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In silico investigations revealed four potential colon cancer drugs from phytochemicals in *Zingiber officinale*

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Abstract

Cancer is a difficult disease to treat, and few effective drugs are available. Hence, it is of great importance to develop effective anti-cancer therapeutic agents with well-defined pharmacokinetic properties. Although, ginger (*Zingiber officinale*) has a number of proven pharmacological activities, its effect on colon cancer has not received much attention. This study therefore investigated potential colon cancer drug in compounds found in ginger. Dihydropyrimidine dehydrogenase was modeled using comparative homology modeling and virtual screening was performed locally on a Linux platform using AutoDock Vina®. The results showed that human dihydropyrimidine dehydrogenase is a homolog of pig dihydropyrimidine dehydrogenase. The leads were beta-sitosterol, 6-Shogoal, Alloaromadedrene, and Zingiberol. They had similar binding site with levamisole for tumor necrosis factor ligand superfamily member 6 with His 148 and Tyr 192 common at their binding site whereas they had different binding sites from 5-fluorouracil for dihydropyrimidine dehydrogenase. The leads had better bioactivities compared with reference drugs (5-flourouracil and Levamisole) approved clinically for the treatment of colon cancer. *In vitro*, *ex vivo* and/or *in vivo* validations of the leads against colon cancer are recommended.

Keywords : Colon cancer; *Zingiber officinale*; virtual screening; Ginger; Molecular docking

Introduction

The development of novel, efficient, selective and less toxic cancer therapeutic molecules has been a challenging goal [1]. Clinical trials in identifying new agents and treatment modalities have been significant with many limitations. This includes side effects induced by the drugs and acquired drug resistance [2]. Thus, the need for the development of effective anti-cancer therapeutic agents with well-defined pharmacokinetic properties is of great importance [1]. Levamisole has anticancer activity in combination with 5-fluorouracil (5-FU) as adjuvant therapy for tumor-node-metastasis (TNM) stage III (Dukes' C) colon carcinoma. The molecular targets of levamisole and 5-FU are tumor necrosis factor ligand superfamily member 6 (FAS-L) and dihydropyrimidine dehydrogenase (DPD) respectively.

It has been reported that a derivative of levamisole ((2-benzyl-6-(4`-fluorophenyl)-5-thiocyanato-imidazo [2,1-b][1,3,4] thiadiazole) treatment activates FAS and FAS-L death receptor pathway, leading to cleavage of CASPASE-8 followed by activation of CASPASE-3. Hegde *et al.,* [1] reported that extrinsic pathway of apoptosis is induced by the levamisole derivative leading to cell death both *in vivo* and *ex vivo* suggesting that the levamisole derivative could be used as a potential cancer therapeutic agent. It has equally been reported that dihydropyrimidine dehydrogenase - mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumor cells and that up to 80% of administered 5-FU are broken down by DPD in the liver [3]. Hence, they are used as targets in the present investigation.

Ginger (*Zingiber officinale*) has a number of proven pharmacological activities such as cardio protective activity, anti-inflammatory activity, anti-microbial activity, antioxidant property, anti-proliferative activity and hepatoprotective activities but effect of ginger on colon cancer has remained obscure [4]. Therefore, the present study investigated the potential colon cancer drug of compounds found in ginger using FAS-L and dihydropyrimidine dehydrogenase as targets and four potential colon cancer agents found in ginger were reported.

Materials and Methods

Materials

The materials used in this work are computer software, online databases, and tools. They include Linux based operating system (Ubuntu 12.04), autodock-vina®, autodocktools, PyMol 1.4.1, UCSF

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Chimera 1.9, Modeller, ModBase, Zinc database, Protein databank, DrugBank, Molinspiration, OpenBabel, Traditional Chinese medicine Systems Pharmacology Database and Analysis Platform (TCMSP).

