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MOLECULAR DOCKING STUDIES OF BIOACTIVE COMPOUNDS FROM 'NJAVA', THE MEDICINAL RICE OF KERALA, AS PHOSPHOLIPASE A₂ INHIBITORS

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ABSTRACT

Molecular modeling helps to design new and more potent drugs against many diseases and conditions quickly and at a lower cost. Snake bite is a serious global problem, especially in tropical countries like India. Phospholipases A₂ (PLA₂s) commonly found in snake venom, are extensively studied due to their pharmacological and physiopathological effects. Numerous plant species are used in folk medicine to treat venomous snake bite without scientific validation. Njavara is a unique medicinal rice variety of Kerala used in Ayurveda for many disease conditions including snake bite pustules. In this paper, bioactive compounds isolated from Njavara are screened as inhibitors, against Indian Russell's viper PLA₂ (PDB id: 1TH6) using molecular docking techniques. Phytochemical investigation of Njavara led to isolation of six compounds including bioactive phenolic acids (ferulic, syringic, vanillic and protocatechuic acid), β -sitosterol, and 24-methylene cycloartanyl ferulate for the first time. Tricin and its flavonolignans reported earlier by the authors' group, were also screened. The results showed a better interaction of bioactive compounds with the enzyme. Tricin 4'-O-(*threo*- β -guaiacylglyceryl) ether was found to be better inhibitor among the compounds, with a binding energy of -10.79 kJ/mol and hydrogen bonding with HIS48 and ASP49, the main amino acid residues at the active site of PLA₂.

Keywords: Njavara, Phenolic acids, Molecular docking, Phospholipase PLA₂, Viper venom

INTRODUCTION

Molecular docking plays an important role in the rational design of drugs. In the field of molecular modeling, docking is a method which predicts the preferred orientation of one molecule to another when bound to each other to form a stable complex. The aim of molecular docking is to achieve an optimized conformation for both the protein and ligand and relative orientation between protein and ligand such that the free energy of the overall system is minimized¹. Today, the process of drug discovery has been revolutionized with the advent of genomics, proteomics, bioinformatics and efficient technologies like, combinatorial chemistry, high throughput screening (HTS), virtual screening, *de novo* design, *in-vitro*, *in-silico* ADMET screening, structure-based drug design and computer-aided drug design techniques that can effectively reduce cost and speed up drug discovery². In recent years, *in-silico* molecular docking has emerged as complementary to high-throughput screening, to discover novel, potential 'lead' compounds and thus play a key role in the process of drug discovery³.

Snake bite venom continues to attract attention as a serious global problem⁴. Phospholipase A₂ (PLA₂) are enzymes that abundantly occur in snake venom which can induce several pharmacological effects such as edema, modulation of platelet aggregation, as well as neurotoxic, anticoagulant and myotoxic effects^{5,6}. PLA₂ plays a major role in the formation of inflammatory mediators. PLA₂ class of enzymes catalyze the hydrolysis of 2-acyl bond of phospholipids resulting in the formation of arachidonic acid-like unsaturated fatty acids and

other lysophospholipids^{7,8}. Arachidonic acid acts as a very important inflammatory precursor and is used as a substrate by COX and LOX enzymes and led to the formation of eicosanoids⁹. These products can have biological actions or further metabolized to form a variety of pro-inflammatory lipid mediators including prostaglandins, leukotrienes and platelet – activating factor and lysophospholipids¹⁰. PLA₂s are of two types-intracellular (high molecular weight and involved in phospholipid metabolism, signal transduction and other cellular process¹¹) and extracellular (of low molecular weight and found in mammalian pancreatic juice, snake and insect venoms¹²). Bioactive compounds from plants possessing anti-snake venom activity might have rational and many of these compounds may prove to be the potential candidates for the development of novel anti-snake venom drugs in future. The review on 'Plant natural products active against snake bite- the molecular approach' by Mors *et al*¹³ indicates the importance of the topic in modern research. Njavara is a plant recommended in Ayurveda to treat snake bitten patients. Njavara is suggested as a safe food for them and the pain due to biting of Viper is reduced and the pustules are cured by applying Njavara rice paste regularly on the pustules formed from bite¹⁴.

Njavara (*Oryza sativa* L.var.njavra) is a distinct medicinal rice variety of very short duration grown in Kerala, India which matures in (60-90 days). Njavara, a special cereal important in Ayurveda, is an effective remedy for rheumatic complaints, neuro muscular disorders, circulatory problems and body rejuvenation. The medicinal and nutritive properties of Njavara have recently received wide recognition all over the world.

