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Research Article

Novel Hexokinase Inhibition by *Glycyrrhiza glabra*: Therapeutic Uses in COVID

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Abstract

Background: The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has been declared a pandemic since it has affected health and economy of the entire world. Glycyrrhizin from *Glycyrrhiza glabra* has the potential to bind to ACE2 receptors and has been proposed as a promising constituent for preventing SARS-CoV-2 infection. The active constituents in *G. glabra* root, glabridin and glycyrrhizin showed in-vitro anti-covid activity and anti-human hexokinase activity. With no specific anti COV-2 drug available, herbal interventions using *G. glabra* extracts appear as promising alternatives for prevention and treatment.

Methods: Molecular mechanism of interaction between glabridin and hexokinase enzyme was predicted using molecular docking techniques and compared with 2-Deoxy-D-glucose, which is known for its potent hexokinase inhibitor activity.

Results: Through systematic molecular docking studies, we observed strong interaction of glabridin and glycyrrhizin with human hexokinase I and II enzymes that was better than the well- known hexokinase inhibitor 2-Deoxy-D-glucose. We disclose properties of a novel herbal dry powder inhaler formulation of the licorice herbal extract and demonstrate its stability and effectiveness in the lung deposition using the twin-impinger system for administration to COVID patients.

Conclusion: Glabridin administration reduces inflammation of intestines, and inhibits the production of inflammatory mediators such as NO, PGE2, and inflammatory cytokines, that advance the disease progression in COVID. With the new findings of hexokinase inhibition, licorice will find its use as effective agents for treatment of COVID, cancer and clinical conditions where hexokinase levels increase significantly more than normal levels.

Keywords: Enzyme(s); Formulation; Inhalation; Lung drug delivery; Molecular modeling; Global Health; COVID

Abbreviations

HIV: Human Immunodeficiency Virus; HCV: Hepatitis C Virus; HSV: Herpes Simplex Virus; HRSV: Human Respiratory Syncytial Virus; HBV: Hepatitis B Virus; COVID-19: Coronavirus Disease 19; SARS-Cov-2: Severe Acute Respiratory Syndrome Coronavirus-2; HPLC: High-Performance Liquid Chromatography; TNF-A: Tumour Necrosis Factor Alpha; IL-6: Interleukin-6; IL-1α: Interleukin-1Alpha; IL-1β: Interleukin-1Beta; ACE2: Angiotensin-Converting Enzyme -2; GLUT: Glucose Transporters; HK: Hexokinase; LGA: Lamarckian Genetic Algorithm; PDB: Protein Data Bank; DPI: Dry Powder Inhaler; UDD: Uniformity Of The Delivered Dose; FPF: Fine Particle Fraction; DUSA: Dosage Unit Sampling Apparatus; 2-DG: 2-Deoxy-D-Glucose; DNA: Deoxy Ribose Nucleic Acid; NO: Nitric Oxide; PG2: Prostaglandin-2; PMDIs: Pressurized Metered-Dose Inhalers

Introduction

SARS-CoV-2 virus has considerably affected the health of people worldwide and more than 263,733,739 patients have been reported to be affected by this virus with a fatality rate of 2% to 3%. Mild pneumonia is common amongst 81% of the mild case category of COVID-19 infection cases where patients recover without any special treatment. A total of 14% of subjects belong to the severe illness category where in addition to dyspnea, disturbances in blood oxygen saturation levels are observed. The critical illness category includes 5% of cases, who experience respiratory failure, septic shock, coagulation disorders, and/or multiple organ dysfunction or failure [1,2].

Therapeutic strategies to deal with COVID infection are merely focused on sustaining a patient's physiological well-being since there are no specific treatments available. Currently, the COVID-19 patients are being treated with drugs such as Fabiravir and Ribavirin, Lopinavir/Ritonavir, Remdesivir, Arbidol, Ivermectin, Chloroquine and hydroxychloroquine, Cyclosporin A, Interferons, Tocilizumab, and plasma therapy, that have been previously used in treating SARS, MERS, and other previous influenza viruses. The antiviral activity of these drugs is based on various modes of inhibition of COVID virus multiplication e.g. inhibition of nucleotide biosynthesis, preventing the binding of virus to host receptors, preventing viral replication through inhibition of viral polymerase and reducing cytokine release [3,4].

Through its spike glycoprotein, the SARS-COV binds to the Angiotensin-Converting Enzyme -2 (ACE2) receptor enabling its entry into the host cell [5]. The ACE2 receptor is localized in various human organs such asoral and nasal mucosa, lungs, kidneys, liver, gut and brain and of these; tongue has the highest levels of ACE2 [6]. These findings clearly indicate that the high risk route for the 2019-nCov infection is through the oral mucosal cavity [7].

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Herbal medicines are known for their activity against coronavirus, coxsackievirus, dengue, enterovirus, hepatitis B virus, hepatitis C virus, herpes simplex virus, human immunodeficiency virus, influenza virus, measles virus, echovirus, and respiratory syncytial virus and this is achieved through various active constituents that have effect on the adsorption, replication, spread, viral polymerase activity and viral inactivation [8]. Some of the plants that exhibit potent anti-viral activities include *Bupleurum* spp., *Heteromorpha* spp., *Scrophularia scorodonia*, *Lycoris radiate*, *Artemisia annua*, *Pyrrosia lingua*, *Lindera aggregata*, and *Glycyrrhiza* roots [9-11].

