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Anti-inflammatory activity and COX 2 inhibition potential of Trois in murine macrophage cell line

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Abstract

The objective of present study was to investigate the anti-inflammatory effects of Trois in lipopolysaccharide (LPS) induced macrophage RAW 264.7 cells. A biomembrane stabilisation assay was also conducted to evaluate its role in biomembrane fluidity during inflammation. Cytotoxicity of drugs on RAW 26-4.7 macrophages cell line was conducted using 3-(4,5-dimet-hylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. mRNA expression of cyclooxygenase-2 (COX-2) was studied using polymerase chain reaction (PCR). Results of our study revealed that IC_{50} of Trois and volini was 2.82 and 1.73 mg/ml, respectively, after 24 hrs treatment. Further, our data proved that Trois had excellent anti-inflammatory activity as it caused reduced production of nitric oxide (9-12%), myeloperoxidase (5-14%) and mRNA of COX-2 (12-33%) in comparision to volini. More-over, Trois had 5.44% more membrane stabilization potential compared to volini. From the above study it can be concluded that Trois can be an excellent option for the treatment of inflammation related diseases.

Keywords: Trois, membrane stabilization, inflammation, cyclo-oxygenase-2 inhibition.

Introduction

Inflammation is a common clinical condition, which is typically characterized by redness, swelling, pain and heat. Majority of the human populations are affected by inflammation related disorders at some point, across the world (Bhagyasri et al., 2015). A growing line of evidences have indicated that inflammation is involved in the pathogenesis of many diseases including arthritis and other life-threatening and debilitating disorders (Finch, 2005; Caruso et al., 2004; Lee et al., 2014; Kumar et al., 2009; Manouri et al., 2015). Among the various causes, oxidative stress is the primary cause of inflammation. It can induce inflammatory cells to produce inflammatory mediators, such as cytokines and chemokines, which enhance tissue damage from the recruitment of more inflammatory cells, eventually resulting in more oxidative stress (Mansouri et al., 2015; Kasama et al., 2005; Wolf et al., 2005; Odobasic et al., 2014). Induction of pro-inflammatory cytokines (IL-6, TNF-α, and NF-κB) can induce nitric oxide (NO) production and cyclo-oxygenase-1 (COX-1) which contribute in inflammation (Kirby et al., 2014; Saraswathi et al., 2015; Alhouayek and Muccioli, 2014). Another inflammatory process is the production of matrix metalloproteinases (MMPs). MMPs are extracellular matrix degrading enzymes that can be secreted by the induction of many pro-inflammatory cytokines and interleukins (Breckwoldt et al., 2008). Several reports have demonstrated the involvement of COX-2, NO and *myeloperoxidase (*MPO) in inflammation of rheumatoid arthritis or spondyloarthropathies (Sharma et al., 2008; Breckwoldt et al., 2008; Saraswathi et al., 2015; Alhouayek and Muccioli, 2014). Lipid peroxidation causes an auto-oxidation of polyunsaturated fatty acids in the membrane leading to reduced membrane fluidity (Chippada et al., 2011).

Inflammation related diseases are commonly treated by nonsteroi-dal anti-inflammatory drugs (NSAIDs). However NSAIDs are not always useful due to their side effects (Bhagyasri et al., 2015; Ki-rby et al., 2014). To avoid side effects of NSAIDs, natural reme-dies can be the alternative option. Natural remedies are known to have less side effects and toxicity (Arif et al., 2009; Chatterjee et al., 2015; Kapewangolo et al., 2015). Medicinal plants have been wide source for the treatment of various diseases and nearly 80% of the world populations rely on medicinal herbs for their primary health care (Jordan et al., 2010). There is increasing evidence that medicinal plants have many biological properties including anti-oxidant, anti-inflammatory, anti-cancer and anti-atherosclerotic effects (Zhen et al., 2015; Bhagyasri et al., 2015; Chatterjee et al., 2015).

