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TUMOR NECROSIS FACTOR RECEPTOR 1 (TNFR1) NEUTRALIZATION AMELIORATED CARRAGEENAN-INDUCED INFLAMMATION IN MICE SUPPLEMENTED WITH HERBAL ANTIOXIDANTS

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Keywords:

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ABSTRACT: TNF-α plays pivotal role in initiation of inflammation *via* its interaction with TNFR1 in an $O_2^{\cdot-}$ induced oxidative stress dependent manner. The present study investigated whether the anti-oxidant effect of hydroalcoholic extract of *Clitoria ternatea* (CTE), could facilitate the anti-inflammatory effect of TNFR1 neutralization in context to carrageenan-induced acute inflammation in mice. Sub-plantar injection of λ-carrageenan (0.1% in saline) elicited profound inflammatory reactions in plantar tissue 0-12 hour (h) post carrageenan injection (p.c.i.), as evident from increased PMN infiltration (myeloperoxidase activity), local and systemic release of cytokines and chemokines, free radical generation and oxidative stress in male Swiss albino mice (20 - 22g, 3 - 4 weeks of age). The inflammatory reactions were attenuated from 6 - 12 h pci in CTE (50 mg/kg) and QG (2.5 mg/kg) pre-treated mice, and were potentiated followed by TNFR1 neutralization with anti-TNFR1 antibody (10 μg/mouse) in terms of significant decreases ($p < 0.05$) in PMN infiltration, pro-inflammatory cytokines and chemokines as well as ROS/RNS generation and oxidative stress in parallel to decreased TNFR1 and NF-κB mediated COX-2 and iNOS expression from 4 - 12 h pci. Results suggested that anti-inflammatory effect exerted by CTE or QG were potentiated following antibody mediated TNFR1 neutralization which might become a reliable combinatorial approach to combat inflammatory diseases in future therapeutic research.

INTRODUCTION: Tumor necrosis factor (TNF)-α is traditionally considered proinflammatory cytokine because of its central role in inflammation, it is also an immunoregulatory molecule [1]. TNFα, the key player in initiation and orchestration of inflammation and immunity, is primarily expressed as a transmembrane protein (membrane TNF) that gets processed into a soluble form (sTNF) and exerts its functions *via* TNF receptor 1 (TNFR1) and TNFR2 [2].

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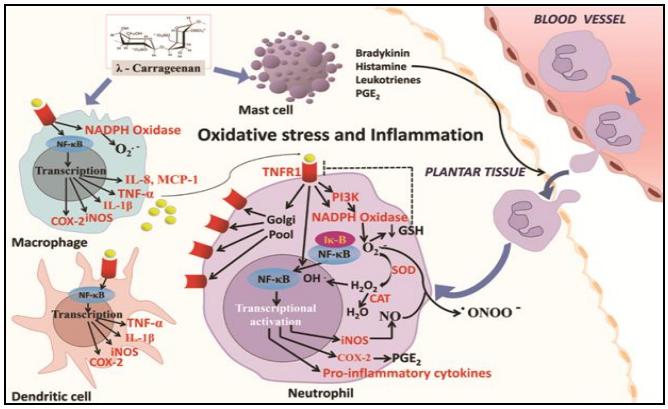
TNFR1 expression is predominantly associated with inflammation and tissue degeneration whereas TNFR2 is mainly implicated in immune modulation and tissue repair [3]. Functional differences between these two receptors in regulating immune system might explain the failure of therapeutic efficiency of TNF inhibitors as therapeutics in the treatment of many disease models [4]. As a consequence, many investigators seek for agents that will target specific TNF-receptors.

There is a limited pattern of cellular expression of TNFR1 and TNFR2 *in-vivo*, and each is highly regulated during inflammation in a wide range of tissues; this is likely to be a result of differences in both the rate of synthesis and shedding of

receptors. The activation of TNFR is associated with oxidative stress during acute inflammatory reactions, and has been linked with the activation of NF-κB mediated transcription of iNOS, COX-2, and other enzymes responsible for generation of reactive oxygen species (ROS) and nitrogen species (RNS) [5]. Previous studies reported that blocking of TNFα/TNFR1 interactions by CAY10500 led to decreased neutrophil (PMN) accumulation [6]. TNFR1 antagonist–neutralizing antibody has also been shown to inhibit apoptotic signals in cells via down-regulation of ASK1, p38, and JNK phosphorylation [7]. Similarly, use of the anti-oxidant N-acetyl-cysteine (NAC) and antagonist anti-TNFR1 antibody also led to reduced cellular ROS [8]. Taken together, those studies illustrated how targeting TNFR1 could be important as a therapeutic during acute inflammation events.

Acute inflammation is characterized by extensive cell infiltration (including PMN) and tissue damage as a result of an increase in synthesis / release of important chemokines and mediators. Activation of a number of different cellular/molecular components of the immune system is associated with generation of ROS and RNS [9] and resulting local / systemic oxidative stress **Scheme 1**.

The role of oxidative stress in tissue degeneration and necrosis, in turn, has been attributed to activation of NADPH oxidases, iNOS, and other enzymes in an inter-linked mechanism [10]. Because ROS/RNS help exaggerate inflammatory damage, this provides a rationale for the use of anti-oxidants in the treatment of inflammatory states or related-disease conditions.



SCHEME 1: ROLE OF TNFα / TNFR1 MEDIATED SIGNALING IN INFLAMMATORY REACTIONS AND OXIDATIVE STRESS IN CARRAGEENAN INDUCED INFLAMMATION IN PLANTAR TISSUE

There is currently a trend for searching of naturally available sources of anti-oxidants and anti-inflammatory agents to replace synthetic ones [11, 12]. It is estimated ≈ 25% of the drugs prescribed worldwide are obtained from plants; this increasing usage might be related to a need to avoid many side-effects associated with conventional synthetic drugs [13]. *Clitoria ternatea* Linn (Family: Fabaceae, commonly known as butterfly pea plant in English; local name is Aparajita) is an evergreen twinning plant which is found in adequate amounts in regions of South-eastern Asia. Besides its potent immunosuppressive, and antioxidant action, decoctions and extracts of *C. ternatea* flower petals has been reported to have potential anti-inflammatory effects [14]. Flower petals of *C. ternatea* Linn are rich source of a huge variety of polyphenols. Three flavonol glycosides, kaempferol-3-β-glucoside, quecetin-3-β-glucoside, and myricetin-3-β-glucoside were isolated from the petals of *C. ternatea*, amongst these particularly quercetin-3β-D-glucosides were identified by NMR studies, and LC/MS studies in highest quantities particularly in methanolic extracts of the *C. ternatea* flower petals [15]. The hydroalcoholic extracts of blue flower petal possess potential anti-oxidant and anti-inflammatory activities, and its disease modifying activities has been correlated to its flavonoid components [16, 17, 18].

Quercetins have emerged as a good therapeutic choice in treatment of inflammatory diseases due to diverse pharmacological effects, *e.g.*, anti-inflammatory, anti-allergic, anti-oxidant, and anti-tumor activities [19, 20]. Although low absorption, extensive metabolism and rapid elimination from body renders the bioavailability of quercetins (aglycone) low, high absorption rate of quercetin glucosides due to presence of water soluble glucose moiety among all other derivatives can be attributed to its bioactivity [21]. Bioactivities of quercetin glucosides include long lasting anti-inflammatory action via inhibition of iNOS, COX and LOX enzyme activities and decreased NF-κB-DNA binding [22].

To better characterize the potential anti-inflammatory effects of CTE and quercetin-3β-D-glucoside (QG), in presence of antibody mediated TNFR1 neutralization the present study have investigated the effects of carrageenan-induced

inflammation in mice pre-treated with hydro-alcoholic extracts *C. ternatea* flower petals (CTE) or QG. Outcomes assessed in treated mice included evaluations of neutrophil infiltration (MPO activity), local and systemic levels of TNFα, interleukins (IL)-1β, -6, -8-, 10, and -12p40, interferon (IFN)-γ, monocyte chemoattractant protein (MCP)-1, and C-reactive protein (CRP), along with measures of local free radical generation ($\bullet O_2^-$ and NO production), redox balance (reduced glutathione [GSH], lipid peroxides [LPO]), an activities of superoxide dismutase (SOD) and catalase (CAT). Local expression of TNFR1, NF-κB, COX-2 and iNOS were also determined. These endpoints were selected as they are parts of the pathways of inflammatory response and oxidative stress associated with TNFR1-mediated (ROS dependent)-pathways involved in carrageenan induced inflammation in the plantar tissues of mice.

MATERIALS AND METHODS:

Animals and Materials: Swiss mice (male, 20 - 22 g, 3 - 4 weeks of age) were obtained from the Chittaranjan National Cancer Institute, Kolkata, India for use here. All mice were housed in separate polystyrene cages in specific pathogen-free facilities maintained at 25 (±2) ºC, with 50-60% relative humidity, and a 12-hr light: dark cycle. All mice had *ad libitum* access to standard rodent diet and filtered tap water. All experiments involving animals were conducted according to the protocols approved by Department of Animal Ethical Committee, Department of Physiology, University of Calcutta [as it is affiliated to the University of Calcutta is called Institutional Animal Ethics Committee (IAEC)], under the supervision of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines (Registration no. IAEC/IV / Proposal / BB-2/2014, dated August 26, 2014), Ministry of Environment and Forest, Government of India.