Glycyrrhiza glabra Linn, cultivated in Italy, Russia, France, UK, USA, Germany, Spain, China, and Northern India, is known for its anti-viral, anti-inflammatory, anti-tumor, anti-microbial, and anti-oxidant properties [12]; it is also used to treat cough owing to its demulcent and expectorant properties. Licorice contains more than 20 triterpenoids and approximately 300 flavonoids; thus, it can effectively treat acidity, jaundice, hiccough, hoarseness, bronchitis, and diarrhea [13]. Among licorice's constituents, Glycyrrhizic acid or Glycyrrhizin has been shown to exhibit anti-viral and anti-microbial activity against HIV, HCV, influenza HSV, rotavirus, coxsackievirus, HRSV, and HBV [14,15]. Licorice extracts are reported to down-regulate the expression of pro-inflammatory cytokines, Tumor Necrotic Factor Alpha (TNFα), Interleukin 1 (IL-1), and Interleukin 6 (IL-6) [16], the levels of which are increased in viral infections such as COVID.

The aqueous *G. glabra* root extracts (flavonoid-rich fraction) exhibits anti-HIV and anti-HSV activities, while the alkaline extract has higher anti-HIV activity [17]. Recently, Glycyrrhizin has been shown to have the potential to bind to ACE2 receptors [18] and since targeting the inhibition of ACE2, which inhibits viral replication in fresh cells, is a promising strategy for preventing SARS-CoV-2 infection [19], there is high potential on use of Glycyrrhizin on COVID infection. In addition, Glycyrrhizin has been reported to induce the production of endogenous interferon [20]; interferons limit the spread of virus by inhibiting replication of both its DNA and RNA at different stages of replicative cycles; therefore, the use of licorice as an agent to increase interferon levels would benefit COVID patients. Interestingly, glycyrrhizin has been used to treat SARS in patients and Hoever et al. [21,22] have shown that addition of 2-acetamido-β-D-glucopyranosylamine group into the glycoside chain of glycyrrhizin resulted in a 10-fold increase in anti-SARS-CoV activity compared to unmodified glycyrrhizin.

Glabridin used as a food and dietary supplement and has potent anti-bacterial activity. Known for enhancing muscle mass [23] and alleviation of hangover associated with drinking [24], this constituent has beneficial effects on cardiovascular protection and obesity [25,26]. Glabridin also inhibits melanogenesis and inflammation in the skin and improves vascular functions [27,28]. Studies by Wu and co-workers demonstrate that glabridin possesses hypoglycemic effects when administered to diabetic animals at 40 mg/kg dose than 10 mg/kg or 20 mg/kg [29].

Inhibition of hexokinase, an intracellular enzyme that phosphorylates hexose sugars such as glucose, fructose and mannose to their corresponding hexose-6-phosphates has previously been shown to be effective against viruses in cell culture and suppressed infection with rhinovirus in mice [30,31]. Recent *in-vitro* studies have shown that with 2-Deoxy-D-Glucose (2DG), one can effectively block glycolysis and prevent SARS-CoV-2 replication in Caco-2 cells [32]. This is achieved through inhibition of cellular uptake of glucose through Glucose Transporters (GLUT) of the mammalian cells causing depletion of ATP, NADH, pyruvate, and nucleotides that are required for cell survival through multiple intermediary metabolites and metabolic pathways. Such a depletion of intracellular ATP, causes necrosis of cells, and cell death eventually due to membrane instability and extracellular ATP release [33].

In the present study, the inhibitory efficacy of glabridin and glycyrrhizin on hexokinase was studied using *in-vitro* hexokinase assay. Molecular mechanism of interaction between glabridin, glycyrrhizin and hexokinase enzyme was predicted using molecular docking techniques and their docking energies compared with the well-known hexokinase inhibitor- 2-Deoxy-D-glucose. This is the first report of glabridin and glycyrrhizin as hexokinase inhibitors. Both *in-vitro* assays and molecular docking study have shown that the glabridin and glycyrrhizin can be used as potent natural inhibitors of hexokinase with better stability and higher bioactivity.

Materials and Methods

Chemicals

Hexokinase calorimetric assay kit (Product no: MAK91) and 2-Deoxy-D-glucose were also purchased from Sigma Aldrich (USA). Glabridin and Glycyrrhizin of >95% purity was procured from Natural Remedies (Bangalore, India). Rest of the reagents used were of analytical grade, unless mentioned otherwise. The glabridin and glycyrrhizin content was estimated in the sample by HPLC as described by Kulkarni et al. [34].

Source and preparation of licorice extracts

The licorice roots were procured from local market from North India and were identified by Mr. P. Santhan, a taxonomist at Durva Herbal, Tamil Nadu, India. The freshly collected samples of licorice roots were washed, air-dried, and stored at 4°C, protected from light and humidity before analysis.

Blending of licorice extracts and micronization

Initially the accurately weighed quantity of licrorice extracts and the excipients were mixed into the mortar and pestle. Both the extracts (acetone and water) were sifted with excipients through 60# sieve. After sifting all the mixtures were blended into the Alphie three-dimensional mixture (Alphie Mixture, Model - Alphie 3, Mumbai, India) at 25 rpm for 30 minutes. The above blend was micronized using air jet mill (Microtech Engineering, Model-M-50, Mumbai, India). Operating parameters of air jet mill were as follows, Milling Pressure 9.0 bar; Feeding Pressure 5.0 bar; Feeder Pressure 2.0 bar; Feed Rate 2.0 gm/min. After milling, the micronized powder was passed through a 60# sieve and mixed in to Alphie three-dimensional mixture at 25 rpm for 30 minutes. The finalized DPI formulation contained both the acetone and water soluble extract of licorice at 40% concentration each along with excipients such as magnesium stearate at 5% concentration. Three batches of such a formulation were made and charged into the size 3 capsules in HDPE bottles as per ICH guidelines. The samples were tested at 1, 3 and 6 months and the 6 months data is represented here.

