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Original Article

IN VITRO AND *IN SILICO* EVALUATION OF THE ANTIDIABETIC EFFECT OF HYDROALCOHOLIC LEAF EXTRACT OF *CENTELLA ASIATICA*

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ABSTRACT

Objective: Aim of the present study was to explore the possible mechanisms through which *Centella asiatica* may be beneficial in managing diabetes and its associated complications by *in vitro* methods and to predict the potential bioactive constituent/s responsible for its anti-diabetic activity through *in silico* docking study.

Methods: Hydro-alcoholic leaf extract of *C. asiatica* was prepared using Soxhlet extraction. Plant extract was evaluated for its *in vitro* antioxidant, anti-inflammatory and anti-diabetic activity. Further, docking screening was performed using Molegro Virtual Docker software to predict potential moiety which may be responsible for its anti-diabetic activity.

Results: Soxhlet extraction resulted in extractive yield of 35.43% and showed high antioxidant potential as demonstrated by its ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (98.72%). This antioxidant activity may be attributed to high phenolic and flavonoid components present in extract (1.004 mg gallic acid equivalent (GAE)/gram and 0.113 mg Rutin equivalent (RE)/gram dried extract respectively). Plant extract inhibited albumin denaturation (81.77%) and stabilized RBCs membrane (66.7%) indicating its high anti-inflammatory potential. *In vitro* anti-diabetic assays revealed that anti-hyperglycaemic activity of this plant can be attributed to its high efficiency to inhibit α-amylase (62.13%) and glucosidase (59.9%) enzymatic activity, which are well established targets for the management of diabetes. Further, through docking studies we predicted that centellasaponin-C, asiaticoside, asiaticoside-E, castilliferol and brahminoside present in this plant might be responsible for the anti-diabetic properties exhibited by this plant.

Conclusion: These results provide a scientific justification for the traditional anti-diabetic use of this plant. It may control diabetes through lowering dietary glucose uptake and may benefit in progression of diabetic complications through reducing oxidative and inflammatory stress. Predicted anti-diabetic molecules need to be screened further for the management of hyperglycemia.

Keywords: *Centella asiatica,* Antioxidant, Anti-inflammatory, Anti-diabetic, Docking

INTRODUCTION

Diabetes Mellitus (DM) is a complex metabolic disorder which is characterized by the persistent hyperglycaemic state. Despite rapid advancement in the diabetic therapeutic, prevalence of DM is increasing at an alarming rate and is expected to double by the year 2040 [1]. DM is generally associated with several complications that arise secondary to diabetes such as neuropathy, nephropathy, retinopathy, cardiovascular complications etc. [2]. During DM glucose is abundantly available, which upon oxidation generates excessive free radicles and saturates body's antioxidant defence (catalase, superoxide dismutase, glutathione etc.). These free radicals interacts with the biomolecules such as protein, lipids, RNA, DNA etc. and render them non-functional, thereby developing persistent oxidative stress in the body which aids in the development of secondary diabetic complications [3]. Further diabetes is known to potentiate inflammatory processes within the body via chronically elevating pro-inflammatory cytokines and chemokines and this inflammatory stress further exaggerate diabetic complications [4]. Furthermore, DM have been demonstrated to be induce genotoxicity, which is primarily attributed to the interaction of highly reactive free radicals with the DNA, resulting in impaired gene expression. Further, interplay of inflammatory and oxidative stress is known to play an important part in the development of insulin resistance and DM [5].

Available anti-diabetic drugs are focused on eliminating excessive glucose from the blood and have negligible effect on the mechanisms which leads to the development of various complications, besides several side effect and necessity to take lifetime medication is there. These complications continue to grow uncontrolled and are responsible for compromising living standards of patients, despite regular treatment with anti-diabetics [6]. Therefore an efficient therapeutic strategy for diabetes would be to supplement anti-diabetic drugs with certain additives which interfere with pathways leading to various complications.

