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In-Vitro And *In Silico* Alpha Amylase And Alpha-Glucosidase Inhibitory Activity Of *Emblica officinalis.*

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ABSTRACT

The present study on *Emblicaofficinalis*, commonly known as Indian gooseberry with various pharmacological properties, provides a description of the antihyperglycemic profile of ethanolic extract of*E.officinalis* fruit (EEEOF) by assessing the inhibitory potential of alpha-amylase and alpha-glucosidase enzymes extracted from whole wheat and barley respectively *in vitro* in combination with themolecular interaction study *in silico*.Themolecular interaction study of the phytoconstituents with the target enzymesalpha-amylase and glucosidase has been performed *in silico* using molegro virtual docker software (version 5.02). The parameters MolDock score, rerank score and H bond interactions wereassessed in the docking study. Acarbose was used as a standard drug for *in vitro* and *in silico*analysis. The IC_{50} value of EEEOF has been found to be 73.0 µg/ml and 78.01 µg/ml for alpha-amylase and alpha-glucosidase inhibition assayrespectivelyin comparison with that of standard drug acarbose which has IC_{50} of 43.78 µg/ml and 69.17 µg/ml. The MolDock Score and hydrogen bond interactions of the selected active constituents were found to be similar to that of the standard drug acarbose.The present study suggested that the *E.officinalis* fruit has the ability to control hyperglycemia by inhibiting the carbohydrate metabolizing enzymes.

Keywords: Amla, *in vitro*, *in silico*, Gout, Emblica officinalis,

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INTRODUCTION

Diabetes Mellitus, a multifactorial disorder which involves alterations in homeostasisof glucose and persistent hyperglycemia leading to several complications [1].Controlling hyperglycemia could be helpful in the management of the disease preventing further complications. A majorpartof therapy includes using inhibitors of intestinal absorption of sugar whichwill not affect the sugar metabolism and aids to control hyperglycemiaina noninvasive manner [2].Starch and sucrosebeing the main components of dietary carbohydrates are firstdisintegrated into oligosaccharides by salivary and pancreatic alpha-amylase.Pancreaticalpha-amylase serves as a key enzyme in the initial step of starch hydrolysis to maltose and lastly to glucose. Hydrolysis of starch takes place hastily which may result in post-prandial hyperglycemia. Alpha-glucosidase, enzyme attached to the intestinal epithelium, catalyzes the breakdown of complex carbohydratesforming glucose [3]. The key enzymes serve as potential avenues in the treatment of the progressive disorder [4].

The aim of oral therapy is to bring down the blood glucose to normal levelthereby preventing later complications. Alpha-glucosidase inhibitors serve as a desirable option in reducing the elevated levels of postprandial glucose representing a noninvasive approach for managing hyperglycemia [5]. The increase in blood glucose for a prolonged period may lead to further complications [6].

Acarbose being the standard drug makes the absorption of carbohydrates slower, by inhibitingintestinalalpha-glucosidases.The conversion of complex carbohydrates can only be delayed by acarbose molecules rather entirely blocked due to the reversible nature of enzyme-inhibitor interactions [7].

Natural products being the rich source ofpolyphenols with actions resembling insulin-glucose utilization, act as potent inhibitors of important enzymes associated with a metabolic disorder like diabetes mellitus and lipid peroxidation in tissues [8]. In recent years, much focus has been given on bioactive compounds derived from natural resources.

Emblica officinalis Gaertn. (syn: *Phyllanthus emblica*L.) (Euphorbiaceae) usuallyidentified as Indian gooseberry (Amla),found mostly in the tropical and subtropicalparts of China, India, and the Malay Peninsula and has been used significantly in Indian tradition. The hydrolyzable tannins and its derivatives found to be the main constituents and emblicanin A and B stated to have significant antioxidant properties [9]. Amla fruits have been showed to possess antimicrobial, antioxidant, adaptogenic, hepatoprotective, antitumor and antiulcerogenic activities [10].

Alpha-glucosidase and alpha-amylase inhibitors derived from natural sources which act by hindering the enzymatic cleavage of oligosaccharides and other derivatives have been recognized as a possibly safe tactic to manageelevated sugar levelsby modulating theabsorption of dietary glucose [11].

