Introduction

*Sapindus trifoliatus* (ST) is a medium sized deciduous tree growing wild in south India that belongs to the family Sapindaceae. It is known locally as soapnut tree. The plant has been reported for its high content of saponins and sugars. The saponin moiety is characterized as the hederagenin group of glycosides. The pericarp is reported for various medicinal properties. It is regarded as a tonic, stomachic, spermicidal and is used in the treatment of hemicrania (migraine) etc. It is evident from the literature that plants like feverfew (*Tanacetum parthenium*) and butter bur (*Petasites hybridus*), which are known for migraine prophylaxis exhibited antinoceptive and antiinflammatory effects in animal models of pain and inflammation. Since *Sapindus trifoliatus* (as intranasal application) has been quoted for its use in hemicrania (migraine pain) in traditional folk medicine and has recently been reported for its antinociceptive and antimigraine activity, the objective of the present study was to investigate its possible antiinflammatory effect in both *in vitro* and *in vivo* experimental models of inflammation.

Materials and Methods

Drugs and chemicals

Arachidonic acid (AA), carrageenan, serotonin, histamine, 12-O-tetradecanoylphorbol 13-acetate (TPA), dinitrofluorobenzene (DNFB), 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (oxazolone), indomethacin, zymosan A (from Saccharomyces cervisiae) and dexamethasone were obtained from Sigma Chemical Co (St. Louis, USA). 1-phenyl-3-pyrazolidinone (phenidone) was from Aldrich, Germany. 5-lipoxygenase (5-LO, human recombinant, specific activity ~ 20 units/mg) was purchased from Caymen Chemicals, Ann Arbor, USA. BWB70C and capsaicin were procured from Tocris, Avonmouth, UK. All other chemicals and reagents were of pure analytical grade and obtained from local suppliers.
Plant material and extraction procedure

The dried pericarps of the fruits of Sapindus trifoliatus Linn, family Sapindaceae were collected from the local market and were authenticated by Dr. A. M. Mujumdar, Agharkar Research Institute, Pune, India. Aqueous extract of ST was prepared as reported. Briefly, one hundred grams of the pericarp was soaked in 400 ml of distilled water for 16 h. The percolate was then decanted, centrifuged and filtered through Whatman (No.1) filter paper to obtain clear extract (300 ml). This process of extraction was repeated again with the same volume of distilled water. The percolates were pooled and lyophilized which yielded a brown colored powder (68% yield). Acid hydrolysis of the extract yielded only one glycone, which was identified as hederagenin. Therefore, estimation of the saponins present in the extract was calculated as hederagenin. The content of hederagenin was estimated in the extract by boiling it with 50% methanolic hydrochloric acid. The entire mixture was evaporated to dryness and reconstituted in methanol. HPLC estimation was carried out using Kromasil C-18 column (5 μm, 250 x 4.6 mm) with gradient elution (0.1% formic acid and acetonitrile in the ratio of 80:20 v/v) and evaporative light scattering detector. The concentration of hederagenin was found to be between 5.61-6.97 % by weight of the extract.

Animals

Adult male Swiss albino mice (18-22 g) and Wistar rats (180-220 g) were obtained from the Research Animal Facility of Poona College of Pharmacy (PCP), Pune, India. On arrival, the animals were placed at random and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24±2 °C and relative humidity of 30-70%. A 12:12 light-dark cycle was followed. All animals had free access to water filtered through Aquaguard® and standard pelleted laboratory animal diet. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of PCP, Pune, India and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry Forests and Environment, Government of India. Rules of CPCSEA are laid down as per ILAR (Institute of Laboratory Animal Resources, USA) guidelines.

In vitro assays

5-lipoxygenase assays

For the evaluation of 5-LO inhibitory activity, the enzymatic activity of 5-LO was measured spectrophotometrically using human recombinant 5-LO and an incubation mixture containing 50 mM sodium phosphate, pH 7.4, 12 μg/ml phosphatidyl choline, 0.2 mM ATP, 0.2 mM GdCl, and 20 μM AA. Different concentrations of ST (dissolved in Milli Q water) or 1 μM of WBW70C, a specific 5-LO inhibitor (dissolved in 10% Dimethyl sulfoxide [DMSO], 0.1% final concentration) were added to the reaction mixture. The reaction was initiated by the addition of an aliquot enzyme (25 units), and the rate of conjugated diene formation was followed at room temperature for 2 min. Enzymatic activity was calculated from the highest linear rate of the diene formation at absorbance 234 nm and percentage inhibition was calculated relative to a control reaction containing Milli Q water or DMSO vehicle.