Human civilization has exploited natural resources for the treatment and prevention of several ailments since time immemorial. A rich heritage of this traditional knowledge of the medicinal values of nature can be reflected form the ancient scholastic work illustrated in the ancient text like Atharvaveda (an Indian religious book), Ayurveda (Indian traditional system of medicine) etc. [7]. In recent time focus of the research has been inclined towards traditional medicinal plants and significant amount of evidence has been accumulated which scientifically justifies traditionally use of these plants. *Centella asiatica* L. (mandukparni or Indian pennywort or jalbrahmi) is a small creeping perennial herbal plant belonging to the family Apiaceae or Umbelliferae, which flourishes abundantly in swampy regions of South-East Asia, Madagascar, and South Africa and South pacific and Eastern Europe. Traditional literature depicts *C. asiatica* L. as an important medicinal herb which has been depicted to be useful during various ailments such as leprosy, lupus, varicose ulcers, eczema, psoriasis, diarrhoea, fever, amenorrhea, diseases of the female genitourinary tract, diabetes, brain tonic and depression [8-10]. Reports from the past few decades depict various biological activities *C. asiatica.* Considerably small amount of work have been focused on its effect on diabetic complications and none of the precious studies depicts possible mechanisms through which it may prove beneficial in countering diabetic complications. Therefore, aim of the present study was to gain preliminary insite into the mechanism through which it can aid in controlling diabetes and its complications through *in vitro* and *in silico* tools. On this ground we evaluated the potential of hydro-alcoholic extract of *C. asiatica* to neutralize oxidative stress and inflammatory stress which plays decisive role in the development and progression of diabetic

complications. Further, potential ant-diabetic mechanism of this plant was experimentally screened through *in vitro* assays and bioactive ant-diabetic constituents which may be responsible for anti-diabetic property were predicted from *in silico* docking studies.

MATERIALS AND METHODS

Materials

All the chemicals used in the present study were procured from Sigma-Aldrich, Loba Chemie, Merck, Sdfine-Chem, Himedia and Spectrochem.

Collection and extraction of plant material

Leaves of the *C. asiatica* were procured from Natural Remedies, Banglore, India (Bath No. ERD 040). Leaves were cleaned under running water and dried in shade. The dried leaves were then made into a coarse powder and were subjected to hydro-alcoholic (30:70) extraction using Soxhlet extractor. Collected extract was filtered while hot, concentrated under reduced pressure using rotary evaporator, lyophilized and stored at 4 °C until used.

Preliminary phytochemical screening

The lyophilized hydro-alcoholic leaf extract of *C. asiatica* (HLEC) was qualitatively tested for the presence of phytochemical constituents such as alkaloids, flavonoids, terpenoids, phenols, tannins etc., as per the previously well-defined and widely used standardized methods [11].

Evaluation of total phenolic content

Folin-Ciocalteu reagent was used to evaluate the total phenolic content of the HLEC using gallic acid as standard [12]. Standard curve of gallic acid was prepared and total phenolic content of HLEC was expressed as mg gallic acid equivalent (GAE) per gram of extracts. Extraction reagent was prepared by mixing 40 ml acetone, 40 ml methanol, 20 ml water and 0.1 ml acetic acid together. Reaction mixture consisted of 100 mg extract, 25 ml distilled water and 25 ml of extraction reagent. Reaction mixture was vortexed gently and heated to 60 °C over water bath for 1h followed by cooling to room temperature under running tap water. 1.6 ml of sodium carbonate (7.5% in deionized water) and 2 ml of Folin Ciocalteu reagent (0.1% in deionized water) was added to 400 µl reaction mixture followed by incubation of 1 h at room temperature. Total phenolic content present in extract was determined spectrophotometrically by UV-spectrophotometer (Shimadzu 265, Japan) at 525 nm.

Evaluation of total flavonoid content

Method of Zhishen *et al.* [13] was used to determine total flavonoids contents in HLEC by taking rutin as a standard. Standard curve of rutin was prepared and total flavonoid content of HLEC was expressed as mg rutin equivalent (RE) per gram of the extract. For this, 0.5 ml of 200 mg/ml extract was added to the test tube containing 75 µl 5% $NaNO_2$ solution and mixture was allowed to stand for 10 min. 150 µl 10% $AlCl_3.6H_2O$, 0.5 ml NaOH (1 M) and 2.5 ml distilled water was then added to each reaction mixture followed by 5 min incubation at room temperature. Total flavonoid content determined by taking absorbance at 510 nm using UV Spectrophotometer.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant potential of *C. asiatica* L. was determined through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as depicted previously by Wang et. al, [14], with slight modifications. This assay is based on the scavenging of purple stable free radical, DPPH, by test compound resulting in a colourless solution which is measured spectrophotometrically. For this, 0.4 mM DPPH solution was prepared in 95% methanol. 10 ml of different concentrations of HLEC extract (250-1000 µg/ml), standard (ascorbic acid) (250-1000 µg/ml) or 95% methanol (blank) were transferred into test tubes containing 3 ml DPPH solution and mixed vigorously followed by incubation in dark at 37 °C for 30 min. Entire experimentation was performed in triplicate and absorbance of reaction mixtures was recorded in 1 cm cuvettes using UV Spectrophotometer at 517 nm. % DPPH radical scavenging activity was calculated as follows:

$$\%\text{DPPH radical scavenging} = 1 - (\text{Abs test}/\text{Abs control}) \times 100$$

