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RESEARCH ARTICLE

Strategies in the design of antidiabetic drugs from *Terminalia chebula* using *in silico* and *in vitro* approach

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ABSTRACT

Diabetes mellitus is the fifth deadliest disease in the developing countries. Even with all the research and new drugs available, combating diabetes is still challenging. There are successes in finding new cost effective drugs without side effects, even if not perfect. In our investigation, we studied the *in silico* binding mechanism of secondary metabolites of *Terminalia chebula,* with the key enzymes used in the diabetes management. It was analyzed that three compounds out of 16 have a higher binding affinity for the target proteins/enzymes. Ellagic acid showed highest binding affinity with alpha amylase, beta glucosidase and alpha glucosidase with lesser binding energies -4.5 kcal/mol, -5.36 kcal/mol and -4.48 kcal/mol respectively. Arjungenin has lesser binding energy of 4.77 kcal/mol with glucokinase while luteolin has a binding energy of -7.25 kcal/mol for enzyme glycogen synthase kinase. These entire compounds interacted with non-covalent interaction. *In vitro* antidiabetic studies revealed that the petroleum ether extract has the significant alpha amylase inhibitory activity, i.e. 51.22% as compared to standard drug (65.99%). Further, TLC analysis revealed the presence of total 9 compounds in different plant extracts one of them might be a lead compound which could be further exploited for the development of novel, safer and potent antidiabetic drug.

Keywords: Diabetes mellitus; Molecular docking; Thin Layer Chromatography; Alpha amylase; Ellagic acid.

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INTRODUCTION

Diabetes mellitus is one of the major global health crises and is the fifth leading cause of the death [1]. It is associated with severe pathological imbalances, including long-term damage, dysfunction and failure in many organs [2, 3]. The prevalence of type II diabetes (T2D) is higher (90%) than that of the type I diabetes (T1D) mellitus [4]. In spite of huge number of data available regarding the development and research on the antidiabetic drugs, search of more safer natural antidiabetic drug still continued [5]. Herbal drugs play a crucial role and are acceptable in the management of the T2D due to their less adverse effects and cost

effectiveness but sill lot much efforts are needed to dig out an efficient drug from these natural sources [6, 7]. 70-95% of the population of developing countries, including India make use of herbal drugs as an effective antidiabetic agent. The reason behind that, these natural agents are easily available, it has very low costs and the most important unavailability of conventional antidiabetic drugs [8]. Herbal drugs are needed to be standardized, ensured for its quality, safety and reproducibility [9].

Terminalia chebula Retz. is popularly called the 'King of Medicine' as it has a remarkable property of healing every kind of disease, including various asthma, candidiasis, diarrhea, vomiting, hiccough, bloody stools, dysentery, ulcer, gout ,wound infection, urinary tract infection, gastroenteritis, skin diseases, etc. [10, 11]. It is a native plant of India and near about 250 species of *T. chebula* are spread all over a world especially in a tropical area [12]. *T. chebula* is a rich source of tannins and other secondary metabolites like tannic acid, gallic acid, chebulinc acid, chebulic acid, chebulagic acid, corrilagin, and triterpenoids [13, 14]. The plant have demonstrated antioxidant and free radical scavenging activity [15], cytoprotective activity [16], anti-carcinogenic activity [17, 18], anticonvulsant activity [19], anti-mutagenic activity [20] anti-ulcerogenic activity [21], anti-tussive activity [22] and anti-caries activity [23]. Herbal formulation known as a 'Triphala', recommended by the Indian System of Medicine (ISM), contains the powder of fruit rind of *T. chebula* as one of the ingredients. Triphala is effective in the treatment of diseases of the mouth, including dental caries, gingivitis, stomatitis, spongy and bleeding gums, etc. [24]. It possesses hepatoprotective property [25] and also used in the treatment of cancer [26].

