

# Anti-inflammatory Effect of Squalene Isolated from *Simarouba glauca* in Experimental Animal Model

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## ABSTRACT

**Background:** Most dynamic area of scientific investigation is to identify the bioactive compounds and establish their potential health effects against chronic diseases. Multi-targeted compounds with fewer side effects has shown to be potential therapeutic agents. **Objectives:** To evaluate the anti-inflammatory effect of isolated Squalene (SQ), a triterpenoid from *Simarouba glauca* in carrageenan-induced acute inflammation. **Materials and Methods:** Squalene (SQ), a triterpenoid fraction was isolated from *Simarouba glauca* and characterized by FT-IR and NMR. Experimental animals were categorized into five groups, group I was control (0.5% DMSO in normal saline), group II received Carrageenan (Carr), group III received SQ and Carr, whereas group IV received diclofenac and Carr. After 1 hr, SQ and diclofenac (20mg/kg) were administered (i.p.). Carrageenan suspension was injected into the sub-plantar tissue of the right hind paw. Paw edema was determined 3 hours post-carrageenan administration. Rats were sacrificed and mRNA expression of TNF- $\alpha$  and IL-6, levels of PGE-2 and TBARS, activities of COX-2, SOD, catalase, GPx, MPO, and the level of nitrite were measured. **Results:** SQ at a dose of 5.0 mg/kg body weight was found to be the minimal dose for maximum edema inhibition. Serum levels of TNF- $\alpha$ , IL-6, PGE-2, NO, COX-2 and levels of TBARS and MPO were significantly reduced ( $p < 0.05$ ). Antioxidant markers such as SOD, Catalase and GPx were increased significantly ( $p < 0.05$ ). **Conclusion:** These results suggest the anti-inflammatory properties of SQ and its multi-targeted mechanism of action, meriting its potential therapeutic efficacy in various inflammatory diseases.

**Keywords:** Antioxidants, Inflammation, MPO, PGE2, Squalene.

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## INTRODUCTION

Inflammation is the response of the immune system of the body which is developed in order to encounter harmful stimuli, such as pathogens, damaged cells, or toxic compounds, and thereby revert to homeostasis.<sup>[1]</sup> Therefore, it is said to be a defense mechanism that is vital to health. However, it is a preventive mechanism; prolonged or unregulated acute inflammatory responses may progress into a chronic condition, which is implicated in the pathogenesis of various chronic disorders such as aging, cancers, neuro-inflammation, cardiovascular dysfunction, and other life-threatening and debilitating disorders.<sup>[2]</sup> Inflammation is one of the common mechanisms of many diseases, which is typically characterized by inflammatory cardinal signs including the development of redness, edema, pain, hyperthermia heat, and loss of function at the injured site.<sup>[3]</sup> Apart from these signs, several

other microcirculatory events were found to be allied with the development of inflammatory responses involving the alteration of leukocyte infiltration, vascular permeability, and excessive release of free radicals and pro-inflammatory mediators. Various studies demonstrated that the generation of oxidative stress is a key element for instigating many inflammatory ailments. Inflammation is closely associated with the over-production of Reactive Oxygen Species (ROS), especially from mitochondria mediates the activation of signaling molecules, which is responsible for the production of inflammatory cytokines<sup>[4]</sup> thereby it activates endothelial cells to express adhesion and chemo-attract molecules to the inflammatory foci. Furthermore, upon an inflammatory stimulus, there is a depletion of the cellular antioxidant system and induces lipid peroxidation by damaging cellular lipids and perturbing the imbalance of ROS status.<sup>[5]</sup>

In spite of the importance of controlling inflammation, the currently prescribed anti-inflammatory drugs including steroidal and non-steroidal drugs present many side effects such as hepato-renal injury and gastrointestinal complications that limit their clinical use.<sup>[6]</sup> Therefore, it is still necessary to develop novel



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anti-inflammatories with minimum side effects and maximum efficiency is necessary. Therefore, the need for a drug with less or no side effects as well as more efficient is much more necessary day by day. This leads to more demand for herbal products. Many phytochemicals and their active components are claimed to have potent anti-inflammatory effects on many chronic inflammatory diseases.<sup>[7]</sup>

*Simarouba glauca*, widely known as Lakshmi Taru in India belonging to the family of Simaroubaceae, is a plant of medicinal wonders. It is otherwise known as Pitomba, Simaba, Bitterwood, Aceituno, etc.,<sup>[8]</sup> Leaves, Bark extract, roots, seeds, and fruit pulp of this plant are effectively used as antimicrobial, antiviral, astringent, antioxidant, and vermifuge agents.<sup>[9]</sup> *Simarouba glauca* is a treasure chamber of many biologically significant compounds like alkaloids, tannins, flavonoids, and phenolic compounds which attributes to its various health benefits. Among the various phytochemicals 'quassinoids' like ailanthinone, glaucarubinone and holacanthone are highly active constituents belonging to the triterpene family.<sup>[10-12]</sup> Studies on this plant concerning its phytochemical constituents and pharmaceutical effects are still going on. The present study aims the isolation of Squalene from *S. glauca* leaves and evaluation of its anti-inflammatory activity on a carrageenan-induced animal model.