Evaluation of anti-inflammatory activity

Inhibition of protein denaturation

In vitro anti-inflammatory activity of HLEC was performed according to previously well-established method [15] with slight modification. Diclofenac sodium was taken as a standard anti-inflammatory drug and all dilutions were made in distilled water. 1 ml different concentration of HLEC and diclofenac sodium (250-1000 µg/ml) or distilled water (blank) were taken in test tubes and equal volume of 1% aqueous solution of bovine albumin was transferred to it. pH of the reaction mixture was adjusted to 6.3 by using 1 N HCl and samples were incubated for 30 min at 37 °C. Further, all the samples were subjected to denaturation temperature of 57 °C for 5 min and were immediately cooling under running tap water. Turbidity of all the samples was recorded spectrophotometrically in triplicate at wavelength of 660 nm. Percentage inhibition of albumin denaturation by HLEC and standard drug was calculated using following equation:

$$\text{Percentage inhibition} = 1 - (\text{Abs test}/\text{Abs control}) \times 100$$

Membrane stabilization test

Membrane stabilization test is another method used for evaluating *in vitro* anti-inflammatory activity [16], and we used this method to evaluate anti-inflammatory potential of HLEC and diclofenac sodium (standard). For this, sufficient amount of blood was taken from rat's tail vein under light ether anaesthesia. Blood was immediately transferred into glass tube having 1.8 mg/ml 5% EDTA solution and was centrifuged at 3000 rpm for 15 min. RBC pellet was washed three times with equal volume of normal saline and 10% v/v suspension was prepared in saline. Prepared suspension was stored at 4 °C and used within 6 h. Reaction mixture consisted of 1 ml phosphate buffer, 1 ml blood suspension and 1 ml of different concentration of HLEC, diclofenac sodium or distilled water (blank). Reaction mixture was incubated at 57 °C for 30 min and then centrifugation at 2500 rpm for 5 min. Supernatant was collected and its absorbance was recorded at 560 nm. Entire experimentation was performed in triplicate and percentage membrane stabilization was calculated by using following equation:

$$\%\text{ membrane stabilization} = 1 - (\text{Abs test}/\text{Abs control}) \times 100$$

Anti-diabetic activity

α-Glucosidase activity

Method of Elya *et al.* [17] was used to determine *in vitro* α-glucosidase inhibitory activity of *C. asiatica* using acarbose as a standard drug. Reaction mixture consisted of 10 µl different concentration of HLEC (250-1000 µg/ml), acarbose or distilled water, 490 µl phosphate buffer (pH 6.8) and 250 µl *p*-nitrophenyl-α-D-glucopyranoside (5 mM). Reaction mixture was incubated at 37 °C for 5 min and then 250 µl α-glucosidase (0.15 unit/ml) was added to each tube followed by 15 min incubation at 37 °C. The reaction was terminated by adding 2 ml Na_2CO_3 solution (200 mM). α-glucosidase activity was determined by measuring the quantity of *p*-nitrophenol released from *p*-nitrophenyl-α-D-glucopyranoside spectrophotometrically at 400 nm. Blank reaction represent 100% enzyme activity and relative activity was determined as per following equation.

$$\text{Relative enzyme activity} = (\text{enzyme activity} / \text{control}) \times 100$$

α-Amylase activity

Inhibition of salivary amylase activity by HLEC was performed according to method described by Gillard *et al.* [18], with some modification. 50 ml saliva was collected from normal individuals with paraffin chewing. Saliva samples were refrigerated within 2 h of collection and left overnight at 4 °C. Samples were centrifuged for 30 min at 2000 rpm and supernatant was used for the study. Reaction mixtures consisted of 500 µl 0.02 M phosphate buffer (pH 6.9; 6 mM of NaCl), 1 ml of saliva (salivary amylase), different concentrations of HLEC (250-1000 µg/ml), rutin (250-1000 µg/ml) or distilled water (blank). After 15 min

incubation at room temperature, 1 ml of 3, 5-dinitrosalicylic acid (10 mM) was added to it. Reaction mixture was incubated at 95 °C over water bath for 1 h and then brought to room temperature. Inhibition of α-amylase activity was determined spectrophotometrically at 540 nm. Blank reaction represent 100% enzyme activity and relative activity was determined as per following equation.