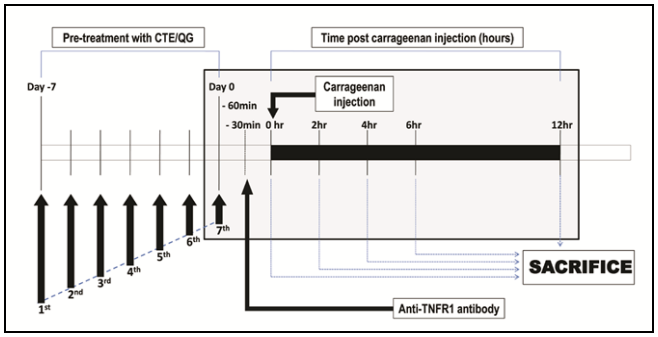
Plant Collection, Extraction, and Acute Toxicity Study: Whole plants of *C. ternatea* were collected from south eastern wild region of West Bengal. Taxonomical identification of the collected plant materials was done by the Central National Herbarium (CNH), Botanical Survey of India (Ministry of Environment, Forest and Climate Chang), Government of India, Shibpur, Howrah and the voucher specimen was deposited, Voucher no. CU/RA/003 (Ref. no. CNH/2017/Tech.II/19). Plant materials were dried under shade, and ten grams of finely ground *C. ternatea* flower petals were soaked into 30 ml methanol at 30 ºC for 12 h with shaking. The methanol was then allowed to evaporate completely under sterile conditions; this extraction protocol of the original materials was repeated thrice. Each final residue was then dissolved into 10 ml methanol and filtered through Whatman no.1 filter paper. Each filtrate was then centrifuged at 2000 rpm for 10 min. Supernatant was collected and air-dried to completeness under sterile conditions.

The final yield was 6.5% for the flower petals of *C. ternatea*. CTE at a dose of 50 mg/kg was used for animal experiments in this study and toxicological safety upon oral administration in animals was confirmed from acute oral toxicity study. The extracts were suspended in sterile saline and a dose regimen of 250, 500, 1000, 2000, 4000, and 8000 mg/kg body weight for CTE was subjected for acute oral toxicity study.

The results showed that dose upto 1000 mg/kg for CTE did not cause death or morbidity for upto 72 hr, and therefore $1/20^{th}$ of this dose, *i.e.*, 50 mg CTE/kg were thus considered as safe for oral administration in this study [23]. Mice were randomly allocated into seven groups (n = 15/group): control (Con), carrageenan (CA) only, CTE + carrageenan (CTE + CA), QG + carrageenan (QG + CA), anti-TNFR1 antibody + carrageenan (CAab), CTE + anti - TNFR1 antibody + carrageenan (CTE + CAab), and QG + anti-TNFR1 antibody + carrageenan (QG + CAab).

Experimental Design: Mice from respective groups were pre-treated with CTE (50 mg/kg) and QG (2.5 mg/kg) by oral gavaging (200 µl) on seven consecutive days before the carrageenan injection; the schedule was adjusted so the final dose was given just 1 hr before carrageenan administration. Control mice were gavaged with saline only. For the latter, 100 µl of 1% (w/v) λ-carrageenan (Sigma, St. Louis, MO) dissolved in normal saline was injected into the sub-plantar region of the right hind leg of all mice and the opposite limb received only sterile saline; controls were injected with saline only in each leg. With the mice in the CAab, CTE + CAab, and QG + CAab groups, 100 µl of

rabbit anti-mouse polyclonal anti - TNFR1 neutralizing antibody (Abcam, Cambridge, MA) dissolved in phosphate-buffered saline (PBS, pH 7.4), to yield a dose of 10 µg/mouse, was intravenously administered 30 min prior to the carrageenan injection (Study design) at 4 ºC. The serum was collected and aliquots were assessed for total protein using the Bradford method [24]; all remaining aliquots were stored at -20 ºC for later analysis. Plantar tissues isolated from each mouse were stored at -20 ºC for later use as well.



SCHEME 2: POSSIBLE MECHANISMS OF ANTI-INFLAMMATORY EFFECT EXERTED BY CTE OR QG FOLLOWING ANTIBODY MEDIATED TNFR1 NEUTRALIZATION

Assessment of MPO Activity in Plantar Tissue: Tissue myeloperoxidase (MPO) activity was analyzed to reflect neutrophil (PMN) infiltration into the plantar tissues [25]. Portions of the isolated tissues (≈ 1 g/mouse) were placed in 1 ml of 20 mM Tris-EDTA-HCl (pH 7.0) buffer supplemented with sucrose and protease inhibitor cocktail and processed using a tissue homogenizer (Royal Scientific, New Delhi, India). The homogenates were then centrifuged at 10000 rpm for 25 min at 4°C and supernatants were then collected. An aliquot of 100 µl was used for protein estimation [24].

Another aliquot (100 µl) of supernatant was then mixed with 2.5 ml of 10 mM potassium phosphate buffer, 100 µl o-dianisidine dihydrochloride (0.167 mg/ml in ethanol), and 10 µl of 0.005% hydrogen peroxide solution (H_2O_2). After incubation at 37 ºC for 5 min in the dark, MPO activity was measured at 405 nm in a UV-1800 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). Activity was expressed as the change in absorbance/min/mg tissue protein.

Assessment of Cytokine and Chemokine Concentrations into the Plantar Tissue and Serum: Portions of the isolated plantar tissues (≈ 1 g/mouse) were lysed in 1 ml lysis buffer (containing 300 mM NaCl, 15 mM Tris [pH 7.4], 2 mM $MgCl_2$, 2 mM Triton X-100, 1 mg pepstatin A/ml, 1 mg leupeptin/ml, and 1 mg aprotinin/ml) and homogenized as above. Thereafter, the sample was centrifuged at 2900 rpm for 15 min at 4 °C; the resulting supernatant was isolated, 100 µl was analyzed for protein content [24], and another 100 µl aliquot was placed at -20 °C until use.

Concentrations of pro-inflammatory cytokines including TNFα, IL-1β, IL-6, IL-12p40, IL-10, and IFNγ, as well as chemokines IL-8 and MCP-1 in the supernatants were then determined using commercial kits (RayBiotech, Norcross, GA), according to manufacturer instructions. The same kits were used to assess cytokine/chemokine levels in serum samples. Levels for each cytokine in a sample were extrapolated from standard curves prepared in parallel. All samples were evaluated using a microplate reader (BioRad, Hercules, CA). All values were reported as pg of cytokine/mg plantar tissue protein in homoge-nate samples or per ml serum. The level of sensitivity (per ml materials placed in kit wells) of the kits was 60 pg TNFα, 5 pg IL-1β, 2 pg IL-6, 2 pg IL-8,45 pg IL-10, 5 pg IL-12, 5 pg IFNγ, and 3 pg MCP-1.

Assessment of CRP Concentration into the Serum and Plantar Tissue: Concentrations of C-reactive protein (CRP) in plantar tissue homogenate and serum were determined using a commercial ELISA kit (Biosource, San Diego, CA) according to manufacturer instructions. Levels of CRP in the same samples analyzed above were extrapolated from a standard curve prepared in parallel. All values were reported as ng CRP/ml serum or ng CRP/mg plantar tissue protein in homogenate. The level of sensitivity of the kit was 2000 ng CRP/ml.

Assessment of Plantar $\bullet O_2^-$ and NO Production: Portions of the isolated plantar tissues (≈ 1 g/mouse) were homogenized in 1 ml ice-cold PBS and homogenized as above. The resulting mixture was centrifuged (12,000 rpm, 30 min, 4 °C), and an aliquot of the resulting supernatant was collected. To determine $\bullet O_2^-$ release, an assay was employed that measured the change in color of cytochrome C (CytC) when reduced by $\bullet O_2^-$. Equal volumes (100 µl) of each aliquot of supernatant were combined with 100 µl of a solution of cytochrome C (2 mg/ml

in PBS) and incubated at 37 °C for 30 min. The reaction was then terminated by placing each tube on ice for 5 min. Total $\bullet O_2^{\cdot -}$ was monitored spectrophotometrically at 550 nm in the UV-1800 spectrophoto-meter against a reference blank that contained the same components except for distilled water in place of sample supernatant. The amount (μM) of $\bullet O_2^-$ production/sample was calculated as mean absorbance at 550 nm × 15.87 [26].

Nitrite concentrations in paw tissues were measured to reflect local NO levels. The same supernatants as above were analyzed for NO production using a modified Griess spectrophoto-metric method as described earlier [27]. Fifty microliters of supernatant was incubated in 40 μM Tris (pH 7.9) containing 40 μM of the reduced form of *β*-nicotinamide adenine dinucleotide phosphate ($NADP^+$), 40 μM flavin adenine dinucleotide (FAD), and 0.05 U/ml of nitrate reductase at 37 °C for 15 min. Reduced samples were incubated with an equal volume of Griess reagent consisting of sulphanilamide (0.25% (w/v) and N-1-naphthylethylenediamine (0.025% (w/v), and the mixture was incubated for 10 min, and the absorbance at 550 nm was measured in a spectro-photometer (UV-1800 UV–VIS spectrophotometer, Shimadju, Japan). The amount (μM) of NO produced/sample was determined by extrapolation from a standard curve prepared in parallel using sodium nitrite.

