Original Research Article

INTEGRATING *INSILICO* WITH *IN VITRO* STUDIES OF RUTIN AS AN ANTI HYPERTENSIVE

ABSTRACT

Rutin was evaluated for its antihypertensive effect using *In vitro* ACE inhibitory activity using Captopril is used as standard. For molecular docking the proteins namely PDB ID: 1UZE, 1O86, 2XY9 and 3L3N are modelled and the qualities of the 3D model were evaluated using the PROCHECK program and assessed using the Ramachandran plot. The glide scores of rutin was found to be more than the glide score of standard drug captopril stating that the compound might have same affinity to bind to the proteins. Proteins with PDB ID 1UZE, 1O86, 2XY9, and 3L3N showed more than 90% favoured region which clearly indicates that the selected models in the present study are of good quality. ADME results revealed the three violations of rutin (like molecular mass, hydrogen donor and acceptors) of five and the standard Captopril has got zero violations which clearly indicated the probability for its higher oral bioavailability. The results of bioactive score revealed that rutin and captopril were found to moderatively active against GPCR ligand, ion channel modulator, kinase inhibitor and nuclear receptor but both rutin and the standard captopril were found to be highly active against protease inhibitor and enzyme inhibitor. The PASS results clearly stated that rutin and captopril as cardio protectant, vasoprotector, vasodilator, antihypertensive. Rutin was predicted with hepatoxicity and nephrotoxicity while captopril was predicted with myocardial infarction, hepatotoxicity and nephrotoxicity. The direct targets of rutin have interventions with Cytochrome P4503A, Carbonic anhydrase II, Sodium /glucose cotransporter/2 and TNF-alpha for direct targets. The possible targets were with vascular endothelial growth factor receptor 1, and Sodium /glucose cotransporter/2. *In vitro* ACE inhibition assay demonstrated that rutin has shown antihypertensive activity by inhibiting angiotensin converting enzyme. From the above it is clear that the rutin can act as novel therapeutic agent for the treatment of hypertension.

Keywords: Rutin, Molecular docking, Swiss ADME, PASS (Predicted activity spectra for substances), bioactive score

1. INTRODUCTION

The compounds like flavonoids are found in plants and are consumed in the form of fruitlets, nuts, vegetables, and derivative foods such as wine and brunette. The diet consumed by the western countries mostly comprise of quercetin [1]. Quercetin is an example of a flavonoid group which is found in nutriments having sugars, chiefly as β-glycosides. Rutin also called rutoside is the glycoside linking the flavonol quercetin and the disaccharide rutinose. This citrus flavonoid is found in a widespread diversity of plants. Rutin, a nutritional flavonoid which has established prodigious consideration, due to their pharmacological properties, including antimicrobial, anti-inflammatory, anticancer, antidiabetic and inter alia [2].

Though flavonoids are lacking typical nutritive value, they are gradually more regarded as valuable dietary constituents that act as probable defenders contrary to human diseases such as coronary heart disease, cancers and inflammatory bowel disease. Rutin turns out to be a quercetin releaser to the gut; moreover, quercetin is widely broken down in the gut, released from rutin and/or its colonic metabolites might play a vital role [3].

To understand the ligand binding properties of rutin with the angiotensin converting enzyme, the test compound rutin was subjected to molecular docking studies. These studies act as a computational tool to expect the probable interactions between rutin and protein. An *in silico* study of rutin was performed by SwissADME to calculate its pharmacokinetics, drug-likeness and medicinal chemistry friendliness of trivial molecules to support drug discovery [4]. PASS (Prediction of Activity Spectra for Substances) online software was performed to evaluate the biological activity, adverse drug reactions, direct and possible targets to establish molecular mechanism of rutin and standard drug captopril. In the present research an attempt is made to study the *insilico* and *invitro* approaches of rutin as an anti hypertensive.

