ISSN NO. 2320-5407

*Journal homepage: http://www.journalijar.com*

*INTERNATIONAL JOURNAL OF ADVANCED RESEARCH*

**RESEARCH ARTICLE**

# Triterpenoids isolated from Euphorbia tirucalli Linn. inhibit lipopolysaccharide mediated inflammatory response in RAW264.7 murine macrophage cell line

**Pramod C[1*], Neethu MV[1], Anjumol VM,[1] Vysakh A[2], Raj S[1]**

**1-**Department of Pharmacology, University College of Pharmacy, M.G University, Kottayam, Kerala, India.

**2-** Department of Biochemistry, St.Thomas College, Palai, Kottayam, Kerala, India

## *Manuscript Info*

***Manuscript History:***

Received: 15 August 2015
Final Accepted: 26 September 2015
Published Online: October 2015

***Key words:***

Triterpenoids, 5-lipoxygenase, Reactive oxygen species, Euphorbia *tirucalli* Linn.

***Corresponding Author***

**Pramod C**

## *Abstract*

The latex of Euphorbia *tirucalli* Linn. (Euphorbiaceae) is traditionally used for treating inflammatory disorders. However, studies of triterpenoids from *E. tirucalli* on inflammatory mediators have been few. The current study aims to investigate the anti-inflammatory effects of the triterpenoid fraction isolated from *E. tirucalli* (TET) in RAW 264.7 cell lines. The TET was prepared from the aerial parts of *E. tirucalli*. Different concentrations of TET (5-25µg/ml) were subjected to *in vitro* study. The inhibitory effect of TET on nitrate, reactive oxygen species (ROS) production, total cycloxygenase (COX), 5-lipoxygenase (5-LOX) activity and COX-2, inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) gene expressions were studied in LPS stimulated RAW 264.7 cells. TET (25µg/ml) inhibited maximum total COX and 5-LOX activity by 60.32% and 76.46% respectively, besides significantly ($p<0.05$) diminished nitrate and ROS generation when compared with LPS treatment alone. Moreover TET down regulated the mRNA expression of TNF-α, IL-6, COX-2 and iNOS against LPS stimulation. Our results demonstrates that TET is able to attenuate inflammatory response possibly via ROS and NO suppression, inhibiting the production of arachidonic acid metabolites and modulation of proinflammatory mediators and cytokines release.



## 1. INTRODUCTION

The inflammation is an essential event; this protective response may lead to potentially damaging consequences. Various autoimmune disorders are characterized by marked inflammation and associated failure of repair process. Inflammation may release or generate a diverse population of pro-inflammatory mediators like bradykinins, serotonin, histamines, prostaglandins and nitric oxide. These substances contribute to the classic clinical picture of heat (calor), redness (rubor), pain (dolor), swelling (tumor) and diminished function associated with inflammation and may produce hyperalgesia or allodynia (Howard et al., 2006). In addition, chronic inflammation can also lead to a number of diseases such as hay fever, periodontitis, rheumatoid arthritis, arteriosclerosis, cardiovascular diseases, diabetes, obesity, pulmonary diseases, neurologic diseases and cancer.

LPS is a potent inducer of systemic inflammatory responses by activation of macrophages, which initiate a diverse series of functional responses such as the production of NO and cytokines as well as the activation of phospholipase $A_2$, which produces lipid metabolites of arachidonic acid (Chiong et al., 2013). Proinflammatory molecules like TNF-α, certain interleukins, prostaglandins and even pathogenic concentration of nitric oxide are instrumental in raising such response (Van der Vliet et al., 2000). High level of different cytokines including TNF-α is known to involve in over expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) genes, which up-regulate these enzymes (Hughes et al., 1999). Constitutive level of these proteins, particularly

iNOS is negligible (Alderton et al., 2001) and is unique to be induced in stress conditions (Madrigal et al., 2001). Similarly, COX-2 undergoes selective upregulation during inflammation (Rohrenbeck et al., 1999). Non-selective and selective (for COX-2) inhibitors of cyclooxygenases are used extensively to combat inflammation (Dhikav et al., 2002). However, their role on nitric oxide production has not been ascertained (Tunctan et al., 2003). A growing body of evidence in recent years implicated nitric oxide and iNOS activity in different inflammatory pathogenesis (Guzik et al., 2003). Nitrosylation of several functionally important proteins involved in cell proliferation and inflammation have been reported (Foster et al., 2003). More interestingly, nitric oxide has been shown to have the ability to stimulate COX-2 showing a potential synergism (Hughes et al., 1999). Thus, nitric oxide appears to be of crucial importance and hence may be considered as a rewarding target for intervention. Many current anti-inflammatory drugs target these mediators at different levels, yet they lack specificity and their untoward effects restrict their long-term use (Dhikav et al., 2002). Hence, there is a constant demand for better therapeutic alternatives. Herbal products are well known for their reputed medicinal properties; however, most of them are empirically used.