Homology modeling of human dihydropyrimidine dehydrogenase

Tumor necrosis factor ligand superfamily member 6 was obtained from protein data bank (pdb) database. Because the 3-D coordinate of dihydropyrimidine dehydrogenase from human was not available in pdb database as at the time of the investigation, comparative homology modeling of its structure was achieved with its amino acid sequence obtained from GenBank (Accession number: AAB51366.1) using ModWeb Server [5]. Briefly, the amino acid sequence was submitted and a total of 389 hits were detected. Number of models due to the sequence were calculated and two best models were selected from the calculated models using ModPipe quality score (MPQS), Estimated native overlap (TSVMOD), Discrete optimized protein energy (DOPE) and LONGEST_DOPE as selection criteria. The models reliability/fold assignments were evaluated using MPQS 1.1, TSVMod No. 35 (estimated native overlap at 3.5 Å) 40 %, GA341 (Model score) 0.7, E-value < 0.0001 and zDOPE < 0 [6]. On this basis, one of the models (model 1) was selected and further evaluated with ModEval in ModWeb Server [5]. Briefly, amino acid sequence and 3-d coordinate of the selected model was submitted to ModEval Server and model evaluation parameters (Predicted RMSD, Predicted native overlap, sequence identity, zDOPE, GA341, z-pair, z-surf, z-combi and DOPE profile were calculated and returned.

Visualization of surface cavities and Loop modeling/optimization

Visualization of the surface cavities of the modeled protein was achieved with UCSF Chimera 1.9 [7]. Minimum cavity score was assigned light blue, medium cavity score were assigned green, maximum cavity score was assigned maroon while cysteine residues on the surface was marked yellow. Also, the volume and area of main surface cavity in the dihydropyrimidine dehydrogenase from the model template (chain A of 1gte) and that of modeled protein were calculated with UCSF Chimera 1.9. Comparative protein structure prediction is limited mostly by the errors in alignment and loop modeling [8]. In many cases, one can obtain better quality loops (at the expense of more computer time) by using the newer DOPE-based loop modeling protocol [9]. Loops in the modeled protein were modeled locally with Modeller 9.14 using DOPE modeling protocol [10]. Briefly, the 3-D coordinate of the protein was used, DOPE modeling protocol was specified and Modeller 9.14 was called from UCSF Chimera 1.9 interface to generate a total of five (5) models. The result was analyzed with UCSF Chimera and one of the models (model 5) with the lowest zDOPE score was used for virtual screening.

Preparation of receptors

AutoDock-vina® [11] was employed to gain insight into the ligand binding to tumor necrosis factor ligand superfamily member 6 (pdb: 4MSV) and the modeled human dihydropyrimidine dehyrogenase. The 3-D atomic coordinates of 4MSV was obtained from Protein Data Bank (PDB) and prepared for docking simulation. Both tumor necrosis factor ligand superfamily member 6 (pdb: 4MSV) and the modeled human dihydropyrimidine dehyrogenase were prepared for docking simulation using UCSF Chimera [7] and MGLTools-1.5.6 [12, 13]. Briefly, chain B (DCR3) of 4MSV and all hetero molecules were deleted and non-polar hydrogens were merged. Grid box sizes of 25 x 25 x 35 and center of -12.189 x 4.798 x -43.121 at 1.0 Å grid spacing and exhaustiveness of 10 was applied to tumor necrosis factor ligand superfamily member 6 (pdb: 4msvA) using MGLTools-1.5.6 [12, 13]. Also, Grid box sizes of 55 x 65 x 80 and center of 64.279 x 66.976 x 82.163 at 1.0 Å grid spacing and exhaustiveness of 12 was applied to the human dihydropyrimidine dehyrogenase.

Preparation of ligands

The ZINC® Database [14] was used to obtain the 3-D coordinates of 5-fluorouracil (Zinc code, 38212689) and levamisole (119839) which were used as positive controls. Likewise azoxymethane (Zinc code, 60286308) and 1,2-dimethylhydrazine (Zinc code, 38147320) were used as negative controls. The 3-D coordinate of sixty four (64) phytochemicals present in *Z. officinale* were obtained from Traditional Chinese medicine Systems Pharmacology Database and Analysis Platform [15]. Energy minimization of the ligands (phytochemicals) was achieved with Chimera 1.9 [7]. Briefly, each phytochemical was subjected to 100 steps of steepest decent and 10 steps of conjugate gradient energy minimization at step size of 0.02 Å. Using autodocktools, all hydrogens were added to each of the energy minimized ligands. Also, rotatable bonds and torsions were assigned to them and were subsequently saved as pdbqt files. The prepared receptor and ligands were used for molecular docking simulation.

