6. Chapter 3

Anti-inflammatory activity of different extracts of *S. zeylanica* (L.) and *S. ovalifolia* (Roxb.) in Wistar Albino Rats

6.1. Introduction

Inflammation is a type of primary non specific immune response in which the body react to any kind of infection, irritation or injury (Ryan and Majno, 1977; Henson 2005). *Calor, rubor, tumor, dolor* and *functio laesa* are the five cardinal signs of inflammation. First four were proposed by Celsus as long as 2000 years ago. *Tumor* is the swelling of the tissue, *Calor* is the elevated tissue temperature, *rubor* is the redness in the affected area due to increased vascularisation and *dolor* is the intensive sensation of a noxious stimulus and *functio laesa* is the impaired function of the organ affected (Rather, 1971). The American Pathologist Menkin (1960) defined *proteolysis* as the sixth cardinal and essential biochemical sign of inflammation.

There occurs a rise in the osmotic pressure due to disturbed metabolic balance in favour of catabolism. This allows influx of extra fluid into the tissue causing oedema. This is followed by an elevation in the tissue temperature on its way out of the body owing to free heat liberated from lytic and other exothermic reactions such as decarboxylation, deamination or glucose fermentation. Oedema can also appear even in the absence of a noticeable *calor* and *dolar* which may be commonly due to direct mechanical pressure of oedematous fluid on surrounding nerves or action of some mediators or as a result of direct irritant action of the causative factor. Accumulation of red blood cells due to increased vascular permeability and/or arteriolar dialation results in rubor (Stankov, 2012).

Inflammations contribute to disease by damaging the very tissues it has evolved to protect. Apparently unrelated disorders such as asthma, alzheimer, multiple sclerosis, inflammatory bowel diseases and rheumatoid arthritis have common inflammatory elements that trigger the disease process. These signs are due to extravasation of plasma and infiltration of leukocytes into the sites of inflammation.

It is evident that at the site of injury, several vasodilating mediators and leukocyte chemotactic factors are produced and induce vasodilation and recruitment of leukocytes to the affected area thereby establishing inflammatory reactions. Types of leukocytes infiltrating to the site of inflammation depends on the types, doses of and time intervals from the tissue injury, raising the possibility of the presence of cell type-specific leukocyte chemotactic factors. Several leukocyte chemotactic factors such as C5a, N-formyl peptides derived from bacteria and leukotriene B4 are known. These assumptions lead to the discovery of a large number of low molecular weight chemokines that exhibit cell type-specific leukocyte chemoattraction (Vane and Botting, 1987).

Inflammatory responses take place in different phases each mediated by different mechanisms. The acute transient phase is characterised by local vasodilation and increased capillary permeability. Infiltration of leukocytes and phagocytic cells occurs in the sub chronic phase. In the chronic proliferative phase, tissue degeneration and fibrosis occurs (Vogel, 2002).

Different animal models are used to study the mechanisms behind and signs of inflammation. Erythema are the very earliest sign of inflammation unaccompanied by plasma exudation and oedema and were well studied using UV induced erythema models in guinea pigs. The development of elevated PGE levels corresponds to the development of the delayed phase of erythema. Prostaglandin E (PGE) levels in the skin have been shown to be elevated during the 24 h period following exposure of guinea pig skin to

ultraviolet radiation from 280-320 nm. This model illustrates the delay in development of UV erythema on Albino guinea pig skin by systemic pre-treatment with clinically equivalent doses of phenylbutazone and other NSAIDs (Patel, 2012).

Vascular permeability testing in rats is used to evaluate the inhibitory activity of drugs against increased vascular permeability which is induced by phlogistic substances. The increase of permeability in Wistar Albino rats can be documented by the infiltration of the injected sites of the skin with the vital dye Evan's blue. Reduction in the vascular permeability by test drugs is indicated by decreased concentration of dye. The quantitative evaluation of the topical and systemic anti-inflammatory activity of a compound following topical administration is determined by a model of delayed contact hypersensitivity called Oxazolone-induced ear oedema in mice. The sensitization with Oxazolone increases the level of Th2 cytokines such as IL-4 and decrease the level of Th1 cytokine in the lesioned skin. Ear oedema induced with Croton-oil is also used to study the activity of anti inflammatory agents on topical application in rats and mice (Patel, 2012).

Granuloma pouch technique in Sprague-Dawley rats is a model of inflammation in which there is a possibility of bringing the test compounds into direct contact with the target cells by injecting them into a subcutaneous air pocket. When an irritant is introduced into a subcutaneous air pocket, granulation tissue begins to proliferate which consists of fibroblasts, endothelial cells, an infiltrate of macrophages and polymorphonuclear leukocytes and gradually covers the inside of the pouch. The major disadvantage of the model is that it does not provide quantitative information on cytotoxicity of the test compounds in vivo (Patel, 2012).

Carrageenan-induced pleurisy in rats is an excellent acute inflammatory model to study the fluid extravasation, leukocyte migration and the various biochemical

parameters. Pleurisy can be induced by several irritants like histamine, bradykinin, prostaglandins, mast cell degranulators, dextran, enzymes, antigens, microbes and nonspecific irritants like turpentine and carrageenan. A single intrapleural injection of 0.1 ml of carrageenan (1%) induces pleurisy in mouse or rats. After 4 hours, the animals are sacrificed under ether anaesthesia. The thorax was opened and the pleural cavity was washed with 1.0 ml of sterile Phospho Buffer Saline (PBS) containing heparin (20 IU per ml). Samples of the pleural wash were analysed for the determination of exudation, myeloperoxidase, adenosine-deaminase activities, nitric oxide and total and differential leukocyte count (Patel, 2012).

The ability of the test drug to reduce oedema formation followed by the administration of a phlogistic agent in the rat hind paw was measured by using rat paw oedema methods. A variety of phlogistic agents are used to induce paw oedema in rats namely Brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, aerosil, sulfated polysaccharides like carrageenan or naphthoylheparamine etc (Patel, 2012).

The proliferative phase of inflammation was studied by using cotton wool granuloma and glass rod granuloma. In cotton wool granuloma, subcutaneous implantation of pellets of compressed cotton produces giant cells and undifferentiated connective tissue along with the fluid infiltration. When cotton pellets are impregnated with carrageenan, more intensive granuloma formation can be observed. Newly formed connective tissue can be measured by weighing the dried pellets after removal.

