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BEHP - A PHTHALATE DERIVATIVE CHARACTERIZED FROM THE SOUTH INDIAN SQUID AND ITS ANTI-HCV LIKE PROPERTY: AN *IN-VITRO* AND *IN-SILICO* ANALYSIS

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ABSTRACT

Over decades, chronic infection with hepatitis C viruses results in liver cirrhosis, hepatocellular carcinoma and eventually, death. Many approved agents for treating viral hepatitis limits their efficacy as they manifest various side effects. Therefore, it is necessary to develop new antiviral drugs with fewer side effects, higher efficacy and with different modes of action against hepatitis viruses. Recent advances in the *in-silico* virtual screening paves way towards efficient discovery of new drugs on the basis of drug target evaluation and analysis. Phthalates and their derivatives extracted from the secondary metabolites of various existing natural sources have proved to possess various bioactive properties. Bis (2-ethyl hexyl) phthalate [BEHP] is a phthalate derivative extracted from various natural sources with bioactive properties and in our earlier studies we have characterised the same phthalate from the black pigmented ink of the squid and it was antimicrobial in nature. In this concern this research work was carried out to assess the anti-HCV like activity by ELISA and have been characterised for the *in-silico* analysis against NS3/4A *Ser protease* target of HCV. Toxicity prediction was made using ADME/Tox tool. The docking scores and the *in-silico* toxicity test results indicate the application of BEHP as potential novel anti-HCV agent and a promising candidate to combat viral hepatitis in near future.

KEYWORDS: Hepatitis C virus, Antiviral agents, Phthalates, Bis (2-ethyl hexyl) phthalate.

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INTRODUCTION

Phthalates and their derivatives are used in a wide variety of industrial and pharmaceutical products as enteric coatings in pills [1], viscosity control agents, lubricants, binding agents and emulsifiers[2]. The most widely used phthalates are the di or bis (2-ethyl hexyl) phthalate (DEHP or BEHP), di-isodecyl phthalates (DIDP) and di-isononyl phthalate (DINP)[3]. Though phthalates and their derivatives in higher concentrations are linked with obesity[4], endocrine disruption and hormonal changes[5], metabolic interference[6], birth defects[7] and hepatotoxicity[8], their bioactive properties were also studied. The antimicrobial and pharmaceutical activities of the phthalates have already been reported. Phthalic esters isolated and identified from *Gibberellafujiikori*is antimicrobial in nature[9]. BEHP isolated from *Streptomycesbangladeshiencis* has shown antibacterial activity against Gram positive and pathogenic fungi[10]. Bis (2-methyl heptyl) phthalate isolated from *Pongammiapinnata* leaves exhibit antiviral activity[11]. BEHP isolated from *Alchorneacordifolia* has shown to decrease anti-inflammatory activity[12]. The antimicrobial constituents of the extracts of *Leea indica* have been studied and have BEHP as one of its constituents [13]. This review background suggests the implementation of BEHP as one of the promising candidates to treat microbial infections.Viral hepatitis is caused by hepatitis B and C viruses and is the prime cause of liver cirrhosis and hepatocellular carcinoma (HCC) [14, 15]. Hepatitis C virus (HCV) is a RNA virus and its occurrence is considered as a global epidemic manifested mainly by chronic liver infection. Many treatments have been approved for HCV infections, including IFN-α, immune-modulators and nucleoside or nucleotide analogues[16]. However, induction of viral mutations and resistance limit their efficacy[17]. Thus, efficient treatment of HCV can be implemented only with the discovery of newer antiviral drugs. Several new drug candidates being in clinical trials for the treatment of chronic hepatitis[18]novel therapies targeting unique molecules with short term efficacy are in need.To implement newer candidates, *in-vitro* analysis along with the *in-silico* analytical tools play a vital role in targeting and designing novel drugs against potent markers of infection. HCV possess various markers that play its role in infectivity[19, 20]. NS3/4A (non-structural 3 and 4 B) protease from HCV plays a key role in the processing of polyprotein precursor the same viral *Serprotease* NS3/4A is studied to cleave some unknown cellular targets involved in innate immunity[21]. NS3/4A thus becomes attractive target for antiviral drug discovery against viral hepatitis.The present study has been designed to analyse the anti-HCV like property of BEHP extracted from the ink of the south Indian squid *Loligo duvauceli* and the virtual screening of BEHP against NS3/4A from HCV. In our earlier studies BEHP, the phthalate derivative was characterised from the pigmented ink of Indian squid *Loligo duvauceli* by gas chromatography mass spectroscopy studies (GC-MS) [data under publication]. In relation with this, the present study throws more light on the anti-HCV like activity of BEHP with NS3/4A of HCV. The auto-docking result will give us suitable clues to implement this phthalate derivative as a good candidate for treating viral hepatitis after designing with the latest bio-informatics tools.

