PROTECTIVE EFFECT OF *CELASTRUS PANICULATUS* SEEDS EXTRACT AGAINST LIPOPOLYSACCHARIDE-INDUCED PAW EDEMA IN RATS

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ABSTRACT

The present study was designed to evaluate the effect of *Celastrus paniculatus* seeds methanolic extract (CPME) against lipopolysaccharide (LPS)-induced paw edema and nociception in rats. Moreover, *in-vitro* test for soluble epoxide hydrolase (sEH) inhibition activity of CPME was conducted to study its possible mode of action.

Celastrus paniculatus seeds were extracted with methanol by cold maceration for 7 days. The obtained dark brown coloured extract was evaluated for preliminary phytochemical screening and subjected for HPTLC analysis. Then the extract was evaluated for its action against LPS-induced paw edema in rats at the dose of 100, 200 and 400 mg/kg body weight by measuring the paw edema, nociceptive bioassays and estimating 5-HETE in serum. In addition, CPME was evaluated for *in-vitro* sEH inhibition activity.

Administration of CPME attenuates LPS-induced paw edema by 22.85% and serum 5-HETE levels by 9.56% (P<0.001) and elevated pain threshold by 112.19% (P<0.001). The CPME inhibits human, mouse and rat sEH activity with IC$_{50}$ of 4.60 µg/mL, 4.17 µg/mL and 5.18 µg/mL, respectively. The present study results indicate that CPME has protective effect against LPS-induced inflammation and pain in rats. The possible mode of action is attenuation of 5-HETE levels and sEH inhibition. However, further research is warranted to elucidate and isolate the active constituents from *Celastrus paniculatus* seeds.

Keywords: Jyotishmati; nociceptive bioassays; Randall-Selitto test; linoleic acid; 5-HETE

INTRODUCTION

Inflammation is a complex biological response of the body to pathogens, injury, irritants or other harmful stimuli and is a part of the body's defense mechanism (**Alam 2020**). The purpose of inflammation is to remove the initial cause of cell injury, clean necrotic cells and damaged tissues and initiate the process of tissue repair (**Rock & Kono 2008**). The persistence of inflammation for a longer duration of time may lead to painful stimuli and discomfort to the sufferers (**Rock & Kono 2008**). In addition, inflammatory responses play an important role in multiple pathological conditions and pathogenesis of many diseases (**Chen *et al.*, 2018**). The conventional therapies for inflammation include steroids and nonsteroid anti-inflammatory drugs (NSAIDs). However, these therapies have shown many side effects and deficiencies. NSAIDs are known to cause unwanted effects like gastrointestinal disturbances, stomach ulcers, headaches, dizziness, allergic reactions, increased risk of CVS and renal complications. The steroids may cause weight gain, difficulty in sleeping, an increased risk of infections and changes in mood and behavior (**Del Grossi Moura *et al.*, 2018**).

Celastrus paniculatus Willd. (Celastraceae) is commonly known as Jyotishmati and is distributed throughout Southeast Asia, India and Taiwan. The seeds of *C. paniculatus* are used in folk medicine for the treatment of analgesia, inflammation and rheumatic pain (**Kothavade *et al.*, 2015, Kulkarni *et al.*, 2015**). In *Ayurveda,* the seeds of *C. paniculatus* are used in the treatment of *vatavyadhi i.e.,* diseases like arthritis and gout (**Anonymous 2004**). The genus *Celastrus* (Celastraceae) plants have been widely used in traditional Chinese medicine and Indian medicine to treat cognitive dysfunction, epilepsy, insomnia, rheumatism, gout and dyspepsia for thousands of years (**Kothavade *et al.*, 2015, Kulkarni *et al.*, 2015**).

It is reported that the seeds of *C. paniculatus* contain chemical constituents such as palmitic, stearic, oleic, linoleic and linolenic acids, sesquiterpene alkaloids (celapanin, celapanigin) and celapagin, sesquiterpene polyol ester and phytosterols including b-sitosterol, campesterol and stigmasterol (**Kothavade *et al.*, 2015, Kulkarni *et al.*, 2015**).

Literature review reveals that flower extracts of *C. paniculatus* has been reported for their significant analgesic and anti-inflammatory activities (**Ahmad *et al.*, 1994**). *C. paniculatus* seeds extracts were evaluated for antiarthritic effect in adjuvant-induced arthritis in rats (**Patil & Suryavanshi 2007, Kothavade *et al.*, 2015**) and carrageenan-induced acute plantar inflammation (**Kulkarni *et al.*, 2015**).

