

In Vivo and in Vitro Approach to Study the Anti-Inflammatory Efficacy of *Eugenia Uniflora* L

S. Syama¹, Meenu Thampi², Dr. M S Latha^{3,*}

^{1,2,3} School of Biosciences, Mahatma Gandhi University, Priyadarshini Hills, Kottayam, Kerala, India.

*Corresponding author: School of Biosciences, Mahatma Gandhi University, Priyadarshini Hills, Kottayam, Kerala, India.

Abstract— Inflammation is the mechanism by which our body protects itself from infections, allergens, burns and toxic chemicals. Due to the side effects of currently used synthetic drugs, it becomes necessary to identify new anti-inflammatory drugs with lesser side effects. The ultimate source has been the plant-derived drugs which are found to be safer than the currently available anti-inflammatory drugs. Most of the plants in our nature consist of pharmacologically active phytochemicals. *Eugenia uniflora*, a shrub belonging to the Myrtaceae family has various traditional medicinal uses and is pharmacologically active with hypoglycemic, anti-oxidant and hypertensive properties. *Eugenia uniflora* is also found to be a rich source of vitamin B complex aiding human health as its leaves are consumed as a tea. *Eugenia uniflora* can be recycled as both foods and also as medicine for different diseases. In the current study an attempt was made to assess the *in vitro* and *in vivo* anti-inflammatory activity of EF18, the *Eugenia uniflora* active fraction obtained from the partial purification of crude ethanolic extract of *Eugenia uniflora*. EF18 obtained was 100 percent ethanolic fraction. Preliminary phytochemical analysis of EF18 fraction revealed the presence of various pharmacologically active phytochemicals. For the *in vitro* studies, RAW 264.7 cell line stimulated with LPS was used. Cyclooxygenases, lipoxygenases, myeloperoxidases, nitric oxide synthase enzyme activity, and cellular nitrate levels were also estimated in *in vitro* studies. EF18 showed a positive effect on the inhibition of enzyme activities thereby regulating inflammation. The expression of COX-2 and Nuclear factor- κ B (NF- κ B) genes were also assessed in LPS stimulated RAW264.7 cells by RTPCR. EF18 reduced the mRNA expression of COX-2 and NF- κ B. *In vivo* carrageenan-induced, chronic inflammatory studies were conducted using male Wistar rats. Diclofenac sodium was used as the standard reference drug. Two doses of EF 18(500mg/kg) and lower dose (250mg/kg) were selected on the basis of acute toxicity studies. Acute oral toxicity study was performed as per OECD-423 guidelines. EF18 reduced the paw edema, restored the hematological and serum biochemical parameters. The histopathological analysis also supported the *in vivo* anti-inflammatory effect of EF18. Our results demonstrated that EF18 was capable to diminish the inflammatory response possibly via inhibiting proinflammatory mediators. EF18 was subjected to HR-LCMS analysis which showed the presence of Gallic acid and Dihydromyricetin having proven anti-inflammatory property. These two pharmacologically active compounds attribute for the anti-inflammatory property presented by the *Eugenia uniflora* active fraction (EF18). It is concluded that *Eugenia uniflora* active fraction (EF18) can be effectively used in regulating inflammation.

Keywords— *Eugenia uniflora*; Inflammation; anti-inflammatory drug; Cyclooxygenases; lipoxygenase. Carrageenan.

I. INTRODUCTION

Medicinal plants play a prominent role in traditional healthcare and medicine. One of the traditional medicinal systems in India is Ayurveda[1]. Medicinal plants have been used as ancient treatment practices. Plants with its rich in source of bioactive molecules are responsible for its limitless therapeutic and pharmaceutical applications. The reactive oxygen species are generated inside our body as a result of the mitochondrial metabolism. An imbalance between the antioxidants and free radicals leads to oxidative stress. Oxidative stress results in the formation of various disorders like Alzheimer's disease, cardiovascular diseases, Parkinson's diseases and diabetic neuropathy [2]. Inflammation is the process where a protective response occurs against the injuries caused by traumas, microbiological agents or noxious chemicals. Inflammatory responses are inevitable to maintain the normal homeostasis of the body [3]. The inflammatory process is somewhat a complicated phenomenon. There are numerous mechanisms in our body to resolve the inflammation. Inflammation involves the participation of various factors like the proliferative, vasoactive and chemotactic at different stages or as targets for anti-inflammatory action [4]. Lipopolysaccharide (LPS) seen in the cell wall of gram-negative bacteria is one of the major stimuli that can enhance the up-regulation of pro-inflammatory proteins. Macrophage forms a link between the innate and the adaptive responses. Macrophages are the major source of inflammatory mediators. LPS binds to the toll-like receptor 4 (TLR4) that is present on the surface of macrophage and in turn, stimulates the downstream signalling pathways along with nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase pathway (MAPK). All these pathways are correlated to LPS-stimulated gene expression of inflammatory mediators. Cyclooxygenase enzyme (COX) exists mainly in two forms COX-1 and COX-2. COX-1 IS produced in cells under normal conditions whereas COX -2 is produced by pro-inflammatory mediators like cytokines, mitogens, etc. Cyclooxygenase is an important enzyme that helps in the formation of prostaglandins.

Inducible COX-2 is responsible for the high level of prostaglandin [5] [6]. Most of the Nonsteroidal anti-inflammatory drugs (NSAIDs) act on both the Cyclooxygenases (COX-1 and COX-2). Acute inflammation includes increased vascular permeability, migration of leukocytes and also infiltration of capillaries. Uncontrolled acute inflammation leads to chronic inflammation. For

chronic inflammation, both the steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs) were used. Extensive usages of synthetic drugs result in various side effects. Some of the side effects caused by the steroidal drugs include alteration in the metabolism of proteins, carbohydrates, salt and water imbalance, and various enzymatic reactions. Whereas the NSAIDs drugs excessive intake results in cardiovascular, gastric and respiratory problems. So the study focuses on herbal anti-inflammatory drugs with more effective and fewer side effects which will regulate the pro-inflammatory conditions.

Different phytochemicals isolated from the plants exert anti-inflammatory actions through the inhibition of various pro-inflammatory mediators. *Eugenia uniflora* is a shrub which possesses various medicinal properties. *Eugenia uniflora* is known as the Brazilian cherry and it belongs to the Myrtaceae family. *Eugenia uniflora* leaf infusions are used for the treatment of stomach and digestive disorders, gout, yellow fever, and hypertension. *Eugenia uniflora* was found effective for reducing blood pressure, weight and it also acts as a diuretic [7]. Essential oils acquired from the leaves of *Eugenia uniflora* exhibited strong anti-fungal property [8]. Preliminary screening studies of *Eugenia uniflora* showed the presence of phytochemicals in it. The main aim of the study was to evaluate the anti-inflammatory activity of EF18 (*Eugenia uniflora* active fraction) on RAW 264.7 macrophage cells and *in vivo* anti-inflammatory study in male Wistar rats by the carrageenan-induced method.