Trois is a nanotechnology based herbal product that contains wintergreen oil (*Gaultheria precumbens*) and eucalyptus oil (*Eucalyptus globulus*) as major ingredients. It is indicated for the treatment of pain and inflammation associated with different types of arthritis including osteoarthritis, gouty

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arthritis, rheum-atoid arthritis, ankylosing spondylitis, sprains and backache. To study its anti-inflammatory effects, murine macrophages cells i.e. RAW 264.7 cells have been used as in vitro inflammatory model in which inflammation was induced by lipopolysaccharides (LPS) (Mosmann, 1983; Seo et al., 2011; Achoui et al., 2010).

This study was aimed to assess the anti-inflammatory effect Trois in LPS induced macrophage RAW 264.7 cells. Biomembrane stabilisation assay was also conducted to evaluate its biomembra-ne fluidity ability during inflammation.

Materials and Methods

Drugs

Following two drugs were used in the study: Trois (Venus Remedies Limited, Baddi, India) and volini (Ranbaxy Laboratories Limited, Paonta Sahib, India). Trois contains wintergreen oil 18.9 % (v/v), eucalyptus oil 6.06 % (v/v), menthol 5.08 % (w/v), *Vit-ex negundo* leaves extract 3.24 % (w/v) and *Apium graveolens* fruit extract 3.24 % (w/v) and volini contains diclofenac diethyl amine 1.16 % (w/w) equivalent to diclofenac sodium (1 %), linseed oil BP (3 % w/w), methyl salicylate IP (10 % w/w) and menthol IP 5 % (w/w).

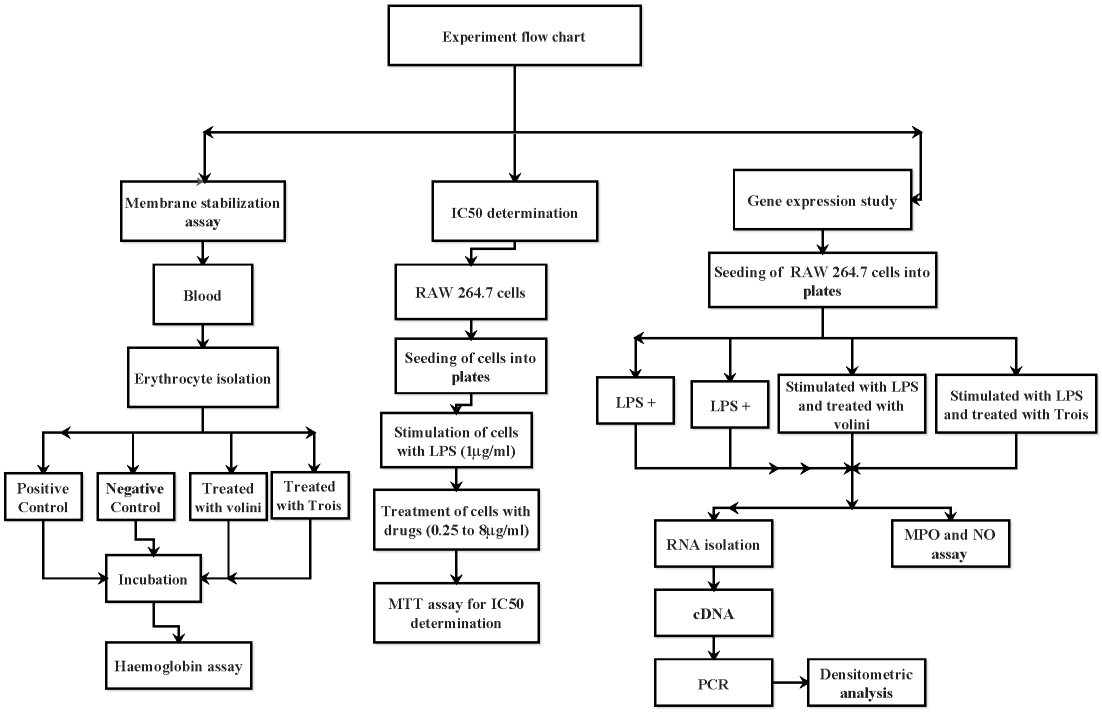

Figure 1: Flow chart of entire experiment.