Based on the information regarding the usefulness of *T. chebula* in medicine without any harm, we hypothesized that one or more of its phyto-constituents may be useful in the diabetes to develop an effective antidiabetic drug. We have investigated the plant secondary metabolites for their *in silico* and *in vitro* antidiabetic effect. For this purpose, we have targeted some of the key enzymes used in the management of diabetes [27-31] and analyzed that up to what extent our secondary metabolites are effective in modulating the activity of these enzymes.

MATERIALS AND METHODS

In-silico approach - molecular docking

The 3-D crystal structure of the all five enzymes (Alpha amylase-PDB ID: 1HNY, beta glucosidase-PDB ID: 2JFE, glycogen synthase kinase-3β-PDB ID: 4ACD, glucokinase-PDB ID: 1V4T and alpha-glucosidase-PDB ID: 3W37) which can be regulated to cure diabetes, retrieved from the protein data bank (http://www.rcsb.org/pdb/). Total sixteen phytochemicals were screened against these target proteins.

With the help of literature survey of *T. chebula*, 16 phytochemicals (shown in Table 1) were found out for docking analysis [23, 32-35]. Structures of 16 compounds were obtained in .sdf file format from PubChem database and was converted to .pdbqt file which is required ligand file format with the aid of Open Babel software. Energy minimization of the ligands was done and carried forward for further docking analysis with Auto dock module available in PyRx Version 0.8 software (http://pyrx.sourceforge.net/) and the results were analyzed with the help of LigPlot (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/)[36].

In vitro approach

Plant material and extract preparation

Seeds of *T. chebula* were purchased from local market of Ahmednagar, (MS), India and authenticated by the, Department of Botany, Padmashri Vikhe Patil College, Pravaranagar (Loni), Tal. Rahata, District: Ahmednagar, (MS), India. Then the seeds were crushed manually to a powder with a mortar and pestle at room temperature in the absence of sunlight. Powdered material was subjected to successive extraction by maceration in petroleum ether, chloroform, ethanol and water (increasing order of their polarity). The extract was then filtered and evaporated to obtain concentrate [37].

In-vitro alpha amylase inhibitory activity assay

The assay was carried out according to published protocol [38, 39]. The total reaction mixture containing 250 µl of 0.02M sodium phosphate buffer, 250 µl of enzyme (α-amylase, 0.5mg/ml in 0.02M sodium phosphate buffer, pH 6.9 with 0.006M sodium chloride) and the plant extracts at a different concentration of 100-1000 µg/ml, were incubated at 25^0C for 10 min followed by the addition of 250 µl of 1% starch. After 10 min incubation at 25^0C, the reaction was terminated with addition of 500 µl of di-nitro salicylic acid color reagent and all tubes were then placed in boiling water bath for 5 min, cooled to room temperature, diluted up to 5 ml with distilled water and the absorbance was measured at 540 nm). The control samples were without any plant extracts showing 100% enzyme activity.

The % inhibition of the assay was calculated using the formula:

% Inhibition = [(Abs control-Abs extracts)/Abs control] x 100

Chromatographic separation

Chromatoplates were prepared by silica gel G coating in 1:2 ratios with distilled water. Thickness of the coat was ≈0.5 mm. Plates were allowed to dry at room temperature and activated in an oven at 110^0C for 1 hr. 10 µl of the extract was spotted and kept in a developing chamber containing appropriate solvent (Table 3). After development, plate were brought out, allowed to dry and analyzed by exposure to iodine vapor [40]. Rf value was calculated by using the following formula:

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

RESULT AND DISCUSSION

Molecular docking analysis

The workflow was directing towards the screening of bioactive compound from 16 secondary metabolites obtained from the *T. chebula*, which would prove a potent antidiabetic agent by getting docked with the said target enzymes and interacting favorably with them. Molecular docking is the computational method for structure-based drug designing which gives an idea about the proper and stable conformation of ligand and target protein and also tells about suitable protein ligand interactions [41]. The binding energy is nothing but the binding strength of the ligand which not only help predicting the stable conformation of ligand-protein complex but also optimize the newly formed bonds [42]. Docking result based on their binding energies is shown in the Table 1. Analysis of docking results shows binding energies in a range of -4.48 Kcal/mol to -7.25kcal/mol. Three secondary metabolites found to have best docking interaction with the target proteins. Ellagic acid showed highest binding affinity with alpha amylase, beta glucosidase and alpha glucosidase with binding energies -4.5 kcal/mol, -5.36 kcal/mol and -4.48 kcal/mol respectively. Arjungenin has the lesser binding energy of -4.77 kcal/mol with glucokinase. Among all these metabolites luteolin has highest binding affinity with the much lesser binding energy of -7.25 kcal/mol for enzyme glycogen synthase kinase-3β (GSK). Promising binding poses of all theses enzymes are shown in Figure1b and Figure 3. The non-covalent interactions are conventional methods and are proven to be effective in prediction of different binding modes of protein ligand complexes [41]. Non-covalent interactions include hydrophobic interactions, hydrogen bonding, van der waals interaction and the electrostatics interaction [43]. The residues take part in non-covalent interactions of luteolin with GSK via hydrogen bonding and hydrophobic interactions are Ser215, Tyr216, Arg220, Ile228, Arg223, Asp260, Ser261, Gly262 and Gln 265.Out of these only two residues of B chain Ser215 and Ile228 are involved in hydrogen bonding with the bond distance 2.94 A^0 and Ile225 A^0 respectively while others interact with hydrophobic interactions (Figure1a). Ellagic acid forms two hydrogen bonds with a single amino acid residue, Lys227 of A chain of alpha amylase (Figure 2A).

Table 1. Binding energies of *T chebula* 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-glucosidases
1	Ethylgallate	-2.9	-3.09	-5.42	-2.28	-2.43
2	Arjungenin	-4.38	-4.42	-0.15	**-4.77**	-4.45
3	Castalagin	-2.85	-3.28	7000	-2.29	-2.43
4	Terflavin A	1.4	0.36	3500	1.19	-0.12
5	Chebullagic acid	-1.2	-2.21	1630	-0.92	-1.14
6	Gallic acid	-2.57	-2.43	-5.31	-3.49	-2.76
7	ChebulosideII	-4.19	-2.58	99.63	-1.54	-2.09
8	Terchebin	1.3	0.29	1430	0.62	0.04
9	Punicalagin	-2.81	-3.14	7680	-2.19	-2.38
10	Arjunglucoside1	-3.15	-3.37	449.91	-1.87	-3.06
11	Luteolin	-4.31	-4.53	**-7.25**	-3.91	-3.44
12	Chebulinic acid	1.63	2.4	1730	1.3	2.27
13	Ellagic acid	**-4.5**	**-5.36**	-6.98	-3.91	**-4.48**
14	Quercetin	-0.94	-0.12	3.56	-0.55	-0.8
15	Chebulic acid	-2.38	-2.54	-5.56	-1.26	-1.95
16	Flavogallonic Acid	-1.42	-1.91	-2.53	-1.03	-2.2
17	Acarbose	-0.46	2.72	2.66	1.87	2.05