II. MATERIALS AND METHODS

A. Plant collection

Eugenia uniflora leaf samples were collected from Chenganoor (9.3183°N, 76.6111°E) Alappuzha district, Kerala, India. The plant material was authenticated by a taxonomist and a voucher specimen SBSBRL 25 was kept at School of Biosciences, M.G. University, Kottayam, Kerala, India.

B. Preparation of crude ethanolic extract of *Eugenia uniflora*

Eugenia uniflora leaves collected were washed properly and then shade dried and powdered and were stored in an airtight container for further analysis. *Eugenia uniflora* powder (50g) was extracted (72 hours) in a soxhlet apparatus with 500ml of various solvents with increasing polarity. The solvents used were petroleum ether, chloroform, ethyl acetate, ethanol, methanol, and water. The extracts were then filtered through whatmann No 1 filter paper and were concentrated to dryness using a rotary evaporator and stored at 4°C in sterile vials for further studies. The most active crude ethanolic extract of *Eugenia uniflora* was obtained by the phytochemical screening, evaluation of the antioxidant and anti-inflammatory activity. It was collected in large quantities and stored for further analysis.

C. Preparation of *Eugenia uniflora* active fraction (EF18)

For the preparation of *Eugenia uniflora* active fraction (EF18), the crude ethanolic extract of *Eugenia uniflora* was subjected to column chromatography partial purification technique using solvents with increasing polarity. The mobile phases used comprises of petroleum ether,

chloroform, ethyl acetate, and ethanol. The stationary phase used was silica gel of mesh size 60-120nm. The fractions obtained (EF1 to EF18) were exposed to phytochemical analysis, antioxidant, and anti-inflammatory activity. The results showed that among various fractions of crude ethanolic extract of *Eugenia uniflora* the EF18 fraction was found to be the most active fraction and was subjected to further *in vitro* studies. This EF18 fraction was 100% ethanolic fraction.

D. HR-LCMS analysis of EF18

HR-LCMS analysis of EF18 was performed using an Agilent 1290 infinity series HPLC instrument (Agilent technologies USA) which was equipped along with a binary pump (G4220B, USA) and an autosampler (G4226A, USA) connected to a column compartment (G1316C, USA). The effective chromatographic separation was done with the Agilent Zorbax C-18 column (2.1x50 mm, 18µm). The mobile phase consists of 0.1% formic acid in water which was used as the solvent A and 90% acetonitrile {CH₃CN +10% formic acid(0.01%)} in water as solvent B in a gradient elution mode. The mobile phase gradient was started with solvent A (95%): solvent B (5%) with a flow rate of 0.3ml per minute for about 18 minutes. After that the solvent was changed to solvent A with 0%: solvent B (100%) for 7 minutes. And finally, it was returned to solvent A 95%: solvent B (5%). The spectra of the analysis were obtained by using UHPLC PDA detector in a mass range of 120 to 1000(m/z) at the rate of 1 spectra/second.

The LCMS analysis was performed using a quadrupole time-of-flight (Q-TOF) mass spectrometer (Q-TOF LC/MS 6550 I Funnel Q-TOFs series G6550A, Agilent Technologies, USA) equipped with an ESI source. Mass spectral data were collected from an Agilent 6200 series TOF and 6500 series Q-TOF LC/MS system B.05.01 (B5125).

E. Cell line culture and treatments

The RAW macrophage cells (264.7 cells) were primarily obtained from the NCCS (National Centre for Cell Sciences, Pune, India). These cells were then maintained in a special medium named Dulbecco's modified Eagles medium (DMEM) purchased from Sigma Aldrich, USA [9].

The cells were cultured using a 25 cm tissue culture flask with DMEM medium supplemented with L-glutamine, 10%FBS, sodium bicarbonate from Merck, Germany. An antibiotic solution containing Streptomycin (100µg/ml), Amphotericin B (2.5µg/ml) and Penicillin (100 µg/ml) were also used for this cell culture. All the cultured cell lines were kept at 37 °C in a humidified 5% carbon dioxide incubator (NBS Eppendorf, Germany). The entire grown-up cells up to 60% confluency were shadowed by the enrichment of lipopolysaccharide (LPS: 1µg/ml). These LPS stimulated RAW 264.7 cells were exposed to various concentrations of diclofenac sodium (standard drug) and EF18 at concentrations 25, 50, 100µg/ml. Diclofenac sodium was considered as the standard anti-inflammatory drug in this study. The LPS stimulated cells were incubated for 24 hours. After the incubation time, all the anti-inflammatory assays were carried out using the cell lysate.

F. MTT assay

The cells cultured in the DMEM medium were seeded in 96 wells and were then treated with various sample concentrations (6.25µg/ml -100µg/ml). Later these cells were then kept for incubation at 37°C for 24 hours. After the treatments, the medium was replaced and 30µl of MTT solution was added subsequently to both the control and sample wells. This was then kept for incubation at 37°C for 4 hours. MTT solution was replaced after incubation. Formazan crystals were then dissolved in 100µl Dimethyl sulfoxide which acts as a solubilization solution. Using an ELISA microplate reader, the extent of reduction of MTT to formazan crystals was quantified by measurement of the absorbance at 570nm.

G. Estimation of Cyclooxygenase (COX) activity

The COX activity was estimated using the method of walker [10]. Here 100µl cell lysate was incubated with Tris-HCl buffer (pH 8), along with glutathione 5mM/L, and hemoglobin 5mM/L for about 1 minute at 25°C. Then the reaction was commenced by the addition of arachidonic acid 200mM/L and incubation was carried out at 37°C for 20 minutes. To this 200µl of 10% Trichloroacetic acid in 1N hydrochloric acid was added. After that, the centrifugation was done and 200µl of 1% thiobarbiturate was added, the tubes were then boiled for 20 minutes. Finally, after cooling, the tubes were again centrifuged for three minutes. COX activity was evaluated by reading absorbance at 632 nm.

H. Estimation of Lipoxygenase enzyme (LOX) activity

LOX activity was determined according to the procedure of Axelrod [11]. The reaction mixture was made up to 2 ml final volume that contained Tris-HCl buffer (pH 7.4), and sodium linoleate (200 µl) and 50µl of cell lysate. The absorbance was read at 234nm and this reflects the 5-hydroxyeicosatetraenoic acid formation.