2. MATERIALS AND METHODS

Insilico studies

2.1 Docking studies

Molecular docking is an attractive scaffold to understand drug biomolecular interactions for the rational drug design and discovery, as well as in the mechanistic study by placing a molecule (ligand) into the preferred binding site of the target specific region of the DNA/protein (receptor) mainly in a non-covalent fashion to form a stable complex of potential efficacy and more specificity [5, 6]. The information obtained from the docking technique can be used to suggest the binding energy, free energy and stability of complexes. At present, docking technique is utilized to predict the tentative binding parameters of ligand-receptor complex beforehand. In this study the crystallographic structure of the enzymatic target ACE was obtained from the Protein Data Bank (PDB) database (PDB: 1UZE, 1O86, 2XY9, 3L3N). The molecular docking study was performed using Mcule. The software allows us to virtually screen a database of compounds and predict the strongest binders based on various scoring functions. The collection of enzyme substrate complexes was identified via docking, and their relative stabilities were evaluated using their binding affinities. Ligand fit was used for accurately docking ligands into protein active sites employing a cavity detection algorithm. A high-throughput screening study applied to the ACE receptor is also presented in which ligand fit when combined with LigScore, an internally developed scoring function, yields very good hit rates for a ligand pool seeded with known actives [4].

2.2 SWISS ADME STUDIES USING MOLINSPIRATION

The ADME properties of Rutin and Captopril are evaluated using the tool Molinspiration Cheminformatics server (http://www.molinspiration.com). There are several pharmacokinetic parameters and physicochemical descriptors which were evaluated for several drugs through application of the tool Molinspiration. These properties are mainly hydrophobicity, electronic distribution, hydrogen bonding characteristics, molecule size and flexibility and of course presence of various pharmacophoric features that influence the behaviour of molecule in a living organism, including bioavailability, transport properties, affinity to proteins, reactivity, toxicity, metabolic stability and many others. The Lipinski rule of five deals four simple physicochemical parameter ranges (MWT $\leq$ 500, log P $\leq$ 5, H-bond donor's $\leq$ 5, Hbond acceptors $\leq$ 10). [7]

2.3 BIOACTIVITY SCORE USING MOLINSPIRATION

The bioactivity score of rutin and captopril are also evaluated using the tool Molinspiration Cheminformatics server (http://www.molinspiration.com). In this computational chemistry technique large chemical databases are analyzed in order to identify possible new drug candidates. Only SMILES or SDfile structures of active molecules are sufficient for the training, no information about the active site or binding mode is necessary. This is particularly useful in projects where structure-based approach cannot be applied because information about 3D receptor structure is not available. [8]

2.4 PASS (Prediction of Activity Spectra for Substances).

The new drug development is a very tedious method and is related with a high probability of negative consequences in terms of pharmacological efficacy). In such a scenario, it becomes fundamental that a device is available which ought to predict the pharmacological properties beforehand. It would allow the researchers to streamline the lookup more efficiently. Prediction of activity spectra of substances (PASS) is such a device which can predict the pharmacological homes beforehand and would help in screening pharmacological manageable leads for a particular condition [9, 10]

2.4.1 Input and Output of PASS.

PASS uses as input data a MOL- or SD-file23 representing the structural information about the molecules under study. On the basis of these data, MNA descriptors (Multilevel Neighbourhoods of Atoms) are generated automatically. Based on the statistics of MNA descriptors for active and inactive compounds from the training set, two probabilities are calculated for each activity: Pa - the probability of the compound being active and Pi - the probability of being inactive. Being probabilities, the Pa and Pi values vary from 0.000 to 1.000 (with three relevant decimals being calculated), and in general $Pa + Pi < 1$, since these probabilities are calculated independently. Pa and Pi can be considered to be measures of the compound under study belonging to the classes of active and inactive compounds, respectively, or can be seen as estimates for the first and second kinds of errors in the prediction. All MNA descriptors influence the estimates in the activity prediction. Their influence can be either positive (if the descriptors are found in compounds with the particular activity), or negative (if the descriptors are found in compounds without the

particular activity), or even neutral (if the descriptors are found in both active and inactive compounds). In the last case, they decrease the relative impact of the “positive” and “negative” descriptors.

2.4.2 Interpretation of Predictions.

The PASS predictions can be interpreted, and used, in a flexible manner. The most probable activities, for a given compound, are characterized by Pa values close to 1, and Pi values close to 0. Let us first consider cases where the Pa value is high and is much larger than Pi. If a statistically significant set of samples with predictions obtained with the threshold Pa > 0.9 is selected from a much larger database and assayed, one has to expect to lose 90% of the active compounds, but the fraction of false-positives will be very small. For a cut off of Pa > 0.8, only 80% of the actives will be lost, but the fraction of false positives will be a little bit higher. Finally, if one goes down to the criteria Pa > Pi, the probability of the first kind of error equals the probability of the second kind of error, i.e., one is just as likely to miss true actives as to find false positives.