Hexokinase assay

The Hexokinase (HK) assay was carried out using the HK calorimetric assay kit (Sigma, St. Louis, USA) as per manufacturer's instructions. One unit of HK was defined as that amount of enzyme that generates 1.0 µmole of NADH per minute at pH 8.0. To each well, 50 µL of the sample solution containing inhibitor and HK enzyme

was added followed by 50 µL of appropriate reaction mix. For control samples, similar procedure was followed without addition of the inhibitor solution. The relative HK activity was calculated by taking the observed HK activity without inhibitor solution as 100%.

Statistics

Experiments were done in triplicates and the values are represented as mean ± SD. Statistical analysis was done on Graph-pad prism (version 9) software by applying ONE-WAY ANOVA method including multiple comparisons. To identify significant differences between groups. $P<0.05$ was considered to be statistically significant and $p<0.001$ as highly significant.

Molecular docking studies

Molecular docking of HK I and II with various ligands was performed by AutoDock 4.2.6 program, using the implemented empirical free energy function and the Lamarckian Genetic Algorithm (LGA). In all the dockings, a grid map with 126 × 84 × 82 points and a grid-point spacing of 1.000 Å was applied and the grid maps were calculated using AUTOGRID, version 3.0. The hydrogen bond were depicted using Discovery studio 2020 Client and Chimera software and interactions analyzed using Pymol software, UCSF Chimera and Accelrys Discovery Studio Visualizer software.

The analysis of the docked protein-ligand complexes was carried out for determining the comparative binding energies along with the dissociation constant (K_D) of the docked molecular complexes. For ligand preparation, the 3-D structure of the compounds namely 2-Deoxy-D-glucose, glabridin and glycyrrhizin with their respective PubChem CID were redeemed and saved in SDF format. Furthermore, ligand preparations were continued by taking the 3-D structure of all the ligands and were introduced in Pymol software for conversion of 3-D structure from SDF to PDB format. Using Pymol software, metals were also removed from the ligands structure for an appropriate docking study and further docking studies were carried out using the prepared ligands saved in PDB format. For the Hexokinase I and II, the crystal structure of target proteins hexokinase I protein (PDB: 1CZA) and hexokinase II protein (PDB: 1NZT) was retrieved from Protein Data Bank (PDB) with PDB IDs and was carried further for more studies of docking process. The ligands were docked against with high Auto Dock V4.2 software.

Cytotoxicity and anti-covid assay

The SARS-CoV-2 assay was done at Regional Center of Biotechnology, Faridabad, India following protocols required for handling BSL-3 virus like COVID-2. The assay was done in two parts where at first the vero cells were subjected to cytotoxicity with the compounds to be tested and then the concentration at which the compounds were not cytotoxic, were chosen for the antiviral assay.

The cytotoxicity assay was done in a 96-well plate format in triplicates.1×10^4 Vero E6 cells were plated per well and incubated at 37°C overnight for formation of a monolayer. The grown cells were then incubated with the Test Substance (TS) at different concentrations and 48 h, the grown cells were stained with Hoechst 33342 and Sytox orange dye. 10X images were taken in such a way that the 16 images per well covered >90% of well area using Image Xpress Microconfocal (Molecular Devices). Hoechst 33342 nucleic acid stains all the live and dead cells while Sytox orange dye stains nucleic acids in cells with compromised membranes and is an indicator of cell death.

The anti-covid *in-vitro* assay was done with the monolayered 1×10^4 Vero E6 cells grown in a 96 well for 16 hours after which, the cells were treated with the Test Substance (TS) at a selected non-cytotoxic concentration. The infection with SARS-CoV-2 was initiated by addition of the virus at a MOI of 0.01 and after 48 h, the viral RNA was extracted from the culture supernatant. qRT-PCR for N and E genes of CoV-2 virus was done in duplicates and their respective Ct values determined. The % inhibition of the virus multiplication was calculated by comparing the fold change in the Ct value between the treated and untreated samples. Remdesivir at 10 µM served as a positive control for the study.

Chromatographic conditions

To determine the amount of glabridin and total flavonoids and glycyrrhizin, method of Kulkarni et al. [34] was followed (data not shown).

T1/T2 for determination of licorice DPI

The licorice DPI was subjected for the aerodynamic assessment of fine particles [35], with the twin impinger over Next Generation Impactor (NGI) in this study to avoid problems of herbal extract to clog the NGI parts and thus, affecting the drug recovery. The Glass Twin Impinger assembly, that separates respirable and non-respirable portions of the drug, was placed vertically and the jet-spacer peg of the lower jet assembly was made to touch the bottom of the lower impingement chamber and with the help of a suitable pump. The airflow was adjusted at 60 ± 5 liters per minute and the pump was switched on for 5 seconds and each capsule with the licrorice DPI was pierced by means of the device. This procedure was repeated twice. The samples, T1 and T2 were prepared using diluent (methanol 80: water 20) and used for HPLC analysis after filtering the solution through 0.45 µm nylon filter. The part B, C and D represent sample T1 while part E, F, H and G form T2.

The Uniformity of the Delivered Dose (UDD) was calculated by DUSA apparatus at an airflow rate Q, which produces a pressure drop of 4.0 kPa (40.8 cm H_2O) over the capsule to be tested at duration T, consistent with the withdrawal of 4 liters of air through capsule following the method described by Gondhale et al. [36].

Ethical statement

There are no animal or human experiments conducted to generate data that are described in this paper. Hence there is no ethical statement to declare.

Results

Table 1 gives the % inhibition of COVID multiplication done using pure glabridin and glycyrrhizin at 30 nM and 10 nM final concentrations respectively. Figure 1 shows the *in-vitro* inhibition of hexokinase by 2-DG, pure glabridin, pure glycyrrhizin and licorice DPI. The docking energy data of 2-DG, glabridin and glycyrrhizin against Hexokinase I and II is shown in Table 2.