Based on glume colour difference, two types of Njavara are recognized, the black and golden yellow types¹⁵. In Ayurveda, Njavara rice is always recommended as brown rice (with bran). In our previous studies, Njavara extracts, especially of the bran portion, showed higher antioxidant and anti-inflammatory effects¹⁵. There were no previous reports on bioactive compounds of Njavara till 2011. The first phytochemical study on Njavara reported three compounds (tricin and flavonolignans – triclin 4'-O-(*erythro*- β -guaiacylglyceryl) ether and triclin 4'-O-(*threo*- β -guaiacylglyceryl) ether) and their occurrence at higher concentration in Njavara bran than in staple varieties¹⁶. In the present study, isolation of more bioactive compounds from Njavara bran is reported and all these compounds and previously reported compounds *viz.* triclin and its flavonolignans are screened by molecular docking using AutoDock 4.0, as inhibitors of snake venom PLA₂. This paper is the first report of molecular docking study of Njavara compounds, against Russell's viper venom PLA₂.

MATERIALS AND METHODS

Instruments and Materials

UV-visible spectroscopy 1601, HPLC (Shimadzu co-operation), Autodock 1.5.4, PyMol softwares for docking. Fresh samples of Njavara black paddy (rice with husk i.e. rough rice), were collected from the certified ECO FARM – 'Karukamanikalam' producing Njavara. The authenticity of the sample [specimen voucher no:- Njavara Black (IC 539968)] was verified by Dr. Maya C. Nair, Department of Botany, Government Victoria College, Palakkad, Kerala-678506. All samples were milled and the bran was stabilized by heating 100 g lots, at 100°C for 30 min in air oven.

Molecular Docking

The crystal structure of PLA₂ was obtained from protein databank (PDB)¹⁷ (PDB ID: 1TH6). A standard docking procedure for a rigid protein and a flexible ligand was performed with AutoDock 4. The Lamarckian genetic algorithm was held rigid. The polar hydrogen atoms were added for 1TH6 using the AutoDock tools, and Kollman united atom partial charges were assigned. A grid box (60 X 60 X 60) with spacing of 1.0 Å⁰ was created and centered on the mass center of the ligand. Energy grid maps for all possible ligand atom types were generated using Autogrid 4 before performing the docking. The resulting conformations were clustered using a root-mean-square deviation (RMSD) of 2.0 Å° and the clusters were ranked in order of increasing binding energy of the lowest binding energy conformation of each docked molecule. The output from AutoDock was rendered with PyMOL.

Extraction and Isolation

Scheme I represents the isolation procedure followed. Njavara bran (100 g) was subjected to Soxhlet extraction using petroleum ether and the residue with methanol. The methanolic extract (4.1 g) again fractionated with diethyl ether to get 0.688 g diethyl ether extract (HDE). The rice bran (100 g) was also extracted with ethanol at room temperature. Ethanol extract (6.4 g) was again fractionated using petroleum ether and diethyl ether to get corresponding extract RPE and RDE in 3.64 g and 0.968 g yield. The procedure was repeated to get sufficient quantity higher amount for doing further column. The RPE extract (2 g) was chromatographed on silica column using solvent system [hexane – ethyl acetate (100:0 to 0:100)] to yield 2.6 mg of compound I. 4 g of diethyl ether extract was

subjected to column chromatography using hexane- ethyl acetate (100:0 to 0:100) to yield 15 pooled fractions (A₁-A₁₅). 1.8 g of A₂ was again subjected to subcolumn using same solvent yielded 7 fractions (B₁-B₇). Fraction B₅ on HPLC using chloroform yielded 7 mg of compound II. The HDE extract (4.5 g) was chromatographed on silica column [hexane – ethyl acetate (100:0 to 0:100)] to yield 3 main fractions. The second fraction was again subjected to subcolumn using same solvent to get 14 pooled (C₁-C₁₄) fractions. Fractions C₄, C₇, C₉ and C₁₂ on HPLC with acetonitrile-water (30:70) solvent system for all fractions yielded compound III, Compound IV, Compound V and compound VI in 4 mg, 2.7 mg, 3.5 mg and 2.2 mg in respective yields.