The antidiabetic profile of *E. officinalis* has been investigated in animal models *in vivo* and *in vitro* using amylase and glucosidase enzymes derived from microbial sources. The current work was pointed to examine the ability of ethanolic extract of *E.officinalis* to inhibit enzymes which metabolize carbohydrates such as alpha-amylase and alpha-glucosidase derived from whole wheat and barley respectively, and to predict the binding potential of bioactive compounds of *E.officinalis* with the target enzymes by molecular docking analysis *in silico* using Molegro Virtual Docker (MVD).

METHODS

Preparation of extract:

Fresh fruits of *E.officinalis* from Tirupati, Chittoor district, Andhra Pradesh were collected, and pharmacognostically identified and authenticated by. Madavachetty, Assistant Botanist, Sri Venkateswara University, Tirupati.

The fresh fruits were cleaned by washing it under running water and driedby keeping undershade. Powderfinelyby using a mechanical grinder and extracted with 90% ethanol by using soxhlet apparatus at the temperature of about 60ºC. The solvent was evaporated by rotary evaporator and brownish gummy exudates

were obtained. The crude ethanolic extract was used to evaluate amylase and glucosidase inhibitory properties. The percentage yield of ethanolic extract was calculated by using the formula.

$$\% \text{ yield} = \frac{\text{the weight of the crude extract}}{\text{the weight of the raw material}} \times 100$$

The percentageyield of ethanolic extract of *E. officinalis*fruit (EEEOF) was found to be 5.65%w/w.

Extraction of alpha-amylase from wheat:

To one liter of calcium acetate solution (0.2% w/v), malted whole wheat flour (500 g) was added slowly and continuously stirred for 2 hourswith a homogenizer at room temperature. Then the suspension was centrifuged at 12000g for 10minutes at 4°C. The resultingclearsupernatant brown color containing the enzyme was kept at 2-3°C before the heat treatment. The supernatant was heated at 70°C (15 minutes)in order to inactivate beta amylase in the enzyme mixture since alpha-amylase has resistance to heat in the pH range of 6.5to 8.02.Cold ammonium hydroxide (4% v/v) was used to adjust the pH of the enzyme extract to 6.6. Heat treatment was carried out in a water bath with continuous stirring at 72 to 74°C. The enzyme extract was then storedat 2-3°C for further use [12].

***In vitro* alpha amylase enzyme inhibition assay:**

It was doneas per the method is given by Sindhu. S. Nair *et al*, (2013). The reaction mixture comprising 200 µl of 0.02M sodium phosphate buffer, 20 µl of enzyme extract with EEEOF at different concentration (31.25, 62.5, 125, 250, 500, 1000 µg/ml) were incubated for 10 mins at room temperature and thestarch (200 µl) was added as substrate.400 µl of DNS reagent was added to terminate the reaction and kept in hot water bath for 5 minutes, cooled andthen diluted with 10 ml of distilled waterand absorbance was noted at 540 nm. Percentage inhibition of alpha-amylaseenzymewas calculated according to the formula

$$\text{Percentage inhibition} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}} \times 100$$

Where $\text{Abs}_{\text{control}}$and $\text{Abs}_{\text{samples}}$ the absorbance of control and samplerespectively.

Extraction of alpha-glucosidase:

100 g barley flour added to 500ml of 0.02 M sodium phosphate buffer (pH 8.0) which contains 0.002M of L-cysteine was mixed for a period of two and half hours at room temperature. The extractwas centrifuged at 4° C at 10,000 rpm for 5 mins. The clear supernatant containing the enzyme was stored at 2-3° C for further use [13].

***In vitro*alpha-glucosidaseenzyme inhibition assay:**

This was performed based on the protocol of J.R. Stark*et al*, (1987) with minor alterations [13]. The reaction mixture comprising of extracted enzyme (0.5 ml), EEEOF (0.5 ml) at different concentrations (31.25 - 1000µg/ml) and 0.5mlof substrate solution (3mg/ml maltose) in sodium acetate buffer (pH 4.7) was incubated at 37° C for 60 mins. 0.5ml of1M sodium bicarbonate solutionwas added to stop the progression of the reaction.At 420 nm the absorbance was measuredagainst the appropriate blank. Percentageinhibition of alpha-glucosidaseenzymewas calculated and compared to that of the standard drug acarbose.

