CHAPTER VI

ANTI-INFLAMMATORY STUDIES OF COX AND 5-LOX DUAL INHIBITORY DAMMARANE TRITERPENOID 1

6.1. AIM AND OBJECTIVES

- To evaluate anti-inflammatory activity of COX and 5-LOX dual inhibitory dammarane triterpenoid 1 in rats using carrageenan-induced paw edema model.

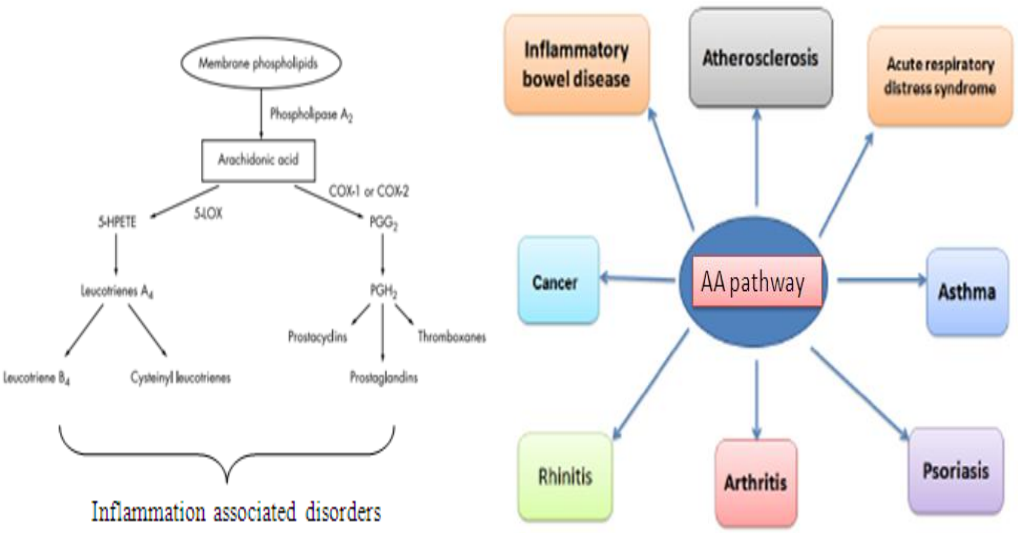
- To evaluate the effect of COX and 5-LOX dual inhibitory dammarane triterpenoid 1 on prostaglandins and leukotrienes production in lipopolysaccharides-stimulated THP1-human monocytes.

- To evaluate the effect of COX and 5-LOX dual inhibitory dammarane triterpenoid 1 on proinflammatory cytokines in lipopolysaccharides-stimulated THP1-human monocytes.

6.2. INTRODUCTION

Inflammation is a protective response to injury and infection in the body. It is a complex process involving many pathways including arachidonic acid pathway. Chronic inflammation may cause various diseases includes asthma, psoriasis, arthritis, rhinitis, cancer, inflammatory bowel disease and atherosclerosis. Arachidonic aid pathway and its mediators orchestrate the inflammatory response. 5-LOX synthesizes leucotrienes which play an important role in the inflammatory process. Prostaglandins are synthesized by cyclooxygenases involve in occurrence of inflammation. COX-2 inhibitors exert antiinflammatory activity by reducing the production of prostaglandins. In arachidonic acid pathway, arachidonate substrate acts by 5-LOX leads to production of LTs. The inflammation occurs as a result of metabolic pathway diversion. 5-LOX inhibition causes over activity of COX pathway. Metabolic diversion of arachidonic acid pathway is major problem with COX-2 or 5-LOX inhibitors. COX-2 and 5-LOX inhibitors can be better therapeutic agents for prevention and therapy of inflammation-associated disorders. Several studies suggest that COX and 5-LOX inhibitors effective in inhibition of inflammatory response as well as inhibition of activation and migration of inflammatory cells, especially neutrophils and monocytes (Martel et al., 2003). Several studies demonstrated that COX inhibitors cause damage of gastrointestinal mucosa. 5-LOX and COX inhibitors

