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RESEARCH ARTICLE

ALLOIMPERATORIN FROM AEGLE MERMELOS -A PROMISING LEAD MOLECULE FOR HEPATOPROTECTION- *IN VIVO* AND *IN SILICO* APPROACH

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ABSTRACT

The present paper describes *in vivo* and *in silico* studies on hepatoprotective potency of aqueous and ethanol fruit pulp extracts of *Aegle mermelos* (Rutaceae). *in vivo* study was done using CCl_4 induced hepatic damage model. Various biochemical parameters like serum total bilirubin, total protein, alanine transaminase, aspartate transaminase and alkaline phosphatase activities were studied. Results indicated significant decrease in the levels of serum markers and increase in total protein indicating the recovery of hepatic cells in the animal group treated with test drug. Histological study of ethanol extract treated animals revealed normal hepatic cords without any cellular necrosis and fatty infiltration. Among the two extracts tested, ethanol fruit pulp extract at the dose of 100 mg/ml could afford significant protection against CCl_4 induced hepatocellular injury. For *in silico* studies, lamivudin and tenofovir were used as standard to procure the targets. It was found that Gag-pol polyprotein was a target of paramount importance in the cure of hepatitis. Its structure was retrieved from PDB (ID: 1VRU). Thirty three compounds occurring in *Aegle mermelos* were studied, out of which only fifteen satisfied Lipenski's criteria. These fifteen molecules were docked with the PDB ID: 1VRU in Hex docking tool. Alloimperatorin showed the best docking score with the binding energy -224.76 kcal/mol. Furthermore, the active site analysis of PDB ID: 1VRU was done using Q-site finder and the active site sequence was retrieved. Molecular property evaluation of the Alloimperatorin was done using Osiris property explorer which gave a positive drug score. From the present study, it can be concluded that, *Aegle mermelos* could offer a promising herbal drug for the cure of hepatitis.

INTRODUCTION

Aegle mermelos is a red listed, endangered medicinal plant of the family Rutaceae[1]). The plant is a medium-moderate sized tree of religious importance. The plant is commonly called as Bilva (Sanskrit), Bael tree (English) and Bilva pathre (Kannada)[1]. The plant is known for its medicinal uses viz., Flower possess analgesic property, fruits were known as aphrodisiac, leaves as appetizer, astringent, febrifuge, laxative, fruit pulp and seeds as restorative and used in treating abdominal pains, anasarca, in cardiac diseases, deafness, in diarrhoea, dysentery, dyspepsia, gonorrhea, in hyphchondriasis, inflammations, in intermittent fevers, jaundice, ophthalmia, piles, thirst and urinary disorders[2]. Plant contain many active ingradients viz., marmesin, imperatorin, alloimperatorin, xanthotoxol, scopoletin, umbelliferone, skimmin, psoralen, marmelide marmalosin, aegelenine, umbelliferone, phellandrene, imperatarin, β-sistosterol, β-phellandrene, sistosterol, ferroquinoline, dictamine, dihydrofurocoumarin, marmasin and β-sistosteorl[3]. marmenol, stembark, protolionoid 4,5. Several pharmacological activity viz., antidiabetic, antihyperlipidemic, antioxidant, antidiarrhoeal, gastroprotective, protection against radiation and isoprenaline induced myocardial infraction, anticancer, asthma, hypoglycemic, antiinflammatory, antipyretic, analgesic, antimicrobial, antiviral, antifungal, in treating shigellosis were studied [6-33]. Review of the literature revealed that, though this plant is known for its hepatoprotective activity by the tribal group of Western Ghat, scientific studies on its hepatoprotective potency and the possible lead molecule responsible was lacking. Hence in the present proposal *in vivo* and *in silico* studies were conducted to evaluate the hepatoprotective potency and to identify the lead molecule.