Relative enzyme activity = (enzyme activity / control) × 100

In silico docking study

In order to predict the active constituent present in *C. asiatica* which may be responsible for the anti-diabetic activity possessed by this plant, we screened all the compounds that were previously reported to be present in the plant through *in silico* docking studies on insulin receptor protein (PDB: 1IR3) by using Molegro Virtual Docker (MVD) software. 3-D structures of all the molecules were prepared by using Marvin sketch software and 1IR3 crystalline structure was obtained from protein databank. Ligands were imported to MVD and their energies were set to minimum possible. Solid surface of insulin receptor protein was created and maximum of 5 different binding cavities were selected on it followed by its energy minimization. Screening process was then initiated by docking 5 different poses of 10 ligand conformation on to insulin receptor. Result of the best run was taken as the final observation, ligand receptor interaction image was generated and docking score was expressed as ligand receptor-interaction energy (kcal/mol) [19-21].

Statistical analysis

Data in the present study was expressed as mean±standard deviation of triplicate recordings using XLSTAT 2015.1. One-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test were used to analyse the data. Significance among samples were expressed at significance level of $p<0.05$.

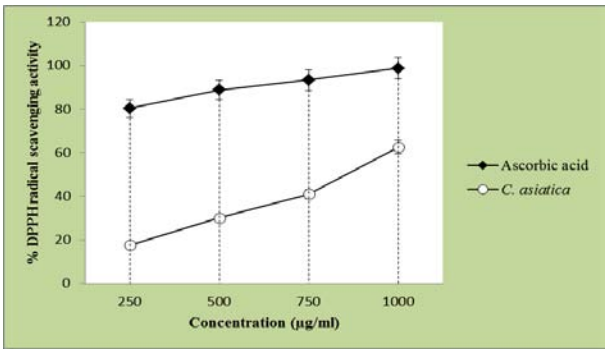

Fig. 1: DPPH radical scavenging activity amylase of hydroalcoholic extract of *C. asiatica*. Data are expressed as means±SD (n = 3)

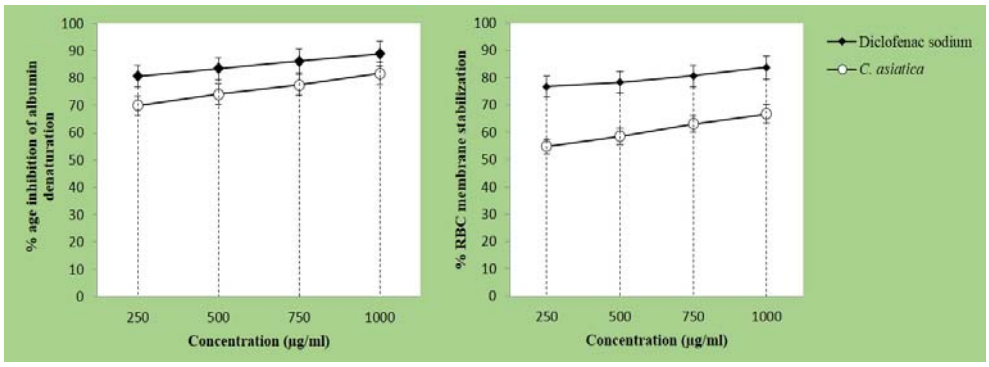

Fig. 2: % inhibition of protein denaturation (a) and % RBC membrane stabilization (b) activity of hydroalcoholic extract of *C. asiatica*. Data are expressed as means±SD (n = 3)