Along with the wet and dry weights or newly formed connective tissue, chemical composition and mechanical properties can be studied extensively using glass rod granuloma model of inflammation. Glass rods with a diameter of 6 mm and length of 40 mm are introduced into a subcutaneous tunnel in the caudal region in cranial direction with a closed blunted forceps and the wound is closed by sutures. The animals are treated

with test drugs and control drugs during whole period of the study of 20 or 40 days or during the last 10 or 20 days. At the end of the study, the glass rods together with the surrounding connective tissue were removed under ether anaesthesia and the glass rod is extracted from the granuloma. The wet and dry weights of granuloma were measured and the amount of collagen and glycosaminoglycans were determined by biochemical analysis (Vogel, 2002).

A number of medicinal plants are pharmacologically investigated for their anti inflammatory properties by means of in vivo and in vitro studies. These studies extensively helped to demonstrate their anti-inflammatory properties which helped into the management of inflammatory diseases (Just *et al*., 1998). A variety of plants like *Radix gentianae macrophyllae* (Kwak *et al.,* 2005 and Yu *et al.,* 2004), *Rhizoma coptidis* (Tse *et al.*, 2006) *Citri unshiu pericarpium* (Higashi *et al*., 2002 and Kim *et al*., 1999), *Leucas aspera*, *Heliotropium indicum* (Srinivas and Rao, 2000) rhizome of *Curcuma amada* (Mujumdar, 2000), *Strobilanthus callosus, Strobilanthus ixiocephala* (Agarwal and Ranagari, 2003), *Wrightia tinctoria* (Tharkar *et al.,* 2010). *Oscillatoria willei* (Rajavel, 2009), *Aconitum heterophyllum* (Verma *et al.,* 2010). *Crallia brachiata* (Krishnaveni, 2009) and *Corallocarpus epigaeus* (Uthrapathy, 2011) were studied for their anti-inflammatory effects in various experimental animal models.

Rat paw oedema and cotton pellet induced granuloma models are selected for the present study to investigate the anti inflammatory properties of different extracts of *S. zeylanica* and *S. ovalifolia*.

6.2. Materials and Methods

6.2.1. Experimental animals

Eight weeks old male Wistar Albino rats were randomly selected as control and experimental groups with six rats in each group. They were kept in the polypropylene

cages under standard environmental conditions of 12 hours dark-light cycle at a temperature of 24±1^{o}C. Animals were fed with standard diet cakes prepared in the laboratory and allowed to freely access clean fresh water *ad libitum*. Prior to dosing all animals were kept in the experimental conditions for about 7-10 days to allow them to be acclimatized. The sterilized paddy husk was used as the bedding material. All experimental protocols were approved by the institutional animal ethics committee (SBCP/2011-2012/1AEC/CPCSEA/3) of Sankaralingam Bhuvaneswari Pharmacy College, Sivakasi. Once the inflammation was induced, food and water were served at the bottom of the cage as severely inflammed rats might have difficulty in obtaining feed from the top of the cage.

6.2.2. Drugs and Chemicals Used

All chemicals used in the study were of analytical grade. Diclofenac and Indomethacin were used as the standard drugs and were obtained as a gift from the Micro Labs Pvt Ltd, Bangalore, India.

6.2.3. Preparation of Doses

Extracts were prepared as mentioned in the Chapter 1 by soaking plant materials in respective solvents. The filtered extracts were concentrated by evaporation of solvents in the rotary flash evaporator. All doses were prepared shortly prior to administration. About 200mg/kg and 400 mg/kg of extracts were prepared by dissolving the extracts in 2.5% Tween 80. The volume administered did not exceed 2ml/100gm of animal. Dose selection of the test extracts were based on the toxicity studies conducted in the earlier part of the research work. It was found that all the test extracts did not cause mortality until a dose of 5000mg/kg bwt.

6.2.4. Experimental Set Up

Animals were divided into fifteen groups with six rats in each group and were orally administered with the vehicle, standard drugs and different test extracts which is tabulated in Table 24.

Table 24: Experimental Set Up for anti-inflammatory studies

	Experimental Groups	Orally administered with	Dose (mg/Kg Bwt)
I	Vehicle Control	Tween 80	2.5 (%)
II	Diclofenac	Diclofenac	200
III	Indomethacin	Indomethacin	400
IV	SOAE 200	Aqueous extract of *S.ovalifolia*	200
V	SOAE 400	Aqueous extract of *S.ovalifolia*	400
VI	SOEE 200	Ethanol extract of *S.ovalifolia*	200
VII	SOEE 400	Ethanol extract of *S.ovalifolia*	400
VIII	SOCE 200	Chloroform extract of *S.ovalifolia*	200
IX	SOCE 400	Chloroform extract of *S.ovalifolia*	400
X	SZAE 200	Aqueous extract of *S.zeylanica*	200
XI	SZAE 400	Aqueous extract of *S.zeylanica*	400
XII	SZEE 200	Ethanol extract of *S.zeylanica*	200
XIII	SZEE 400	Ethanol extract of *S.zeylanica*	400
XIV	SZCE 200	Chloroform extract of *S.zeylanica*	200
XV	SZCE 400	Chloroform extract of *S.zeylanica*	400

6.2.5. Carrageenan Induced Acute Paw Inflammation

After 10 days of acclimatization, animals were grouped into fifteen cages; six in each group as described under experimental set up and fasted overnight with access to water *ad libitum*. All test doses as well as the standard drug doses were prepared prior to the administration. Prior to dosing, baseline paw volume was recorded using mercury plethysmometer. Group I was administered with 2 ml of 2.5% Tween 80 by using an oral gavage and Group II and III also were fed with same volume (2ml) of Diclofenac and Indomethacin respectively. Group IV- XV were fed with different extracts of *S. zeylanica* and *S. ovalifolia* at two different dose levels as mentioned above. Sixty minutes after the administration of the test extracts, standard drugs and vehicle, paw oedema was induced

by subcutaneous injection of 0.1 ml of freshly prepared λ-carrageenan (1%) in normal saline into the left hind paw of the animals. All the animals were observed for a period of 6 hours and paw volumes were recorded at 30minutes 1h, 3h and 6h after challenging with carrageenan.