I. Estimation of Myeloperoxidase

Myeloperoxidase activity was determined by the method of Bradley [12]. Myeloperoxidase estimation involves a cell lysate that was homogenized in a solution of 50mM potassium phosphate buffer and 0.57% hexadecyltrimethyl ammonium bromide (HTAB). These samples were then centrifuged at 2000rpm for 30 minutes at 4°C. After the centrifugation supernatant was taken and then analyzed for its Myeloperoxidase activity. In the sample, Myeloperoxidase was activated by the addition of 50 mM phosphate buffer of pH 6 (1.67mg/ml guaiacol and 0.0005% H₂O₂). Absorbance was monitored at 460 nm. Myeloperoxidase activity can be represented as units per ml of cell lysate. One unit of myeloperoxidase activity was equivalent to 1µM of peroxide degradation in one minute at 25°C.

J. Estimation of nitric oxide

Nitric oxide synthase was evaluated by the method of Salter [13]. Here the cell lysate was homogenized in 2ml of HEPES buffer. The assay system contained substrate 0.1ml L-Arginine, 0.1ml manganese chloride, 0.1ml 30µg dithiothreitol (DTT), 0.1ml NADPH, 0.1ml tetrahydropterin, 0.1 ml oxygenated haemoglobin and 0.1ml enzyme (sample). An increase in absorbance was recorded at 401nm.

K. Estimation of cellular nitrate

The cellular nitrite level was assessed by the method of Lepoivre [14]. Sodium nitrite solution was used as the standard for this reaction. In this procedure sulphosalicylic acid (0.1 ml) was added to cell lysate (0.5 ml) and was vortexed for 30 minutes. After that, the samples were centrifuged for 15 minutes at 5000 rpm. The supernatant (protein-free) was used for the evaluation of nitrite levels. 30µl of 10% NaOH along with 300µl of Tris-HCl buffer was added to supernatant (200µl). All these were mixed well and 530µl of Griess reagent was added to this mixture. This was then kept for incubation in dark for 10-15 minutes. Absorbance was measured at 540nm against the blank (Griess reagent). From the standard curve obtained the nitrite level was estimated.

L. Gene expression studies

Gene expression studies were carried out using raw macrophage cells (264.7 cells). The expression of NF-kB and COX-2 gene was evaluated. The positive control used was GAPDH. The primers used for the study are depicted in Table 1. RAW 264.7 cells were placed in a 6-well plate. After attaining 70% confluency of cells in 6 well plates (approximately 4 x 10⁵ cells), the cells were then treated with EF18 of concentration 100µg/ml and was incubated for 24 hours. A set of untreated control cells was also incubated at 37°C for 24 hours in a CO₂ incubator. After the incubation period, DMEM media was removed aseptically and 200µl of TRIZOL reagent was added to culture well plate and incubated for 5 minutes [15]. These were then moved to a fresh and sterile eppendorf. To this 200µl of chloroform was added and shaken for 15 seconds. After that it was incubated at room temperature for 2-3 minutes. Then centrifugation was carried out for 15 minutes at 4°C at 14000rpm. The top aqueous layer was collected and 500µl of 100% isopropanol was added to that. This was then incubated at room temperature for 10 minutes and after that centrifuged at 4°C for 15 minutes at 14000rpm. After centrifugation the supernatant was discarded and pellet was taken. Pellet taken was washed using 200µl of 75% ethanol and centrifuged again for 5 minutes at 14000rpm at 4°C in a cooling centrifuge. The RNA pellet was then dried and suspended in a TE buffer. The cDNA synthesis was carried out by using Thermo scientific verso cDNA synthesis kit with the method followed by Joshi [16]. To the RNase free tube 4µl of 5 X cDNA synthesis buffers, dNTP mix (2µl), anchored oligo dT (1µl), RT enhancer (1µl), Verso enzyme mix(1µl) and 5µl of RNA template (1nano gram of total RNA) were added. By the addition of distilled water which was sterile, total reaction volume was made up to 20µl. The solution was then mixed properly by using the technique of aspiration. The thermal cycler was programmed to undergo cDNA synthesis. For cDNA synthesis at one cycle, the temperature was maintained at 42°C, for 30 minutes. The temperature of inactivation for one cycle was maintained for 2 minutes at 95°C. The amplification was carried out using the Thermo scientific amplification kit. Primary denaturation for 3 minutes at 95°C was followed by the denaturation for the 30s at 95°C, annealing at temperature for about 30s and extension at 72°C for 1 minute which was continued for 35 cycles and the final extension was at 72°C for 5 minutes. After the amplification,

the PCR product was detached by Agarose gel electrophoresis. The stained gel was visualized using a gel documentation system of E gel imager, Invitrogen.

TABLE I. Primers used in Agarose Gel Electrophoresis

| Oligo name | Primers used in Agarose Gel Electrophoresis | | | |
|------------|---|------|------------------------------|------|
| | FORWARD | | REVERSE | |
| | SEQUENCE (5' ->3') | Tm | SEQUENCE (3' ->5') | Tm |
| GAPDH | ACTCAGAAGA CTGTGGATGG (20) | 57.3 | GTCATCATACTT GGCAGGTT(20) | 55.3 |
| COX 2 | GGAGAGACTA TCAAGATAGT | 51.0 | ATGGTCAGTAG ACTTTTACA | 48.6 |
| NF- kb | CCCACACTATG GATTTCTACT TATGG | 57.3 | CCAGCAGCATC TTCACGTCTC | 54.8 |

M. Animals

Male Wistar rats about 150g ± 5 were taken for the study. Animals were obtained from the Small animal breeding station, Mannuthi Trissur. Studies were performed according to the regulations of the Institute Animal ethics committee that was approved by the committee for the control purpose and supervision of experimental animals (CPCSEA) Reg: number: B11042016-7/MGU/SBS/IAEC/11-04-2016.

N. Carrageenan induced anti-inflammatory study

Carrageenan induced study was evaluated using a standard procedure [17]. Dried EF18 fraction was given orally to the rats. The standard drug used for the study was Diclofenac sodium (4mg/ml). The dried EF18 fraction was given one hour before the carrageenan induction. 0.1ml of 1% carrageenan in saline was induced sub plantar. The animals were divided into 5 groups of 6 animals each. Group I serves as the normal. Group II includes carrageenan-induced animals treated with EF18 lower dose (250mg/kg), Group III includes carrageenan-induced animals treated with a higher dose of EF18 (500mg/kg), Group IV carrageenan-induced animals treated with diclofenac sodium and Group V as toxic control group (carrageenan induced only).

Paw volume was measured before carrageenan induction and for the 1st, 3rd and 5th hour of injection. Percentage inhibition was evaluated. After 5 hours the animals were sacrificed. Parameters analyzed include ESR, Erythrocyte, Leukocytes, and Platelets. Serum biochemical parameters were assessed. Histopathological analysis of tissue samples was performed.

