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ORIGINAL RESEARCH

Structural analysis and prediction of potent bioactive molecule for eNOS protein through molecular docking

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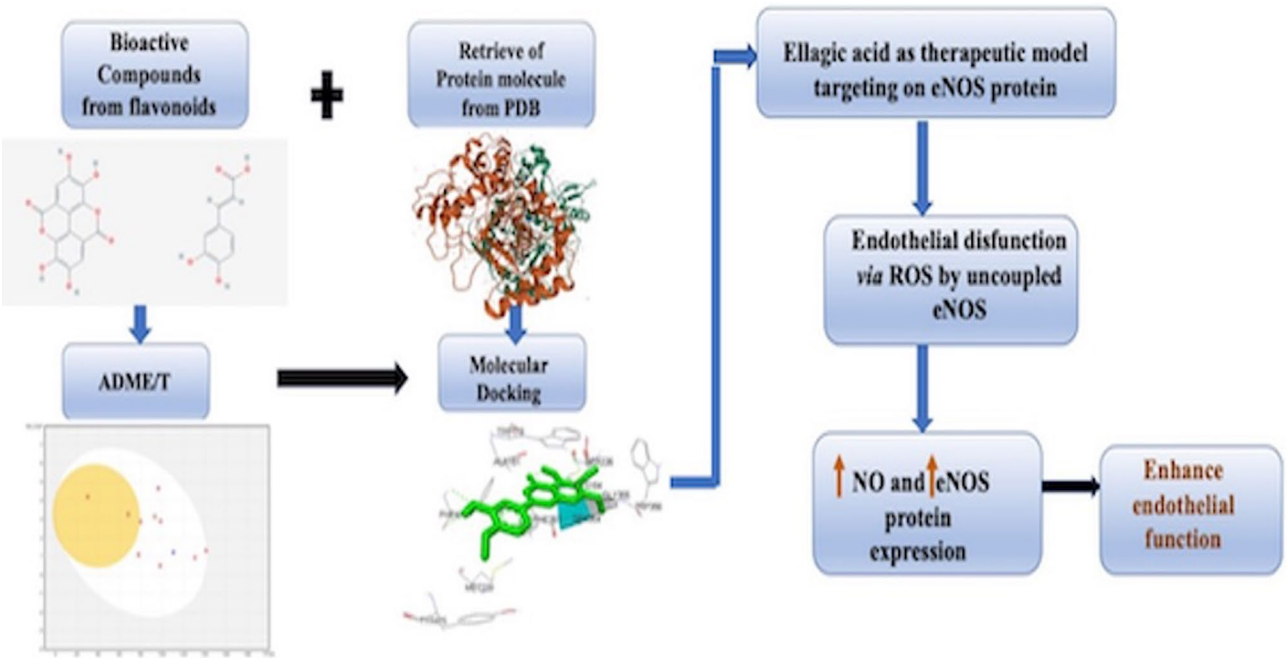
Abstract

Reactive oxygen species by uncoupled eNOS is linked to endothelial dysfunction. Ellagic acid (EA), a polyphenol possesses numerous biological activities including radical scavenging. whether EA exerts a vasculo-protective effect via antioxidant mechanisms in blood vessels remains unknown. Molecular docking provides an initial model of protein and molecular interactions in various physiological and/or pathological functions. To identify a eNOS modulatory biomolecule through molecular docking as possible vascular protective agent. On the basis of binding affinities and other physicochemical features, a molecular docking-based approach was used to classify and evaluate eNOS binding micronutrients found in natural sources, Lipinski's rule was used taking into account their adsorption, delivery, metabolism, and excretion (ADME). An *insilico* approach focused on the ligand–protein interaction technique to determine the therapeutic potential of certain phytochemical-based drugs for the vascular remodelling.20 bioactive molecules were screened, docking analysis on human eNOS proteins was performed. The best poses for target protein was established based on binding energy and inhibition constant. EA and caffeine acid are the strongest candidates for eNOS protein functional norms. This provides a novel insight into the interaction properties of known human eNOS protein with EA and used as a therapeutic agent in various pathologies.

Graphic abstract

Predicting interaction of ellagic acid with eNOS protein by molecular docking in endothelial dysfunction.



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Keywords Ellagic acid · eNOS · Vascular dysfunction · Reactive oxygen species · Docking · Virtual screening

Introduction

Several cardiovascular functions are carried out by nitric oxide (NO), it is an important smallest inter and intracellularsignaling molecule (Kawashima and Yokoyama 2009).It is produced by three homologous isoforms of nitric oxide synthase (NOS) in diverse mammalian tissues. These three isoforms are nNOS, iNOS and eNOS. They are encoded by distinct genes and share 50–60% sequence identity, differ in size,intracellular location, regulation, also catalytic and inhibitory properties (Devika et al. 2014).