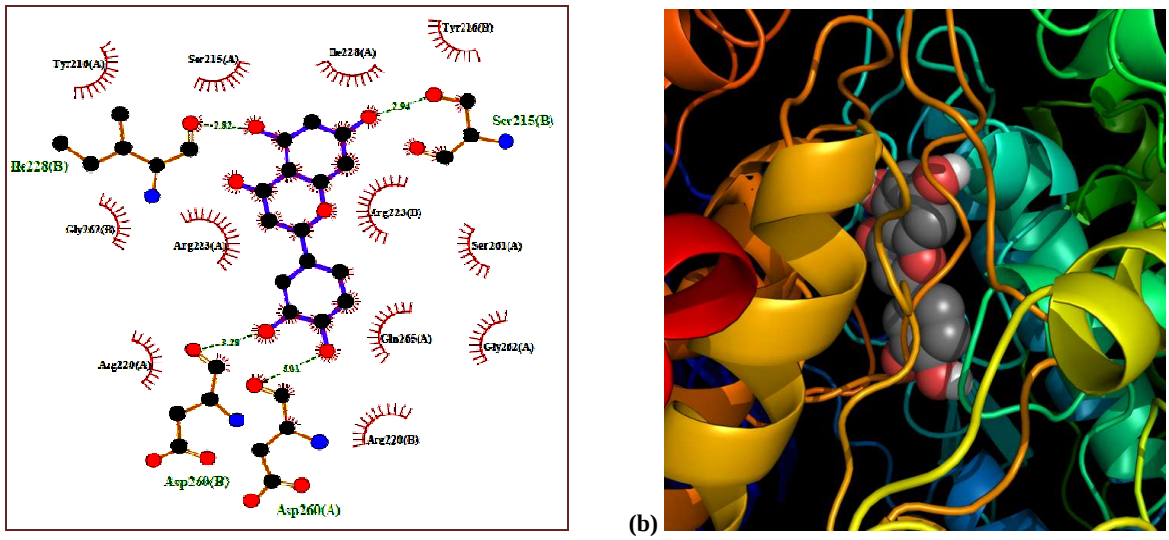


Figure 1. (a) Hydrophobic and hydrophilic interactions of luteolin with Glycogen synthase kinase-3β residues (GSK). Brown colored half circle indicates the hydrophobic reactions of luteolin with the target enzymes GSK. Green dotted lines indicate the hydrogen bond while green colored value indicates their bond length. (b) Promising binding pose of luteolin with GSK. Figure 1a was drawn with LigPlot software while figure 1b was drawn with PyMOL software.