O. Histopathological analysis

Paw tissue specimens from normal and other treated groups were removed and fixed in 10% formalin. For histopathological analysis, the paraffin-embedded paw tissue sections were stained with Hematoxylin-eosin (H&E) followed by the examination and photographed under the light microscope for observation of structural abnormality. [18]

III. RESULTS

A. HR-LCMS analysis of EF18

HR-LCMS analysis revealed the presence of various bioactive compounds in the EF18. Out of the different bioactive compounds obtained Gallic acid and Dihydromyricetin exhibited anti-inflammatory activity. Preliminary phytochemical screening studies of EF18 fraction revealed the presence of various secondary metabolites like alkaloids, flavonoids, tannins, saponins, and terpenoids in it. Phytochemicals exhibited the ability to impede the regulation of inflammatory mediators that have been considered as the impending aspirants for anti-inflammatory drugs. HR-LCMS analysis of EF18 revealed the presence of Gallic acid and Dihydromyricetin as shown in Fig 1, Fig 2 and 3. Both of them exhibited anti-inflammatory properties [19]. Gallic acid has a role in the induction of apoptosis and also acts as a potent antioxidant [20][21]. Dihydromyricetin also known as ampelopsin used as novel anti-alcohol intoxicant [22].

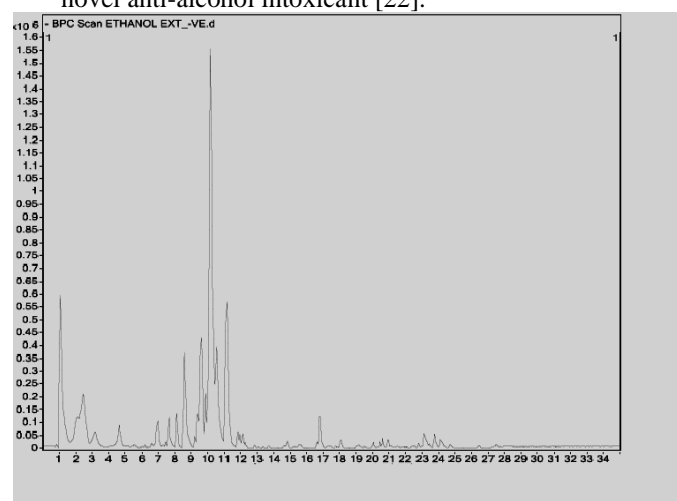


Fig. 1. Total ion chromatogram of EF18

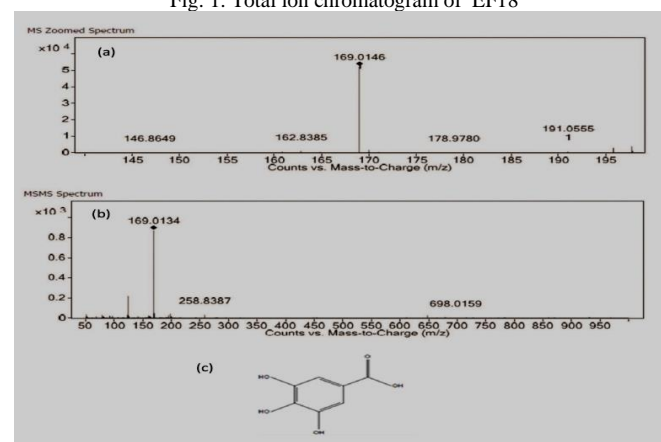


Fig. 2. a) LCMS spectrum of Gallic acid from EF18; b) MSMS spectrum of Gallic acid from EF18; c) Structure of Gallic acid

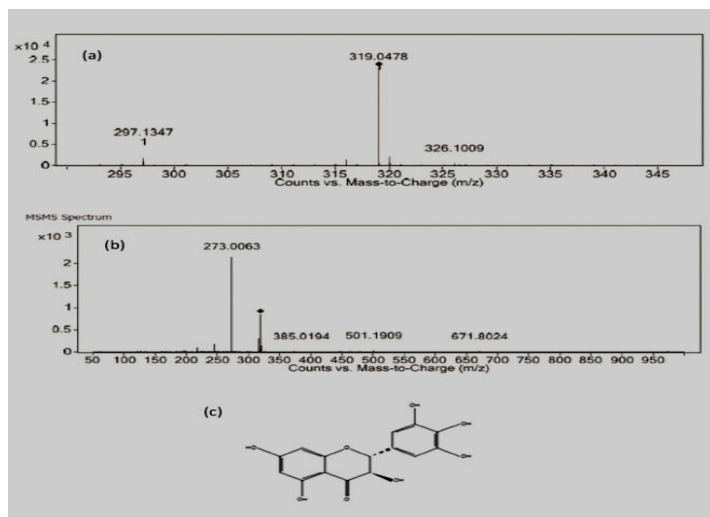


Fig: 3. a) LCMS spectrum of Dihydromyricetin from EF18; b) MSMS spectrum of Dihydromyricetin from EF18; c) Structure of Dihydromyricetin

B. Evaluation of cell viability by MTT assay

The effect of EF18 on cell viability was evaluated using MTT assay and the results revealed that EF18 at a concentration of 100µg/ml showed a cell viability of 80.7%. This demonstrated that EF18 was not at all cytotoxic up to the concentration of 100µg/ml. Therefore the sample concentrations of 25,50 and 100µg/ml were taken for further in-vitro studies (Fig: 4). The outcome of the MTT assay showed that RAW 264.7 cells treated with EF18 did not cause any cytotoxicity up to the concentration of 100µg/ml. So this concentration of EF18 was taken for further studies.

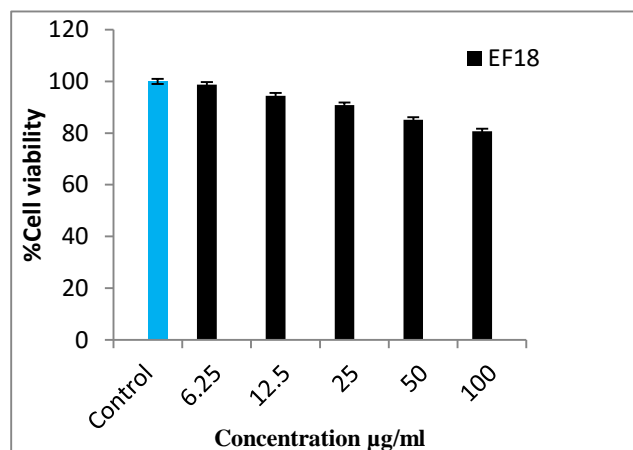


Fig 4. Showing the effect of EF18 on the RAW 264.7 cells by MTT assay. Values are expressed in terms of mean ±SD (n=3).