However, maximizing Pa values for the desired activity is not the only criteria for selection of the most promising compounds. Another aspect might be the novelty of a compound. If Pa is very high, the compound might be a close analogue of known pharmaceutical agents. Thus, if one is interested in finding new leads, especially New Chemical Entities (NCE), one may want to choose compounds for which the specified activity is predicted with lower probability, say, 0.5 < Pa < 0.7. In this case, the probability of false positives is likely to be higher, but if the activity will be confirmed in the experiment, one has a higher chance of having obtained an NCE. [11,12,13]

2.5 *In vitro* ACE inhibitor activity

In vitro ACE inhibitor activity was measured using the hippurylhistidyl-leucine (HHL), as substrate, ACE (EC 3.4.15.1). Rutin at different concentrations (40 µL) was incubated with 100 µL of 0.1 M borate buffer (pH 8.3) containing 5 mM HHL and 0.3 M NaCl and with 20 µL of ACE (2 mU) at 37°C for 30 min. The reaction was terminated with 150 µL of 1 M HCl. The hippuric acid formed was extracted with ethyl acetate (1000 µl) and centrifuged at 1500 rpm for 10 min, and 750 µL of the organic phase was evaporated. The residue

was made up to 800 µL with distilled water, and the absorbance at 228 nm was measured. Triplicates were performed for each sample. Inhibitory activity was expressed as the protein concentration (Pierce, Rockford, IL, USA) using bovine serum albumin as standard needed to inhibit 50% of ACE activity (half maximal inhibitory concentration (IC_{50}) [14].

3. RESULTS AND DISCUSSION

3.1 Molecular docking studies

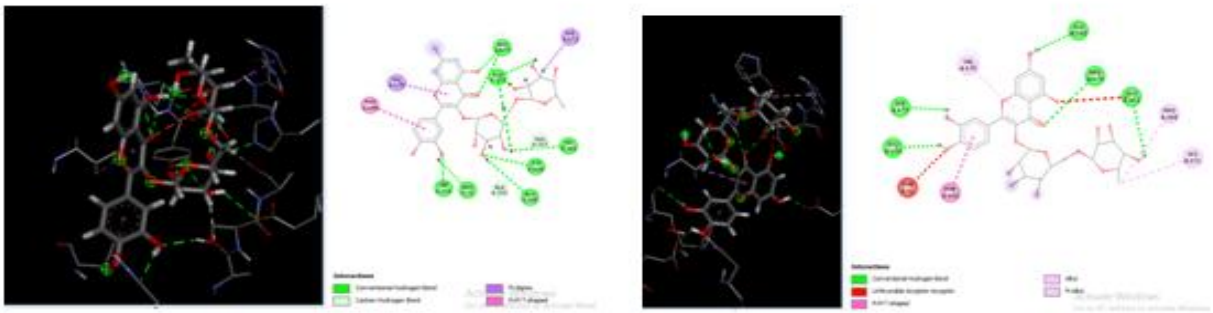
3.1.1 Structure based drug design

Initially the protein downloaded from the PDB was prepared by discovery studio. Water molecules present in the chains are removed. Energy minimization was done. Later, molecules smile image pasted in Mcule online software tool and the structures were docked against protein.

3.1.2 MCULE -docking results

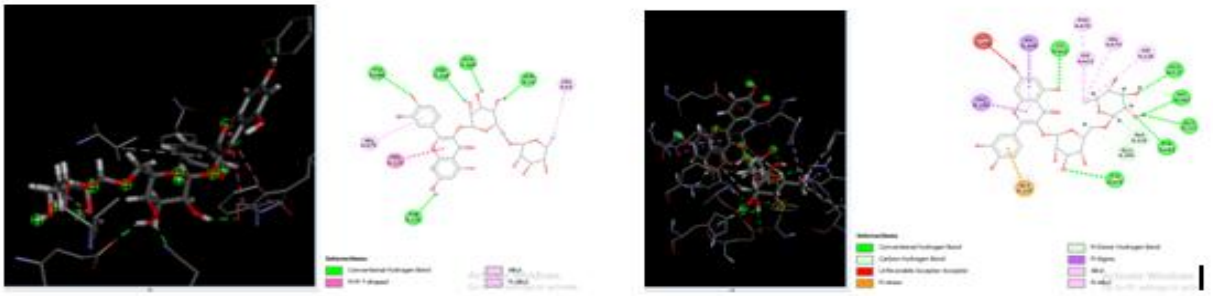
Table 1: Docking Studies of Rutin and Captopril