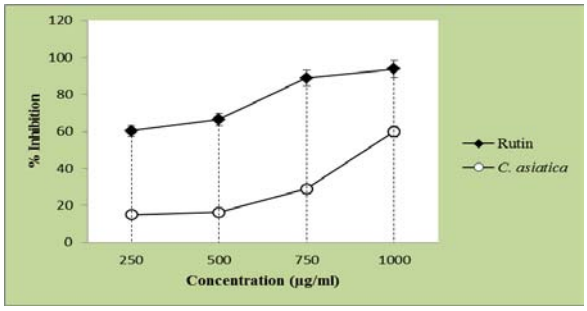

Fig. 3: % inhibition of α-amylase of hydroalcoholic extract of *C. asiatica*. Data are expressed as means±SD (n = 3)

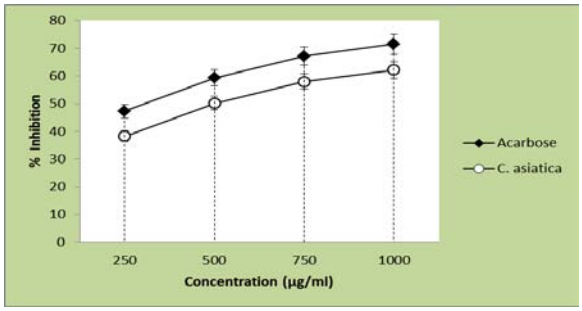

Fig. 4: α-glucosidase inhibitory assay of hydroalcoholic extract of *C. asiatica*. Data are expressed as means±SD (n = 3)

Table 1: Results of the docking interaction study between different compounds present in *C. asiatica* and insulin receptor (PDB: 1IR3)

S. No.	Name of compound (Energy in kcal/mol)	Ligand-protein binding energy	Mol. dock score (kcal/mol)	No. of interaction with protein
1	Centellasaponin-C	249.68kCal/mol	-181.704	15
2	Asiaticoside	228.15kCal/mol	-159.243	10
3	Asiaticoside-E	215.25kCal/mol	-148.455	16
4	Castilliferol	76.73kCal/mol	-142.653	10
5	Brahminoside	271.51kCal/mol	-141.695	15

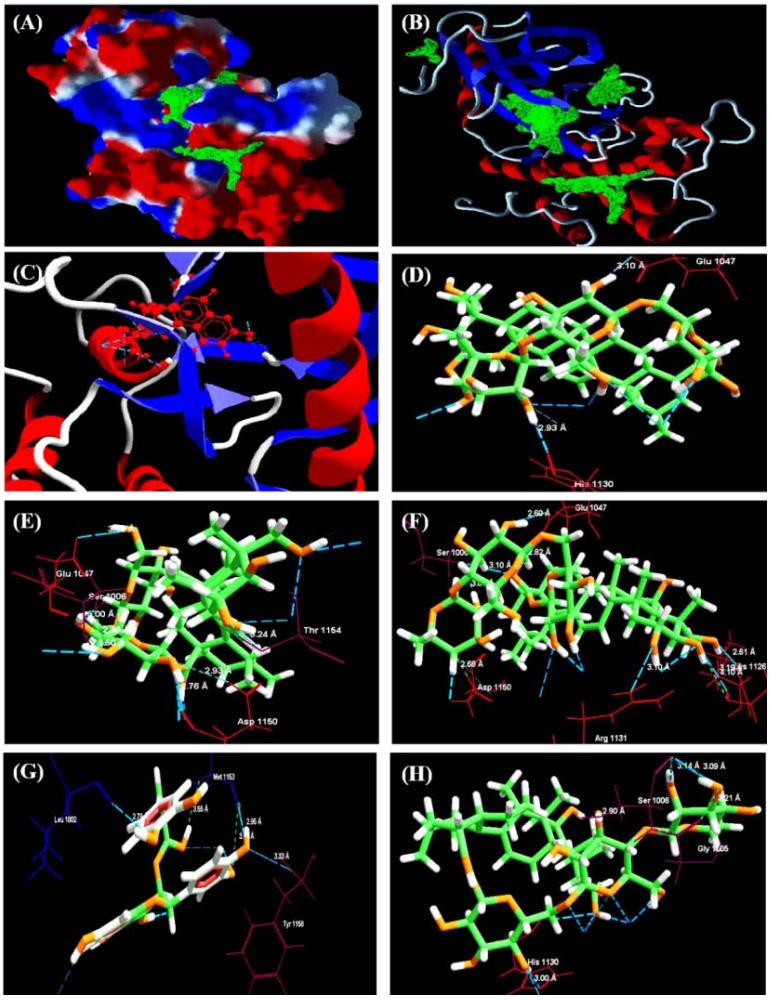

Fig. 5: Docking interaction of different constituents of *C. asiatica* on active site of 1IR3 PDB. A-protein surface; B-secondary structure of 1IR3 protein; C-secondary protein structure with interacting molecule; D-H depicts interaction of centellasaponin-C, asiaticoside, siaticoside-E, castilliferol and brahminoside with 1IR3 PDB respectively