Molecular Docking Study:

In silico docking, the analysiswas done usingtherecently introduced softwareMolegro Virtual Docker (MVD), which has received attention among researchers. It is one of the fastest docking program having flexibility andrenders the probable ligand conformation with the target which can be a macromolecule or an enzyme. It is created using a heuristic search algorithm which combines differential evolution with a cavity prediction algorithm [14]. Docking studies were performed as an additional proof too *in vitro* amylase and glucosidase inhibitory activity by showing the molecular interactions of phytocomponents with the target enzymes.

Preparation of Ligand:

From the RCBS database, the three-dimensional structural data of the phytoconstituents were attained. The major active phytoconstituents of EEEOF namely, Phyllembilic acid B, Ethyl gallate, Gallic acid, 5-Hydroxymethylfurfural, Ascorbic acid, 1, 2, 3-benzenetriol, Ethyl alpha-d-glucopyranoside and b-cyclocitral [15-19] were selected. From the PubChem chemical database, the three-dimensional structures of the selectedphytochemical elements were obtained and saved in .mol format. The ligands were brought in to the workspace of MVD and the molecules were prepared for the docking study. The parameters of docking study for the phytochemical constituents were compared to that of the standardacarbose which was obtained from the drugbankin.mol format.

Preparation of Enzyme:

The enzyme α – amylase (5EMY) and α – glucosidase (3TON) were obtained from RCSB protein databank.

MolDock Optimizer

The guided differential evolution algorithm of MVD has certain parameters in which the number of runswas selected as 5,population size as 50, the maximum number of repetitions as 2000,crossover rate as 0.9, and scaling factor as 0.5. The most appropriate mode of binding in the binding cavity was ensured by employing the pose clustering which leads to multiple modes of binding.

MolDockscore:

To disregard the atoms which are far from the docking site the ignore-distant-atoms option was chosen. Furthermore, H-bond direction between the impending donors and acceptors was checked. The cavity was selected with a radius of 25 Å in the binding site of the target made in the directions X, Y and Z.

Rerank Score:

Rerank scoring functions are used to evaluate the chemical properties (e.g. QSAR) by generating and predicting the models. The rerank scoring function is computationally exclusive compared to the scoringemployedduring docking simulation. The rerank score accurately rank the dissimilar poses of individual ligands. The rough estimate about the poses with the highest rank was obtained subsequently from the measure of binding affinity.

Statistical analysis:

The *in vitro* analyses were done in triplicate. Using GraphPad Prism software (Ver. 5.02), the results were expressedas mean ± SEM and IC_{50} value has been calculated.

RESULTS AND DISCUSSION

Prolonged hyperglycemiabeing a risk factor maylead to chronic diabetic complications. The principal goal of managingdiabetes is to reduce the elevated blood sugarlevels to a normal level [20]. Several plant extracts are known for their antidiabetic effects and were being usedfor the management of diabetes for a long time.

***In vitro*alpha amylase enzyme inhibition assay:**

Inhibiting the activity of alpha-amylasebydelaying the hydrolysis and absorption of carbohydrates limits postprandial glucose levels. The naturally derived antioxidants promote thestimulation of endogenous antioxidant systems to deactivate oxidative stress [21].

Different concentration of EEEOF was tested on alpha-amylaseextracted from the malted wheat *in vitro*. Andthe EEEOF exhibited a potential inhibitory effect on the alpha-amylase enzyme (IC_{50} –73.0 µg/ml) like

that of the standard drug acarbose (IC_{50} - 69.17µg/ml) in a concentration-dependent manner [Table 1 &Figure 1].

***In vitro* alpha-glucosidase enzyme inhibition assay:**

Alpha-glucosidase inhibitors prevent the function of an alpha-glucosidase enzyme essential for carbohydrate metabolism in a competitive manner. The alpha-glucosidases in the intestines metabolize oligosaccharides to glucose and other monosaccharides [22].

From thealpha-glucosidase inhibition assay*invitro*,the EEEOF showed the potential inhibitory effect on alpha-glucosidase enzyme extracted from barley *in Vitro* with an IC_{50} value of78.01µg/ml compared tothat of the standard drug acarbose (IC_{50} – 43.78µg/ml) [Table 1 &Figure 2].