Cell culture

RAW 264.7 mouse macrophages cell line was obtained from National Centre for Cell Sciences (NCCS), Pune, India. It was grown in Dulbecco's Modified Eagle's Medium (DMEM, HiMedia, Mumbai, India) supplemented with 10% fetal bovine serum (FBS, Bio Gene, India) and 1% antibiotic / antimyotic solution at 37°C in an atmosphere of 5% CO_2 in a humidified incubator. Cells of 70-90 % confluent flasks with >90% viability were used for further study. The flow chart of entire experiment is shown in Figure 1.

Cell viability

Viability of the cells was assessed using the 3-(4,5-dimethy-lthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described earlier (Mosmann, 1983). The percentage of cell viability was calculated by the following for-mula:

$$Cell\ viability\ (\%) = OD_{570sample}\ X\ 100\ /\ OD_{570control}$$

IC50 determination of drugs

To evaluate the effect of drugs on LPS stimulated inflammation on macrophage RAW 264.7 cell, $5x10^3$ cells/well seeded into 96 well plate and grouped as follows: A, B, C, D, E, F, G and H. Cells in group A were treated with PBS which served as negative control. Cells in group B to H were stimulated with LPS (1 µg/ml) and cells in group B served as positive control. Cells in C to H groups were simultaneously treated with various concentrations of Trois (0.25 to 8 µg/ml). Only cell culture media in another group I served as blank. The same drug treatment procedure was adopted for volini with drug concentrations ranged 0.25 to 8 µg/ml in another 96 wells plate. After drug treatment, plates were incubated for 24 hrs and processed for IC_{50} determination. The cytotoxic doses that kill cells by 50% (IC_{50}) was determined from cell viability (%) versus concentration curve. All the experiments were done in triplicate.

RNA isolation

Total mRNA from LPS stimulated RAW 264.7 cells (positive control; LPS+), without LPS stimulated cells (negative control; LPS-) and LPS stimulated and drugs treated (Trois and volini) cells with half of IC_{50} values was isolated using Trizole (Invitrogen, Banglore, India). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA. A ratio

of ~2.0 is generally accepted as "pure" for RNA. RNA was stored at -70 °C until use.

Complementary deoxyribonucleic acid (cDNA) synthesis

Total RNA (2 µg) of each treatment group was converted to first strand cDNA as follows: Two microgram of RNA was added to 1 µl of oligo dT primer and 9.2 µl of water and the mixture was incubated at 65 ºC for 5 min. After incubation, following reagents were added sequentially: 4.0 µl of 5X RT buffer, 1.0 µl of 0.1M DTT, 0.5 µl of 10 mM dNTPs and 0.3 µl of 20 U moloney murine leukaemia virus reverse transcriptase (MMLVRT), mixed well and the mixture was subsequently incubated at 37°C for 60 min. The reaction was stopped by heating at 70°C for 10 min. The resultant solution was cDNA which can be used for gene expression study by PCR.