Molecular docking simulation

AutoDockVina® has reported high accuracy in predicting binding free energies by setting the receptor rigid while appraising flexible ligands with a comparatively low standard error [11, 16]. Therefore, tumor necrosis factor ligand superfamily member 6 and dihydropyrimidine dehyrogenase conformational flexibility were neglected by rigid receptor docking. The ligands were docked into tumor necrosis factor ligand superfamily member 6 and dihydropyrimidine dehyrogenase using AutoDockVina® and the

virtual screening was done locally in quadruplet on Linux platform using a script and configuration file containing information on the prepared receptors and ligands.

Lead identification/optimization

The results of virtual screening were ranked according to their affinities to identify the hits. The hits were further screened for Absorption Distribution Metabolism and Excretion (ADME) compliance using an in-house database created with Traditional Chinese Medicine Systems Pharmacology database and Analysis Platform [15], Molinspiration web based software available at www.molinspiration.com and obabel-2.3.2. Briefly, the hits were screened for parameters such as oral bioavailability (OB) of 30%; blood brain barrier (BBB) of -0.3 to +0.3 for moderate penetrating (BBB±) or ＞0.3 for strong penetrating (BBB+); Half-life (HL) of 4 hours for fast-elimination group, between 4-8 hours for mid-elimination group or 8 hours for slow-elimination group; topological polar surface area (tPSA) of less than 60 angstroms squared for cell membrane permeable; and number of rotatable bonds (RBN) of 10 or fewer for good oral bioavailability [15]. All the hits with the mentioned parameters were ranked according to their affinities for dihydropyrimidine dehydrogenase and tumor necrosis factor ligand superfamily member 6. Then, the leads were identified and characterized according to their biological activities (GPCR ligand, ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease inhibitor and enzyme inhibitor). The biological activities of the leads were compared with positive and negative controls.

Data analysis

The binding affinities were calculated and reported as mean ± SD. Visualization and analysis of the virtual screening results (the leads) was achieved with MGLTools-1.5.6 and UCSF Chimera 1.9. Also, structural alignment and analysis of the binding site of the dihydropyrimidine dehyrogenase template (chain A of 1gte) was done with Pymol-1.4.1

Results and Discussion

Comparative Homology Modeling

The result showed that the human modeled dihydropyrimidine dehydrogenase (DPD) is a homolog of pig dihydropyrimidine dehydrogenase (Table 1, Figure. 1A). The sequence identity of the human modeled enzyme and the pig enzyme was 93 % (Table 1). This suggests that human DPD is an ortholog of pig form of the enzyme. Structural alignment of the human DPD and chain A of pig DPD showed that iodouracil have the same binding site for both enzymes (Figure. 1B). It was also observed that the human DPD showed a main surface volume of 120.90e^3 and an area of 44.90e^3 compared with the template (1gteA) which showed main surface volume and area of 121.40e^3 and 44.22e^3 respectively. Also, the DPD had a minimum cavity score (light blue), medium cavity score (green), and cysteine residues on surface (yellow) (Figure 2).

Table 1 Comparative homology modeling parameters using amino acid sequence of human dihydropyrimidine dehydrogenase

Parameter	Model 1	Model 2
Target region	2-1017	654-733
Protein length	1025	1025
Template pdb code	1gteA	2ze3A
Template region	2-1017	29-110
Sequence identity	93.00%	19.00%
E-value	0	0
GA341	1.00	0.35
MPQS	2.06762	0.274449
z-DOPE	-0.65	-0.68
TSVMod Method	MTALL	MSALL
TSVMod RMSD	1.571	5.935
TSVMod NO 35	0.935	0.463

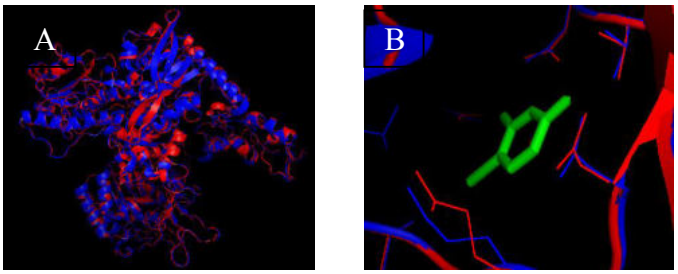

Figure 1. (A) Structural alignment of the 3-D coordinate of template (1gteA) (Blue color) and human model (Red color) of dihydropyrimidine dehydrogenase. (B) Structural alignment of the same enzymes showing iodouracil (green color) at their binding sites.