Table 1: Alpha amylase& alpha-glucosidase inhibition by EEEOF

	Concentration (µg/ml)	31.25	62.5	125	250	500	1000
Alpha amylase inhibition (%)	Acarbose	28.8±0.75	46.77±0.57	57.81±0.73	68.11±0.66	74.06±0.36	79.56±0.31
	EEEOF	21.72±0.37	35.52±0.47	48.04±0.38	57.63±1.20	67.56±1.11	71.31±0.54
Alpha glucosidase inhibition (%)	Acarbose	33.17±0.54	48.18±0.95	58.39±0.44	66.58±0.44	74.27±0.92	81.19±0.64
	EEEOF	27.7±0.42	40.02±1.03	52.83±1.28	60.65±0.48	68.67±1.33	75.27±0.52

Results were expressed in terms of percentage inhibition. Each value represents mean±SEM (n=3).

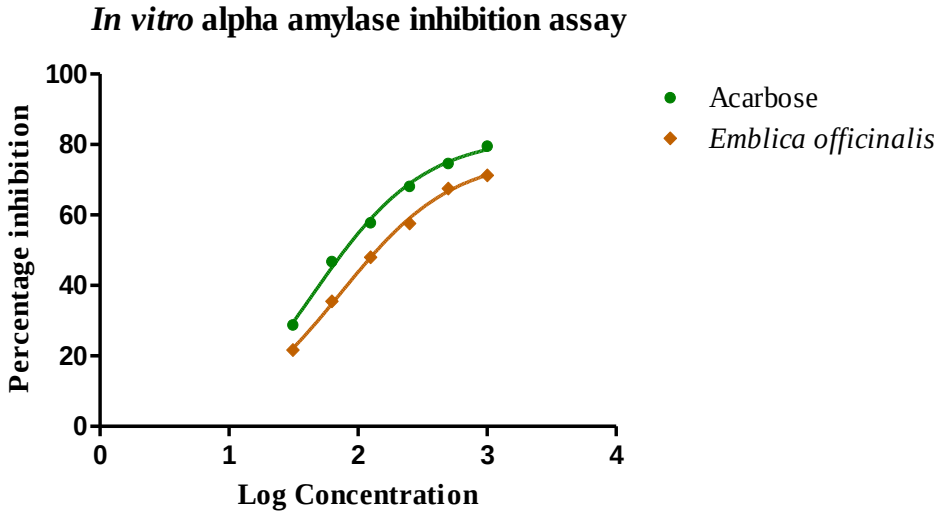

Figure 1: *In vitro*alpha-amylase inhibition assay of *E. officinalis* fruit extract

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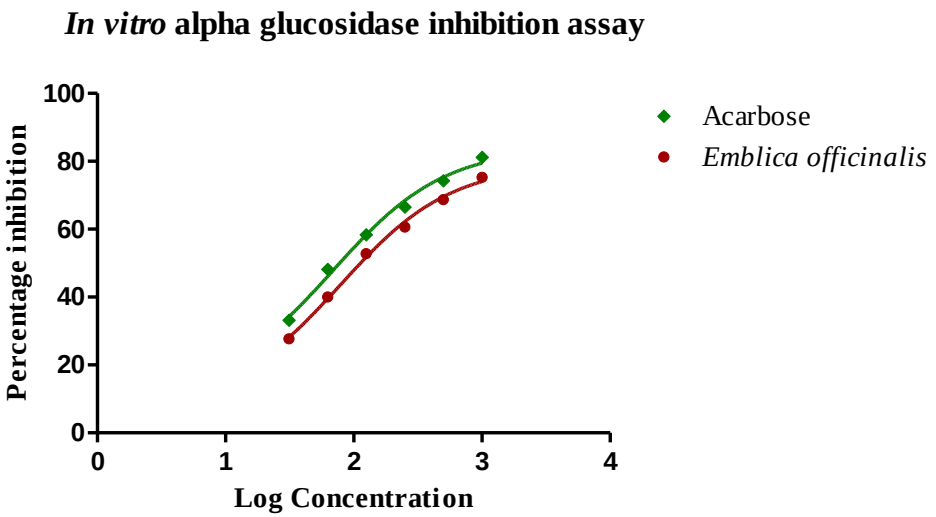

Figure 2: *In vitro*alpha-glucosidase inhibition assay of *E. officinalis* fruit extract

TABLE 2: DOCKING ANALYSIS OF PHYTOCONSTITUENTS FROM *EMBLICA OFFICINALIS* USING ALPHA AMYLASE (PDB ID: 5EMY) RANKING BASED ON MOLDOCK SCORE.