MATERIALS AND METHODS

Source of BEHP

Bis (2-ethyl hexyl) phthalate was characterised from the south Indian squid *Loligo duvauceli* by crude solvent extraction, Thin layer chromatography, Silica gel column chromatography and Gas chromatography and mass spectrometry analysis [data under publication]. In our earlier studies we have reported the antibacterial effects of the crude extracts of the squid ink against various clinical bacteria and yeast[22] and also against drug resistant bacteria[23].

Anti-HCV like activity of BEHP by ELISA

The third generation enzyme immunoassay was performed to detect the binding of the 5mg/ml concentration of fractionated BEHP to the preincubated HCV antigens in the well using the

SP-NANBASE C-96 (3.0) ELISA kit (General Biologicals Corp, Taiwan). 200µl of the specimen diluent was added to each of the preincubated wells. 10µl of the positive and negative controls were included from the kit to each specific wells and the plate was incubated at 37°C/60min. After incubation the wells were washed with 1:20 dilution wash buffer. 100µl of diluted conjugate to each well was added and incubation was done at 37°C/30min. 50µl of TMB substrate A & B was added and incubated at RT/30 min. Finally 100µl of 2N sulphuric acid was added as stop solution into each well and the absorbance was determined using the referred wavelength of the kit in an ELISA reader.

In-silico virtual screening of anti-HCV like property of BEHP

The molecular docking was performed using the standard bio-informatics tools and databases. The selection of the ligand and the drug targets and their interaction is as follows:

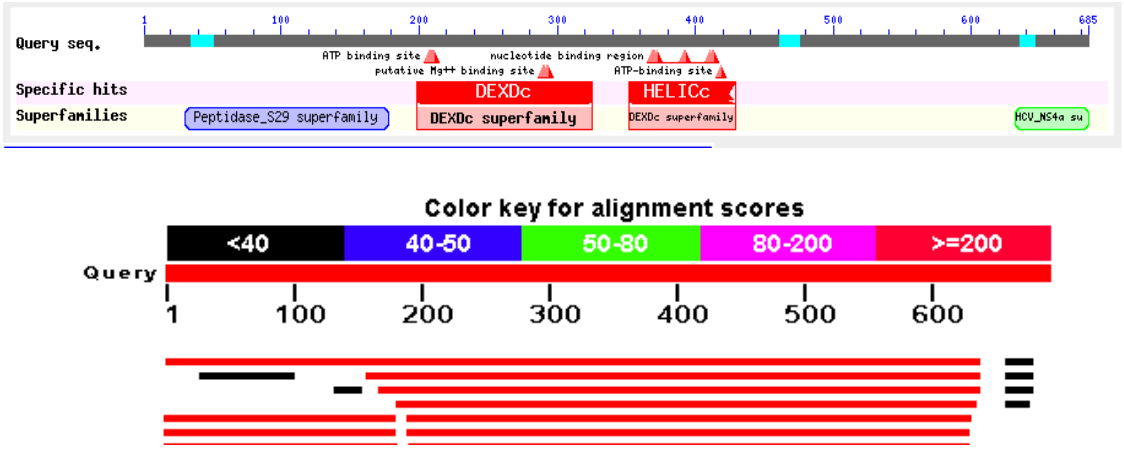
(i) Retrieval of target protein sequence