6.2.6. Formalin Induced Sub Chronic Paw Inflammation

The sub chronic anti- inflammatory activity was assessed by formalin induced rat paw oedema method. After 10 days of acclimatization, the animals were grouped into fifteen cages with six in each group as described above in the acute inflammation using carrageenan. Prior to dosing, baseline paw volume was recorded using mercury plethysmometer.

Oedema was produced by the method described by Brownlee *et al.*, (1950). Thirty minutes after the administration of the test extracts, standard drugs and vehicle, paw oedema was induced by subcutaneous injection of 0.1 ml of freshly prepared formalin (2%) under plantar aponeurosis of right hind paw of the rat of the animals. All animals were closely observed for two hours and the volume (in millilitres) of the inflamed paw was measured by standard volumetric technique using a calibrated plethysmometer at 1, 21 and 43 hours after formalin challenge.

The paw was immersed up to the tibiotarsic articulation in a cylinder filled with mercury. The increased level, consequent on the increase of the mercury meniscus was measured from the increase of dyed ethanol in a glass tube connected to the surface of the mercury so that variation of the mercury level corresponded to increases in the dyed ethanol in a calibrated glass tube. The increase in volume of the paw was calculated by subtracting the initial volume from the volume obtained after formalin administration and expressed as paw volume increase over time (ml ± SD) and was obtained as follows.

$$\text{Percentage Inhibition}(\%) = \frac{(Vt - V0)\ \text{Control} - (Vt - V0)\ \text{Treated}}{(Vt - V0)\ \text{Control}} \times 100$$

V0: average volume of right paw before injection of formalin *i.e.* at 0 h and Vt: average volume of right paw after injection of formalin.

6.2.7. Induction of Granuloma Formation by Subcutaneous Cotton Pellet Implantation

The test animals were divided into fifteen groups, six in each group. All animals were numbered consecutively with picric acid on the fur. All animals were maintained under aseptic condition for the entire duration of the study and fasted overnight prior to study with access to water *ad libitum*.

After overnight fasting, all groups received vehicle or test extracts or standard drugs as mentioned in earlier studies. Thirty minutes after administration of drug/vehicle, the animals were anaesthetized with diethyl-ether and sterile cotton pellets of weight 30±1 mg were implanted subcutaneously, bilaterally under the axilla. The drug treatment was continued for 6 days and on the seventh day, rats were sacrificed and cotton pellets surrounded by granuloma were dissected out. The cotton pellets were weighed and then dried at 60^{o}C till a constant weight was recorded at two consecutive time points. The difference between the initial and post- implantation weight was considered to be the dry weight of granuloma tissue.

6.3. Results and Discussion

There are several methods employed to screen new anti inflammatory agents. The ability of the agent to reduce local oedema induced in the rat paw by an injection of an irritant agent is most widely used method (Chakraborty *et al*., 2004).

6.3.1. Carrageenan Induced Paw Oedema

In the present study, a significant change in the paw volume (Table 25) in all the groups were observed when compared to the control group from the 30 minutes after carrageenan challenge in the hind paw of Wistar rats. All the extracts of *S. zeylanica* exerted a dose dependent significant change in paw volume as the standard drugs, Diclofenac and Indomethacin when compared to the control groups. As time prolonged the highest doses of all extracts of *S. zeylanica* as well as the standard drugs exhibited reduction in the paw oedema when compared to the vehicle control group. After 30 minutes of carrageenan challenge, SZEE 400 offered 65.45% protection against inflammation where as Diclofenac and Indomethacin exerted 61.13% and 64.07% protection respectively. The highest concentration of ethanolic extract of *S. zeylanica* could offer 58% of inhibition in the paw oedema when compared to the control group. The standard drug Diclofenac could exhibit the highest percentage of protection from 60 min onwards and could achieve a protection rate of 89% after 6 hour challenge with the irritant. The SZEE 400 group also could offer a protection rate of 85.64% after 6 hours. A dose dependent protection was observed in both aqueous as well as ethanol extracts of *S. zeylanica*, whereas the chloroform extracts could achieve a protection rate below 50% (Table 25 & Figure 8).

A reverse pattern of protection was observed with the extracts of *S. ovalifolia*. Both the aqueous as well as the ethanol extract inhibited the paw oedema in the early phase of inflammation and the rate of protection was reduced later noticeably when

compared to the other treated groups. The SOEE 400 group exerted the highest inhibition (66.53%) of paw oedema at the very early stage, whereas the chloroform extract of *S. ovalifolia* offered the least protection (Figure 9 &Table 26).

Carrageenan induced paw oedema is a useful model in assessing the contribution of mediators involved in vascular changes associated with acute inflammation. Carrageenan-induced rat paw oedema is a simple and routine animal model for evaluation of pain at the site of inflammation without any injury or damage to the inflamed paw (Sugishita *et al*., 1981; Henriques *et al.,* 1987*;* Jain *et al.,* 2001; Paschapur *et al.,* 2009*;* Petersson *et al.,* 200*1;* Sini *et al.,* 2010*).* The development of oedema in the rat hind paw following the injection of carrageenan has been described as a biphasic. The first detectable mediators in the early phase are histamine, serotonin and bradykinin. The initial phase of oedema which is less inhibited by non steroidal anti-inflammatory drugs (NSAIDs) such as Indomethacin and Diclofenac has been attributed to the release of histamine, 5-hydroxytryptamine (5-HT) and bradykinin (Nantel *et al.*, 1999). Aqueous and ethanolic extracts of *S. ovalifolia* (200&400 mg/kg) inhibited the initial phase of inflammation significantly ($P<0.001$) suggesting their ability to inhibit the early phase mediators of inflammation like histamine, serotonin and bradykinin. In the carrageenan-induced rat paw oedema model, the late phase is further induced by the biosynthesis of prostaglandin and other autacoids production of prostanoids through the serum expression of COX-2 by a positive feedback mechanism (Nantel *et al.*, 1999).