## MATERIALS AND METHODS

All biochemicals used in this study were bought from Sigma Chemical Company, St Louis, MO, USA. Commercially available diclofenac (Novartis, Hyderabad, India) was used as the standard anti-inflammatory medicine for the study. Deuterated detergents for NMR were bought from Merck and other chemicals and detergents used were of logical grade from SRL chemicals, Mumbai, India. <sup>1</sup>H(600 MHz), and <sup>13</sup>C(125 MHz), were recorded CDCl<sub>3</sub> by using a Bruker AMX 500 spectrometer (Bruker Avance II 500) with TMS as the internal standard.

### Collection of plant material

Fresh plant leaves were collected from the Institute premises and authenticated by Prof. K. Shankara Rao, curator, herbarium JCB, Indian Institute of Science (IISc), Bangalore, India.

### Extraction and isolation of Squalene

Dried and powdered leaves of *Simarouba glauca*, (5 kg) was extracted with Hexane and Methanol (MeOH). Successively, MeOH extract was fractionated over silica gel 60 (70-230 mesh) into three fractions: CH<sub>2</sub>Cl<sub>2</sub>, ethyl acetate, and MeOH. The methanol fraction (82 g) was dissolved in H<sub>2</sub>O-MeOH (3:7) and successively partitioned using solvents of increasing polarity (hexane, CH<sub>2</sub>Cl<sub>2</sub>, and EtOAc). The CH<sub>2</sub>Cl<sub>2</sub> sub fraction (2 g) was chromatographed over silica gel (230-400 mesh) using an eluent CH<sub>2</sub>Cl<sub>2</sub>-EtOAc (9:1 and gradient) to furnish 7 fractions (F1-F7).

Squalene (20 mg) was obtained from the F2 fraction. The presence was confirmed by NMR analysis.

Squalene as a Colourless, liquid oil (20mg): R<sub>f</sub> = 0.85 (20% ethyl acetate: chloroform). <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): δ 5.09-5.15 (6H,=CH), 1.60 (18H, allylic CH<sub>3</sub>, cis), 1.68 (6H, allylic CH<sub>3</sub>, trans), 1.96-2.10 (20H, allylic CH<sub>2</sub>). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 135.1, 134.9, (C-6, C-10), 131.2 (C-2), 124.4, 124.30, 124.27 (C-3, C-7, C-11), 39.72, 39.75 (C-5, C-9), 28.3 (C-12), 26.8, 26.7 (C-4, C-8), 25.7 (C-1), 17.7 (C-2'), 16.02, 15.98 (C-6', C-10').

### FT-IR analysis

To characterize the functional group present in the extracts, Thermofisher Scientific's Fourier Transform Infrared spectroscopy model Nicolet iS50 FT-IR operating in transmission mode at a resolution of 4 (0.482 cm<sup>-1</sup>) was used. The liquid sample



Chemical structure of triterpenoid-Squalene (C<sub>30</sub>H<sub>50</sub>)

was directly placed on the ATR (Attenuated Total Reflectance) module.

### Animal Experiments

Adult male wistar rats (weighing 150 ± 10g) bred in the host department animal facility were used for this study. They were kept in a controlled environment for temperature (24-26°C), humidity (55-60%), and photoperiod (12:12 hr light-dark cycle). A commercial laboratory-balanced diet (Amrut laboratory Animal feeds, Maharashtra, India) and filtered and purified water was provided *ad libitum*. All animals received humane care, in agreement with the host institutional animal ethics guidelines. All experiments were conducted as per the guidelines of the animal ethics committee CPCSEA (Registration No. CAF/446/2015) according to the Government of India's accepted principles for laboratory animal use and care.

### Dose-response and toxicity studies

A dose-response study for the anti-inflammatory activity of Squalene against carrageenan-induced paw edema model was carried out in the range of 1-10 mg/kg Body Weight (BW). From this, a dose of 5 mg /kg BW was found to be the minimal concentration possessing maximal edemainhibition. A toxicity curve for Squalene was carried out in the dose range of 1-100 mg/kg body weight and toxicity parameters like GOT, GPT, and LDH were analyzed. Squalene was found to exhibit no toxicity effect up to 100mg/kg bwt (data not shown). Hence the minimal effective

dose of 5mg/kg body weight was selected for further evaluation of the anti-inflammatory effect of Squalene.

### Treatment protocol and experimental design for acute inflammation

Anti-inflammatory activity was measured using carrageenan-induced rat paw edema assay.<sup>[13]</sup> The male wistar rats were randomly divided into five groups with six rats in each group.

Group 1: Control (0.5% DMSO in normal saline),

Group 2: Carrageenan, Carr

Group 3: Carrageenan + Squalene(5 mg/kg bwt), Carr + SQ

Group 4: Carrageenan + Diclofenac (20 mg/kg bwt), Carr + Dic

The diclofenac dose (20mg/kg BW) used for the current study has been previously used in our laboratory as a standard anti-inflammatory drug. 20 mg/kg bwt of diclofenac was found to significantly induce paw edema inhibition, with very little toxic effects in rat.<sup>[14]</sup> Squalene and standard drug diclofenac were given intraperitoneally, followed by injection of 1% carrageenan suspension in 0.9% NaCl solution after 1 hr into the sub-plantar tissue of the right hind paw. Paw volume was measured at hourly intervals for 3hr by using a paw edema meter (Marsap Pvt. Ltd., USA). Anti-inflammatory activity was measured as the percentage reduction in edema level when the extract was treated, as compared to the control.

$$\text{Activity} = 100 - (100 \times \text{average of drug-treated} / \text{average for control}).$$

At the end of the third hour, animals were sacrificed by euthanasia. Blood and paw tissue were collected for various biochemical analyses. Paws from experimental rats were excised above the ankle, degloved, and snap-frozen in liquid nitrogen. Frozen paws were pulverized in a liquid nitrogen bath and divided into aliquots for extraction and analysis of COX-2, MPO, TBARS, and cytokine mRNAs. Samples were processed immediately or stored at -70°C for use within 24 hr.

