

In Vitro Penetration Rate and Efficacy of Trois in Arthritis Induced Rat Model

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Abstract: The study was performed to evaluate the comparative efficacy of trois with other anti-inflammatory drugs in arthritis induced rat model. Total fifty four wister rats were selected and divided into nine groups of six rats each. Arthritis was induced in knee joint cavity of the right hind leg by intra-articular injections of 0.1mL of 1% carrageenan in all the groups except control group for 10 days. The control (normal) group was injected with 0.1 ml of saline in knee joint cavity of the right hind leg. After induction of arthritis in all groups, the treatment was started with respective drugs twice a day for 10 days in all groups except control and arthritis induced without treatment group. The blood samples were collected at the end of experiment and various biochemical parameters such as total protein (TP), uric acid, hemoglobin (Hb) and alkaline phosphatase (ALP) were estimated. Antioxidant enzymes such as catalase along with free radical mediated damage malondialdehyde (MDA) were also measured in the blood sample of all groups. Our finding showed that the level of all biochemical parameters were significantly increased as well as MDA level along with decreased level of hemoglobin and catalase enzyme activity in arthritis induced group. After treatment with respective drugs, all biochemical parameters along with free radical mediated damage MDA levels were significantly improved in all treated groups as compared with arthritis induced group. When trois treated group was compared with all other treated groups, these biochemical parameters were showed better improvement in trois treated group. On the basis of findings, we concluded that trois is effective anti-inflammatory drug that improved inflammation, pain along with biochemical alteration and MDA level during arthritis.

Key words: Arthritis, biochemical parameters, free radical, antioxidant enzymes, trois.

INTRODUCTION

Chronic pain due to arthritis or neuropathy is a major clinical problem globally. Arthritis is a chronic progressive autoimmune disorder characteristic by symmetric erosive synovitis (Recklies *et al.*, 2000). The exact etiology of arthritis remains unknown. It has assumed that either a foreign agent or some alteration in control of cellular responses is involved in the chronic persistent synovial inflammation (Krone and Simon, 1986). Carrageenan has been used as a model of chronic inflammation in the rats and it is a considerable relevance of pathophysiological and pharmacological control of inflammatory processes, as well as the evaluation of analgesic potential and anti-inflammatory effect of drugs.

Arthritis is characterized by persistent of inflammation in the synovial membrane of joint, associated with migration of activated phagocytes and other leukocytes into synovial and periarticular tissue (Mulherin *et al.*, 1996). During phagocytosis, monocytes, neutrophils and macrophages generate superoxide radicals, hydrogen peroxide and the highly reactive hydroxyl radicals (Rowley *et al.*, 1984). These cytotoxic reactive oxygen species (ROS) may cause oxidative damage in the cells (Parke and Sapota, 1996). Activated oxygen intermediates together with highly reactive radicals, such as the hydroxyl radicals, are able to destroy membrane lipids, proteins, deoxyribonucleic acid, hyaluronic acid, and cartilage (Biernacki *et al.*, 1986). In normal cell, there is proper balance between oxidant and antioxidant system. Any imbalance between these system may causes several kinds of pathophysiological state.

Trois is a massage micro-emulsion that contain wintergreen oil (*Gaultheria procumbens*), Camphor oil (*Cinnamomum camphora*), Eucalyptus oil (*Eucalyptus globulus*) and menthol as major ingredients. These

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ingredients have an anti-inflammatory and analgesic properties. The aim of present study was to evaluate the comparative penetration rate and efficacy of trois with other anti-inflammatory drugs in arthritis induced rat model.

MATERIALS AND METHODS

Chemicals:

All of the biochemical reagents used in the present study were procured from Sigma, St. Louis, MO (USA). Other chemicals purchased locally were of analytical grade.

Drugs:

Newly formulated trois drug was obtained from Venus Remedies Ltd, Baddi, H.P. India. Six other drugs were purchased from a pharmacy (Chandigarh) and given in coded form as A, B, C, D, E and F respectively (Table 1).

Table 1: Active ingredients of various coded form drugs

S. No.	Codes	Form	Active Ingredients
1	A	Ointment	Wintergreen oil (Methyl salicylate)
2	B	Gel	Wintergreen oil & Menta arvensis
3	C	Ointment	Wintergreen oil & Menta arvensis
4	D	Gel	Methyl salicylate & Aceclofenac
5	E	Gel	Methyl salicylate & Diclofenac
6	F	Gel	Methyl salicylate & Diclofenac
7	Trois	Microemulsion	Wintergreen oil, Camphor oil and Eucalyptus oil

Appendix 1: Skin irritation Score Card System

Skin reaction	Score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grade of erythema	4
Edema Formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edge of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm extending beyond the area of exposure)	4

Skin Irritation Study:

Total six male wister rats (weighting 100 to 105 gm) were selected for the testing of skin irritation. All the animals were maintained on standard feed and had free access to water. The animals were kept under standard condition. The animals were anesthetized by ketamine injection (10mg/100g of body weight). The hair on the back of the animals were shaved with sterilized razor blades and wiped with methylated spirit and area of 1.0 cm² was marked on the both sides, one side served as control group and other side was test group. 0.25 ml Trois drug was applied topically twice a day for seven days and site was observed for any sensitivity and any reaction. The adopted score card system for skin reactions are shown in appendix 1.

In vitro Anti-inflammatory Study:

The *in vitro* anti-inflammatory activity of trios and other drugs were performed in carrageenan induced arthritis induced rat model. The hind paw edema was measured in arthritis induced rat group as well as in all treated group and compared with control normal saline group. The hind paw volume was measured every three hours on 7th and 10th day in arthritis induced group as well as in all treated groups by using a plethysmograph.

In Vitro Diffusion Profile Study:

Comparative release of methyl salicylate from various drugs were studied according to the permeation apparatus described by Kumar *et al* 2009. A permeation cell was designed in our laboratory. A glass cylinder with both side open, 10.5 cm height and 3.5 cm outer diameter was used as a permeation cell. A cellophane membrane (0.8 µm pore size, cut to suitable size, boiled in distilled water for 1 hour and soaked in phosphate buffer pH 7.4) was fixed to one end of the cylinder by adhesive type. One gram of every coded form drug

was taken in the permeation cell (donor compartment) and the cell was immersed in a beaker containing 150 ml phosphate buffer of pH 7.4 (receptor compartment). The cell was immersed in to a depth of 1 cm below the surface of buffer, which was agitated by a magnetic stirrer and the temperature was maintained at 37 °C ±1 °C throughout the experiment. Suitable aliquots were withdrawn from the receptor compartment periodically (0, 5, 10, 15, 20 and 25 minutes) and methyl salicylate concentration was determined by high performance liquid chromatography(HPLC).