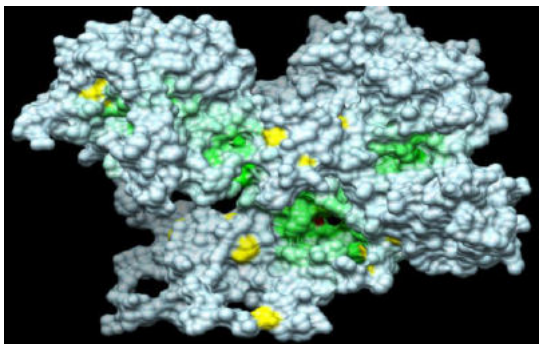

Figure 2. Surface cavity of human model of diphydropyrimidine dehydrogenase (DPG) Minimum cavity score was assigned light blue, medium cavity score was assigned green, maximum cavity score was assigned maroon while cysteine residues on the surface were marked yellow.

Model Evaluation

Table 2 shows the TSVMod and Modeller scoring results of human model of dihydropyrimidine dehydrogenase (DPD) while Figure. 3 shows the DOPE profile of human DPD. The result reveled that human model of dihydropyrimidine dehydrogenase (DPD) was reliable because it exhibited the following reliable fold assignment parameters; MPQS 1.1, TSVMod No. 35 (estimated native overlap at 3.5 Å) 40 %, GA341 (Model score) 0.7, E-value < 0.0001 and zDOPE < 0 [6]. Comparative protein structure prediction is limited mostly by the errors in alignment and loop modeling [8]. Therefore, better quality loops in human model of DPD was achieved at the expense of more computer time by using DOPE-based loop modeling protocol [9]. The result showed that model 5 had a better quality loop compared to other models because it has the lowest zDOPE (Table 3).

Figure 3. DOPE profile of human model of dihydropyrimidine dehydrogenase

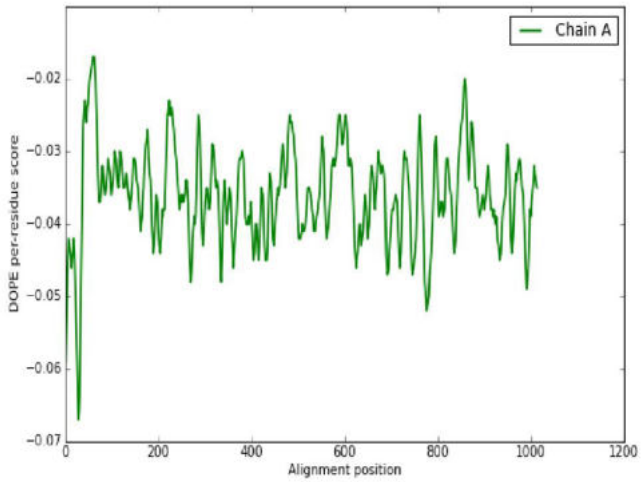

Table 3 Loop modeling parameters for human model of dihydropyrimidine dehydrogenase

Model	GA341	zDOPE
1	1.00	-0.53
2	1.00	-0.55
3	1.00	-0.52
4	1.00	-0.50
5	1.00	-0.58

Virtual screening results

The ligands were ranked according to their mean affinities and compared with negative (azoxymethane and 1,2-dimethylhydrazine) and positive (5-fluorouracil or levamisole) controls. The affinities of azoxymethane and 1,2-dimethylhydrazine for dihydropyrimidine dehydrogenase were -3.2 ± 0.0 and -3.1± 0.0 kcal/mol respectively while that of 5-fluorouracil was -5.4 ± 0.0 kcal/mol. Forty one (41) compounds in ginger were hits for dihydropyrimidine dehydrogenase while twenty three (23) were hits for tumor necrosis factor ligand superfamily member 6. Further screening of the hits according to their Absorption Distribution Metabolism and Excretion (ADME) properties, showed that only fifteen (15) compounds were potential colon cancer drug for dihydropyrimidine dehydrogenase target (Table 4) while five (5) were potential cancer drug for tumor necrosis factor ligand superfamily member 6 (Table 5). The leads (beta-sitosterol, 6-Shogoal, Alloaromadedrene, and Zingiberol) had similar binding site with levamisole for tumor necrosis factor ligand superfamily member 6 with His 148 and Tyr 192 mainly common at their binding site (Table 6, Figure. 4 B, and 5) whereas they had different binding site with 5-fluorouracil for dihydropyrimidine dehydrogenase (Table 7, Figure. 4 A, and 6).