PCR analysis

PCR was performed using the cDNA as template. The primers used in this study were obtained from sigma Aldrich (Chemicals Private Limited, Bangalore, India). The following sequences of primers were used. For COX-2-F-5-ATGCTCCTGCTTGAGTATGT-3; COX-2-R-ATGCTCCTG CTTGAGTATGT-3'. β-actin-F-5 GTGGGCCGCCCTAGGCA CC AG-3', for β-actin-R-5-GGA-GGAAGAGGATGCGGC AGT-3'. For PCR amplifications, about 3 µl of cDNA was added to 20 µl mixture containing 0.5 mM of dNTPs, 1.25 µM of each primer and 3.0 U of Taq polymerase (Banglore Genei) in 1x PCR buffer. Amplification was performed in an Eppendorf thermocycler (Germany) with following cycling parameters: initial denaturation at 94°C for 5 min followed by 35 cycles (45 seconds of denaturation at 94°C, 45 second of anneali-ng at 60-62 °C and 1 min of extension at 72ºC) and final extension at 72ºC for 5 min. PCR products were then electrophoresed on a 1.5 % agarose gel containing ethidium bromide. The expected amplicon size for COX-2 gene was 696 base pairs and β-actin gene was 603 base pairs. PCR products were then electrophoresed on a 1.0 % agarose gel containing ethidium bromide and intensity of each amplified band was analyzed using the image J software.

Measurement of myeloperooxidase (MPO)

MPO activity was measured using 3,3,5-5 tetramethylbenzidine (TMB) as described elsewhere (Suzuki et al., 1983). About $5x10^3$ cells/well were seeded into 96 well plate and grouped as follows: negative control (without LPS stimulated cells; LPS-), positive control (LPS stimulated cells; LPS+), drug treatment groups (treated with drugs at half of IC_{50} values following LPS stimulation) and blank (only media). The plates were then incubated for 24, 48 and 72 hrs in CO_2 incubator (37 °C, 5 % CO_2). After incubation at indicated time intervals, 10 µl of cell culture media from each well was withdrawn and used for MPO activity measurement. Cell culture media was used as a blank. The released MPO from cells was determined by comparing the values to a standard curve generated using known concentrations of 10 mM hypochlorous acid. The percentage of MPO inhibition were calculated as follows:

$$\% \text{ Inhibition} = MPO_{control} - MPO_{sample} / MPO_{control} \; X100$$

Measurement of nitric oxide (NO)

NO production was assayed by measuring the level of nitrite in the supernatant of cultured RAW264.7 cells as described earlier (Kang et al., 2008). Grouping and treatment of cells were made as described in the above MPO section. The plates were then incubated for 24, 48 and 72 hrs at 37 °C, 5% CO_2. Following incubation at indicated time intervals, 100 µl of cell culture medium was withdrawn and used for NO assay. Fresh culture medium was used as a blank in every experiment. The concentrations of nitrite were then determined by comparing the values of test samples to a standard curve generated using known concentrations of sodium nitrite (µM). All experiments were performed in triplicate. The percentages of NO inhibition were calculated as follows:

$$\% \text{ Inhibition} = 100 \; X \; NO_{2control} - NO_{2sample} / NO_{2control}$$

Human red blood cells (HRBC) membrane stabilization assay

Erythrocytes are considered to be an excellent model for membrane stabilization assay. This assay was performed according to the method described earlier (Ananthi and Chitra, 2013). Briefly, 5 ml of blood from healthy human was collected in EDTA vacutainer tube. Then, the blood was centrifuged at 3000 rpm for 10 min and packed RBCs were washed two times with isosaline (0.9 % NaCl) and 10 % suspension of RBCs was made. For membrane stabilization assay, reaction system consisted of 88.9 µl of hyposaline (0.25 w/v NaCl), 44.4 µl of 0.15 M phosphate buffer (pH 7.4) 22.2 µl of 10 % RBC suspension and test solution (half of IC_{50}) in isosaline. For positive control, 44.4 µl of distilled water was added into reaction system instead of hyposaline to produce 100 hemolysis while negative control was lacking RBC suspension. All the tubes were incubated at 37°C for 15, 30, 60 and 120 minutes. At every interval of time, the tubes were centrifuged at 3000 rpm for 20 minutes. The haemoglobin content in the suspension was then estimated using spectrophotometer at 560 nm. The percentage of HRBC membrane stabilization was calculated using following formula:

% Protection= 100 - (Optical density of sample/Optical density of control X 100)

Statistical analysis

Data was analyzed using Graph pad prism 5.01 and expressed as mean ± standard deviation (SD). The continuous variables were tested with one-way analysis of variance (ANOVA) and turkey test. Values of $P>0.05$ were considered not to be significant.