Plantar Anti-oxidant, Peroxide, and SOD/CAT Enzyme Activity Status: Portions of the isolated plantar tissues (≈ 1 g/mouse) were homogenized in ice-cold 50 mM potassium phosphate buffer (pH =7.4). Homogenates were then centrifuged (9000 rpm, 20 min, 4 ºC), supernatants were collected, and aliquots assessed for protein content [24]. To assess anti-oxidant status, levels of reduced GSH were estimated using a spectrophotometric method that employs DTNB (5, 5'-dithio-bis-[2-nitrobenzoic acid]); Ellman's reagent) [28]. A 300 μl aliquot of sample supernatant was mixed with an equal volume of 10% tricholoracetic acid (TCA) solution and then centrifuged at 5000 rpm for 10 min. The supernatant was isolated and a 250 μl aliquot was obtained, which was mixed with 500 μl Tris HCl buffer (pH 6.5), and 25 μl 10 mM DTNB and incubated for 5 min at 37 °C in the dark. Thereafter, the sample absorbance was measured in the spectrophotometer at 412 nm (*vs*. solution containing all components, with saline in place of homogenate). All outcomes were expressed as μM GSH/mg tissue protein in homogenate sample by taking the mean absorbance / 14,150 $M^{-1}\bullet cm^{-1}$.

The tissue contents of lipid peroxidation products were determined from the generation of thiobarbituric-reactive substances (TBARS) from the isolated homogenates [29]. In brief, 1 ml sample supernatant was mixed with equal volume of TCA-TBA-HCl mixture and heated in a boiling water bath for 15 min. The mixture was then cooled to room temperature and centrifuged at 10000 rpm (10 min, 4 ºC). The resultant supernatants were collected and the absorbance of each was measured at 532 nm in the spectrophotometer (*vs*. solution containing all components [with saline in place of homogenate] processed in parallel). All outcomes were expressed as nM TBARS / mg plantar tissue protein in homogenate sample by taking the mean absorbance/1.56 x 10^5 $M^{-1}\bullet cm^{-1}$.

SOD enzyme activity in sample supernatants were measured as the amount of enzyme capable inhibiting the auto-oxidation of pyrogallol [30]. Specifically, a solution (A) containing 100 μl sample supernatant and 1.5 ml Tris-EDTA-HCl buffer (pH 8.5) was prepared. In parallel, a solution (B) containing all components (but with saline in place of homogenate material) was prepared. When ready, 100 μl of 7.2 mM pyrogallol was added to each tube, and all samples were incubated at 25 ºC for 10 min. The reaction in each was terminated by addition of 50 μl 1 M HCl to each tube and the absorbance at 420 nm was measured in the spectrophotometer (*vs*. solution containing all components [with saline in place of pyrogallol] processed in parallel). Based on the equation % inhibition = $100 \times (1 - [Abs_{(A)}/Abs_{(B)}])$. One unit of SOD activity was defined as the amount of enzyme that reduced the rate of auto-oxidation of pyrogallol by 50% under these experimental conditions. Ultimately, activity was expressed as U/mg tissue protein in homogenate sample.

Catalase activity in the sample supernatants were determined spectrophotometrically by measures of the decrease in hydrogen peroxide (H_2O_2) [31]. A cuvette received 200 μl of sample supernatants to which 2 ml of potassium phosphate buffer (pH 6.5)

containing 10 mM H_2O_2 was then added. The absorbance in the sample was then immediately monitored at 240 nm in a spectrophotometer against a blank that contained all materials except for homogenate. Measures of absorbance were taken at 15 sec interval after addition of the H_2O_2-buffer. The rate of change in absorbance was calculated and the activity expressed in terms of μM H_2O_2 consumed/min/mg tissue protein in homogenate sample.

Immunoblot Analysis for Plantar TNFR1, NF-κB, COX-2 and iNOS Expression: Portions of the isolated plantar tissues (≈ 1 g/mouse) were placed into RIPA buffer (0.5 M EDTA, 1 M Tris buffer, 5 M NaCl, 10% sodium deoxycholate, 10% SDS supplemented with 1% Triton X-100) and homogenized as mentioned above. After isolating supernatant and evaluating protein content by the Bradford assay [24], 60 μg tissue lysate / mouse was denatured at 100 °C for 5 min and were then resolved over a 10 % SDS-PAGE gel separately for each of TNFR1, NF-κB, COX-2 and iNOS.

Thereafter, all materials were separately electro-transferred to three separate sets of nitrocellulose membranes. After blocking for 2 h at 4 °C in TBST (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% [v/v] Tween-20) containing 5% bovine serum albumin (BSA), the membranes were washed three times with TBST and then separately incubated overnight at 4 °C in TBS containing appropriate rabbit anti-mouse-primary antibodies, *i.e.*, anti - TNFR1 (Abcam, USA), anti-NF-κB, anti-COX-2, or anti-iNOS antibody (Biorbyt Ltd., Cambridge, UK), at 1:100 dilution.

In each case, antibody against β-tubulin was used for equal sample loading. Blots were then washed three times in TBST before then being incubated for 2 hr at 4 °C in TBST containing horseradish peroxidase-conjugated secondary antibody (1:5000 dilution; Biorbyt Ltd., Cambridge, UK). Final signals were developed using Super Signal-chemi-luminescent - substrate (Thermo Scientific, Pittsburgh, PA). Each blot was then exposed to X-Omat BT films (Kodak, Windsor, CO) and bands were quantified using Quantity One software (Bio-Rad, Hercules, CA) in a BioRad GS-900 densitometer.

Statistical Analysis: All results were expressed in means ± SD (n = 3 / group / time point). Assessments of significant differences between groups were performed using a one-way analysis of variance (ANOVA). A Scheffe's F-test post-hoc test for multiple comparisons of the different groups was done when significant p-values were obtained. A p-value < 0.05 was considered significant. All analyses were done using Origin Pro 8 software (Origin Lab Corporation, Northampton, MA).

RESULTS:

Effects on Footpad Swelling and MPO Activity: Effect of oral treatments with 50 mg CTE/kg or 2.5 mg QG/kg for seven consecutive days, followed by TNFR1 neutralization with anti-TNFR1 antibody 30 min before induction of inflammation by sub-plantar injection of carrageenan, was assessed by measures of local swelling and MPO activity, as a measure of PMN infiltration (**Fig. 1A** and **1B**). Induction of acute inflammation in carrageenan-injected mice resulted in a significant increase in footpad swelling; MPO activity continued to rise upto 12 hr pci. In TNFR1-neutralized groups although paw swelling and MPO activity was significantly ($p < 0.05$) inhibited even starting at 2 hr pci, in general, inflammation still was evident up to 12 hr pci. When TNFR1-neutralized mice had also received CTE or QG for the prior 7 days, significant inhibition of swelling ($p < 0.05$) and MPO activity (relative to that by TNFR alone) were observed starting at 6 hr pci. These co-treatments also hastened resolution of swelling such that by 6-12 hr, levels neared those seen in mice that had not received any carrageenan at all.

Effects on Plantar Tissue Cytokines and Chemokines: Alterations in local levels of cytokines TNF-α, IL-1β, IFNγ, IL-6, IL-12p40, and IL-10, and of chemokines IL-8 and MCP-1 **Fig. 2A - 2H**, respectively) after carrageenan injection were also evaluated. In terms of pg/mg tissue protein, levels of TNFα, IL-1β, IL-6, IL-12p40, IFNγ, and MCP-1 were found to have increased several-fold *vs*. control host tissue levels starting at 4 pci and remaining so out to 12 hr pci; significant ($p < 0.05$) elevation in IL-8 was observed from starting from 2 hr pci. In contrast, levels of anti-inflammatory IL-10 were significantly ($p < 0.05$) decreased starting from 4 h pci and remaining so out to 12 hr pci.

