in solvent system chloroform: ethyl acetate (3:1), petroleum ether extracts showed total five spots indicating the presence of five compounds.



(a) **(b)**

Figure 1: (a) Hydrophobic and hydrophilic interactions of kaempferol with Glycogen synthase kinase-3β residues (GSK). Brown colored half circle indicates the hydrophobic reactions of kaempferol with the enzymes GSK. Green dotted lines indicate the hydrogen bond and green colored value indicates their bond length. (b) Promising binding mode of kaempferol with GSK.

The five compounds are lipophilic in nature as lipids compounds dissolve into petroleum ether. The chloroform extract showed four spots in the solvent system chloroform: ethyl acetate (4:6) while ethanol extract showed five spots in the solvent system ethyl acetate: methanol: Water (5:1.1:1). Finally aqueous extract shows only one spot in the solvent system toluene: Ethyl acetate (4:1). In vitro study cleared that the aqueous extract has the highest potential in inhibiting alpha amylase activity which reveals that the single spot observed in the TLC may be of a bioactive compound. Further identification and characterization is needed for this spot which can give an idea about the best drug for antidiabetic activity.

CONCLUSION

All the metabolites studied from *S. persica* were found to have effective anti-diabetic activity. From *in silico* study conclusion is drawn that kaempferol, a dietary flavonoid found to be a more efficient metabolite in alteration of the activity of regulatory enzyme in the diabetes management. *In vitro* study revealed that the aqueous extract has a greater ability to inhibit alpha amylase enzyme and thereby reducing the blood glucose level. There are different compounds present in the plant which shows this activity and they can be separated on the basis of their polarity. Further investigation is necessary for the purification of bioactive compound (kaempferol) and its characterization.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

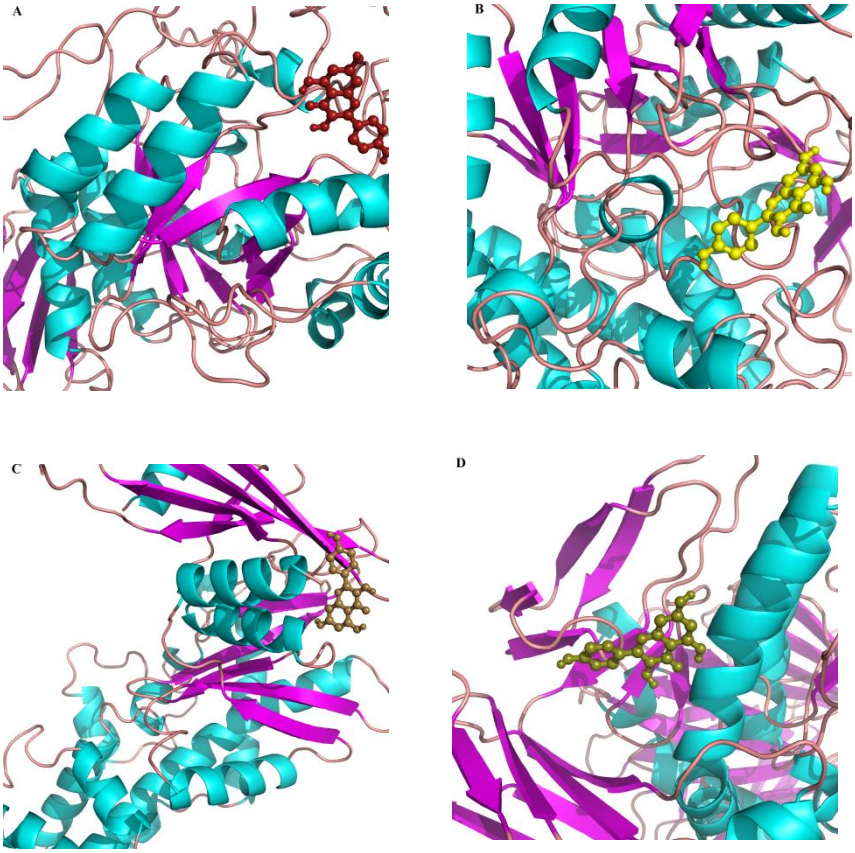


Figure 2: Promising binding modes of kaempferol with A. Alpha amylase, B.Beta glucosidase,C.Glucokinase D. Alpha glucosidase

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

Sr. No.	Concentration (µg/ml)	% Inhibition				
		Acarbose	SPPE	SPCE	SPEE	SPAE
1	100	42.15 ± 0.68	34.12 ±0.19	29.85±0.11	35.31±0.56	45.95±0.79
2	200	57.88 ± 2.42	34.28 ±0.06	30.74±0.06	35.68±0.79	44.85±0.95
3	400	62.24 ± 0.53	37.23 ±0.71	32.56±0.71	37.07±0.33	48.58±1.83
4	600	63.76 ± 0.96	41.79 ±2.49	38.70±5.91	38.65±2.43	54.26±3.44
5	800	64.66 ± 1.26	44.72 ±1.70	39.37±6.23	40.93±3.00	57 ±2.12
6	1000	65.99 ± 1.76	46.73 ±1.10	39.56±0.03	41.35±2.95	72.39±4.53
IC_{50} values (µg /ml)		52	1183	1715	2141	376

