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ORIGINAL ARTICLE

In silico Analysis for Predicting Fatty Acids of Black Cumin Oil as Inhibitors of P-Glycoprotein

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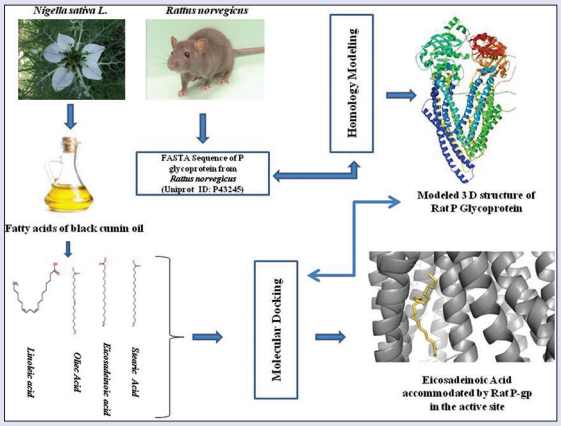
ABSTRACT

Background: Black cumin oil is obtained from the seeds of *Nigella sativa* L. which belongs to family Ranunculaceae. The seed oil has been reported to possess antitumor, antioxidant, antibacterial, anti-inflammatory, hypoglycemic, central nervous system depressant, antioxidant, and immunostimulatory activities. These bioactivities have been attributed to the fixed oil, volatile oil, or their components. Seed oil consisted of 15 saturated fatty acids (17%) and 17 unsaturated fatty acids (82.9%). Long chain fatty acids and medium chain fatty acids have been reported to increase oral bioavailability of peptides, antibiotics, and other important therapeutic agents. In earlier studies, permeation enhancement and bioenhancement of drugs has been done with black cumin oil. **Objective:** In order to recognize the mechanism of binding of fatty acids to P-glycoprotein (P-gp), linoleic acid, oleic acid, margaric acid, cis-11, 14-eicosadienoic acid, and stearic acid were selected for *in silico* studies, which were carried out using AutoDock 4.2, based on the Lamarckian genetic algorithm principle. **Materials and Methods:** Template search with BLAST and HHblits has been performed against the SWISS-MODEL template library. The target sequence was searched with BLAST against the primary amino acid sequence of P-gp from *Rattus norvegicus*. **Results:** The amount of energy needed by linoleic acid, oleic acid, eicosadienoic acid, margaric acid, and stearic acid to bind with P-gp were found to be – 10.60, –10.48, –9.95, –11.92, and – 10.37 kcal/mol, respectively. The obtained data support that all the selected fatty acids have contributed to inhibit P-gp activity thereby enhances the bioavailability of drugs. **Conclusion:** This study plays a significant role in finding hot spots in P-gp and may offer the further scope of designing potent and specific inhibitors of P-gp.

Key words: Binding affinity, black cumin oil, fatty acids, *in silico* analysis, P-glycoprotein

SUMMARY

- Generation of 3D structure of fatty acid compounds from Black cumin oil and 3D homology modeling of Rat P glycoprotein as a receptor.
- Rat P-gp structure quality shows 88.5% residues in favored region obtained by Ramchandran plot analysis.
- Docking analysis revealed that Some amino acids common for all compounds like Ser221, Pro222, Ile224, Gly225, Ser228, Ala229, Lys233, Tyr302, Tyr309, Ile337, Leu338 and Thr341 in the P-gp and ligands binding patterns.
- Eicosadeinoic acid has highest binding affinity with P-gp as the amount of energy needed to bind with P-gp was lowest (-11.92 kcal/mol).