Figures 2 and 3 depict the molecular docking studies of 2-DG with hexokinase I and hexokinase II respectively. There are four amino acids of HK I involved in a hydrophobic interaction with 2-DG (Asp79, Pro149, Lys 147, Tyr 461) which is seen in Figure 2D, while the three amino acids involved in a hydrophobic interaction with 2-DG are Glu79, Pro149, Lys 147 as seen in Figure 3D. Figures 4 and 5 depict the molecular docking studies of Glabridin with hexokinase I and hexokinase II protein respectively. The five amino acids of HK I involved in a hydrophobic interaction with glabridin are His 99, lys146, leu148, pro149 and Ala458 as depicted in Figure 4D while

Table 1: Effect of Glabridin and Glycyrrhizin on in-vitro multiplication of COVID-2 virus.

Compound	Concentration	Cell viability % after 48 h	% inhibition of virus replication (E), 48 h	% inhibition of virus replication (N), 48 h
Remdesivir*	10 µM	94.37	99.64	99.76
Glabridin	10 nM	93.59	69.02	62.37
Glabridin+ Glycyrrhizin	10 nM each	90	91.16	91.39

*Remdesivir was used as a positive control.

Table 2: Comparative evaluation of the binding site residues for Glabridin, Glycyrrhizin and 2-DG on predicted protein Hexokinase I and II.

Protein	Ligand	Binding Energy (kcal/mol)	No. of H Bonds	Interacting residue	Final Intermolecular Energy (kcal/mol)	vdW+Hbond+desolv Energy (kcal/mol)	Electrostatic Energy (kcal/mol)	Torsional Free Energy (kcal/mol)
1CZA	2-Deoxy-D-Glucose	-3.32 kcal/mol	03 (H1=2.13Å, H2=2.39 Å, H3=2.74Å,)	TYR:461(H1), LYS:147(H2), PRO:149, ASP:79(H3)	-3.42 kcal/mol	-3.42 kcal/mol	-0.12 kcal/mol	2.68 kcal/mol
1CZA	Glabridin	-5.77 kcal/mol	1 (H1=2.71Å)	ALA:458(H1), PRO:149, LYS:146, LEU:148, HIS:99	-7.25 kcal/mol	-2.73 kcal/mol	-4.52 kcal/mol	+4.47 kcal/mol
1CZA	Glycyrrhizic acid	-2.78 kcal/mol	03 (H1=1.58Å), (H2=2.48Å), (H3=2.19Å)	LYS:880(H1), LYS:481(H2), LYS:885(H3)	-7.25 kcal/mol	-2.73 kcal/mol	-4.52 kcal/mol	+4.47 kcal/mol
2NZT	2-Deoxy-D-Glucose1	-3.40 kcal/mol	02 (H1=2.52Å, H2=2.43Å)	LYS:147(H1), PRO:149, GLU:79(H2)	-3.52 kcal/mol	-3.42 kcal/mol	-0.09 kcal/mol	+2.68 kcal/mol
2NZT	Glabridin	-6.15 kcal/mol	3 (H1=1.84Å, H2=2.83Å, H3=2.99Å)	PHE:334(H1), SER:415(H2), LYS:419, LYS:337(H3)	-7.04 kcal/mol	-6.74 kcal/mol	-0.30 kcal/mol	+0.89 kcal/mol
2NZT	Glycyrrhizic acid	-4.14 kcal/mol	02 (H1=1.78Å), (H2=1.85Å)	LYS:425(H1), ARG:432(H2), ALA:424	-8.61 kcal/mol	-7.14 kcal/mol	-1.48 kcal/mol	+4.47 kcal/mol

Protein name: 1CZA (Hexokinase); Organism: Homo sapiens; Resolution: 1.90 Å; Gene: *HK1*; Sequence Length: 917; Protein name: 2NZT (Crystal structure of Human Hexokinase II); Organism(s): Homo sapiens; Resolution: 2.45 Å; Gene: *HK2*; Sequence Length=902

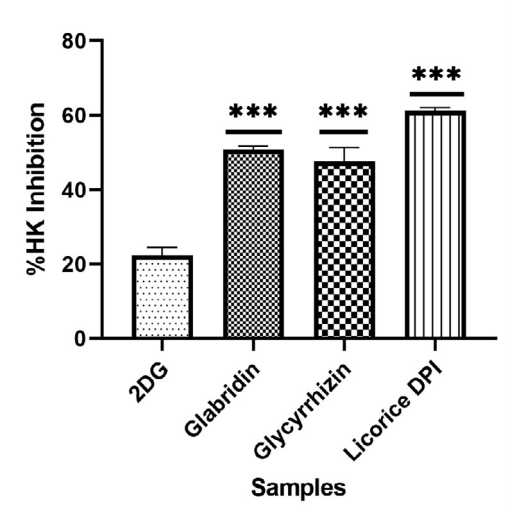

Figure 1: Hexokinase inhibition potential of 2-deoxy-2-glucose (2-DG), Glabridin, Glycyrrhizin and Licorice DPI. All values are mean ± SD, n=3. ***P<0.001 as compared to 2-DG.