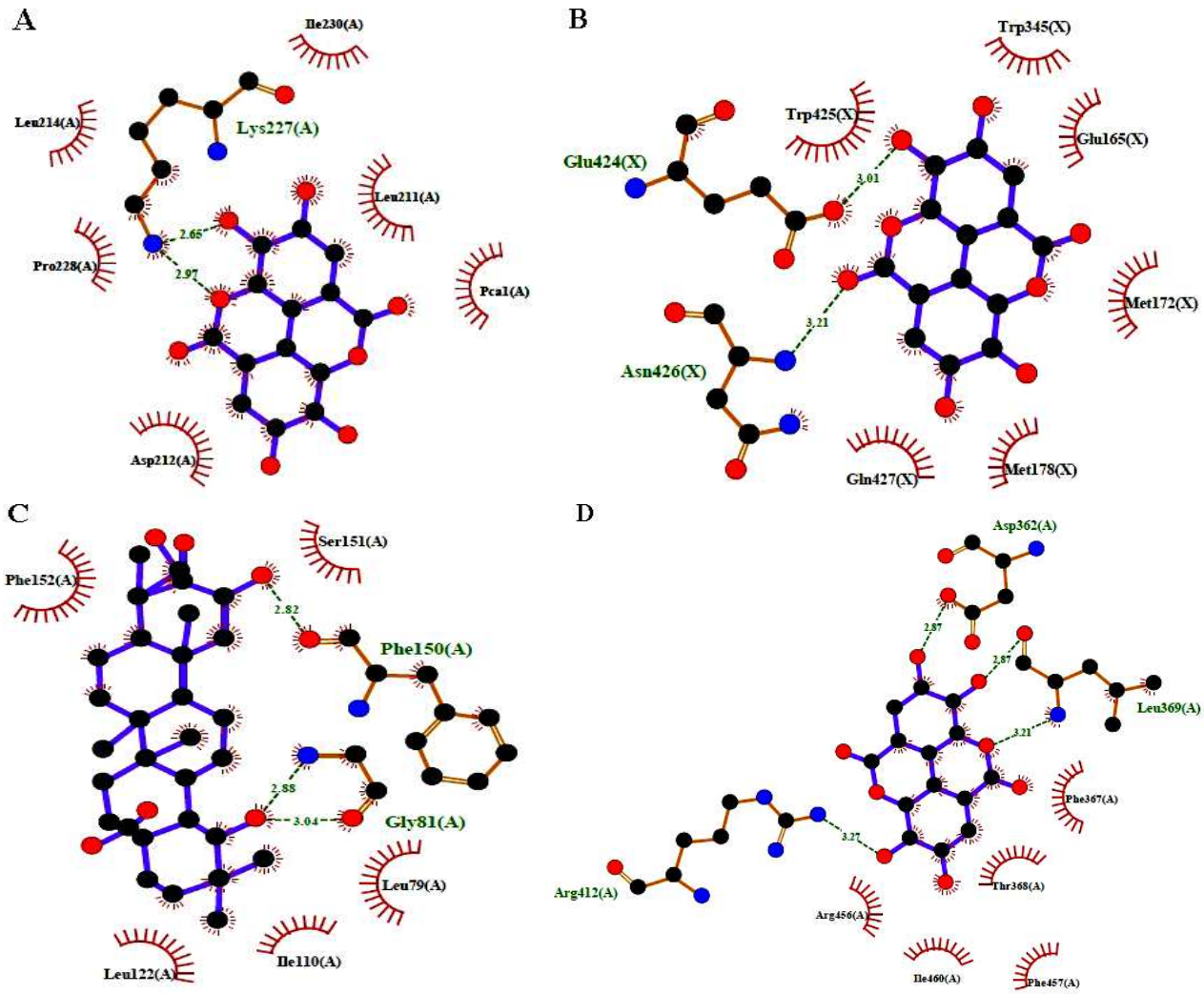


Figure 2. Hydrophobic interactions and hydrogen bonds of ellagic acid with amino acid residues of A. Alpha amylase, B. Beta glucosidase and D. Alpha glucosidase while that of arjungenin with C. Glucokinase. Brown colored half circle indicates the hydrophobic reactions of compounds with the target enzymes. Green dotted lines indicate the hydrogen bond while green colored value indicates their bond length. Figures were drawn with LigPlot software.