*E.tirucalli*. (Euphorbiaceae), pencil tree is an ornamental, succulent plant naturally distributed in paleotropical region of Madagascar, Cape region (South Africa), East Africa, but now it has become acclimatized and grows freely in all parts of India (Gupta et al., 2013). *E. tirucalli* produces and stores abundant amounts of latex in so-called laticifers and it contains high amounts of sterols and triterpenes (Hastilestari et al.,2013). Traditionally the latex of the plant has been used as an application for warts, asthma, rheumatism, neuralgia, tumors and tooth ache in India (Khaleghian et al., 2011).

*E. tirucalli* has been reported to present numerous pharmacological activities such as the latex of this plant exhibited strong oxytocic activity against isolated strips of the gravid rat uterus (Mwine et al., 2013). Furthermore, *E. tirucalli* latex has pesticidal properties against pests such as mosquitoes (*Aedes aegypti* and Culex quinquefasciatus), bacteria (Staphylococcus aureus), molluscs (Lymnaea natalensis) and nematodes such as Haplolaimus indicus, Helicotylenchus indicus and Tylenchus filiformis besides a dose-dependent latex toxicity to parasitic nematodes such as *Haplolaimus indicus*, *Helicotylenchus indicus* and *Tylenchus filiformis in vitro* (Hastilestari et al.,2013). A recent study demonstrated that the crude latex of *E. tirucalli* modulates the cytokine response of leukocytes, especially CD4+ T lymphocytes (Avelar et al.,2011) and it has a promising activity in modulation of myelopoiesis there by enhancing the resistance of tumor-bearing mice (Valadares et al.,2006). However efficacy of triterpenoid fraction isolated from *E. tirucalli* has not been validated for the mediators associated with chronic inflammatory condition. In the current investigation was aimed to evaluate the anti inflammatory effect of triterpenoid fraction isolated from *E. tirucalli* in LPS stimulated RAW 264.7 cells.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and reagents

All chemical used in this study are of analytical reagent grade. Biochemical reagents were purchased from Merck, India. Tissue culture plates were purchased from Tarson, India. RT-PCR kit was purchased from Eppendorf India Ltd, Chennai.

### 2.2 Isolation of Triterpeniod fraction from *E. tirucalli*

*E. tirucalli* was collected from Karunagappally area of Kollam in March 2014 and confirmed taxonomically by Rojimon Thomas, Assistant Professor, Department of Botany, C.M.S College, Kottayam. A voucher specimen (voucher No:275**)** was preserved at Department of Pharmaceutical Science, Cheruvandoor Campus, M.G University, Kottayam district, Kerala. The freshly collected1000g stem bark of *E. tirucalli* was soaked in 1.5L of 95% of ethanol. After 7 days the extract was filtered and concentrated. Then it was partitioned between ethyl acetate and water. The former fraction was further partitioned into hexane to isolate triterpenoids. The concentrated hexane fraction was then weighed. The percentage yield of hexane fraction of *E. tirucalli* Linn. was found to be as 0.984%w/w.

### 2.3 Cell culture and treatments

The RAW264.7 murine macrophage cell line was purchased from NCCS Pune. The cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (penicillin/streptomycin) under a humidified atmosphere of 5% $CO_2$ at 37 $^0$C. The extract was dissolved in DMSO and applied to the cell cultures at final concentrations of 5, 10, 15, 20 and 25µg/ml alone or with 1µg/ml of LPS. The cells were incubated for 24 hours after and harvested and lyses the cells by using cell lysis buffer and performed various assays that determine the anti-inflammatory effect of extract.

**2.4 Measurement of total COX activity**

The total COX activity was measured by method previously described by (Shimizu et al.,1981). The assay mixture contained Tris HCl buffer, glutathione, hemoglobin and enzyme. The reaction was initiated by the addition of arachidonic acid followed by incubation at $37^0$C for 20 minute. The reaction was terminated by addition of 0.2ml of 10% TCA in 1N HCl. The above reactants were mixed, 0.2ml of TBA was added and contents were heated in a boiling water bath for 20 minute. The contents were cooled and centrifuged at 1000 rpm for 3 minute. The absorbance of the supernatant was measured at 632 nm.

**2.5 Measurement of 5-LOXactivity**

Determination of 5-LOX activity was done by the method previously described by (Axelrod et al.,1981). The reaction was carried out in a quartz cuvette at $25^0$c with 1cm light path. The assay mixture contained 2.75ml of tris buffer of pH 7.4, 0.2ml of sodium linoleate and 50µl of sample. The increase in O.D was measured in 234nm.

**2.6 Measurement of nitrate and ROS production in RAW 264.7 cells**

Nitrite level was determined by Greiss reaction described by Gillum et al. 1993). Intracellular ROS was measured by 2'7'dichlorofleuresin diacetate assay. The pictures were taken in Blue excitation (Excitation wavelength 450-480nm: Emission wavelength 515nm) Olympus CKX41 epifluorescent microscopy with Optika (Italy) imaging system.

**2.7 Estimation of cellular iNOS level**

The assay mixture contained 2ml HEPES buffer, substrate 0.1ml L-Arginine, 0.1ml manganese chloride,0.1ml 30µg dithiothreitol (DTT),0.1ml NADPH, 0.1ml tetrahydropterin,0.1ml oxygenated haemoglobin and 0.1ml enzyme (sample). Absorbance was recorded at 401nm.