Abbreviations used: P-gp: P-glycoprotein

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INTRODUCTION

Black cumin oil is a fixed oil and generally regarded as safe by the Food and Drug Administration.[1] The oil is obtained from the seeds of *Nigella sativa* L. (*Ranunculaceae*), an annual flowering plant. *Nigella* is indigenous to South-west Asia and especially found in the Mediterranean region. In India, *N. sativa* is found as a weed in Punjab, Himachal Pradesh, Bihar and Assam and commonly known as "Kalajira" or "Kalongi." The seeds are considered carminative, stimulant, diuretic, emmenagogual, and galactagogual, whereas their oil is applied externally for skin eruptions as antiseptic.[2] Seed oil is beneficial to treat eczema and boils and to prevent cold symptoms.[3,4] The seed oil has been reported to have antitumor,[5] antioxidant,[6] antibacterial,[7-9] anti-inflammatory,[10] hypoglycemic,[11] central nervous system depressant,[12] antioxidant, and immunostimulatory activities.[13,14] These activities have been attributed to the fixed oil, volatile oil, or their components. Seed oil consisted of 15 saturated fatty acids (17%) and 17 unsaturated fatty acids (82.9%). Linoleic acid (50.2%), oleic (19.9%), margaric acid (10.3%), cis-11, 14-eicosadienoic acid (7.7%), and stearic acid (2.5%) were the major components.[15]

P-glycoprotein (P-gp), an ATP-dependent active transporter belongs to ABC transporter superfamily, occurs not only in cancer cells but also in the plasma membrane of many normal tissues.[16,17] P-gp was reported

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as a possible site of interaction during the intestinal absorption.[18] Improved clinical efficacy of various drugs observed by P-gp inhibition in intestine, brain, liver, and kidneys, which has been hypothesized and emphasized by many researchers in recent years.[19] Long chain fatty acids (oleic and linoleic acid) and medium chain fatty acids (caprylic and capric acid) have been reported to increase oral bioavailability of peptides, antibiotics, and other important therapeutic agents.[20] The oral bioavailability of cinnarizine was greatly enhanced by oleic acid.[21] A concentration-dependent increase in the oral bioavailability of polar high molecular weight drugs such as glycyrrhizin in rats has been found with fatty acids.[22] Fatty acids have also been reported to produce a dose-dependent increase in the concentration of norfloxacin in rabbits.[23] Fatty acids act as absorption enhancers by increasing the fluidity of the apical and basolateral membranes.[24] *Nigella* oil interacted with carvedilol and amoxicillin when co-infused and increased the permeation and absorption across the gut wall. The hexane extract of *Nigella* seeds affected the intestinal absorption that might be attributed to the presence of fatty acids in it. Linoleic acid, oleic acid, margaric acid, cis-11, 14-eicosadienoic acid and stearic acid were identified as main fatty acids.[25,26] Although these studies lack information on their exact mechanism of action, a great interest is growing in order to understand the molecular mechanisms. Most of the drugs inhibit P-gp function by blocking drug binding sites and enhance the bioavailability. Then, the question is raised how the inhibitors are separated at the molecular level and block the binding sites of P-gp. Molecular docking is a method, which predicts the preferred orientation of two molecules when bound to each other and form a stable complex. Docking is frequently used to investigate the binding affinity and activity of the small molecule candidates to their protein targets receptor of known three-dimensional (3D) structure.[27] Thus, in the present study, we did a molecular docking analysis to investigate the mechanism how the fatty acids of black cumin oil inhibit the multi-drug resistance transporter P-gp at the molecular level and increase bioavailability of drugs.

MATERIALS AND METHODS

Three-dimensional modeling of rat P-glycoprotein receptor

Template search

Template search with BLAST and HHblits has been performed against the SWISS-MODEL template library (SMTL, last update: October 08, 2014, last included protein data bank (PDB) release: October 03, 2014). The target sequence was searched with BLAST[28] against the primary amino acid sequence of P-gp from *Rattus norvegicus* (Uniprot ID: P43245) contained in the SMTL.[29] A total of 137 templates were found. An initial HHblits profile has been built using the procedure outlined in Remmert *et al.*,[30] followed by one iteration of HHblits against NR20. The obtained profile has then been searched against all profiles of the SMTL. A total of 3270 templates were found.

Template selection

For each identified template, the template's quality has been predicted from features of the target template alignment. The templates with the highest quality have then been selected for model building. After analyzing obtained results, we have selected PDB ID: 3G60 (ABCB1 A of *Mus musculus*) as a template for the 3D model building of Rat P-gp.

