CHAPTER V

IN VITRO AND *IN SILICO* STUDIES OF DAMMARANE TRITERPENOID 1 ON ARACHIDONIC ACID PATHWAY METABOLIZING ENZYMES

5.1. AIM AND OBJECTIVES

- To evaluate the effect of isolated dammarane triterpenoid 1 on arachidonic acid metabolizing enzymes (PLA2, COX-1&2 and 5-LOX) using *in vitro* methods
- To determine the binding orientation and binding interactions of dammarane triterpenoid 1 on arachidonic acid metabolizing enzymes (PLA2, COX-1&2 and 5-LOX) crystal proteins using molecular docking (*in silico*) studies

5.2. INTRODUCTION

PLA_2s (EC 3.1.1.4) are initial enzymes of AA pathway, which hydrolyze the *sn*-2 acyl bond of membrane bound phospholipids upon several stimuli and form the free fatty acid, mostly AA and LPLs (Figure 1.1). COXs or prostaglandin (PG) endoperoxide H synthases (EC 1.14.99.1), important enzymes, catalyze AA into PGs and thromboxanes (TXA) (Carol and Lawrence, 2009, Claus and Ambra, 2011). 5-lipoxygenase (5-LOX), a member of the lipoxygenase family of enzymes, transforms EFAs into leukotrienes and has been considered as target for therapeutic intervention in various inflammatory and oncologic diseases. 5-LOX converts arachidonic acid to 5-hydroperoxyeicosatetraenoic acids which is further metabolized to the physiologically and pathologically important products, 5-hydroxyeicosatetraenoic acid (5-HETE), 5-oxo-eicosatetraenoic acid (5-oxo-ETE), and leukotrienes (LTB4, LTC4, LTD4, and LTE4).

PLA2 inhibition would theoretically prevent release of arachidonic acid (Meyer et al., 2005). However, several clinical studies demonstrated that some PLA2 inhibitors are ineffective in suppression of arachidonic acid metabolites (Titsworth et al., 2008). COX-2

selective inhibition is more advantageous because COX-1 is a constitutive enzyme. However, recent reports demonstrated that both COX-1 and COX-2 enzymes play vital roles in carcinogenesis. Recent studies demonstrated that COX inhibition causes increase in leukotrines production from arachidonic through the 5-LOX pathway as a result of metabolic pathway diversion (Kit et al., 2012; Cianchi et al. 2006). In the same way, 5-lipoxygenase inhibitors, such as zileuton, might conversely increase arachidonic acid availability to cyclooxygenase enzyme and thus increase production of PGs. Only COX or 5-LOX inhibitors are ineffective and cause adverse effects. Thus development of safer and effective drugs that inhibit both COX-2 and 5-LOX would be advantageous due to blocking of both PGs and LTs metabolic pathways (Kit et al., 2012; Cianchi et al. 2006).

Several COX and 5-LOX inhibitors have been developed by several pharmaceutical industries and academic institutions. An acyphloroglucinol was isolated from *Melicope ptelefolia,* inhibited significantly 5-LOX and COX-2 activities with IC_{50} of 0.42 and 0.40 μM, respectively (Shaari et al. 2011). Further molecular docking studies demonstrated that acyphloroglucinol exhibited good binding affinity on 5-LOX and interacts crucial amino acid residues which chelate with the metal ion in the active site (Shaari et al. 2011). Chebulagic acid was isolated from *Terminalia chebula* as a COX-2/5-LOX dual inhibitor with IC_{50} of 2.1±0.057, 15±0.288 and 0.92±0.011 μM for 5-LOX, COX-1 and COX-2 respectively (Reddy et al. 2009). Dammara-20,24-dien-3-one was isolated from *Chisocheton polyandrus* and showed dual inhibition of both human 5-LOX and COX-2 with IC_{50} of 24.27±2.92 and 3.17±0.90 μM, respectively (Kit et al. 2012). In present study, 5-LOX and COX inhibition studies of dammarane triterpenoid 1 are performed using *in vitro* and *in silico* methods.