RESULTS

Molecular Docking

PLA₂ plays a major role in the formation of inflammatory mediators. There are reports of binding of non-steroidal anti-inflammatory drugs with PLA₂ and COX enzymes¹⁸. Indomethacin has been reported long time ago as a non-selective COX-2 inhibitor¹⁹. In recent studies, it has also been reported that indomethacin inhibits PLA₂²⁰. Hence, there is a possibility of molecules having anti-inflammatory effect, to be PLA₂ inhibitors also. *In-silico* docking is one of the widely applied methods for screening molecules against target protein. To deduce the promising compounds, all were docked into the identified binding pocket of PLA₂ using the Auto Dock 4.0 program. As a reference drug, indomethacin was also included here for docking studies. The compounds being reported in this paper namely, β -sitosterol, 24-methylene cycloartanyl ferulate, syringic acid, vanillic acid, protocatechuic acid and ferulic acid and also the compounds which were isolated and reported previously from Njavara¹⁶ - Tricin, Tricin 4'-O-(*erythro*- β -guaiacyl glyceryl) ether and Tricin 4'-O-(*threo*- β -guaiacylglyceryl) ether were chosen for docking studies. Indomethacin, which was reported to be PLA₂ inhibitor, was also screened as a reference compound using the same software. Russell's viper PLA₂ (PDB id-1TH6) was chosen as the target protein. Russell's viper is one of the most dangerous, commonly seen Indian viper. The interaction of compounds with amino acid residues of Russell's viper venom with hydrogen bonding is shown in Table 1. The molecular docking of the compounds studied, with the enzyme, is given in Figure 1. Most of the compounds showed hydrogen bonding with amino acid residues in the active site (Table 1).

The drug-active site (target) interaction generally comprises hydrogen bonding, hydrophobic and electrostatic interactions. The molecular docking studies showed that these compounds are having favourable interactions with amino acids at the active site of Russell's viper PLA₂, lending support to the ancient wisdom of using Njavara for reducing inflammation and healing of wounds of the snake bite. The active site of enzyme is formed by HIS48, ASP49, TYR52, ASP99, LYS69, GLY30 and TYR22 amino acid residues²¹. β -sitosterol was found to interact with TYR22 while 24-methylene cycloartanyl ferulate interacted with TYR52. Tricin showed interaction with ASP49 and HIS48. Tricin 4'-O-(*erythro*- β -guaiacyl glyceryl) ether and triclin 4'-O-(*threo*- β -guaiacyl glyceryl) ether were found to interact with ASP49, HIS48, and LYS69. The phenolic acids identified in Njavara, also showed interaction with amino acid residues. Vanillic acid and protocatechuic acid interacted with GLY30. But syringic acid showed interaction with HIS48, in addition to GLY30 and ferulic acid interacted with LYS34. Indomethacin, the reference compound, interacted with LYS69 and TYR22.

The hydrogen bonding interactions are shown in Figure 1. Among the molecules, tricetin and the flavonolignans - *erythro* and *threo*- showed H-bonding interaction with ASP49. This bond could promote the destabilization of the calcium coordination and it could cause a displacement of this cation from the calcium binding loop which is essential for the enzymatic activity, as it helps to polarize the SN_2 ligation of the glycerophospholipids that will be hydrolyzed²². Also, these three compounds and syringic acid presented a hydrogen bond with HIS48 that blocks water activation, which is important for catalyzing the hydrolysis of glycerophospholipids²³.

Isolation of compounds from Njavara

One flavonoid (tricetin) and two flavonolignans (tricetin 4'-O-(*erythro*- β -guaiacylglyceryl) ether and tricetin 4'-O-(*threo*- β -guaiacylglyceryl) ether) were previously reported from Njavara bran by the corresponding author's group¹⁶. Further, Njavara bran (100 g) was subjected to Soxhlet extraction (hot extraction) using petroleum ether and subsequently with methanol, and room temperature (r.t.) extraction using ethanol. The isolated compounds reported here are β -sitosterol, 24-methylene cycloartanyl ferulate, ferulic acid, syringic acid, vanillic acid, protocatechuic acid.

Scheme 1: Extraction and Isolation of Compounds I-VI from Njavara bran.

Figure 1: Molecular docking of A) tricetin 4'-O-(*threo*- β -guaiacylglyceryl) ether and B) Tricetin 4'-O-(*erythro*- β -guaiacylglyceryl) ether with PLA₂ using two types of representation

Figure 2: Chemical structure of compounds isolated from Njavara bran (compound I-VI, tricrin, tricrin 4'-O-(threo-β-guaiacylglyceryl)ether and tricrin 4'-O-(erythro-β-guaiacyl glyceryl) ether) and reference compound indomethacin selected for docking studies