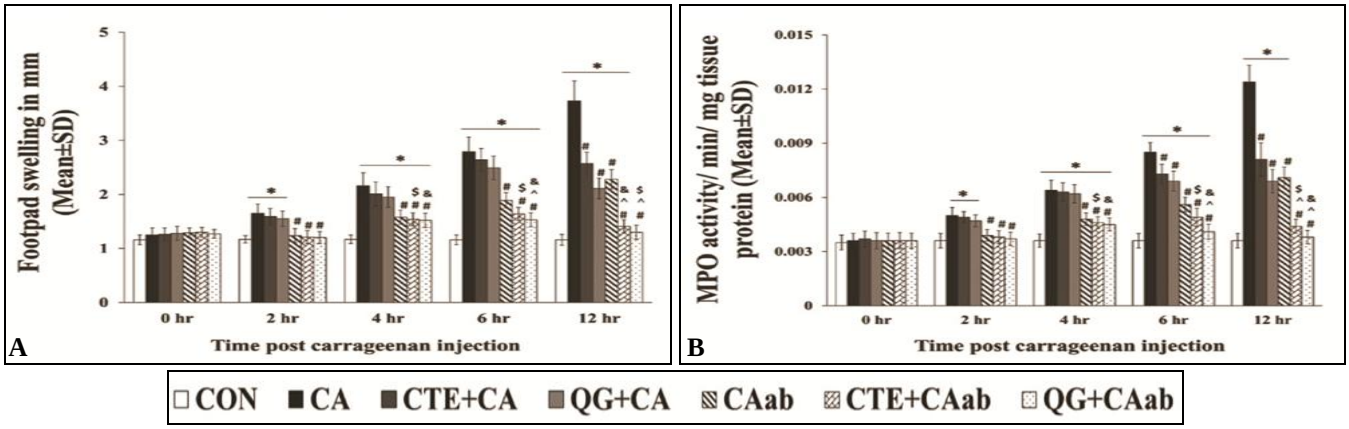
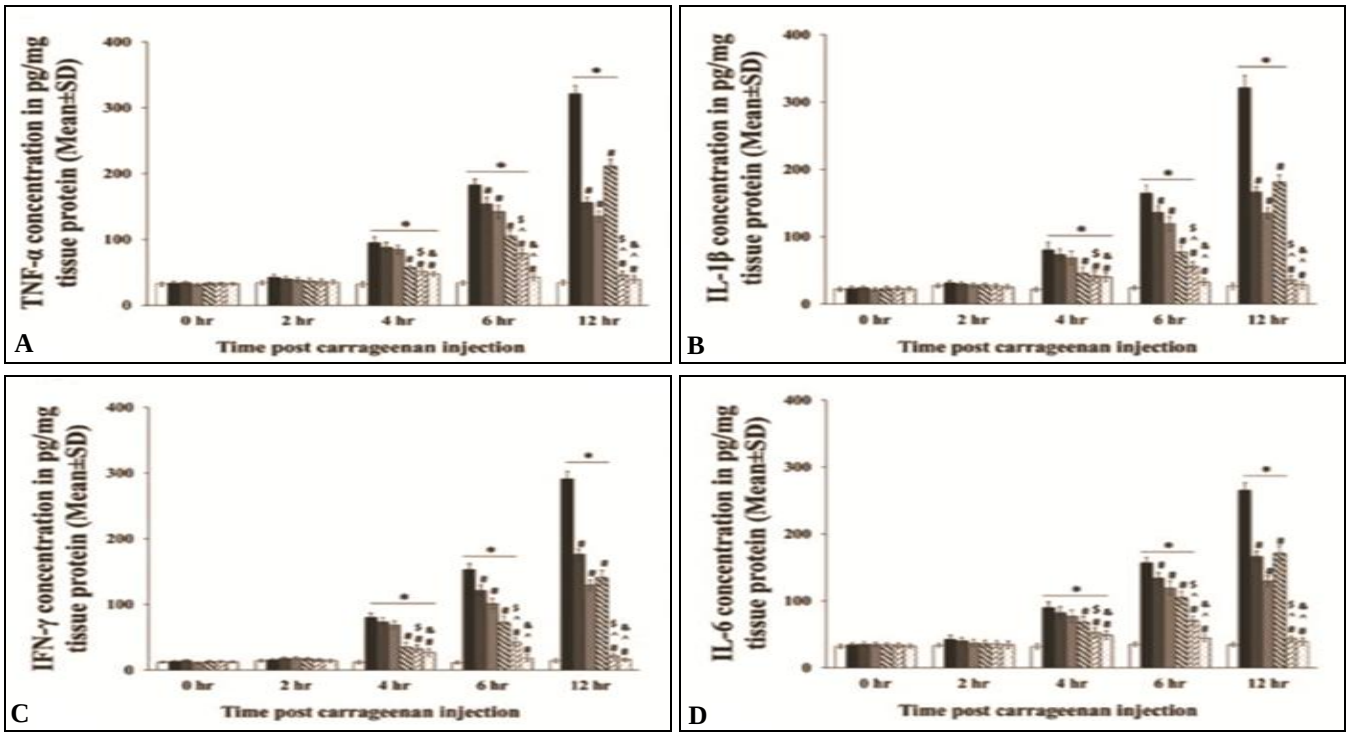


FIG. 1: FOOTPAD SWELLING OF FOOTPAD AND PMN INFILTRATION

Results were reproduced in three independent experiments. Values shown are means ± SD (mm for swelling and activity/min/mg tissue protein for MPO activity); (n = 3 / group / time point). *indicates significantly different ($p < 0.05$) compared to control; #indicates significantly different ($p < 0.05$) compared to CA group (CA; isotype control antibody); ^indicates significantly different ($p < 0.05$) compared to TNFR1 neutralized CA group (CAab; anti TNFR1 antibody); $indicates significantly different ($p < 0.05$) CA group supplemented with 50mg CTE/kg (CTE+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 50mg CTE/kg (CTE+CAab; anti-TNFR1 antibody); &indicates significantly different ($p < 0.05$) CA group supplemented with 2.5 mg QG/kg (QG+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 2.5 mg QG/kg (QG+CAab; anti-TNFR1 antibody).

CTE or QG alone did not produce any significant ($p < 0.05$) ameliorating effects on these changes until 6 - 12 hr pci, with effects differing between the two with regard to potency, although QG produced better resolution significantly ($p < 0.05$) than CTE. TNFR1 neutralization alone often afforded significant ($p < 0.05$) remediation, but the onset of significance ($p < 0.05$) varied across the various cytokines / chemokines - starting at 2 hr pci with IL-8, at 6 hr with IL-10, and at 4 hr for all the other measured proteins. Among the mice that received the anti-TNFR1 and either CTE or QG, the ameliorating effects were even greater, with levels attaining significant ($p < 0.05$) differences even from the anti-TNFR1 group itself starting moreover at 6 hr pci. In almost every case, levels by 12 hr pci in these mice neared those of untreated (no carrageenan) mice. Interestingly, levels of IL-10 in these mice actually became supra-elevated relative to those controls and very significantly ($p < 0.05$) elevated even compared to those in mice that received only the antibody treatment.