MATERIALS AND METHODS

For *In vivo* studies

Collection of plant materials: Fruits of *Aegle mermelos* were collected from the Ayurvedic garden at Gajanur, Shimoga district, maintained by the Karnataka state forest, during March 2010.

Preparation of extracts: The fruit pulps were shade dried, powdered mechanically (sieve no. 10/44) and stored in airtight

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containers. About 250g of the powdered material was subjected to soxhlation and exhaustively extracted with 70% ethanol for 48 hrs. The solvent was distilled off at low temperature under reduced pressure using rotory flash evaporator (Buchi, Flawil, Switzerland). The yield was 40% w/w. Another 250g of the powdered material was macerated with distilled water for 7 days with intermittent shaking, filtered and concentrated to get the aqueous extract. The yield was 25.5% w/w. Both the extracts were subjected to preliminary phytochemical tests [34].

Drug formulation: Oral suspensions containing 50mg/ml and 100 mg/ ml of the aqueous and ethanol fruit pulp extracts were prepared in 1% w/v gum tragacanth.

Animals: Male Wistar albino rats weighing 150-200g were procured from the National College of Pharmacy, Shimoga. The animals were housed in polypropylene cages and were maintained at 27º±2^{O}C, relative humidity 60±5% and 12h light/dark cycle, they were fed with commercial diet (Hindustan Lever Ltd., Bangalore) and water *ad libitum* during the experiment. The study was permitted by the Institutional Animal Ethical Committee with Reg No 144 /1999/ CPCSEA/ SMG.

Acute toxicity studies: Earlier reports reveals that fruit extract was non-toxic up to a dose of 1750 mg/kg b. wt [21]. So in the present study 50 mg and 100 mg/ kg. p.o. dose was selected to assess the hepatoprotective activity.

Evaluation of hepatoprotective activity: The animals were divided into seven groups of six rats each. The animals in group I served as control and received the vehicle 1ml/kg/day of 1% w/v gum tragacanth p.o. for 14 days. All the animals of group II to VII received 0.1ml/kg/day i.p. of CCl_4 (E-Merck, Mumbai, India) in 1:1 olive oil for 14 days. Group III animals received the standard drug silymarin (Ranboxy Lab, Dewas) in the dose of 100mg/kg/day P.O. for 14 days. Aqueous and ethanol fruit pulp extracts of *Aegle mermelos* were administered to the animals of group IV, V, VI and VII in the dose of 50mg/kg/day p.o. and 100 mg/kg/day p.o. respectively for 14 days. The CCl_4, silymarin and the extracts were administered concomitantly to the respective groups of animals. The animals of all the groups were sacrificed on 14th day under light ether anaesthesia. The blood sample of each animal was collected separately by carotid bleeding into sterilized dry centrifuge tubes and allowed to coagulate for 30min at 37ºC. The clear serum was separated at 2500rpm for 10min and were subjected to biochemical investigation viz., total bilirubin, total protein, serum alanine transaminase, aspartate transaminase and alkaline phosphatase [35-38]. Results of biochemical estimations are reported as mean ± SE of six animal in each group. The data was subjected to one way ANOVA followed by Student's *t*-test. P≥0.01 was considered as statistically significant.

Histopathology: The liver samples were excised from the experimental animals of each group and washed with the normal saline. Initially the materials were fixed in 10% buffered neutral formalin for 48h and then with bovine solution for 6h. They were processed for paraffin embedding. The sections were taken at 5µ thickness using microtome, processed in alcohol-xylene series and were stained with alum-haematoxylin and eosin [39]. The sections were examined microscopically for the evaluation of histopathological changes.

For *In silico* studies

Data mining, Target and Lead Identification

The data mining process was carried out to find the compounds identified in *Aegle mermelos* [51-53]. In order to find the target, two standard drugs namely lamivudin and tenofovir was considered for the study [49, 50]. The common target for both lamivudin and tenofovir was chosen as a lead target [51]. Each compound was then subjected to Lipenski's rule to check its druglikeness. Only the compounds following the Lipenski's criteria were considered for further analysis [54].