Results

Cytotoxicity assay

To examine the cytotoxic effects of drugs, MTT assay was performed on RAW 264.7 cells. Results of our study revealed that IC_{50} of Trois and volini was 2.82 mg/ml and 1.73 mg/ml, respectively after 24 hrs treatment. For further study half of IC_{50} values were used.

NO assay

Stimulation of RAW264.7 cells with LPS caused 12 to 14 folds increase in nitrite concentration in comparison to LPS unstimulated cells (negative control; LPS-) at 24, 48 and 72 hrs. Following treatment of cells with half of IC50 of Trois led to 80 to 95 % inhibition of NO production in comparison to LPS stimulated cells (positive control; LPS+) at 24, 48 and 72

hrs. Similarly, volini produced 69 to 86 % inhibition of NO in comparison to positive control (LPS+ve) at the same time intervals. When we compared the inhibition percentage of Trois and volini, Trois inhibited 9 to 12 % more NO production (Figure 2).

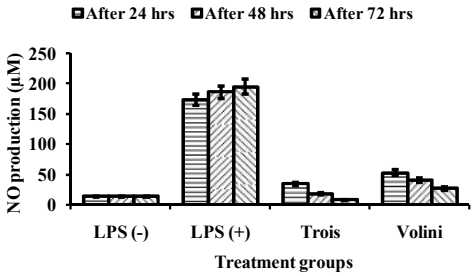

Figure 2: Effect of Trois and volini on NO production in LPS induced RAW264.7 macrophages cell.

The production of NO was assayed in the culture medium of cells stimulated with LPS for 24, 48 and 72 hrs. Values are mean ±SD of triplicate experiments.

MPO assay

Similar to NO, cells stimulated with LPS produced about 9 fold increase in MPO concentration compared to LPS unstimulated cells (negative control; LPS-) at 24, 48 and 72 hrs. Our results showed that Trois markedly inhibited LPS induced MPO production by 62 to 92 % in comparison to LPS stimulated cells (positive control; LPS+) whereas volini produced only 57 to 81 % inhibition of MPO. Trois inhibited 5 to 14 % more MPO production compared to volini (Figure 3).

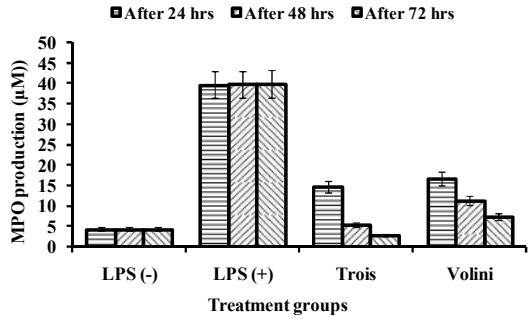

Figure 3: Effect of Trois and volini on MPO production in LPS induced RAW264.7 macrophages cell.

The production of MPO was assayed in the culture medium of cells stimulated with LPS for 24, 48 and 72 hrs. Values are mean ±SD of triplicate experiments.

HRBC assay

Treatment of cells with Trois produced stabilization percentage of 56.8, 85.8, 89.9 and 92.0 % where as volini showed 41.4, 71.0, 76.5 and 83.7% stabilization after 15, 30, 60 and 120 min respectively. From these results, it is clearly evident that Trois had 8 to 15% more membrane stabilization potential compared to volini. Trois showed 15.4% more membrane stabilization potential compared to volini after 15 min indicating onset of action and free radical neutralization is faster in case of Trois (Figure 4).