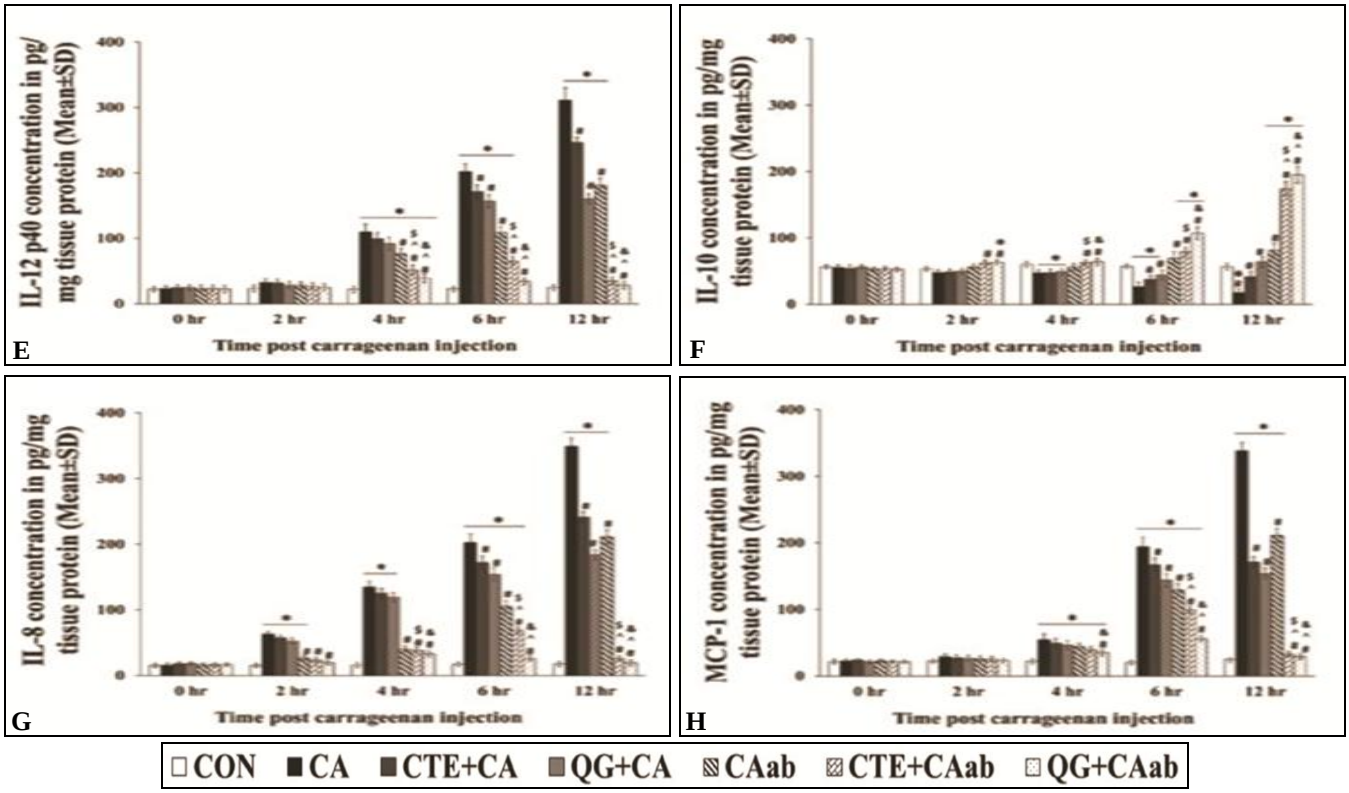


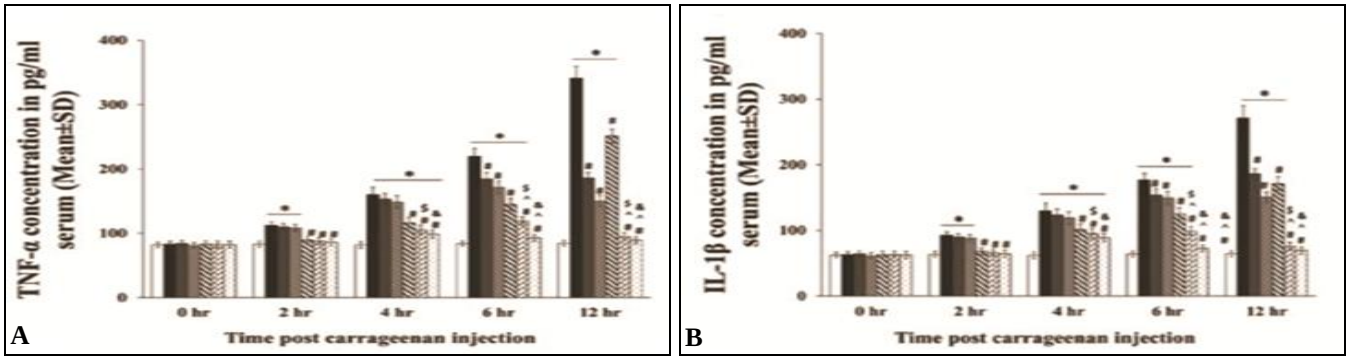
FIG. 2: CHANGES IN LOCAL PRODUCTION OF CYTOKINES AND CHEMOKINES IN PLANTAR TISSUES

(A) TNFα, (B) IL-1β, (C) IFNγ, (D) IL-6, (E) IL-12, (F) IL-10, (G) IL-8, and (H) MCP-1. Results were reproduced in three independent experiments. Values shown are means ± SD pg/mg tissue protein (n = 3 / group / time point). *indicates significantly different ($p < 0.05$) compared to control; #indicates significantly different ($p < 0.05$) compared to CA group (CA; isotype control antibody); ^indicates significantly different ($p < 0.05$) compared to TNFR1 neutralized CA group (CAab; anti TNFR1 antibody); $indicates significantly different ($p < 0.05$) CA group supplemented with 50mg CTE/kg (CTE+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 50mg CTE/kg (CTE+CAab; anti-TNFR1 antibody); &indicates significantly different ($p < 0.05$) CA group supplemented with 2.5 mg QG/kg (QG+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 2.5 mg QG/kg (QG+CAab; anti-TNFR1 antibody).

Effects on Systemic Levels of Cytokines and Chemokines: Systemic levels of TNF-α, IL-1β, IFNγ, IL-6, IL-12p40, IL-10, IL-8, and MCP-1 **Fig. 3A - 3H**, respectively) after carrageenan injection were estimated in serum isolated at each time-point. In general, patterns of elevation / remediation noted in tissues were reproduced in the sera.

Effects on Plantar Tissue Concentration and Circulatory Level of CRP: Plantar tissue and systemic levels of acute phase C-reactive protein (CRP) after carrageenan injection were estimated at each time-point. CRP levels in serum **Fig. 4A** and the plantar tissues **Fig. 4B** were increased several-fold after carrageenan injection starting in each case at 2 hr pci and remaining so thereafter.

Treatment of mice either with CTE or QG had no significant ($p < 0.05$) local or systemic Impact until 6 - 12 hr pci.



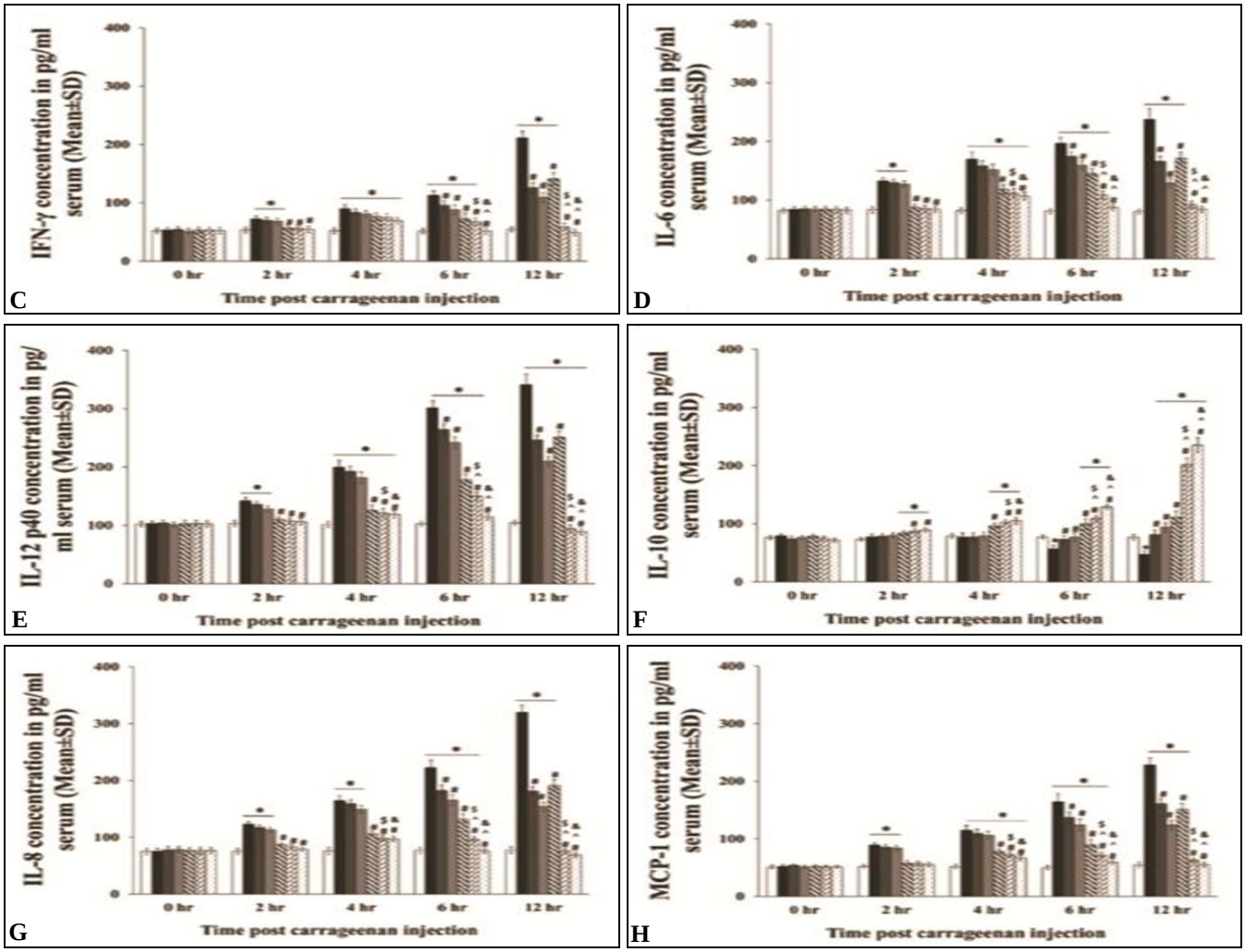


FIG. 3: CHANGES IN SYSTEMIC LEVELS OF CYTOKINES AND CHEMOKINES

(A) TNFα, (B) IL-1β, (C) IFNγ, (D) IL-6, (E) IL-12, (F) IL-10, (G) IL-8, and (H) MCP-1. Results were reproduced in three independent experiments. Values shown are means ± SD pg/ml serum (n = 3 / group / time-point). *indicates significantly different ($p < 0.05$) compared to control; #indicates significantly different ($p < 0.05$) compared to CA group (CA; isotype control antibody); ^indicates significantly different ($p < 0.05$) compared to TNFR1 neutralized CA group (CAab; anti TNFR1 antibody); $indicates significantly different ($p < 0.05$) CA group supplemented with 50mg CTE/kg (CTE+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 50 mg CTE/kg (CTE+CAab; anti-TNFR1 antibody); &indicates significantly different ($p < 0.05$) CA group supplemented with 2.5 mg QG/kg (QG+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 2.5 mg QG/kg (QG+CAab; anti-TNFR1 antibody)