In the present investigation, ethanolic as well as aqueous extracts of *S. zeylanica* showed anti-oedematous activity from first hour and maximum activity at 3^{rd} and 6^{th} hr suggesting that the anti-inflammatory activity might be due to the inhibition of mediators of the inflammation such as histamine, serotonin and bradykinin released during the first

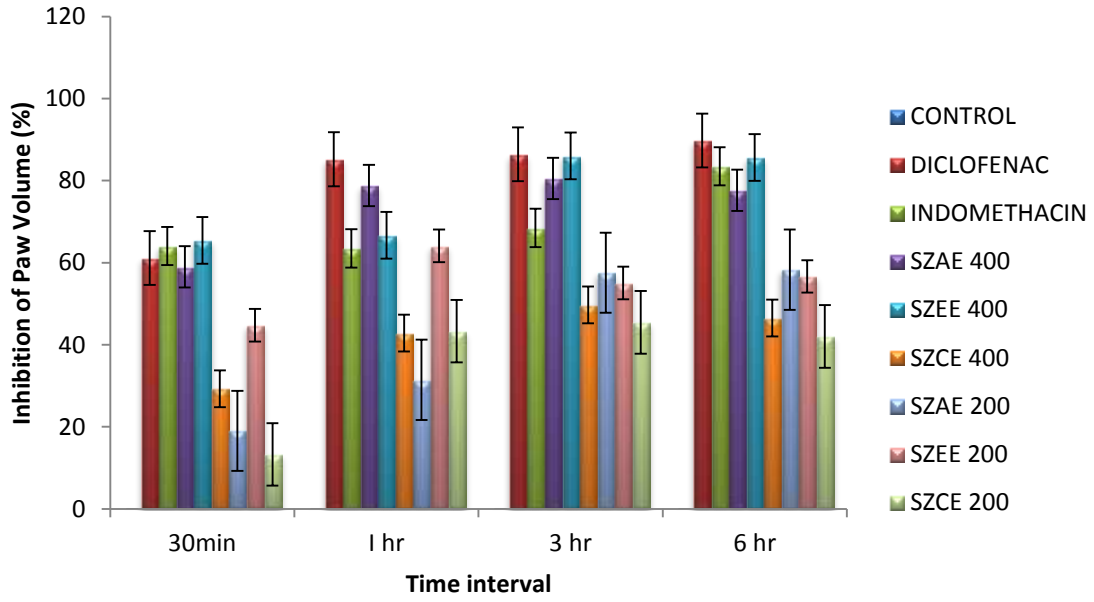
phase of inflammation and prostaglandins which released during the second phase of inflammation (Ueno *et al.*, 2000).

Table 25: Mean reduction in paw volume in carrageenan induced paw oedema by different extracts of *S. zeylanica* and standard drugs in Wistar Albino rats

	Initial	30 Minutes	I Hour	3 Hour	6hours
Control	0.251±0.00	0.30±0.033	0.35±0.035	0.51±0.049	0.51±0.042
Diclofenac	0.25±0.002	0.27±0.008*	0.30±0.042**	0.35±0.043**	0.51±0.034**
Indomethacin	0.25±0.00	0.26±0.047*	0.27±0.041**	0.26±0.041**	0.28±0.045*
SZAE 200	0.25±0.01	0.27±0.00**	0.26±0.060**	0.28±0.056**	0.33±0.061*
SZEE 200	0.25±0.00	0.27±0.002**	0.27±0.04**	0.27±0.021**	0.30±0.015**
SZCE 200	0.25±0.00	0.27±0.032*	0.27±0.044**	0.28±0.031	0.29±0.029*
SZAE 400	0.24±0.10	0.29±0.020**	0.27±0.099**	0.30±0.010**	0.37±0.033**
SZEE 400	0.25±0.00	0.28±0.000**	0.29±0.057**	0.32±0.028**	0.36±0.033**
SZCE 400	0.26±0.20	0.296±0.02*	0.28±0.05**	0.30±0.057**	0.37±0.032*

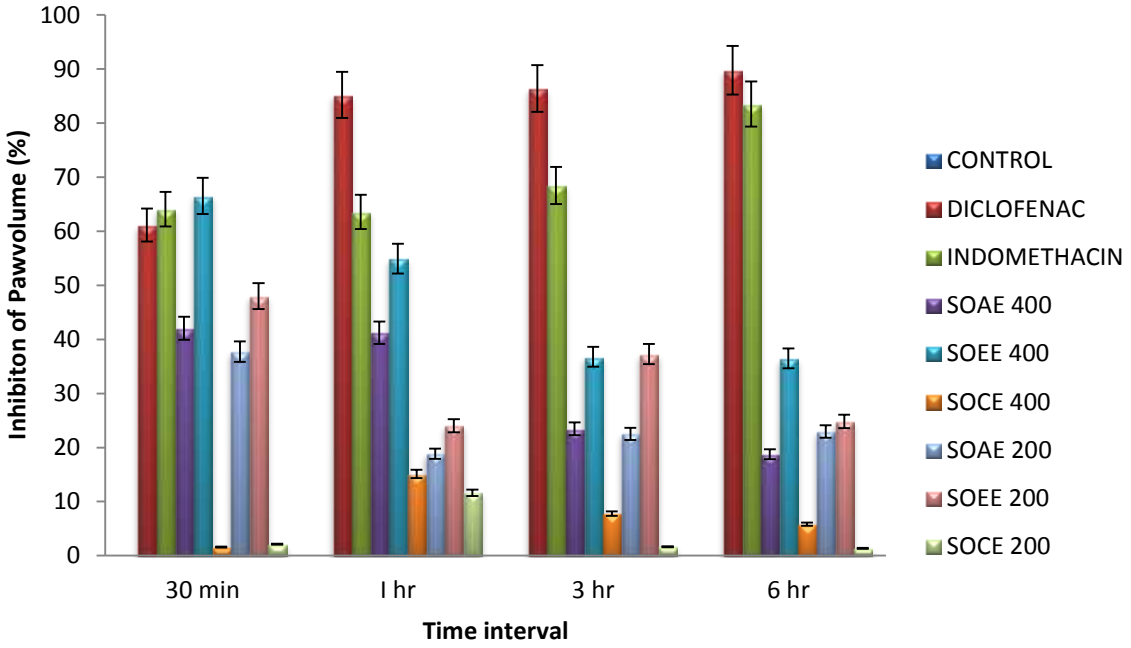
Oneway ANOVA followed by Dunnett's test; Number of animals used in each group (n=6), *P value < 0.05, **P value<0.001.