Analysis:

Apparatus:

Chromatographic separation was performed on Agilent 1200 series liquid chromatographic system equipped with G1311A quaternary pump, Agilent variable UV/visible detector and a G1329A auto injector. EZ Chrome Elite software was employed for data collecting and processing.

Chromatographic Conditions:

Prepare a filter and degassed solution of 0.1ml of (0.1%) orthophosphoric acid in 100 ml of distilled water. Mobile phase used in the analysis was buffer and acetonitrile in ratio of 40 :60. The mobile phase was passed through membrane filter (Millipore corp.), 0.45 µm pore size and de-aerated under reduced pressure. Standard preparation : 25.0 mg methyl salicylate was dissolved in 25 ml mobile phase (stock solution). Take 5.0ml of stock solution and diluted up to 25 ml with mobile phase

Methyl Salicylate Analysis:

For the analysis of analysis MS, take 200 µl supernatant and added 150 µl of mobile phase and shaken vigorously. The chromatographic separation of MS was performed by high performance liquid chromatography with a mobile phase containing buffer and acetonitrile. A column C-18 hypersil ODS (5 µ, 4.6 x 250 mm) was used for the analysis of active ingredient methyl salicylate. The flow rate and column temperature were maintained at 1.2 ml/min at 25°C respectively. After an equilibration of column with mobile phase for 2 hour, 20 µl of sample was injected and detection of methyl salicylate was performed at 280 nm UV wavelength. Under these chromatographic conditions, the retention time of methyl salicylate was found to be 4.5 minute

In Vivo Experiment Design:

Induction of Arthritis and Treatment:

Total fifty four wister rats (weighting 105-110 gm) were randomly selected and divided into nine groups of six rats each. They were housed at controlled temperature and humidity in an alternating 12-hr light and dark cycle with free access to food and water. The study was approved by the institutional animal ethical committee. The arthritis was induced in knee joint cavity of the right hind leg by intra-articular injections of 0.1mL of 1% carrageenan in all groups except control group. The animals were given as below.

- Group I (n=6) Control normal saline treated
- Group II (n=6) arthritis induced (0.1mL of 1% carrageenan)
- Group III (n=6) arthritis induced plus A treated group
- Group IV (n=6) arthritis induced plus B treated group
- Group V (n=6) arthritis induced plus C treated group
- Group VI (n=6) arthritis induced plus D treated group
- Group VII (n=6) arthritis induced plus E treated group
- Group VIII (n=6) arthritis induced plus F treated group
- Group IX (n=6) arthritis induced plus Trois treated group

After induction of arthritis in all groups for 10 days, the drugs were immediately applied topically twice a day for 10 days, except group I & II. 1.0 ml blood samples were collected in sodium citrate containing vials by retro-orbital sinus on 7th and 10th day for estimation of biochemical parameters and antioxidant enzyme activity in all groups.

0.1 ml blood samples were diluted 10 times with chilled distilled water, left for at least 1 hr at 0- 4°C, before the estimation of enzyme assay and rest part of samples were centrifuged at 6600 rpm for 15 minutes and plasma was aspirated out for the measurement of biochemical parameters.

Biochemical Parameters:

Uric acid, ALP and total protein were estimated by using commercially available diagnostic kits (Bayer Diagnostics India Ltd, Baroda, Gujrat India).

Estimation of Catalase Assay:

Catalase enzyme activity was measured by the method of Luck *et al.*, (Luck 1957). The reaction mixture consisted of 0.3 ml phosphate buffer, (0.2M pH 6.8), 0.02 ml H₂O₂ (1M) and water to make the final volume to 3.0 ml. The reaction was started by adding the 5 µl of diluted blood sample. The change in the absorbance was recorded at 15 sec. interval for one minute at 240nm at 25°C. Suitable control was run simultaneously. One Unit of enzyme activity was defined as the amount of enzyme that liberates half of the peroxide oxygen from H₂O₂ in 100 sec at 25°C.

Measurement of Free Radical Mediated Damage:

Free radical mediated damage was assessed by the measurement of the extent of lipid peroxidation in the term of malondialdehyde (MDA) formed, essentially according to Ohkawa *et al.* (1979). It was determined by thio barbituric reaction. The reaction mixture consisted of 0.20 ml of diluted blood, 0.20 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of (20%, pH 3.5) acetic acid, 1.5 ml of 0.8% thio barbituric acid (TBA) and distilled water to make up the final volume of 4.0 ml. The tubes were boiled in water bath at 95 °C for one hour and cooled immediately under running tap water. This was followed by the addition of 1.0 ml of water and 5.0 ml of mixture of n-butanol and pyridine (15:1 v/v) was added and the mixture was vortexed then the tubes were centrifuged at 3500 rpm for 20 minutes. The upper layer was aspirated out and optical density was measured at 532 nm. The reference standard used was 1,1, 3,3 tetraethoxypropane.

Statistical Analysis:

The resulting data was analyzed statistically. All values are expressed as mean ± SD. One-way analysis of variance (ANOVA) with student-Newman-Keuls comparison test was used to determine statistical difference between control *vs* arthritis induced group and arthritis induced group *vs* treated groups. p values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Effect of Trois on Skin Irritation:

Animals treated with trois did not show any toxic skin reaction for 1 to 7 days following the exposure of the test substance on the testing sites. The skin irritation was calculated as zero. The skin irritation value is zero, therefore, it is concluded that the trois, was non-irritant to skin of rats. In the study, there was no any changes in skin such as edema, erythema and eschar formation after twice daily topical application for seven days treatment in animal. The results are represented in table 2.