Molecular docking is a method which predicts the binding orientation of one molecule to another molecule which may be an enzyme when bound to each other to form a stable complex (Lengauer and Rarey, 1996). Knowledge of the binding orientation in turn may be used to predict the strength of association or binding affinity as well as binding interactions between the molecule and the enzyme. Prabhakaran et al. (2012), performed molecular docking studies of withanolides on COX-2 enzyme to determine their binding

affinity. Natural product α-amyrins bound with both active sites of COX-2 and 5-LOX enzymes there by inhibit these enzyme activities (Ranjbar et al. 2016).

In the present study, effect of dammarane triterpenoid 1 on PLA2, COX-1, COX-2 and 5-LOX was examined in cell free system and molecular docking studies of dammarane triterpenoid 1 were performed on PLA2, COX-1, COX-2 and 5-LOX crystal proteins to determine its binding affinity and interactions.

5.3. MATERIALS AND METHODS

5.3.1. Chemicals

Indomethacin, NDGA, Thioetheramide-PC, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), *N,N,N,N*,-tetra methyl-p-phenylene diamine (TMPD), linoleic acid are purchased from Sigma-Aldrich, USA.

5.3.2. Arachidonic acid metabolizing enzymes inhibitory studies

5.3.2.1. In vitro phospholipase A2 assay

PLA2 assay was performed using PLA2 enzyme activity kit (Cayman Chemical, Ann Arbor, Michigan, USA) according to the manufacturer's instructions (Deng et al., 2013). The detailed protocol was mentioned in Chapter 3.3.4.1.

5.3.2.2. In vitro 5-lipoxygenase assay

5-LOX assay was performed according to modified UV-kinetic method (Reddanna et al., 1990). The detailed protocol was mentioned in Chapter 3.3.4.2.

5.3.2.3. In vitro cyclooxygenase 1 and 2 assays

COX-1 and COX-2 assays were performed according to methods described by Reddy et al., 2000. Indomethacin was used as positive control. The detailed protocol was mentioned in Chapter 3.3.4.3.

5.3.3. Molecular docking studies

All X-Ray crystal structures of proteins used in docking studies were obtained from Protein Data Bank. Cyclooxygenase 1(PDB ID: 2OYE), cyclooxygenase 2 (PDB ID: 4COX) and 5-lipoxygenase (PDB ID: 308Y) were used in docking studies (36-38). Co-cystalized ligands and water molecules were removed from target protein using Argus lab. Ligands are prepared using chemoffice (Cambridge). Energy minimization was done using molecular mechanics. The minimization was executed until root mean square value reached below 0.001 Kcal/mol. Such energy minimized ligands and receptors were used for docking studies using GEMDOCK (Generic Evolutionary Method for molecular DOCKing) is a generic evolutionary method with an empirical scoring function for the protein–ligand docking, which is a problem of paramount importance in structure-based drug design, combines both continuous and discrete search mechanisms. A population size of 300 with 70 generations and 3 solutions were used in docking accuracy setting. PyMol is used for better visualization of interactions ((Jinn et al., 2004; Yang and Chen, 2004; Seeliger and DeGroot, 2010).

5.4. RESULTS AND DISCUSSION

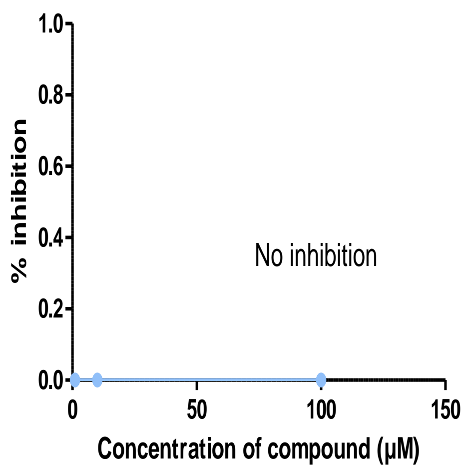
5.4.1. 5-LOX AND COX dual inhibitory activities of isolated dammarane triterpenoid 1 *in vitro*