Among three isoforms, eNOS is the major source of vascular NO and important factor for vascular homeostasis. It controls systemic blood pressure, angiogenesis, regulates gene transcription and mRNA translation, vascular remodeling, and smooth muscle relaxation (Förstermann and Sessa 2012).

The nitric oxide synthase (NOS) present in vascular endothelial cells is a multi-domain enzyme (Fleming and Busse 1999).Under physiological conditions, it is effectively regulated at the transcriptional, posttranscriptional, and posttranslational levels (Rafikov et al. 2011).

eNOS coding gene is located on chromosome 7 which is responsible for synthesis of NO (Nitric Oxide). Any anomaly in the gene may cause reduction in NO level, more generation of ROS and endothelial dysfunction (Lima et al. 2019).

This dysfunction appears as a reduced expression of eNOS, rather to a reduced mechanical or chemical activation of eNOS or to an excessive removal of NO through reaction with ROS (Gliemann et al. 2017). As a consequence, an increased production of ROS by uncoupled eNOSis possibly to share significantly to vascular oxidative stress and endothelial dysfunction Shear stress in the microcirculation (Xia et al. 2016).

Polyphenols from the wide ranging family of phytochemicals possess many medicinal properties like antidiabetic, hypolipdemic, antioxidant activity (Kanthe et al. 2021). Identification and Synthesis of active micronutrients from many phytoconstituents for the promising field of medicine is of great drive among researchers.

In recent years, many small molecules have been identified, those have potential to interact with eNOS protein and similarly may enhance eNOS expression. Ellagic Acid is a micronutreint phenol, primarily known as an antioxidant which has an ability to donate hydrogen atoms or electrons. Ellagic acid effectively lowers the levels of plasma lipids, reduces oxiadtive stress and inhibits lipid peroxidation (Majid et al. 1991). Ellagic Acid can be a promising drug in combating against the vascular complications.

There is an emerging trend in identifying a novel bioactive molecules with its compatible protein and use as a therapeutic agent in various pathologies. Functions of proteins are finely correlated by interactions with other molecules and rightly connected with its structural and internal dynamics. Bioinformatics studies have implied on various work to show interactions of various structures of molecules with different proteins. On this basis, molecular docking provides an initial model of protein and molecular interactions in view of various physiological and/or pathological functions (McWilliam et al. 2013).

Hence, the present study was aimed to find a potent phytochemical-based drug molecule that enhances NO production by up-regulating eNOS expression The Cheminformatics pipeline approach was used to screen for promising drug molecules. These molecules were investigated further using molecular docking, protein–ligand interactions, and in silico ADMET studies.The ellagic acid had strong binding energy, fulfilled the Lipinski rule of five, and had no toxicity, according to the molecular docking review.

Materials and methods

Target identification and sequence retrieval

The FASTA sequence of selected organisms were retrieved from UniProt database. These sequences were used for further analysis purpose.

Multiple sequence alignment and phylogenetic analysis

The multiple sequence alignment and phylogenetic tree of selected proteins was performed in CLUSTAL OMEGA of EBI which is online tool for MSA (Boratyn et al. 2013).

Protein domain identification and target validation

The conserved domains of human eNOS protein were identified by Pfam database and the results were cross-checked with NCBI CDD-tool.To find out the catalytic domain, a significant protein match was prepared by using the BLAST(Basic Local Alignment Search Tool) provided by NCBI (Boratyn et al. 2013).

Prediction of subcellular localization, signal peptide, and physicochemical characterization

For human eNOS protein subcellular localization prediction was carried out by CELLO tools. The presence of signal peptide was checked by Signal P 4.1server and physicochemical

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characterization was done by the ExPASyProt Param tool (Yu et al. 2004).

Phosphorylation profile analysis and prediction of accessible surface area (ASA)

Phosphorylation sites is post translation modification and it is important for proteins and their transportation and functions. Phosphorylation profile analysis of eNOS protein was carried out by Netphos 2.0 server and Accessible surface area was predicted through Net Surf (Petersen et al. 2011).

Generation of secondary structure and Ramachandran plot

The secondary structure of eNOS was predicted by using PDBsum server. To find the percentage of favourable residues the Ramachandran plot was generated in RAMPAGE (Gasteiger et al. 2005).