A. Docking studies

The structure of the target protein was retrieved from PDB and its PDB ID was noted. Compounds clearing the Lipenski's barrier were individually prepared for docking through their energy minimization in Marvin Sketch [55]. Docking analysis for each energy minimized compound was done in Hex 5.1 docking tool. Energy value (E-value) for each ligand docked was noted down. Compound requiring minimum energy to bind to the target was identified as lead [56-58].

B. Binding Site Analysis

Identification and evaluation of surface binding-pockets and occluded cavities are initial steps in protein structurebased drug design. Characterizing the active site's shape as well as the distribution of surrounding residues plays an important role for a variety of applications such as automated ligand docking or *in situ* modeling. After the docking step, the protein was subjected to binding site analysis in Q site Finder [59].

RESULTS AND DISCUSSION

In CCl_4 induced toxic hepatitis a toxic reactive metabolite, trichloromethyl (CCl_3) was produced by the microsomal oxidase system cytochrome P450. This activated radical binds covalently to the macromolecules of the lipid membranes of the adipose tissue and causes peroxidative degradation. As a result fats from the adipose tissues are translocated and accumulated in the hepatocytes[40]. The extent of hepatic damage is assessed by the elevated level of biochemical parameters which is attributed to the generation of trichloromethyl free radical which in turn causes peroxidation of lipids of cellular membrane [41]. Hepatocellular necrosis leads to very high level of aspartate transaminase and alanine transaminase released from liver to blood. Among the two, alanine transaminase is a better index of liver injury, as its activity represents 90% of total enzyme present in the body. The decrease in serum transaminase concentration indicates the stabilization of plasma membrane and protection of hepatocytes against the damage caused by CCl_4[42]. ALP activity on the otherhand is related to the functioning of hepatocytes and increase in its activity is due to its increased synthesis in presence of increased biliary pressure[43]. Effect of aqueous and ethanol fruit pulp extract of *Aegle mermelos* on CCl_4 induced liver damage in rats with reference to biochemical changes in serum is shown in the Table 1. At the

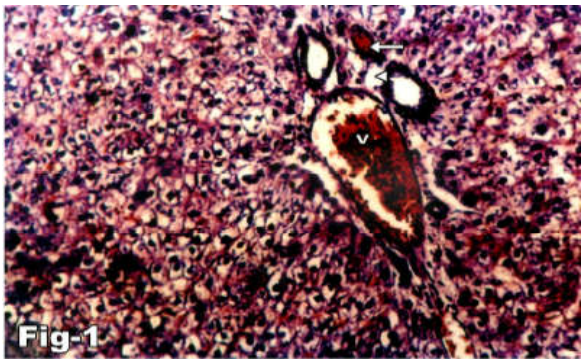

Fig 1: Normal Hepatocytes of Control Animals

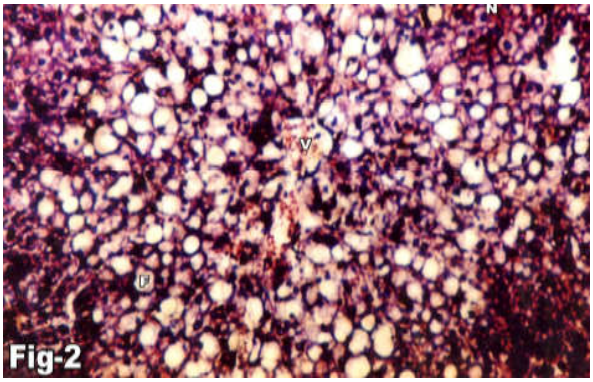

Fig 2: Centrilobular Necrosis, vacuolisation and macrovesicular fatty changes in Group-2 Animals