The protein sequences of the external core antigen NS3/4A from Hepatitis C virus possessing the potential drug targets were retrieved from the protein sequence database of SwissProt [http://www.expasy.ch/sprot/]. The accession number of the retrieved proteins was NS3/4A for HCV. Similarly the structure of the ligand Bis (2-ethyl hexyl) phthalate [BEHP] was also retrieved from the Swissprot database.

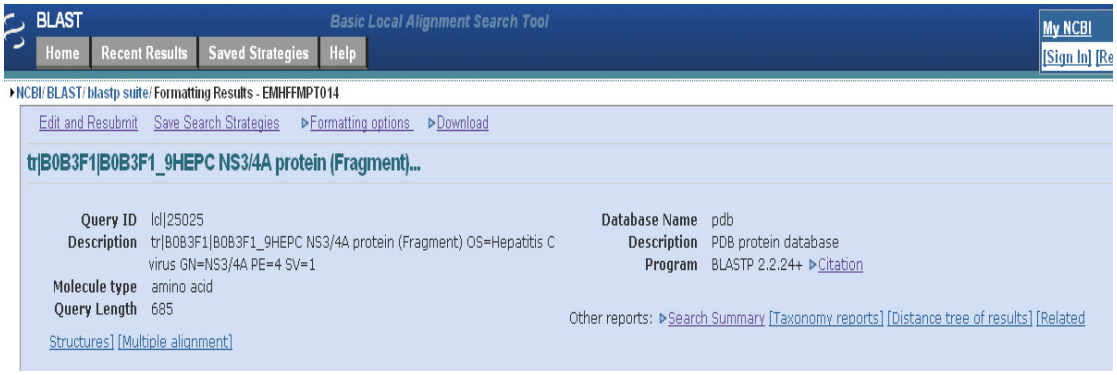
(ii) Template identification

The structure availability and suitable templates were identified using BLAST-P similarity search tool [http://blast.ncbi.nlm.nih.gov/Blast.cgi] [Figure 1].

Figure 1
BLAST result for target sequence



Structure availability and suitable template identified using BLAST p (PDB Database)



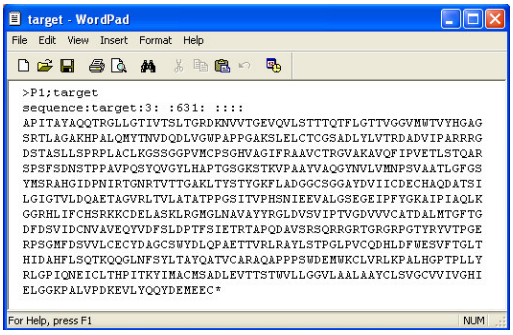
Thus the alignment of the template results in the selection of 1CU1A chain from NS3/4A of HCV. The structure of the identified proteins was viewed in RASMOL viewer [http://rasmol.org/].

(iii) Model generation

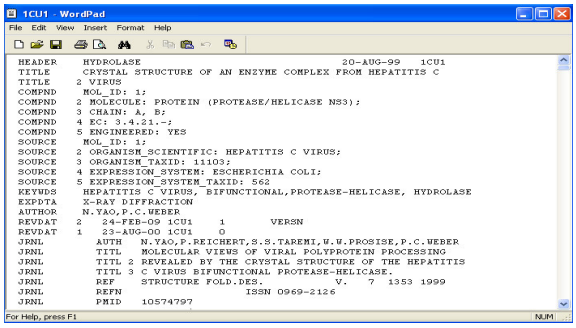
The three dimensional structure of the template chains has been predicted using MODELLER 9V-5 [http://www.salilab.org/modeller/9v5/release.html]. The script "align2d.py" has been employed to perform an alignment between the target and template sequence [http://www.webqc.org/moleculerformatsconverter.php]. A rough 3D model was then obtained using the script "model-default.py" based on the generated alignment [Figure 2].