Other in vitro assays

The enzyme inhibition assays for cyclo-oxygenases (COX – 1 and COX–2), leukotriene B₄ (LTB₄), nitric oxide synthase (NOS) were carried out by NovaScreen Biosciences Corporation, USA. The studies were done as per the standard protocols found elsewhere (www.novascreen.com). The details of the receptor source, ligands and reference compounds used are listed in Table 1.

In vivo assays

Dose response study of ST with the range of doses of 10, 20, 40, 80, 100 and 160 mg/kg, was carried out and the minimal effective dose of ST to cause effect was found to be 20 mg/kg, i.p. There was no incremental dose-related response with 40 and 80 mg/kg. However at 100 mg/kg, i.p the response was maximal. This could be attributed to the nature of the extract. Hence only two doses 20 and 100 mg/kg, i.p were employed throughout the in vivo experiments.

Carrageenan, histamine or serotonin-induced rat pedal inflammation

Pedal inflammation in rat was produced according to the method of Winter et al.[9] and Kaur et al.[10] Rats were treated intraperitoneally with ST (20 and 100 mg/kg) or indomethacin (20 mg/kg) or saline (10 ml/kg). Fifteen minutes after the treatment, rats were injected with 0.1 ml of carrageenan or histamine or serotonin (all 1% solution in saline) into the right hind paw under the subplantar tissue. Paw volume measurements were carried out immediately before and various time points after phlogistic agent injection using a plethysmometer (Ugo Basile 7140).

Zymosan A-induced mouse paw inflammation

The method of Rouleau et al.[11] was followed with minor modifications. Inflammation was induced in mice by subplantar injection of 25 μl of 2% zymosan A suspension in saline into the left hind paw. The animals were killed 4 h later and the hind paws were cut off at the ankle and weighed in an analytical balance. ST (20 or 100 mg/kg, i.p.) or indomethacin (20 mg/kg, i.p.) or saline was injected 15 min prior to zymosan injection.

Acute single application of tetradecanoylphorbol acetate (TPA)–induced mouse ear inflammation

The method of Recio et al.[12] was followed. Inflammation was induced on the right ear by topical application of 20 μl of TPA in acetone (2.5 μg/ear) with a micropipette. Ear thickness was measured before and 4 h after induction of inflammation using a micrometer (Mitutoyo, Japan). ST was dissolved in 80% acetone and applied topically (1 and 5 mg/ear in 20μl) simultaneously with TPA. The standard drug indomethacin was administered at a dose of 0.5 mg/ear. The effect of the extract on inflammation was expressed as per cent inhibition from the mice treated with 80% acetone.

Mouse ear inflammation-induced by multiple topical applications of TPA

The method of Stanley et al.[13] was followed. Chronic inflammation was induced by topical application of 20 μl of TPA
(2 μg/ear x 5 times) to both the inner and outer surface of both the ears of each mouse with a micropipette on alternate days. ST (1 and 5 mg/ear) or dexamethasone (0.05 mg/ear) was applied topically twice daily for four days in the morning immediately after TPA application and 6 h later. The ear thickness was measured 4 h after the last TPA application.

**Capsaicin–induced mouse ear inflammation**

The method of Martione and Rodriguez\[15\] was followed with minor modifications. Inflammation was induced on the right ear by topical application of 20 μl of capsaicin in acetone (100 μg/ear) with a micropipette. Ear thickness was measured before and 1 h after induction of inflammation using a micrometer (Mitutoyo, Japan). ST was dissolved in 80% acetone and applied topically (1 and 5 mg/ear in 20 μl) 15 min prior to capsaicin application. The standard drug indomethacin was administered at a dose of 0.5 mg/ear. The effect of the extract on inflammation was expressed as per cent inhibition from the mice treated with 80% acetone.

**Arachidonic acid (AA)–induced mouse ear inflammation**

The method of Giner et al \[17\] was followed. ST (1 and 5 mg/ear in 20 μl) dissolved in 80% acetone was applied 30 min before the application of AA (2 mg in 20 μl) in the right ear. Thickness of the ears was measured before and 1 h after the induction of inflammation using the micrometer. A reference group was treated with phenidone (1 mg/ear).