Table 2: Skin Irritation study of trois in wister rat model

<i>Skin reaction of trois for seven day</i>		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day7
Animals (n=6)	Symptoms							
	Erythema	0	0	0	0	0	0	0
	Eschar formation	0	0	0	0	0	0	0
	Edema	0	0	0	0	0	0	0

Skin Irritation Index = 0

Diffusion Profile:

In the present study, *in vitro* release pattern of methyl salicylate was obtained higher in trois drug at different time intervals (0,5,10,15,20 and 25 minutes) in comparison to other respective drugs at same time intervals. The maximum concentration of methyl salicyate (1.69 µg/ml) was found higher in trois drug in comparison to other respective drug. The result are represented in figure 1 and chromatograms (1-9).

Body Weight Changes in Arthritis Induced Animals:

In the present study, there was slight increased in body weight of arthritis induced group on 7th and 10th day, after administration of carrageenan when compared with control normal saline group. After administration of various drugs topically twice a day for 10 days treatment, the body weight was gradually decreased in all arthritis induced plus treated groups on 7th and 10th day as compared with arthritis induced group. The body weight of trois treated group was found to be significantly lowered and comes near to control group in comparison to other treated groups on 7th and 10th day treatment. The results are represented in table 3.

In vitro diffusion profile of various drugs as well as trois drug

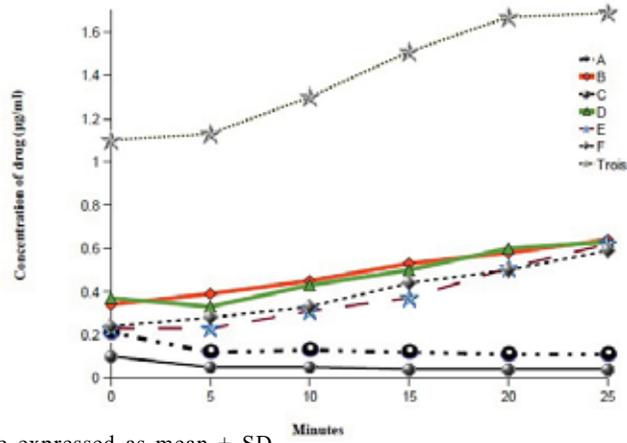
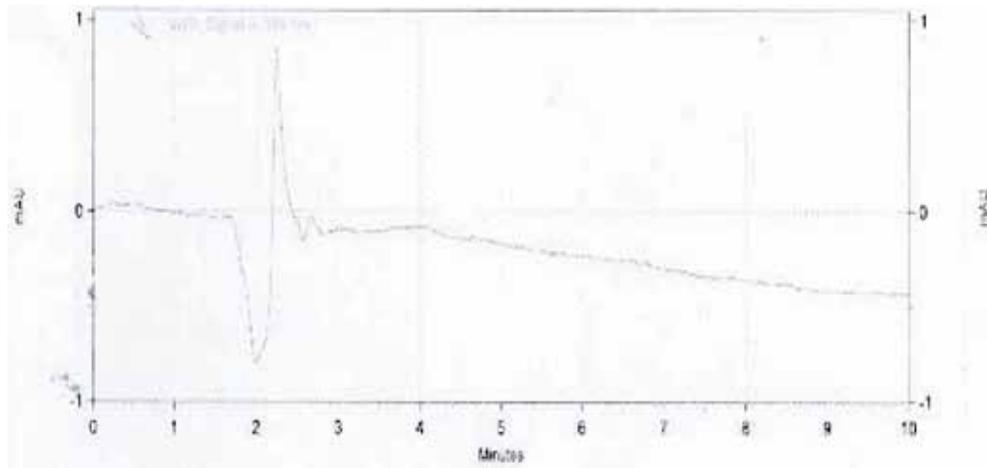
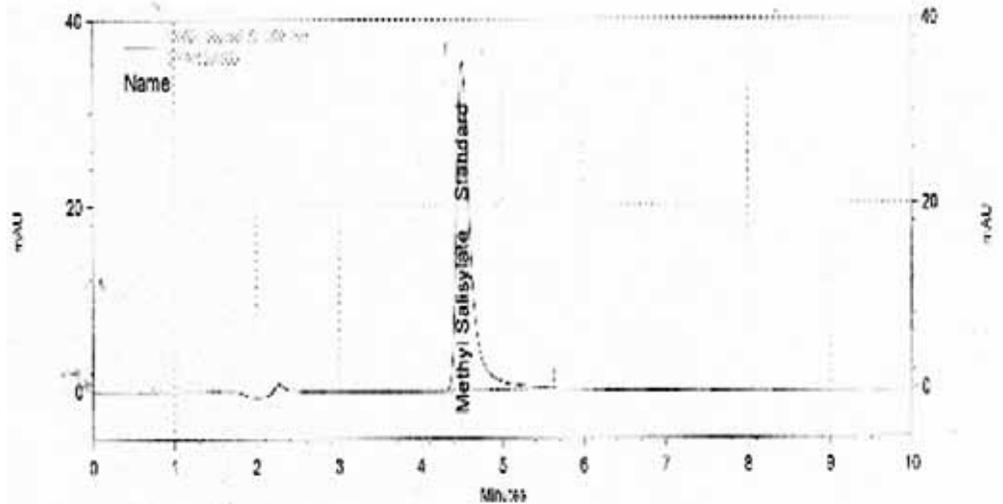


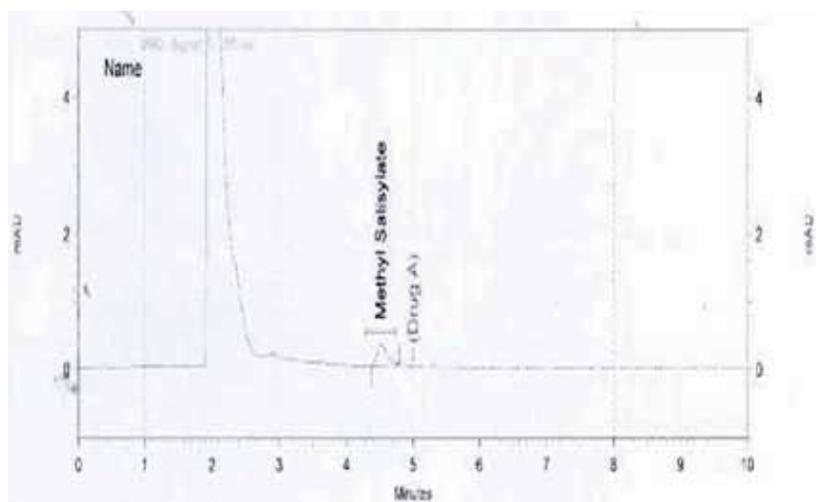
Fig. 1: All values were expressed as mean \pm SD.