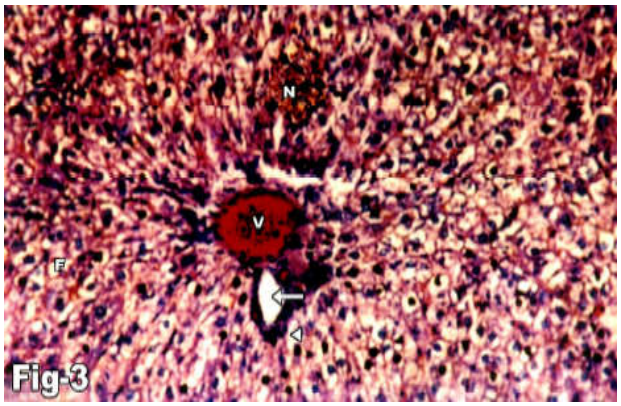

Fig 3: Normal hepatic architecture of Silymarin treated Animals

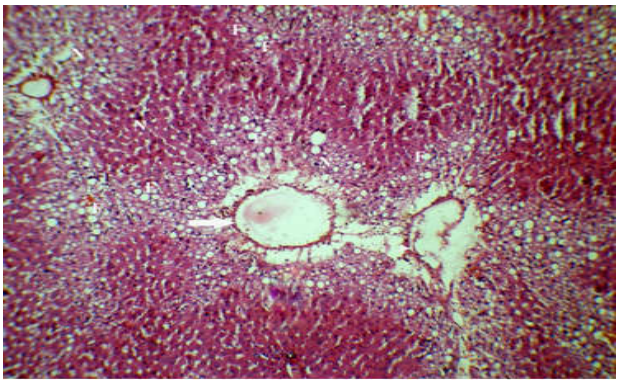

Fig 4: Liver protection in Animals treated with Ethanol

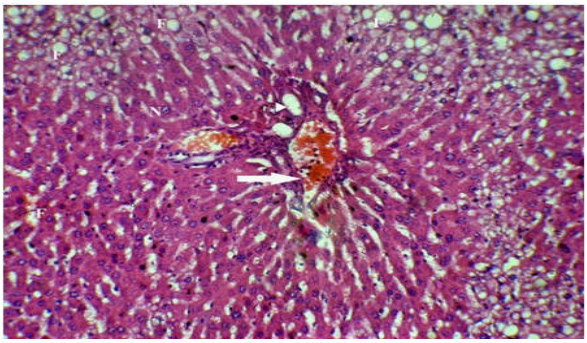

Fig 5: Liver protection in Animals treated with Aqueous Extract

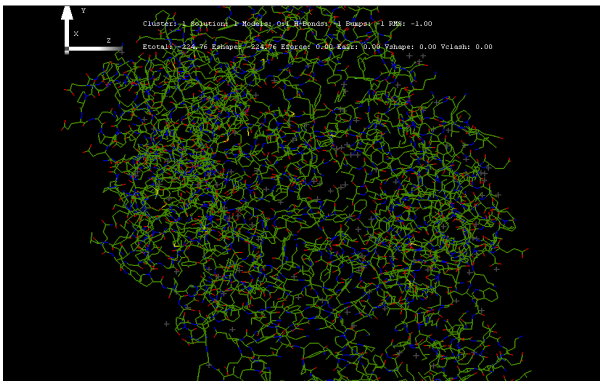

Fig 6: Docking Result for Alloimperatorin against protein with PDB ID 1VRU in Hex 5.1