S.NO	Name	Ligand	MolDock Score	Rerank Score	HBond
1.	[00]Corilagin	Corilagin	-160.839	-135.878	-18.4691
2.	[00]Acarbose	Acarbose	-129.787	-103.119	-10.6892
3.	[00]Rutin	Rutin	-118.4	-88.6417	-12.8966
4.	[00]Beta- Sitosterol	Beta- Sitosterol	-112.475	-81.2896	-2.5
5.	[00]Trigalloyl glucose	Trigalloyl glucose	-111.016	-91.7649	-9.41433
6.	[00]campesterol	campesterol	-94.1997	-76.5999	-5
7.	[00]Phyllembilic acid B	Phyllembilic acid B	-83.3313	-89.1124	-7.42459
8.	[00](2,6,6-Trimethylcyclohex-1-enylmethanesulfonyl) benzene	(2,6,6-Trimethylcyclohex-1-enylmethanesulfonyl) benzene	-79.1313	-70.5254	-2.09542
9.	[00]n-Hexadecanoic acid	n-Hexadecanoic acid	-74.7292	-59.6224	0
10.	[00]Ascorbic acid	Ascorbic acid	-71.6554	-63.8955	-7.25785
11.	[00]Ethyl .alpha.-d-glucopyranoside	Ethyl .alpha.-d-glucopyranoside	-69.3475	-63.2582	-7.60668
12.	[00]Gallic acid	Gallic acid	-68.7966	-65.2747	-7.10358
13.	[00]5-Hydroxymethylfurfural	5-Hydroxymethylfurfural	-68.4974	-58.7118	-4.39676
14.	[00]Ethyl gallate	Ethyl gallate	-67.3025	-63.6145	-6.13913

TABLE 3: DOCKING ANALYSIS OF PHYTOCONSTITUENTS FROM *EMBLICA OFFICINALIS* ON ALPHA AMYLASE (PDB ID: 5EMY) - RANKING BASED ON HBOND.

S.NO	Name	Ligand	MolDock Score	Rerank Score	HBond
1.	[00]Corilagin	Corilagin	-160.839	-135.878	-18.4691
2.	[00]Rutin	Rutin	-118.4	-88.6417	-12.8966
3.	[00]Acarbose	Acarbose	-129.787	-103.119	-10.6892
4.	[00]Trigalloyl glucose	Trigalloyl glucose	-111.016	-91.7649	-9.41433
5.	[00]Ethyl .alpha.-d-glucopyranoside	Ethyl .alpha.-d-glucopyranoside	-69.3475	-63.2582	-7.60668
6.	[00]Phyllembilic acid B	Phyllembilic acid B	-83.3313	-89.1124	-7.42459

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7.	[00]Ascorbic acid	Ascorbic acid	-71.6554	-63.8955	-7.25785
8.	[00]Gallic acid	Gallic acid	-68.7966	-65.2747	-7.10358
9.	[00]Ethyl gallate	Ethyl gallate	-67.3025	-63.6145	-6.13913
10.	[00]campesterol	campesterol	-94.1997	-76.5999	-5
11.	[00]5-Hydroxymethyl furfural	5-Hydroxymethyl furfural	-68.4974	-58.7118	-4.39676
12.	[00]Beta- Sitosterol	Beta- Sitosterol	-112.475	-81.2896	-2.5
13.	[00](2,6,6-Trimethylcyclohex-1enylmethanesulfonyl) benzene	(2,6,6Trimethylcyclohex-1-enylmethanesulfonyl) benzene	-79.1313	-70.5254	-2.09542
14.	[00]n-Hexadecanoic acid	n-Hexadecanoic acid	-74.7292	-59.6224	0

TABLE 4: DOCKING ANALYSIS OF PHYTOCONSTITUENTS FROM *EMBLICA OFFICINALIS* ON ALPHA GLUCOSIDASE (PDB ID: 3TON) - RANKING BASED ON MOLDOCK SCORE.