Sl.no	Compounds	Glide Score			
		1UZE	**1O86**	**2XY9**	**3L3N**
1	Rutin	-9.2	-9.4	-9.8	-11.1
2	Captopril	-5.7	-5.8	-5.4	-5.4



Rutin with PDB ID: 1UZE (-9.2)

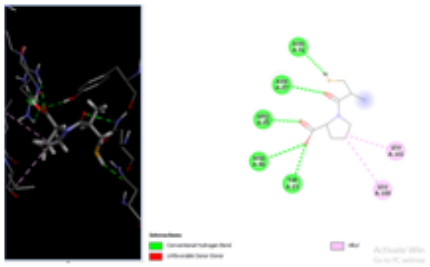
Rutin with PDB ID: 1O86 (-9.4)



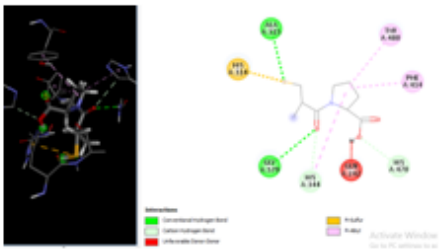
Rutin with PDB ID: 2XY9 (-9.8)

Rutin with PDB ID: 3L3N (-11.1)

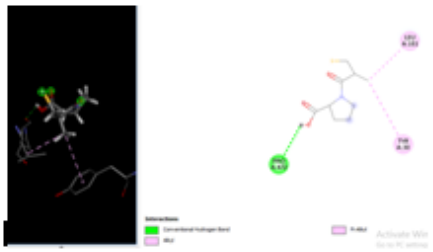
Figure 1: 3D & 2D structures of Rutin with PDB ID: **1UZE, 1O86, 2XY9, and 3L3N** with glide scores interactions



Captopril with PDB ID: 1UZE (-5.7)



Captopril with PDB ID: 1O86 (-5.8)

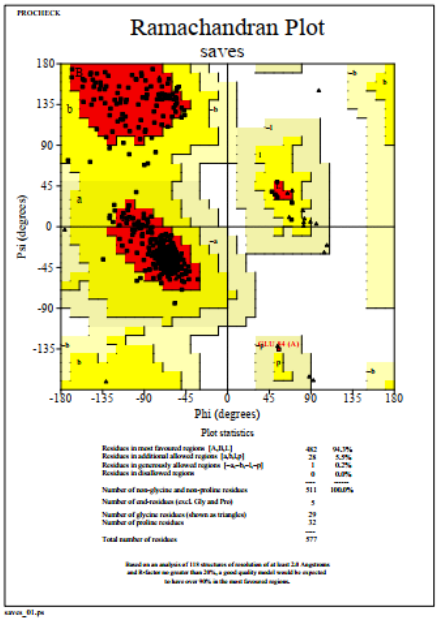


Captopril with PDB ID: 2XY9 (-5.4)

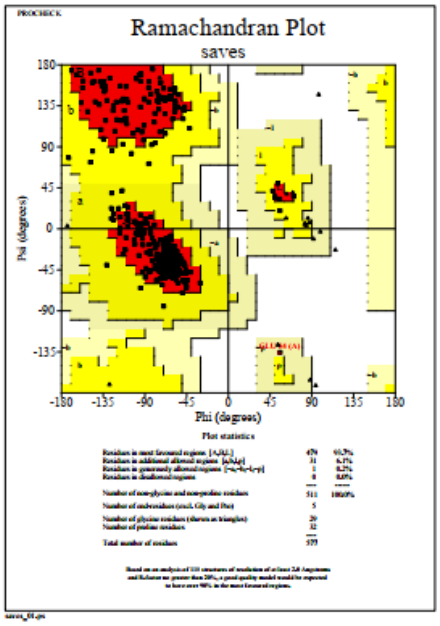
Captopril with PDB ID: 3L3N (-5.4)

Figure 2: 3D & 2D structures of Captopril with PDB ID: **1UZE, 1O86, 2XY9, and 3L3N** with glide scores interactions