Selection of ligand, receptor, and active site prediction

Based on literature review, 20 bioactive molecules were selected and their 3D structures were downloaded from Pubchem and NPACT (Blom et al. 1999). All ligands were prepared for molecular docking which included the formation of tautomers and ionization states, addition of hydrogen atoms, neutralisation of charged groups and optimisation of the geometry of the ligands. To explore the binding sites of ligands (inhibitors) on the eNOSstructure, its 3D-structure was retrieved from RCSB-PDB (Fig. 1). Active sites of human ACE were retrieved from PDBsum (Parvatikar and Madagi 2018).

Preparation of ligands and receptor

Ligands and receptor were prepared for docking by minimizing their energy and then 3D by removing solvent molecules (water) and other sites on eNOS, facilitating the interaction of only bioactive compounds or ligands with the selected receptor.

ADME property analysis

ADME property analysis was performed in SwissADME tool. In this tool Egan BOIED-Egg method (Brain Or IntestinaLEstimateD permeation predictive model), gives threshold value and graphical representation about characterised of drug molecule.

Molecular docking

For molecular docking purpose Autodock 4.2 was used. Using genetic algorithm, extra precision docking was performed with the prepared protein and the ligands (Pinto-Junior et al. 2017). Structures of ligands were kept flexible to generate different conformations. Receptor grid generation workflow was used to define a grid (box) around the ligand, to keep all the functional residues in the grid. A receptor grid of $10 \times 10 \times 10$ Å was generated around the active site residues of crystal structure. All the ligands were docked into

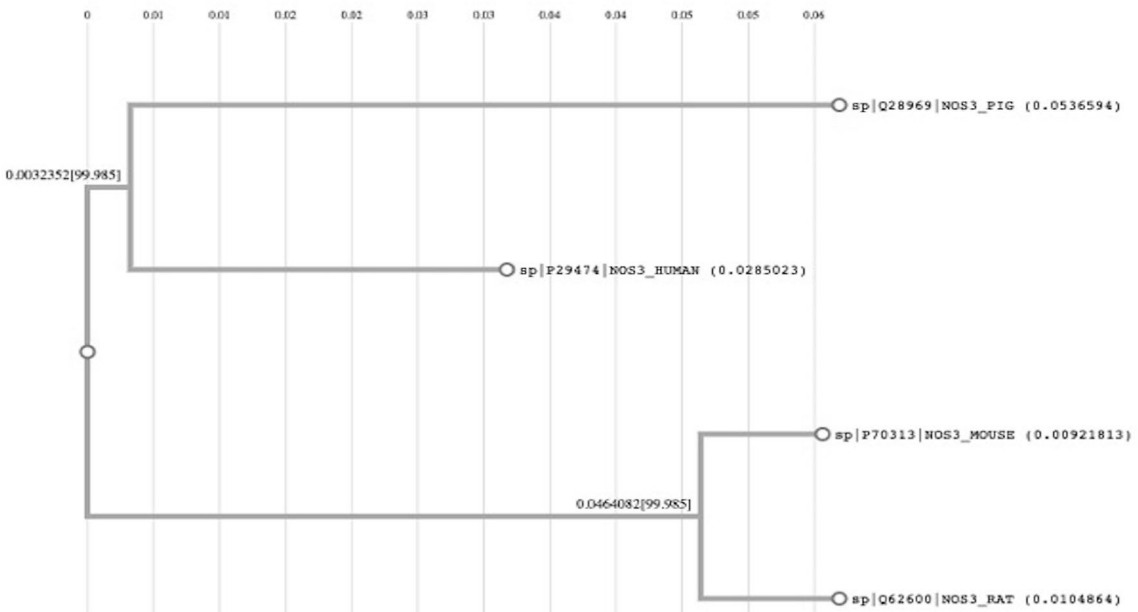

Fig. 1 Phylogenetic analysis of eNOS protein for selected organisms

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the binding pocket of a target and then complexes with high docking scores were forwarded to the next steps. Docking was performed on Intel® Core™ i3-Dell laptop with 8 GB RAM, Windows 8Pro operating system. All the results were visualized in Discovery studio.

Result

Target identification and sequence retrieval

The amino acid sequence of the TG2 protein was retrieved from Uniprot after a protein database scan. The target protein is prevalent in mammals, and the research will aid in understanding the conserved domain and evolutionary relationship of human eNOS with the selected mammals. One of the goals of the research was to determine the suitability of various animals as drug screening models (Table 1).