Figure 2
Template identification

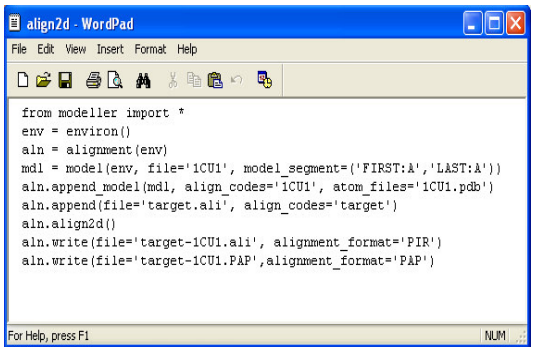
a.target.ali



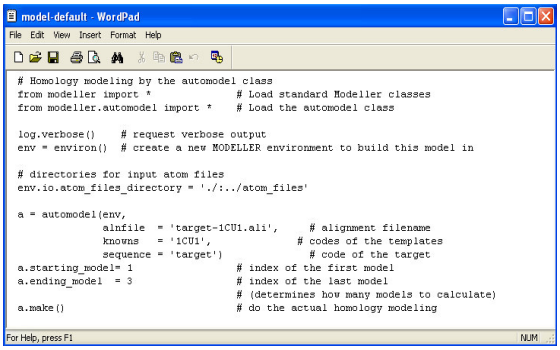
b. template.pdb



c. align2d.py



d. model-default.py



(iv) Evaluation

The backbone conformation of the rough model was inspected using the Phi / Psi Ramachandran plot obtained in the PROCHECK server [http://nihserver.mbi.ucla.edu/SAVES_3/saves.php]. The results of Ramachandran plot indicate that the rough model generated had no residues in the disallowed region.

(v) Domain analysis

The functional analysis of the target proteins was predicted using Pfam Database (http://pfam.sanger.ac.uk/)

(vi) Active site prediction

After obtaining the final model, the possible binding sites of the target proteins were predicted using Q SITEFINDER [http://www.modelling.leeds.ac.uk/qsitefinder/].

(vii) Auto-docking

BEHP was docked with the 1QGT-A and 1CU1-A using the Lamarckian Genetic Algorithm (LGA) provided by the AutoDock Program, version 3.0 http://autodock.scripps.edu/]. Polar hydrogens were added to the receptor, kollaman charges were assigned and salvation parameters were added with the "Addsol" option in AutoDock. The internal degree of freedom and torsions were defined using the "Ligand torsions" menu option of AutoDock. The grid maps representing the protein were calculated using the "AutoGrid" option. The protein was centered on the geometric centre prior to docking. Docking simulations were carried out with an initial population of 50 individuals, and a maximum number of 25,000 energy evaluations were used as the docking parameters for obtaining the final docked structures. In addition to returning the docked structure, autoDock also calculates an affinity constant for each ligand-receptor configuration. The best ligand-receptor structure from the docked structures was chosen based on lowest energy and minimal solvent accessibility of the ligand. The docked molecules were visualized by Pymol tool [http://pymol.org/].

(viii) Toxicity prediction

The toxicity for the ligand BEHP was identified using ADME/Tox web tool [http://pharma-algorithms.com/webboxes/]. The different parameters such as oral bioavailabilty, pka, logD, P-gp substrate and inhibitor specificity, solubility in pure water and in buffer, Abraham salvation parameters, active transport properties, absorption, physiochemical properties, solubility and P-gp specificity was analyzed for the selected ligand.