Table 1: Hydrogen bonding of compounds with amino acid residues of protein

Compounds	Hydrogen bonding
β-sitosterol	TYR22 (2.74Å°)
24-methylene cycloartenyl ferulate	TYR52 (3.17Å°)
Tricin	ASP49 (3.215Å°) HIS48 (3.022Å°)
Tricin 4'-O-(erythro-β- guaiacyl glyceryl) ether	ASP49 (3.502Å°) HIS48 (2.854Å°) LYS69 (3.237Å°)
Tricin 4'-O-(threo-β- guaiacylglyceryl) ether	HIS48 (2.998Å°) LYS69 (2.932Å°) ASP49 (3.542Å°)
Vanillic acid	GLY30 (3.033Å°)
Syringic acid	HIS48 (3.252Å°) GLY30 (3.072Å°)
Protocatechuic acid	GLY30 (3.140Å°) CYS45 (3.117Å°)
Ferulic acid	LYS34 (2.0644Å°)
Indomethacin	LYS69 (2.981Å°) TYR22 (2.920Å°)

Table 2: Binding energy and drug likeness model score of compounds

Compounds	Binding energy (kJ/mol)	Drug-likeness model score
β -sitosterol	-9.76	0.88
24-methylene cycloartanyl ferulate	-12.03	0.82
Tricin	-8.04	0.18
Tricin 4'-O-(<i>erythro</i> - β -guaiacyl glyceryl) ether	-10.19	1.46
Tricin 4'-O-(<i>threo</i> - β -guaiacyl glyceryl) ether	-10.79	1.46
Vanillic acid	-4.94	-0.59
Syringic acid	-4.72	-0.76
Protocatechuic acid	-5.10	0.74
Ferulic acid	-5.83	-0.44
Indomethacin	-8.51	0.79

DISCUSSION

The best docking was confirmed by binding energy (the energy released due to formation of complex between the inhibitor and the protein) of the interaction of the compounds with the active site of the enzyme. The complex is considered more stable when it is formed with the least energy. In the present study, 24-methylene cycloartanyl ferulate – PLA₂ complex showed the least energy, indicating the higher stability among the complex of other compounds. The compound is already reported to have strong anti-inflammatory activity on mice ear edema²⁴. 24-methylene cycloartanyl ferulate followed by the flavonolignans, triclin 4'-O-(*erythro*- β -guaiacylglyceryl) ether and triclin 4'-O-(*threo*- β -guaiacylglyceryl) ether showed least binding energy. *Threo*- was reported to have higher anti-inflammatory effect compared to *erythro*-¹⁵. The order of binding energy of the compounds is 24-methylene cycloartanyl ferulate < triclin 4'-O-(*threo*- β -guaiacylglyceryl) ether < triclin 4'-O-(*erythro*- β -guaiacyl glyceryl) ether < β -sitosterol < triclin < protocatechuic acid < vanillic acid < syringic acid (Table 2).

Based on binding energy, 24-methylene cycloartanyl ferulate is found to be the best inhibitor of phospholipase enzyme followed by *threo*- and *erythro*- flavonolignan isomers of triclin. However on considering hydrogen bonding, 24-methylene cycloartanyl ferulate is not showing interaction with the two important amino acid residues HIS48 and ASP49, while the flavonolignans are showing the hydrogen bonding. Between flavonolignans, *threo*- (-10.79 kJ/mol) is having least binding energy than *erythro*- (-10.19 kJ/mol). Indomethacin (reference) is showing a binding energy of -8.51 kJ/mol and hydrogen bonding with LYS69 and TYR22. Hence the docking studies indicate triclin 4'-O-(*threo*- β -guaiacylglyceryl) ether is the most probable inhibitor of Russell's viper PLA₂, among the studied including indomethacin. In addition to the binding energy properties, molecular properties, drug likeness score, toxicity and bioactivity of the compounds were noted. Phenolic acids showed risk towards mutagenic and tumorigenic effects, while all other compounds showed no toxicity against mutagenic, tumorigenic, irritant and reproductive effect. On the basis of binding energy also vanillic acid, syringic acid and protocatechuic acid are showing less binding. So the *threo*- lignan can be considered as a good inhibitor of PLA₂.

Nirmal *et al* reported the molecular modeling of herbal compounds (acaluphin, chlorogenic acid, tectoridin, stigmasterol, curcumin) and marine compounds (gracilin A and aplysulphurin 1) against Russell's viper PLA₂ (pdb id: 1TH6) and bovine pancreatic PLA₂ (PDB id: 1O2E) using Molecular Operating Environment (MOE) software and compounds showed favourable interactions with amino acid residues at the active site²⁵. Pereanez *et al* reported the docking of polyphenolic

compounds (ferulic acid, gallic acid, epigallocatechingallate, propylgallate, caffeic acid and tannic acid) inhibited PLA₂ (PDB id: 2QOG) using Molegro Virtual Docker (MVD) and among the compounds epigallocatechingallate was found to be showing high interaction while ferulic acid showed less²⁶.