Multiple sequence alignment and phylogenetic analysis

The result of multiple sequence alignment by using the CLUSTAL OMEGA showed that the amino acid residues of eNOS protein were conserved throughout the sequence. The alignment score of 44,080 suggested that this protein can act as a good target protein for further work (Fig. 1).

After multiple sequence alignment, the phylogenetic tree was constructed by using CLSTAL OMEGA. The entire sequences were used to understand the overall evolutionary pathway for diversification. The method taken for this analysis was Phylogram; neighbour-joining tree without distance correction. The results reveal that eNOS protein is paralogus; they may derive from the same ancestral sequence. The paralogs protein result from a gene duplication (Fig. 1).

Physiochemical properties of eNOS

Human eNOS was found to exhibit a molecular weight of 133,143.59 Daltons and isoelectric pH 6.98. It is a stable protein with an aliphatic index of 79.54, whereas its instability index was predicted to be 52.53 The prediction of GRAVY value of −0.351 demonstrates that eNOS is a hydrophilic peptide (Table 2).

Membrane topology of eNOS

Signal p4.1 was predicted that eNOS is an extracellular enzyme, present outside of the cell membrane (Fig. 2).

NCBI Conserved Domain Database has shown that human eNOS has three domain and belongs to the family nitric oxide synthase, ferredoxin reductase and flavodoxin. However, MEME predicted that eNOShas the three motiffs. The 3D structure of human eNOS protein was retrieved from the PDB (PDB ID: 3NOS) online protein database and visualized with a desktop tool, i.e., Discovery studio (Fig. 3). eNOS was predicted to have 6 active sites for interactions using InterPro (EMBL-EBI, Cambridgeshire, UK) (Table 3).

Phosphorylation profile analysis and prediction of accessible surface area

Phosphorylation generally occurs on serine, threonine, tyrosine and histidine residues in eukaryotic proteins. Regions of human eNOSsequence showed extensive phosphorylation on serine and threonine residues, while low phosphorylation capability was predicted at tyrosine residues.As per NetSurfP tool found that human eNOS has a combination of both buried and exposed amino acid residues which signify the presence of transmembrane segments in this human TG2 protein.

Generation of secondary structure and Ramachandran plot

PDBsum analysis of human eNOS wiring diagram showed that secondary structure consists of 7 sheets, 2 beta-alpha unit, 5 beta-hairpins, 2 beta bulges, 18 strands, 20 helices, 19 helix-helix interfaces, 30 beta turns, 4 gamma turns and 1 disulphide bond (Fig. 3). The ramchandran plot releveled that about 89.4% of amino acids and 50 glycine, 56 proline

Table 1 Uniprot-Id and length of protein sequence of selected organisms

Sr. no	Organism name	Uniprot-Id	Length of protein
1	Homo sapiens	P29474	1203
2	Rattus norvegicus	Q62600	1202
3	Mus musculus	P70313	1202
4	Sus scrofa (Pig)	Q9TUX8	1205

Table 2 Physicochemical properties of human eNOS protein (Uniprot id: P29474)

Sr. no	Property	Value
1	Number of amino acids	1202
2	Total number of atoms	18,639
3	Molecular weight	133,143.59
4	Theoretical pI	6.98
5	Extinction coefficient	161,950
6	Instability index	53.52
7	Aliphatic index	79.54
8	GRAVY index	−0.351

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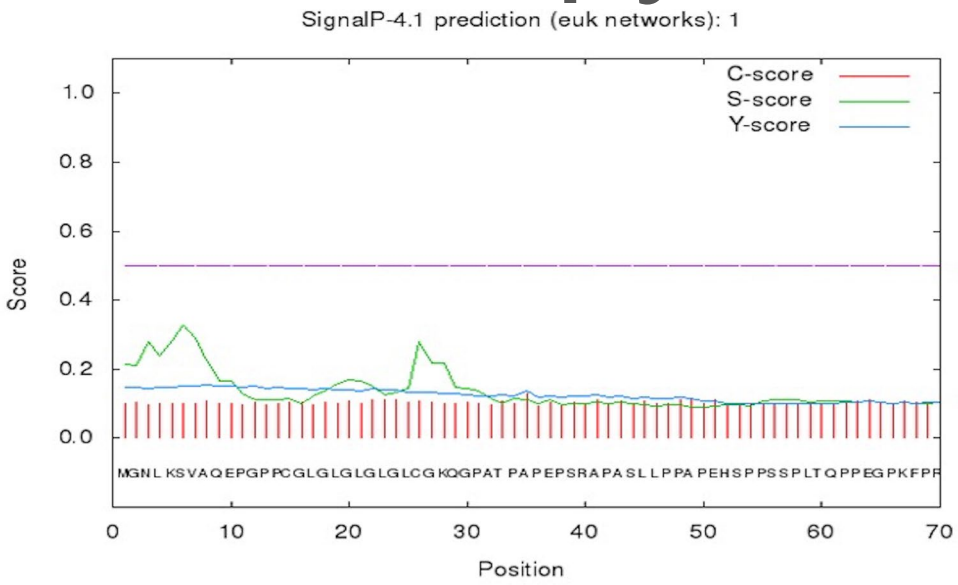