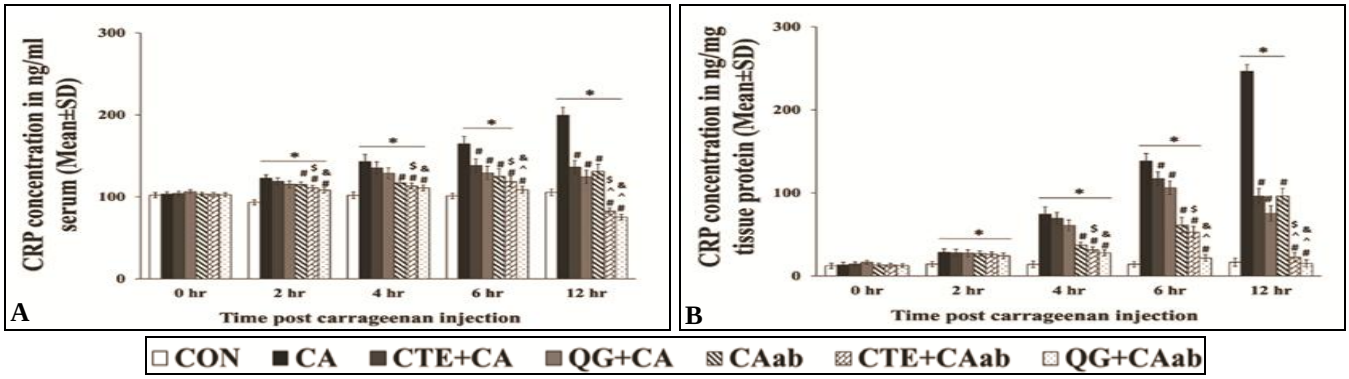


FIG. 4: CHANGES IN THE SYSTEMIC CRP LEVELS AND CRP LEVELS IN PLANTAR TISSUES

(A) Serum CRP and (B) plantar tissue CRP were determined by ELISA in. Results were reproduced in three independent experiments. Values shown are means ± SD ng/ml serum or ng/mg tissue protein (n = 3 / group / time-point). *indicates significantly different ($p < 0.05$) compared to control; #indicates significantly different ($p<0.05$) compared to CA group (CA; isotype control antibody); ^indicates significantly different ($p < 0.05$) compared to TNFR1 neutralized CA group (CAab; anti TNFR1 antibody); $indicates significantly different ($p < 0.05$) CA group supplemented with 50mg CTE/kg (CTE+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 50mg CTE/kg (CTE+CAab; anti-TNFR1 antibody); &indicates significantly different ($p < 0.05$) CA group supplemented with 2.5 mg QG/kg (QG+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 2.5 mg QG/kg (QG+CAab; anti-TNFR1 antibody).

Neutralization of TNFR1 alone resulted in detectable significant ($p < 0.05$) reductions in CRP levels starting at 2 hr pci in the blood and at 4 hr pci in the plantar tissue. Among mice that had also received CTE or QG, even greater decrements in CRP levels from those with the antibody regimen alone were evident, most dramatically in the plantar tissues themselves. By 6 - 12 hr pci, the CTE + CAab and QG + CAab mice had values in both the serum and tissues that were even significantly ($p < 0.05$) lower than those in mice that received the antibody alone. As with many of the other endpoints above, by 12 hr pci, levels of this marker protein had returned to near-baseline levels seen in control (no carrageenan) mice.

Effects on Plantar Tissue Free Radical Generation: Local ROS/RNS levels were also measured after the carrageenan injection. Significant ($p < 0.05$) increases in $\bullet O_2^-$ (starting at 4 hr pci, **Fig. 5A**) and NO (starting at 2 hr pci, **Fig. 5B**) in paw tissues were notable up to 24 hr pci in mice treated with carrageenan only. TNFR1 neutralization alone did not start to induce significant ($p < 0.05$) decreases in generation of either ROS/RNS until 4 h pci. Supplementation with either CTE or QG alone reduced production of either agent significantly ($p<0.05$) starting sometime after 6 - 12 hr pci. Providing anti-TNFR1 neutralizing antibody to mice already treated with CTE or QG further significantly ($p<0.05$) reduced the ROS/RNS levels (*vs.* levels seen in hosts that received only anti-TNFR1) starting at 4 hr pci and remaining so out to 12 hr pci. In the case of NO, these values had returned to near-baseline levels seen in control (no carrageenan) mice.

Effects on Plantar Tissue Anti-oxidant Status: Levels of select agents / enzyme activities that reflect local anti-oxidant status in the plantar tissues were also measured after the carrageenan injection. Injection of the carrageenan alone led to significant reductions in reduced GSH content (**Fig. 6A**) and in catalase (CAT) activity (**Fig. 6D**) starting at 4 hr pci. Significant increases in TBARS, reflecting local oxidative damage (**Fig. 6B**); and in superoxide dismutase (SOD) activity (**Fig. 6C**) become evident starting in the period 4 - 6 hr pci.

Treatment with CTE alone (data not shown) induced significant remediation (vs. that from CA alone) starting at 6 hr pci with CAT, 12 hr with GSH and TBARS, and at 12 hr with the SOD. In comparison, treatment with CTE alone (data not shown) induced significant remediation (*vs.* that from CA alone) starting at 6 hr pci with CAT and TBARS and at 12 hr with both GSH and SOD. Treatment with anti-TNFR1 alone seemed to induce significant ($p < 0.05$) reversals of effects on all these endpoints primarily starting in the period 4 - 6 hr pci. Interestingly, use of the extract + TNFR1 neutralization regimens seems to cause even faster amelioration of the changes induced by the CA alone.

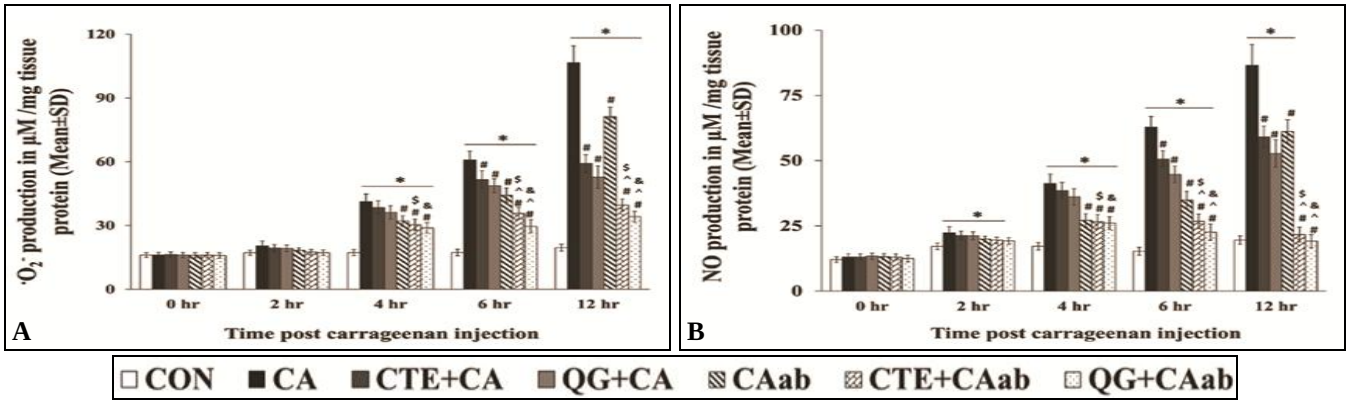


FIG. 5: CHANGES IN OXYGEN / NITROGEN SPECIES (O_2^- AND NO) PRODUCTION IN PLANTAR TISSUES

(A) $\bullet O_2^-$ (µM/mg tissue). (B) NO (µM/mg tissue). Results were reproduced in three independent experiments. Values shown are means ± SD (n = 3/group/timepoint). *indicates significantly different ($p < 0.05$) compared to control; #indicates significantly different ($p < 0.05$) compared to CA group (CA; isotype control antibody); ^indicates significantly different ($p < 0.05$) compared to TNFR1 neutralized CA group (CAab; anti TNFR1 antibody); $indicates significantly different ($p < 0.05$) CA group supplemented with 50 mg CTE/kg (CTE+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 50 mg CTE/kg (CTE+CAab; anti-TNFR1 antibody); &indicates significantly different ($p < 0.05$) CA group supplemented with 2.5 mg QG/kg (QG+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 2.5 mg QG/kg (QG+CAab; anti-TNFR1 antibody).

