Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 3, 2014

Original Article

IN SILICO SCREENING TO ELUCIDATE THE THERAPEUTIC POTENTIALS OF ASPARAGAMINE A

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Received: 17 Feb 2014, Revised and Accepted: 03 Mar 2014

ABSTRACT

Objective: *Asparagus racemosus*, the source of Asparagamine A, has been known for its multifaceted therapeutic actions. But these actions have hardly been attributed to Asparagamine A, a polycyclic pyrrolizidine alkaloid.

Methods: In the present study, a molecular docking of Asparagamine A with critical proteins associated with many diseases. Farnesyl Pyrophosphate Synthase (FPPS) in osteoporosis, Plasmepsin II in malaria, HIV1 proteases in AIDS, CmaA2 and PKnB in tuberculosis, Trypanothione Reductase (TR) in Trypanosomiasis and Leishmaniasis, Insulin Receptor (IR), Vascular Endothelial Growth Factor Receptors (VEGFR) and Peroxisome Proliferator Activated Receptors (PPAR) in cancer, are few of the proteins being targeted for their associated diseases. Lamarckian Genetic Algorithm was applied for molecular docking using Autodock4.2. The metabolite structures were retrieved from KNApSAcK-3D database. PreADMET server was used for Toxicity and ADME predictions.

Results: Asparagamine A was found to exhibit good drug-likeness score, good ADME properties with no carcinogenicity and toxicity. Asparagamine A showed a higher affinity with the above mentioned proteins than standard commercially available drugs. Thus, the phytochemical Asparagamine A can be a potential therapeutic molecule.

Conclusion: Asparagamine A gave a high affinity for crucial drug targets involved in many diseases, thus providing a clue for design of lead molecules with better specificity and affinity. Further, *in vitro* and *in vitro* studies needs to be carried out.

Keywords: *Asparagus racemosus*, Asparagamine A, Molecular Docking, ADME & Toxicity.

INTRODUCTION

Asparagus racemosus belongs to the family of Asparagaceae and commonly known as Shatavari, is found in tropical and subtropical regions of Asia, Africa and Australia [**1**]. In traditional medicinal systems like *Ayurveda, Unani and Siddha*, this plant has been reported to possess potential to treat variety of diseases and disorders. The plant is widely known for its excellent rejuvenating activity on female reproductive system. In addition, extracts from various plant parts of *Asparagus* are well known to possess anti-ulcer, anti-bacterial, anti-depressant, anti-inflammatory, antiprotozoal, antiviral, antioxidant and anticancer properties [**1-4**]. Such a great variety of therapeutic usefulness of this plant is due to the wide variety of phytochemicals present in it. The major phytochemicals present in the *Asparagus racemosus* include Shatavarins (I-IV), Idaein, Immunoside, Racemosides, Diosgenin, Sarasasapogenin, Asparagamine A etc. [**1, 3-5**]. Among these, Shatavarin IV has already been known to have anti-cancerous activity [**6**]. Keeping in view, the wide therapeutic activity of *Asparagus racemosus*, not much work has been done to attribute these therapeutic benefits to any specific phytochemical. Interestingly, when the structures of all the phytochemicals of this plant were evaluated, Asparagamine A, a polycyclic pyrrolizidine alkaloid, found to have a very unique cage like structure [**7-8**]. Though this unique structured Asparagamine A was isolated and characterized two decades ago by Sekine *et.al.*, yet not much have been reported about its potential activities in the scientific literatures [**7**]. This compound has been reported to possess excellent antioxidant, anti-oxytocin, antitumor activities [**8-10**], but the effectiveness and efficacy of the compound was never evaluated over wide range of diseases against which *Asparagus racemosus* has shown therapeutic benefits. In the present study, we have tried an *In silico* approach to explore the therapeutic potentials of Asparagamine A over osteoporosis, AIDS, tuberculosis, cancer, malaria, leishmaniasis and trypanosomiasis. The critical proteins which are considered to be essential for the development and progression of these diseases were isolated computationally and were docked with Asparagamine A using AutoDock4.2 tool (version 1.5.6).

MATERIALS AND METHODS

Protein structure retrieval

The three dimensional crystal structures of target proteins were retrieved from Protein Data Bank (http://www.rcsb.org/pdb/). The proteins selected for targeting the diseases have been compiled in Table 1. Miscellaneous ligands and other hetero-atoms such as water, ions, etc. were removed from the protein models for active site predictions and further docking studies using Argus Lab Software.