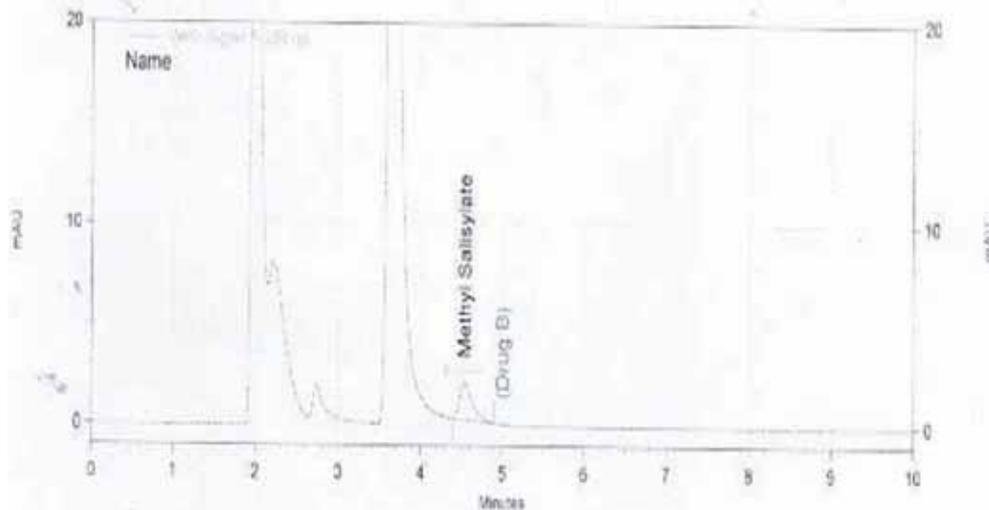
Chromatogram 1: Blank



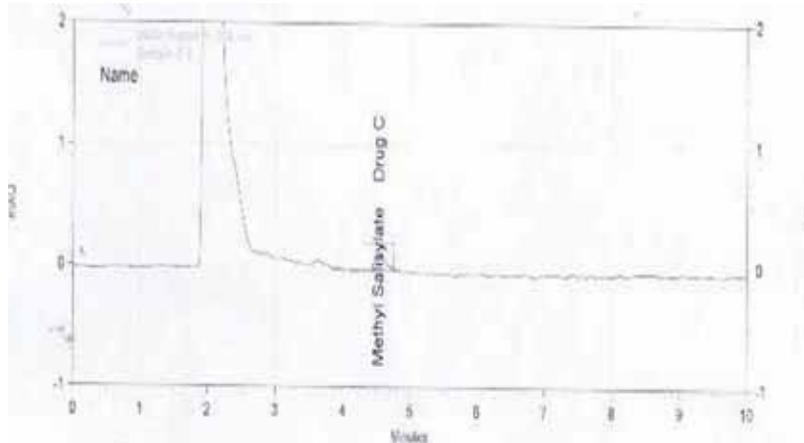
Chromatogram 2: Standard peak of methyl salicylate with retention time 4.5 minutes.



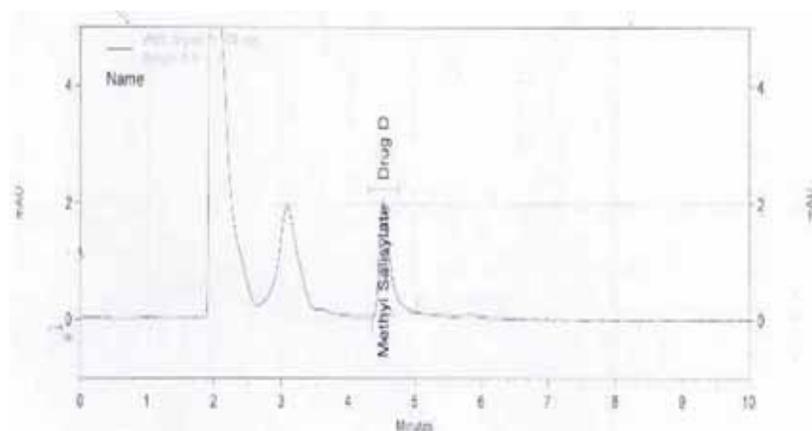
Chromatogram 3: Peak of methyl salicylate in drug A with retention time 4.5 minutes.



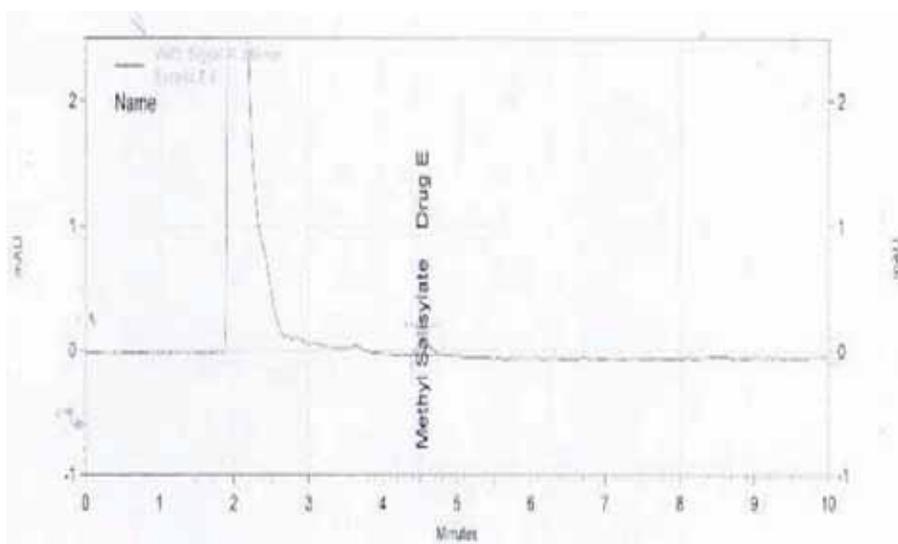
Chromatogram 4: Peak of methyl salicylate in drug B with retention time 4.5 minutes.