C. Inhibitory effect of EF18 on total Cyclooxygenase (COX) activity

RAW 264.7 cell treatment with LPS showed an increased level of total COX activity and this was decreased on treatment with EF18. It was observed that as the concentration of EF18 was increased the percentage inhibition also increased simultaneously. The percentage inhibition of COX activity by EF18 occurred in a dose-dependent manner. In the study, EF18 showed total COX inhibition of 61.59% at 100µg/mL

concentration in RAW 264.7 cells which is depicted in Fig.5.

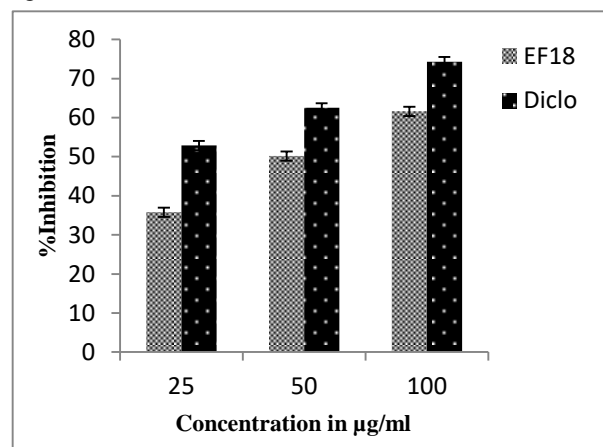


Fig.5. Effect of EF18 on cyclooxygenase enzyme activity in RAW 264.7 cells.

EF18: *Eugenia uniflora* active fraction and Diclo: Diclofenac sodium. Values are expressed in terms of mean ±SD (n=3).

D. Inhibitory effect of EF18 on Lipoxygenase (5- LOX) activity

The treatment of RAW 264.7 cell with LPS (1µg/mL) was performed prior to the EF18 treatment at various concentrations (25, 50 and 100µg/mL). LPS treated cells showed an increased level of 5- LOX activity and this was decreased upon the treatment with different doses of EF18. It is evident from the study that EF18 at 100µg/mL concentration showed 5- LOX inhibition of about 72.23%. It was observed that EF18 treatment causes 5- LOX activity inhibition in a dose-dependent manner. The results are shown in Fig.6.

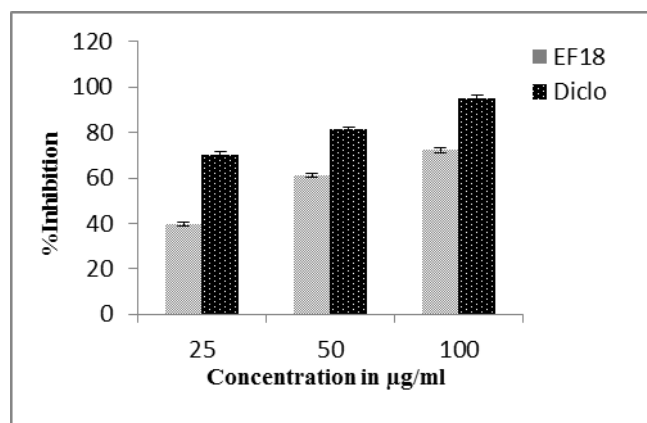


Fig. 6: Effect of EF18 on 5- LOX in RAW 264.7 cells.

EF18: *Eugenia uniflora* active fraction and Diclo: Diclofenac sodium. Values are expressed in terms of mean ±SD (n=3).

E. Effect of EF18 on Myeloperoxidase (MPO) enzyme activity

The effect of EF18 on Myeloperoxidase enzyme activity was assessed. The treatment of RAW 264.7 cells with LPS (1µg/mL) was carried out before the treatment with EF18 and diclofenac sodium at concentrations 25, 50 and 100µg/mL. The myeloperoxidase activity was found

significantly reduced on treatment with 100µg/mL concentration of EF18. The MPO level on treatment with EF18 was compared with that of standard diclofenac sodium. It was noted from the study that as the concentration of EF18 increases the myeloperoxidase activity decreases. The results are shown in Fig.7.

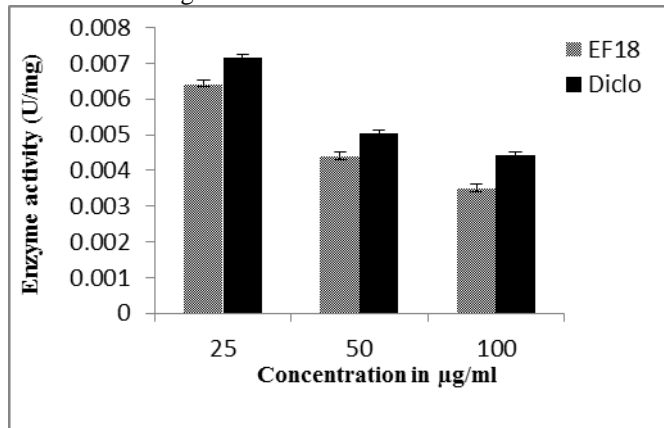


Fig. 7: Effect of EF18 on MPO activity in RAW 264.7 cells

EF18: *Eugenia uniflora* active fraction and Diclo: Diclofenac sodium. Values are expressed in terms of mean ±SD (n=3).

F. Effect of EF18 on inducible nitric oxide synthase (iNOS) enzyme

The effect of EF18 on the inducible nitric oxide synthase was evaluated. Treatment of RAW 264.7 cells with LPS enhances the level of iNOS whereas the treatment of these cells with EF18 and Diclofenac sodium reduces the iNOS level. The enzyme activity was reduced significantly on EF18 treatment at a concentration of 100µg/mL when compared to that of Diclofenac sodium. The results are illustrated in Fig.8.

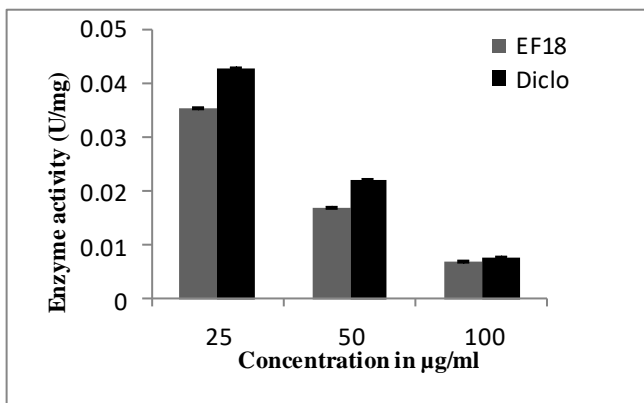


Fig 8. Effect of EF18 on iNOS activity in RAW 264.7 cells.