Protein Active Site Predictions

The active sites of target proteins were predicted by using CASTp Calculations (Computed Atlas of Surface Topography of proteins). Among the predicted site, the site having the catalytic amino acids was chosen for the docking of the ligand. The catalytic amino acids of the each protein were analyzed from UniProt (http://www.uniprot.org/).

Substrate selection

The three dimensional structure of Asparagamine A was screened from KNApSAcK-3D database (http://knapsack3d.sakura.ne.jp/). The PDB structure of the ligand was deduced using PRODRG Server (davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg). Ligand optimization and energy minimization was further done using Argus lab software. Apart from this, the three dimensional structures of standard inhibitors for all the aforesaid proteins were also deduced from PubChem database (http://pubchem.ncbi.nlm.nih.gov/) and were used as reference molecules.

Molecular Properties and Drug likeness

The Asparagamine A was further examined for its drug likeness and molecular properties using FAF-Drug2 (http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=FAF Drugs#forms:: FAF-Drugs2) and PreADMET (http://preadmet.bmdrc.org/).

Molecular Docking

The computational docking of Asparagamine A and standard inhibitors were performed into the active site of corresponding

protein models using AutoDock4.2 software (version 1.5.6). While docking, polar hydrogen's were added to protein models using the hydrogen's module and thereafter, Kollman united atom partial charges were assigned. Docking of ligand was carried out using Lamarckian Genetic Algorithm with standard docking protocol on the basis a population size of 150 randomly placed individuals; a maximum number of $2.5*10^7$ energy evaluations, a mutation rate of 0.02, a crossover rate of 0.80 and an elitism value of 1 [11]. Fifteen independent docking runs were carried out for each ligand and results were clustered according to the 1.0 Å rmsd criteria. The grid maps representing the proteins were calculated using autogrid and grid size was set to 60*60*60 points with grid spacing of 0.375 Å. The coordinate of the docked protein along with the ligand was visualized using UCSF chimera and LigPlot+ [12].

RESULTS AND DISCUSSION

Millions are being affected and millions are dying each year because of the diseases like cancer, AIDS, malaria, tuberculosis, obesity, osteoporosis, leishmaniasis and trypanosomiasis. A number of drugs exist commercially that could be used for treatment of these diseases. But still the search of new leads continues because of the side-effects posed by the existing drugs; emergence of the drug resistant forms of microorganisms and the need to develop drug with better efficacy and potency. The drugs developed against the above mentioned diseases are usually targeted towards some of the crucial proteins associated with the diseases or the causative microorganisms (Table 1). In this study, Asparagamine A was targeted against those proteins to analyze its inhibitory effect over aforesaid diseases and the result was compared with the inhibitory efficiency of standard inhibitors.

Table 1: Potential Drug targets to target various diseases

Diseases	Proteins	Notations	Organism	PDB ID	Ref.
Osteoporosis	Farnesyl Diphosphate Synthase	FPPS	*Homo sapiens*	2F8C	[**13**]
Cancer	Peroxisome Proliferator Activated Receptor ?	PPAR alpha	*Homo sapiens*	3ET1	[**14**]
	Peroxisome Proliferator Activated Receptor ?	PPAR delta	*Homo sapiens*	3ET2	
	Peroxisome Proliferator Activated Receptor ?	PPAR gamma	*Homo sapiens*	3ET3	
	Insulin Receptor Kinase Domain	IR	*Homo sapiens*	1IRK	[**15**]
	Vascular Endothelial Growth Factor Receptor 1 Kinase Domain	VEGFR1K	*Homo sapiens*	3HNG	[**16**]
	Vascular Endothelial Growth Factor Receptor 2 Kinase Domain	VEGFR2K	*Homo sapiens*	3VHE	
	Insulin like Growth Factor 1 Receptor Kinase Domain	IGF1RK	*Homo sapiens*	3LW0	[**17**]
Malaria	Plasmepsin II	PII	*Plasmodium falciparum*	1SME	[**18**]
Tuberculosis	Mycolic Acid Cyclopropane Synthase	CmaA2	*Mycobacterium tuberculosis*	1KPI	[**19**]
	PKnB Kinase Domain	PKnB	*Mycobacterium tuberculosis*	1O6Y	[**20**]
HIV 1	HIV1 Protease	H1P	HIV	1AJV	[**21**]
Leishmaniasis	Trypanothione Reductase	LTR	*Leishmania infantum*	2JK6	[**22**]
Trypanosomiasis	Trypanothione Reductase	TTR	*Trypanosoma cruzi*	1BZL	

