https://ijrps.com

ISSN: 0975-7538
Research Article

Isolation and Insilico evaluation of artemisinin from ethanolic extract of *Artemisia indica willd* for antidiarrhoeal activity

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ABSTRACT

The present study investigates the Isolation and insilico evaluation of Artemisinin from ethanolic extract of *Artemisia indica Willd* for antidiarrhoeal activity is a medicinal plant which has been valued for centuries in ayurvedic medicine. Phyto-chemical screening of Artemisia indica plant extracts revealed the presence of various active constituents such as alkaloids, flavonoids, flavanones, Carbohydrates, steroids and terpenoids. Since terpenoids have remarkable anti-diarrhoeal activity, our present work aims at evaluating the anti -diarrhoeal activity of Artemisinin which is a sesquiterpenoid lactone isolated from Artemisia indica by thin layer chromatography and eval uated by docking studies. The results of our studies suggests that artemisinin showed more anti -diarrhoeal activity than the standard drug loperamide when binded with the μ opioid receptors. Therefore our results support the use of artemisinin in treating diarrhoea in animal studies. We hope it might become a good antidiarrhoeal drug in future.

Keywords: *Artemisia indica*; Artemisinin; Antidiarrhoeal studies; docking studies; Loperamide; Sesquiterpenoid lactone.

INTRODUCTION

Diarrhoea is one of the most important dysfunction in gastro intestinal tract which is produced by some infections. Diarrhoea is caused by different entero toxigenic bacteria like Enterotoxigenic Escherichia coli (Ecoli),Salmonella typhimorium, Clostridium difficile ,Clostridium freundii , Aeromonas hydro phyla, Yersinia entero colitica, Campylobacter jejuni and Vibrio cholera.. Diarrhoeal diseases caused several million of deaths in the world annually, (Field M 2003). In developing countries they are the most common causes of morbidity and mortality Therefore, the World Health Organization has initiated the diarrhoea disease control program, which includes studies of traditional medicine practices together with the evaluation of health education and prevention approaches (Mukherjee pk et al., 1995)

The main aim of present study was to confirm the presence of artemisinin constituent in *Artemisia indica* which is effective in the treatment of diarrhoea. The action is compared with the standard drug Loperamide which is already established in the therapy by using insilico evaluation method.

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Received on: 03-09-2015
Revised on: 02-10-2015
Accepted on: 07-10-2015

Molecular docking technique is the famous in structural bioinformatics to solve the problems in protein and ligand interaction studies. In the context of docking, energy evaluations are usually carried out wi th the help of a scoring function and developing these is a major challenge facing structure based drug design (Viet et al., 1998). In the market there are various docking software's available among them Insight-II, MOE, GOLD and sybyl (Tripos) but most some of the software's are academic free software's like DOCK, Auto dock , FlexX. From the literature we found Auto dock is widely using software by academic institutions.

Auto dock

The program Auto Dock was developed to provide an automated procedure for predicting the interaction of ligands with bio macromolecular targets. The motivation for development of Auto dock software arises from problems in the design of bioactive compounds, and in particular the field of computer-aided drug design (CADD).

MATERIAL AND METHODS

The fresh aerial parts of Artemisia indica were collected in months of April from surroundings of kadapa dist, Andhra Pradesh state. The plant was authentified by a Botanist, Sri krishnadevaraya University, Anantapuramu. The plant material was air-dried at room temperature and grinded in to a fine powder.

Reagents and chemicals

The following chemicals were used: 95 % Ethanol, Ethyl acetate, cyclo hexane, iodine crystals. All the other chemicals and reagents used were of analytical grade obtained from SD fine chem. Limited, Mumbai.

Extraction procedure

The 300gms powder was subjected to extraction with 1100 ml of 95 % ethanol in soxhlet apparatus at (60 -70 °C) and the marc concentrated to obtain ethanolic extract of Artemisia indica (EEAI). The yield of the extract obtained is 38.5 gms. The extract was stored in amber colored bottle and refrigerated.

Phytochemical screening

Various Phytochemical tests were carried out on the extract (EEAI) to detect the presence of bioactive constituents (flavonoids, saponins, tannins, alkaloids etc) of the plant material (Trease and Evans, 2004)

METHODS FOR ISOLATION

Fractionation of Extract

The most viscous extract containing artemisinin was identified by a thin layer chromatography (TLC) with standard artemisinin comparison. In thin layer chromatography, the stationary phase used 60 F254 silica gel and mobile phase is ethyl acetate: cyclo hexane (3:97). Fractions containing artemisinin separated. Fractions with the same profile spots combined and for the next concentrated then re-chromatography (preparative TLC) to obtain a fraction with a single spot containing artemisinin (Deliana Dahnum et al., 2012)

DOCKING STUDIES

Hardware Components

In present work all the calculations were carried out with high frequency computational analysis such as molecular modeling, energy minimizations, design and optimization of lead molecules, protein ligand interaction studies by molecular docking etc., a Hi -end server (Pentium IV 3.4 MHz, AMD Athlon 64 bit, Dual processor with 1 GB RAM) manufactured by HCL Corporation, Pondicherry, India was used.