Fig. 2 Signal p output showed that human eNOS cleavage site (C-score 0.427), signal score (S-score 0.950) and combined cleavage Site (Y-Score 0.586)

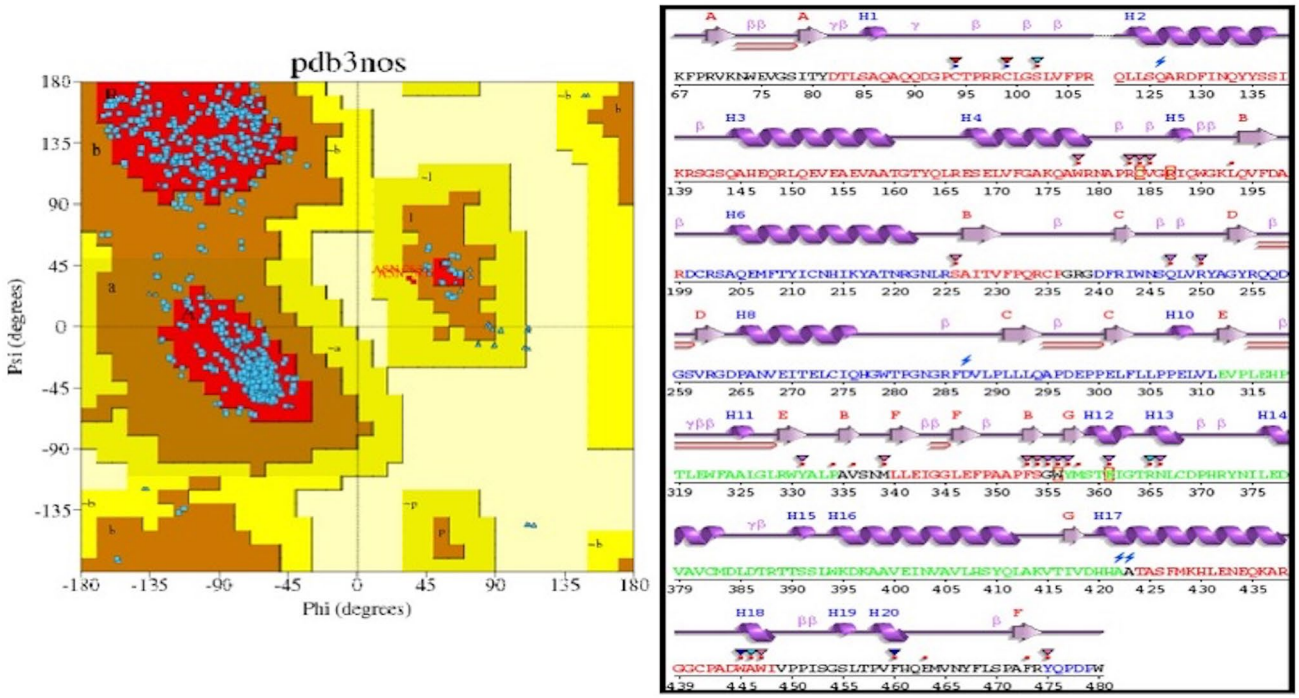

Fig. 3 Ramchandran plot analysis andSecondary structure analysis of human eNOS protein. Helices and sheets ( ) labelled by H1, H2…, strands and by their sheets A,B… motifs by β, γand, turn ( ) for the beta hairpin

residues are in favouredregions. The Φ and Ψ distributions of the Ramachandran plots of glycine, proline residues are summarized (Fig. 3). The score of Phi-psi distribution is -0.14 while Chi distribution -0.18 which suggest that human eNOS protein is suitable for further interaction.

Selection of bioactive molecules

The 2D structures of bioactive molecules from polyphenol group were downloaded from NPACT in SDF format (Table 4).