S.NO.	Name	Ligand	MolDock Score	Rerank Score	HBond
1.	[00]Corilagin	Corilagin	-125.583	-108.351	-12.9896
2.	[00]Acarbose	Acarbose	-108.288	-73.8178	-15.8702
3.	[00]Trigalloyl glucose	Trigalloyl glucose	-92.9835	-90.3237	-14.7984
4.	[00]Rutin	Rutin	-86.0583	-78.2912	-9.73685
5.	[00]campesterol	campesterol	-85.5094	-68.5215	-2.5
6.	[00]Beta- Sitosterol	Beta- Sitosterol	-85.2876	-68.3883	-5
7.	[00]Ethyl gallate	Ethyl gallate	-73.3818	-68.3231	-11.3492
8.	[00]5-Hydroxymethylfurfural	5-Hydroxymethylfurfural	-60.8764	-51.6385	-5
9.	[00]Ascorbic acid	Ascorbic acid	-59.445	-52.3105	-11.7511
10.	[00]Ethyl .alpha.-d-glucopyranoside	Ethyl .alpha.-d-glucopyranoside	-57.9836	-55.8911	-11.9607
11.	[00](2,6,6-Trimethylcyclohex-1-enylmethanesulfonyl)benzene	(2,6,6-Trimethylcyclohex-1-enylmethanesulfonyl)benzene	-56.8598	-48.47	0
12.	[00]Gallic acid	Gallic acid	-53.3777	-53.4056	-8.39482
13.	[00]n-Hexadecanoic acid	n-Hexadecanoic acid	-52.0085	-35.8675	-0.148177
14.	[00]Phyllembilic acid B	Phyllembilic acid B	-45.14	-57.8742	-12.5472

TABLE 5: DOCKING ANALYSIS OF PHYTOCONSTITUENTS FROM *EMBILICA OFFICINALIS* ON ALPHA GLUCOSIDASE (PDB ID: 3TON) - RANKING BASED ON HBOND.

S.NO.	Name	Ligand	MolDock Score	Rerank Score	HBond
1.	[00]Acarbose	Acarbose	-108.288	-73.8178	-15.8702
2.	[00]Trigalloyl glucose	Trigalloyl glucose	-92.9835	-90.3237	-14.7984
3.	[00]Corilagin	Corilagin	-125.583	-108.351	-12.9896
4.	[00]Phyllembilic acid B	Phyllembilic acid B	-45.14	-57.8742	-12.5472
5.	[00]Ethyl .alpha.-d-glucopyranoside	Ethyl .alpha.-d-glucopyranoside	-57.9836	-55.8911	-11.9607
6.	[00]Ascorbic acid	Ascorbic acid	-59.445	-52.3105	-11.7511
7.	[00]Ethyl gallate	Ethyl gallate	-73.3818	-68.3231	-11.3492
8.	[00]Rutin	Rutin	-86.0583	-78.2912	-9.73685
9.	[00]Gallic acid	Gallic acid	-53.3777	-53.4056	-8.39482
10.	[00]5-Hydroxymethylfurfural	5-Hydroxymethylfurfural	-60.8764	-51.6385	-5
11.	[00]Beta- Sitosterol	Beta- Sitosterol	-85.2876	-68.3883	-5
12.	[00]campesterol	campesterol	-85.5094	-68.5215	-2.5
13.	[00]n-Hexadecanoic acid	n-Hexadecanoic acid	-52.0085	-35.8675	-0.148177

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14.	[00](2,6,6-Trimethylcyclohex-1-enylmethanesulfonyl)benzene	(2,6,6-Trimethylcyclohex-1-enylmethanesulfonyl)benzene	-56.8598	-48.47	0

***In silico* docking analysis:**

E. officinalis extract has shown inhibitory activity against amylase and glucosidase enzymes *in vitro* henceforth, a molecular interaction of phyto constituents of *amla* fruit with alpha-amylase(PDB ID: 5EMY) and alpha-glucosidase (PDB ID: 3TON) has been investigated in this study using Molegro Virtual Docker software. The binding prototype of the ligands was analyzed by means ofthein-built ligand energy inspector tool of MVD.

The phytoconstituents were ranked on the basis of MolDockscore (Table 2), and-Bond(Table 3) targeted on the enzyme α-amylase and on α-glucosidase based on the MolDock score (Table 4), and H-Bond(Table 5). It was found that the binding patterns were like the standard drug acarbose, possessing maximum MolDock score as well as rerank scorein comparison to the standard.

Thefindings of the study demonstrated that the corilagin possesses a potent binding affinity with the targeted enzymes [Figure 3 & 5] compared to that of the standard acarbose [Figure 4 & 6] and Trigalloyl glucose, Phyllembilic acid B,Rutin, Ascorbicacid showed good binding interactions *in silico*.