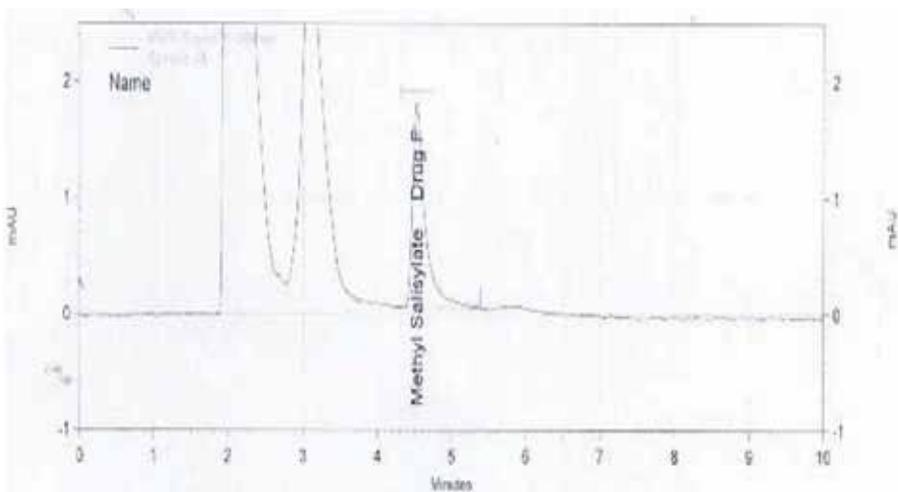
Chromatogram 5: Peak of methyl salicylate in drug C with retention time 4.5 minutes.



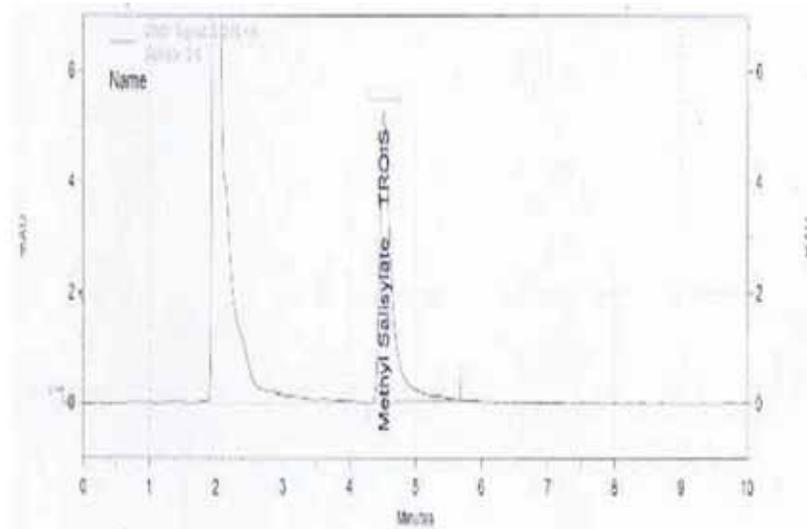
Chromatogram 6: Peak of methyl salicylate in drug D with retention time 4.5 minutes.



Chromatogram 7: Peak of methyl salicylate in drug E with retention time 4.5 minutes.



Chromatogram 8: Peak of methyl salicylate in drug F with retention time 4.5 minutes.



Chromatogram 9: Peak of methyl salicylate in Trois drug with retention time 4.5 minutes.

Knee Diameters in Arthritis Induced Animals:

There was significantly increased in the knee diameter of right leg of arthritis induced groups as compared with control group after administration of carrageenan for 10 days. After treatment with respective drugs twice a day for 10 days, the diameter of knee was found to be reduced in all treated groups as compared with arthritis induced group. When trois treated group was compared with other treated groups, the knee diameter was significantly lowered in trois treated groups on 7th and 10th day treatment and comes near to control normal saline treated group (Fig.2)

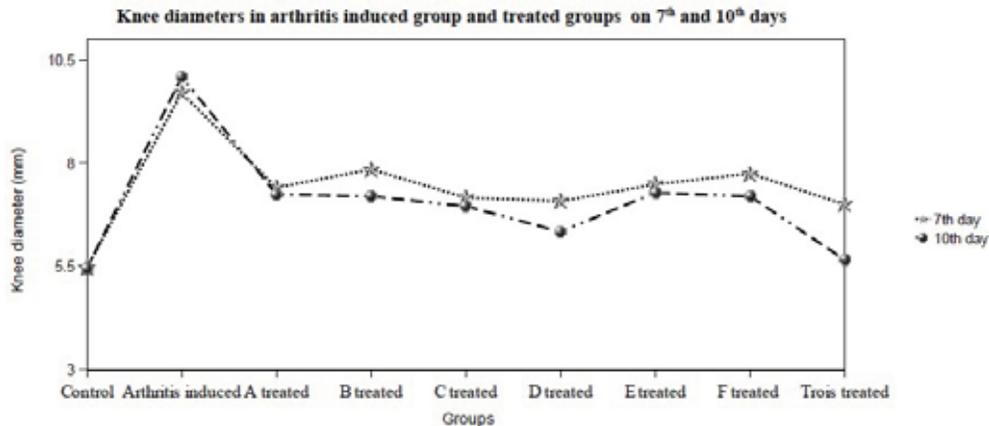


Fig. 2: All values were expressed as mean ± SD. The figure showed that the knee diameters were significantly reduced in trois treated group in comparison to other treated groups on 7th and 10th day. Each groups contain total six numbers of animals.

Paw Volume Changes in Arthritis Induced Animals:

Paw volume was found to be significantly higher in arthritis induced group as compared with control normal saline treated group after administration of carrageenan on 7th and 10th day. After topical administration of respective drugs for 10 days treatment, the paw edema was markedly decreased in all arthritis induced plus treated group on 7th and 10th day treatment as compared to arthritis induced group. On comparative study of trois treated group with other treated groups, the paw edema was found to be significantly lowered in trois treated group (Fig 3).