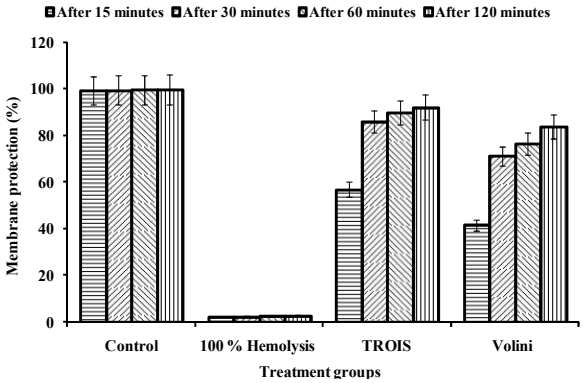

Figure 4: Biomembrane protection percentage.

COX-2 gene expression study

The expression of COX-2 mRNA was elevated in cells treated with LPS (positive control; LPS+) compared to LPS unstimulated cells (negative control; LPS-). Following treatment of cells with

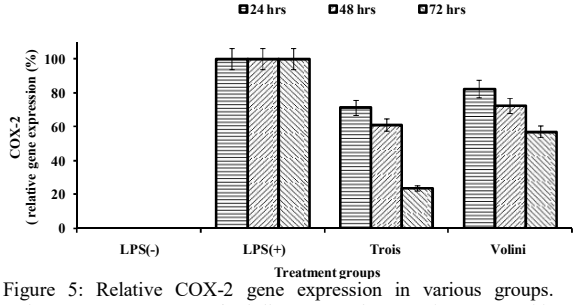

Figure 5: Relative COX-2 gene expression in various groups. Values are mean ±SD of triplicate experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

drugs, Trois produced 28.7, 38.7, 76.2 % reduction in COX-2 m-RNA expression after 24, 48 and 72 hrs of treatment, respectively which correspond to **, $p<0.01$;***, $p<0.001$ and ***, $p<0.001$ in comparision to positive control. On the other hand, volini produced 17.5, 27.5, and 42.8 % reduction in COX-2 mRNA expression after 24, 48 and 72 hrs of treatment, respectively which correspond to *, $p<0.05$;**, $p<0.01$ and ***, $p<0.01$ in comparison to positive control (Figure 5 and Figure 6, A,B &C). Further, a 33.4% more COX-2 inhibition by Trois in comparison to volini after 72 hrs indicating the long acting action of Trois.

Discussion

Increasing number of natural plants have been used across the globe to treat various kinds of acute and chronic diseases (Pan et al., 2006; Bhagyasri et al., 2015; Margaret et al., 2014; Kapewangolo et al., 2015). The natural products which are derived from plants reduce the risk of developing pathological conditions, including cancer, nervous system disorders, cardiovascular, genetic, and inflammatory diseases (Jurenka, 2009; Newman and Cragg, 2007; Chatterjee et al., 2015, Kelly et al., 2014). Various drugs which contain wintergreen oil (Gaultheria precumbens), camphor oil (Cinnamomum camphora), eucalyptus oil (Eucalyptus globulus) and menthol as major ingredients have shown to have anti-inflammatory and analgesic properties (Chippada et al., 2011; Hajhashemi et al., 2003; Salleh et al., 2012; Alsaad et al., 2015; Mota et al., 2015).