RESULTS AND DISCUSSION

Extractive yield and phytochemical screening

Hydro-alcoholic extraction of *C. asiatica* resulted in the crude extractive yield of 35.43%. Lyophilized crude extract was then subjected to preliminary phytochemical screening which mainly showed the presence of flavonoids, alkaloids, glycosides, carbohydrates, acidic compounds and saponins the extract. Tests for volatile oil, proteins, amino acids and lignin did not showed positive results.

Antioxidant activity

Total phenolic content present in the extract was determined in term of GAE from the standard curve of gallic acid. High correlation (r^2 = 0.997) between concentration and corresponding absorbance was observed from the calibration curve of gallic acid (y = 0.9748x-0.2487). Total flavonoid content of the crude extract was determined in term of RE from the standard curve of rutin. Linear regression equation of y = 0.1174x+0.0052 and high degree of correlation (r^2 = 0.9983) between concentration and corresponding absorbance was observed from the calibration curve. Total phenolic content in the HLEC was calculated to be 1.004 mg GAE/gram crude extract and total flavonoids content was found to be 0.113 mg RE/gram crude extract.

DPPH radical scavenging assay is a well-established and highly reliable method used to screen antioxidant potential of the herbal extracts [22]. In methanol solution, DPPH exist as a purple coloured free radicle which upon reduction by antioxidant agents gets converted into stable and colourless reduced form. This discoloration demonstrates the presence of antioxidants in the plant extract and degree of discoloration is measured spectrophotometrically at 517 nm [22]. In the present study HLEC was subjected to DPPH radicle scavenging assay at different concentrations and antioxidant activity was compared to ascorbic acid. Concentration depended scavenging activity was observed for both standard and HLEC (fig. 1). 62.51±1.78% and 98.72±0.95% DPPH radicle scavenging activity was observed for HLEC and ascorbic acid at 1000 µg/ml concentration respectively.

Phenolic compounds and flavonoids are well known antioxidants and antioxidant properties possessed by the plant or extract are primarily attributed to the total phenolic and flavonoids present in it. Antioxidants have previously been demonstrated to be beneficial in the management of diabetes and its complications [23]. Recent advancement in diabetic research has demonstrated that severe oxidative stress is inflicted during hyperglycaemia, which is primarily responsible for the development and progression of diabetic complications [24]. HLEC possessed high content of phenolic and flavonoid compounds and good DPPH free radical scavenging potential, which may aid in the prevention of excessive oxidative stress induced damage during hyperglycaemic conditions and thereby progression of diabetic complications.

Anti-inflammatory activity

Inhibition of albumin denaturation is simple and rapid method used to screen synthetic or natural compounds/extract for their anti-

inflammatory property [16]. Albumin possesses well defined and specific functions within the living system which is somewhat guided by the complex structure. In response to several external or internal stressors, proteins may losses their well-organized structure and thereby rendering them non-functional. Protein denaturation causes inflammation and several researches depicts that non-steroidal anti-inflammatory drugs (NSAIDs) have excellent ability to inhibit albumin denaturation [25]. On this ground we evaluated anti-inflammatory potential of HLEC in term of its ability to inhibit denaturation of albumin and results are depicted in fig. 2 (a). HLEC showed concentration dependent high potential to prevent denaturation of albumin with maximum inhibition of 81.77±2.27% at 1000 µg/ml concentration which was comparable to diclofenac sodium (88.96±1.72%).

Cellular membrane initiates inflammatory response by releasing arachadonic acid from membrane phospholipids which generates various inflammatory mediators through cyclooxygenase (COX) and lipoxygenase (LOX) pathway. Therefore it is assumed that drugs that are capable of stabilizing cellular membrane will reduce the generation of inflammatory mediators and thus suppress inflammation [26]. For *in vitro* evaluation, RBC membrane is considered to be ideal since it resembles lysosomal membrane, structurally as well as functionally, which is involved in inflammatory reactions within the body. NSAIDs are well documented to stabilize cellular membrane and thereby preventing the release of hydrolytic enzymes stored within lysosome and alleviating inflammation [27]. Results of the effect of HLEC on RBC membrane stabilization were compared to diclofenac sodium and are depicted in fig. 2(b). Consistent dose dependent increase in the membrane stabilization was observed for extract treatment from 250 to 1000 µg/ml concentration. Maximum stabilization of cellular membrane was observed at maximum concentration (66.72±4.15%) which was comparable to that observed for standard drug, diclofenac sodium (83.78±3.14%).