Figure 8: Percentage inhibition of paw volume by *S. zeylanica* in Carrageenan Induced Oedema



Values are expressed as mean ± standard deviation, N=6.

Figure 9: Percentage Inhibition of Paw Volume by *S. ovalifolia* in Carrageenan Induced Oedema



Values are expressed as mean ± standard deviation, N=6.

Table 26: Mean reduction in paw volume in carrageenan induced paw oedema by different extracts of *S. ovalifolia* and Standard drugs

	Initial	30 Minutes	I Hour	3 Hour	6hours
Control	0.251±0.00	0.30±0.033	0.35±0.035	0.51±0.049	0.51±0.042
Diclofenac	0.25±0.02	0.27±0.008*	0.30±0.042**	0.35±0.043**	0.51±0.034**
Indomethacin	0.25±0.00	0.26±0.047*	0.27±0.041**	0.26±0.041**	0.28±0.045*
SOAE 200	0.25±0.02	0.28±0.027*	0.31±0.061	0.45±0.017*	0.46±0.017
SOEE 200	0.25±0.002	0.27±0.032*	0.30±0.061*	0.42±0.024*	0.42±0.029*
SOCE 200	0.25±0.004	0.30±0.039	0.34±0.041	0.49±0.031	0.50±0.044
SOAE 400	0.24±0.002	0.28±0.018**	0.34±0.030*	0.45±0.018*	0.45±0.037
SOEE 400	0.25±0.012	0.27±0.012**	0.33±0.018**	0.41±0.047*	0.44±0.047*
SOCE 400	0.26±0.002	0.30±0.024*	0.34±0.027	0.50±0.022	0.51±0.030

Oneway ANOVA followed by Dunnett's test; Number of animals used in each group (n=6), *P value < 0.05, **P value<0.001.

6.3.2 Formalin Induced Sub Chronic Paw Inflammation

In the present study, it was found that the aqueous and ethanolic extracts of *S. zeylanica* and *S. ovalifolia* as well as the standard drug Diclofenac showed significant changes in the mean paw volume when compared to the control (Tables 27,28 and Figures 10,11). The chloroform extract also significantly reduced the mean paw volume ($P<0.05$) when checked after 12 hours of formalin challenge. The highest concentration of ethanol extract and Diclofenac exhibited significant reduction in the paw volume ($p<0.05$) from the 3rd hour after formalin challenge. The SOEE group could achieve 83.84% protection against formalin induced paw oedema after 12hrs of formalin challenge which is the highest rate of protection followed by 78.38% protection by Diclofenac ($p<0.001$). There was a slight decrease in the rate of protection in the sixth hour by Diclofenac, Indomethacin and SZEE (200mg/kg) groups. In all the other experimental groups, the protection rate increased with time. A dose dependent protection rate was observed with the chloroform extracts of both herbs.

Anti-inflammatory activity of Smilax species are well studied earlier both in vivo and in vitro models. Shu *et al.,* (2006) reported the anti-inflammatory effect of aqueous extract of the tuber of *Smilax china* by egg-albumin-induced oedema, where the aqueous extracts at dose level 1000 mg/kg bwt exerted a significant anti-nociceptive and anti-inflammatory effect compared to physiological saline and the anti-inflammatory activity was similar to acetylsalicylic acid (200 mg/kg bwt). The extract also inhibited both COX-2 activity and COX expression in lipopolysaccharide (LPS)-induced mouse macrophage cells. Shu *et al.,* (2006) reported that the ethyl acetate extract of *S. china* 50-100 mg/kg bwt could significantly decreased the rat paw oedema by egg-albumin with inhibition of ear oedema by xylene, increased vascular permeability and foot oedema by formaldehyde in mice.

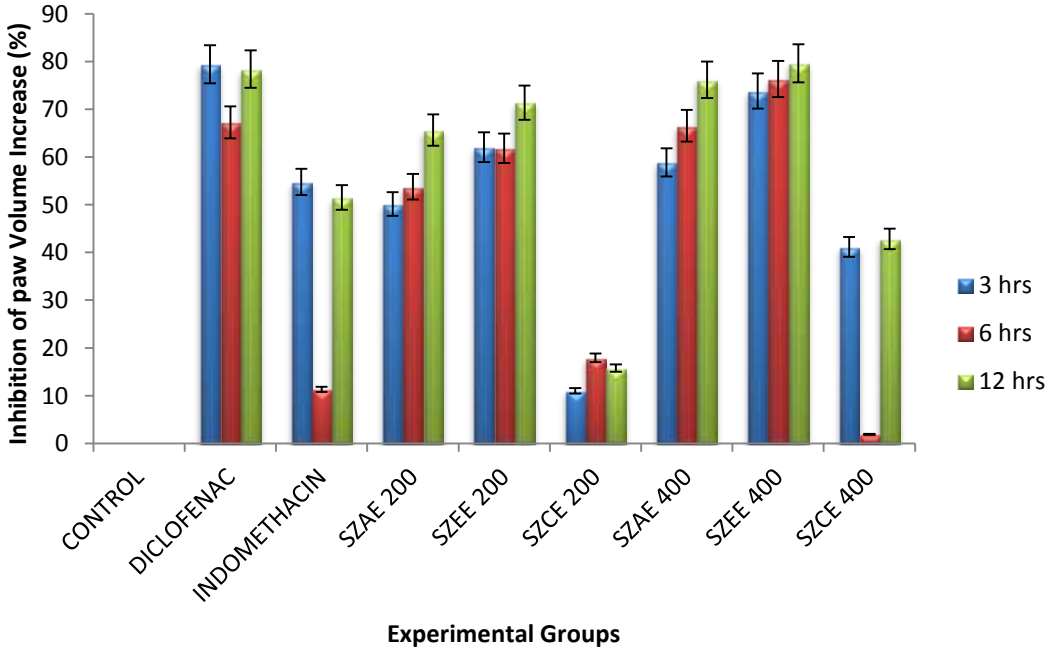
Arthritis induced by formalin is a model used for the evaluation of an agent with probable anti-proliferative activity (Banerjee *et al*., 2000). Oedema induced by formalin in rats is one of the most suitable test procedures to screen anti-inflammatory and anti-arthritic agents as it closely resembles human arthritis (Greenwald *et al*., 1991). The formalin induced inflammation in the rat's foot may be conveniently divided into two parts, the first involving 5-hydroxytryptamine as mediator and the second some mediator which is unrelated to 5-hydroxytryptamine. The extracts produced significant inhibition in the late phase of formalin induced inflammation. It seemed that the total response produced by the extracts were due to the prevention of the 'second mediator'. The second mediator can be prevented by treatment with certain analgesic-antipyretic drugs and other substances like the hydroxybenzoates, the pyrazolones, the flavone and flavanone glycosides and are inactive against 5-hydroxytryptamine-induced inflammation but they produced their action against an formalin-induced inflammation by inactivating the second factor (Northover *et al*., 1962). The present study revealed the ability of *S. ovalifolia* as well as *S. zeylanica* extracts to inhibit chemical mediators of inflammation. As the test extracts significantly inhibited this model of inflammation it can be thought to possess anti proliferative and anti arthritic activities similar to Diclofenac and Indomethcin.