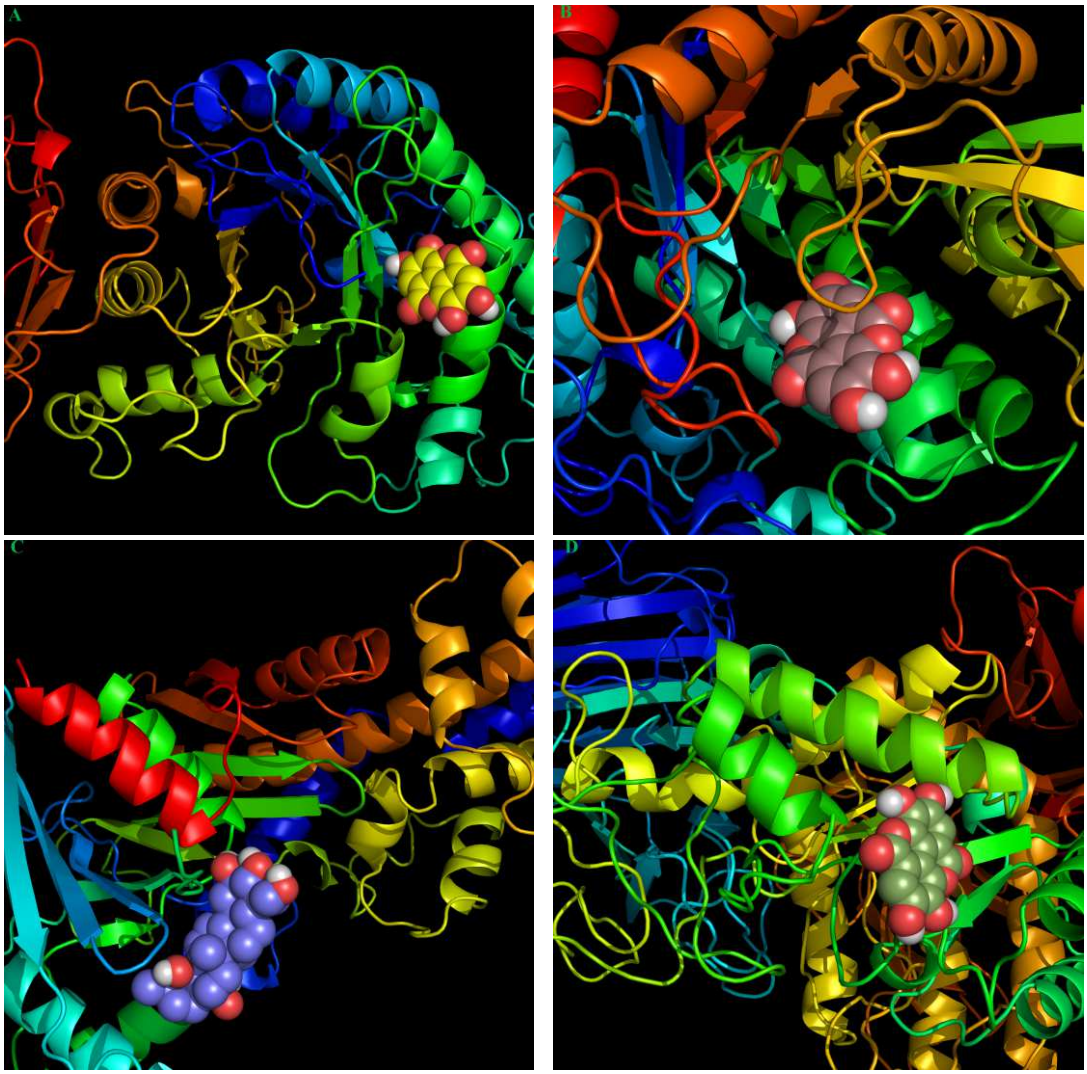


Figure 3. Promising binding poses of ellagic acid with A. Alpha amylase, B. Beta glucosidase and D. Alpha glucosidase and arjungenin with C. Glucokinase. Figures were drawn with PyMOL software.

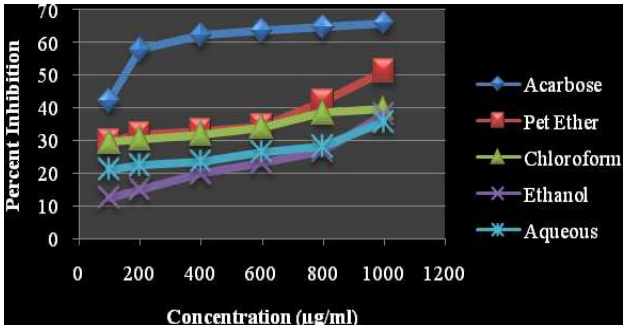
The bond length of these two bonds is 2.66A^0 and 2.97 A^0. Ellagic acid also formed a hydrogen bond with beta glucosidase at the distance of 3.01 A^0 with amino acid residue Glu424 (Figure 2B). Two hydrogen bonds are formed in between ellagic acid and alpha glucosidase through Leu369 and Arg412 amino acid residues and the bond length is 3.21 A^0 and 3.27 A^0 respectively (Figure 2D). Arjungenin also involved in hydrogen bond formation via gly81 residue of glucokinase and forms a bond from the distance 2.88 A^0 away (Figure 2C). It is clear from the above result that three phytochemicals ellagic acid, luteolin and arjungenin interacted well with the key regulatory enzymes and hence can prove to have an ability to be a future drug.

In vitro alpha amylase inhibitory activity assay analysis

Inhibition of digestion of dietary carbohydrates in the small intestine is a widely used method in the management of diabetes mellitus [44]. Inhibition of these carbohydrates by alpha amylase is one of the therapeutic approaches taken to reduce postprandial glucose level [45]. Keeping the same approach in mind, we proceeded to find out the potential alpha amylase inhibitory activity from different solvent extract of *T chebula*. All plant extracts have shown a great potential antidiabetic activity in comparison with standard drug Acarbose (Table 2). TCPE showed the 51.22% inhibition for alpha amylase at the concentration of 1 mg/ml while at the same concentration standard drug showed 65.99% inhibition. TCCE showed the 39.71% percent inhibition value while TCEE showed 38.03% inhibition of alpha amylase activity. TCAE inhibited alpha amylase activity 35.59%. Graph 1 shows the comparison of all this plant extracts with the standard drug Acarbose. Comparing the extracts among them showed that TCPE showed a significant capacity to inhibit the enzyme and thus may contain one of three compounds (ellagic acid, luteoline, arjungenin) as a bioactive compound.