Model building

Models are built based on the target template alignment using Promod-II. Co-ordinates which are conserved between the target and the template are copied from the template to the model. Insertions and deletions are remodeled using a fragment library. The side-chains are then rebuilt. Finally, the geometry of the resulting model is regularized by using a force field. In case loop modeling with ProMod-II[31] does not give satisfactory results, an alternative model is built with Modeller.[32]

Model quality estimation

The global and per-residue model quality has been assessed using the QMEAN scoring function.[33]

Model validation

The model validation completed by rampage Ramchandran Plot analysis server. We have found that 88.5% (1097) residues were lying in favored region, 8.5% (105) were in allowed region and 3.0% (37) were in the outer region.

Preparation of receptor molecule

Modeled 3D structures of rat P-gp were submitted to minimization process. Chimera 1.10 was used for energy minimization, removal of steric collision with the steepest descent steps 1000, steepest descent size 0.02 Å, Conjugated gradient steps 1000 and the conjugate gradient step size 0.02 Å for the conjugate gradient minimization.[34,35]

Preparation of three-dimensional structure of ligand

The.*mol* files of fatty acids from black cumin oil Linoleic acid, oleic acid, margaric acid, cis-11, 14-eicosadienoic acid and stearic acid were obtained from ChemSpider database. They were converted it into. *pdb* files using Accelrys Software Inc., Discovery Studio Modeling Environment, Release 4.0, (San Diego: Accelrys Software Inc, 2013). Discovery Studio makes it easier to examine the properties of large and small molecules. Further, the ligands were submitted for minimization using Chimera version 1.10 (Chimera development by the UCSF Resource for Biocomputing, Visualization, and Informatics is funded by the National Institutes of Health) using with Genetic Algorithm Steps 2000 and 0.5 grid units Optimized.[36]

Docking studies

Docking studies were performed by MGL tools version 1.5.6 Autodock 4.2 (MGLTools is a software developed at the Molecular Graphics Laboratory (MGL) of The Scripps Research Institute for visualization and analysis of molecular structures)[37,38] and Cygwin interface was used in the Microsoft Windows 7 professional service pack 1, operating System on Intel® Core™(Microsoft Corporation) i5, 3230M CPU at 2.60 GHz, 64-bit and 4.0 GB of RAM of Lenovo machine. We adopted molecular docking methods followed by searching the best conformation of P-gp and natural compounds complex based on total internal binding energy. Water molecules were removed from the protein structures before docking and hydrogen atoms were added to all target proteins. Kollman united charges, and salvation parameters were added to the proteins. Gasteiger charge was added to the ligands. Grid box was set to cover the maximum part of proteins and ligand. The values were set to 60 Å ×60 Å ×60 Å in X (25.427), Y (38.034) and Z (98.248) axis of a grid point. The default grid points, spacing, was 0.375 Å. Lamarckian Genetic Algorithm (LGA)[39] was used for proteins ligands flexible docking calculations. The LGA parameters such as population size (ga_pop_size), energy evaluations (ga_num_generation), mutation rate, crossover rate and step size were set to 150, 2500000, 27000, 0.02, 0.8 and 0.2 Å, respectively. The LGA runs were set at 10 runs. All 10 conformations of the receptor and ligands complex were analyzed and the interactions and binding energy of the docked structure using Accelrys Software Inc., Discovery Studio Modeling Environment, Release 4.0, (San Diego: Accelrys Software Inc, 2013) also graphics generated by PyMol.

Table 1: Detailed information of selected compounds

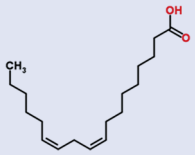
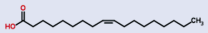
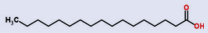
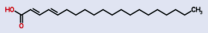
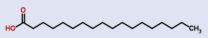
ChemSpider ID	Compounds	2D structure	Chemical formula	Average mass (Da)	SMILES
4444105[41]	Linoleic acid		$C_{18}H_{32}O_2$	280.445	CCCCC/C=C\C/C=C\CCCCCCCC(=O)O
393217[42]	Oleic acid		$C_{18}H_{34}O_2$	282.461	CCCCCCCC/C=C\CCCCCCCC(=O)O
10033[43]	Margaric acid		$C_{17}H_{34}O_2$	270.451	CCCCCCCCCCCCCCCCC(=O)O
9658485[44]	Eicosadienoic acid		$C_{20}H_{36}O_2$	308.499	O=C (O)\C=C\C=C\CCCCCCCCCCCCCC
5091[45]	Stearic acid		$C_{18}H_{36}O_2$	284.477	CCCCCCCCCCCCCCCCCC(=O)O