### Myeloperoxidase (MPO) activity assay

The neutrophil marker enzyme, Myeloperoxidase (MPO), was measured using the method of Bradley *et al.* 1982.<sup>[15]</sup> All the paw tissue was homogenized in 50 mM K<sub>2</sub>HPO<sub>4</sub> buffer (pH 6.0) containing 0.5% Hexadecyl Trimethylammonium Bromide (HTBA) using a homogenizer. After freeze-thawing three times, the samples were centrifuged at 2500 x g for 30 min at 4°C to obtain the supernatant. MPO activity was measured by mixing 40 µL of supernatant with 960 µL of 50 mM phosphate buffer pH 6, containing 0.167 mg ml<sup>-1</sup> O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured spectrophotometrically. One unit of MPO

activity was defined as that amount of enzyme degrading 1 µmol of peroxide per minute at 25°C and activity is expressed as units per mg of tissue.

### Superoxide dismutase activity assay

SOD activity was measured according to the method of Kakkar *et al.*, 1984.<sup>[16]</sup> The assay mixture contained 0.1 mL of supernatant, 1.2 mL of sodium pyrophosphate buffer (pH 8.3; 0.052 M), 0.1 mL of phenazine methosulphate (186 µM), 0.3 mL of nitroblue tetrazolium (300 µM) and 0.2 mL of NADH (750 µM). The reaction was started by the addition of NADH. After incubation at 30°C for 90 sec, the reaction was stopped by the addition of 0.1 mL of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0 mL of *n*-butanol. The colour intensity of the chromogen in the butanol layer was measured spectrophotometrically at 560 nm. One unit of enzyme activity was defined as that amount of enzyme which caused 50% inhibition of NBT reduction/mg protein.

### Catalase activity assay

CAT activity was measured by the method of Aebi, 1984.<sup>[17]</sup> An aliquot (5 µL) of each tissue supernatant was added to a cuvette containing 1.995 mL of 50 mM phosphate buffer (pH 7.0). The reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H<sub>2</sub>O<sub>2</sub>. The rate of decomposition of H<sub>2</sub>O<sub>2</sub> was measured spectrophotometrically at 240 nm.

### Glutathione peroxidase assay

Paw tissue GPx was assayed in a 1 mL cuvette containing 0.890 mL of 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN<sub>3</sub>, 0.2 mM NADPH, 1 U/mL GSH reductase, and 1 mM GSH. 10 µL of each paw tissue homogenate was added to make a total volume of 0.9 mL. The reaction was initiated by the addition of 100 µL of 2.5 mM H<sub>2</sub>O<sub>2</sub>, and the conversion of NADPH to NADP<sup>+</sup> was monitored spectrophotometrically at 340 nm for 3 min. GPx activity was expressed as nmoles of NADPH oxidized to NADP<sup>+</sup>/min/mg protein, using a molar extinction coefficient of 6.22 × 10<sup>6</sup> (cm<sup>-1</sup>M<sup>-1</sup>) for NADPH.<sup>[18]</sup>

### Determination Measurement of Thiobarbituric Acid Reactive Substance (TBARS)

TBARS levels were measured by the double heating method.<sup>[19]</sup> The method is based on spectrophotometric measurement of the purple color generated by the reaction of Thiobarbituric Acid (TBA) with MDA. Briefly, 0.5 mL of paw tissue homogenate was mixed with 2.5 mL of trichloroacetic acid (TCA, 10%, w/v) solution followed by boiling in a water bath for 15 min. After cooling to room temperature, the samples were centrifuged at 3000 rpm for 10 min and 2 mL of each sample supernatant was transferred to a test tube containing 1 mL of TBA solution (0.67%, w/v). Each tube was then placed in a boiling water bath

**Table 1: Sequences of primers used for RT-PCR.**

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
IL-6	TGGTCCTTAGCCACTCCTTC	CCACTGCCTTCCCTACTTCA
TNF- $\alpha$	GACTCCAAAGTAGACCTGCCC	GTCGTAAACCACCAAGC
GAPDH	ATCCCATCACCATCTTCCAG	CCTGCTTCACCACCTTCTTG

for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm with respect to the blank solution.

### Determination of Cyclooxygenase-2 (COX-2) activity in paw

COX-2 activity was checked using a colorimetric assay kit purchased from Cayman Chemicals, USA. COX-2 activity assay utilizes the peroxidase component of cyclooxygenases. Peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N, N, N', N'- tetramethyl-p-phenylenediamine (TMPD) at 590 nm. Briefly, the paw tissue after the experimental period was dissected and rinsed with Tris buffer (pH 7.4) to remove any red blood cells and clots. Tissue was homogenized in cold 0.1 M Tris-HCl (pH 7.8) containing 1mM EDTA and spun at 10,000g for 15 min at 4°C. The supernatant obtained was used for COX assay. A COX-1-specific inhibitor, SC560 was used in the assay to eliminate non-COX-2 activity.