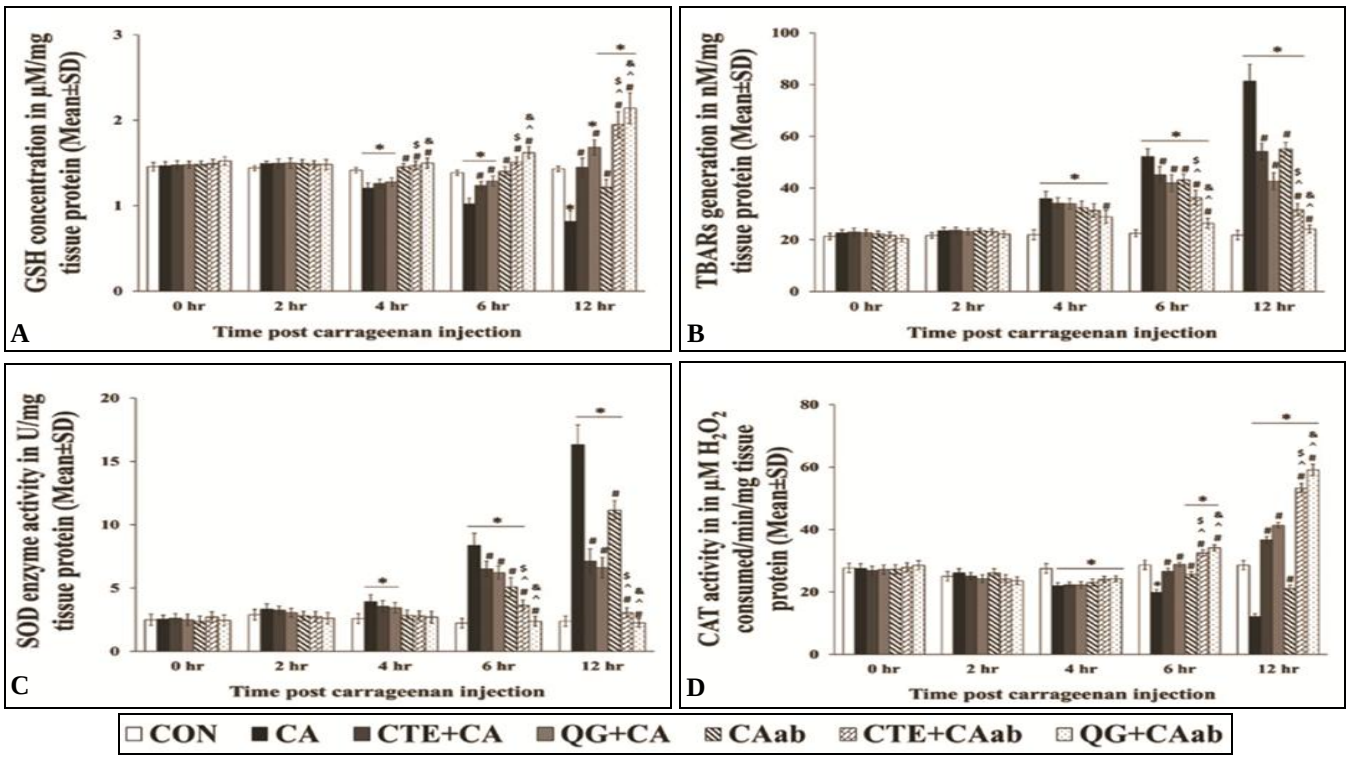


FIG. 6: CHANGES IN ANTI-OXIDANT STATUS IN PLANTAR TISSUES

Levels of (A) GSH (µM/mg tissue protein), (B) LPO (nM/mg tissue protein), (C) SOD (U/mg tissue protein), and (D) catalase activity (µM H_2O_2 consumed/min/mg tissue protein). Results were reproduced in three independent experiments. Values shown are means ± SD; (n = 3 / group / time-point). *indicates significantly different ($p < 0.05$) compared to control; #indicates significantly different ($p < 0.05$) compared to CA group (CA; isotype control antibody); ^indicates significantly different ($p < 0.05$) compared to TNFR1 neutralized CA group (CAab; anti TNFR1 antibody); $indicates significantly different ($p < 0.05$) CA group supplemented with 50 mg CTE/kg (CTE+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 50 mg CTE/kg (CTE+CAab; anti-TNFR1 antibody); &indicates significantly different ($p < 0.05$) CA group supplemented with 2.5 mg QG/kg (QG+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 2.5 mg QG/kg (QG+CAab; anti-TNFR1 antibody)

By 4 hr pci, the co-treatments had already caused even more significant ($p < 0.05$) reversals of the effects on GSH than did the antibody treatment alone; this was also the case by 6 hr pci with regard to CAT activity. By 6 hr pci, the use of QG + CAab had already given rise to even more significant ($p < 0.05$) reversals of the effects on SOD activity and TBARS levels than the antibody alone. It is interesting to note that while these co-treatment regimens led to 12 hr pci values for SOD and TBARS that were near control (no carrageenan) levels, they also somehow led to an over-induction/presence of GSH and of CAT activity at this time-point. The effect on the latter was even evident beginning as early as 6 hr pci.

Effects on Plantar Tissue TNFR1, NF-κB, COX-2 and iNOS Expression: Based on Western blot analyses, sub-plantar carrageenan injection significantly ($p < 0.05$) increased the local expression of TNFR1, NF-κB, COX-2 and iNOS at 6 hr pci **Fig. 7A**. Supplementation either with CTE or QG alone however did not cause any significant decrease in TNFR1 expression **Fig. 7B**, but that of NF-κB **Fig. 7C**, COX-2 **Fig. 7D** and iNOS **Fig. 7E** were significantly ($p < 0.05$) decreased *vs.* levels in tissues of time-matched carrageenan-only mice.

Use of anti-TNFR1 alone, or in conjunction with CTE or QG further significantly decreased the local expression of all three proteins. As with some of the other endpoints reported above, these co-treatment values were even significantly lower than those attained with the anti-TNFR1 alone.

DISCUSSION: In the present study, a single intravenous dose of anti-TNFR1 antibody regulated the local edema and PMN accumulation and hastened the resolution phase in mice supplemented with multiple doses of the CTE natural extracts or QG. It has been reported that TNFα increases Mac-1 and intercellular adhesion molecule-1 (ICAM-1) expression, and subsequently induces neutrophil rolling on and adhesion to vascular endothelial

cells, leading to infiltration and accumulation of neutrophils in tissues [32]. TNFα/TNFR1-mediated chemokine production is also reported to contribute to neutrophil accumulation in tissues; therefore, decreased local MPO activity (PMN infiltration) in mice treated with anti-TNFR1 antibody in the present study could be correlated with a decrease in local edema in the plantar tissues. As PMN infiltration and edema in the tissues was not achieved in mice pre-treated with anti-TNFR1 antibody alone, the failure to regulate inflammation by TNFR1 neutralization after 4 hr pci was suggestive that pre-treatment with anti-TNFR1 antibody could regulate the early phases of inflammation *via* some regulation of PMN accumulation.