Table 27: Mean reduction in paw volume in formalin induced paw oedema by different extracts of *S. zeylanica* and standard drugs

	Initial	3 hrs	6hrs	12 hrs
Control	0.255±0.171	0.313±0.050	0.326±0.016	0.427±0.016
Diclofenac	0.250±0.123	0.268±0.025*	0.273±0.013**	0.287±0.014**
Indomethacin	0.247±0.000	0.271±0.010	0.307±0.052	0.328±0.461*
SZAE 200	0.253±0.003	0.282±0.012	0.286±0.01**	0.312±0.007**
SZEE 200	0.253±0.002	0.275±0.013	0.280±0.020**	0.302±0.020**
SZCE 200	0.256±0.005	0.307±0.036	0.314±0.040	0.400±0.002*
SZAE 400	0.253±0.002	0.277±0.002	0.277±0.002**	0.294±0.002**
SZEE 400	0.248±0.013	0.263±0.01*	0.265±0.01**	0.283±0.01**
SZCE 400	0.254±0.003	0.288±0.012	0.323±0.020	0.352±0.040*

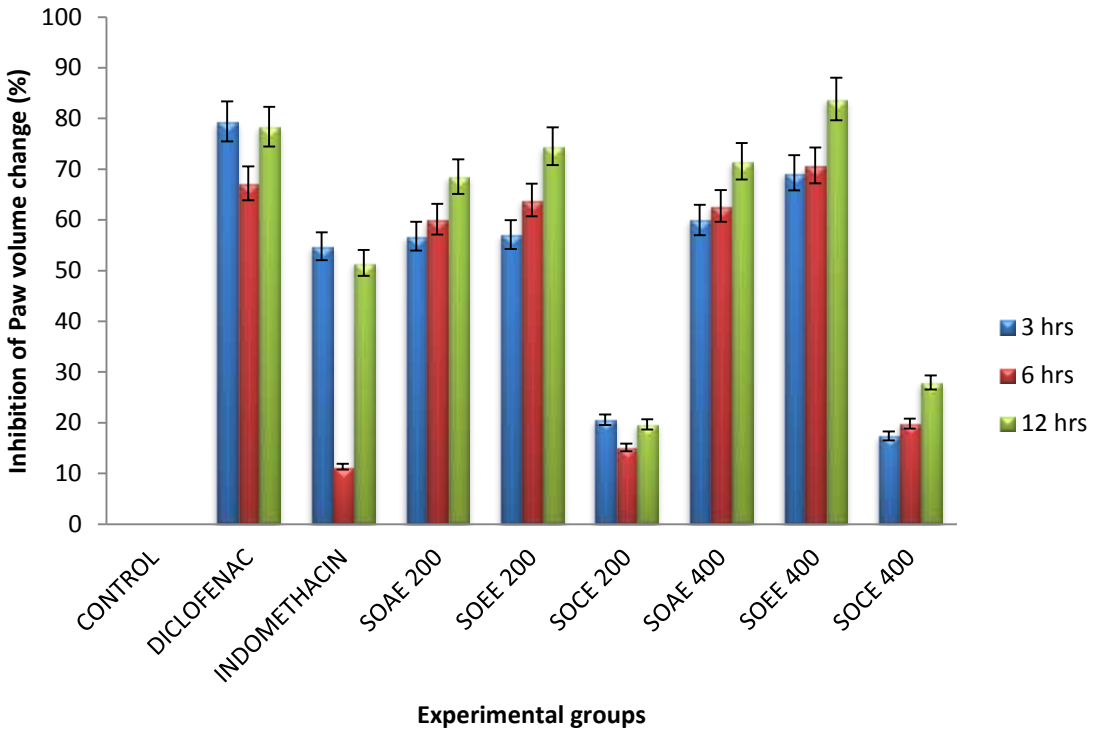
One way ANOVA followed by Dunnett's test; Number of animals used in each group (n=6). *P value < 0.05, **P value<0.001.

Figure 10: Percentage inhibition of increase in paw volume by *S. zeylanica* in formalin induced paw oedema



Values are expressed as mean ± standard deviation, n=6.

Figure 11: Percentage inhibition of increase in paw volume by *S. ovalifolia* in formalin induced oedema



Values are expressed as mean ± standard deviation, n=6.

Table 28: Mean reduction in paw volume in formalin induced paw oedema by different extracts of *S. ovalifolia* and standard drugs

	Initial	**3 hrs**	**6hrs**	**12 hrs**
Control	0.255±0.171	0.313±0.050	0.326±0.016	0.427±0.016
Diclofenac	0.250±0.123	0.268±0.025*	0.273±0.013**	0.287±0.014**
Indomethacin	0.247±0.000	0.271±0.010	0.307±0.052	0.328±0.46*
SOAE 200	0.254±0.008	0.279±0.012	0.282±0.012**	0.308±0.020**
SOEE 200	0.251±0.013	0.275±0.01	0.276±0.012**	0.294±0.016**
SOCE 200	0.251±0.012	0.296±0.022	0.311±0.022	0.388±0.030*
SOAE 400	0.250±0.042	0.273±0.010	0.276±0.023**	0.299±0.014**
SOEE 400	0.254±0.010	0.272±0.00	0.275±0.023**	0.282±0.001**
SOCE 400	0.259±0.004	0.306±0.01	0.315±0.013	0.382±0.152*

One way ANOVA followed by Dunnett's test; Number of animals used in each group (n=6). *P value < 0.0 , **P value<0.001.