Software Components

Most of the software's used were either Windows or Linux plat form based which were well accepted and referred in various publications at high rated research journals.

PyMOL

It is an open-source, user-sponsored, molecular visualization system, which is well suited to produce high quality 3D images of small molecules and biological macromolecules such as proteins. (http://www.delanoscientific.com/)

Auto dock Tool

Auto Dock is a suite of automated docking tools designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure.

On line Tools

In addition to the above software, various on line computational tools used in the present study were as denoted below.

PDB

The Protein Data Bank (**PDB**) is a repository for 3-D structural data of proteins and nucleic acids.

Mol inspiration Server

JME Molecular Editor is a Java applet which allows to draw / edit molecules and reactions (including generation of substructure queries) and to depict molecules directly within an HTML page. Editor can generate Daylight SMILES or MDL mol file of created structures. Mol inspiration can also offer help with installation and deployment of the JME ·(http://www.molinspiration.com/docu/webme/)

Preparation of files for Auto dock

The advanced molecular docking program Auto Dock 4.0, (Morris, G. M et al., 1999) which uses a powerful Lamarckian genetic algorithm (LGA) (Morris et al., 1999) method for conformational search and docking, was applied for the automated molecular docking simulations.

Protein–Lead molecules binding studies using Auto Dock Tool

Auto Dock 4.0/ADT (Goodsell, *et al*., 1998) was used for the docking interactions of lead molecules on to the 4dkl. In the present thesis Auto Dock has been used exclusively along with one of its search method called Lamarckian genetic algorithm (LGA) In order to run Auto Dock the PDB file of the protein and ligand will be converted into PDBQS file by assigning partial charges, and the ligand PDBQT file has been obtained from PRODRG2 Server .All the atoms in the ligand file were also checked if needed, and then auto grid was set for docking. In the docking matrix the file were saved as grid parameter file (gpf). Into the PDBQS file of the enzyme genetic algorithm parameters and local search parameters were set in such a way that a population size of 150 individuals were chosen. These 150 individuals were calculated at 100 different runs (100 dockings) and saved as docking parameter file (dpf) and the docking program was run. After the completion of the docking the interactions were generated in the form of dock log file (dlg) which shows the interactions of ligand molecules to the protein.

The interactions are represented in the form of mean docked energy, lowest docked energy and RMSD.

Figure 1: TLC OF ARTEMISININ

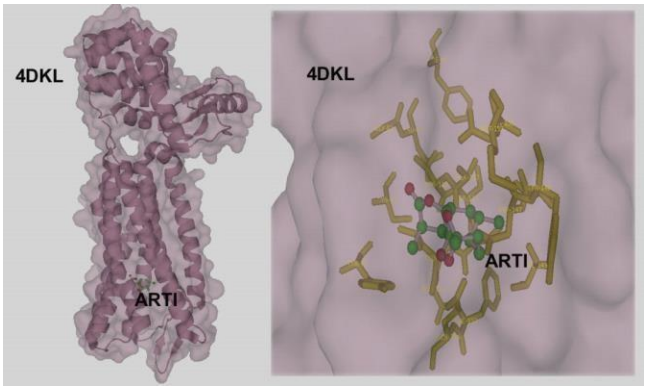

Figure 2: Interaction of Artemisinin molecule (ball and stick) with active site amino acids (yellow of 4DKL).

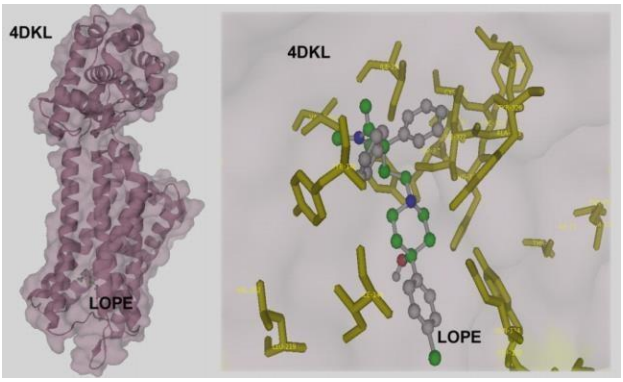

Figure 3: Interaction of Loperamide molecule (ball and stick) with active site amino acids (yellow of 4DKL).

Docking log file (dlg file) shows best interactions among all in the form of histogram. Represented in table 1.

Basing on the dlg file the best docking interactions of the ligand were observed by using PMV viewer 1.4.5 (http://autodok.scripps.edu/).

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical analysis of the crude extract has shown the presence of Alkaloids, Carbohydrates, Steroids, Flavonoids, Flavanones and Terpenoids.