exhibit good gastrointestinal safety profile (Martel et al., 2003). Several COX and 5-LOX inhibitors have been developed by several research groups of academic institutions and industries throughout the world and some of them are under clinical trials. Flavocoxid, a dual COX and 5-LOX inhibitor, inhibited the expression of NF-κB, COX-2, 5-LOX, TNF-α and IL-6 as well as PGs and LTs levels in mice with sepsis (Bitto et al., 2012). COX and 5-LOX dual inhibitory psoralidin, a coumestan derivative was isolated from seed of *Psoralea corylifolia*, inhibited the ionizing radiation-induced COX-2 expression and PGE(2) production through regulation of PI3K/Akt and NF-κB pathway (Yang et al., 2011). Recent clinical studies demonstrating that COX and 5-LOX inhibitory licofelone exhibited potential anti-inflammatory activity with gastrointestinal safety profile (Martel et al., 2003). In present study, we have evaluated anti-inflammatory effect of COX and 5-LOX dual inhibitory dammarane triterpenoid 1 in rats as well as its effect on secretion of proiflammatory cytokines (TNF-α/IL-1β/IL-6) in lipopolysaccharides-stimulated THP1 monocytes.



(A) (B)

Figure 6.1. (A) Arachidonic acid metabolic pathway and its associated inflammatory diseases. (B) Arachidonic acid metabolic pathway and its products involve in inflammatory diseases.

6.2. MATERIALS AND METHODS

6.2.1. Chemicals and reagents

λ-carrageenan, indomethacin, nordihydroguaiaretic acid (NDGA), dexamethasone, lipopolysaccharide (LPS), hematoxylin and eosin were procured from Sigma-Aldrich Chemical Company, (St. Louis, MO, USA).

6.2.2. *In vivo* anti-inflammatory activity by carrageenan induced rat paw edema model

Carrageenan-induced paw edema model is the most widely used for the evaluation of anti-inflammatory activity (Morris, 2003). Male wistar albino rats weighting 150-200 g were obtained from M/S Mahavir Enterprises (Hyderabad, Telangana, India). The animals were housed under standard conditions (Temperature of 22±10 ^{0}C with an alternating 12 h light-dark cycle and relative humidity of 60±5%, before and also during the experiment. The animals were fed with standard laboratory diet, which was purchased from M/S Rayans Biotechnology pvt. Ltd. (Hyderabad, Telangana, India). During the experiment, the rats were allowed water and food ad libitum. Animal experiments were conducted according to CPCSEA guidelines. Animal experimental protocol was approved by institutional animal ethical committee (IAEC) of GITAM University (IAEC no. GU/GIS/IAEC/2013/PROTOCOL NO.03/2013).

The animals were divided into three groups (n = 3 - 6). Group I served as control and received saline at 1 ml/kg body weight (b.w) *per* os (p.o). Group II served as standard, received indomethacin at a dose of 5 mg/kg b.w, p.o. and group III served as test and received isolated dammarane triterpenoid 1 at a dose of 5 mg/kg b.w, p.o, respectively. The animals were treated with dammarane triterpenoid 1 and indomethacin before 1 h to inject (subcutaneous) 0.1 ml of 1% λ-carrageenan solution with saline into sub-plantar region of left hind paw of each rat. The right hind paw of same rat was treated with 0.1 ml of saline alone in manner as control. Before induction of edema, the dorsiventral thickness of both the paws of each was measured using mercury displaced glass plethysmometer and also the measurements were taken at 1st, 2nd, 3rd, 4th and 6th hour after carrageenan injection (Fereidonia et al., 2000).

For histopathological studies, the right hind paws (4^{th} hour after carrageenan injection) of the animals were surgically removed under anesthesia (Chi et al. 2012). Tissue slices were fixed in 10% formalin for few days, decalcified overnight and kept in paraffin. The tissues were cut into thin tissue sections (5 μm), which were stained with hematoxylin and eosin (HE stain) and observed under a microscope (Olympus) for acute inflammatory exudates and various inflammatory cells (Chi et al. 2012).