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Table 3 Amino acid residues present in active site

Amino acid	Position
GLU	316
Trp	356
Tyr	357
Arg	250
Asn	366
Val	336

Table 4 Selected bioactive compounds

Sr. no	Name	Molecular weight Gm/mol	Hydrogen acceptor	Hydrogen donar	ADME/T
1	Caffeic acid	180.16	4	3	Yes
2	Gallic acid	170.12	5	4	Yes
4	Epicatechin	290.27	6	5	Yes
5	Gallocatechin	306.27	7	6	Yes
6	Candenatenin A	316.3	6	5	Yes
7	4 bromoflavone	301.13	1	2	Yes
8	Acacetin	284.26	5	2	Yes
9	Curcumin	368.4	6	2	Yes
10	Burttinone	438.5	6	3	Yes
11	Flavone	222.24	1	2	Yes
12	Eupatorin	344.3	7	2	Yes
13	Ellagic acid	302.19	8	4	Yes

ADME analysis

The ADME analysis is crucial for determining an inhibitor's suitability. Ellagic acid, on the other hand, showed only a slight development. Basically, ADME is founded on Lipinski's rule of five, which aids in inhibitor screening. Low ADME properties are unfavourable for a biological system and are the primary cause of most medicines failing in clinical trials. The bioactive molecules chosen for this study follow Lipinski's rule, and an Egan's egg graph for the bioactive compounds was generated with SwissADME. According to the graph, only ellagic acid, a herbal compound, is absorbed by the brain, though in an acceptable range. So, on the basis of Egan's boiled-egg rule threshold values, only ellagic acidandcandenatenin A penetrates the blood–brain barrier, though within acceptable limits. The blue dots indicate molecules predicted to be effluated from the CNS by P-glycoprotein, and the red dots indicate molecules predicted not to be effluated from the CNS by P-glycoprotein. These rules define molecular properties that are important for a drug's pharmacokinetics in the human body, such as absorption, distribution, metabolism, and excretion (ADME). If a ligand fails to meet the Ro5 parameters, it is highly likely that it will cause problems if consumed (Attique 2019). BOILED-egg results demonstrating the possibility of inhibitor absorption and penetration in the GI tract and brain using WLOGP and TPSA parameters are presented in (Fig. 4).

Molecular docking

Molecular docking determines how closely the lowest energy pose (binding confirmation) can fit and it was predicted by the object scoring function. A $20 \times 20 \times 20$ Å grid was generated around the centroid of binding site residues of human eNOS. All of the selected bioactive compounds were found in the target protein's pocket, indicating a possible interaction with eNOS. The interaction

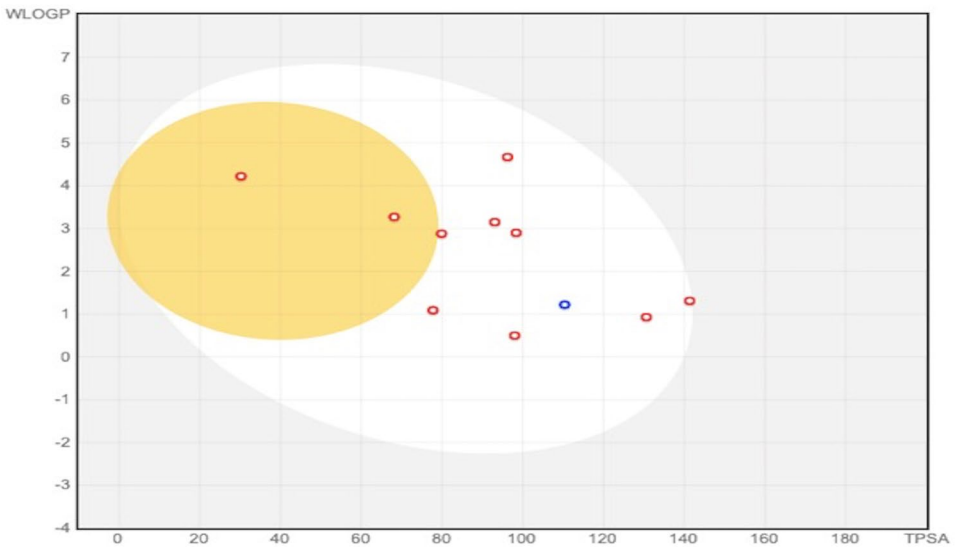

Fig. 4 ADME/T property of selected bioactive molecule by boiled egg method

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score and binding energy were used to evaluate the results of the interactions. Bioactive compounds with the lowest score tend to interact strongly with eNOS at specific active sites. Following in silico docking, we identified a ligand with the lowest binding energy of all the inhibitors. Ellagic acid, which is present in most of the berries, showed a minimum binding energy of-6.61; therefore, it establishes the strongest interaction with human eNOS among all the bioactive compounds with binding energy77.17 kcal/mol, interacted with ASN338,GLN 476 (Fig. 5; Table 5).