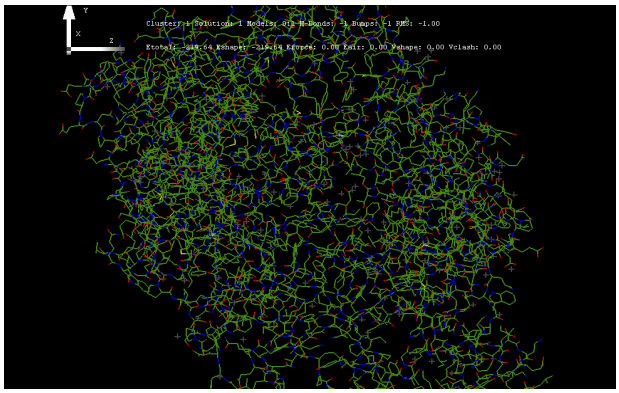

Fig 7: Docking Result for Marmelosin against protein with PDB ID 1VRU in Hex 5.1

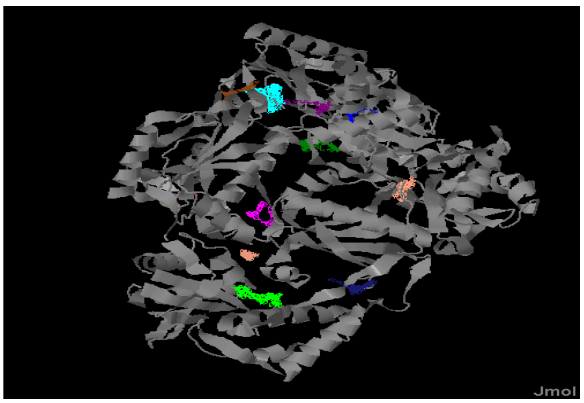

Fig 8: Prediction of active sites in protein with PDB ID 1VRU by Q-site finder

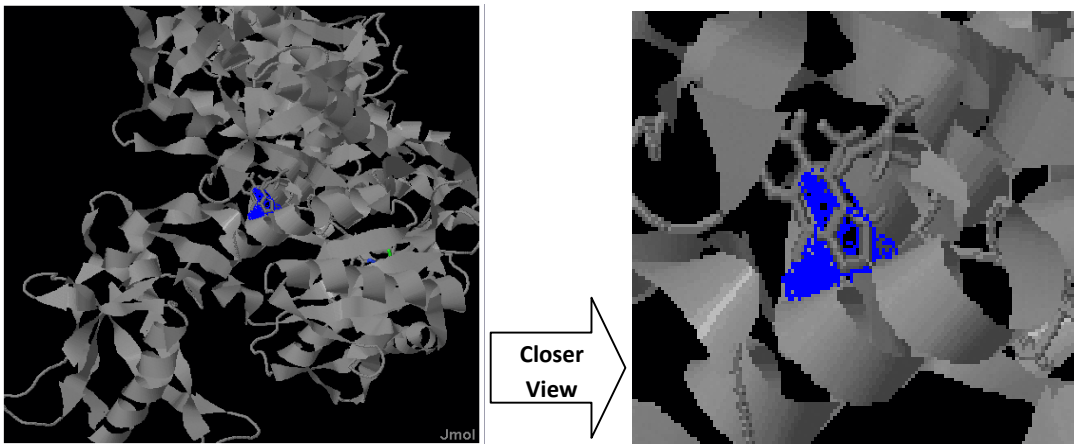

Fig 9: Prediction of active site to which alloimperatorin binds with its closer view