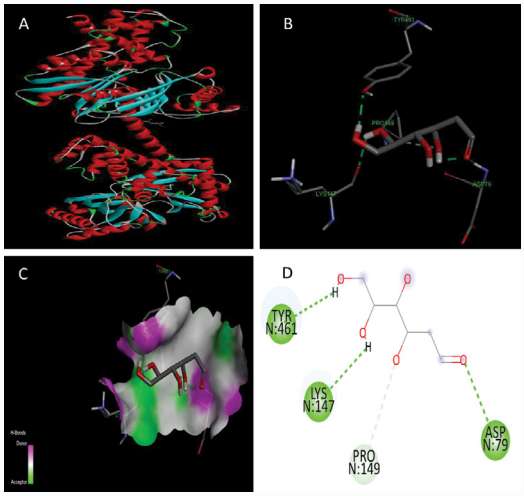

Figure 2: Molecular docking of 2-Deoxy-D-Glucose binding with Hexokinase I (PDB ID: 1CZA) shows 3D model of the interactions and the 2D interaction patterns and H-bond interaction. The red spiral represents the Alpha Helix, the cyan blue spiral represents the beta pleated sheet, and the green string represents the Turns of Coils. Panel A shows the interaction of the ligand (2-DG) with protein HK I; Panel B shows the 3D representation of the ligand 2-DG; Panel C shows the overall 3D surface view of the modeled protein HK I represented to display all the possible H-bond donor (pink color) and acceptor group (green color) when complexed with 2-DG and panel D shows the 2D representation for the docked complex of the 2-DG. The different colors (Vander Waals- green; purple) covalent bonds have been used for showing the different types of molecular interactions involved.

the amino acids of HK II involved in a hydrophobic interaction with glabridin were Phe334, lys337, Ser415 and Lys419 (Figure 5D).

The molecular docking studies of glycyrrhizin with hexokinase I and II is given in Figures 6 and 7 respectively. The Discovery studio visualizer enabled us to get the 2D and 3D interactions of the docking results and aided in identifying significant interaction between the ligands and the receptor binding site of the Hexokinase I and II. The seven amino acids of HK I involved in a hydrophobic interaction with glycyrrhizin were lys481, Arg853, pro884, Gln877, Lys880, Glu881, Lys 885 as can be seen from Figure 6D while the nine amino acids of HK II involved in a hydrophobic interaction with glycyrrhizin were Lys418, Pro421, Tyr417, His420, Ala424, Lys 425, His428, Arg 432, Arg444 (Figure 7D). The unique stability of glabridin in the binding site of HK I and II is attributed to the Pi-interactions. The Pi-alkyl

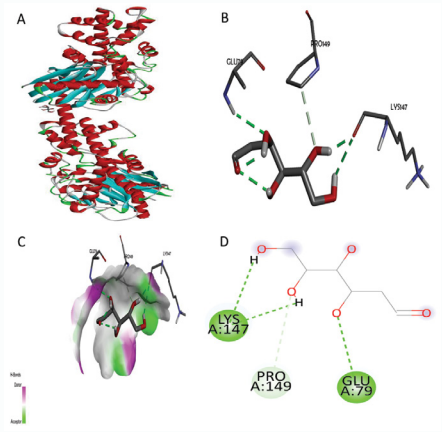

Figure 3: Molecular docking of 2-Deoxy-D-Glucose with Hexokinase II (PDB ID: 2NZT) shows H-bond interaction. The red spiral represents the Alpha Helix, the cyan blue spiral represents the beta pleated sheet, and the green string represents the Turns of Coils. Panel A shows the interaction of the ligand (2-DG) with protein HK II; Panel B shows the 3D representation of the ligand 2-DG; Panel C shows the overall 3D surface view of the modeled protein HK I represented to display all the possible H-bond donor (pink color) and acceptor group (green colour) when complexed with 2-DG and panel D shows the 2D representation for the docked complex of the 2-DG. The different colors (Vander Waals- green; purple) covalent bonds have been used for showing the different types of molecular interactions involved.

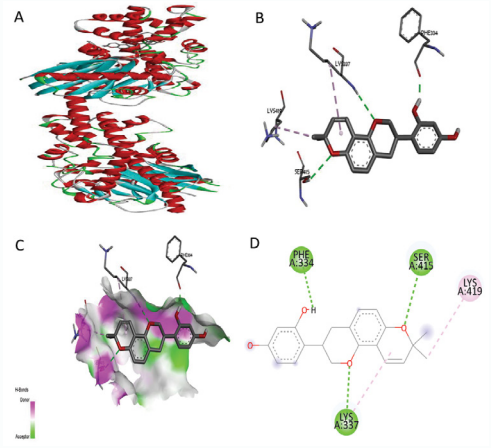

Figure 5: Molecular docking of Glabridin binding with Hexokinase II (PDB ID: 2NZT) shows 3D model of the interactions and the 2D interaction patterns and H-bond interaction. The red spiral represents the Alpha Helix, the cyan blue spiral represents the beta pleated sheet, and the green string represents the Turns of Coils. Panel A shows the interaction of the ligand (Glabridin) with protein HK II; Panel B shows the 3D representation of the ligand glabridin; Panel C shows the overall 3D surface view of the modeled protein HK II represented to display all the possible H-bond donor (pink color) and acceptor group (green colour) when complexed with glabridin and panel D shows the 2D representation for the docked complex of the glabridin. The different colors (Vander Waals- green; purple) covalent bonds have been used for showing the different types of molecular interactions involved.