Discussion

It is well documented that impaired vascular function in hypertension, diabetes and ageing is concerened to a dysfunctional NO system. This dysfunction appears to be related to a reduced expression of eNOS, or to an excessive removal of NO via reaction with reactive oxygen species (Ulrich Förstermann 2006). Endothelial NOS is also crucial for adaptive vascular remodeling to chronic changes in flow. Hence endothelial NO synthase (eNOS), is a key regulator of vascular wall homeostasis, produces NO under normal physiological conditions (Meza et al. 2019). An increased production of ROS by uncoupled eNOS is likely to contribute significantly to vascular oxidative stress and endothelial

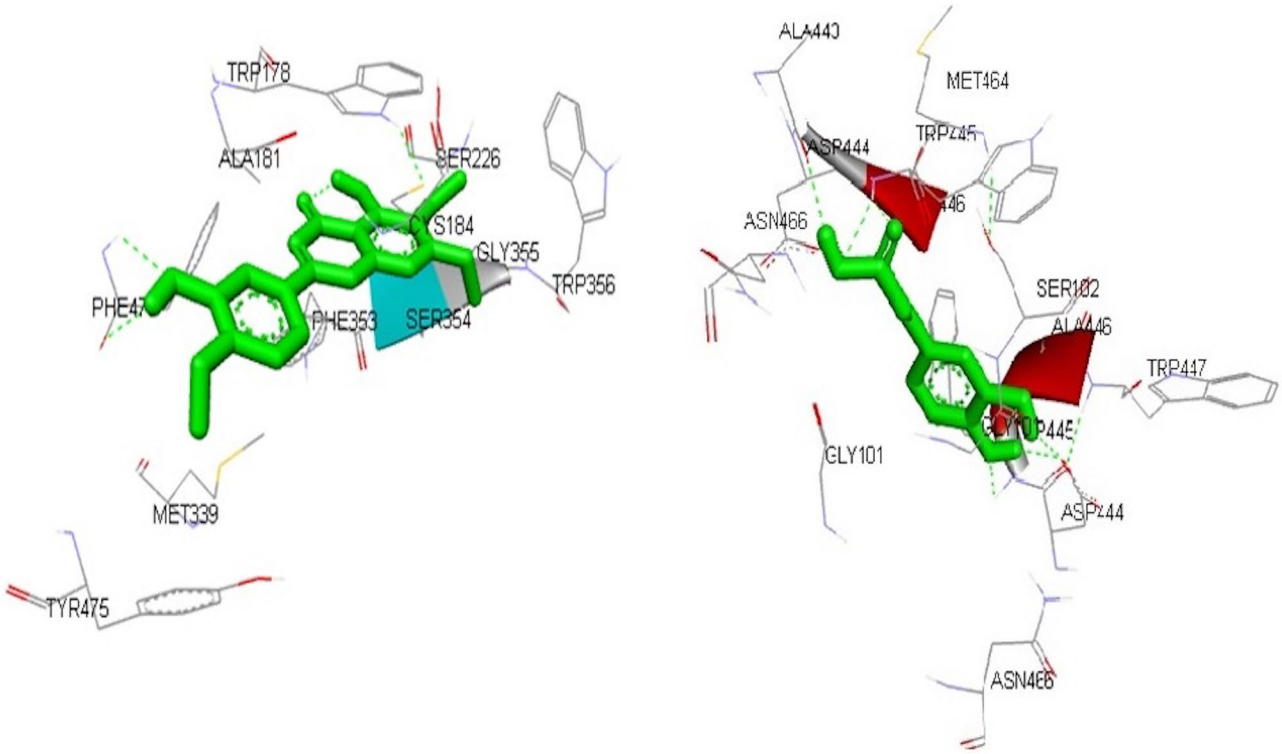

Fig. 5 Binding interaction of human eNOS protein with ellagic acid and caffeic acid

Table 5 Multiple docking interaction of human eNOS protein with top four bioactive molecules

Sr. no	Name	Binding energy	Inhibition constant Kcal/mol	Interacting amino acids	Interacting hydrogen atoms
1	Caffeic acid	– 6.2	132.15	SER 226, PHE47	H19,H20
2	Ellagic acid	– 5.29	28.68	ASN446, ASP444, TRP445, GLY101	H16, H15, H11, H21
3	Gallic acid	– 4.84	283.91	ARG365	H21,H11
4	Epicatechin	– 5.38	113.56	ARG365	H21

dysfunction (Kuo et al. 2011a). And how it is changed in disease has been poorly reported in vivo and the available data in humans are also insufficient.