In vitro 5-LOX studies demonstrated that isolated dammarane triterpenoid 1 showed excellent 5- LOX inhibition with IC_{50} of 9.7±0.02 μM, where as NDGA (positive control) inhibited with 15.1±0.03 μM (Figure 5.4 and Table 5.1). COX-1 and COX-2 were inhibited by dammarane triterpenoid 1 with IC_{50} values of 92.7±0.05 μM and 218±0.28 μM, respectively, whereas indomethacin inhibited with IC_{50} values of 6±0.03 μM and 48±0.09 μM, respectively (Figures 5.2 & 5.3). COX-2 selectivity point of view, dammarane triterpenoid 1 showed better selectivity ratio (2.35) than indomethacin (8.0). It was also noted that dammarane triterpenoid 1 did not show any inhibitory effect on PLA2 activity in cell free system (Figure 5.1).

Table 5.1. 5-LOX and COX inhibitory activities of dammarane triterpenoid 1 *in vitro*.

***In vitro* efficacy**		
Arachidonic acid metabolizing enzymes	IC_{50} (μM)	
	Dammarane triterpenoid 1	Positive control*
COX-1	92.7±0.05	6±0.03
COX-2	218±0.28	48±0.09
COX-2/COX-1 (Selectivity ratio)	2.35	8
5-LOX	9.7±0.02	15.1±0.03

*Nordihydroguaritic acid (NDGA) for 5-LOX; Indomethacin for COX (1 & 2).



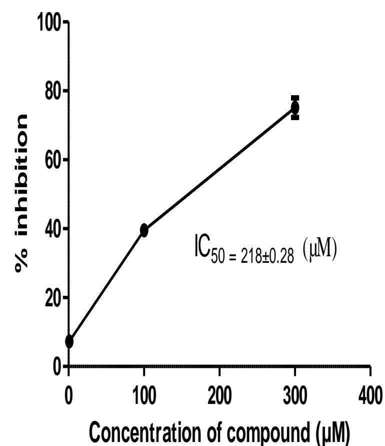
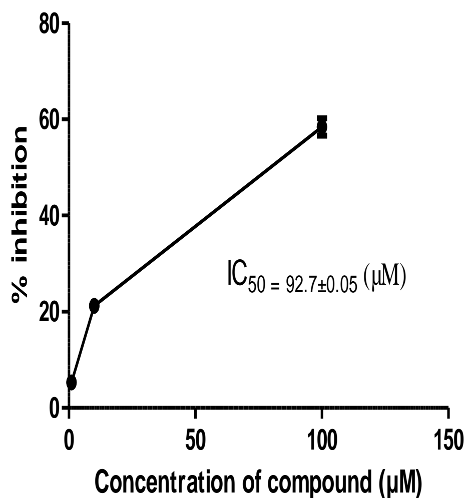