The identified leads for colon cancer (beta-sitosterol, 6-Shogoal, Alloaromadedrene, and Zingiberol) were further characterized according to their bioactivities and compared with the negative and positive controls. The results showed that the identified leads of anti-colon cancer agents had better bioactivities compared with reference drugs (5-flourouracil and Levamisole) approved for clinical use and negative controls (azoxymethane and 1,2-dimethylhydrazine) (Table 8). This suggests that the leads will have better pharmacological response than the approved clinical drugs.

Previous study has shown that β-sitosterol is used for many diseases including prevention of cancer, cervical cancer, benign prostatic hyperplasia and modulating of immune system [17]. It has been reported that 6-gingerol and 6-shogaol showed significant anticancer activities toward HeLa cancer cell lines at certain concentration without being toxic to normal cells [18]. Studies in animal models have shown that ginger and its phenolic constituents (6-gingerol) suppress carcinogenesis in the skin, gastrointestinal tract, colon, breast [19-22]. It has been suggested that 6-shogaol is an effective therapeutic agent for treating neurodegenerative diseases [23]. The chemopreventive mechanisms of ginger are not well understood but are thought to involve the up-regulation of carcinogen-detoxifying enzymes, antioxidant and anti-inflammatory activity [23]. The present study has validated beta-sitosterol and 6-Shogoal as anticancer agents and has predicted alloaromadedrene, and zingiberol as anticancer agents.

Table 4 Hits for colon cancer drug for dihydropyrimidine dehydrogenase target

Mol ID	Molecule name	MWT (g/mol)	XLogP	Hdon	Hacc	OB (%)	BBB	DL	TPSA (Å^2)	RBN	HL (Hour)	Affinity (Kcal/mol)
MOL000358	beta-sitosterol	414.79	8.08	1	1	36.91	0.99	0.75	20.23	6	5.36	-8.375±0.09
MOL000066	alloaromadedrene	204.39	4.22	0	0	53.46	2.1	0.10	0	0	12.51	-7.40±0.000
MOL006123	zingiberol	222.41	4.18	1	1	37.24	1.25	0.07	20.23	4	4.43	-6.80±0.000
MOL002495	6-shogaol	276.41	4.55	1	3	31	0.49	0.14	46.53	9	4.07	-6.13±0.263
MOL000118	(L)-alpha-Terpineol	154.28	2.42	1	1	48.8	1.72	0.03	20.23	1	11.35	-5.95±0.404
MOL001217	()-Bornyl acetate	196.32	2.35	0	2	65.55	1.59	0.08	26.3	2	6.94	-5.90±0.082
MOL000119	Nerolidol	222.41	4.56	1	1	40.34	1.31	0.06	20.23	7	4.73	-5.875±0.05
MOL002042	thymol	150.24	3.24	1	1	41.47	1.68	0.03	20.23	1	11.33	-5.875±0.05
MOL000234	L-Limonen	136.26	3.5	0	0	38.09	2.13	0.02	0	1	11.64	-5.775±0.45
MOL000608	()-Terpinen-4-ol	154.28	2.55	1	1	81.41	1.66	0.03	20.23	1	10.81	-5.78±0.222
MOL000206	isoeugenol	164.22	2.5	1	2	70.1	1.28	0.04	29.46	2	0.65	-5.775±0.05
MOL001254	(S)-p-Mentha-1,8-dien-7-al	150.24	2.67	0	1	39	1.57	0.03	17.07	2	2.22	-5.75±0.300
MOL000202	Moslene	136.26	3.45	0	0	33.02	2.05	0.02	0	1	11.08	-5.60±0.216
MOL000122	1,8-cineole	154.28	2.15	0	1	39.73	2.06	0.05	9.23	0	11.29	-5.58±0.150
MOL000268	(1S,5S)-1-isopropyl-4-methylenebicyclo[3.1.0]hexane	136.26	2.93	0	0	46.21	2.18	0.04	0	1	11.47	-5.55±0.404

Octanol water partion coefficient (XLogP), Number of hydrogen bond donors (Hdon), Number of hydrogen bond donors (Hacc), Topological polar surface area (tPSA), molecular weight (MwT), Oral bioavailability (OB), Drug likeness (DL), Blood brain barrier (BBB), Number of rotatable bonds (RBN), Half-life (HL)