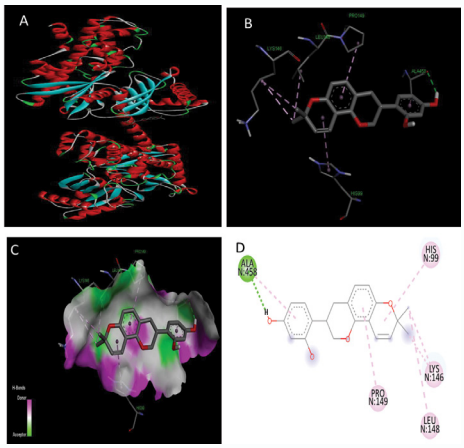

Figure 4: Molecular docking of Glabridin binding with Hexokinase I (PDB ID: 1CZA) shows 3D model of the interactions and the 2D interaction patterns and H-bond interaction. The red spiral represents the Alpha Helix, the cyan blue spiral represents the beta pleated sheet, and the green string represents the Turns of Coils. Panel A shows the interaction of the ligand (Glabridin) with protein HK I; Panel B shows the 3D representation of the ligand glabridin; Panel C shows the overall 3D surface view of the modeled protein HK I represented to display all the possible H-bond donor (pink color) and acceptor group (green colour) when complexed with glabridin and panel D shows the 2D representation for the docked complex of glabridin. The different colors (Vander Waals- green; purple) covalent bonds have been used for showing the different types of molecular interactions involved.

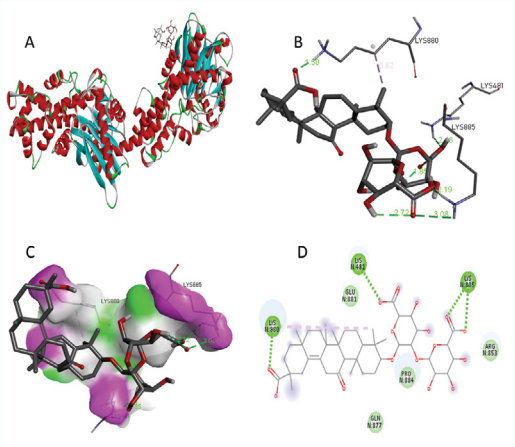

Figure 6: Molecular docking of Glycyrrhizin binding with Hexokinase I (PDB ID: 1CZA) shows 3D model of the interactions and the 2D interaction patterns and H-bond interaction. The red spiral represents the Alpha Helix, the cyan blue spiral represents the beta pleated sheet, and the green string represents the Turns of Coils. Panel A shows the interaction of the ligand (Glycyrrhizin) with protein HK I; Panel B shows the 3D representation of the ligand glycyrrhizin; Panel C shows the overall 3D surface view of the modeled protein HK I represented to display all the possible H-bond donor (pink color) and acceptor group (green colour) when complexed with glycyrrhizin and panel D shows the 2D representation for the docked complex of the glycyrrhizin. The different colors (Vander Waals- green; purple) covalent bonds have been used for showing the different types of molecular interactions involved.

interaction of glabridin was seen with Lys146, Leu 148, His99 and Pro 149 of HK-I and with Lys419 of HK-II. With glycyrrhizin, the Pi-alkyl interactions was seen only with HK-II with Ala424 (Figure 7D) and no such interactions with HK-I (Figure 6D). Since such Pi-interactions are not seen of 2 DG with HK I and II, its interaction with HK appears weakest amongst the ligands tested.

Figure 8 shows the stability data of the licorice DPI at various temperatures for glabridin while stability of the licorice DPI in terms of content of glycyrrhizin is shown in Figure 9.

Discussion

In accordance with WHO recommendations, detection of genes namely E gene and N gene allow to detect viruses from the beta-coronavirus group (*E gene*), as well as to identify SARS-CoV-2 virus (*N gene*). Our observations of >90% inhibition of COVID multiplication

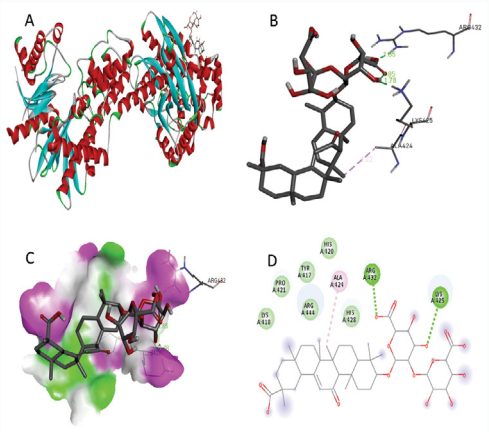

Figure 7: Molecular docking of Glycyrrhizin binding with Hexokinase II (PDB ID: 2NZT) shows 3D model of the interactions and the 2D interaction patterns and H-bond interaction. The red spiral represents the Alpha Helix, the cyan blue spiral represents the beta pleated sheet, and the green string represents the Turns of Coils. Panel A shows the interaction of the ligand (Glycyrrhizin) with protein HK II; Panel B shows the 3D representation of the ligand glycyrrhizin; Panel C shows the overall 3D surface view of the modeled protein HK II represented to display all the possible H-bond donor (pink color) and acceptor group (green colour) when complexed with glycyrrhizin and panel D shows the 2D representation for the docked complex of the glycyrrhizin. The different colors (Vander Waals- green; purple) covalent bonds have been used for showing the different types of molecular interactions involved.

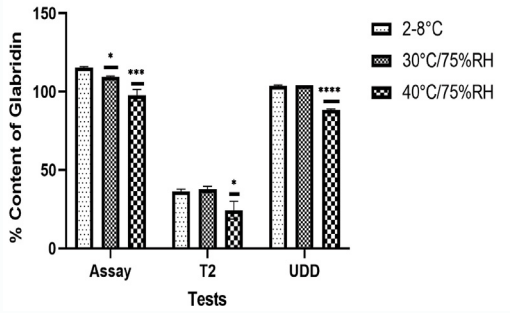

Figure 8: Stability studies of Licorice DPI with respect to Glabridin in HDPE pack. All values are mean ± SD, n=3. ****P<0.001, ***P<0.001 and **P<0.01 for 30°C/75%RH and 40°C/75% RH conditions when compared to 2°C-8°C.