ADME & Toxicity predictions were initially done to evaluate the pharmacokinetics and the toxicity of the phytochemicals/ligands. These predictions demonstrated that Asparagamine A did not have any mutagenicity and carcinogenicity. Asparagamine A was found to follow Lipinski's rule of five which allow the evaluation of pharmacokinetics of the drug including absorption, distribution, metabolism and excretion (ADME). The predicted percentage absorption of Asparagamine A through oral route was found to be 90.94%. High absorption of the drug allows it to achieve high concentration at the target site. Also, it was found to bind with plasma proteins weakly, making the majority of the unbound molecules available for diffusion, distribution and deposition across the body. The molecular properties as well as the three and two dimensional structure of Asparagamine A are mentioned in Table 2 and Figure 1 respectively.

Molecular docking using the computational approach has proved to be an effective methodology to analyze the interaction patterns of the ligands with the proteins models. The interaction of drug with protein could bring structural changes that may change or block its potential activity. A known fact is that the active site of protein plays a critical role in protein's activity and the computational designed drugs, usually targets these active sites. Docking analysis revealed that Asparagamine A possessea a very high affinity towards the Plasmepsin II and HIV-1 proteases with estimated Ki values of 1.06 nM and 4.58 nM respectively. Both these proteins belong to the class of aspartic proteases and are usually targeted for drug designing against malaria and AIDS respectively [**23-24**]. Plasmepsins assist in degrading the hemoglobin, a critical metabolic need of plasmodium. On the other hand, HIV-1 protease catalyzes the proteolytic cleavage of the polypeptide precursors into mature enzymes and structural proteins of the virus [**25**].

HIV infection has significantly contributed to worldwide re-emergence of tuberculosis incidences. The emergence of multi drug resistant (MDR) and extensively drug resistant (EDR) strains of *Mycobacterium tuberculosis* has drawn much of the attention towards the development of novel drugs [**26-27**]. In this regard, it is desired to design lead molecules that can simultaneously target both the diseases. Interestingly, Asparagamine A showed a binding energy of -9.48Kcal/mol and -8.54Kcal/mol towards CmaA2 and PKnB respectively; which have recently been identified as potential drug targets to combat tuberculosis [**19-20**]. CmaA2 is known to be a trans–cyclopropane synthase for both the methoxy- and keto-mycolates which are the major components of the cell envelope of *M. tuberculosis* and helps in the interaction of these bacteria with the human host [**28**]. PKnB is known as a functional kinase that is autophosphorylated on serine/ threonine residues and is also able to phosphorylate the peptide substrate myelin basic protein[**29**].

Another protein Trypanothione Reductase (TR) is unique to Trypanosomes & Leishmania and absent from mammalian cells, is considered as a potential drug target to combat Leishmaniasis and Trypanosomiasis [**22, 30**]. Asparagamine A was found to have high affinity towards Trypanothione Reductases derived from both, *Leishmania infantum* (Ki: 17.75 nM) and *Trypanosoma cruzi* (Ki: 37.93 nM) with estimated binding energies of -10.57 Kcal/mol and -10.12 Kcal/mol respectively.

Farnesyl Pyrophosphate Synthase (FPPS) is an enzyme which regulates a variety of cell processes which are important for osteoclast functioning, membrane ruffling, endosomes trafficking, and apoptosis [**31-32**]. The literature suggests that the inhibition of FPPS leads to the suppression of bone resorption which is a key feature of diseases such as osteoporosis, cancer associated bone disease and Paget's disease of bone [**33**]. Asparagamine A was found to have a binding energy of -9.12 Kcal/mol (Ki: 205 nM) against FPPS.