6.3.3. Cotton Pellet Induced Granuloma

The cotton pellet method is extensively used to evaluate the transudative and proliferative components of the chronic inflammation. In cotton pellet induced granuloma model, granuloma develops within several days and this model of inflammation is used to study the proliferative phases of inflammation. Granuloma is formed due to the proliferation of macrophages, neutrophils and fibroblasts which are the basic sources of granuloma formation. Granulomas are histologically giant cells seen with undifferentiated connective tissue along with accumulated fluid. Newly formed connective tissues can be measured. The wet weight of the cotton pellets associate with the exudate; the dry weight of the pellets correlates with the amount of the granulomatous tissue.

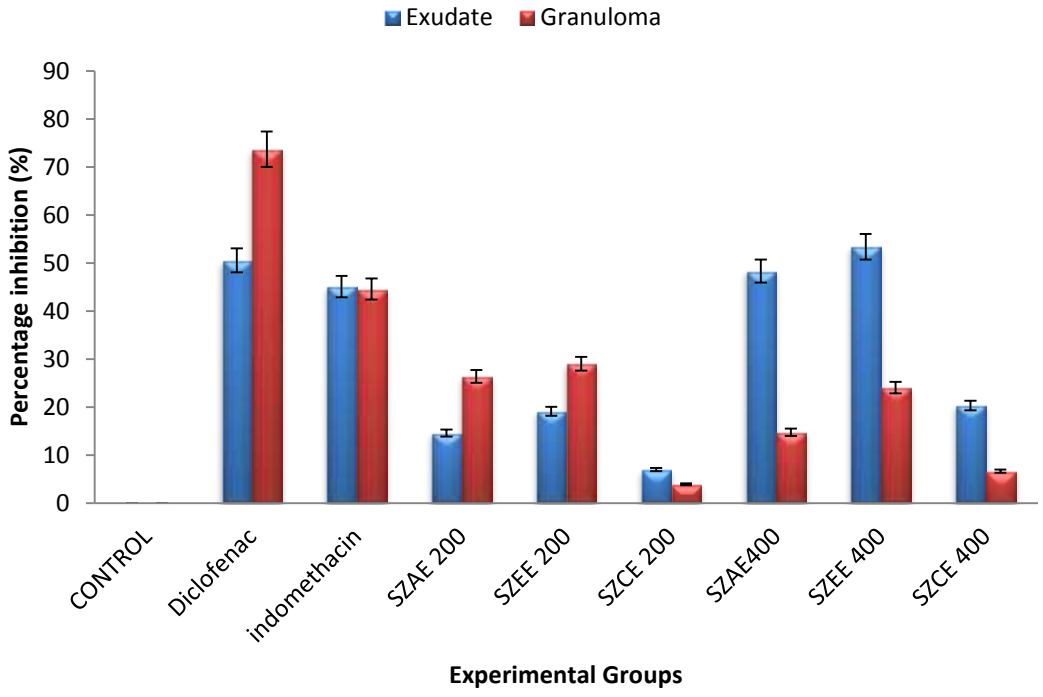
In the present study it was found that the ethanol and aqueous extracts of *S. zeylanica* and *S. ovalifolia* significantly (200& 400 mg/kg bwt) reduced the wet weight of cotton pellets as the standard drugs Indomethacin and Diclofenac (Tables 29 &30). The Diclofenac reduced the wet weight of cotton pellet to 57.74% followed by Indomethacin (40.76%). In case of *S. ovalifolia,* the ethanol extracts produced a greatest percentage of inhibition followed by the aqueous extracts (Figures 12 &13). The chloroform extracts of both plants at both dose levels produced a protection but were not statistically significant. Ethanol and aqueous extracts of *S. zeylanica* at higher dose level conferred 33.66% and 26.9% inhibition of wet weight formation (Plate 10).

Table 29: Effect of *S. zeylanica* and standard drugs on cotton pellet induced granuloma

Experimental groups	Wet weight (mg)	Percentage inhibition (%)	Dry weight (mg)	Percentage inhibition (%)
Control	220.83±21.39	-	132.17±8.21	-
Diclofenac	93.33±16.82**	57.74	49.5±13.34**	62.55
Indomethacin	130.83±24.85**	40.76	82.15±14.53**	37.85
SZAE 200	178.33±9.31**	19.25	102.58±6.43**	22.39
SZEE 200	171.33±7.94**	22.42	99.62±9.56**	24.63
SZCE 200	219.25±26.28	0.72	117.78±10.31	10.89
SZAE400	161.43±4.64**	26.9	115.62±13.53**	12.52
SZEE 400	146.5±6.57**	33.66	105.18±8.64**	20.42
SZCE 400	195.33±20.27	11.55	124.7±9.07	5.65

One way ANOVA followed by Dunnett's test; Number of animals used in each group (n=6). *P value < 0.05, **P value<0.001.

Figure 12: Effect of *S. zeylanica* and standard drugs on cotton pellet induced granuloma