Isolation of artemisinin

The best separation which artemisinin spots well separated from other spots on the concentration of ethyl acetate: cyclo hexane (3:97) with Rf value of 0.2 using iodine vapor as a detecting agent which is simi lar to that of standard Artemisinin (Rf value is 0.2). Later we have observed it in UV Spectrophotometer and the absorbance and concentration of sample artemisinin is very nearer to that of standard artemisinin, and is located at 240 nm with the absorbance of 1.756. Spot was obtained 1.0 mg. Based on the research done, artemisinin compound was obtained 1.0 mg (0.08% w / w) by ethanol extraction and TLC methods. Typical chromatogram can be seen in fig 1.

Docking of loperamide and artemisinin on to the μ - opioid receptor such as 4DKL

The crystal structure of μ - opioid receptor such as 4DKL (PDB id) retrieved from RCSB PDB; the pdb file was edited and used for docking. The 4DKL was docked

Clus-ter Rank	Lowest Docked Energy	Run	Mean Docked Energy	Num in Clus	Histogram 5 10 15 20 25 30 35
1	-10.59	44	-10.59	1	#
2	-8.94	46	-8.94	1	#
3	-8.63	45	-8.42	2	##
4	-8.42	19	-7.98	5	#####
5	-8.39	33	-7.83	3	###
6	-8.24	6	-7.55	18	##################
7	-7.85	48	-7.57	7	#######
8	-7.80	7	-7.79	2	##
9	-7.67	32	-7.44	3	###
10	-7.66	4	-7.50	2	##
11	-7.59	9	-7.59	1	#
12	-7.57	39	-7.57	1	#
13	-7.33	8	-7.08	2	##
14	-7.33	34	-7.33	1	#
15	-6.61	13	-6.61	1	#

Figure 4: Protein–Lead molecules binding studies

Table 1: Docking energy values of 4DKL with the LOPE and ARTI.

Receptor	Compound	Docking energy (kal\mol)	Final Intermolecular Energy kcal/mol	Inhibition constant uM	Torsional Free Energy kcal/mol
μ-opioid receptor 4DKL	LOPE (Loperamide)	-4.83	-8.72	466.76	+2.47
	ARTI (Artemisinin)	-7.20	-7.20	5.27	+0.00

with standard drug loperamide (LOPE) (Table 2). Among all the 50 docked runs, LOPE shown lowest docking energy value i.e. -4.45 kcal/mol and inhibition constant of 466.76 uM it is selected as standard for comparative docking.

Loperamide and artemisinin are docked on to 4DKL. All the docking calculations were carried out using the program Auto Dock 4.0. The program searches for best conformation and best place of binding of the ligand within a fixed protein structure. The auto grid module enables to set a grid map to each ligand molecule with x, y and z parameters (default 60x60x60). The space between the grid points is 0.375A^0. The number of energy evaluations and docking runs were set to 2,000,000 and 200. The mutation rate and cross over rate were 0.02 and 0.8 respectively. For flexible docking process the genetic algorithm parameter was chosen with a population size of 300 individuals. Each of the lead molecules was set with docking stimulations of 50 for docking on to 4DKL and thus was used efficiently to study the ligand binding process with the active sites. All the ten selected lead molecules which were docked have shown to interact well with the active site amino acids. The final docking result has generated a "Docking log file" (dlg). Analysis of the DLG file of each molecule gives us a clear picture of the 15 best stimulations among all the 100 stimulations. These 50 docking stimulations were observed for each i.e. LOPE and ARTI ligands on to the 4DKL.

Each molecule has shown different mode of interactions with the active site residues of the 4DKL at different runs. The interactions are due to the formation of hydrogen bonds or vanderwal forces. The interactions shown by both the molecules were shown in the following Fig 2 and Fig.3**.** The docking results of these molecules were represented in the Table 2 based on docking energy and other values. ARTI has shown best interactions with Tyr 148, Tyr75, Tyr228, Asp147, Ser119, thr118, ala117, gly82, cys89, pro322, ile144, ile141 and His323 of 4DKL (Fig.2). During all these interactions hydrogen bond is found to play a vital role between ligand and active site residues of 4DKL. Both the molecules showed good binding conformations with the 4DKL. The rank of each ligand molecule was based on free energy of binding, lowest docked energy, final Intermolecular energy, inhibition cons tant and torsional free energy values (Table 2). Among both docked molecules, ARTI had shown best predicted binding energy of -7.20 Kcal/mol, final inter molecular docked energy of -7.20 Kcal/mol to the 4DKL (Table 2). Similarly LOPE had shown binding energy of -4.83 Kcal/mol, final inter molecular docked energy of -8.72 Kcal/mol to the 4DKL (Table 2).

CONCLUSION

With this data we explain that ARTI is having best docking energy than that of LOPE, this concludes that ARTI is having the more antidiarrhoeal activity than that of LOPE. This detailed analysis helps to understand the binding modes of 4DKL and its ligands and avoid obvious pitfalls in the detection of new ligands.

ACKNOWLEDGMENTS

The authors are grateful to the SKU College of pharmaceutical sciences, Anantapuramu for providing labora-

tory facility for this research work and we also thank Dr. Madhu department of bioinformatics for performing docking studies. Not but not the least we convey our sincere thanks to almighty who blessed us immensely to carry out this work.

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