Table 2: Molecular Properties of Asparagamine A

Molecular properties	
Molecular Weight	385.45
Log P	2.24
Log Sw	-3.44
tPSA	66.02
HB Donors	1
HB Acceptors	6
Lipinski's Violations	0
Solubility (mg\l)	12322.28
Oral Bioavailability (VEBER)	Good
Oral Bioavailability (EGAN)	Good
AMES Test	Non Mutagen
Carcinogenicity	Non Carcinogen
Human Intestinal Absorption (%)	90.947
In Vitro Plasma Protein Binding (%)	44.936

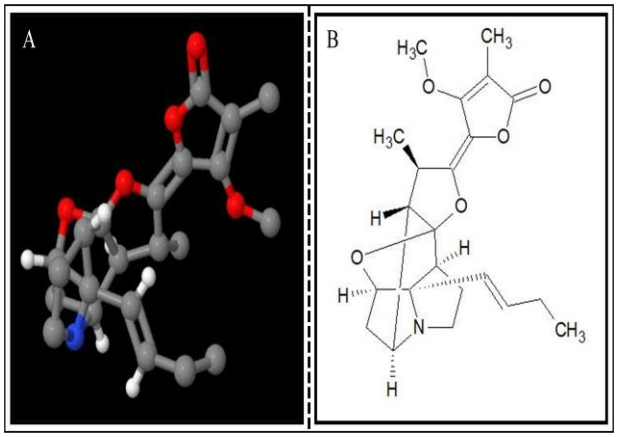

Fig. 1: Three dimensional (A) and two dimensional (B) structure of Asparagamine A.

The anti-tumor and anti-cancer potentials of Asparagamine A have already been mentioned in the literatures. But still the mechanism through which such an effect is exerted is unknown. The analysis suggests that the anticancer and antitumor activity of Asparagamine A could be because of the inhibition of either angiogenesis or the critical signaling pathways involved in the survival of cancerous cells. Asparagamine A bound efficiently with kinase domains of VEGFR1 (Ki: 389.75 nM) and VEGFR2 (Ki: 913.59 nM).

The signaling cascades activated through these receptors are crucial for the angiogenesis [**16, 34-35**]. It is also important to mention that the insulin receptors (IR) and insulin like growth factor 1 receptor (IGF1R) surface expression increases under malignant conditions which in turn mediates invasion and metastasis of the cancerous tissue [**17, 36-37**]. Asparagamine showed a good affinity towards both the receptor proteins, IR (Ki: 272.24 nM) and IGF1R (Ki: 142.08 nM).

Peroxisome Proliferator-Activated Receptors (PPARs) are known to play a critical role in regulating lipid metabolism. PPARs have also been acknowledged for being associated with disorders like cancer and diabetes. Heightened expression of PPARβ/γ has already been reported by Jaeckal *et al*, in the neck and head squamous carcinomas [**38**]. Furthermore, certain agonists of PPARγ are found to enhance the expression of VEGF in colorectal tumor cell lines HT29, thus promoting the tumor growth [**39**].

Over expression of PPARδ in turn have an inhibiting effect over PPARγ activities in regulating tumor cell death. It has also been reported that inhibition of PPARγ, prevents the proliferation of human colon cancer cells (HT-29) [**40**]. Therefore, PPAR's are identified as a critical drug targets for treatment of cancer [**14, 41-43**]. Asparagamine A showed a high affinity towards PPAR γ and PPAR α and potentially good affinity towards PPAR δ (Ki: 1620 nM).

It is highly desirable that a ligand must possess a high affinity for the protein to modulate the protein activity. The affinity of the ligand for the protein correlates with the binding energy of the protein ligand complex. Thus, lower the binding energy of the complex, higher the affinity of the ligand for the protein.

The analysis of ligand's affinity towards the protein is insufficient to predict stable docking. Thus, it is essential to analyze the complete interaction profile of ligand with amino acid residues of the protein. The interactions that are usually accounted in protein-ligand complex are hydrogen bonds and hydrophobic interactions. The presence of these hydrogen bonds and hydrophobic interactions increases the stability of protein ligand complex. In this regard, the probable hydrogen bonds and hydrophobic interactions formed between proteins and Asparagamine A was illustrated through Chimera (Table 3) and LigPlot+ (Table 4 & Figure 2).

A comparative analysis of the binding affinity of Asparagamine A and the reference molecules towards the corresponding proteins suggested that the alkaloid Asparagamine A could be developed into potential therapeutic agent for the treatment of many diseases (Table 5 and 6). The analogues of Asparagamine A could also be developed for the improvement of its specificity and affinity towards a protein target. The chemical synthesis of Asparagamine A was reported by Brüggemann M. *et.al.*, also makes it a suitable candidate for much desirable mass scale production of the ligand and its analogues [**44**].