6.2.3. Estimation of TNF-α, IL-1β and IL-6 secretion levels in LPS induced THP1 human monocytes

Cell viability or the cytotoxic effect of compound in THP-1 human monocytes was determined by MTT assay (Mosmann, 1983). The compound did not show any cytotoxicity up to 50 μM concentration thus various doses of compound were fixed between 1-50 μM. Cells were incubated with compound alone or in combination with purified LPS (100 ng/mL) from *Escherichia coli* serotype O127 : B8 (Sigma) for 4 h. THP-1 cell culture supernatants were collected from individual wells by centrifugation and concentration of TNF-α/IL-1β/IL-6 were measured using cytokine-specific sandwich quantitative ELISA (enzyme linked immunosorbent assays) according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA). The 96-well microtiter plates (Corning Inc, Corning, NY, USA) were coated overnight with mouse anti-human TNF-α/IL-1β/IL-6 antibody as capture antibodies. The plates were washed with PBST (0.05% Tween 20) to remove excess capture antibody. To reduce non-specific binding, wells were blocked for 2 h with bovine serum album (BSA) (1% (w/v) of BSA; Sigma) and washed with PBST. Recombinant human TNF-α/IL-1β/IL-6 was used as standard at the concentration of 15.6, 31.5, 62.5, 125, 250, 500, and 1000 pg/ml. Cell culture supernatants were added to appropriate wells and plates were incubated overnight. After washing, biotinylated goat anti-human TNF-α antibodies were added as detection antibodies and the mixture was incubated for 2 h. The plates were washed with PBST and incubated with streptavidin-horseradish peroxidase conjugate for 20 min. The tetramethylbenzidine (TMB) substrate was added to the plates as a colour indicator and was incubated for 20 min. H_2SO_4 was added to stop the reaction. The optical density was read at 450 nm. TNF-α concentration in each well was quantified from a standard curve and expressed as pg/ml of culture medium (Singh et al., 2005; Brown et al., 2013). Dexamethasone was used as the positive

control (Steer et al., 2000). LPS-induced TNF-α/IL-1β/IL-6 concentration in untreated monocytes was considered as control while LPS induced TNF-α/IL-1β/IL-6 concentration in compound treated monocyes was considered as test. The results were expressed as percent inhibition of TNF-α/IL-1β/IL-6, which was calculated using a formula:

% Inhibition = ((Concentration of TNF-α/IL-1β/IL-6 in Control) – (Concentration of TNF- α/IL-1β/IL-6 in Test)) / (Concentration of TNF-α/IL-1β/IL-6 in Control)) X 100

6.2.4. Quantification of Leukotriene B4

Leukotriene B4 levels were quantified in cell culture supernatant using leukotriene B4 competitive ELISA kit (Cayman chemical, USA). The assay is based on the competition between LTB4 in the sample and acetylcholinesterase conjugated LTB4 (LTB4-AChE) for LTB4 antibodies which are bound to an anti-Rabbit IgG precoated 96-well plate. The amount of LTB4-AChE captured by the coating antibody decreases when the concentration of LTB4 in the sample increases. Therefore, there is an inverse relationship between optical density (OD) and the amount of LTB4 in the sample. Ellman's Reagent, contains the substrate to AChE, is added to the well. The reaction can be read at 412 nm (Maclouf, 1987).

6.2.5. Quantification of Prostaglandin E_2

Prostaglandin E2 (PGE2) levels were quantified in cell culture supernatant using Prostaglandin E2 competitive ELISA kit (Cayman chemical, USA). The assay is based on the competition between PGE2 in the sample and acetylcholinesterase conjugated PGE2 (PGE2-AChE) for PGE2 monoclonal antibodies which are bound to an anti-Rabbit IgG precoated 96-well plate. The amount of PGE2-AChE captured by the coating antibody decreases when the concentration of PGE2 in the sample increases. Therefore, there is an inverse relationship between optical density (OD) and the amount of PGE2 in the sample. Ellman's Reagent, contains the substrate to AChE, is added to the well. The reaction can be read at 412 nm (Hamberg and Samuelsson, 1971).

6.2.6. Statistical analysis

Experimental results were expressed as mean (n = 3-6) ± standard error mean (S.E.M). The difference between experimental groups was compared by one-way analysis of variance (ANOVA). The *p* value ≤0.05 was considered as significant. IC_{50} was calculated using using Prism software.

6.3. RESULTS

6.3.1. *In vivo* carrageenan-induced paw edema inhibition activity of isolated dammarane triterpenoid 1 in rats

In vivo oral anti-inflammatory efficacy of dammarane triterpenoid 1 (5 mg/kg body weight) was tested on group of rats using carrageenan-induced paw edema model and paw volumes (ml) of treated (dammarane triterpenoid 1 and indomethacin) and untreated (vehicle control), were presented in Figure 6.2. Dammarane triterpenoid 1 (5 mg/kg body weight) showed 70.10±1.52%, 73.81±1.25% edema inhibition at 3rd h and 4th h respectively, where as indomethacin (5 mg/kg body weight) showed 72.87±1.79%, 75.98±1.34% edema inhibition at 3rd h and 4th h respectively. Dammarane triterpenoid 1 was exhibited comparable *in vivo* oral anti-inflammatory activity with indomethacin (positive control).