Table 2: Results obtained from docking analysis

Compounds	H bonds (common residue LYS 233 in bonding)	Hydrogen bonds distance (angstrom)	Amino acid involved in hydrophobic regions	Final intermolecular energy (vdW + H bond + desolve energy+electrostatic energy) (kcal/mol)	Inhibition constant (uM)
Linoleic acid	A: LYS233:HZ3 -:UNK1:O19	1.80324	Ser221, Pro222, Ile224, Gly225, Ser228, Ala229, Lys233,	−10.60	32.46
	A: LYS233:HZ3 -:UNK1:O20	2.0929	Tyr302, Tyr309, Ile337, Leu338, Thr341, Ile344, Gly345, Ala348		
Oliec acid	A: LYS233:HZ1 -:UNK1:O19	2.47896	Ser221, Pro222, Ile224, Gly225, Ser228, Ala229, Ala232,	−10.48	65.36
	A: LYS233:HZ1 -:UNK1:O20	1.63207	Lys233, Tyr302, Tyr309, Ile337, Leu338, Thr341		
	A: LYS233:HZ3 -:UNK1:O19	2.20812			
Margaric acid	A: LYS233:HZ1 -:UNK1:O19	2.32646	Ser221, Ile224, Gly225, Ser228, Ala229, Lys233, Tyr302,	−9.95	159.33
	A: LYS233:HZ3 -:UNK1:O18	1.84493	Tyr309, Ile337, Leu338, Thr341, Ile344, Gly345, Ala348		
	A: LYS233:HZ3 -:UNK1:O19	2.15184			
Eicosadeinoic acid	A: LYS233:HZ3 -:UNK1:O1	2.2499	Ile217, Leu218, Ser221, Pro222, Gly225, Ser228, Lys233,	−11.92	9.48
	A: LYS233:HZ3 -:UNK1:O3	1.70361	Tyr302, Ile337, Leu338, Thr341, Ile344, Gly345, Ala348		
Stearic acid	A: LYS233:HZ1 -:UNK1:O19	2.22793	Thr198, Gly202, Ser221, Gly225, Ser228, Ala229, Lys233,	−10.37	130.78
	A: LYS233:HZ3 -:UNK1:O19	2.32921	Tyr302, Tyr309, Ile337, Leu338, Gly340, Thr341, Ile344, Ala348		
	A: LYS233:HZ3 -:UNK1:O20	1.74899			