Table 3: Measurement of body weight in arthritis induced group as well as in treated groups

Mean body weight (gm)			
S. No	Groups	7 th day	10 th day
1	Control (n=6)	106.15 ± 2.48	106.93 ± 2.20
2	Arthritis induced (n=6)	129.48 ± 5.09	137.05 ± 4.40
3	A treated (n=6)	126.35 ± 2.46	125.16 ± 2.28
4	B treated (n=6)	121.95 ± 1.97	119.81 ± 1.13
5	C treated (n=6)	120.82 ± 2.23	117.92 ± 2.09
6	D treated (n=6)	119.29 ± 1.34	118.50 ± 0.68
7	E treated (n=6)	117.65 ± 3.89	116.78 ± 3.09
8	F treated (n=6)	118.41 ± 3.30	117.49 ± 3.77
9	Trois treated (n=6)	109.49 ± 2.34	107.68 ± 2.43

All values were expressed as mean ± SD. N = numbers of animals in each groups.

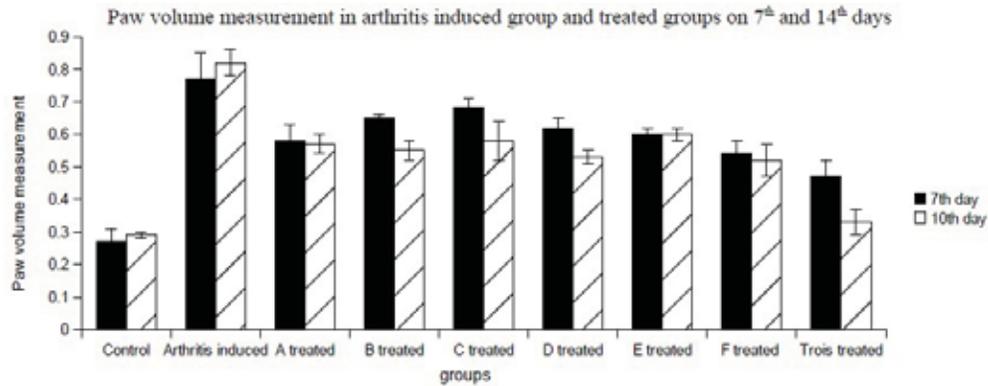


Fig. 3: All values were expressed as mean ± SD. The figure showed Paw volume was elevated in arthritis induced group as compared to treated groups on 7th and 10th day. The volume was decreased in trois treated group in comparison to other treated group Each groups contain total six numbers of animals.

Change in Hemoglobin Level:

There was statistically insignificant ($p > 0.05$) decreased hemoglobin level in arthritis induced group as compared with control normal saline treated group on 7th and 10th day. When arthritis induced group was compared with all respective treated groups on 7th and 10th day, the level of hemoglobin was increased but insignificant ($p > 0.05$) in all treated groups. When trois treated group was compared with other treated groups, the hemoglobin level was elevated in trois treated group on 7th and 10th day (Fig 4).

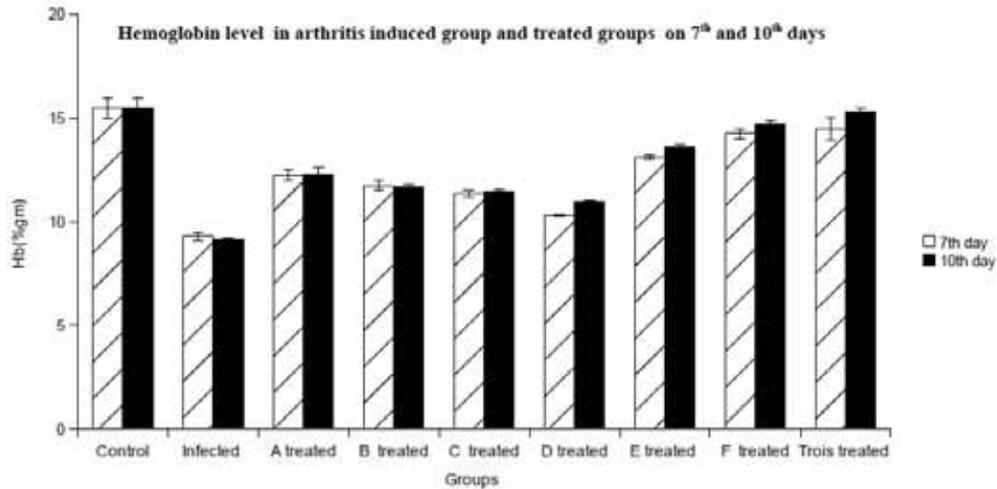


Fig. 4: All values were expressed as mean ± SD. The figure showed that the hemoglobin were significantly elevated in trois treated group in comparison to other treated groups on 7th and 10th day. Each groups contain total six numbers of animals.

Change in Alkaline Phosphatase Total Protein, and Uric Acid Levels:

The levels of protein as well as uric acid were significantly increased ($p < 0.001$) in arthritis induced group as compared with control group on 7th and 10th day. When arthritis induced group was compared with respective treated groups, the protein and uric acid level were decreased ($p < 0.001$) significantly in all treated group after treatment with respective drugs. The level of alkaline phosphatase also significantly increased in arthritis induced group as compared to control normal saline treated group. After treatment with respective drugs, ALP level was found to be lowered ($p < 0.001$) in all treated group as compared with arthritis induced group. When trois treated group was compared with all other treated groups on 7th and 10th day, the level of these above parameters were significantly improved in trois treated group (Fig 5-7).