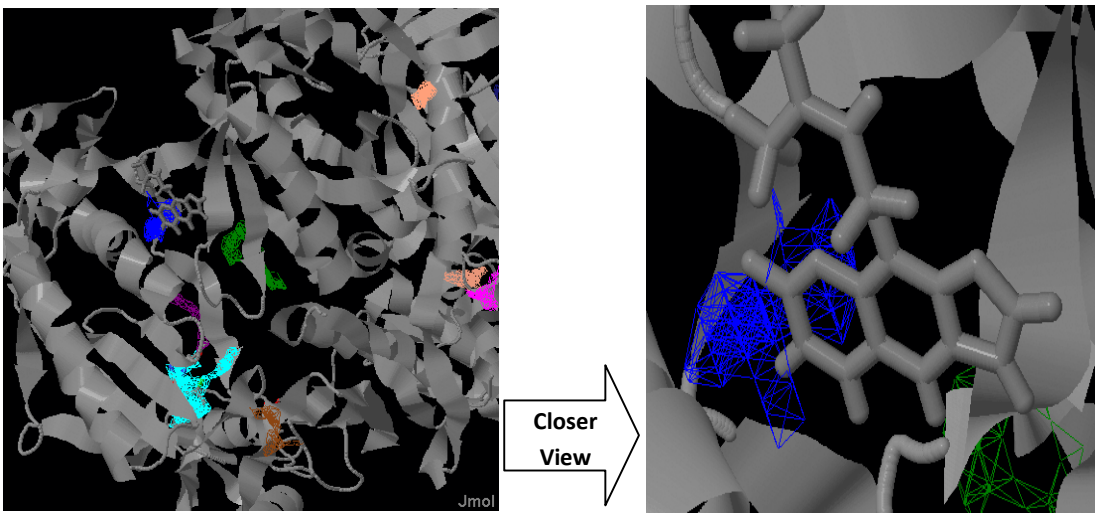

Fig 10: Prediction of active site to which marmelosin binds with its closer view

TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	GLU	GLU	GLU	GLU	VAL	VAL	VAL	VAL	VAL	VAL
GLN	GLN	GLN	PRO	PRO	ALA	ALA	ALA	ALA	ALA	ILE	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
GLN	SER	SER	SER	SER	SER	SER	SER	SER	SER	THR	THR	THR	THR	THR	THR	THR	LYS	LYS	LYS
LYS	LYS	LYS	LYS	LYS	LYS	ILE	ILE	GLY	GLY	GLY	GLY	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
PRO	PRO	PRO	PRO	PRO	PRO	GLY	GLY	GLY	GLY	ARG	ARG	ARG	ARG	ARG	ARG	ARG			

The active site sequence

Table 1. Effect of aqueous and ethanol fruit pulp extract of *Aegle mermelos* on CCl_4 induced hepatotoxicity in rats

Group (N)	Total Bilirubin (mg/dl)	Total Protein (gm%)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Control	0.452±0.013	9.110±0.049	136.13±0.550	55.43±1.040	178.23 ±1.133
CCl_4	2.557±0.102*	5.804±0.227*	2288.86±4.115*	1410.5±2.133*	512.53±2.380*
CCl_4+silymarin	0.559±0.021$^{=}$	8.895±0.025$^{=}$	187.22±0.160$^{=}$	72.01±0.212$^{=}$	198.13±0.324$^{=}$
CCl_4 + aqueous extract(50mg/kg/day p.o.)	1.897±0.006$^{=\varpi}$	7.788±0.003$^{=\varpi}$	289.40±0.120$^{=\varpi}$	195.32±0.170$^{=\varpi}$	314.3±0.190$^{=\varpi}$
CCl_4 + aqueous extract(100mg/kg/day p.o.)	0.886±0.004$^{=\varpi}$	7.218±0.005$^{=\varpi}$	245.30±0.100$^{=\varpi}$	165.11±0.130$^{=\varpi}$	298.1±0.110$^{=\varpi}$
CCl_4 + ethanol extract.(50mg/kg/day p.o.)	0.732±0.001$^{=\varpi}$	7.871±0.013$^{=\varpi}$	223.40±0.220$^{=\varpi}$	118.30±0.239$^{=\varpi}$	216.3±0.310$^{=\varpi}$
CCl_4 + ethanol extract.(100mg/kg/day p.o.)	0.572±0.012$^{=\varpi}$	8.141±0.013$^{=\varpi}$	195.30±0.120$^{=\varpi}$	78.10±0.189$^{=\varpi}$	206.2±0.150$^{=\varpi}$

N = 6 animals in each group: *P≤0.01 indicates significant when compared to control: $^{=}$P≤0.01 indicates significant when compared to CCl_4; $^{\varpi}$P≤0.01 indicates significant when compared to silymarin; Values are expressed as mean ± SE.