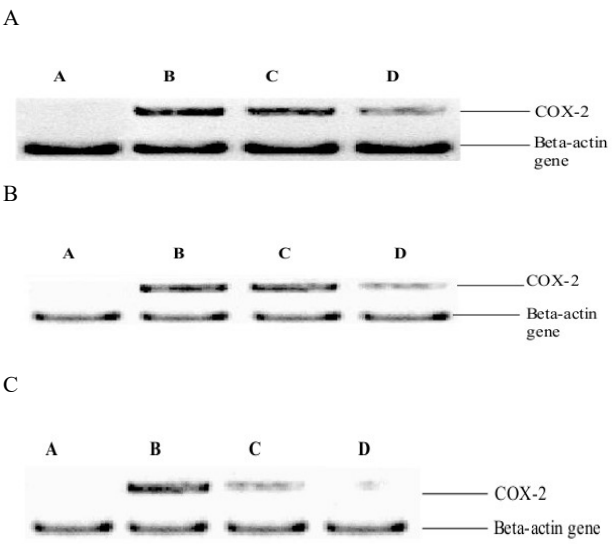

Figure 6: Effect of Trois and volini on the mRNA expression of COX-2 in RAW264.7 cells. A-(A) without stimulation (B) stimulated with LPS (C) stimulated with LPS in presence of volini for 24 hrs (D) stimulated with LPS in presence of Trois for 24 hrs. B- (A) without stimulation (B) stimulated with LPS (C) stimulated with LPS in presence of volini for 48 hrs (D) stimulated with LPS in presence of Trois for 48 hrs. C- (A) without stimulation (B) stimulated with LPS (C) stimulated with LPS in presence of volini for 72 hrs (D) stimulated with LPS in presence of Trois for 72 hrs.

In this study, we established invitro anti-inflammatory activity of Trois, a nanotechnology based herbal product in comparison to volini (a diclofenac and menthol based product) in murine macrophage RAW264.7 cells. It has been reported that LPS induces iNOS transcription and transduction with subsequent NO production (MacmMicking et al., 1997). NO production has been implicated in a variety of pathological processes including various forms of inflammation and inflammatory disorders (Pan et al., 2006; Kitisin et al., 2015). Our results revealed that Trois inhibited 9 to 12 % more LPS induced NO production in RAW cells compared to volini indicating higher anti-inflammatory mechanism of Trois may be through the greater NO production inhibition. Furthermore, Vigo et al. (2004), suggested the role of Eucalyptus globulus in NO inhibition. Moreover, it has been indicated that Apium graveolens which containe apiin inhibit the nitrite (NO) production and also iNOS expression (Mencherini et al., 2007). Naithani et al. (2011) also demonstrated that Trois strongly inhibited the NO production.

MPO plays a central role in oxidant production by neutrophils. Also, it is the most abundant pro-inflammatory enzyme (Pulli et al., 2013; Marlous et al., 2014) which is secreted during inflammatory response and contributing to arthritis development (Breckwoldt et al., 2008; Garcia et al., 2014). Our results clearly showed that Trois inhibited 5 to 14 % more MPO production in RAW cells compared to volini. Previous studies have also demonstrated that many plants may inhibit MPO activity (Nastasijevic et al., 2012; Vieira et al., 2011; Leskovac et al., 2013), therefore, anti-inflammatory effect of Trois may be due to inhibition of MPO. Data indicated that NO has profound effect on COX-2 catalytic activity (Wu 1995). However, the exact mechanism needs to be explored. One possibility is that lipid peroxidation initiated by peroxynitrite the product of the reaction of NO with superoxide, liberates arachidonic acid from the cell membrane which in turn activates COX-2 (Davidge et al., 1995; Esposito and Cuzzocrea 2007). Our data further indicated that Trois exhibited significant down regulation in COX-2 mRNA expression indicating that the action of Trois occurs at transcriptional level. Several studies have demonstrated that drugs targeting COX2 enzyme might provide a better anti-inflammatory activity (Ronchetti et al., 2009; Turini and DuBois 2002).

Erythrocyte membrane is an analogue to the lysosomal membrane (Breckwoldt et al., 2008). Stabilization of membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as proteases which cause further membrane damage up on extracellular release (Murugaesan et al., 1981). Our data demonstrated that Trois has enhanced membrane stabilization potential which is evident by higher protection of erythrocyte membrane from damage.

Conclusions

The anti-inflammatory property of Trois is strongly supported by its ability to reduce the NO, MPO and mRNA of COX-2 production as these are regarded as potent inflammatory mediators at concentration that were not toxic to cells. Therefore, Trois can be termed as more safe topical preparation for management of inflammatory disorders.

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