### Reverse transcription-polymerase chain reaction

Levels of TNF- $\alpha$  and IL-6 mRNA were measured by semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from paw tissues using an RNA isolation mini kit (Sigma Aldrich, USA) according to the manufacturer's instructions. The sequences of the primers used are shown in Table 1. GAPDH primers were used as an internal control for RNA loading. RT-PCR was performed in an Eppendorf thermocycler using a two-step RT-PCR kit where reverse transcription and DNA amplification was done separately. 2  $\mu$ g of total RNA was used as a template in the first reaction that included dNTPs, oligo (dT), and reverse transcriptase enzyme. The second reaction included appropriate primers, PCR enzyme, cDNA formed in the first step, and dNTPs. PCR conditions were as follows: denaturation at 94°C for 4 min; 35 cycles at 94°C for 30 sec, primer annealing for 30 sec, 72°C for 1 min; and then a final extension for 3 min at 72°C. 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.

### Determination of PGE<sub>2</sub> level

Paw tissues were added to 4 mL/g tissue of lysis solution containing 80% methanol, 20% saline, and 1 mM indomethacin. Tissues were homogenized on ice with a polytron and centrifuged at 5000x g for 15 min. Supernatants were stored at -70°C until

analyzed. PGE<sub>2</sub> level was determined using a PGE<sub>2</sub> EIA kit (Cayman Chemicals, Ann Arbor, MI, USA). The sensitivity of the PGE<sub>2</sub> assay was 15 pg/mL as per the manufacturer's instructions.

### Determination of NO concentrations in serum

NO was measured as its breakdown product of nitrite by using the Griess method.<sup>[20]</sup> In the presence of H<sub>2</sub>O, NO is rapidly converted into nitrite and nitrate. The total production of NO therefore may be determined by measuring the stable NO metabolite nitrite (NO<sub>2</sub><sup>-</sup>). Equal volumes of paw tissue supernatant and Griess reagent (1% sulphanilamide and 0.1% N-[naphthyl]ethylenediamine dihydrochloride: 1:1) were mixed and absorbance measured at 550 nm. The amount of nitrite was calculated from a NaNO<sub>2</sub> standard curve.

### Assay of protein

Protein was assayed by the method of Lowry *et al.*<sup>[21]</sup>

### Statistical Analysis

All statistical calculations were carried out with the Statistical Package for Social Sciences (SPSS) software program (version 11.0 for windows). The values are expressed as the mean  $\pm$  SD. Statistical evaluation was done using the one-way ANOVA and a significant difference was determined using Duncan's test at the level of  $p \leq 0.05$ .

## RESULTS

### NMR analysis

The isolated compound from the extract was identified as squalene which was characterized by <sup>1</sup>H NMR and <sup>13</sup>C NMR.

#### <sup>1</sup>H NMR

All the peaks that appeared in proton NMR were in the aliphatic region, which evidently confirms there are no aromatic units in this compound. The peak that appeared at 3.75, 3.25 ppm indicates the presence of alkaline (double bond unit). A sharp peak that appeared at 3.25 indicates the presence of methyl units. Peaks appeared before 3.0 ppm indicating which contains aliphatic chains. These shreds of evidence show the compound could be squalene.

#### <sup>13</sup>C NMR

Squalene was additionally confirmed by <sup>13</sup>C NMR. The peak that appeared around 39 ppm indicates the presence of the



double-bonded unit. There is no aromatic carbon, which is clearly visible in the  $^{13}\text{C}$  NMR spectrum. All the peaks that appeared in this NMR show the compound should be aliphatic chained. The above result also showed that the compound could be *squalene* (Figure 1).

### FT-IR analysis

FTIR confirms the signature peaks for squalene at 735.57, 1041.7, 1177.11, 1366.28, 1377.01, 1463.46, 2868.38, 2923.85, and 2953.83  $\text{cm}^{-1}$ . As per Figures 3, 4, 5 and 6, the squalene peaks are almost matching with that of plant extract 1 and extract 2. Only a peak at 1044  $\text{cm}^{-1}$  was not found in the compounds isolated from the extracts. The presence of most of the squalene peaks in the extracts proves the presence of squalene in the plant extracts Figure 2.

### Effect of SQ on Antioxidant Enzymes

Activities of SOD, GPx, and CAT in the 3<sup>rd</sup> hr following the intravenous paw injection of carrageenan in rats were decreased significantly compared to the control group, as shown in Figure 7. However, pre-treatments with SQ boosted the antioxidant levels of SOD, GPx, and CAT activities significantly.

### Effect of SQ on MPO Activity

As shown in Figure 8, when compared to the control group, the MPO activity of the carrageenan-control group was significantly increased. However, administration with SQ markedly suppressed MPO activity.