RESULTS

Detailed information of selected compounds and *in silico* results were documented in Table 1 and 2. Results showed that fatty acids exhibited interactions with P-gp and were found to bind easily in the active site with a slight conformational difference [Figures 1-4]. The amount of energy needed by Linoleic acid, Oliec acid, Eicosadeinoic acid, Margaric acid and Stearic acid to bind with P-gp were found to be – 10.60, –10.48, –9.95, –11.92 and – 10.37 kcal/mol respectively. All compounds showed binding energy values ranging between – 11.92 to – 9.95 kcal/mol. In the formation of complex for fatty acids with P-gp, involved amino acids were Ser221, Pro222, Ile224, Gly225, Ser228, Ala229, Lys233, Tyr302, Tyr309, Ile337, Leu338, Thr341, Ile344, Gly345, Ala348 for Linoleic acid, Ser221, Pro222, Ile224, Gly225, Ser228, Ala229, Ala232, Lys233, Tyr302, Tyr309, Ile337, Leu338, Thr341 for Oleic acid, Ser221, Ile224, Gly225, Ser228, Ala229, Lys233, Tyr302, Tyr309, Ile337, Leu338, Thr341, Ile344, Gly345, Ala348, for Margaric acid Ile217, Leu218, Ser221, Pro222, Gly225, Ser228, Lys233, Tyr302, Ile337, Leu338, Thr341, Ile344, Gly345, Ala348 [Figure 1] for Eicosadienoic acid and Thr198, Gly202, Ser221, Gly225, Ser228, Ala229, Lys233, Tyr302, Tyr309, Ile337, Leu338, Gly340, Thr341, Ile344, Ala348 for Stearic acid, respectively. Some amino acids are found to be common for all compounds such as Ser221, Pro222, Ile224, Gly225, Ser228, Ala229, Lys233, Tyr302, Tyr309, Ile337, Leu338, and Thr341. Linoleic acid and Eicosadienoic acid involved in the building of 2 hydrogen bonds with the minimum distance of 1.70361 Å, while Oleic acid, Margaric acid and Stearic acid involved in the formation of three hydrogen bonds with the minimum distance of 1.63207 Å. Inhibition constant was also predicted for fatty acids, which bring about additional information along with energy values. Inhibition constant for Linoleic acid, oleic acid, margaric acid, cis-11, 14-eicosadienoic acid and stearic acid were found to be 32.46 µm, 65.36 µm, 159.33 µm, 9.48 µm and, 130.78 µm, respectively [Table 2].

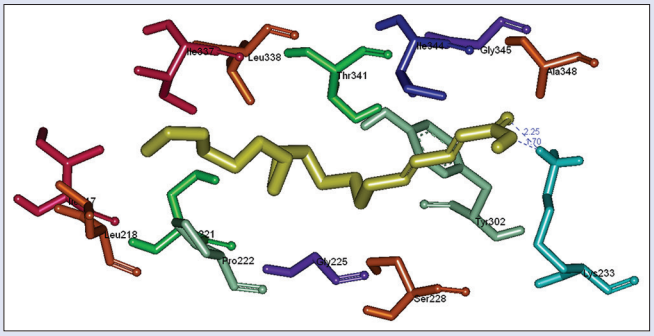


Figure 1: Amino acids involved in hydrophobic region found in rat P-glycoprotein and eicosadienoic acid (in golden color) interaction. Blue dotted line shows hydrogen bond and their distance in angstrom. Graphic generated by Discovery Studio Visualizer

DISCUSSION

Linoleic acid, oleic acid, margaric acid, cis-11, 14-eicosadienoic acid and stearic acid were selected for *in silico* docking studies to understand the mechanism of binding interaction between fatty acids and P-gp.

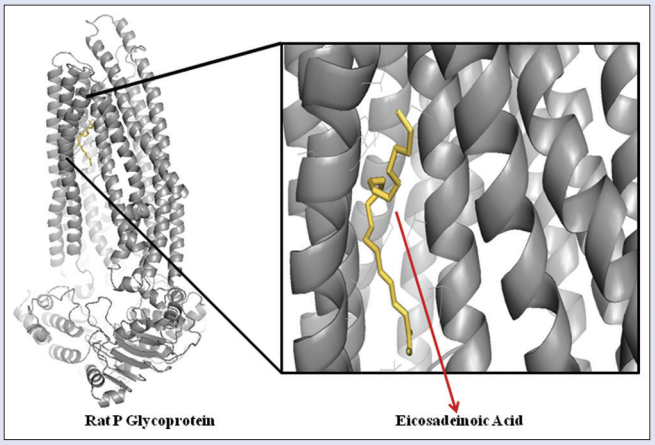


Figure 2: Eicosadienoic acid (in golden color) accommodated by active site of rat P-glycoprotein. Graphics generated by PyMol three-dimensional structure visualization software