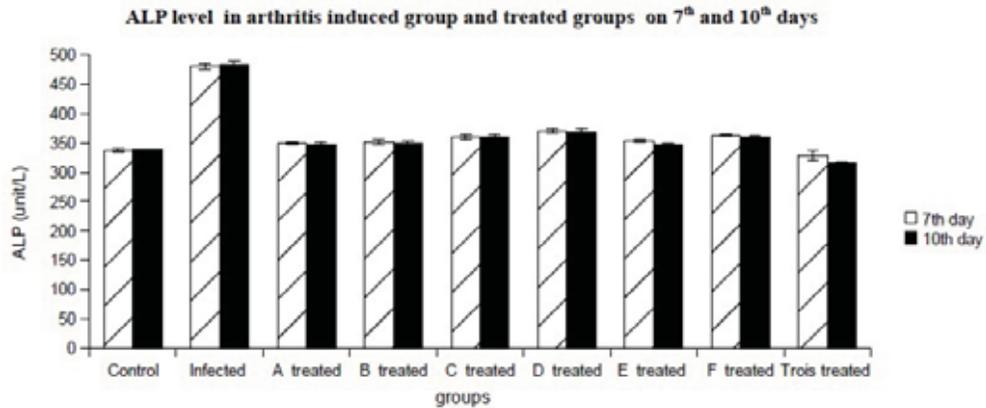


Fig. 5: All values were expressed as mean \pm SD. The figure showed that the alkaline phosphatase level was significantly lowered in trois treated group in comparison to other treated groups on 7th and 10th day. Each groups contain total six numbers of animals.

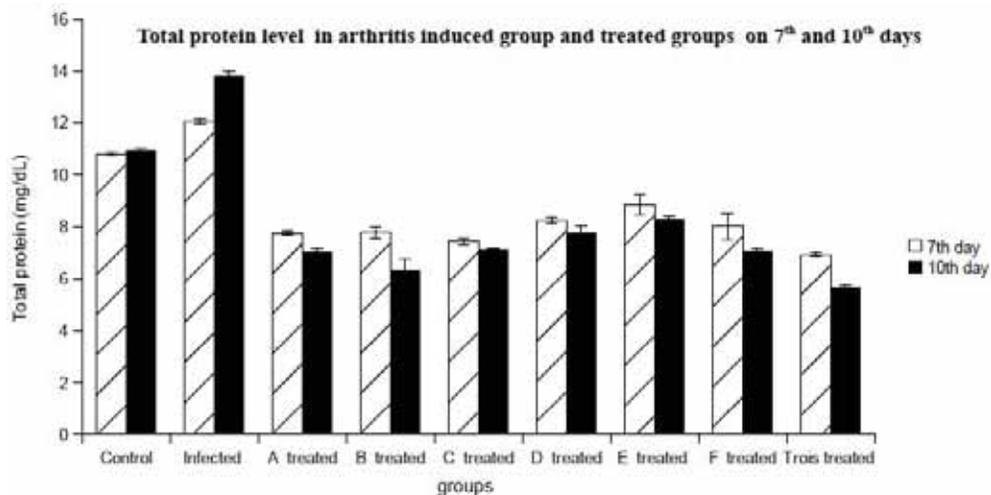


Fig. 6: All values were expressed as mean \pm SD. The figure showed that the total protein level was significantly lowered in trois treated group in comparison to other treated groups on 7th and 10th day. Each groups contain total six numbers of animals.

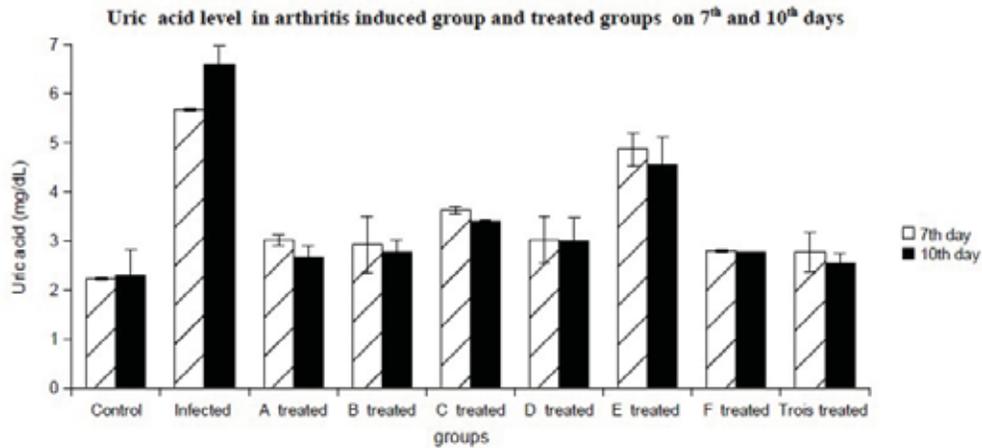


Fig. 7: All values were expressed as mean \pm SD. The figure showed that the uric acid level was significantly lowered in trois treated group in comparison to other treated groups on 7th and 10th day. Each groups contain total six numbers of animals.

Changes in Catalase Enzyme Activity and MDA Level:

There was significant ($p < 0.001$) decreased in catalase enzyme activity along with significantly ($p < 0.001$) increased malonalldialdehyde level in arthritis induced group on 7th and 10th day as compared with control normal saline treated group. After topical application of respective drugs, the level of malonalldialdehyde along with catalase enzyme activity were significantly ($p < 0.001$) improved in all treated group as compared with arthritis induced group. When all treated groups were compared with trois treated group, these parameters were significantly improved in the trois treated group on 7th and 10th day treatment (Fig 8-9).

Status of catalase enzyme activity in arthritis induced group and treated groups on 7th and 10th days

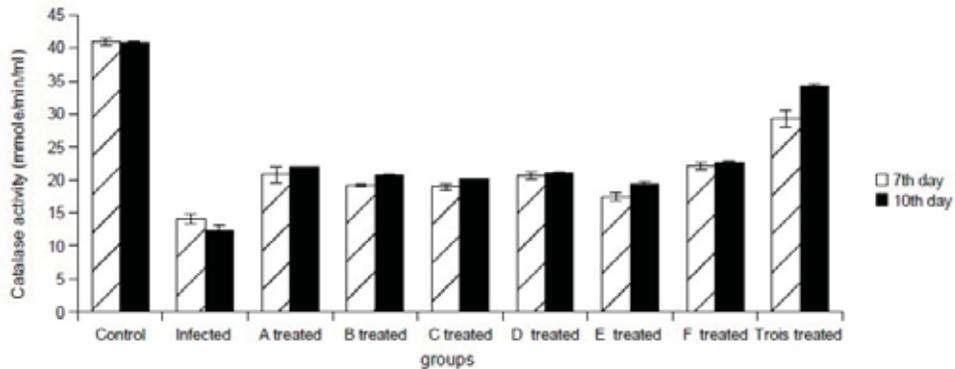


Fig. 8: All values were expressed as mean \pm SD. The figure showed that catalase enzyme activity was significantly increased in trois treated group in comparison to other treated groups on 7th and 10th day. Each groups contain total six numbers of animals.