**Oxazolone or dinitrofluorobenzene (DNFB)–induced mouse ear inflammation**

Mice were sensitized by topical applications on the shaven ventral abdomen of 50 μl of 2% solution of oxazolone in acetone or 0.2% (v/v) solution of DNFB in olive oil and acetone mixture (1:4) on two consecutive days. Challenge was performed on Day 6 by application of either 30 μl of 2% oxazolone or 0.2% DNFB. ST (1 and 5 mg/ear in 20 μl) or dexamethasone (0.5 mg/ear) was applied 30 min before the challenge. Ear thickness was measured 24 h after the challenge.\[18,19\]

**Statistical analysis**

Inflammations are expressed as mean±SEM. The inhibition percentages are calculated from the differences between the treated and control groups. Statistical significance of differences between the mean values was analyzed by ANOVA and Dunnett’s test. A P value of < 0.05 was considered to be significant.

**Results**

**5-LO assays**

ST (50–400 μg/ml) caused a concentration -dependent inhibition of the human recombinant 5-LO. At the concentration of 400 μg/ml the inhibitory effect (49.37% inhibition) was found to be statistically significant (P<0.05). The specific 5-LO inhibitor BW B 70C exhibited 48.13% inhibition at 1μM (Figure 1).

**Other in vitro assays**

The percent inhibition exhibited by ST (at 250 μg/ml) on COX –1, COX – 2, NOS (constitutive-neuronal) and LTD\(_4\) are listed in Table 1.

**Carrageenan, histamine, serotonin or zymosan A-induced paw inflammation**

Intraplantar injection in the hind paw with carrageenan induced a progressive inflammation reaching a maximum at 3 h. Animals treated intraperitoneally with aqueous extract of ST at doses 20 and 100 mg showed a significant inhibition of inflammation in all phases of the experiment. The

**Table 1**

<table>
<thead>
<tr>
<th>Receptor/enzyme</th>
<th>Source</th>
<th>Ligand</th>
<th>Reference compound</th>
<th>% inhibition at 250 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS (constitutive neuronal)</td>
<td>Rat cerebellum</td>
<td>[3H]Arginine</td>
<td>L-Arginine</td>
<td>84.10</td>
</tr>
<tr>
<td>COX-1</td>
<td>Bovine seminal vesicle</td>
<td>Arachidonic acid</td>
<td>SC560</td>
<td>29.00</td>
</tr>
<tr>
<td>COX-2</td>
<td>Bovine seminal vesicle</td>
<td>Arachidonic acid</td>
<td>DuP697</td>
<td>34.00</td>
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<tr>
<td>LTB(_4)</td>
<td>Guinea pig spleen</td>
<td>[3H]LTB(_4)</td>
<td>LTB(_4)</td>
<td>79.00</td>
</tr>
</tbody>
</table>

*The radio-ligand binding studies were carried out by NovaScreen Biosciences Corporation, USA*
Antiinflammatory effect at the 100 mg dose was comparable to that of indomethacin (20 mg/kg, i.p) (Figure 2).

At the lower dose (20 mg/kg, i.p.) ST extract did not exhibit significant inhibition of paw inflammation induced by intraplantar injection of histamine or serotonin or zymosan A. However, at 100 mg dose ST significantly inhibited the pedal inflammation caused by histamine or serotonin or zymosan A, which was similar to that of indomethacin (20 mg/kg, i.p.) (Figures 3 – 5)

**TPA-induced acute mouse ear inflammation**

Topical application of aqueous extract of ST inhibited the TPA-induced acute mouse ear inflammation by 37.31% and 52.08 % at doses 1 and 5 mg/ear respectively. The reference drug indomethacin (0.5 mg/ear) exhibited 59.90% inhibition in this inflammatory model (Table 2).

**Capsaicin-induced acute mouse ear inflammation**

Topical application of aqueous extract of ST significantly (P<0.001) inhibited the capsaicin-induced acute mouse ear inflammation by 26.13% and 41.15 % at doses 1 and 5 mg/ear respectively. The antiinflammatogenic effect exhibited by ST was comparable to that of reference drug indomethacin (0.5 mg/ear, 42.39% inhibition) in capsaicin-induced mouse ear inflammation (Table 2).