### Effect of SQ on TBARS Level

In the carrageenan group, TBARS level in the carrageenan-induced edema paw remarkably increased compared to the

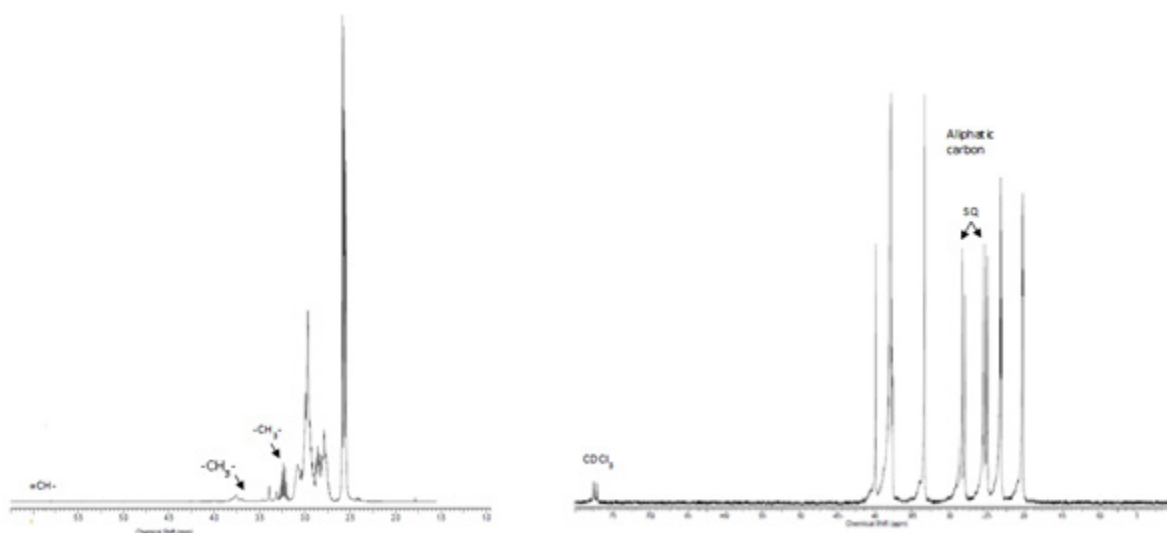


Figure 1: NMR analysis for Squalene.

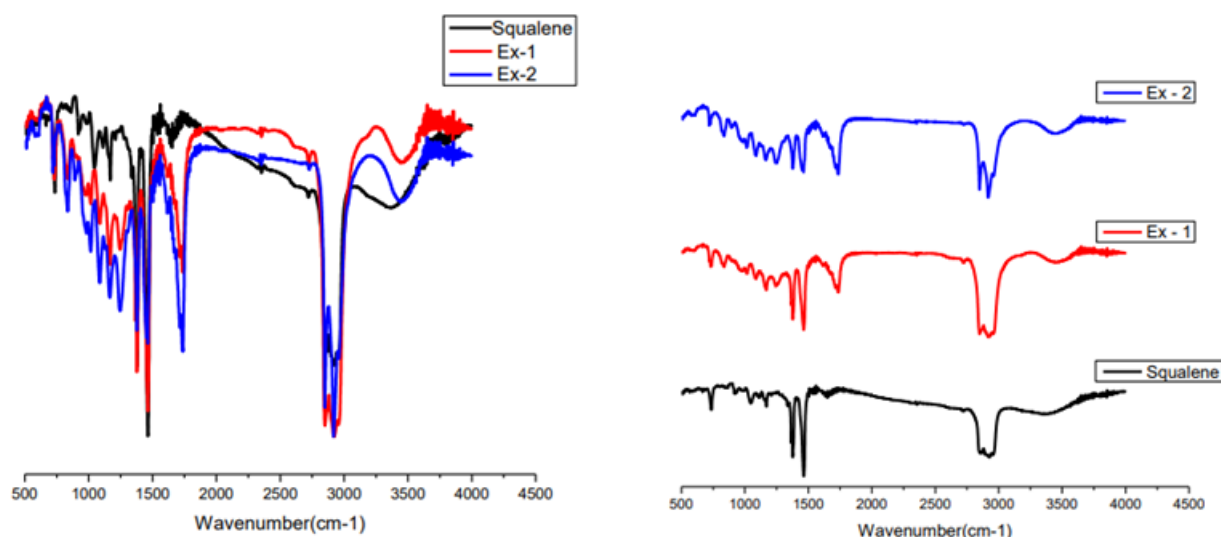


Figure 2: IR spectra of pure squalene and the plant extracts.