Table 3: Binding Interactions of Asparagamine A – Docking and Chimera Analysis

Proteins	Minimum Binding Energy (Kcal/mol)	Ki (nM)	Hydrogen Bonds	Interacting Amino Acids
FPPS	-9.12	205	8	Lys57, Arg60, Arg112(4), Lys257, Asp243
PPAR alpha	-10.02	45.4	3	Ser280 (2), Cys276
PPAR delta	-7.9	1620	0	-
PPAR gamma	-9.97	48.88	3	Ser289 (2), Cys285
IRK	-8.96	272.24	1	Asp1150
VEGFR1K	-8.74	389.75	0	-
VEGFR2K	-8.24	913.59	1	Asp1046
IGF1RK	-9.34	142.08	2	Asp1153 (2)
PII	-12.24	1.06	4	Asp34, Val78 (2), Ser79
CmaA2	-9.48	111.94	0	-
PKnB	-8.54	553.05	3	Lys40 (2), Asp156
H1P	-11.38	4.58	2	Ile50, Asp128
LTR	-10.57	17.75	6	Thr51, Asp327 (3), Met333, Thr335,
TTR	-10.12	37.93	6	Ser15, Asp36 (2), Thr52 (2), Cys53

Table 4: Molecular Interactions of Asparagamine A – LigPlot⁺ Analysis

Proteins	Interacting Amino Acids via	
	Hydrogen Bonds	Hydrophobic Interactions
FPPS	Arg60, Arg112 (3), Lys257	Gly56, Lys57, Gln96, Leu100, Asp103, Asp107, Asp174, Lys200, Thr201, Tyr204, Gly240, Asp243, Asp261, Lys266
PPAR alpha	Cys276, Ser280 (2)	Phe273, Thr279, Ile317, Tyr314, Leu321, Met330, Leu331, Val332, Ile354, Met355, His440, Val444, Leu460, Tyr464
PPAR delta	Thr253	Cys249, Thr252, Ile290, Phe291, Met293, Leu294, Ile297, Leu303, Ile327, Ile328, Lys331, His413
PPAR gamma	Cys285, Ser289, His323	Phe282, Arg288, Leu330, Ile326, Leu340, Ile341, Met364, His449, Leu453, Leu465, Leu469, Tyr473
IRK	Asp1150	Phe1054, Val1059, Leu1123, Phe1128,His1130, Asp1132, Asn1137, Ile1148, Gly1149, Gly1152, Met1153, Thr1154, Tyr1162
VEGFR1K	Asp1040	Ala874, Thr877, Glu878, Ile881, Leu882, Val892, Cys1018, His1020, Ile1038, Cys1039, Gly1042
VEGFR2K	Asp1046(2)	Val848, Ala866, Lys868, Glu885, Ile888, Leu889, Ile892, Val899, Leu1035, Phe1044, Cys1045, Phe1047, Val1916
IGF1RK	-	Glu1050, Met1054, Val1062, Val1063, Met1079, His1133, Arg1134, Asp1135, Ile1151, Gly1152, Asp1153
PII	Asp34, Val78 (2), Ser79	Ile32, Gly36, Ser37, Met75, Asn76, Tyr77, Ile123, Leu131, Tyr192, Gly216, Asp214, Thr217
CmaA2	-	Tyr24, Tyr41, Gly145, Ile184, Ile210, Leu211, Phe215, Gly218, Leu220, Tyr247, Trp254, Tyr280,Cys284, Phe288
PKnB	Lys40, Asp138, Asp156	Phe19, Gly20, Met22, Ser23, Val25, Met155, Gly158, Thr159
H1P	Asp 124, Asp 128, Ile50	Asp25, Gly27,Ala28,Gly48, Gly49, Gly126, Ala127, Asp129, Val131, Ile146, Gly147, Ile149,Pro180,Val181, Ile183
LTR	Asp327 (2), Thr335	Gly13, Ser14, Gly50, Thr51, Cys52, Gly56, Cys57, Ala159, Gly161, Ser162, Tyr198, Arg287, Gly326, Met333, Leu334
TTR	Thr52 (2)	Ile11, Gly12, Ala13, Ser15, Ile 35, Asp 36, Val37, Ser47, Gly51, Gly126, Gly128, Ala160, Ser161, Gly162, Arg291, Ala338