**Arachidonic acid (AA)-induced mouse ear inflammation**

Administration of AA to mouse produced a short inflammatory response. Topical application of ST extract showed a significant (P<0.001) inhibition of AA-induced ear inflammation at doses 1 mg/ear (25.44% inhibition) and 5 mg/ear (40.40% inhibition) (Table 2).

**Mouse ear inflammation induced by multiple topical application of TPA**

ST at 1 mg/ear did not exhibit a significant inflammation reduction in prolonged inflammation induced by multiple applications of TPA to mouse ear. However, at the 5 mg/ear dose...
the extract showed mild antiinflammagenic effect on TPA-induced chronic mouse ear inflammation. The glucocorticoid dexamethasone (0.05 mg/ear) exhibited a statistically significant (P<0.05) reduction of inflammation (Table 2).

**Oxazolone and DNFB-induced contact-delayed type hypersensitivity mouse ear inflammation**

ST failed to show any significant effect on oxazolone and DNFB-induced mouse ear swelling at 1 mg/ear dose. At 5 mg/ear dose a mild antiinflammagenic effect was observed (8.59% inhibition in oxazolone and 10.23% inhibition in DNFB) (Table 2).

**Discussion**

The present study established the antiinflammatory activity of the aqueous extract of ST. The extract produced marked inhibition of carrageenan-induced rat paw inflammation, a test which has a significant predictive value for antiinflammatory agents acting by inhibiting the mediators of acute inflammation.\[11\] Also, carrageenan-induced paw inflammation is a test largely used to study both steroidal and non-steroidal antiinflammatory drugs. Carrageenan induces an inflammatory reaction in two different phases. The initial phase, which occurs between 0 and 2 h after injection of carrageenan, has been attributed to the release of histamine, serotonin and bradykinin on vascular permeability.\[20\] The inflammation volume reaches its maximum approximately 3 h post-treatment after which it begins to decline.\[21\] The late phase, which is also a complement-dependent reaction, has been shown to be due to overproduction of prostaglandin in tissues.\[22\] ST extract inhibited the inflammation from the first hour, acting on both the early as well the late phases. ST extract also effectively inhibited the inflammation produced by histamine and serotonin, which suggests that the antiinflammatory activity of ST is possibly mediated by inhibiting the action of these mediators. In addition to these mediators, NO also plays an important role in carrageenan-induced paw inflammation.\[23\] Inducible nitric oxide synthase (iNOS) expression and subsequent production of NO maintains the inflammation. In radioligand binding studies, at a concentration of 250 μg/ml ST has an 84.10% inhibition of NOS (constitutive neuronal) (Table 1), which may support to the present *in vivo* findings.

Zymosan, an insoluble fraction of yeast cell wall produces an inflammatory response through multiple factors which include generation of anaphylotoxins that induce histamine release from mast cells, biosynthesis of eicosanoids by neutrophil macrophages and generation and release of platelet-activating factors, oxygen free radicals and lysosomal enzymes.\[24,25\] ST at 100 mg/kg dose significantly inhibited the zymosan-induced paw inflammation in mice, suggesting that ST could involve inhibition of one or more of the above mentioned inflammatory mediators.

Topical application of TPA offers a model of skin inflammation appropriate for evaluating antiinflammatory agents. TPA produces inflammation by activating phospholipase A\(_2\) (PLA\(_2\)) which subsequently activates the release and metabolism of AA. The COX and 5-LO inhibitors are very effective in suppressing TPA-induced ear inflammation indicating the role of prostaglandins and leukotrienes respectively.\[18\] Topical application of ST extract significantly inhibited TPA-induced acute ear inflammation. *In vitro* inhibition of COX (Table 1) and 5-LO (Figure 1) by ST could be corroborated with the results of the *in vivo* acute inflammatory model.

It is reported that, capsaicin-like molecules affect C-fiber thin primary afferent neurons that are connected to distinct sensory receptors.\[26\] Stimulation of sensory nerves in the skin causes an inflammatory reaction comprising arteriolar dilatation, increase in vascular permeability and recruitment of