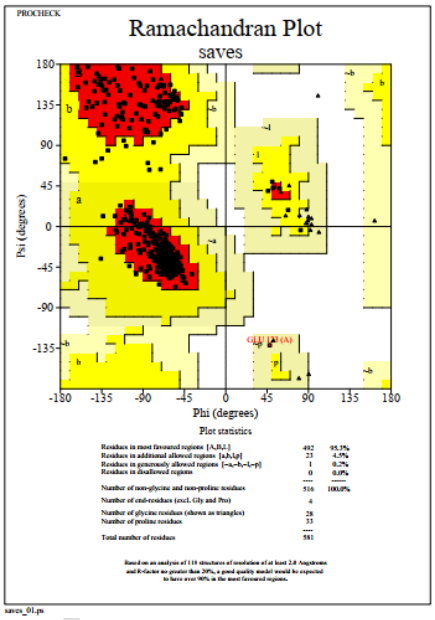
3.2 Ramachandran Plot



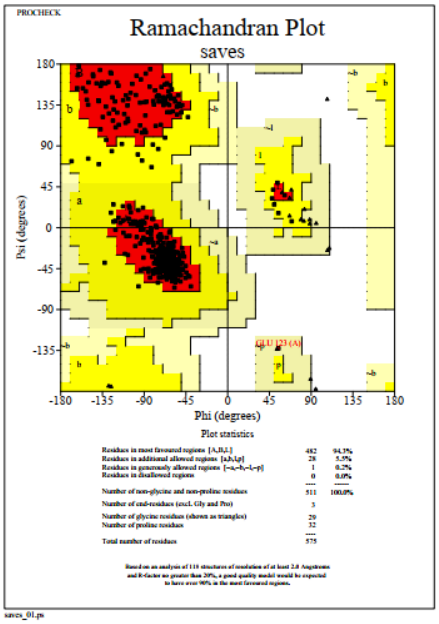
PDB ID: 1UZE



PDB ID: 1O86



PDB ID: 2XY9

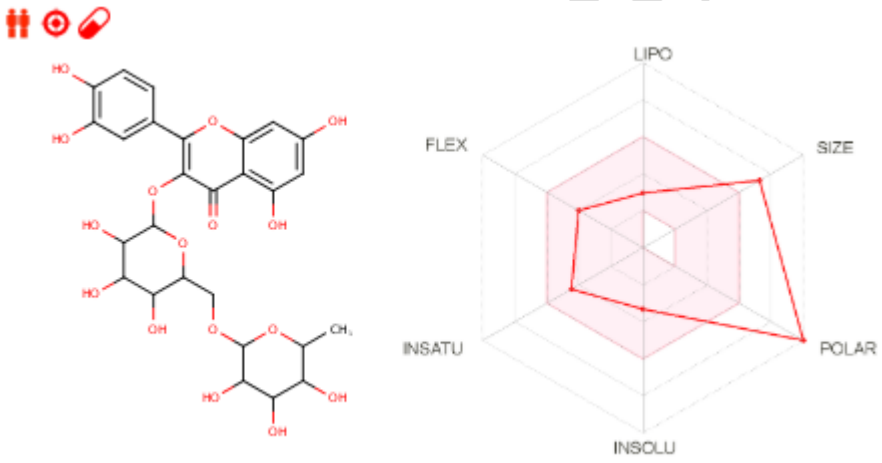


PDB ID: 3L3N

Figure 3: Ramchandran Plot with PDB ID: **1UZE, 1O86, 2XY9, and 3L3N**

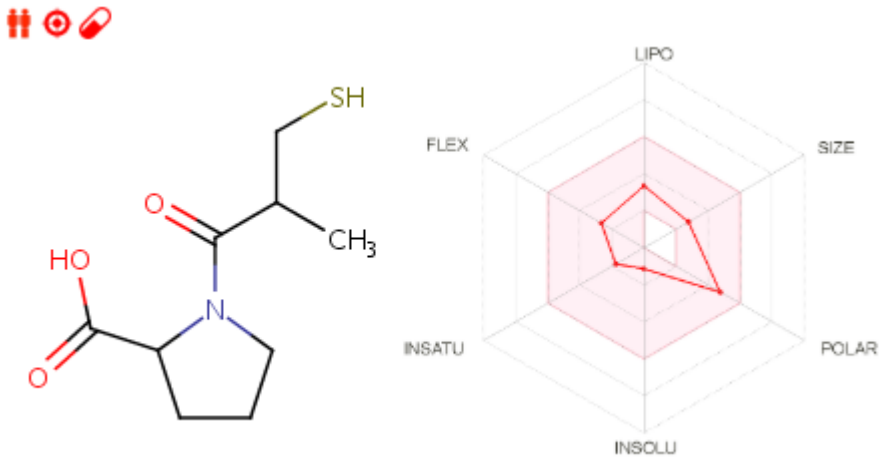
Table 2: Profile of Rutin and Captopril using SWISS ADME Software