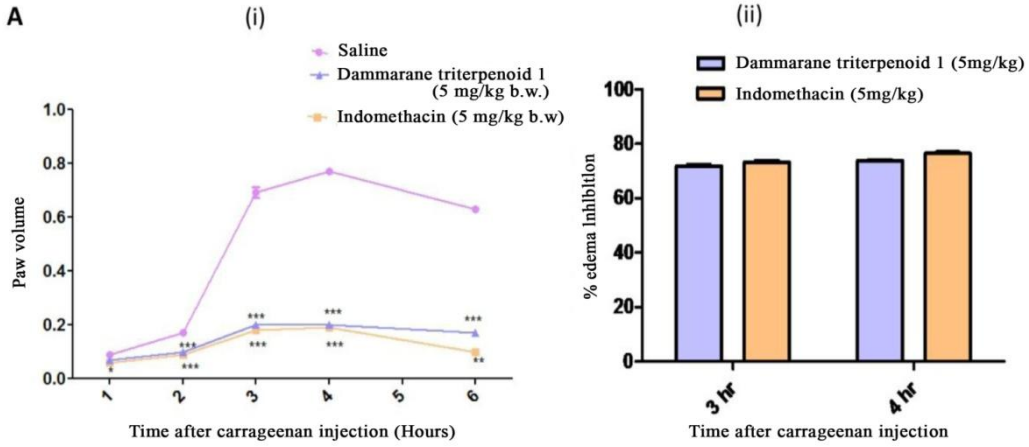


Figure 6.2. Antiinflammatory activity of dammarane triterpenoid 1 in rats (i) Represents the rat paw edema volumes (ml) of treated (dammarane triterpenoid 1 and indomethacin) and untreated (saline) at various time intervals (1, 2, 3, 4 and 6 hrs) after carrageenan injection. Experimental results are expressed as mean (n = 3-6) ± standard error mean (S.E.M). The difference between experimental groups was compared by one-way analysis of variance (ANOVA). The *P* value of results less than 0.05 (P<0.05) versus control was considered as

significant (*represents $p<0.05$, **$p<0.001$, ***$p<0.0001$). (ii). Dammarane triterpenid 1 showed comparable activity with indomethacin (positive control) and exhibited maximum edema inhibition between 3rd and 4th h after carrageena injection.

6.3.2. Histopatholigcal analysis

In histological studies, reduced edema volume and decreased inflammatory cells population (neutrophiles and macrophages) were observed in dammarane triterpenoid 1 treated edema containing tissue slides (4th h after carrageenan injection) as compared to the control group (untreated) tissue slides (Figure 6.3).

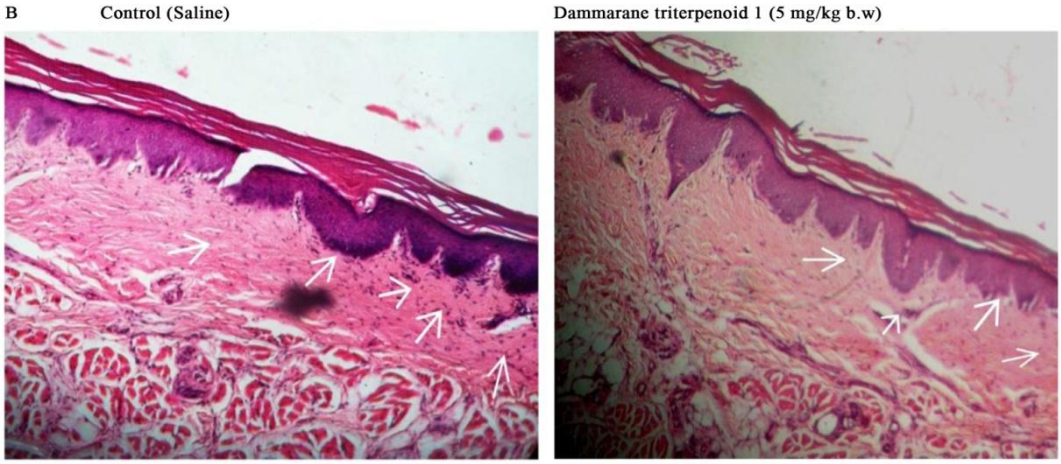
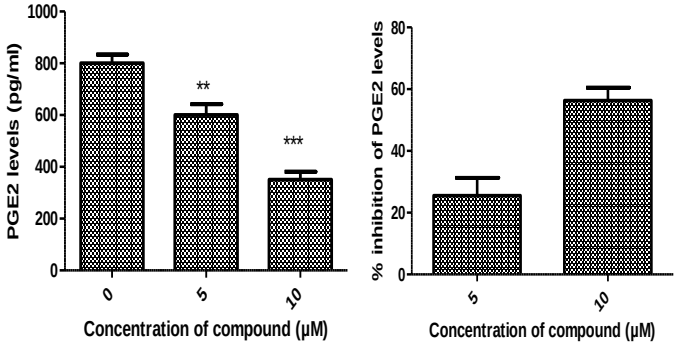