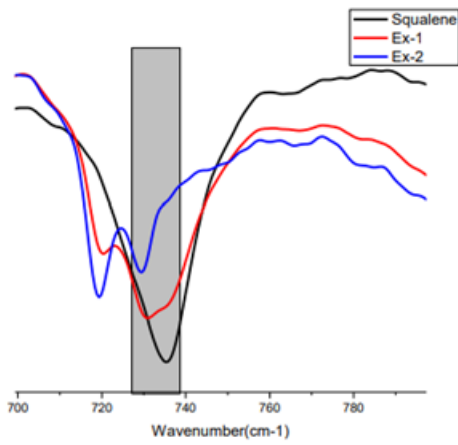


Fig.3

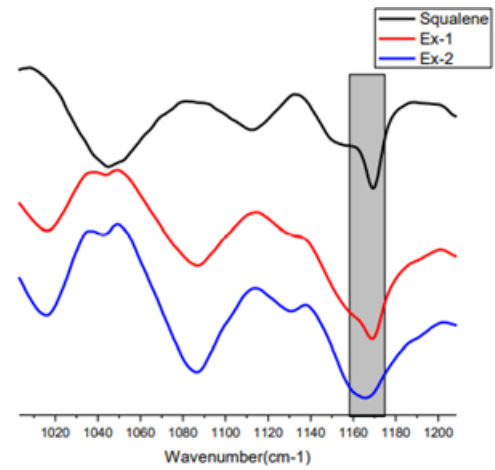


Fig. 4

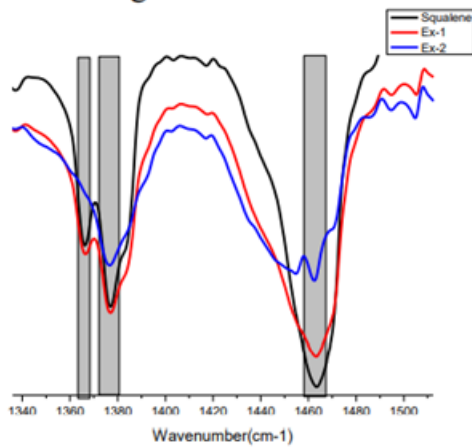


Fig. 5

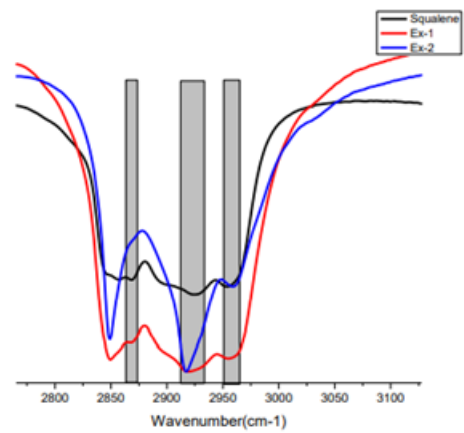


Fig.6

Figure 3-6: Represents the comparisons of the pure squalene and the plant extracts. The data have been processed by origin 8.5 software.

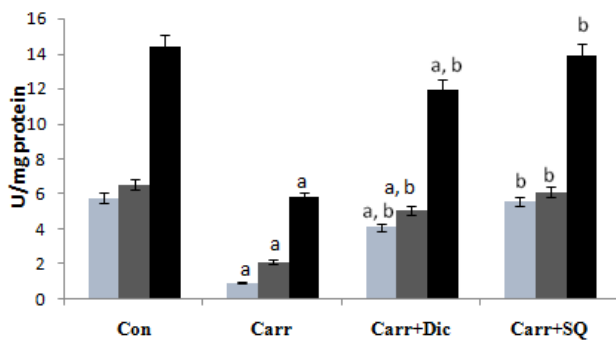


Figure 7: Effect of SQ on the activity of SOD, CAT, and GPx level in Carr-induced rats. The values are expressed as the mean± SD of six rats in each group. a - Statistical difference with the normal control group at  $p < 0.05$ . b - Statistical difference with carr-induced rats at  $p < 0.05$ . SOD: U-enzyme concentration required to inhibit chromogen production by 50% in 1 min. Catalase: U- $\mu\text{mol H}_2\text{O}_2$  decomposed/min. GPx: U- $\mu\text{mol NADPH}$  oxidized/min. Carr-Carr-induced arthritis, DIC-Diclofenac, SQ-Squalene.

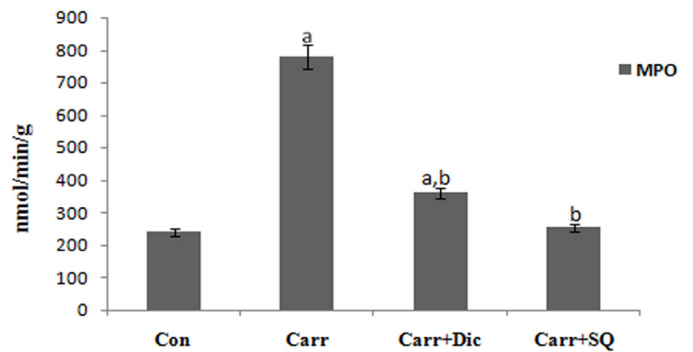


Figure 8: Effect of SQ on the activity of MPO in paw tissue. The values are expressed as the mean± SD of six rats in each group. a - Statistical difference with the normal control group at  $p < 0.05$ . b - Statistical difference with carr-induced rats at  $p < 0.05$ . Carr-Carr-induced arthritis, DIC-Diclofenac, SQ-Squalene.

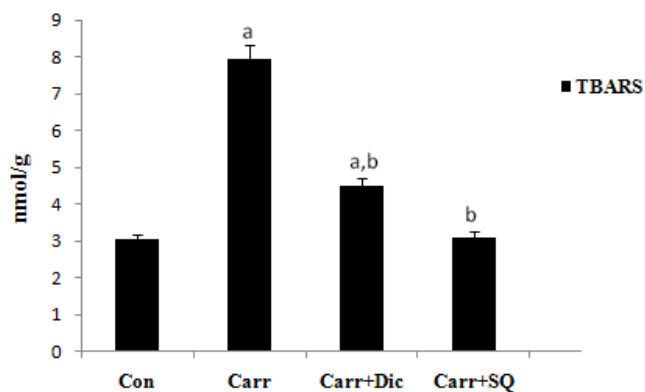
control group. However, the TBARS level decreased significantly in the group treated with SQ as well as the group treated with 20 mg/kg diclofenac (Figure 9).