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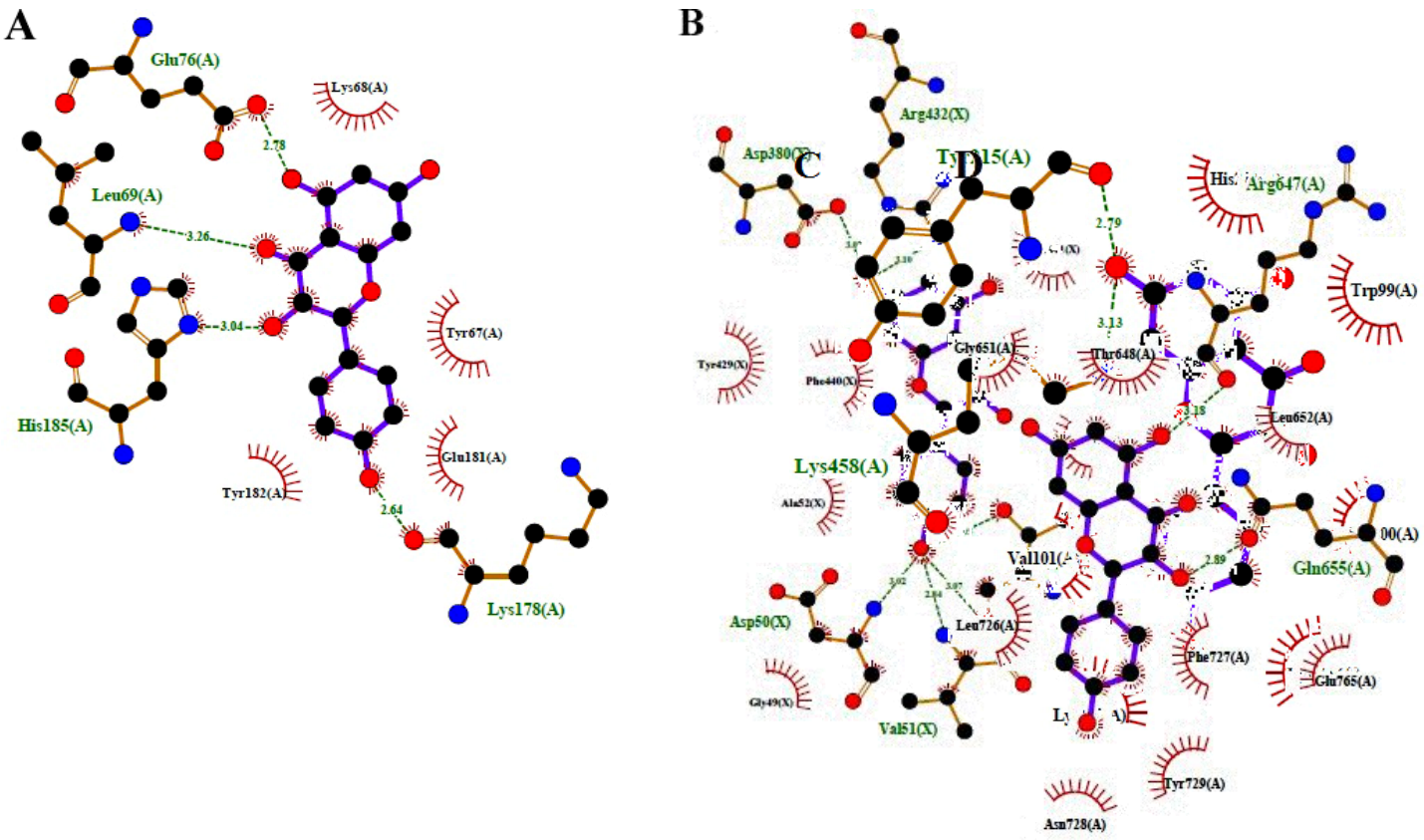
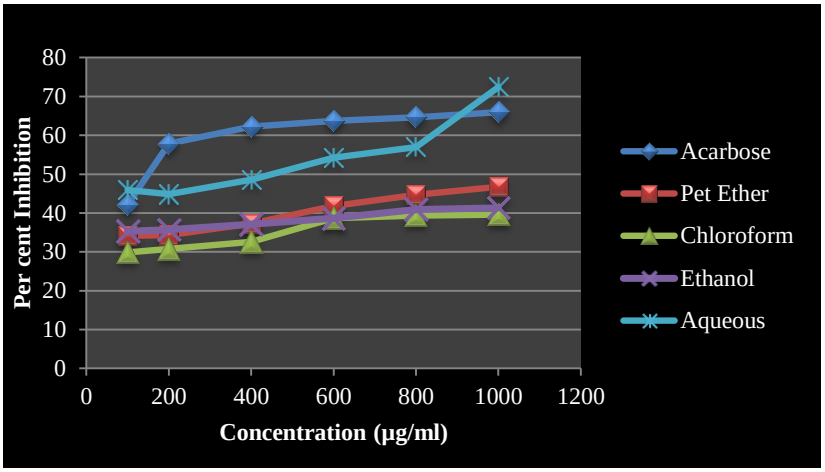


Figure 3: Hydrophobic interactions and hydrogen bonds of kaempferol with amino acid residues of A. Alpha amylase, B. Beta glucosidase C.Glucokinase D. Alpha glucosidase. Brown colored half circle indicates the hydrophobic reactions of kaempferol with the target enzymes. Green dotted lines indicate the hydrogen bond along with their bond length (Values o\in green color).



Graph 1: The enzyme inhibitory activity of different extracts of *S. persica* leaf extract on α-amylase

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

Sr No	Extract	Solvent system used	Rf value
1	Petroleum ether	Chloroform: ethyl acetate (3:1)	0.85, 0.57, 0.46, 0.20, 0.14
2	Chloroform	Chloroform: ethyl acetate (4:6)	0.95, 0.88, 0.68, 0.17
3	Ethanol	Ethyl acetate :Methanol: Water (5:1.1:1)	0.94, 0.81, 0.70, 0.51, 0.24
4	Aqueous	Toluene: Ethyl acetate (4:1)	0.12

AUTHORS CONTRIBUTIONS

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

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