Recent reports demonstrated that diabetes is associated with increased levels of inflammatory mediators in the body. Inflammatory stress intensifies diabetes mediated cellular damage and thereby aid in the development and progression of secondary diabetic complications [28]. Several agents that reduces inflammatory stress during hyperglycaemic state have been demonstrated to benefit diabetes and its associated complications [29]. Here we demonstrated that HLEC possesses high potential to cope up with inflammatory stress through inhibiting protein denaturation and stabilizing of cellular phospholipid membrane. Therefore it may be beneficial in managing the development and progression of diabetic complications.

Anti-diabetic activity

α-glucosidase and α-amylase are responsible for the conversion of unabsorbable dietary polysaccharides into monomer, especially D-glucose, in the gut which are then absorbed and reached blood circulation thereby elevating blood glucose level. Inhibition of these enzymes is associated with reduction in hyperglycaemia through reduction in dietary uptake of glucose [26,30]. This process is mimicked *in vitro* to screen plant extracts for their potential anti-diabetic activity and these are one of the best known and extensively used *in vitro* anti-diabetic assays. In our study we screened HLEC for its potential anti-diabetic activity through α-glucosidase and α-amylase inhibition assay and results are depicted in fig. 3 and fig. 4. Extract treatment resulted in appreciable and concentration dependent inhibition of the activity of both enzymes. HLEC induced maximum inhibition of 62.13±1.04% and 59.9±1.51% for α-glucosidase and α-amylase enzymes at 1000 µg/ml concentration. Standard drug, acarbose, showed much higher inhibitory activity which was observed to be 71.45±1.54% and 93.7±3.11% for acarbose and rutin respectively at 1000 µg/ml. From these results it can be concluded that plant extract possesses appreciable potential to inhibit the activity of these enzymes and thereby may be useful additive to diabetic therapeutic for managing dietary inflow of glucose.

***In silico* docking studies**

Molecular docking is a very efficient tool used for predicting receptor specific activity of molecules by evaluating their binding interaction with the target protein. Docking outcome provide us with the ligand-protein interaction energy (kcal/mol), number of interactions and amino acids involved in it, based on which biological activity of various molecules is predicted [31]. MVD software is used for *in silico* docking studies and we used it to screen compounds present in *C. asiatica* for their interaction with insulin receptor (1IR3). Compounds having minimum ligand-receptor binding energy and maximum interactions with the receptor (<6 Å bond lengths) were predicted to be most effective. Results of the docking studies showing best 5 molecules are depicted in table 1. Compounds showing interaction with insulin receptor may be efficient in modulating hyperglycaemia through insulin signalling. Centella saponin-C, asiaticoside, asiaticoside-E, castilliferol and brahminoside were predicted to be most efficient molecules as depicted by lowest interaction energy, bond length and number of ligand-protein interaction (fig. 5). These molecules showed exceptionally good interaction with 1IR3 and can be considered as potential molecules that may prove to be beneficial in diabetes through their direct action on insulin receptor. However these molecules need to be further screened extensively through *in vitro* and *in vivo* experimentation before reaching any deceive conclusion.

CONCLUSION

These results provide a scientific justification for the traditional anti-diabetic use of this plant. HLEC showed high amount of phenolic and flavonoids which corresponds to its excellent antioxidant activity, as determined by DPPH radical scavenging assay. HLEC inhibited denaturation of protein and stabilized cellular phospholipid membrane thereby exhibited excellent ant inflammatory potential. Further, HLEC inhibited α-glucosidase and α-amylase activity and thereby may control diabetes through lowering dietary glucose uptake. It may benefit in progression of diabetic complications through reducing oxidative and inflammatory stress. Anti-diabetic potential of HLEC may be attributed to the presence of centella saponin-C, asiaticoside, asiaticoside-E, castilliferol and brahminoside, as predicted through *in silico* dicking study against insulin receptor, and needs to be screened further.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest amongst themselves

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