### Effect of SQ on PGE2 Production and Activity of COX-2

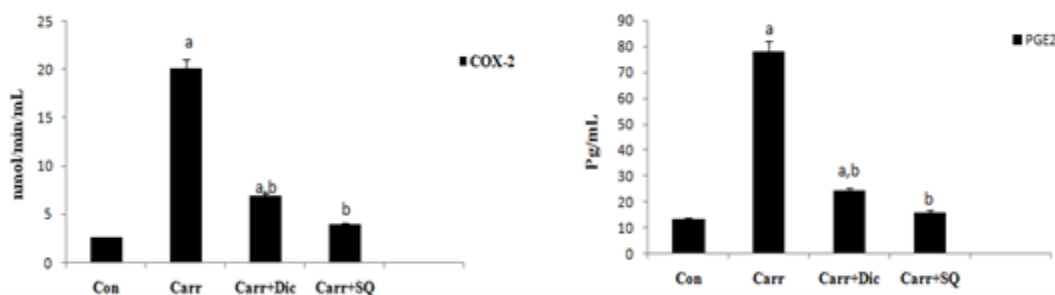
The effect of SQ on the PGE2 production and the COX-2 level was shown in Figure 10. Upon treatment with carrageenan, there were increased levels of PGE2 and COX-2 as compared to the control group. However, the level of inflammatory markers like PGE2 and COX-2 in the SQ administrated groups was significantly suppressed when compared to the carrageenan group.

### Effect of SQ on NO level

Carrageenan intoxicated group of rats evidenced a significant increase ( $p < 0.05$ ) in serum NO when compared with normal control rats. Administration of SQ however significantly attenuated ( $p < 0.05$ ) the serum marker levels toward normalcy (Figure 11). Isolated squalene, a triterpenoid compound offered better protection against inflammation.



**Figure 9:** Effect of SQ on the concentration of TBARS in experimental rats. The values are expressed as the mean  $\pm$  SD of six rats in each group. a - Statistical difference with the normal control group at  $p < 0.05$ . b - Statistical difference with carr-induced rats at  $p < 0.05$ . Carr-Carr-induced arthritis, DIC-Diclofenac, SQ-Squalene.



**Figure 10:** Effect of SQ on COX-2 activity. The values are expressed as the mean  $\pm$  SD of six rats in each group. a - Statistical difference with the normal control group at  $p < 0.05$ . b - Statistical difference with carr-induced rats at  $p < 0.05$ . Carr-Carr-induced arthritis, DIC-Diclofenac, SQ-Squalene.

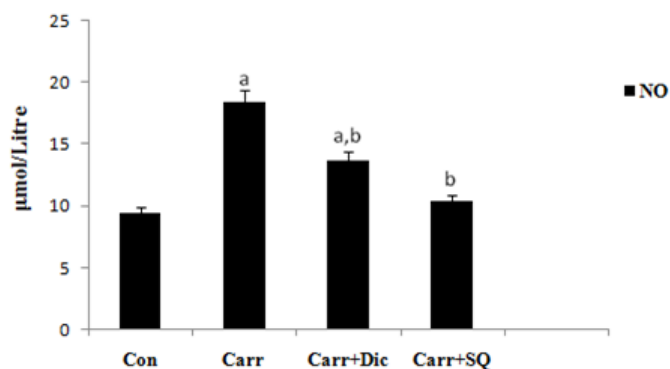
### Effect of SQ on TNF- $\alpha$ and IL-6 Production

When compared with the control, the protein levels of TNF- $\alpha$  and IL-6, and in carrageenan-induced paw edema of the carrageenan-control group were remarkably raised. Administration with SQ had inhibitory effects on TNF- $\alpha$  levels. In addition, SQ down-regulated the protein level of IL-6 as compared to the carrageenan group. The results were shown in Figure 12.

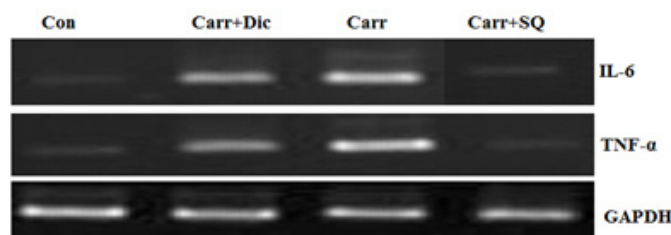
## DISCUSSION

The isolated compound was chemically characterized using NMR and FTIR spectroscopy. NMR analysis gave confirmation that there are no aromatic bonds and that the compound is composed of aliphatic chains like that of standard squalene. Through FTIR analysis it is again established that the isolated compound from the extract possesses most of the major peaks as that of the standard squalene representing the presence of most of the major functional groups in the isolated compound also, proving it is squalene.

It is widely accepted that ROS could also be important both as a triggering signal upon initial tissue injury and within the development of tissue damage. An increased ROS and insufficient antioxidant activity is associated with inflammation. A variety of antioxidants were collectively required for the entire removal of free radicals to guard the body against adverse effects of ROS, generated from inadvertent exposure to carrageenan challenge.<sup>[22]</sup> Assessment of anti-inflammatory activity of natural products from plants using Carrageenan-induced local inflammation (paw edema or pleurisy) in the rat paw is the most common assay and also this method is to evaluate the efficacy of Non-Steroidal Anti-Inflammatory Drugs (NSAID) and also in determining the role of mediators involved in vascular changes associated with acute inflammation.<sup>[23,24]</sup> In the current study, isolated Squalene (SQ), a triterpenoid from *Simarouba glauca* revealed significant anti-inflammatory activity at a dose of 5 mg/kg body weight than the standard drug-treated group. The inflammatory process is characterized by the production of PGs, leukotriene, histamine,



**Figure 11:** Effect of SQ on the concentration of nitrite in experimental rats. The values are expressed as the mean  $\pm$  SD of six rats in each group. a - Statistical difference with the normal control group at  $p < 0.05$ . b - Statistical difference with carr-induced rats at  $p < 0.05$ . Carr- Carr-induced arthritis, DIC-Diclofenac, SQ- Squalene.