Compound name	Physiochemical Properties					Lipophilicity WLOGP
	Molwt g/mol	TPSA Å^2	No rotb	No H bond Acceptors	No H bond donors	
Rutin	610.52	269.43	6	16	10	-1.69
Captopril	217.29	96.41	4	3	1	0.25



Structure of Rutin **b. Violations from Lipinski depicted in red line**

Fig 4 : Physiochemical properties of Rutin



a. Structure of captopril **b. Zero violations from Lipinski depicted in redline**

Fig 5: Physiochemical properties of Captopril

Lipinski's rule of five is to evaluate drug likeness or determine if a chemical compound with a certain pharmacological or biologicalactivity has chemical properties and physical properties that would make it an orally active drug in humans. In the present study rutin has three violations (like molecular mass, hydrogen donor and acceptors) of five. Captopril got zero violations which clearly indicated the probability for its higher oral bioavailability. Lipinski violations of rutin and zero violations of standard captopril were depicted in fig. 4 and 5. Topological polar surface area (TPSA) allows prediction of transport properties of drug candidates in the intestines and blood–brain barrier [15]. Rutin has TPSA of 269.43 and this high score of TPSA suggested that this molecule preferentially act as hydrophilic in nature and cannot easily transport through the blood brain barrier when compared to captopril which has TPSA score of 96.41clearly indicating lipophilic in nature.

The three violations of rule five for Rutin indicated the probability for its less oral bioavailability [15]. The ADME profile of rutin and captopril was given in table 2.

3.3 Bioactivity Score of rutin and captopril

Table 3: Bioactivity Score of rutin and captopril using molinspiration

Compounds	GPCR ligand	Ion channel	Kinase inhibitor	Nuclear receptor	Protease inhibitor	Enzyme inhibitor

		modulator		**ligand**		
Rutin	-0.05	-0.52	-0.14	-0.23	-0.07	0.12
Captopril	-0.14	-0.08	-0.98	-0.55	0.97	0.50

3.4 PASS

Table 4. Antihypertensive activity predicted for Rutin and standard Captopril using PASS (prediction of activity spectra for substances).

Sl.No	Compound	Probable Activity (Pa)	Probable Activity (Pi)	Biological Activity
1.	Rutin	0,988	0,001	Cardioprotectant
		0,980	0,001	Vasoprotector
		0,740	0,006	Vasodilator
2.	Captopril	0,559	0,014	Antihypertensive
		0,661	0,010	Vasodilator, coronary

Table 5. Adverse effects predicted for the Rutin and standard captopril using PASS (Prediction of Activity Spectra for Substances)

Sl no	Compound	Pa	Pi	Adverse Effect
1.	Rutin	0.384	0.271	Hepatotoxicity
		0.247	0.228	Nephrotoxicity
2.	Captopril	0.923	0.004	Myocardial infarction
		0.808	0.010	Nephrotoxicity
		0.686	0.106	Hepatotoxicity

Table 6. Direct and possible target Prediction for Rutin and Captopril using PASS (prediction of activity spectra for substances).