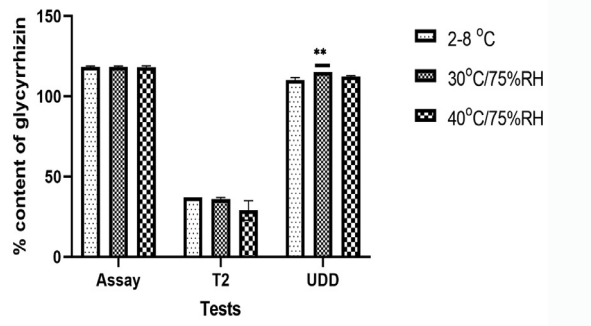

Figure 9: Stability studies of Licorice DPI with respect to Glycyrrhizin in HDPE pack. All values are mean ± SD, n=3. ****P<0.001, for 40°C/75% RH condition when compared to 2°C-8°C.

with an equal mixture of glabridin and glycyrrhizin at 30 nM and 10 nM final concentration respectively in the *in-vitro* assay shows promising potential of use of licorice extract for COVID control.

2-Deoxy-D-glucose, a glucose mimic molecule, through hexokinase inhibition forms 2-Deoxy-d-Glucose-6-Phosphate (2-DG6P), in place of glucose 6 phosphate and induces cell death. It is reported that the glycolytic and other associated pathways is up regulated by Coronavirus (SARS-CoV-2) to obtain substrates vital for its structure, function, and replication. Hence, hexokinase inhibitors have promising potential for affecting COVID multiplication. Also, the increased levels of pro inflammatory cytokine IL-1β in COVID-19 patients is through generation of 3-phosphoglycerate, a by-product of glycolysis suggesting a direct link of Covid to glucose metabolism [37]. Hence, in COVID affected patients with metabolic diseases, targeting the glucose metabolism could serve an effective way to control viral multiplication and this could also support organs that are affected due to COVID.

Our results with Licorice DPI indicating 60% inhibition of hexokinase activity when 0.022% of the licorice powder is used in the hexokinase assay is novel and opens up a new possibility of usage of this health supplement for addressing COVID. Interestingly, pure glabridin and glycyrrhizin, which are the major flavonoids of licorice extracts, showed inhibition of hexokinase at 1 mM final concentration attributing to the hexokinase inhibition by licrorice DPI to these two active constituents glabridin and glycyrrhizin. This is the first report on observations of glabridin and licorice in inhibition of human hexokinase and opens up the possibility of using these as effective anti-cancerous agents since it affects basic respiration of cells.

The *in-vitro* hexokinase inhibition by glycyrrhizin and glabridin supported by the AutoDock runs which has shown the binding energy scores from -2.8 kcal/mol to -5.77 kcal/mol in the order glabridin>2-DG>glycyrrhizin, where we used 2-DG, a well-known hexokinase inhibitor as a positive control. Among the three inhibitors selected for docking studies, glabridin showed best binding with HK I while with HK II, the best binding followed the order glabridin>glycyrrhizin>2-DG. The docking score had a good correlation with experimentally reported most potent inhibition of hexokinase (50%) showed a highest dock score (-6.1 kcal/mol) than the well-studied inhibitor 2-DG (20% hexokinase inhibition with a docking score of -3.4 kcal/mol).

Hexokinase inhibition is reported to induce apoptosis of the cells through intrinsic and extrinsic pathways [38]. The activity of hexokinase-2, is suggested to be dependent on physical interaction with Mitochondrial Antiviral-Signaling Protein (MAVS) [39] and agents that affect such interaction finally increases type I IFN production, that helps to combat viral infection [40]. Hence, hexokinase inhibitors are promising alternative for inhibiting viral infections including COVID.

2-deoxy-D-glucose affects synthesis of both the host cell DNA and viral DNA which in turn affects the Herpesvirus replication [41] and hence our results on COVID replication inhibition by pure glycyrrhizin and glabridin support such literature reports. The drugs such as 2-deoxyglucose, 3-bromopyruvate and metformin are found to be HK II inhibitors [42], which block glucose metabolism in cancer cells, inhibit its proliferation with minimum side effects [43], hence it is tempting to speculate the possible use of such constituents as anti-cancer agents. Earlier studies reported the potential of 2-deoxyglucose to inhibit novel Coronavirus by acting on HK II [33,44]. Hence, our observations of inhibition of glabridin and glycyrrhizin on COVID multiplication opens up new therapeutic uses of licorice in such clinical conditions.

Glabridin has been reported to have anti-viral activity against HBV [45,46]. The oral administration of glabridin has been shown to be effective in reducing inflammation of intestines, reducing the incidence of septic shock, and inhibiting the production of inflammatory mediators (e.g., NO, PGE2, and inflammatory cytokines) that are essential in COVID infection.(47) Because NO and PGE2 production is essential in the pathogenesis of COVID, and PGE2 expression is essential for the viral replication of a positive-strand RNA virus [48], it may be useful to administer glabridin in therapeutic and control disease progression in COVID patients.

In mammalian tissues, four isoforms of HK-I, HK-II, HK-III, and HK-IV are known [49] and of these, the HK with least affinity for glucose is HK-IV. Being predominant in cancer cells, HK-I and HK-II inhibition would be potential anti-cancer agents [50,51]. It is tempting to speculate that both glabridin and glycyrrhizin might find use in cancer treatment based on the hexokinase inhibition studies.

Due to low toxicity and lesser side effects, herbal drugs have been the drugs of choice by nearly 80% of world population [52]. There are, however, some limitations such as poor aqueous solubility, susceptible to degradation by low gastric pH, oxidization of some of the herbal medicines which causes irregular absorption from oral solid forms. Hence, alternate routes of delivery of herbal drugs/actives, such as pressurized Metered-Dose Inhalers (pMDIs) and Dry Powder Inhalers (DPIs), appear attractive.