Table 5: Hits for colon cancer drug for tumor necrosis factor ligand superfamily member 6 target

Mol ID	Molecule name	MWT (g/mol)	xLogP	Hdon	Hacc	OB (%)	BBB	DL	TPSA (Å^2)	RBN	HL (Hour)	Affinity (Kcal/mol)
MOL000358	beta-sitosterol	414.79	8.08	1	1	36.91	0.99	0.75	20.23	6	5.36	-5.7±0.327
MOL002495	6-shogaol	276.41	4.55	1	3	31.00	0.49	0.14	46.53	9	4.07	-5.30±0.14
MOL000066	alloaromadedrene	204.39	4.22	0	0	53.46	2.10	0.10	0.000	0	12.51	-5.10±0.00
MOL006123	zingiberol	222.41	4.18	1	1	37.24	1.25	0.07	20.23	4	4.43	-5.08±0.05
MOL000119	Nerolidol	222.41	4.56	1	1	40.34	1.31	0.06	20.23	7	4.73	-4.80±0.08

Table 6: Binding site of lead colon cancer drug in *Z. officinale* for tumor necrosis factor ligand superfamily member 6 target

Levamisole	Beta-sitosterol	6-shogaol	Alloaromadedrene	zingiberol
His 148	Val 146	Val 146	Val 146	Arg 144
Tyr 192	His 148	His 148	Tyr 279	Val 146
	Ile 168	Tyr 192		Tyr 192
	Tyr 192	Tyr 244		Tyr 279
	Tyr 279	Tyr 279		

Table 7: Binding site of lead colon cancer drug in *Z. officinale* for dihydropyrimidine dehydrogenase target

5-Fluorouracil	Beta-sitosterol	6-shogaol	Alloaromadedrene	zingiberol
Gly 281	Asp 60	Glu 68	Phe 157	Glu 68
Gly 479	Thr 65	Phe 157	Val 856	Phe 157
Asp 480	Glu 68	Glu 160		Glu 180
	Phe 157	Val 161		Pro 853
	Val 161	His 858		Thr 855
	Pro 236			Val 856
	Pro 853			His 858
	Thr 855			

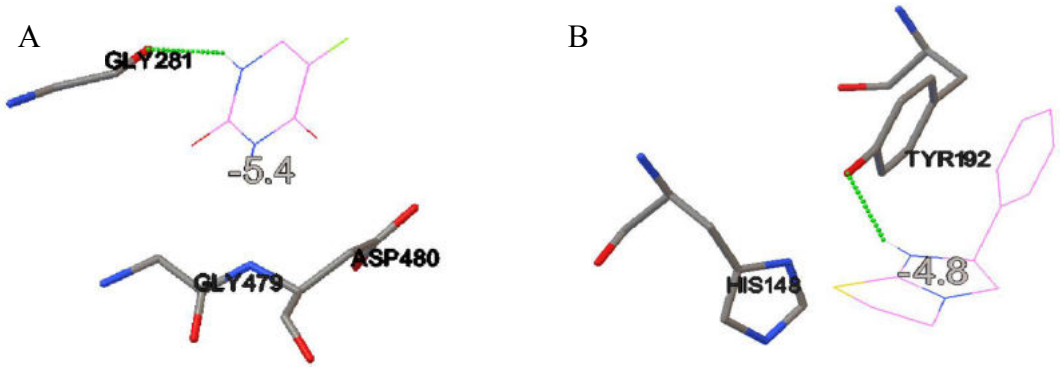

Figure 4: Amino acids at the binding sites of (A) DPD and (B) FASL for 5-flourouracil and Levamisole respectively. The amino acids are represented as sticks while the drugs are represented as lines. Green dots are the hydrogen bonds between the the ligands and the amino acids.