RESULTS

In-vitro anti-HCV like activity of BEHP

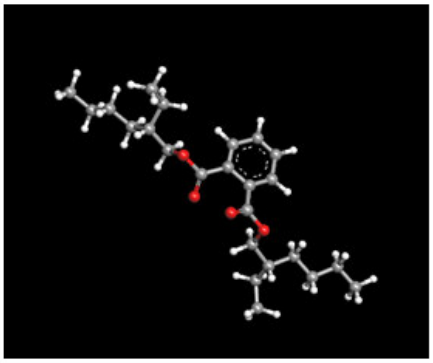
The anti-HCV activity was studied by SP-NANBASE C-96 (3.0) ELISA. The results yielded a negative reaction for the positive control added in the BEHP pre-incubated wells. The cutoff value was calculated and it indicated the nearness of the values towards the negative value. ELISA thus yielded negative reaction in the performed assay. Based on the cut off values analyzed BEHP had a promising anti-HCV like activity.

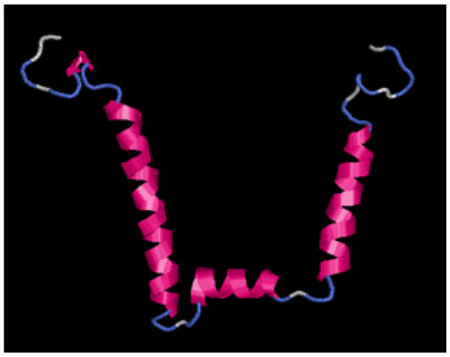
Homology modeling of 1CU1A (HCV)

The result of the alignment of the template ID 1CU1 A chain with NS3/43 of HCV resulted in 83% identity indicating 91% of the amino acids were similar. The positives showed 91% of the aligned amino acids are of similar group with 0% gaps. The predicted protein structure was viewed with RASMOL viewer with three different colored chains. The best modeled structure which is displayed below have MOF value of 1680.90356. The structure of BEHP was also visualized using Rasmol tool (Figure 3a & b).