Figure 5.1. Effect of dammarane triterpenoid 1 on PLA2 activity

Figure 5.2. Effect of dammarane triterpenoid 1 on COX-1 activity



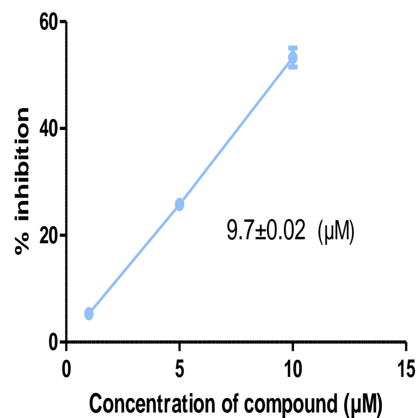


Figure 5.3. Effect of dammarane triterpenoid 1 on COX-2 activity

Figure 5.4. Effect of dammarane triterpenoid 1 on 5-LOX activity

5.4.2. Molecular docking studies (*in silico*) of isolated dammarane triterpenoid 1 on 5-LOX and COX crystal proteins

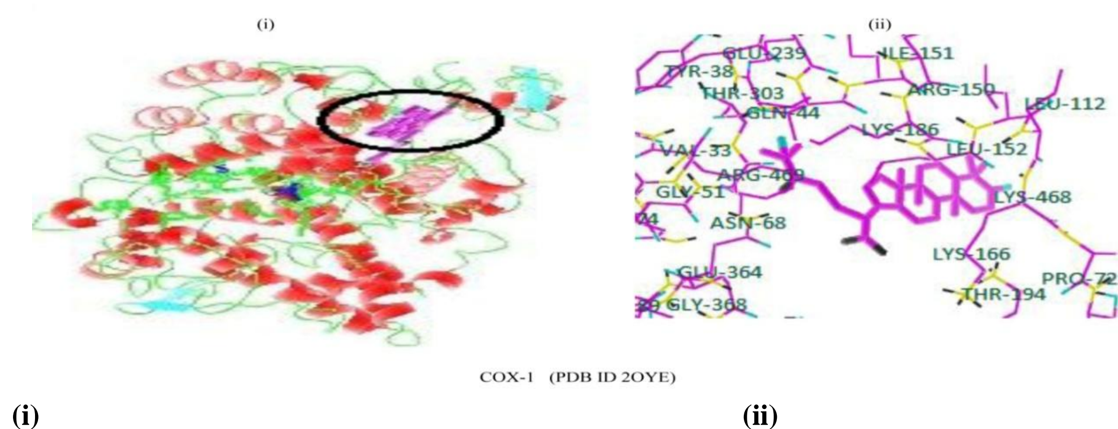
Molecular docking studies demonstrated that dammarane triterpenoid 1 showed good binding affinity on 5-LOX crystal protein with binding energy of -220.43 Kcal/mol and binds in the vicinity of Cys^{159}, Arg^{138}, Glu^{134}, Lys^{133}, Val^{109} amino acid residues (Figure **5.7**). In molecular docking studies on COX (1 & 2), dammarane triterpenoid 1 docked COX-1 and COX-2 crystal protein with the binding energies of -174.34 and -150.24 respectively whereas indomethacin was docked with the binding energies of -189.45 and -168.33 respectively (Figures **5.5** &**5.6,** and Table.**5.2**). Dammarane triterpenoid 1 interacts with active site residues of COX-1 (Lys^{468}, Leu^{152}, Arg^{439}, Asn^{68}, $Lys^{166)}$ and COX-2 crystal proteins (Lys^{1570}, Arg^{1571}, Gln^{1144}, Glu^{1567}, Lys^{1732}, $Asn^{1149)}$. The strong binding affinity of dammarane triterpenoid 1 on active site amino acids of 5-LOX and COX may be responsible for inhibition of enzyme activity (Table.**5.2**).

Table 5.2.

Molecular docking studies of dammarane triterpenoid 1 on COX (1&2) and 5-LOX

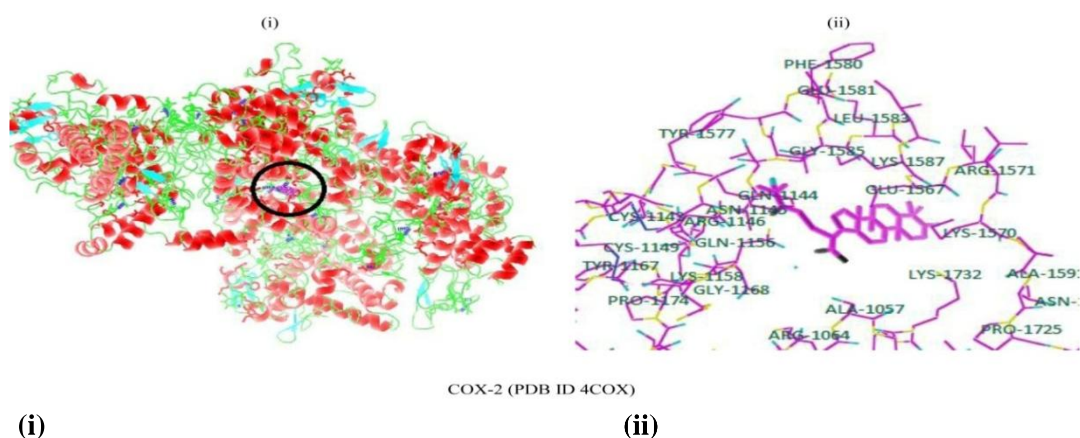
	***In silico* analysis**	
	Binding energy (-Kcal/mol)	
Enzyme	Dammarane triterpenoid 1	Positive control*
COX-1	-170.34	-189.45
COX-2	-150.24	-168.33
5-LOX	-220.43	-100.32