Figure 6.3. Histological analysis of 4th h carrageenan induced edema containing paw tissue with dammarane triterpenoid 1(5 mg/Kg b.w. p.o.) slightly reduced with edema fluid and inflammatory cells like neutrophiles (white arrow marked) as compared to the control rat group (untreated).

6.3.3. Effect of dammarane triterpenoid 1 on production of PGs in LPS induced THP-1 human monocytes

As shown Figure 6.4, dammarane triterpenoid 1 inhibited 25.50±5.80% and 56.27±4.2% production of PGs in LPS-induced THP-1 human monocytes at 5 and 10 µM concentrations respectively. The results were statistically significant.

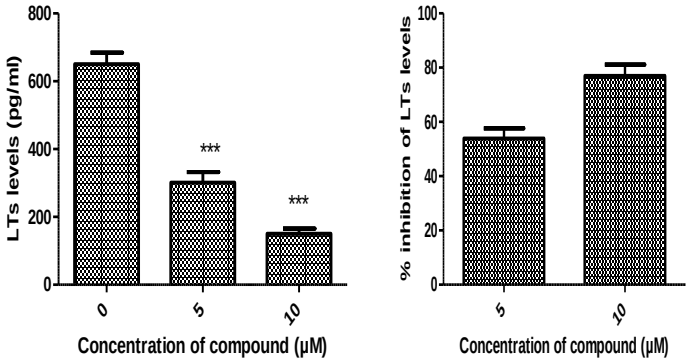


(i) (ii)

Figure 6.4. The effect of dammarane triterpenoid 1 on PGs secretion levels represented in pg/ml concentration (i) and percent inhibition (ii) in LPS induced THP-1 human monocytes

6.3.4. Effect of dammarane triterpenoid 1 on production of LTs in LPS - induced THP-1 human monocytes

As shown Figure 6.5, Dammarane triterpenoid 1 inhibited 53.84±3.8% and 76.92±4.2% production of LTs in LPS-induced THP-1 human monocytes at 5 and 10 µM concentrations respectively. The results were statistically significant.

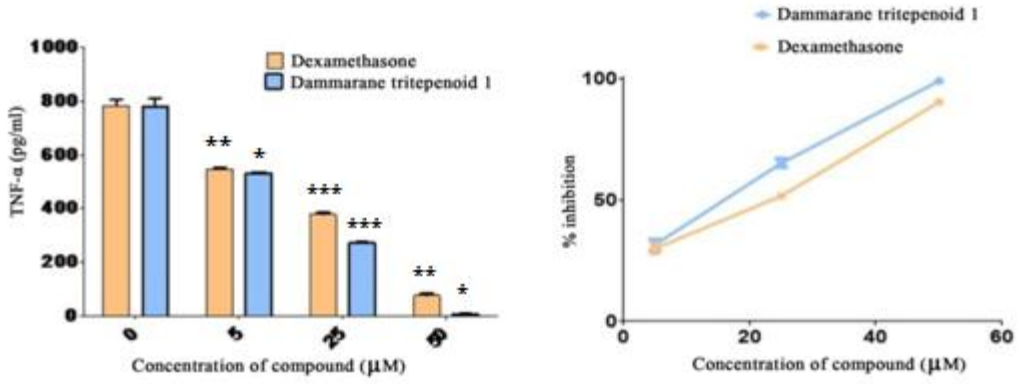


(i) (ii)

Figure 6.5. The effect of dammarane triterpenoid 1 on LTs secretion levels represented in pg/ml concentration (i) and percent inhibition (ii) in LPS induced THP-1 human monocytes.