EF18: *Eugenia uniflora* active fraction and Diclo: Diclofenac sodium. Values are expressed in terms of mean ±SD (n=3).

G. Estimation of cellular nitrate level

The effect of EF18 on cellular nitrate level in RAW 264.7 cells was measured. Results revealed that as the concentration of EF18 and Diclofenac sodium increases the cellular nitrate level decreases. Observations from EF18 treated cells were compared with that of diclofenac sodium treatment. The results were shown in Fig.9.

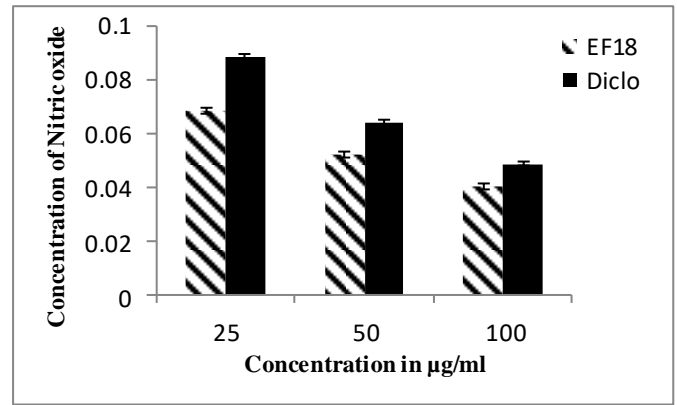


Fig.9: Effect of EF18 on cellular nitrate level in RAW 264.7 cells

EF18: *Eugenia uniflora* active fraction, Diclo: Diclofenac sodium. Values are expressed in terms of mean ±SD (n=3).

H. Gene expression studies

Expression of the NF-kB and COX-2 genes in LPS stimulated raw macrophage cells (264.7 cells) on the treatment of EF18 was assessed. This was achieved by RT-PCR. LPS treatment enhanced the expression of NF-kB and COX-2 while treatment of cells with EF18 down-regulated the expression as shown in Fig.10.

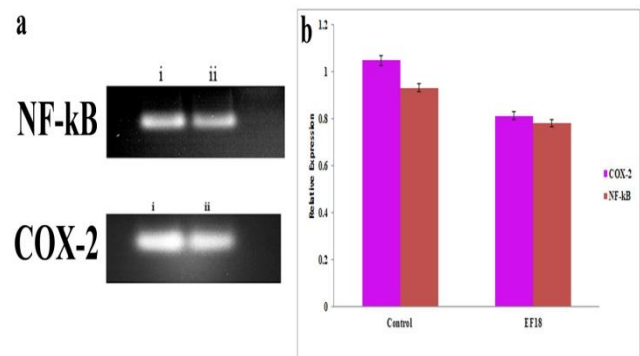


Fig.10: Effect of EF18 on the expression of NF-kB and COX-2.

a) Agarose gel visualization; B: The intensity of the bands. NF-kB is one of the important transcription factors involved in the regulation of proinflammatory mediator synthesis like iNOS, IL- 6 and TNF-α. NF-kB inactivation is considered to be the major target for the reduction of inflammation. EF18 also inhibited the NF-kB that was activated during the LPS stimulation, signifying that it plays a critical role in the downregulation of inflammatory response in LPS signalling as shown in Fig 10.

Treatment of cells with EF18 blocked the NF-kB signal transduction pathways which in turn results in the inhibition of COX enzyme activity [23]. When the cells were treated with EF18 it inhibited the effects due to LPS stimulation. These findings demonstrated that EF18 fraction exhibited anti-inflammatory properties due to the pharmacologically active compounds present in it and this was achieved by the inhibition of pro-inflammatory mediator's synthesis. The EF18 was 100% ethanolic fraction obtained from the partial purification of crude ethanolic extract using column chromatography. The preliminary

phytochemical analysis also revealed that it EF18 fraction of *Eugenia uniflora* exhibited the highest phytochemical content. These phytochemicals are responsible for the pharmacological properties exhibited by *Eugenia uniflora* active fraction (EF18).

I. In vivo carrageenan-induced anti-inflammatory study - Paw volume changes

The effect of EF18 on Carrageenan induced paw edema was evaluated. The paw volume was measured using a vernier caliper just before the Carrageenan induction and after at 1st, 2nd, 3rd, 4th, 5th and 24 hours. The result of the study revealed that EF18 especially it's higher dose had a positive effect on the paw volume of Carrageenan induced groups. The results depicted in TABLE II.

TABLE II. Effect of *Eugenia uniflora* active fraction on Carrageenan induced paw edema

| Time | Normal | Toxic control | EF18 (250mg/kg) | EF18 (500mg/kg) | Diclofenac sodium |
|------|-----------------|------------------|-------------------|-------------------|-------------------|
| 1h | 0.024± 0.03 | 0.044± 0.04* | 0.041± 0.005*# | 0.037± 0.004*# | 0.036± 0.005# |
| 2h | 0.026± 0.002 | 0.048± 0.003* | 0.046± 0.004*# | 0.038± 0.004*# | 0.034± 0.004# |
| 3h | 0.026± 0.002 | 0.056± 0.003* | 0.047± 0.003*# | 0.040± 0.003*# | 0.032± 0.003# |
| 4h | 0.024± 0.001 | 0.056± 0.002* | 0.032± 0.002*# | 0.030± 0.001*# | 0.028± 0.003# |
| 5h | 0.025± 0.001 | 0.054± 0.002* | 0.030± 0.001*# | 0.028± 0.001*# | 0.027± 0.001# |
| 24h | 0.004± 0.001 | 0.054± 0.002* | 0.017± 0.001*# | 0.015± 0.002*# | 0.011± 0.001# |

Values are the mean ± SD (n=6). * denotes p < 0.05 when compared with normal control, #denotes p < 0.05 when compared with toxic control.

J. Effect of EF18 on percentage inhibition of carrageenan-induced paw edema.

The percentage inhibition by EF18 is shown in TABLE III. All the results obtained were compared with that of the standard reference drug and it was found that a higher dose of EF18 showed more inhibition of edema in 1st, 2nd and 3rd hour than the lower dose of EF18.

TABLE III. Effect of EF18 on percentage inhibition of carrageenan induced paw edema

| Groups | Treatment | %inhibition at 1 st h | %inhibition at 3 rd h | %inhibition at 5 th h |
|-----------|-------------------|----------------------------------|----------------------------------|----------------------------------|
| Group II | EF18 (250mg/kg) | 2.44 | 1.086 | 0.466 |
| Group III | EF18 (500mg/kg) | 25.93 | 46.81 | 54.72 |
| Group IV | Diclofenac sodium | 38.59 | 60.2 | 76.8 |
| Group V | Toxic control | 50 | 66.96 | 87.3 |

Values are the mean ± SD (n=6).