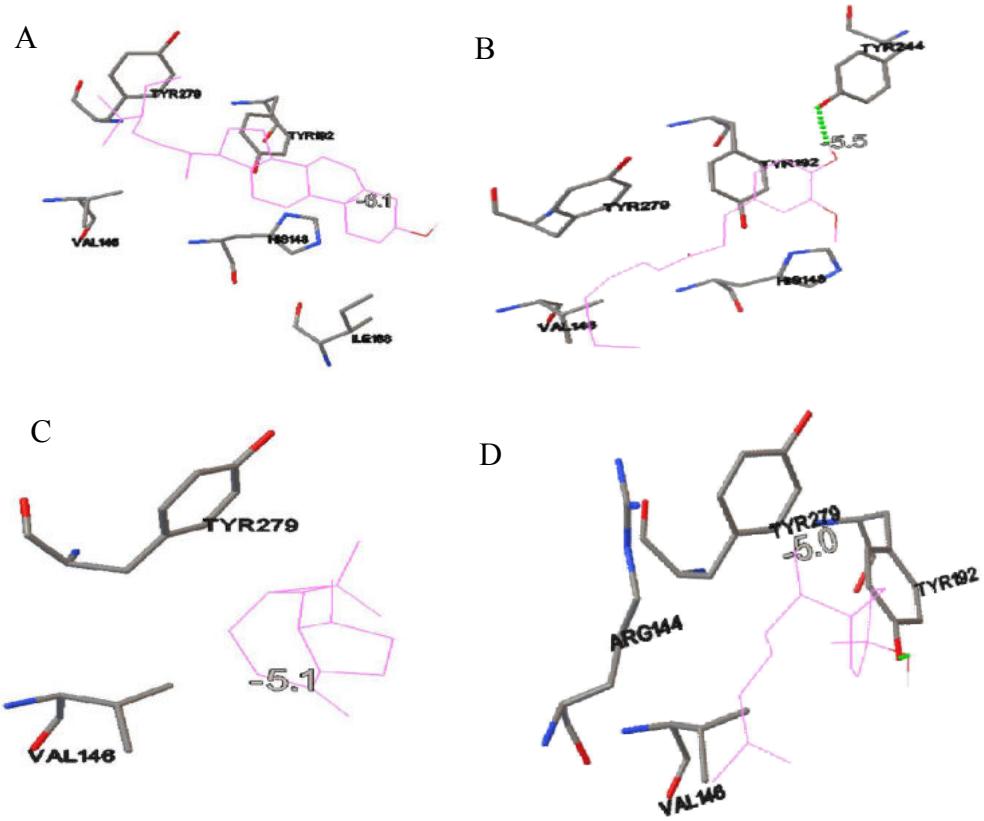

Figure 5: Binding sites of some leading anti-colon cancer agents from ginger for FASL (A) beta-sitosterol, (B) 6-Shogoal (C) Alloaromadedrene, and (D) Zingiberol. The amino acids are represented as sticks while the ligands (phytochemicals) are represented as lines. Green dots are the hydrogen bonds between the the ligands and the amino acids.