The stage 2 results of >40% of glycyrrhizin and glabridin from the licrorice DPI capsules are in broad agreement with the stability results for similar products obtained with the twin impinger stages I and 2 [53]. The stability data of the samples (Figures 8 and 9) show that the assay for glabridin and glycyrrhizin content was similar in all the stability conditions and the Uniformity of Delivered Dose (UDD) and T2 fraction also remained constant at all temperatures. The reduced % of assay in stage 2 of samples charged at 40°C is possibly due to the DPI powder becoming sticky when exposed to the temperature since both glycyrrhizin and glabridin are stable at 40°C, which is substantiated by the stable assay results of both these constituents at all temperatures.

Due to their effect on COVID multiplication and hexokinase inhibition, we believe both the both glycyrrhizin and glabridin constituents from *G. glabra* root extract would prove effective on COVID patients. Because DPI devices enable delivery of the drugs directly into the lungs, the developed licorice DPI formulation would be a cost-effective method to treat Coronavirus and alleviate other symptoms.

GCSF therapy has been determined to benefit critically ill patients with severe sepsis or septic shock [54]. Because sepsis shock is common in COVID patients [55] and licorice extract induces GCSF secretion [56], one can hypothesize that GCSF may have an anti-inflammatory effect [57], in COVID patients when licorice extracts are administered. Licorice ethanolic extract has been reported to inhibit LDL oxidation by a mechanism involving scavenging of free radicals [58] and the immune-boosting properties of glycyrrhizin have been previously reported [59]; hence the use of licorice DPI will contribute to enhancing the overall immunity in COVID patients.

Prevention of blood clots that appear in 20% to 30% of the critically ill COVID-19 patients [60], using blood thinners appears to be inconsistent and results in their death owing to stroke as a result of clot blockage in the brain. *G. glabra* has been reported to be a thrombin inhibitor [61]; thrombin inhibitors are often used as blood thinner alternatives to heparin and warfarin; therefore, it is possible to reduce the formation of blood clots in COVID patients using *G. glabra* extract.

Oxygen consumption is affected during COVID infection. Breath tests performed during ergometer exercises after taking a single dose of 600 mg of licorice glabra polyphenols (Glavonoid™) have shown that the mean value of oxygen consumption (VO2) of subjects in the licorice glabra polyphenols (Glavonoid™) group was significantly higher than that in the placebo group, which corresponded to enhanced caloric consumption [28]. Thus, glabridin will improve oxygen consumption in COVID patients.

Licorice root extract reduces liver inflammation and alleviates hepatic injury, which normalizes the level of hepatic enzymes [62]; therefore, the use of licorice extract benefits COVID patients. The oral administration of glabridin has been shown to be effective in reducing inflammation of intestines, reducing the incidence of septic shock, and inhibiting the production of inflammatory mediators (e.g., NO, PGE2, and inflammatory cytokines) that are essential in COVID infection [47]. Because NO and PGE2 production is essential in the pathogenesis of COVID, it may be useful to administer glabridin in therapeutic doses. PGE2 expression is essential for the viral replication of a positive-strand RNA virus [48]. The ability of glabridin to reduce production of nitric oxide and prostaglandin E2 may help control disease progression in COVID patients [63].

The hydrophobic flavonoids and glabridin of licorice have been proven safe at the dose of 300 mg/person -1200 mg/person [64]. Since delivering an effective concentration of the drug into the lungs is critical for an effective therapy for COVID, we believe that a formulation that provides drugs directly into lungs of COVID patients would be beneficial. The ACE2 receptor on airway epithelial cells is essential for viral infectivity since it helps in transport of the virus. Hence, the current article showing the delivery of licorice active constituents to lungs directly could help in reduction in the progression of the COVID infection.

Also, metformin, the drug most widely used to treat type 2 diabetes, has been shown to selectively inhibit HK isoforms I and II and hence has proven to be a potent anti-cancer agent [65]. The anticancer properties of glabridin is reported through caspase -3, -8, and -9 activation and poly (ADP-ribose) polymerase cleavage [66] and hence we were curious to look at hexokinase inhibition by glabridin. Before the in-vitro experiments, we carried out molecular docking studies between hexokinase 1 and 2 with pure glabridin and glycyrrhizin and based on docking studies, we carried out in-vitro hexokinase enzyme inhibition studies.

Formulation as a dry powder for inhalation is an ideal method to deliver drugs directly to lungs. Our current disclosure of preparing a simple DPI method of licorice extract outweighs the method described by Vishwanathan et al. [67]. Since herbal extracts are prone to moisture absorption, any formulation that is in dry state would always be beneficial. Our results of >90% inhibition of COVID replication by 10 nM of glabridin and glycyrrhizin is far superior to reports shown by other compounds such as Clemastine [68] and Ivermectin [69], opens up the possibility of further research on these lines. A recent study by Li et al. [70] propose that due to its easy absorption and inhibition activity on a few metabolic enzymes, glabridin can improve the pharmacokinetic characteristics of other medicines, hence this

research article will be immensely useful for researchers in academics and industry alike.

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Author Contributions

Contributor SP was responsible for conceptualization, methodology, investigation and writing the original draft. PK was responsible for carrying out hexokinase inhibition studies, and statistical analysis and investigation of the data. RB carried out standardization of the hexokinase assay and SY was responsible for resources and preparation of licorice extracts used for the formulation studies. VM was responsible for developing formulation of the dry powder inhaler described in this study while PG carried out analysis of the DPI formulation using T1/T2 twin impinger system. All authors approved the manuscript to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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