**2.8 Reverse transcription-polymerase chain reaction**

The gene level expression of COX-2, TNF-α, iNOS, IL-6 mRNA was measured by semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR. For cDNA synthesize, two step RT-PCR kit was used following manufacturers procedure. RT-PCR was performed in an Eppendorf thermocycler by using forward and reverse primers of COX-2, TNF-α, iNOS, IL-6. GAPDH primers were used as an internal control. The sequences of the primers used are shown in table 1.The PCR products were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide, visualized under a UV- transilluminator and the relative intensities of bands of interest were measured on a GelDoc 2000 scanner (Bio-Rad, CA, USA) with scan analysis software.

**2.9 Statistical analysis**

The results obtained are presented as mean ± standard error. One-way ANOVA was performed for comparison test of significant differences among groups. Pair fed comparisons between the groups was made by Duncan's multiple range tests. $P < 0.05$ was considered significant. All the statistical analysis were carried out using SPSS/PC+, version 11.0 (SPSS Inc., Chicago, IL, USA) statistical software.

## 3. RESULTS

**3.1 Inhibitory effect of TET on total COX activity**

RAW 264.7 murine macrophage cells were pretreated with LPS (1µg/ml) for 1 hour and then incubated with TET at various concentrations (5-25µg/ml) for 24 hours. Data were represented as mean ± SEM of triplicate determination. The percentage inhibitions obtained from different concentration were depicted in figure1. In this assay TET showed total COX inhibition activity with value of $IC_{50}$ 21.03µg/ml.

**3.2 Inhibitory effect of TET on 5-LOX activity**

RAW 264.7 were pretreated with LPS (1µg/ml) for 1 hour and then incubated with TET at various concentrations (5-25µg/ml) for 24 hours. Data were represented as mean ± SEM of triplicate determination. The percentage inhibitions obtained from different concentration were presented in figure 2. In this assay TET showed 5-LOX inhibition activity with value of $IC_{50}$ 13.77µg/ml.

**3.3 Effect of TET on nitrate level**

RAW 264.7 murine macrophage cells were pre-treated with LPS (1µg/ml) concentration for 1hr and then incubated with TET (15, 20, 25µg/ml) and standard drug DFC at a concentration of 10µg/ml for 24 hrs. The amount of nitrate produced in cell culture supernatant was measured at 24 h of treatment, showed that LPS induced nitrate production is statistically reduced in TET at a concentration of 25 µg/ml when compared to control (treated with LPS alone). Results were depicted in figure 3.

**3.4 Effect of TET on intracellular ROS generation**

RAW 264.7 cells were pre-treated with LPS (1µg/ml) concentration for 1hr and then incubated with TET (25µg/ml) and DFC at a concentration of 10µg/ml for 24 hrs. ROS produced in cell supernatant was measured by

fluorescent microscope showing significant reduction as compared control (treated with LPS alone). Results were depicted in figure 4.

**3.5 Effect of TET on iNOS in RAW (264.7)**

RAW (264.7) were pretreated with LPS (1µg/ml) concentration for 1 hour and then incubated with TET at various concentrations (µg/ml) for 24 hours. Data were represented as mean ± S.D of triplicate determination. The percentage inhibitions obtained from different concentration were shown in figure 5. In this assay, TET showed iNOS inhibition activity with an $IC_{50}$ value of 17.35µg/ml.

**3.6 Effect of TET on IL-6, COX-2, TNF-α and iNOS gene expression**

Inhibitory effect of TET and DFC on the expression of the proinflammatory markers like COX-2, iNOS and cytokines like IL-6, TNF-α was determined by reverse transcriptase-PCR. GAPDH was used as a control. The gene expression of COX-2, iNOS, IL-6, TNF-α in LPS stimulated group is up regulated. Down regulated expression of COX-2 and iNOS IL-6, TNF-α were seen in TET treated group as compared to LPS treated group. Results were shown in figure 6.