Discussion:

Arthritis is a chronic inflammatory disorder and the inflammation involves the release of mediators like cytokines, interferons and PGDF that are responsible for the pain, destruction of bone and cartilage that can lead to severe disability (Eric and Lawrence, 1996). The determination of paw swelling is apparently simple, sensitive and quick procedure for evaluating the degree of inflammation and assessing of therapeutic effects of drugs. In the present study, the paw swelling was observed higher in arthritis induced group after

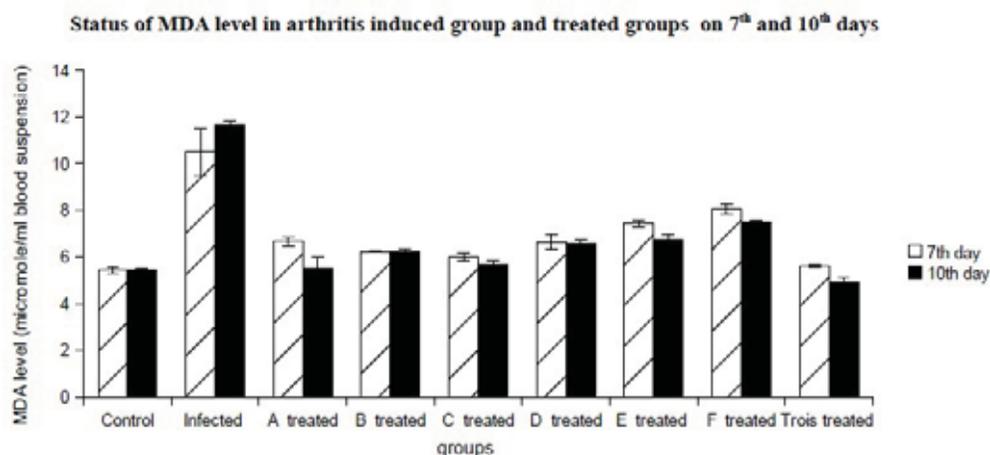


Fig. 9: All values were expressed as mean \pm SD. The figure showed that MDA level was significantly lowered in trois treated group in comparison to other treated groups on 7th and 10th day. Each groups contain total six numbers of animals.

administration of 1% carrageenan injection. After topically administration of respective drugs for 10 days, the paw swelling was reduced significantly in all arthritis induced plus treated groups. Paw swelling was found be significantly reduced in trois treated group in comparison to other treated drugs. The swelling was reduced due to fast release of methyl salicylate from trois drug. Trois is a microemulsion that contains winter green oil, in which 98% methyl salicylate is a major constituent. Methyl salicylate show anti-inflammatory and analgesic activity that is responsible for inhibition of platelet aggregation along with increased blood flow. It also inhibit the prostaglandin synthesis. In the present study, the concentration of methyl salicylate was found higher in comparison to other drugs by the study of *in vitro* diffusion.

Arthritis is partly related to the excessive generation of reactive oxygen species (free radical). There are complex network relationships among super-oxide anion, neutrophils and inflammatory cytokines, and they are more complicated *in vivo*. Neutrophils can be primed and activated by interleukin (IL- β) and tumor necrosis factor (TNF- α) to immigrate to inflamed sites which release the active oxygen species (Filippin *et al.*, 2008). It has been clinical evidenced that the level of antioxidant enzymes were decreased along with increased in the oxidative stress in arthritis induced patients (Karatas *et al.*, 2003; Vijayakumar *et al.*, 2006). In our studies the level of free radical mediated damage malondialdehyde (MDA) were increased along with decreased catalase enzyme activity in arthritis induced group as compared with control normal saline treated group. After treatment with respective drugs for 10 days, the level of MDA and catalase were significant improved on 7th and 10th day as compared with arthritis induced group. When trois treated group was compared with other groups the free radical mediated damage along with antioxidant enzyme activity were improved in trois treated group than other treated groups. Catalase enzyme activity in the erythrocytes is to protect hemoglobin against oxidation. The main function of catalase enzyme is detoxification of hydrogen peroxide. This result indicates that trois act as a free radical scavenger. Anemia is a commonly noted in chronic arthritis patients (Glen *et al.*, 1977). In our study, the level of hemoglobin was decreased in arthritis induced group as compared with control group. After treatment with all drugs, the hemoglobin level was found increased in all treated groups but in case of trois treated group, it was found higher in comparison to other groups. The hemoglobin level was decreased due to changes in bone marrow during arthritis that prevents the release of iron for incorporation into red blood cells. Several researchers have been reported that the hemoglobin level was decreased in arthritis (Jaijesh *et al.*, 2009). The level of alkaline phosphatase, total protein and uric acids were significantly increased in arthritis induced group as compared with control group. After administration of respective drugs on 7th and 10th day, these levels were improved in all induced plus treated groups. These levels were significantly improved in trois treated group as compared to other treated groups. Alkaline phosphatase activity has been reported to increase during the morphological and functional development of bone. It is a present mainly in blood vessel, pia arachnoid and choroid plexus. Active bone resorption is accompanied by concomitant bone formation and rise in serum alkaline phosphatase. There are several report suggesting that the ALP level was increased in arthritis condition (Raj Kapoor *et al.*, 2007). Uric acid was increased due to

alteration in purine metabolism. Excessive release of uric acid in the blood causes gout. Arthritis also causes alteration in the protein level that causes inflammatory response. Chemistries are normal in arthritis with a exception of a slight decrease in albumin and increase in total protein level reflecting the chronic inflammatory process. In the present study, the level of total protein was increased. It suggests that protein level increases due to inflammatory process. Trois contain major ingredient such as wintergreen oil (*Gaultheria procumbens*), Camphor oil (*Cinnamomum camphora*), Eucalyptus oil (*Eucalyptus globulus*) and menthol.

On the basis of above findings, it has been concluded that trois has fast skin penetration rate without any adverse effect which lead to reduces inflammation and pain along with decreased oxidative stress as well as improves antioxidant enzyme activity as well as other biochemical parameters in the arthritis.

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