Sl.no	Compounds	Direct target	Confidence	Possible target	Confidence
1.	Rutin	Cytochrome P4503A	0.3519	Vascular endothelial growth factor receptor 1	0.4503
		Carbonic anhydrase II	0.3198		

		Sodium /glucose cotransporter/2	0.1088	Sodium /glucose cotransporter/2	0.0821
		TNF-alpha	0.2162		
2	Captopril	Angiotensin converting enzyme	0.1368	Endothelin converting enzyme-1	0.0222
		Renin	0.0491	Angiotensin II type 2 (AT-2) receptor	0.0081
		Angiotensin II type 2 (AT-2) receptor	0.0486		
		Endothelin converting enzyme	0.0218	Vasopressin V2 receptor	0.0107

3.5 ANTI HYPERTENSIVE ACTIVITY

3.5.1 *In vitro* ACE inhibition assay

Rutin was tested for angiotensin converting enzyme inhibitory activity using ACE inhibition assay method. The concentrations and percentage inhibition of rutin and standard drug captopril were recorded. From the percent inhibition, IC_{50} values were calculated and reported in table 1. The standard drug captopril was tested at different dose levels and found to be linear, which substantiates the usefulness of captopril for comparison of the test doses. IC_{50} of rutin was 66.01 µg/mL and captopril was 20.31 µg/mL.

Table 7: Effect of Rutin on Angiotensin converting enzyme (ACE) inhibition assay

Rutin			**Captopril**		
Concentration (µg/mL)	**% Inhibition** (mean±SEM)	**IC_{50}** (µg/mL)	**Concentration** (µg/mL)	**% Inhibition** (mean±SEM)	**IC_{50}** (µg/mL)
100	77.25±0.22	**66.01**	-	-	**20.31**
50	35.50±0.08		50	85.20±0.62	
25	19.50±0.87		25	59.82±0.88	
12.5	10.25±0.19		12.5	30.12±0.01	

Assay was performed in triplicate. The above results show that rutin and standard compound captopril has antihypertensive activity.

Rutin has shown antihypertensive activity by angiotensin converting enzyme inhibitory action (IC_{50}=66.01 µg/mL). *In vitro* studies have revealed the antioxidant activity of rutin and the role of rutin in reducing oxidative stress associated with hypertension.

4. Discussion

4.1 Molecular docking studies

Molecular docking is a computational technique that predicts the noncovalent interaction between two macromolecules or more repeatedly in a macro and small molecules. Drug discovery, molecular docking, and virtual screening, offering multi-user capability, enhanced accuracy. In this study, the search of flavonoids in the molecular basis for binding to active site of ACE Inhibitors is revealed by computer aided docking analysis. With a growing number of known experimental structures of target molecules, computational methods have been used successfully to supplement and speed up drug discovery [16]. The docking analysis of rutin and captopril were carried out using mcule software. The rutin and captopril were subjected to docking against PDB ID: 1UZE, 1O86, 2XY9, and 3L3N using Mcule online software tool. The highest glide scores were observed with rutin and captopril with almost all the selected proteins with PDB ID: 1UZE, 1O86, 2XY9, and 3L3N. The glide scores of rutin was found to be more than the glide score of standard drug captopril stating that the compound might have same affinity to bind to the proteins. These results clearly indicate that the Rutin might have shown similar mechanism to that of the standard drug captopril in reducing hypertension. The proteins identified namely1UZE, 1O86, 2XY9, and 3L3N are modelled and the qualities of the 3D model were evaluated using the PROCHECK program and assessed using the Ramachandran plot. It is evident from the Ramachandran plot that predicted models have most favourable regions, additionally allowed regions, generally allowed regions and disallowed regions. Such a percentage distribution of the protein residues determined by Ramachandran plot shows that the predicted models are of good quality. According to Ramachandran plot a good quality model would be expected to have over 90 % in the most favoured region. Proteins with PDB ID 1UZE, 1O86, 2XY9, and 3L3N showed almost 90% favoured a region which clearly indicates that the selected models in the present study are of good quality.

4.2 SWISS ADME analysis:

Molecular properties were calculated on the basis of Lipinski's rule and its components. Lipinski's rule of five is to evaluate drug-likeness or determine if a chemical compound with a certain pharmacological or biological activity has chemical properties and physical properties that would make it an orally active drug in humans. In the present study rutin has three violations (like molecular mass, hydrogen donor and acceptors) of five. Captopril got zero violations which clearly indicated the probability for its higher oral bioavailability. The molecular weight of captopril is 217.29 g/mol and rutin is 610.52. With less molecular weight the standard captopril might have easily absorbed, diffused and transported and this might be responsible for its high oral bioavailability. Topological polar surface area (TPSA) allows prediction of transport properties of drug candidates in the intestines and blood–brain barrier [16]. Rutin has TPSA of 269.43 and this high score of TPSA suggested that this molecule preferentially act as hydrophilic in nature and cannot easily transport through the blood brain barrier when compared to captopril which has TPSA score of 96.41clearly indicating lipophilic in nature. Any compound with less than 140 exhibits better permeability into the tissues.