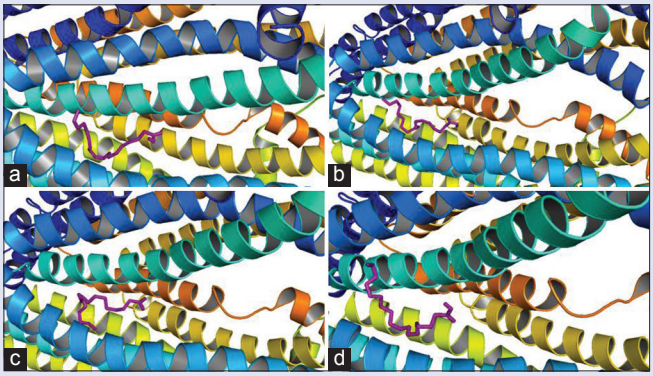


Figure 3: Conformational visualization of rat P-glycoprotein (shown in ribbon) interaction with (a) linoleic acid (b) oleic acid (c) margaric acid and (d) steric acid. All compounds shown in stick purple color. Graphics generated by PyMol

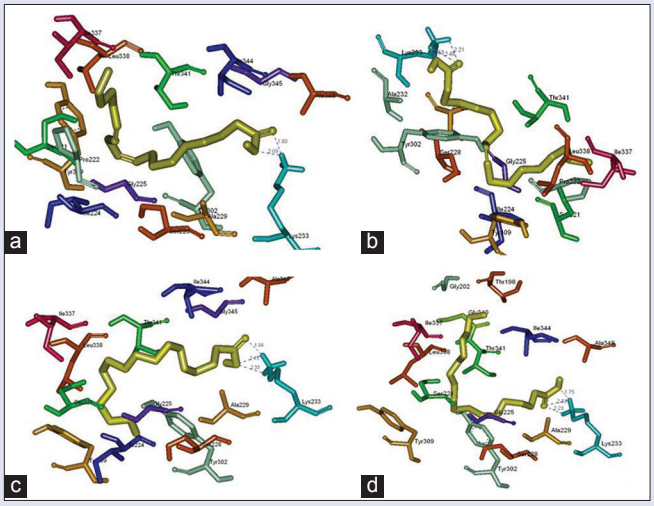


Figure 4: Amino acids of rat P-glycoprotein (shown in stick with multiple colors) involved in hydrophobic interaction with (a) linoleic acid, (b) oleic acid, (c) margaric acid, and (d) steric acid. The distances of hydrogen bonds (in angstrom) shown by blue dotted lines. All compounds shown in stick golden color. Graphics generated by Discovery Studio Visualizer

Unfortunately, the 3D structure of rat P-gp was not available in the PDB so we have modeled the structure using homology modeling approach from SWISS-MODEL server. The most suitable templates were searched using the BLAST program again PDB. We selected PDB ID: 3G60 (ABCB1 A of *Mus musculus*) as a template for the 3D model building of rat P-gp. The model quality estimation was done by the Q-mean scoring function. The obtained QMEAN Z-score was − 8.46 kcal/mol. Further, the model was validated by RAMPAGE (Ramachandran plot) (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) analysis server. We have found that 88.5% (1097) residues were lying in the favored region, 8.5% (105) were in allowed region and 3.0% (37) were in the outer region. Active sites of proteins are often associated with structural pockets in the protein. The identification of such substrate binding sites in enzymes helps us to understand their binding interactions with substrates and other small molecules. The drug binding site of P-gp was taken from the template structure that was used for homology modeling. The important parameters determined were binding energy, inhibition constant and intermolecular energy. All selected fatty acids involved in building of hydrogen bonds. Hydrogen bond interactions play a significant role in predicting the binding affinity and help in describing drug permeability.[40] The obtained data supports that all the selected fatty acids have contributed to inhibit P-gp activity thereby enhances the bioavailability of drugs. Eicosadienoic acid has a highest binding affinity with P-gp as the amount of energy needed to bind with P-gp was lowest (−11.92 kcal/mol).

CONCLUSIONS

Complete understanding of P-gp efflux mechanisms would offer an opportunity for not only enhancing the bioavailability of life-saving drugs such as paclitaxel and saquinavir but also improve their pharmacokinetics.[19] This manuscript provides details of interactions between fatty acids and P-gp varying in their chemical nature and binding affinity, which has helped in understanding the mechanism how fatty acids inhibit P-gp through binding and increase the bioavailability of drugs. This study may offer further scope of designing potent and specific inhibitors and play a vital role in finding hot spots in P-gp.

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Nil.

Conflicts of interest

There are no conflict of interest.

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