Given the beneficial effects of endothelial NO, enhancement of NO production by up-regulating eNOS expression could be of prophylactic or therapeutic interest. In present scenario researchers have reported about identification of many small molecules those have potential to prevent vascular oxidative stress. Ellagic Acid is a micronutreint phenol, mainly recognised as an antioxidant. Ellagic acid effectively lowers the levels of plasma lipids, reduces oxiadtive stress and inhibits lipid peroxidation (Kuo et al. 2011b). It would be intriguing to know the underlying molecular mechanisms by which ellagic acid exerts its effects by eNOS uncoupling and at the same time enhance eNOS expression.

A recent study has reported that ellagic acid lowered blood pressure in hypertensive rats through an antioxidant effect of ellagic acid which includes increased nitric oxide synthase (NOS) expression and decreased ROS levels in the plasma (Ou et al. 2010). In individuals with coronary artery disease, catechins were reported to diminish platelet aggregation and reverse endothelial dysfunction, implying antiatherosclerotic effects (Mazumder et al. 2019). Similarly, polyphenols from the tropical plant Terminalia have been shown to activate eNOS in a calcium-dependent manner (Stromsnes et al. 2020). Fruit extracts of Camelia japonica (CJF), a plant widely distributed in Asia and well known for its antioxidant effects have been shown to increase NO generation in endothelial cells via Akt pathways and to activate eNOS via phosphorylation at Ser1179 (Forte et al. 2016).

Concerning the effects on vascular physiology, several studies indicate that polyphenols influence NO signalling and metabolism, improving eNOS expression and activity while decreasing eNOS uncoupling (Park et al. 2015). One of the current limitations in the characterization of the molecular pathways activated by polyphenols is that most experiments have been conducted with total extracts of food, such as wine, cocoa powder, or olive leaf extracts; as a result, it is frequently difficult to identify the specific compound exerting protective effects. Even so, some studies looked at the effects of single compounds like resveratrol, quercitin, or curcumin (Marella et al. 2020). Molecular docking is a powerful computational modeling tool in evaluating binding of a ligand (phytochemicals or others) to the active site of an enzyme or receptor. So, the present study was primarily focused on screening of novel micronutrients those can specifically bind eNOS protein. We identified the key amino acid residues involved in the interactions for selected proteins and screened the lead compound hits for proteins from the NPACT using, virtual screening, and molecular docking along with in silico ADMET analysis. When developing bioactive compounds as medicinal treatments, high oral availability is frequently a factor to consider. The drug-like properties of the selected optimum compounds were investigated. The PreADME web-based technique was used to conduct an in silico investigation for the prediction of pharmacokinetic parameters (Nikfarjam et al. 2021). In addition to the rule of five, in silico filters can identify toxicity factors, which are necessary for excluding compounds with no drug-like properties or undesired hazardous consequences from the screening process. The availability of current inhibitors, combined with bioinformatics tools and databases, greatly aids in the difficulty of finding prospective inhibitors/activators among a huge number of gene/protein surveys. Ellagic acid molecule HN group interacts with one hydrogen bond with binding energy −5.29 and amino acid ASN446, ASP444, TRP445, GLY101. Caffeic acid interact with 2 amino acid residues SER 226, PHE47 with binding energy −6.2. The in silico ADMET results revealed that all the top two bioactive molecules are virtually safe and active. The novelty of our study lies in screening and molecular docking of ellagic acid which can specifically bind eNOS protein which exerts as an antioxidant effect in an altered vascular environment. These novel compounds may merit further investigation for safety and efficacy in vitro and in vivo.

Conclusion

This current study may provide important insights into the identification of Ellagic Acid and caffeic acid as new strategy in preventing the development and/or retarding the progression of vascular complications. Although continuous research is going on, but presently very few bioactive drugs are in the pipeline due to their limitations associated with a molecule to satisfy the ADME profile. The present in silico study shows Ellagic acid and Caffeic acid are the best candidates for eNOS protein functional norms. However, before validating further studies are required to carry out using in vivo models and to declare these compounds as potent drugs.

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Declarations

Conflict of interest Authors declare no conflict of interest.

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