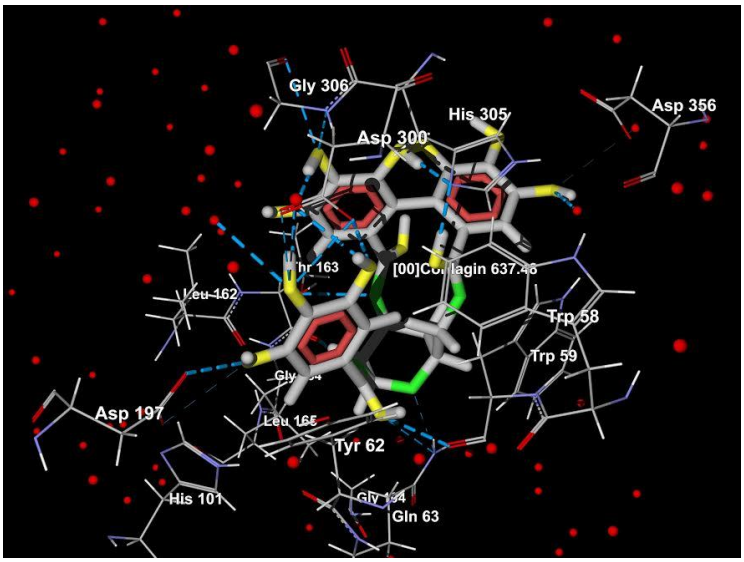

FIGURE 3: DOCKED VIEW OF CORILAGIN WITH THE ENZYME ALPHA AMYLASE (PDB ID: 5EMY)

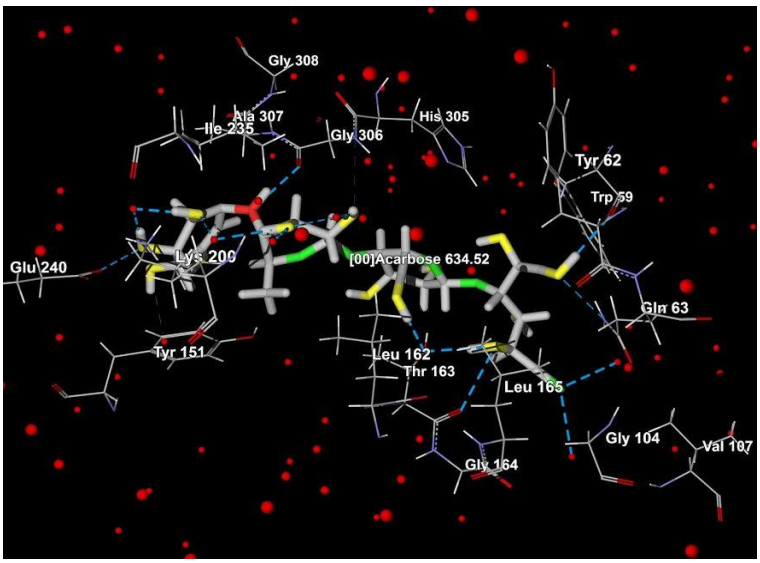

FIGURE 4: DOCKED VIEW OF ACARBOSE WITH THE ENZYME ALPHA AMYLASE (PDB ID: 5EMY)

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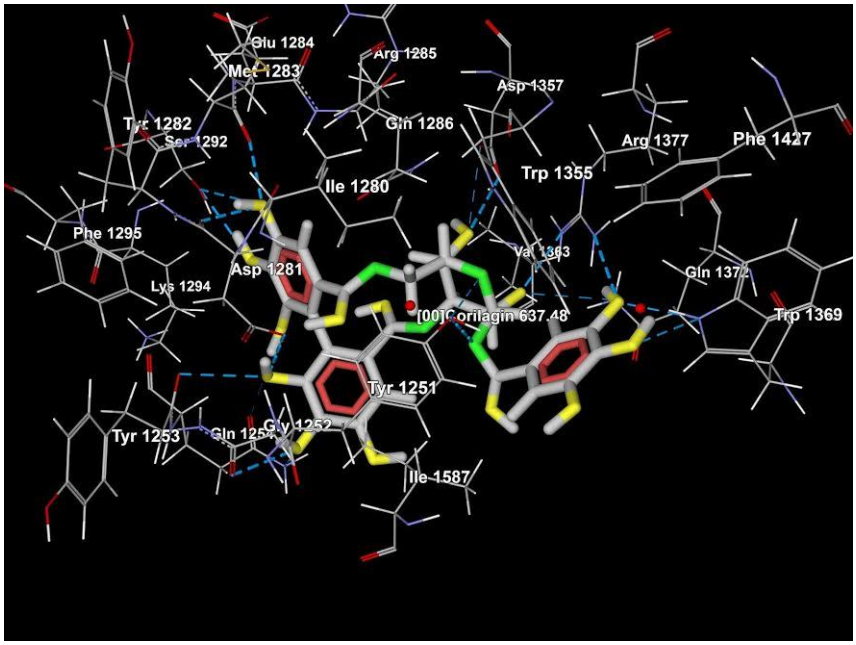