6.3.5. Effect of dammarane triterpenoid 1on TNF-α secretion in LPS - induced THP-1 human monocytes

Dammarane triterpenoid 1 showed dose dependent inhibition of TNF-α secretion in THP-1 monocytes and IC_{50} found at 11.36±0.14 µM, whereas dexamethasone (positive control) inhibited with IC_{50} of 18.71±0.26 µM (Figure **6.6**). The results were statistically significant. Dammarane triterpenoid 1 did not show any cytotoxicity up to 50 µM concentration. Therefore, it was proved that TNF-α inhibitory activity of dammarane triterpenoid 1 is non cytotoxic.

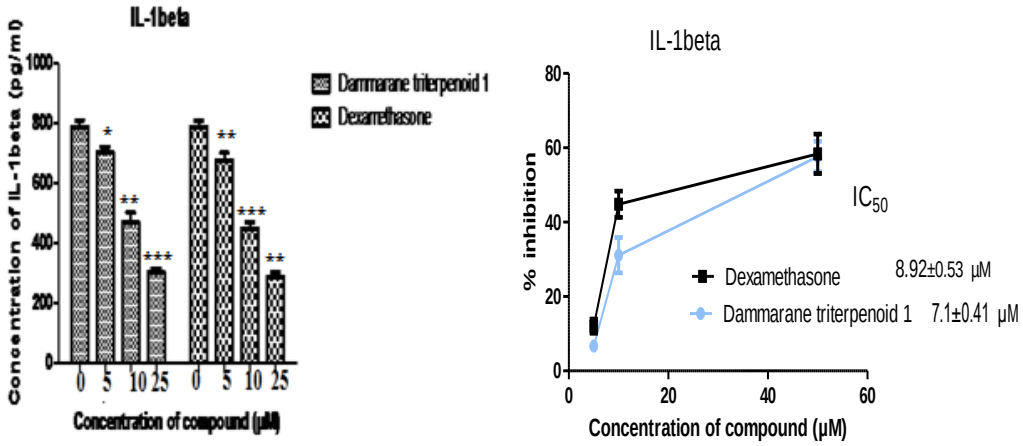


(i) **(ii)**

Figure 6.6. The effect of dammarane triterpenoid 1 on TNF-α secretion levels represented in pg/ml concentration (i) and percent inhibition (ii) in LPS induced THP-1 human monocytes in comparing with dexamethasone (positive control).

6.3.6. Effect of dammarane triterpenoid 1 on secretion of IL-1β in LPS - induced THP-1 human monocytes

Dammarane triterpenoid 1 showed dose-dependent inhibition of IL-1beta secretion in THP-1 monocytes and IC_{50} found at 7.1±0.41µM, whereas dexamethasone (positive control) inhibited with IC_{50} of 8.92±0.53 µM (Figure 6.7).



(i) (ii)

Figure 6.7. The effect of dammarane triterpenoid 1 on IL-1β secretion levels represented in pg/ml concentration (i) and percent inhibition (ii) in LPS induced THP-1 human monocytes in comparing with dexamethasone (positive control).

6.3.7. Effect of dammarane triterpenoid 1 on secretion of IL-6 in LPS - induced THP-1 human monocytes

Dammarane triterpenoid 1 showed dose-dependent inhibition of IL-6 secretion in THP-1 monocytes and IC_{50} found at 16.55±0.92 µM, whereas dexamethasone (positive control) inhibited with IC_{50} of 23.52±1.33 µM (Figure 6.8).