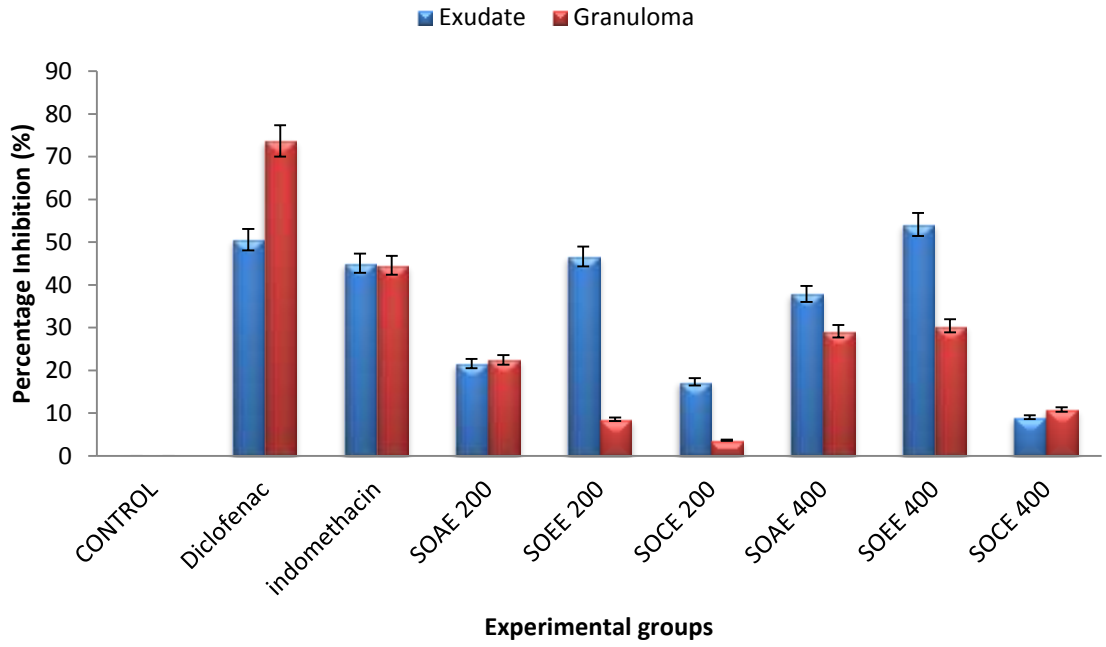
Values are expressed as mean ± standard deviation, n=6.

Table 30: Effect of *S. ovalifolia* and standard drugs on cotton pellet induced granuloma

Experimental groups	Wet weight (mg)	Percentage inhibition (%)	Dry weight (mg)	Percentage inhibition (%)
Control	220.83±21.39	-	132.17±8.21	-
Diclofenac	93.33±16.82**	57.74	49.5±13.34**	62.55
Indomethacin	130.83±24.85**	40.76	82.15±14.53**	37.85
SOAE 200	176.47±5.74**	20.09	106.97±14.83**	19.07
SOEE 200	169.87±3.43**	23.08	122.58±15.73**	7.26
SOCE 200	201.37±14.86	8.81	128.07±21.71	3.1
SOAE 400	154.55±3.21**	30.01	99.48±16.4**	24.73
SOEE 400	138.67±12.71**	37.2	98.03±10.83**	25.83
SOCE 400	200.67±23.08	9.13	120.02±34.89	9.19

One way ANOVA followed by Dunnett's test; number of animals used (n=6);*P value<0.05, **P value<0.001

Figure 13: Effect of *S. ovalifolia* and standard drugs on cotton pellet induced granuloma



Values are expressed as mean ± standard deviation, n=6.

Plate 10: Anti-inflammatory Activity of Different Extracts of *S. zeylanica* and *S. ovalifolia* in cotton pellet induced granuloma

a: Control , b: Diclofenac, c: Indomethacin, d: SZAE, e: SZEE, f: SZCE, g: SOAE, h: SOEE, i: SOCE

The standard drug Diclofenac reduced the dry weight to 62.55% followed by Indomethacin (37.85). Ethanol and aqueous extracts of *S. ovalifolia* exerted a protection rate of 25.83% and 24.75% respectively at their higher concentrations whereas the chloroform extract of the same produced only 9.19 % of protection at 400mg/kg bwt dose level. When compared to *S. ovalifolia*, *S. zeylanica* extracts offered lesser inhibition of dry weight (20.42 & 24.63% for ethanol extracts and 12.52 & 22.39% for aqueous extracts respectively). *S. ovalifolia* exerted significant inhibition of dry weight and wet weight of cotton pellet when compared to the control group.

Cotton pellet-induced granuloma formation is considered to be a reliable experimental model for evaluation of the effects on macrophage dysfunction and granuloma formation. It was found that the ethanol and aqueous extracts of both plants significantly reduced the granuloma formation whereas the chloroform extracts produced a lesser extent of inhibition of granuloma. Wet and dry weights of cotton pellets directly corresponded to the amount of exudates and granuloma formed during inflammation. Hence, the decrease in the weight of granuloma indicated the ability of the test extracts in reducing the synthesis of proteins, collagen, activation, infiltration and aggregation of macrophages. Smilax genus is widely used in Ayurveda formulations. The phyto constituents of the *S. ovalifolia* and *S. zeylanica* are found to be alkaloids, glycosides, flavanoids, saponins etc. and these components are reported to possess potent anti-inflammatory effects through inhibiting prostaglandin pathways (Patwardhan and Hopper, 1992).

Similar results were also reported by Verma *et al*., (2010), where *A. heterophyllum* extract inhibited the weight of wet cotton pellet in a dose dependent manner and the inhibitory action of the higher dose of *A. heterophyllum* was very close to the inhibitory effect of Diclofenac sodium. Non steroidal anti inflammatory drug like Diclofenac sodium inhibit the prostaglandins synthesis at the late phases of inflammation (Smith and Dewitt, 1995) and thereby decrease granuloma tissue, prevent the formation of collagen fibre and suppress mucopolysaccharides. Srinivas *et al.*, (2000) also demonstrated significant anti inflammatory activity of *H. indicum* and *L. aspera in* carrageenan induced paw oedema and cotton pellet induced granuloma models of inflammation. The present study showed significant anti-inflammatory activity in cotton pellet induced inflammation by ethanol and aqueous extracts of both plants and hence it is concluded that these test extracts are effective in chronic inflammatory conditions by

inhibiting the increase in the number of fibroblasts and synthesis of collagen and mucopolysaccharides during granuloma tissue formation. Apart from these, a variety of indigenous herbs are reported to possess anti inflammatory activity *viz.*, ethanolic extracts of *C. amada* Roxb. (Mujumdar *et al.,* 2000), Wrightia *tinctoria* R.Br. (Tharkar *et al.,* 2000), *Oscillatoria willei* (Rajvel *et al.*, 2009), *Semecarpus anacardium* Linn.(Nadkarni, 1954). *Benincasa hispida* (Rachchh *et al.*, 2011), *Corollocarpus epigaeus* (Uthrapathy *et al.*, 2011) etc.

Efficacy of the test extracts in the reduction of granuloma and exudates suggests their role in the reduction of macrophage derived pro-inflammatory cytokines like IL-6 and IL-1β and TNF α. Based on the results it can be concluded that the test extracts possessed significant role in inhibiting the both acute and chronic phases of inflammation in Wistar Albino rats.