Figure 3
Visualization of 3D structure of the ligand and target

a. Bis (2 ethyl hexyl) phthalate b. NS3/4A chainof HCV

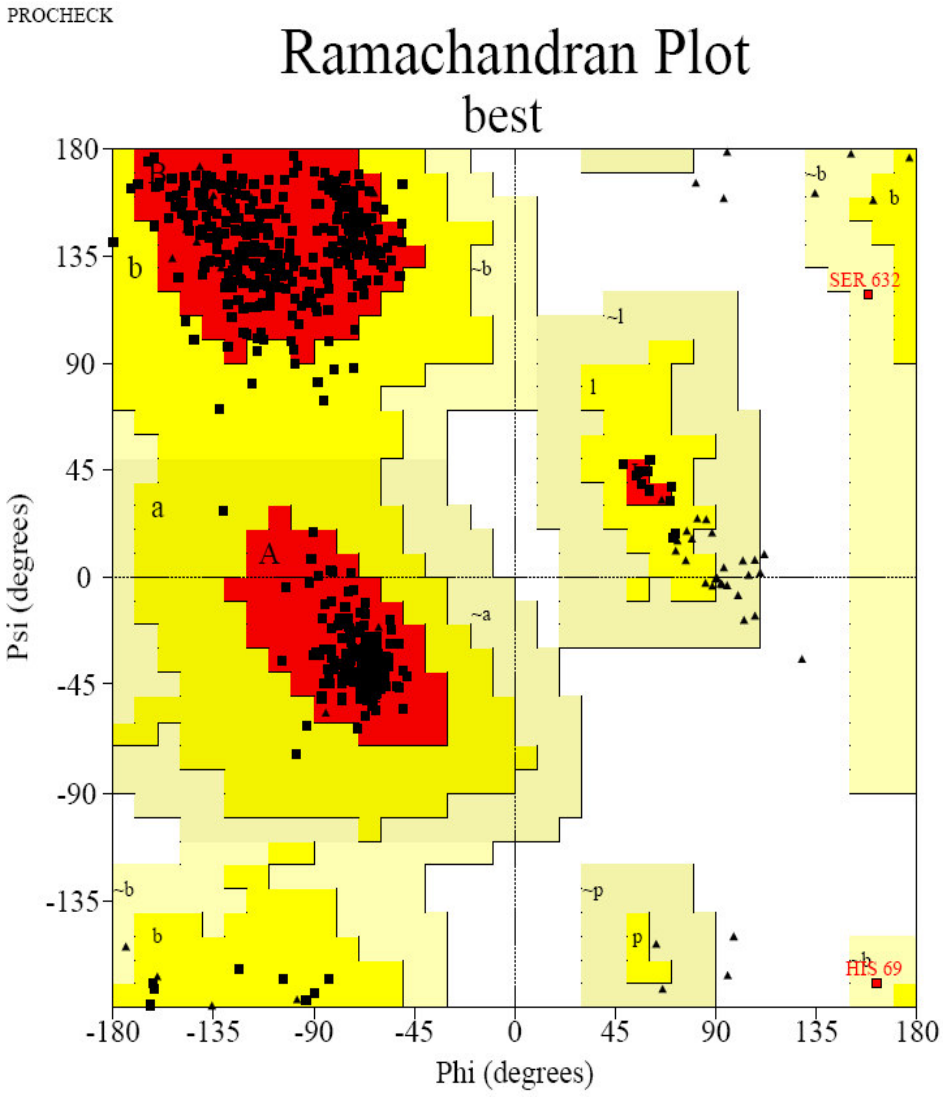




Validation

The 3D structure generated by the modeler was validated using Ramachandran validation methods by PROCHECK program in SavsServer. The Ramachandran plot showed 93.9% residues for NS3/4A of HCV in most favored regions and no residues in disallowed region (Figure 2). NS3/4A of HCV showed GLN9, THR10, ARG11, GLY12, LEU13,THR16, SER20, ASP25, VAL35, LEU36, SER37, THR38, THR42, PHE43, THR54, GLY58, ALI59, ARG62, THR63, LEU64, ALA65, TRP85, ARG10 [Figure 4].

Figure 4
Ramachandran validation plot



Plot statistics

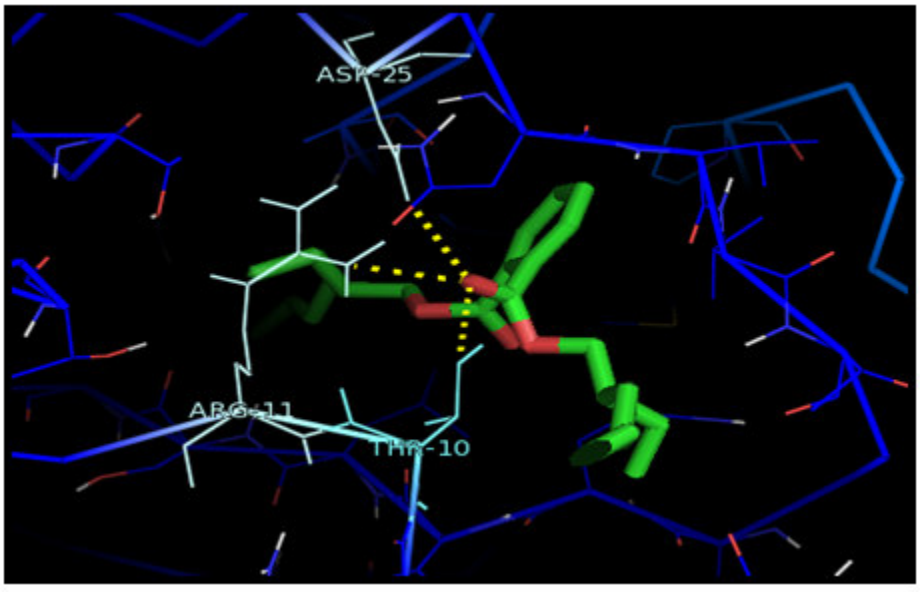
Residues in most favoured regions [A,B,L]	538	93.9%
Residues in additional allowed regions [a,b,l,p]	33	5.8%
Residues in generously allowed regions [~a,~b,~l,~p]	2	0.3%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	573	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	67	
Number of proline residues	43	
Total number of residues	685	