**Figure 12:** The inhibitory effect of SQ on the expression of the cytokines like IL-6, and TNF- $\alpha$  was determined by reverse transcriptase-PCR. GAPDH was used as a control. Carr- Carr-induced arthritis, DIC-Diclofenac, SQ-Squalene.

bradykinin, platelet-activating factor, and the release of chemicals from tissues and migrating cells.<sup>[25,26]</sup> The initial phase of inflammation (edema, 0-1 hr) has been attributed to the discharge of histamine, 5-hydroxytryptamine, and bradykinin, followed by a late phase (1-6 hr) mainly sustained by PG production by COX activation in the tissue.<sup>[27]</sup>

Myeloperoxidase (MPO) is considered a hallmark of cell infiltration (mainly neutrophils) in inflammation. During a Carr-induced acute inflammation event, paw tissue loses normal muscle architecture and shows important amassing of infiltrating inflammatory cells and increased inter-fiber space during microscopic observation.<sup>[28]</sup> Our results indicated that SQ reduces inflammation by inhibiting neutrophil recruitment. Hence, SQ-decreased infiltrating inflammatory cells are also demonstrated by the significant decrease in MPO activity.

MDA is one of the most known secondary products of lipid peroxidation, and it is mostly used as a marker of cell membrane injury.<sup>[29]</sup> Additionally, another important biomarker, in the pathogenesis of inflammation, is NO which is produced by inducible nitric oxide synthase during the formation of l-citrulline from l-arginine.<sup>[30]</sup> The present study showed that SQ at a dose of 5 mg/kg was in grade to significantly diminish lipid peroxidation and NO formation.

ROS are the culprit of cellular damage in inflammatory disorders. MDA and NO, produced by lipid peroxidation, are essential oxidative stress markers.<sup>[31]</sup> Antioxidant enzymes can act as a cellular defense mechanism.<sup>[32]</sup> A current study showed that the administration of rats with SQ before carrageenan caused a significant reduction in lipid peroxidation and nitrate levels compared to the carrageenan group while causing a significant elevation in antioxidant enzymes such as SOD, CAT, and GPx during carrageenan induction.

COX-2 is the key enzyme that catalyzes the conversion of arachidonic acid to PGE2 in the biosynthesis of the prostaglandin (PGE2) pathway. It is a very important mediator of all types of inflammation and is responsible for increased prostaglandin production in inflamed tissue. During carrageenan treatment, the liberation of bradykinin, which induces prostaglandin production, is responsible for the formation of inflammatory exudates.<sup>[33]</sup> The present study revealed that SQ significantly inhibited the production of PGE2 and moreover, it was confirmed through the inhibition of the COX-2 activity in the paw tissue. Therefore, SQ has an anti-inflammatory effect by reducing inflammatory mediators such as NO, COX-2, and PGE2 in rat paw tissue.

TNF- $\alpha$  is a major mediator in inflammatory responses, which is capable of inducing innate immune responses by activating T cells and macrophages and can stimulate the secretion of other inflammatory cytokines. TNF- $\alpha$  has been shown to be one of the pro-inflammatory mediators of carrageenan-induced inflammatory reaction and was able to induce a further release of kinins and leukotrienes, with a possible role in the prolongation of a long-lasting nociceptive response. Interleukin-6 (IL-6) is a cytokine that is produced during inflammation and that plays an important role in host defenses to invasive infection.<sup>[34,35]</sup> IL-6 is a multifunction cytokine that is produced by a range of cells, usually at sites of tissue inflammation. IL-6 stimulates the production of most acute phase proteins in inflammatory reactions.<sup>[34]</sup> In this study, SQ markedly suppressed TNF- $\alpha$  and IL-6 expression in paw tissue significantly. This finding has great potential for advocating that an isolated natural triterpenoid-Squalene (SQ) from *Simarouba glauca* produces an anti-inflammatory effect.

## CONCLUSION

Our data demonstrated that isolated SQ from *Simarouba glauca* has potential anti-inflammatory effect in acute inflammatory model system. SQ was shown to inhibit the production of MPO, Lipid peroxidation, COX-2, NO, PGE2, TNF- $\alpha$ , and IL-6 in paw tissue. Hence, isolated SQ, a triterpenoid from *Simarouba glauca* can be considered a powerful inhibitor of inflammation and can be considered as a potential therapeutic agent against a number of inflammatory diseases.



## ANIMAL ETHICS APPROVAL

Animal experiments were conducted and approved as per the guidelines of the animal ethics committee CPCSEA (Registration No: CAF/446/2015) Indian Institute of Science (IISc.) Bangalore, Karnataka, India according to Government of India accepted principles for laboratory animals' use and care.

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## CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**SQ:** Squalene; **i.p.:** Intraperitoneal; **hr:** Hour; **min:** Minute; **sec:** Second; **BW:** Body weight; **Kg:** Kilogram; **mg:** Milligram.

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