*Nordihydroguaritic acid (NDGA) for 5-LOX; Indomethacin for COX (1 & 2)



(i) **(ii)**

Figure 5.5. Molecular docking studies of dammarane triterpenoid 1 on of COX-1 crystal protein. Binding orientation of dammarane triterpenoid 1 on X- ray crystal structure of COX-1 (PDB ID: 2OYE) in ribbon model (i) and vicinity of amino acid residues at active site were Lys^{468}, Leu^{152}, Arg^{439}, Asn^{68}, Lys^{166} (ii).



(i) **(ii)**

Figure 5.6. Molecular docking studies of dammarane triterpenoid 1 on of COX-2 crystal protein. Binding orientation of dammarane triterpenoid 1 on X- ray crystal structure of COX-2 (PDB ID: 4COX) in ribbon model (i) and vicinity of amino acid residue at active site were Lys^{1570}, Arg^{1571}, Gln^{1144}, Glu^{1567}, Lys^{1732}, Asn^{1149} (ii).

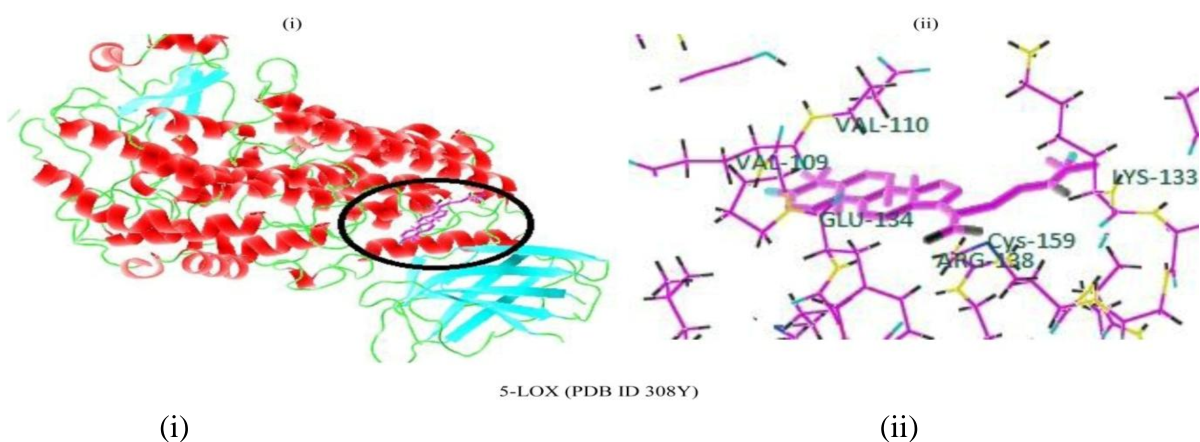


Figure 5.7. Molecular docking studies of dammarane triterpenoid 1 on of 5-LOX crystal protein. Binding orientation of dammarane triterpenoid 1 on X- ray crystal structure of 5-LOX (PDB ID: 308Y) in ribbon model (i) and vicinity of amino acid residue at active site were Cys^{159}, Arg^{138}, Glu^{134}, Lys^{133}, Val^{109} (ii).

5.5. CONCLUSIONS

Dammarane triterpenoid 1 substantial 5-LOX inhibitory activity and considerable COX (1 & 2) inhibitory activity *in vitro*. It was also noted that dammarane triterpenoid 1 did not show any inhibitory effect on PLA2 activity in cell free system. *In silico* studies demonstrated that dammarane triterpenoid 1 strongly binds with 5-LOX and COX (1&2). The strong binding affinity of dammarane triterpenoid 1 on active site amino acids of 5-LOX and COX may be responsible for inhibition of enzyme activity. Dammarane tritepenoid 1 showed comparable or better 5-LOX inhibition activity than NDGA (positive control).