Molinspiration ADME enables the computation of key physicochemical, pharmacokinetic, drug-like and related parameters for one or multiple molecules. Number of H-bond acceptors should be in a range of 0-10 and number of H-bond donors should be 0-5. The Number of H-bond acceptors for rutin and captopril are 16 and 3 and the number of H-bond donors for rutin and captopril are 10 and 1. The score of the captopril was found to be within the range. A negative value for logP means the compound has a higher affinity for the aqueous phase (it is more hydrophilic); when logP equals 0 the compound is equally partitioned between the lipid and aqueous phases; a positive value for logP denotes a higher concentration in the lipid phase (i.e., the compound is more lipophilic).

4.3 Bioactivity score

Rutin and Captopril were subjected to bioactivity score using molinspiration. The scores for the selected compounds can be interpreted as Active (bioactivity score > 0), moderately active (bioactivity score: -5.0-0.0) and inactive (bioactivity score < -5.0). Rutin and captopril were found to moderatively active against GPCR ligand, ion channel modulator, kinase inhibitor and nuclear receptor but both rutin and the standard captopril were found to be highly active against

protease inhibitor and enzyme inhibitor.

4.4 PASS software:

Prediction of activity spectra of substances (PASS) is a valuable interface that should be adopted as an archetypal tool for predicting the potential molecules and to predict the biological activity of Rutin and standard captopril as anti hypertensive. To understand the molecular mechanisms of rutin and captopril as anti hypertensive these were subjected to prediction studies by applying PASS online software. Rutin and captopril were predicted by engaging the canonical simplified molecular-input line-entry system obtained from PubChem.com followed by using PASS online.

On the other hand, several new paths were predicted in which the *in vitro* and *in vivo* evaluation of several drugs which can be made on the basis of PASS predicted activities. It would allow the researchers to streamline the lookup more efficiently. Prediction of activity spectra of substances (PASS) is such a device which can predict the pharmacological homes beforehand and would help in screening pharmacological manageable leads for a particular condition [9,10]

The results of rutin and captopril like probable activity (Pa) and probable inactiveness (Pi) and biological activity were given in table 4. The possible interventions of rutin and captopril were found to be Cardio protectant, Vasoprotector, Vasodilator, antihypertensive and Vasodilator, coronary. Rutin and captopril were subjected to pass software for adverse effects. Rutin was predicted with hepatoxicity and nephrotoxicity while captopril was predicted with myocardial infarction, hepatotoxicity and nephrotoxicity.

Rutin and captopril were subjected to pass software for direct and possible targets. Rutin was found to have interventions with Cytochrome P4503A, Carbonic anhydrase II, Sodium /glucose cotransporter/2 and TNF-alpha for direct targets. The possible targets were with vascular endothelial growth factor receptor 1, and Sodium /glucose cotransporter/2. Captopril was found to have interventions with Angiotensin converting enzyme, Renin, Angiotensin II type 2 (AT-2) receptor and Endothelin converting enzyme for direct targets. The possible targets were with Endothelin converting enzyme-1, Angiotensin II type 2 (AT-2) receptor and Vasopressin V2 receptor. From the above PASS is an important tool for effectively showing the compounds of interest for the biological actions of interest. This helps the researchers to rationalize the research.

4.5 *In vitro* ACE inhibition assay

Rutin has shown antihypertensive activity by angiotensin converting enzyme inhibitory action (IC_{50}=66.01 µg/mL). Recently it has been hypothesized that oxidative stress is a key player in the pathogenesis of hypertension. A reduction in superoxide dismutase and glutathione peroxidase activity has been observed in newly diagnosed and untreated hypertensive subjects, which are inversely correlated with blood pressure. Hydrogen peroxide production is also higher in hypertensive subjects. *In vitro* studies have revealed the antioxidant activity of rutin and the role of rutin in reducing oxidative stress associated with hypertension. If oxidative stress is indeed a cause of hypertension, then, rutin as an antioxidant has beneficial effects on hypertension control and reduction of oxidative damage might have resulted in a reduction in blood pressure.

5. CONCLUSION

Finally it is concluded that the *In silico* and *in vitro* studies clearly demonstrated the anti hypertensive action of Rutin.

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