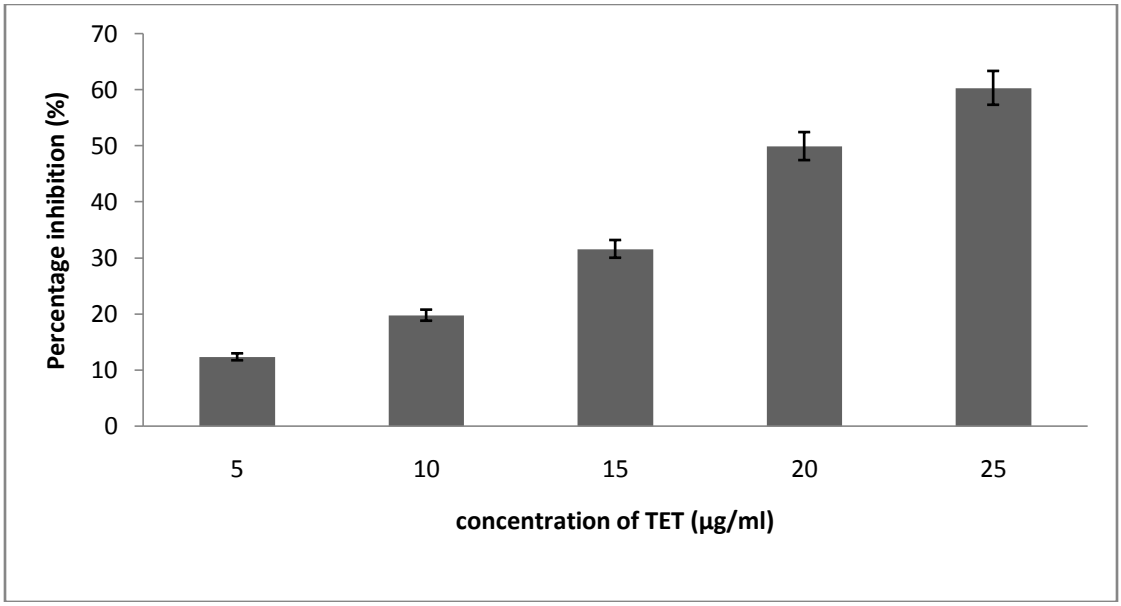


**Figure 1. Effect of TET on total COX activity in RAW 264.7 cells:** RAW 264.7 cells were pre-treated with LPS (1µg/ml) concentration for 1hr and then incubated with TET at various concentrations (µg/ml) for 24 hrs. Data were represented as mean ± S.EM of three triplicate determinations.

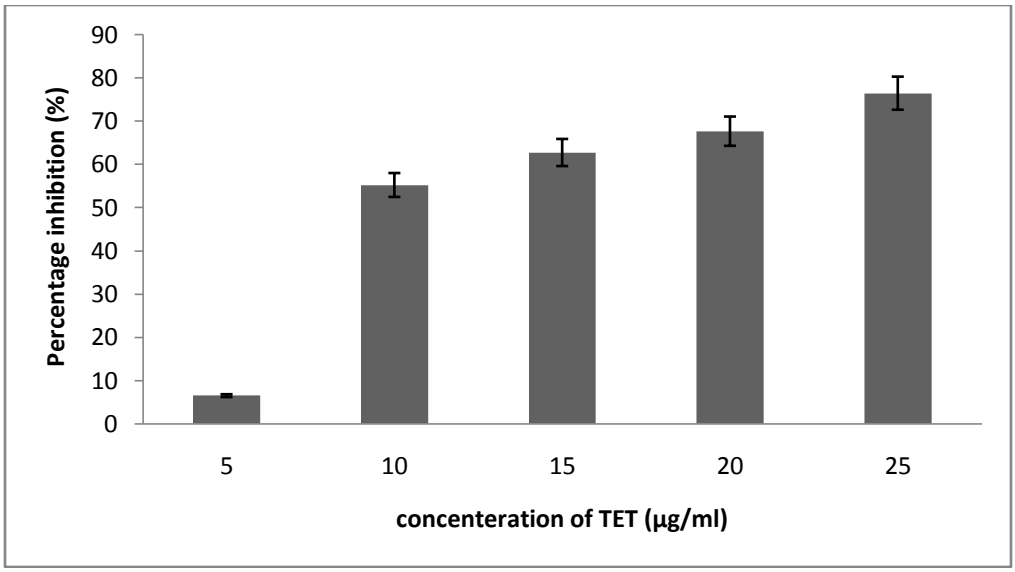


**Figure 2. Effect of TET on total 5-LOX in RAW 264.7 cells:** RAW 264.7 cells were pre-treated with LPS (1µg/ml) concentration for 1hr and then incubated with TET at various concentrations (µg/ml) for 24 hrs. Data were represented as mean ± S.EM of three triplicate determinations.