end of 14 days treatment, blood samples of CCl_4 treated animals showed significant increase in the levels of total bilirubin (2.557±0.102), alanine transaminase (1410.5±2.133), aspartate transaminase (2288.86±4.115) and alkaline phosphatase (512.53±2.380) compared to normal control groups but the total protein level decreased (5.804±0.227) reflecting the liver injury caused by CCl_4. Whereas, the animals treated with aqueous and ethanol fruit pulp extracts of *Aegle mermelos,* at the dose of 100mg/ml/kg/p.o. showed significant decrease in the levels of serum markers. Among the test groups, animal treated with ethanol extract at the dose of 100mg/Kg/Po showed significant reduction in total bilirubin (0.572±0.012), alanine transaminase (78.10±0.189), aspartate transaminase (195.30±0.120) and alkaline phosphatase (206.2±0.150) and significant increase in total protein (8.141±0.013) to the near normal which are comparable to the values registered in the standard drug treated group of animals, indicating the protection of hepatic cells.

The animals group treated with 50mg/ml/kg/*p.o*. and 100mg/ml/kg/*p.o*. aqueous extract and 50mg/ml/kg/*p.o.* ethanol extract treated group recorded moderate decrease in the levels of serum markers and moderate increase in total protein. Among the two extracts ethanol leaf extract at the dose of 100mg/ml/kg/*p.o*. showed significant protection against CCl_4 induced hepatic damage. Histological profile of control animal showed normal hepatocytes (Fig.1), the section of liver of the group II animals exhibited severe intense centrilobular necrosis (N), vacuolisation and macrovesicular fatty changes (F) (Fig.2). The liver sections of silymarin treated animals showed normal hepatic architecture (Fig.3). The liver sections of the animals treated with ethanol extract (100mg/ml/kg/*p.o.*) exhibited significant liver protection against CCl_4 induced liver damage as evident by the presence of normal hepatic cords, absence of necrosis and fatty infiltration (Fig.4). However moderate accumulation of fatty lobules (Fig.5) was observed in the liver sections of aqueous extract treated animals (100mg/ml/kg/p.o). It has been evident that several phytoconstituents have the ability to induce microsomal enzymes there by accelerating the excretion of CCl_4 or inhibiting the lipid peroxidation induced by CCl_4 [48]. Phytoconstituents like, flavonoids, triterpenoids and alkaloids are well known for their antioxidant and hepatoprotective activities [44-47]. *Aegle mermelos* contains many constituents and with the aim to identify the lead molecule responsible for the heaptoprotection, *In silico* studies was conducted. Two standard hepatitis curing drugs namely Lamivudin and Tenofovir having a common target protein i.e Gag-pol polyprotein was selected for the study. The structure of this protein was obtained from PDB batabase which had the PDB ID 1VRU, of the 33 compounds, 15 compounds met the Lipenski's criteria for druglikeness. Energy minimization of these compounds was done in Marvin Sketch. The energy minimized compounds which were subsequently docked in Hex 5.1 were checked for the least Energy value (E-value). It was found that alloimperatorin had the least E-value of -224.76 (Fig.6) followed by marmelosin with E-value -219.94 (Fig.7). The docking results show that alloimperatorin and marmelosin has a very high binding affinity towards the protein with PDB ID 1VRU. The active site analysis carried out in Q-site finder gave prominent active sites in the protein PDB ID 1VRU. The active sites are illustrated in Fig.8 as colored sites. The protein-ligand interaction studies using Q-site finder gave the exact binding site to which alloimperatorin and marmelosin binds which is shown in Fig.8 & 9. The same active site sequence is depicted in fig 6. It can be concluded from the *In silico* analysis that *Aegle mermelos* have the potential of displaying hepatoprotective activity and the constituents alloimperatorin and marmelosin has very good prospects of acting as drug that target Gag-pol polyprotein for curing Hepatitis B.

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