K. Effect of EF18 on hematological parameters during carrageenan-induced acute inflammation

Hematological parameters showed a substantial increase during the Carrageenan injection (TABLE IV). A significant dose-dependent decrease was observed in the ESR and leukocyte count in rats treated with EF18 especially its higher dose. The effect of EF18 can be comparable with that of diclofenac sodium.

TABLE IV. Effect of EF18 on hematological parameters during Carrageenan induced acute inflammation

| Parameters | Normal | Toxic control | EF18 (250mg/kg) | EF18 (500mg/kg) | Diclofenac sodium |
|--|-----------------|----------------|-----------------|-----------------|-------------------|
| ESR (mm/hr) | 4.4± 0.082 | 6.9± 0.094 | 6.7± 0.090* | 5.4± 0.092* | 5± 0.086* |
| Erythrocytes (X 10 ⁶ /μL) | 5.510± 0.059 | 7.81± 0.078 | 7.4± 0.075* | 6.36± 0.066* | 6.23± 0.062* |
| Leukocytes (X 10 ³ /μL) | 5.865± 0.039 | 7.5± 0.060 | 7.1± 0.058* | 6.32± 0.047* | 6.1± 0.042* |
| Platelets(X 10 ³ /mm ³) | 816.5± 4.435 | 902.8± 6.32 | 848.3± 5.42* | 836.7± 4.58* | 822.3± 4.42* |

Values are the mean ± SD (n = 6). * denotes p < 0.05 compared with toxic control group.

L. Effect of EF18 on serum biochemical parameters during Carrageenan induced acute inflammation

In the case of liver marker enzymes, there was a significant increase in groups treated with Carrageenan when compared to normal groups (Table V).EF18 higher dose (500mg/kg) and lower dose (250mg/kg) treated groups showed a significant dose-dependent decrease in ALT, AST and in ALP. There was a decrease in the total protein level and it was regained by EF18 treatment. Diclofenac sodium treated groups improved the protein level and restored the level of liver marker enzymes.

TABLE V. Effect of EF18 on serum biochemical parameters during Carrageenan induced acute inflammation

| Parameters | Normal | Toxic control | EF18 (250mg/kg) | EF18 (500mg/kg) | Diclofenac sodium |
|------------|------------------|-------------------|-------------------|------------------|-------------------|
| ALT (U/L) | 76± 2.954 | 103.5± 1.292* | 93± 1.827*# | 85± 1.293*# | 80± 3.365*# |
| AST (U/L) | 220.8± 4.103 | 239.4± 2.37* | 237± 1.826*# | 228± 1.825# | 226.9± 1.891*# |
| ALP (U/I) | 633.5± 2.0833 | 6.46.4± 2.219* | 640± 1.290*# | 637.9± 1.706# | 635± 2.3# |
| TP (g/dl) | 7.7± 0.080 | 6.822± 0.098* | 7.124± 0.094*# | 7.274± 0.07# | 7.6± 1.43# |

Values are the mean ± SD (n = 6). * denotes p < 0.05 compared with normal control group and # denotes p<0.05 when compared with toxic control.

M. Histopathological analysis of Carrageenan induced study

Histopathological analysis of edema tissue was performed (Fig.11). In the case of normal paw tissue, it was free from inflammation or edema and the epidermis and dermis were observed as normal. The results revealed that mild hyperkeratosis and epithelial hyperplasia were found in tissues. Both the Dermis and hypodermis are edematous. In toxic control group hyperkeratosis with epithelial hyperplasia was noted in the epidermis. Hypodermis showed inflammation, edema, and tissue necrosis are absent. In the EF18 lower dose (250mg/kg) treated group mild hyperkeratosis with epithelial hyperplasia was observed in the epidermis. Hypodermis showed signs of slight inflammation. In EF18 higher dose (500mg/kg) treated group mild hyperkeratosis and epithelial hyperplasia was noted in the epidermis. Dermis and hypodermis were edematous. No inflammatory cells infiltration. In the Diclofenac treated group, the hypodermis was free from inflammation.

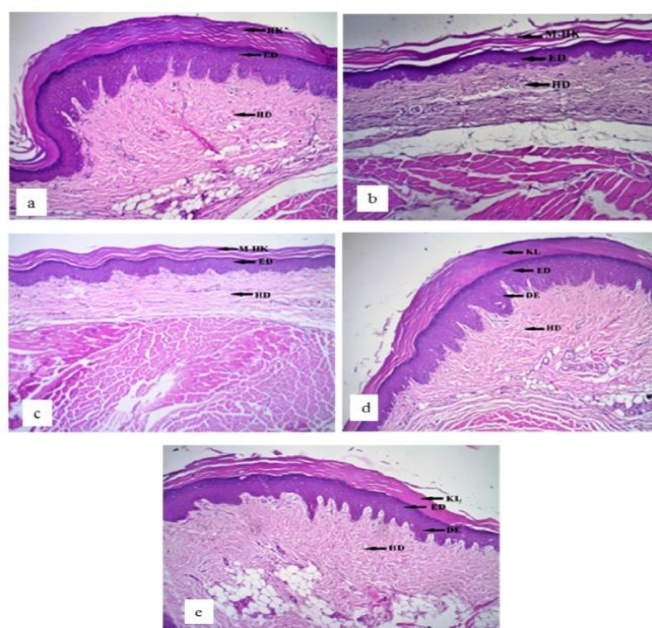


Fig.11.Effect of EF18 on histopathological analysis of Carrageenan induced paw tissue (H& E staining)

a) Carrageenan induced group (Toxic control) (b) Carrageenan +EF18 (250mg/kg) (c) Carrageenan +EF18 (500mg/kg) (d) Diclofenac treated Carrageenan induced group (e) Normal paw tissue.

EH, =Epithelial hyperkeratosis, M.HK=Mild hyperkeratosis, ED= Epidermis, MA=Mitotic activity, HD=Hypodermis, HK=Hyperkeratosis, DE=Dermis, KL=Keratin layer, IN= Inflammation, NC= Necrosis, NP=Nuclear pleomorphism.

IV. DISCUSSION

The present study was based on the *in vitro* and *in vivo* evaluation of the anti-inflammatory property of EF18 which was obtained from the partial purification of crude ethanolic extract of *Eugenia uniflora*. Inflammation is facilitated by a group of chemical mediators that have some specific role in different stages of inflammation [24]. The effect of EF18 fraction on pro-inflammatory mediators like Cyclooxygenase (COX), Lipoygenase (LOX), Myeloperoxidase, Inducible

nitric oxide synthase and Cellular nitrite level was analyzed in the study.