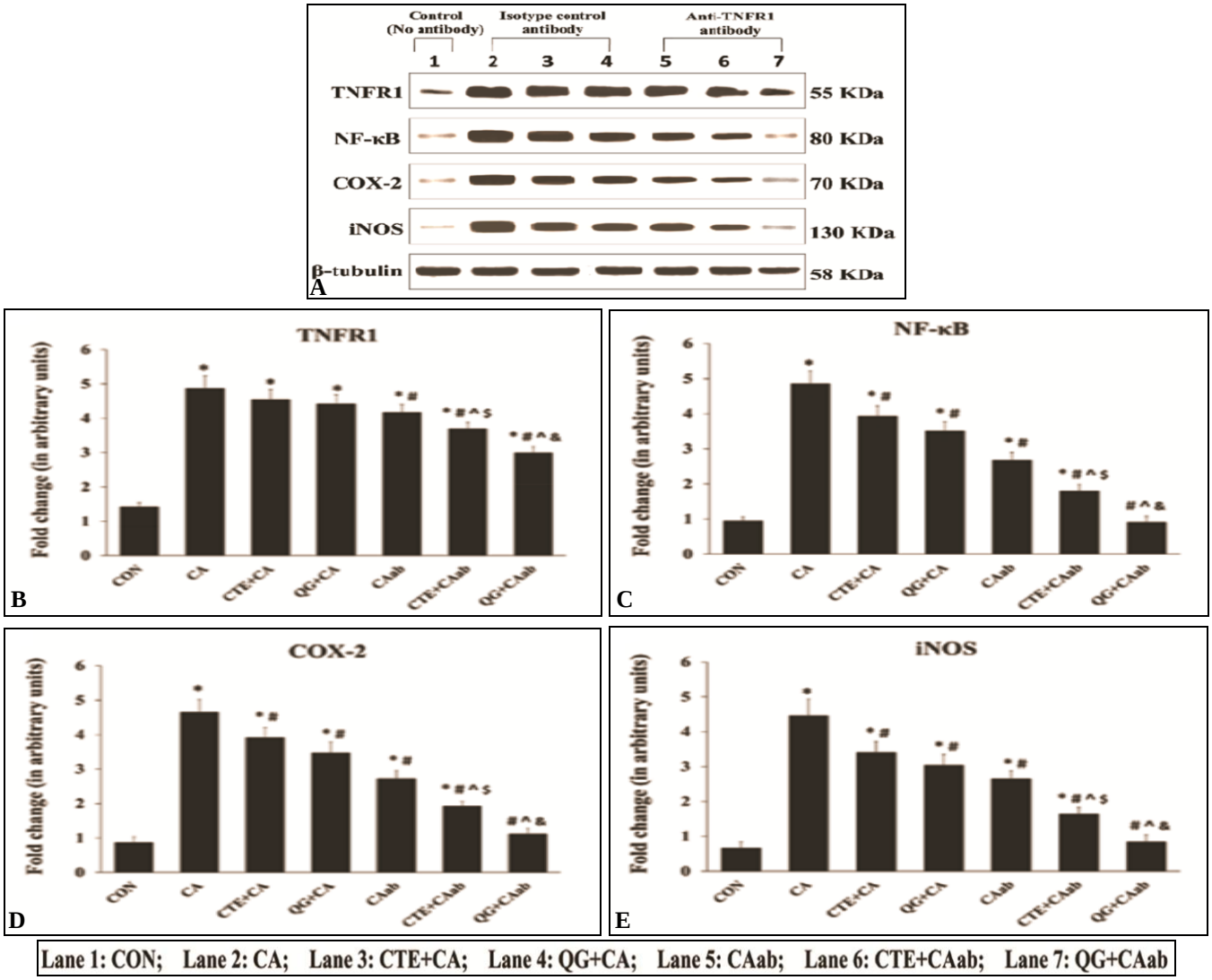


FIG. 7: CHANGES IN TNFR1, NF-KB, COX-2 AND INOS EXPRESSION IN PLANTAR TISSUES

(A) Representative panel of immunoblots. Expression levels of (B) TNFR1, (C) NF-κB, (D) COX-2, and (E) iNOS at 6 hr pci were determined by immunoblotting and respective fold-changes (in arbitrary units normalized to control [no carrageenan] mouse levels) based on densitometric analyses of the blots are provided. Results were reproduced in three independent experiments. Values shown are mean ± SD; (n = 3 / group / time-point). *indicates significantly different ($p < 0.05$) compared to control; #indicates significantly different ($p < 0.05$) compared to CA group (CA; isotype control antibody); ^indicates significantly different ($p < 0.05$) compared to TNFR1 neutralized CA group (CAab; anti TNFR1 antibody); $indicates significantly different ($p < 0.05$) CA group supplemented with 50 mg CTE/kg (CTE+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 50 mg CTE/kg (CTE+CAab; anti-TNFR1 antibody); &indicates significantly different ($p < 0.05$) CA group supplemented with 2.5 mg QG/kg (QG+CA; isotype control antibody) compared to TNFR1 neutralized CA group supplemented with 2.5 mg QG/kg (QG+CAab; anti-TNFR1 antibody).

Neutrophils contribute to the majority of the cellular infiltrate after carrageenan injection into plantar tissues and these cells, along with macrophages are responsible for generation of severe oxidative stress in the tissues and it is also reported that NET (neutrophil extracellular traps) release by PMN during inflammation results from NADPH oxidase-dependent ROS generation [33].

Like many other cells, PMN express TNFR1 on their surface and a cross-talk of TNFR1 with IL-1/IL-8 receptors regulates expression of many genes responsible for inflammation, apoptosis, and oxidative stress via NF-κB and MAPK pathways [34]. Therefore, TNFR1 expression at high levels in plantar tissues after a carrageenan injection was likely due in great part to increased infiltration of inflammatory cells into the tissue, and in part by self-perpetuation of TNFR1 secondary to cell interactions with TNFα released from resident macrophages and other cells. Specifically, TNFR1 can then signal the release of TNFR1 from a pool stored inside Golgi bodies [35].

In this study, a decrease in TNFR1 expression by neutralizing antibody could potentially be explained in terms of decreased PMN infiltration and/or decreased release from the Golgi pool of TNFR1 in PMN, macrophages, and other cells in the plantar tissues.

Signaling *via* TNFR1 is reported to be dependent on local production of superoxide anion, and in turn, TNFR1-p38-MAPK-PI3K pathways increase NADPH oxidase (NOX) activity used to generate $\bullet O_2^-$ [36]. Since NADPH oxidase-mediated ROS has been reported to affect cytokine/chemokine production by macrophages [37, 38]; decrease in local production of TNFα, IFNγ, IL-1β, IL-6, IL-12, IL-8, and MCP-1 noted in mice that received anti-TNFR1 antibody and the extracts anti-oxidants could be related to overall decreases in TNFR1-NADPH-oxidase mediated $\bullet O_2^-$ production. In the current study, carrageenan-induced inflammation was accompanied by an early increase in tissue TNFα, IFNγ, IL-1β, and IL-6.

The TNFR1-NF-κB signal circuit regulates synthesis of pro-inflammatory cytokine and therefore increases the release of TNFα, IL-1β, IL-6, and IL-8 [35]. This rise in local IL-8 production, a chemokine for neutrophils, in the plantar tissue could be directly correlated to the local PMN accumulation and edema [39]. It is speculated here that TNFR1 neutralization and anti-oxidant supplementation somehow hastened this resolution phase in part by down-regulating IL-8 production. Changes in systemic levels of TNFα, IL-1β, IL-6, and IL-12p40 have been reported to mediate the adherence of neutrophils to injured dermal endothelium in the tissues of rats injected with carrageenan [40]. This overall decrease in synthesis of pro-inflammatory cytokines in plantar tissues and concurrent decreases in these cytokines in the systemic circulation as a result of the anti-TNFR1 antibody treatment in combination with the extracts indicated this combinatorial approach might one day have a thera-peutic potential against inflammation in other host systems.

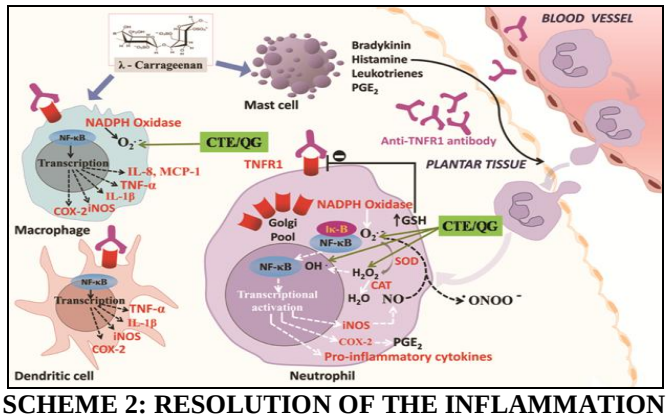
Redox balance was greatly compromised in the plantar tissues during the carrageenan-induced inflammation. The local increases in $\bullet O_2^{\cdot -}$ were in turn associated with likely increases in hydroxyl radical ($OH^{\cdot}$) generation, increases in lipid peroxide generation, and depletion of cell reduced GSH stores [41]. Generation of lipid peroxide as a result of over-production of ROS can lead to activation of NF-κB, and inositol phosphate (IP_3)-mediated activation of Akt and mTOR can lead to inflammatory gene activation [42]. Cellular GSH depletion has been correlated with TNFα/TNFR1-elicited oxidative stress [43]; and has been reported to sensitize some cell types to TNFα-induced apoptosis and impair survival pathways mediated by TNFα-NF-κB (*via* both IKK-dependent or -independent mechanisms) [44].

As such, regimens that lead to a restoration in GSH content (here, in plantar tissue) can be linked to modulation of TNFα signaling towards an overall resolution of inflammation. The data from the current study indicating restoration of CAT activity and decreases in SOD activity in parallel with significant decreases in edema / PMN accumulation supports the fact that anti-oxidant supplementation (in conjunction with the anti-TNFR1 antibody) can bring about a subtle change in redox balance and provide significant protection against tissue damage associated with antigen-induced inflammation.

RNS generation *via* cNOS activity first, and then activation of iNOS by NF-κB signaling pathways inside phagocytes are greatly responsible for the production of NO, a key regulator molecule of inflammation [45]. In turn, released NO can activate inducible isoforms of cyclooxygenases (COX) by increasing nitrosation of specific cysteine residues in the catalytic domain [46].

The current findings showed that the carrageenan enhanced expression of iNOS and COX-2 in the plantar tissues was attenuated by host anti-TNFR1 pre-treatment along with CTE or QG, but that neither the antibody nor the CTE or QG alone induced such declines. This indicated to us that during carrageenan-induced inflammation, iNOS and COX-2 activities were only partly regulated by TNFα/TNFR1 signaling (likely *via* TRAF-RIP-1-NF-κB-mediated signaling) [47] and partly by TNFR1-mediated oxidative stress [48].

CONCLUSION: In summary, supplementations with CTE or QG were seemed to ameliorate any disruption in redox balance and decreased the oxidative stress that would have been associated with ROS produced post-activation of TNFR1 pathways. Because anti-TNFR1 antibody interfered with TNFR1 signaling and decreased NF-κB-mediated transcription of genes encoding several inflammatory mediators, it was unsurprising NOX activity (and NOX-mediated $\bullet O_2^-$ production) was compromised. This effect of TNFR1 neutralization did not last long, probably due to subsequent disengagement of the neutralizing antibody from the receptor. However, TNFR1 neutralization in mice supplemented with either CTE or QG regulates both the TNFR1 induced inflammatory signals and oxidative stress into plantar tissue following carrageenan injection to bring down most of the parameters to its baseline and hastened resolution of the inflammation **Scheme 2**.



SCHEME 2: RESOLUTION OF THE INFLAMMATION

ACKNOWLEDGEMENT: Author B.B. and R.A. designed the study and designed the protocols. R.A., A.N., and S.S. performed all the experiments and analysis. R.A. and B.B. managed the literature searches. R.A. undertook all the statistical analysis. R.A. wrote the manuscript and B.B. approved the final manuscript. This work was supported and funded by Department of Science and Technology (DST), Government of West Bengal, Calcutta, India [Grant number. 117(Sanc)/ST/P/S&T/9G-08/ 2012].

CONFLICT OF INTEREST: The authors declare that they have no conflicts of interests.

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