Table 2. Alpha amylase inhibition by *T. chebula* using different solvent extracts. Tests were carried out in triplicate manner and values are expressed as the mean ± SD. The IC50 value is the concentration of inhibitor which inhibits 50% of its activity under the assayed conditions (TCPE - *T. chebula* petroleum ether extract, TCCE - *T. chebula* chloroform extract, TCEE - *T. chebula* ethanol extract, TCAE - *T. chebula* aqueous extract).

Sr. No.	Concentration (µg/ml)	% Inhibition				
		Acarbose	TCPE	TCCE	TCEE	TCAE
1	100	42.15 ± 0.68	29.55±1.15	29.45±1.46	12.5±3.09	21.17±1.71
2	200	57.88 ± 2.42	31.83±1.37	30.36±0.71	15.07±4.20	22.305±1.22
3	400	62.24 ± 0.53	32.88±0.05	31.83±1.45	19.79±1.50	23.535±1.54
4	600	63.76 ± 0.96	34.31±1.40	33.92±1.48	23.22±1.53	26.465±2.02
5	800	64.66 ± 1.26	41.88±5.37	38.49±6.26	26.62±1.23	28.09±2.02
6	1000	65.99 ± 1.76	51.22±4.29	39.71±6.21	38.03±4.71	35.59±7.76
IC50 values (µg /ml)		52	1115	1712	1589	2168



Graph 1. The enzyme inhibitory activity of different solvent extracts of *T. chebula* seed extract on α-amylase.

Table 3. TLC results of different extracts of *T. chebula* visualized by iodine chamber.

Sr No	Extract	Solvent system used	Rf value
1	Petroleum ether	Chloroform: ethyl acetate (3:1)	0.56, 0.41
2	Chloroform	Chloroform: ethyl acetate (4:6)	0.94, 0.54, 0.90
3	Ethanol	Ethyl acetate: Methanol: Water (5:1.1:1)	0.8, 0.63
4	Aqueous	Toluene: Ethyl acetate (4:1)	0.17, 0.10

Thin layer chromatography analysis

Thin layer chromatography is one of the analytical techniques which is being developed to standardize the natural products isolated from the medicinal plants [46]. To analyze the bioactive compound(s) present in the mixture of different solvent extracts and to document the phytoconstituents in these extracts we performed TLC. A different solvent system used for analysis showed better resolution for different plant extracts (Table 3). In solvent system, chloroform: ethyl acetate (3:1), petroleum ether extract of *T. chebula* showed two spots /compounds with Rf value 0.56, 0.41 while in solvent system, chloroform: ethyl acetate (4:6) of chloroform extract showed three spots showing Rf values, 0.94, 0.54, 0.90. Ethanol extract in the solvent system ethyl acetate: methanol: water (5:1.1:1) showed two spots with Rf values 0.8, 0.63. Finally, aqueous extract gave better results in toluene: ethyl acetate (4:1) mobile phase and the Rf values calculated were 0.17, 0.10.Totally 9 compounds were isolated by TLC method one or all them containing potential for antidiabetic potentially.

CONCLUSION

Our study confirmed that the secondary metabolites of *T. chebula* extract specially ellagic acid, luteolin and arjungenin possess a great potential of antidiabetic activity. One of the probable mechanisms of these phytoconstituents for the antidiabetic action is the inhibition of carbohydrate metabolizing enzyme, alpha amylase. Alpha amylase inhibitory activity is higher in petroleum extract which contain two compounds (TLC analysis). Further analysis is necessary to purify the bioactive compound and study its potential for antidiabetic activity.

AUTHORS' CONTRIBUTION

TSB designed the study, wrote the protocol, carried out all laboratories work and wrote the first draft of the manuscript. BKS managed the literature searches and edited the manuscript. Both the authors read and approved the final manuscript.

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