Cyclooxygenase helps in the formation of prostaglandins that have a prominent role in immune function regulation [25]. These prostaglandins act as mediators of inflammation. The result from the present study showed that EF18 had the potential to inhibit COX-2 and reduce the inflammation via blockage of the arachidonic acid pathway of prostaglandin synthesis. LPS, an inducer of pro-inflammatory mediators up-regulate the pro-inflammatory mediator COX-2. The upregulation of these genes was found to be suppressed by the treatment of EF18 at a concentration of 100µg/mL.

Similarly, Lipoygenase enzymes are responsible for the conversion of arachidonic acid into leukotriene [26]. EF18 showed a dose-dependent maximum inhibition of 5-LOX at 100µg/ml. This revealed the role of EF18 in blocking leukotrienes biosynthesis through the 5-LOX activity. EF18 exhibited lipoygenase inhibition activity and this effect may be attributed due to the presence of compounds like Gallic acid and Dihydromyricetin in EF18 fraction.

Myeloperoxidase is a peroxidase enzyme that contains heme and seen abundantly in neutrophils [27]. Myeloperoxidase is an enzyme that is seen in neutrophil, macrophages and in monocytes. Myeloperoxidase forms a complex on reacting with hydrogen peroxide which is formed as a result of the respiratory burst. LPS treatment significantly enhances the MPO activity whereas EF18 treatment significantly inhibited MPO synthesis in a dose-dependent way.

Nitric oxide (NO) which is one of the important cellular signaling molecules is synthesized from L arginine by nitric oxide synthases [28]. The expression of nitric oxide synthases was triggered by LPS treatment. In the current study, the LPS stimulation up-regulated the NO production and iNOS level in RAW 264.7 cells which were down-regulated by the EF18 treatment. This result showed the effect of EF18 in curing the inflammatory mediators.

Treatment of cells with EF18 blocked the NF-kB signal transduction pathways which in turn results in the inhibition of COX enzyme activity [29]. When the cells were treated with EF18 it inhibited the effects due to LPS stimulation.

In vivo, carrageenan-induced edema is mainly used to evaluate the impact of natural products to repel the biochemical changes associated with the acute inflammation [30]. The time period of edema development in Carrageenan induced paw edema model in rats can be represented as a biphasic curve [31]. The first phase of Carrageenan inflammation was mainly due to the pain of the injection and also because of the serotonin and histamine release [32]. Results of the paw volume changes confirmed that EF18 exhibited anti-inflammatory activity in a dose-dependent manner. EF18 restored the paw volume that undergoes variation due to the injection of Carrageenan to the normal range. In the current study, there was no significant percentage inhibition in the 1st hour of Carrageenan injection. Percentage inhibition obtained for EF18 lower dose (250mg/kg) was 25.93% and for EF18 higher dose (500mg/kg) was 38.59%. So it can be concluded that there was substantial inhibition for serotonin and histamine. Diclofenac sodium exhibited a percentage inhibition of about

50% at 1st hour. In rats the carrageenan-induced paw edema model is sensitive to the cyclooxygenases enzyme inhibitors. Therefore this method has been used mainly to assess the anti-inflammatory activity of non-steroidal anti-inflammatory agents as these cyclooxygenases were mainly involved in prostaglandin synthesis inhibition [33]. The EF18 holds a major role in the development of secondary phase of inflammation which was measured at the 3rd hour. The percentage inhibition values acquired by EF18 higher dose and lower dose at the 3rd hour was 46.81% and 60.2% and that of Diclofenac sodium 66.96% respectively. At the 5th hour higher dose of EF18 exhibited a percentage inhibition of 76.8% and for lower dose the percentage inhibition was 54.72%. Diclofenac sodium displayed a percentage inhibition of 87.3%. A significant $p < 0.05$ percentage inhibition of the paw edema was displayed by the higher dose and lower dose of EF18. Hence it can be concluded that EF18 holds an inhibitory effect against the carrageenan-induced inflammatory models and the effect was due to the inhibition of prostaglandin synthesis by cyclooxygenases. The higher dose of EF18 was superior to the lower dose on the basis of the results obtained and the values obtained by the higher dose can be compared with that of Diclofenac sodium.

Results of the hematological analysis of Carrageenan induced rats showed an increased level of ESR, and leukocytes during the Carrageenan injection was decreased to the normal level by the treatment of EF18. The result also points out that EF18 exhibited a protective role during Carrageenan induced inflammation.

EF18 higher dose treatment restored the level of platelet and erythrocyte to the normal range. The values obtained by the higher dose can be compared with that of the Diclofenac sodium. EF18 employs its effects by the suppression of leukocytes towards the area where the inflammation was formed. The reticuloendothelial system was also stabilized by the EF18 [34]. On the treatment of EF18 on the Carrageenan induced animal groups, the level of serum marker enzymes transaminases and phosphatases were restored to the normal level. This may be because of the membrane-stabilizing property of EF18. Since increased nitric oxide production results in chronic inflammation [35]. Present study revealed that EF18 can regulate the synthesis of excess nitric oxide thereby reducing inflammation.

Histopathological analysis of paw tissue revealed improvements in the paw histology of EF18 treated carrageenan-induced groups. An increase in the paw volume which was associated with the infiltration of neutrophils was inhibited [36]. There was no sign of inflammation in paw tissue, edema and necrosis were absent in EF18 higher dose treated group when compared to toxic control group. In EF18 lower dose treated group hypodermis showed signs of slight inflammation in paw tissue. In Diclofenac sodium treated group the hypodermis appeared to be normal as there was no evidence of inflammation or necrosis in paw tissue. It can be suggested that EF18 acts in a dose-dependent way. As the dosage of the fraction was increased the anti-inflammatory activity was also enhanced.

These findings demonstrated that EF18 fraction exhibited *in vitro* and *in vivo* anti-inflammatory properties due to the pharmacologically active compounds present in it and which was achieved by the inhibition of pro-inflammatory mediators synthesis. The EF18 was 100% ethanolic fraction obtained from the partial purification of crude ethanolic extract using column chromatography. The preliminary phytochemical analysis also revealed that EF18 fraction of *Eugenia uniflora* exhibited the highest phytochemical content. These phytochemicals are responsible for the pharmacological properties exhibited by *Eugenia uniflora* active fraction (EF18). From the above context it is clear that *Eugenia uniflora* active fraction (EF18) exhibited potent anti-inflammatory activity and can be a source of anti-inflammatory drug in pharmaceutical industry.

Further studies are needed to determine the exact mechanism of the Gallic acid and Dihydromyricetin present in EF18 fraction in regulating inflammation. In this direction, studies are leading in our laboratory

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