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**Table 2**

Effect of aqueous extract of *Sapindus trifoliatus* on ear inflammation induced by various inflammatory agents in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TPA</th>
<th>Capsaicin</th>
<th>Arachidonic acid</th>
<th>Multiple TPA</th>
<th>Oxazolone</th>
<th>DNFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone control</td>
<td>0.35±0.02</td>
<td>0.32 ± 0.02</td>
<td>0.27 ±0.01</td>
<td>0.18±0.01</td>
<td>0.26±0.01</td>
<td>0.34±0.03</td>
</tr>
<tr>
<td>ST 1 mg/kg</td>
<td>0.22±0.01**</td>
<td>0.24 ± 0.02**</td>
<td>0.20±0.01**</td>
<td>0.17±0.00</td>
<td>0.28±0.01</td>
<td>0.32±0.02</td>
</tr>
<tr>
<td>(37.31)</td>
<td>(26.13)</td>
<td>(25.44)</td>
<td>(3.41)</td>
<td>(0.00)</td>
<td>(6.04)</td>
<td></td>
</tr>
<tr>
<td>ST 5 mg/kg</td>
<td>0.17±0.00**</td>
<td>0.19 ± 0.01**</td>
<td>0.16±0.01**</td>
<td>0.16±0.00 *</td>
<td>0.24±0.01</td>
<td>0.31±0.02</td>
</tr>
<tr>
<td>(52.08)</td>
<td>(41.15)</td>
<td>(40.40)</td>
<td>(10.98)</td>
<td>(8.59)</td>
<td>(10.23)</td>
<td></td>
</tr>
<tr>
<td>Indomethacin 0.5 mg/kg</td>
<td>0.14±0.00**</td>
<td>0.19 ± 0.01**</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>(58.90)</td>
<td>(42.39)</td>
<td>-</td>
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<tr>
<td>Phenidone 1 mg/kg</td>
<td>-</td>
<td>-</td>
<td>0.14±0.00**</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>(48.88)</td>
<td></td>
<td></td>
<td>(19.32)</td>
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<tr>
<td>Dexamethasone 0.05 mg/kg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.14±0.00*</td>
<td>-</td>
<td></td>
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<tr>
<td>(22.35)</td>
<td></td>
<td></td>
<td>(19.32)</td>
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<tr>
<td>Dexamethasone 0.5 mg/kg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.20±0.01</td>
<td>0.25±0.02*</td>
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<tr>
<td>(26.51)</td>
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<td>(26.51)</td>
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</table>

Compounds were applied topically (see text for details). Ear thickness (in mm) is expressed as mean±SEM and percentage inhibition in parentheses. n=5 in each group. *P<0.05, **P<0.001 compared to acetone control. ST- Sapindus trifoliatus, TPA-12-O-tetradecanoylphorbol 13-acetate, DNFB-2, 4-dinitrofluorobenzene.
leukocytes.[27] This neurogenic inflammation arises from the release of vasoactive peptide transmitters such as substance P (SP) and calcitonin-gene related peptide (CGRP) from the peripheral endings of the afferent nerve fibers.[28] It is possible that the antiinflammatory effect of ST observed in this model could be related to inhibition of the above peptide mediators.

According to Young et al.,[29] AA provokes a rapid intense inflammatory response in the mouse ear inflammation that is affected by lipoxygenase inhibitors, and the COX inhibitors are generally not active in this model. Effect of ST on AA-induced ear inflammation could be correlated with its 5-LO inhibitory activity observed in vitro (Figure 1).

Repeated application of TPA causes a measurable increase in the ear skin mastocyte population.[17] Although glucocorticoids are the most active drugs against the chronic inflammation caused by repeated application of TPA, other pharmacological agents have also been found to be effective, e.g. 5-LO inhibitors.[30] We thus propose that the mild inhibition by ST could be due to its combined inhibition of 5-LO, LTB

Topical oxazolone or DNFB-induced delayed type hypersensitivity (mouse ear inflammation test) is applied as a tool to discover new drugs that could inhibit the inflammation and tissue destruction. It has been reported that non-steroidal antiinflammatory drugs are fairly inactive in this model.[31] No potential inhibitory effect of ST was observed in the above models of chronic inflammation.

In conclusion, ST a phytotherapy traditionally used in the treatment of hemicephaly (a neurovascular disorder due to neurogenic inflammation and vasoactive peptides) exhibited antiinflammatory activity in various in vitro and in vivo models of inflammation. These results suggest that cyclo-oxygenase and lipoxygenase pathways could be involved in the antiinflammatory activity of ST like feverfew, a well-known prophylactic antimigraine herb.[32] Saponins, particularly the hederagenin type are known to have antiinflammatory, anticoagulant and antirheumatic activities.[33] It is therefore probable that the saponin component of the extract may contribute in part for the observed pharmacological activities.

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