Domain analysis

The functional regions of the target proteins were predicted and docking was performed with the ligand BEHP. The bio-molecular interactions between the bis (2-ethyl hexyl) phthalate with the target antigens selected through the tools were finally analyzed by autodocking (Figure 5).

Figure5

VisualizationofmolecularinteractionsbetweenHCVandBis (2-ethylhexyl) phthalate



The final docked conformations obtained for the selected ligand was evaluated based on the docking score and the number of hydrogen bonds formed between active site and inhibitory ligand (Table 1& 2).

Table 1

Docking Results for NS3/4a with Bis (2-Ethyl Hexyl) Phthalate

NS3/4A		Bis (2-Ethyl hexyl) phthalate	Distance Å
Residue	Atom		
ARG 11	HH12	O	2.3

Table 2

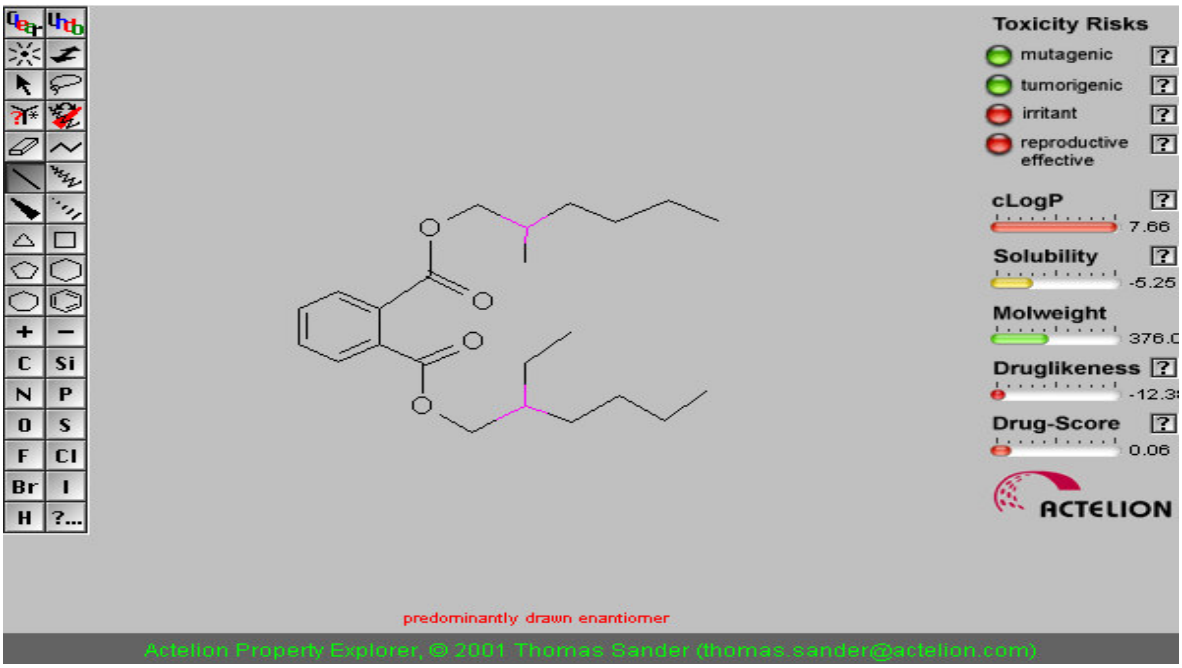
Docking Score for the BEHP

Drug target	Compound name	Docking Score (Kcal/Mol)	No. of Hydrogen bonds formed
NS3/4A	Bis (2-Ethyl hexyl) phthalate	-13.01	3

Toxicity prediction

The toxicity property of BEHP analyzed by ADME/Tox web tool and was displayed in Figure 6.