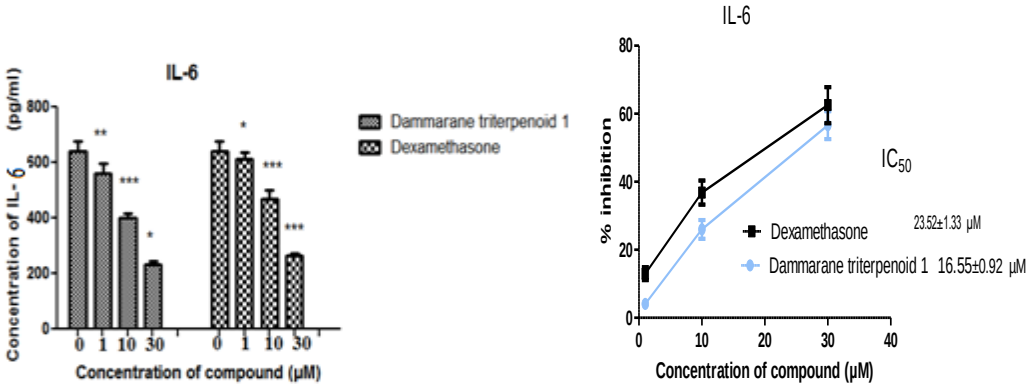


Figure 6.8. The effect of dammarane triterpenoid 1 on IL-6 secretion levels represented in pg/ml concentration (i) and percent inhibition (ii) in LPS induced THP-1 human monocytes in comparing with dexamethasone (positive control).

6.4. DISCUSSION

In vivo acute inflammatory studies by inflammogen (carrageenan) induced paw edema model demonstrated that dammarane triterpenoid 1 showed maximum edema inhibition between 3rd and 4th h after inflammogen (carrageenan) injection. It was known that COX-2 and 5-LOX are high in 3rd to 4th hours, which is called as second phase of acute inflammation. This demonstrated that *in vivo* anti-inflammatory activity of dammarane triterpenoid 1 may be due to COX-2 and 5-LOX inhibition. Further, histopathological studies of tissues of 4th h paws (maximum edema inhibition occur in 4th h) demonstrated that decreased inflammatory cells populations in tissues of treated rat paws compared to untreated paw tissues. This denotes that COX and 5-LOX inhibitors may block the recruitment of inflammatory cells in site of inflammation during the inflammation. However, detailed investigations are required on influence of COX/LOX inhibitors in recruitment of inflammatory cells at inflammation site.

PGs and LTs are produced in monocytes by the activities of COX and 5-LOX respectively and involve in inflammatory response. Dammarane triterpenoid 1 inhibited secretion of PGs and LTs in LPS-stimulated THP1-human monocytes. This is due to COX and 5-LOX dual inhibitory potential of dammarane triterpenoid 1.

Pro-inflammatory cytokines (TNF- α, IL-1β and IL-6) are key orchestrators of inflammation. Pro-inflammatory cytokines (TNF- α, IL-1β and IL-6) and arachidonic acid cascade enzymes relation also has been established. Mani *et al*., (2008) reported that *Acanthus ilicifolius* leaf extract inhibited carrageenan-induced rat paw edema and inhibited COX and 5-LOX activities. The extract also inhibited the production of proinflammatory cytokines (TNF alpha and IL-6) in LPS-treated peripheral blood mononuclear cells (PBMCs). Chen et al. (2010) reported that 3-(4-bromophenyl)-6-nitrobenzo(1.3.2)dithiazolium ylide 1,1-dioxide was a novel dual COX/5-LOX inhibitor that also inhibited proinflammatroy cytokines (TNF- α, IL-1β and IL-6) secretion. In this background, proinflammatory inhibition studies of dammarane triterpenoid 1 were carried out and we found that dammarane triterpenoid 1 significantly inhibited TNF-α production in LPS - induced THP-1 monocytes. It has been reported that COX inhibitors don`t have any effect on TNF-α expression but 5-LOX inhibitors have been reported as TNF-α suppressive agents (Melinda et al., 2004; Devaraj and Jialal, 2005). Earlier findings suggest that TNF- α suppressive effect of dammarane triterpenoid 1 in LPS activated THP-1 human monocytes may be due to its significant 5-LOX inhibitory activity (Melinda et al., 2004; Devaraj and Jialal, 2005; Chen and Lv., 2006). However, further studies are needed to demonstrate the precise molecular mechanism in proinflammatory cytokines suppressive effect of COX and LOX dual inhibitory dammarane triterpenoid 1.

6.5. CONCLUSIONS

5-LOX and COX dual inhibitory dammarane triterpenoid 1 exhibited oral anti-inflammatory activity in rats by inhibiting carrageenan-induced paw edema. It exhibits comparable or more antiinflammaotry activity than indomethacin (standard). Dammarane triterpenoid 1 significantly inhibited prostaglandins and leukotrienes as well as pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) secretion in LPS-stimulated THP1 human monocytes. These results warranted further studies to develop dammarane triterpenoid 1 as an anti-inflammatory agent.