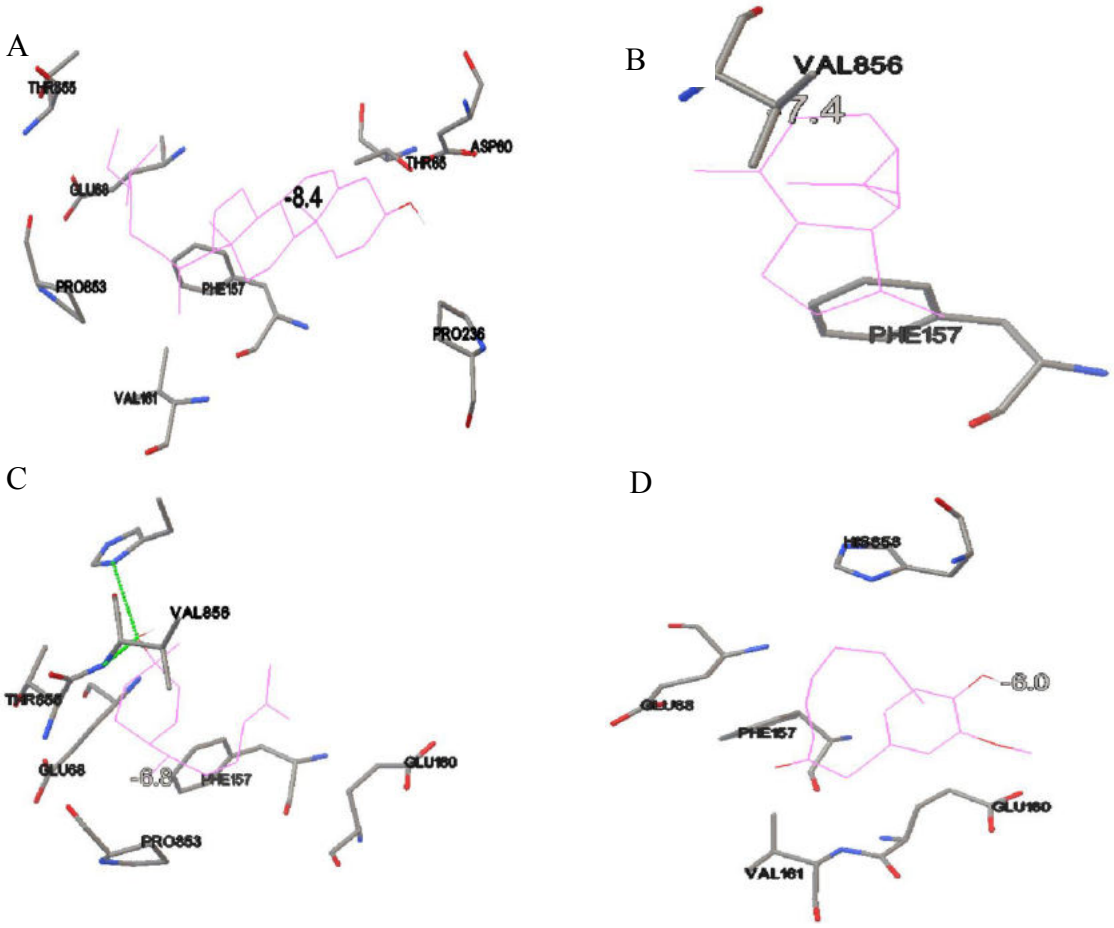

Figure 6: Binding sites of some leading anti-colon cancer agents from ginger for DPD (A) beta-sitosterol, (B) alloaromadedrene, (C) Zingiberol and (D) 6-Shogoal. The amino acids are represented as sticks while the ligands (phytochemicals) are represented as lines. Green dots are the hydrogen bonds between the the ligands and the amino acids.

Table 8: Bioactivity scores of some lead colon cancer drug in ginger compared with the positive and negative controls

Mol ID	Molecule name	GPCR L	ICM	KI	NRL	PI	EI
MOL000066	alloaromadedrene	-0.66	-0.47	-0.98	-0.21	-0.67	-0.3
MOL000358	beta-sitosterol	0.14	0.05	-0.51	0.73	0.07	0.51
MOL002495	6-shogaol	0.06	0.01	-0.5	0.21	-0.05	0.29
MOL006123	zingiberol	-0.04	0.02	-0.66	0.5	-0.29	0.38
38212689	5-fluorouracil	-2.60	-1.95	-2.61	-3.084	-3.15	-1.56
60286308	azoxymethane	-3.97	-3.69	-4.33	-4.68	-4.31	-3.86
119839	levamisole	-0.79	-0.36	-1.24	-1.34	-0.71	-0.54
38147320	1,2-dimethylhydrazine	-4.62	-4.22	-4.21	-4.55	-4.00	-4.22

GPCR L= G-protein coupled receptor ligand, ICM= ion channel modulator, KI= kinase inhibitor, NRL= nuclear receptor ligand, PI= protease inhibitor and EI= enzyme inhibitor

Conclusion

Human dihydropyrimidine dehydrogenase is a homolog of of pig dihydropyrimidine dehydrogenase. The lead potential colon cancer drugs found in ginger were beta-sitosterol, 6-Shogoal, Alloaromadedrene, and Zingiberol. These leads had similar binding site with levamisole for tumor necrosis factor ligand superfamily member 6 with His 148 and Tyr 192 mainly common at their binding site whereas they had a binding site different from that of 5-fluorouracil for dihydropyrimidine dehydrogenase. The leads had better bioactivities compared with reference drugs clinically approved for the treatment of colon cancer (5-flourouracil and Levamisole). The present study validated beta-sitosterol and 6-Shogoal as anticancer agents and has predicted alloaromadedrene, and zingiberol as anticancer agents. *In vitro*, *ex vivo* and/or *in vivo* validations of the leads against colon cancer are recommended.

Author's contribution

FCE conceived of the study, and participated in its design, Homology modeling, molecular docking simulations, coordination and helped to draft the manuscript. ICU participated in design, molecular docking simulations and drafting of the manuscript. CBL participated in data analysis, interpretation of results and drafting of the manuscript. INO participated in data collection and interpretation of results. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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