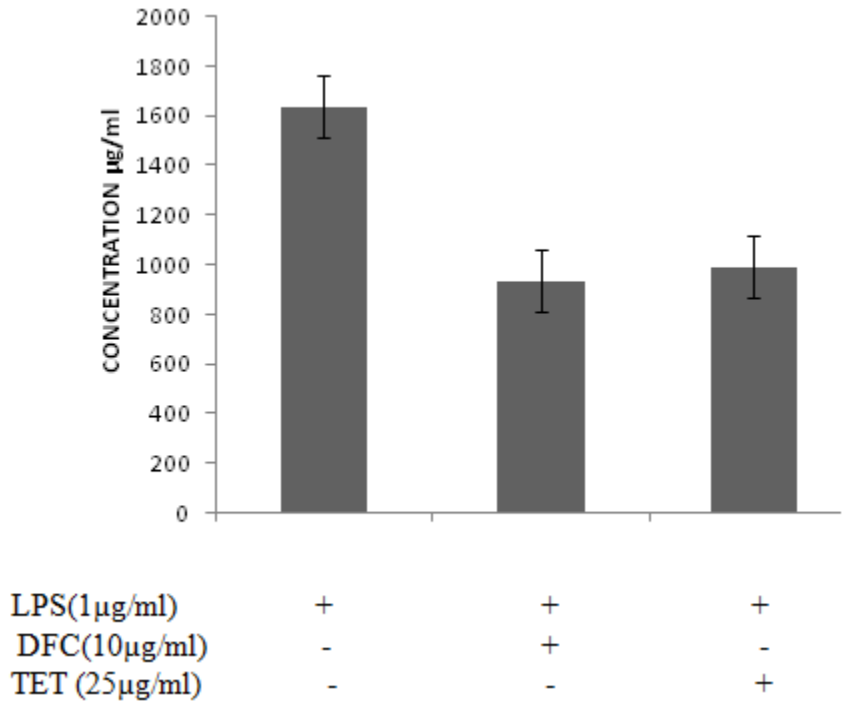


**Figure 3. Effect of TET on nitrate in RAW 264.7 cells:** RAW 264.7 cells were pre-treated with LPS (1µg/ml) concentration for 1hr and then incubated with TET at various concentrations (µg/ml) and DFC (10µg/ml) for 24 hrs. Data were represented as mean ± S.E.M of triplicate determination.; DFC-Diclofenac; TET: Triterpenoid fraction of *E.tirucalli.*

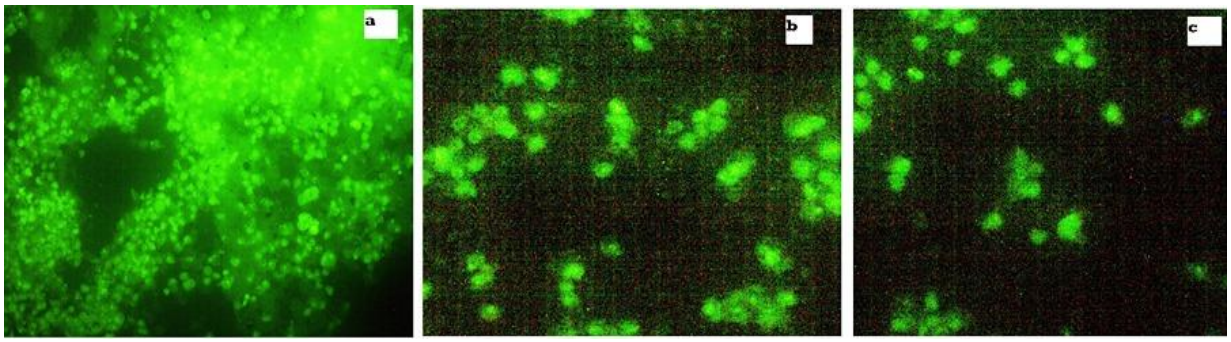


**Figure 4. Fluorescent staining of ROS generation in RAW 264.7 cells**: RAW264.7 cells treated with TET and diclofenac standard (DFC) at a concentration of 10µg/ml. Pictures was taken in Blue excitation (Excitation wavelength 450-480nm: Emission wavelength 515nm). a: LPS + control, b: LPS + DFC (10µg/ml), c: LPS + TET( 25µg/ml). DFC-Diclofenac; TET: Triterpenoid fraction of *E.tirucalli.*

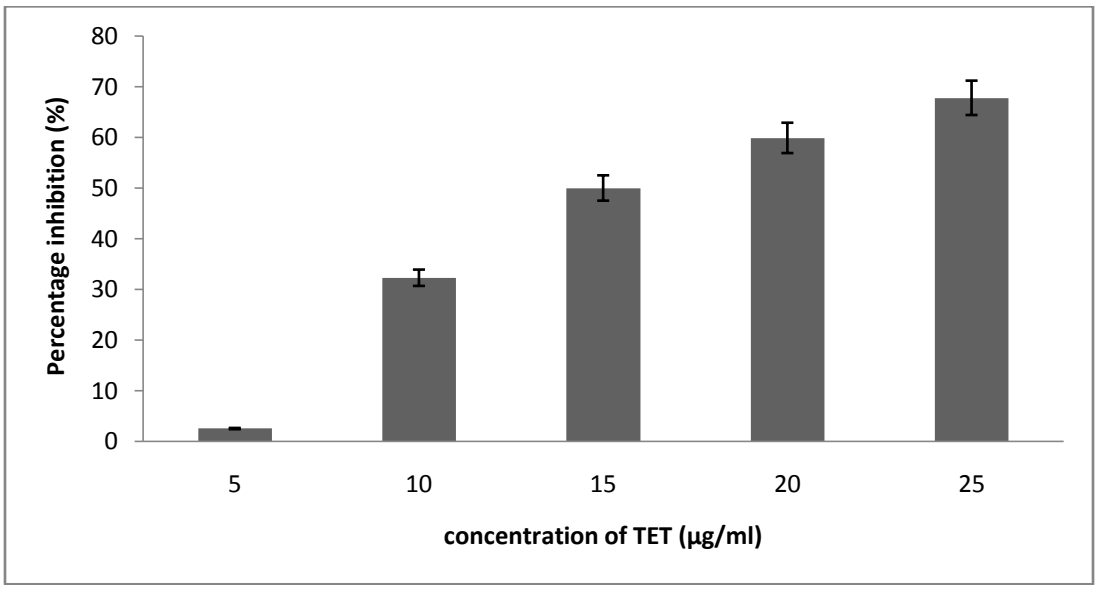


**Figure 5. Effect of TET on iNOS in RAW 264.7 cells:** RAW 264.7 cells were pre-treated with LPS(1µg/ml) concentration for 1hr and then incubated with TET at various concentrations (µg/ml) for 24 hrs. Data were represented as mean ± S.D of triplicate determination.