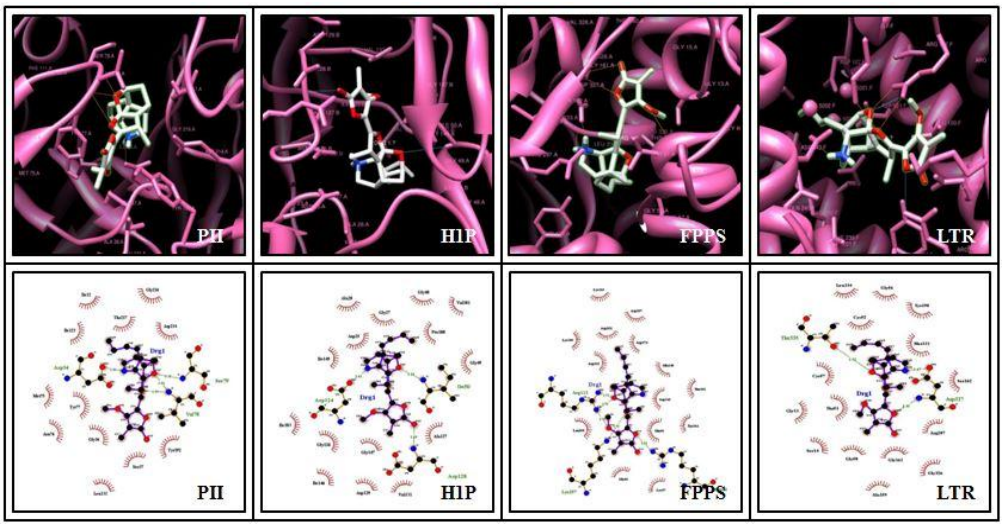

Fig. 2: Molecular interactions of Asparagamine A- UCSF Chimera & LigPlot+

Table 5: Binding Energy of Reference Inhibitors (Standard drugs)

Proteins	Inhibitors	Minimum Binding Energy (Kcal/mol)	Ki (nM)
FPPS	Alendronate [**33**]	-9.32	148
PPAR alpha	MK886 [**45**]	-7.96	1460
PPAR delta	MK886	-3.64	214000
PPAR gamma	MK886	-9.28	158
	1,3-Thiazolidine-2,4-dione [**41**]	-4.01	1140000
IRK	BMS-536924 [**15**]	-6.34	22350
VEGFR1K	Axitinib [**46-47**]	-9.86	58
	Vandetanib [**46-47**]	-8.54	550
VEGFR2K	Axitinib [**46-47**]	-9.94	52
	Vandetanib [**46-47**]	-7.36	4010
IGF1RK	PQIP [**48**]	-9.47	114
PII	Chloroquine [**49**]	-8.44	652
CmaA2	Thioacetazone [**19**]	-6.26	26000
PKnB	2,4-Dichloroquinazoline [**20**]	-4.75	330000
H1P	Indinavir [**21**]	-11.17	6
	Amprenavir [**21**]	-9.62	89
LTR	Ebsulfur [**50**]	-6.96	7970
TTR	Ebsulfur	-6.74	11500

Table 6: Comparison between the standard drugs with Asparagamine A

Proteins	Inhibitors	Ki (nM) [Standard drugs]	Ki (nM) [Asparagamine A]
FPPS	Alendronate	148	205
PPAR alpha	MK886	1460	45.4
PPAR delta	MK886	214000	1620
PPAR gamma	MK886	158	48.88
	1,3-Thiazolidine-2,4-dione	1140000	48.88
IRK	BMS-536924	22350	272.24
VEGFR1K	Axitinib	58	389.75
	Vandetanib	550	389.75
VEGFR2K	Axitinib	52	913.59
	Vandetanib	4010	913.59
IGF1RK	PQIP	114	142.08
PII	Chloroquine	625	1.06
CmaA2	Thioacetazone	26000	111.94
PKnB	2,4-Dichloroquinazoline	330000	553.05
H1P	Indinavir	6	4.58
	Amprenavir	89	4.58
LTR	Ebsulfur	7970	17.75
TTR	Ebsulfur	11500	37.93

CONCLUSION

The complete study was conducted *in silico* to explore the therapeutic potentials of the ligand Asparagamine A derived from *A. racemosus*. The overall analysis suggests that Asparagamine A shows a higher affinity towards the critical proteins, generally targeted to combat aforesaid mentioned diseases. The study provides a clue for the development of a new lead that could be used for the prevention or cure of multiple diseases. Further, *In vitro* and *In vivo* validation of aforesaid therapeutic potentials of Asparagamine A is required.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

"NOTE: The First Author and Second Author have contributed equally."

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