FIGURE 5: DOCKED VIEW OF CORILAGIN WITH THE ENZYMEALPHA GLUCOSIDASE (PDB ID: 3TON)

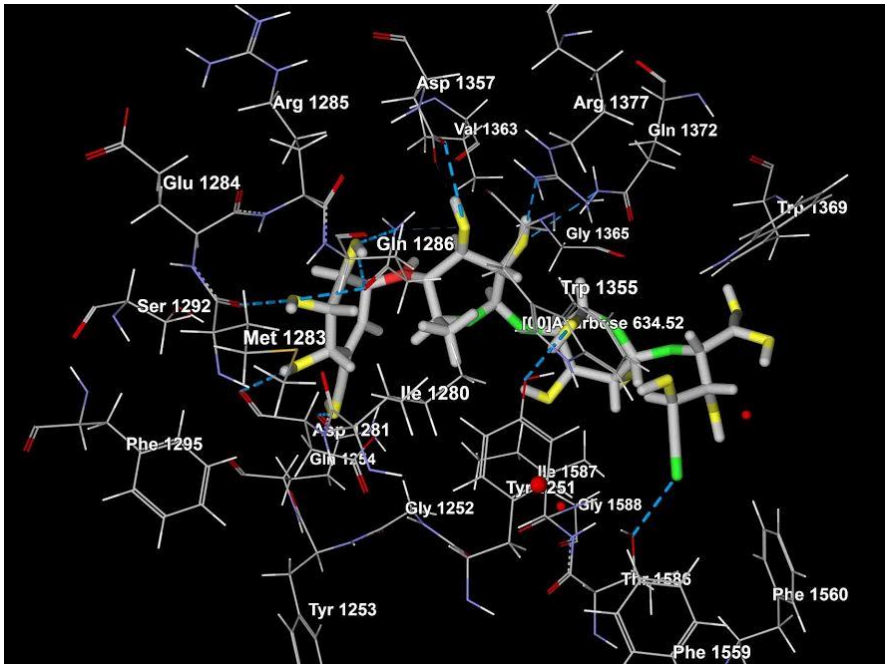

FIGURE 6: DOCKED VIEW OF ACARBOSE WITH THE ENZYME ALPHA GLUCOSIDASE (PDB ID: 3TON)

Alpha-amylase and alpha-glucosidase have been stated moral targets in the treatment of elevated levels ofmeal-derived glucose. Inhibition of these enzymes delays carbohydrate breakdown, decreasing the rate of glucose absorption subsequently blunting the rise in plasma glucose levels. The drugs available currently, as of amylase and glucosidase inhibitors show unintended abdominaleffects such as inflating, abdominal discomfort, flatulence making them less striking as therapeutic agents [23].

Plants mostlycontain secondary metabolites like phenolics, coumarins, flavonoids, terpenoids, saponins, glycosides, and alkaloids with specific distinctivecharacteristics, attributing to their biological properties [21, 24].

Our findings revealed the ability of EEEOF phytoconstituents to interact with the target enzymes involved in carbohydrate metabolism by confirmative docking analysis performed *insilico* with a supportive data showing the potential effect in *in-vitro* inhibition assays using the amylase and glucosidase enzyme derived from natural sources.

CONCLUSION

The present study examined the inhibitory potential of ethanolic extract of E. *officinalis* fruit on the enzymes alpha-amylase and alpha-glucosidase. It was identified by both *in vitro* and *in silico*analysisthat the phytoconstituents of *E.officinalis* can control hyperglycemia by inhibiting the majorcarbohydrate metabolizing enzymes. Future studies may be required to investigate the effect *in vivo* and also to identify the safety and efficacy parameters at both preclinical and clinical stages.

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