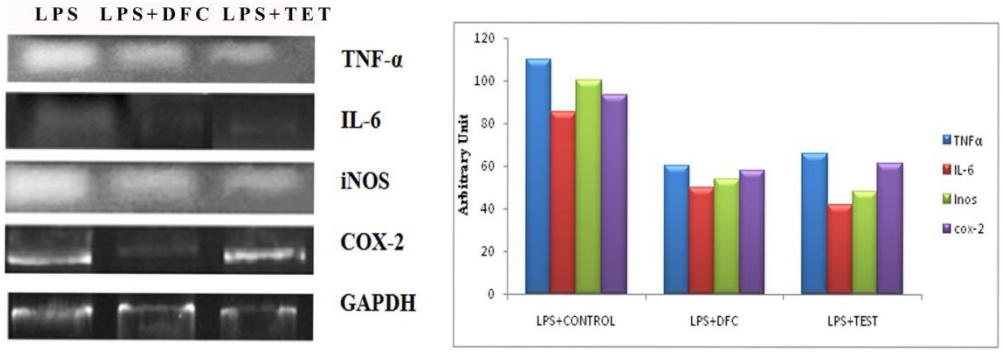


**Figure 6. RT-PCR gel of COX-2, iNOS, IL-6, TNF-α mRNA in RAW 264.7 cells:** Inhibitory effect of TET on the expression of the proinflammatory markers like COX-2, iNOS and cytokines like IL-6, TNF-α was determined

by reverse transcriptase-PCR,. GAPDH was used as a control. The gene expression of COX-2, iNOS, IL-6, TNF-α in LPS stimulated group is up regulated. Down regulated expression of COX-2 and iNOS IL-6, TNF-α were seen in TET treated group as compared to LPS treated group. DFC-Diclofenac; TET: Triterpenoid fraction of *E.tirucalli.*

| Gene | Forward primer | Reverse primer |
|---|---|---|
| COX-2 | 5′ TCTGATCAATGTCATGAGCAAAGG 3′ | 5′TCTGATCAATGTCATGAGCAAAGG 3′ |
| iNOS | 5′ACAACAAATTCAGGTACGCTGTG3′ | 5′TCTGATCAATGTCATGAGCAAAGG3′ |
| TNF-α | 5′ CCAGGGACCTCTCTCTAATCAGC3′ | 5′CTCAGCTTGAGGGTTTGCTACAA3′ |
| IL-6 | 5′ CCTTAAAGCTGCGCAGAATG3′ | 5′ATTCAATGAGGAGACTTGCC3′ |
| GAPDH | 5′ TCCATGACAACTTTGGTATCGTG3′ | 5′ACAGTCTTCTGGGTGGCAGTG3′ |

**Table 1: The sequences of the primers used for study**

## 4. DISCUSSION

Triterpens are ubiquitously distributed in the plant and marine animal kingdom (Alqahtani et al.,2013). They are produced in plant as secondary metabolites and have diverse pharmacological activities such as anticancer, immunomodulatory, anti-anxiety as well as antinociceptive. It is well recognized that herbal products containing triterpens have long been used in many Asian countries to treat or prevent variety of disease by the traditional healers (Parmar et al.,2013). The latex of *E. tirucalli* comprises triterpens are the major constituent (Hastilestari et al.,2013). In the present study we investigated the modulatory effect of triterpenoid fraction isolated from *E. tirucalli* in LPS stimulated RAW 264.7 murine macrophage cells.

Macrophages are one of the important components in the immune defense mechanism. During the progress of inflammation, macrophages actively participate in inflammatory responses by releasing proinflammatory cytokines and mediators such as ROS, NO, iNOS, IL-6 and COX-2. Thease mediators plays a key role in the pathogenesis of many acute and chronic inflammatory diseases (Bak et al.,2013). LPS is a well-studied component from the outer membrane of gram-negative bacteria, is widely considered one of the most potent activators of macrophages (Chiong et al.,2013). When activated by bacterial endotoxin LPS, macrophages produce inflammatory cytokines, which in turn activate other macrophages and other nearby cells to promote more inflammatory cytokines (Xu et al.,2012).

LPS stimulation in RAW 264.7 murine macrophage cells enhances the enzymatic activity of both COX and 5-LOX, which in turn activate the enormous production of prostaglandins and leukotrienes. COX catalyzes the biosynthesis of inflammatory mediators such as prostaglandin, thromboxane and prostacyclin. Inhibition of COX was considered to be partly responsible for the anti-inflammatory activity (Zhao et al., 2009). In the present study triterpene fraction of *E.tirucalli* exhibited significant anti COX activity with an $IC_{50}$ value of 21.03µg/ml. LOXs are a family of non-heme iron-containing dioxygenases catalyzing the biosynthesis of leukotrienes, initiators of inflammation and their inhibition is considered to be partly responsible for the anti-inflammatory activity (Martel-Pelletier et al.,2003). In the present study TET showed significant anti 5-LOX activity with an $IC_{50}$value of 13.77µg/ml.