Figure 6
Toxicity prediction [OSIRIS: ADME Tox Tool]



DISCUSSION

In-vitro analysis of BEHP for the anti-HCV like activity showed a promising anti-HCV like activity. The antiviral property of BEHP isolated from *Pongamia*sp leaves has already been reported to possess antiviral property. This co-relates with the present study that BEHP could possess an anti-HCV like property.*In-silico* drug designing have paved the way towards the efficient discovery and development of new drugs on the basis of drug target evaluation and analysis[24]. This medical bioinformatics research work has given an overview of the novel phthalate derivative BEHP to be applied as an effective antiviral drug for viral hepatitis caused by HCV as they cause a substantial health burden worldwide. Using the computational methods and resources the design and discovery of novel antiviral agents have been highly facilitated. Though an effective antiviral therapy has been established from the mid 1980's the present scenario of combination therapy emphasize the emerging resistance pattern to the routine antiviral agents[25]. BEHP, the phthalate derivative will thus hold good to combat viral hepatitis caused by HCV.The selection of drug targets was mainly based on the availability of the template structures. Thus we could obtain a best template model the NS3/4A chain HCV from the BLAST-P database using the PDB data. These antigens are potent virulent factors in establishing the viral infections in the liver[26, 27]. This target selection will definitely aid in the discovery and designing of novel compounds to fight against the viral hepatitis resulting in HCC[28].The sequence retrieval of the target protein was best achieved from the FASTA was obtained from Swissprot database. The similarity search tool BLAST-P determined and described the template ID, molecular type and the query length from the PDB protein database for antigen NS3/4A of HCV. The alignment of the target chain 1CU1-A chain of NS3/4A from HCV was best observed the graphical BLAST display. Modeler 9V-5 using four input files viz., target.ali, template.PDB, model default –python script (default) and align 2d.py-python script (default) predicted the best homology modeling successfully. RASMOL provided the best three dimensional views of both the ligand BEHP and the drug targets. The pink regions represented the alpha helices, the beta sheets by the yellow regions and the blue and white regions

represented the loops and turns. This 3D analysis provided valuable insight into the molecular level of function and enabled the analysis of its interactions with the selected ligand. Among various conformations generated, the one with the least modeler objective function value was considered to be thermodynamically stable and was chosen for further refinement and validation. The plot statistics evaluated by the Ramachandran validation method showed more amino-acid residues towards the favorable sites. Q-site finder showed nearly 23 aminoacids for the 1CU1-A chain NS3/4A from HCV. These will form the best pocket for the ligand to interact. Docking analysis shows one interaction obtained with hydrogen of ARG 11 residue with the oxygen atom of 1CU1 chain of NS3/4A chain from HCV. The findings of the study reported effective docking with one hydrogen bond between Bis (2-ethyl hexyl) phthalate with NS3/4A chain of HCV.This in-silico virtual screening analysis can assist the synthetic chemists in the designing of novel phthalate BEHP as a potent antiviral agent for treating viral hepatitis.

CONCLUSION

The application of computational technology can yield target specificity with greater accuracy and probability in a positive manner and can be implemented as a novel antiviral agent after proper clinical trials. This study thus concludes by stating that the discovery and development of BEHP from the pigmented ink of the Indian squid and its designing using the latest bioinformatics tools will yield a promisingdrug against Hepatitis C viruses after proper clinical and toxicological studies. This will serve as a cheap and best natural sources to obtain a novel compound for the efficient treatment against the perilious hepatitis infection.

Conflict of Interest

Conflict of interest declared none.

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