Njavara bran (100 g) was subjected to Soxhlet extraction (hot extraction-H) using petroleum ether and subsequently with methanol, and room temperature (R) extraction using ethanol. The methanolic extract (4.1 g) was further fractionated using diethyl ether to get diethyl ether extract (0.688 g) (HDE) and ethanolic extract (6.4 g) was further fractionated using petroleum ether and diethyl ether to get corresponding extracts RPE (3.64 g) and RDE (0.968 g). The procedure was repeated many times to get sufficient quantity for doing further work up. 2 g petroleum ether extract was subjected to column chromatography using silica gel (60 g, 100-200 mesh) with varying composition of hexane - ethyl acetate to get white needles of compound I in 2.6 mg. From the spectral values and by comparison with literature²⁷, compound I was confirmed to be β -sitosterol and the structure is as shown in Figure 2. 4 g of diethyl ether extract was subjected to column chromatography using hexane - ethyl acetate solvent system to yield 15 fractions. 1.8 g of sub-fraction again subjected to column chromatography using hexane - ethyl acetate, followed by HPLC using chloroform to yield compound II in 7 mg. The compound II was confirmed to be 24-methylene cycloartanyl ferulate from all spectral data and by comparison with literature²⁸. Recently Liu *et al.*²⁸ reported a novel method for the separation of two major triterpene alcohol ferulates namely, cycloartanyl ferulate (CAF) and 24-methylene cycloartanyl ferulate (24mCAF), from rice bran oil (RBO) using high performance counter-current chromatography (HPLCC). These authors gave the first report on direct separation of CAF and 24-mCAF from RBO by HPLCC. However, there is no report on direct isolation of individual triterpene alcohol ferulates from RBO. 24-methylene cycloartanyl ferulate was isolated for the first time by column chromatography method in rice bran in this study²⁹. 4.5 g of diethyl ether fraction (HDE) extracted from hot methanolic extract, was subjected to silica gel column chromatography to yield 3 fractions. 2nd fraction was again subjected to column chromatography using hexane - ethylacetate to yield 14 pooled fractions. Four compounds were obtained from different fractions on HPLC with Acetonitrile - Water (30:70) solvent system yielded compound III (4 mg), compound IV (2.7 mg), compound V (3.5 mg) and compound VI (2.2 mg) respectively. It was confirmed from the spectral data and literature, compound III to be 4-Hydroxy-3-methoxycinnamic acid (ferulic acid)³⁰, compound IV to be 4-hydroxy-3,5-dimethoxy benzoic acid (syringic acid)³¹, compound V to be 4-hydroxy-3-methoxy benzoic acid (vanillic acid)³² and compound VI to be 3,4-dihydroxy benzoic acid (protocatechuic acid)³³. The structure of

all isolated compounds is given in Figure 2. The spectral details of all these compounds are given in Supplementary material.

CONCLUSION

Molecular docking is an efficient tool for screening the interaction of a number of molecules with proteins causing diseases or adverse conditions, in less time and at minimal cost. The results of molecular docking of bioactive compounds from Njavara rice bran with Russell's viper PLA₂, snake venom enzyme, showed that all compounds are exhibiting a good interaction. 24-methylene cycloartanyl ferulate, tricin 4'-O-(*threo*-β-guaiacylglyceryl ether, tricin 4'-O-(*erythro*-β-guaiacylglyceryl ether, β-sitosterol and tricin are showing very good interaction compared to phenolic acids. Although 24-methylene cycloartanyl ferulate (-12.03 kJ/mol) is showing a minimum binding energy than tricin 4'-O-(*threo*-β-guaiacylglyceryl ether (-10.79 kJ/mol), the latter is found to have hydrogen bonding with the main amino acid residues (HIS48, ASP49) at the active site of PLA₂. The same compound also showed better interaction than indomethacin in AutoDock 4.0. Hence, we can conclude that among all compounds including indomethacin, tricin 4'-O-(*threo*-β-guaiacylglyceryl ether is a better inhibitor of PLA₂. The bioactive compounds (24-methylene cycloartanyl ferulate, tricin 4'-O-(*threo*-β-guaiacylglyceryl ether, tricin 4'-O-(*erythro*-β-guaiacylglyceryl ether and tricin) are reported to be present in higher amount in Njavara black compared to staple rice varieties. The positive interaction of these compounds against the snake venom PLA₂ may contribute to the therapeutic effect of Njavara, in snake bitten patients and corroborates with the ancient wisdom of using Njavara in such conditions.

SUPPLEMENTARY INFORMATION contains the spectral details of the isolated compounds, additional docking details of all the compounds etc.

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