LPS is also known to enhance cellular oxidative stress via the generation of ROS (Bose et al.,2012). ROS are well documented to function as signaling molecules, stimulating cellular activities ranging from cytokine secretion to cell proliferation, and at higher concentration, they can induce cell injury and death (Bak et al.,2013). TNF-$\alpha$ is a pivotal proinflammatory cytokine and is regarded as an endogenous mediator of LPS induction and has a decisive function in the process of inflammation that could represent the severity of inflammation (Xu et al.,2012).

The reactive free radical NO, which is synthesized by iNOS, is a major macrophage-derived inflammatory mediator and has also been reported to be involved in almost every stage of the development of inflammatory

diseases (Bose et al.,2013). This nitric oxide is spontaneously interacts with the oxygen to produce stable products (nitrates, nitrites), which can be determined using Griess reagent. Generation of NO is enzymatically catalyzed by inducible nitric oxide synthase (iNOS), whose expression is triggered by LPS treatment in many cell types, tissues, and organs (Bose et al.,2013). In the present investigation, stimulation with LPS consistently provoked a marked up regulation in iNOS mRNA expression and NO production in RAW 264.7 murine macrophage cells, which were significantly inhibited when the cells were co treated with TET 25 µg/ml and standard drug DFC (10µg/ml).

Additionally LPS stimulation enhanced the mRNA expression of COX-2 and IL-6. COX-2 is a central mediator in inflammation and IL-6 is pivotal pro-inflammatory cytokine, regarded as an endogenous mediator of LPS induced inflammation (Melnicoff et al., 1989). Usually activated inflammatory cells produces high quantities of NO, necessary for maintaining prolonged COX-2 gene expression (Lee et al.,2013). In the present study, we found that both COX-2 and IL-6 mRNA expression were found to be abrogated during co treatment with TET. NF- κB plays a critical role in the regulation of cell survival genes and coordinates the expressions of pro-inflammatory enzymes and cytokines such as iNOS, COX-2, TNF-α and IL-6. Since the expressions of these pro-inflammatory mediators are modulated by NF-κB, our findings suggest that the transcriptional inhibition of pro-inflammatory mediator production by TET may be occurs via blocking of the NF- κB signaling pathway.

In conclusion our findings indicates that TET was able to attenuate the chronic inflammatory response by suppressing many inflammatory mediators including NO, Prostaglandins, leukotrienes, and ROS in RAW264.7 murine macrophage cells stimulated with LPS. Furthermore TET suppressed the mRNA expressions of inducible enzymes like iNOS and COX-2, whose over expression enable the drastic production of NO and PG, as well as the proinflammatory cytokines such as TNF-*α* and IL-6. These effects might be mediated through the inhibition of NF-*κ*B signaling pathways. From the above context of evidence, triterpenoids from *E.tirucalli* may be used as a potent natural anti-inflammatory therapeutic agent.

**Conflict of interest**

There is no conflict of interest

## REFERENCE

Alderton, W.K.., Cooper, C.E., Knowles, R.G.(2001): Nitric oxide syntheses: structure, function and inhibition. Biochemical Journal., 357:593–615.

Alqahtani, A., Hamid, K., Kam, A. (2013): The pentacyclic triterpenoids in herbal medicines and their pharmacological activities in diabetes and diabetic complications. *Curr Med Chem.,* 20: 908-23.

Avelar, B.A., Avelar, F.J.N., Avelar, R.S.(2011): The crude latex of *Euphorbia tirucalli* modulates the cytokine response of leukocytes, especially CD4+ T lymphocytes. *Braz J Pharmacogn*.,21:662-5.

Axelrod, B., Cheesebrough, T.M,, Laakso, S. (1981): Lipoxygenase from soyabean. *Methods Enzymol.,* 71:441- 4.

Bak, M.J., Truong, V.L., Kang, H.S. (2013): Anti-inflammatory effect of procyanidins from wild grape (*Vitis amurensis*) seeds in LPS-induced RAW 264.7 Cells. *Oxid Med Cell Longv* ., 1-11

Bose, S., Kim ,H. (2013): Evaluation of *in vitro* anti-inflammatory activities and protective effect of fermented preparations of rhizome atractylodis microcephale on intestinal barrier function against lipopolysacharide insult. *Evid Based Complement Alternat Med.,* 1-16.

Chiong, H.S., Yong, Y.K., Ahmad, Z. (2013): Cytoprotective and enhanced anti-inflammatory activities of liposomal piroxicam formulation in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Int J Nanomedicine* ., 1245-10.

Dhikav, V., Singh, S., Anand, K.S. (2002): Newer non-steroidal anti-inflammatory drugs—a review of their therapeutic potential and adverse drug reactions. Journal of Indian Academy of Chemical Medicine., 3:332–338.

Foster, M.W., McMahon, T.J., Stamler, S.J.(2003): S-nitrosylation in health and disease. Trends in Molecular Medicine., 9: 160–167.

Gillum, R.F., Ingram, D.D., Makuc, D.M.(1993): White blood cell count, coronary heart disease, and death: The NHANES I epidemiologic follow-up study. *Am Heart J.,* 125:855-8.

Gupta, N., Vishnoi, G., Wal, A. (2013): Medicinal value of *Euphorbia Tirucalli*. *Syst Rev Pharm.,* 4: 40-6.

Guzik, T.J., Korbut, R., Adamek-Guzik, T.(2003): Nitric oxide and superoxide in inflammation and immune regulation. Journal of Physiology and Pharmacology., 54:469–487.

Hastilestari, B.R., Mudersbach. M., Tomala. F. (2013): *Euphorbia tirucalli* L.–Comprehensive characterization of a drought tolerant plant with a potential as biofuel source. *Plos One.,* 8:1-12.

Howard, L., Weiner., Vijay, K., Kuchroo. (2006):Reciprocal developmental pathways for the generation of pathogenic effector $T_H17$ and regulatory T cells. Nature., 441: 235-238.

Hughes, F.J., Buttery, L.D., Hukkanen, M.V., O'Donnell, A., Maclouf, J., Polak, J.M. (1999): Cytokine induced prostaglandin E2 synthesis and cyclooxygenase-2 activity are regulated both by a nitric oxide dependent and independent mechanism in rat osteoblasts in vitro. Journal of Biological Chemistry., 274:1776–1782.

Hughes, F.J., Buttery, L.D., Hukkanen, M.V., O'Donnell, A., Maclouf, J., Polak, J.M. (1999): Cytokine induced prostaglandin E2 synthesis and cyclooxygenase-2 activity are regulated both by a nitric oxide dependent and independent mechanism in rat osteoblasts in vitro. Journal of Biological Chemistry.,274: 1776–1782.

Khaleghian, A., Nakaya, Y., Nazari, H. (2011): Biodiesel production from *Euphorbia tirucalli* L. *J Med Plants Res.,* 5: 4968-5.

Lee, S.Y., Kim, H., Han, J. (2013): Anti-inflammatory effect of oyster shell extract in LPS stimulated RAW264.7cells. *Prev.Nutr.Food Sci.,18*:23-6.

Madrigal, J.L., Moro, M.A., Lizasoain, I., Lorenzo, P., Castrillo, A., Bosca, L., Leza, J.C.(2001): Inducible nitric oxide synthase expression in brain cortex after acute restraint stress is regulated by nuclear factor kappa B-mediated mechanisms. Journal of Neurochemistry .,76:532–538.

Martel-Pelletier, J., Lajeunesse, D., Reboul, P. (2003): Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. *Ann. Rheum. Dis,*. 62: 501-8.

Melnicoff, M.J., Horan, P.K., Morahan, P.S. (1989): Kinetics of changes in peritoneal-cell populations following acute inflammation. *Cell Immunol.,* 118: 178-191.

Mwine, J., Damme, P.V., Hastilestari, B.R. (2011): *Euphorbia tirucalli* L. (Euphorbiaceae) The miracle tree: Current status of available knowledge. *Sci Res Essays Acad J.,*6:4905-14.

Parmar, S.K., Sharma, T.P., Airao, V.B. (2013): Neuropharmacological effects of triterpenoids. *Phytopharmacol.,* 4:354-18.

Rohrenbeck, A.M., Bette, M., Hooper, D.C., Nyberg, F., Eiden, L.E., Dietzschold, B., Weihe, E. (1999): Up regulation of COX-2 and CGRP expression in resident cells of the Borna disease virus infected brain is dependent upon inflammation. Neurobiology Diseases., 6:15–34.

Shimizu,T., Kondo, K., Hayaishi, O. (1981): Role of prostaglandin endoperoxides in the serum thiobarbituric acid reaction. *Arch Biochem Biophys* ., 206:271-5.

Tunctan, B., Altug, S., Uludag, O., Demirkay, B., Abacioglu, N. (2003): Effects of cyclooxygenase inhibitors on nitric oxide production and survival in a mice model of sepsis. Pharmacological Research., 48:37–48.

Valadares, M.C., Carrucha, S.G., Accorsi, W.(2006): *Euphorbia tirucalli* L. modulates myelopoiesis and enhances the resistance of tumor-bearing mice. *Int Immunopharmacol* .,6:294-9.

Van der Vliet, A., Eiserich, J.P., Cross, C.E. (2000): Nitric oxide: a proinflammatory mediator in lung disease? Respiratory Research .,1: 67–72.

Xu, Z., Zhou, J., Cai, J.(2012): Anti-inflammation effects of hydrogen saline in LPS activated macrophages and carrageenan induced paw edema. *J Inflmm.,* 9:1-8

Zhao, F., Wang, L., Liu, K.. (2009): *In vitro* anti-inflammatory effects of arctigenin, a lignan from *Arctium lappa* L. through inhibition on iNOS pathway. *J Ethnopharmacol* .,122:457- 62.