Carrageenan-induced inflammation is acute and nonimmune inflammatory response (**Morris, 2003**). LPS-induced inflammation is the innate immune system, as well as non-immune cells, stimulated inflammatory response (**Yücel et al., 2017**). Systemic LPS has been identified as a pathogen-associated molecule pattern by higher vertebrates (**Yücel et al., 2017**). By binding to immune cells, LPS activates nuclear factor κB (NFκB) to increase the expression of tumour necrosis factor (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) (**Shaikh et al., 2016**). The cytokines play an important in pathogenesis of inflammatory and pain, cytokine modulating anti-inflammatory actions can be well studied by LPS-induced paw edema model in animals (**Vajja *et al.*, 2004, Kostadinov *et al.*, 2014**). Based on the above facts, the present study was planned to evaluate the effects of methanolic extract of *Celastrus paniculatus* seeds (CPME) against LPS-induced paw edema in rats. A plant derived sEH inhibitor 1-(1-propanoylpiperidin-4-yl)-3-(4-trifluoromethoxy) phenyl) urea (TPPU) and Ibuprofen were used as standard control for reference. Moreover, *in-vitro* test for soluble epoxide hydrolase (sEH) inhibition activity of CPME was conducted to study its possible mode of anti-inflammatory action.

MATERIAL AND METHODS

Chemicals

Lipopolysaccharide (LPS) and Ibuprofen were obtained from Sigma-Aldrich (USA), Rat 5-Hydroxyeicosatetraenoic acid ELISA kit was procured from Fine Test (Fine Biotech, China), A reference drug 1-(1-propanoylpiperidin-4-yl)-3-(4-trifluoromethoxy) phenyl) urea (TPPU) an sEH inhibitor was received from Department of Entomology, University of California. All the other chemicals used were of analytical grade.

Plant material and extraction procedure

The seeds of *Celastrus paniculatus* were collected from the local market of Tirupati and authenticated by Dr K Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateshwara University, Tirupati (India). The voucher specimen (644) was kept in the department herbarium for future reference. The dried seeds of *Celastrus paniculatus* (300 g) were coarsely powdered and extracted with absolute methanol (1000 ml) by cold maceration process for 7 days. The solvent was evaporated and dried at 40°C under reduced pressure using a

rotatory vacuum evaporator to get a dark brown colored residue (yield: 15%). The obtained extract was stored in glass bottles at 5°C until its further use.

Preliminary phytochemical screening of CPME

Qualitative tests

The preliminary qualitative tests for *Celastrus paniculatus* methanolic extract (CPME) were performed for the detection of the presence of alkaloids (Mayer's test, Dragendorff's test, Wagner's test, Hager's test), carbohydrates (Molisch's test, Fehling's test, Benedict's test), glycosides (Legal's test, Libermann-Burchard's), phytosterols (Liebermann- Burchard's test), fixed oils and fats (Spot test), saponins (Foam test), phenolic compounds and tannins (Ferric chloride test, Lead acetate test), proteins and free amino acids (Millon's test, Ninhydrin test), gums and mucilage, flavonoids (Shinoda test, Alkaline reagent test) **(Trease & Evans 1989 & Doss 2009)**.

Quantitative tests

Estimation of Total Flavonoids Content

For estimation of total flavonoids, 1 ml of extract (0.1% w/v in ethanol) is mixed with 1 ml $AlCl_3$ (2% w/v in ethanol) and 1 drop of acetic acid was added to it and made up to 25 ml with distilled water. Similarly, the standard quercetin in 0.1% w/v in ethanol was treated in the same manner as a sample. The sample and the standard were allowed to stand at room temperature for 40 min. The absorbance was measured at 415 nm and the readings were recorded **(Trease & Evans 1989 & Doss 2009)**. Total flavonoid content was calculated using the formula:

$$\text{Total flavanoid} = \frac{A \times M_0}{A_0 \times M}$$

where, A – absorbance of extract, Ao - absorbance of standard, M - weight of extract and Mo - weight of standard.

Estimation of Total Phenolic Content

For the estimation of total phenolic content, 1 ml of extract was mixed with 1.5 ml of Folin Ciocalteu's reagent and 8.5 ml of water. Allow it to stand for 5 min. Then 4 ml of 20% Sodium Carbonate was added to it. The absorbance was measured at 765 nm and total phenolic content (TPC) was estimated using gallic acid as standard **(Trease & Evans 1989 & Doss 2009)**.

Estimation of Total Alkaloidal Content

Total alkaloidal content in CPME was estimated by Gravimetric method. Briefly, quantity of extract was weighed and transferred to a separating funnel. 10 ml of chloroform was added and the contents were shaken well for 30 minutes to extract the alkaloids completely. The contents were dried in a china dish and the residue was weighed to calculate the mass of alkaloids **(Trease & Evans 1989 & Doss 2009)**.

HPTLC analysis of CPME

High-performance thin-layer chromatography (HPTLC) studies were carried out using a Camag HPTLC system with a Linomat V sample applicator, a Camag 3 TLC Scanner and WINCATS 4 software for interpretation of the data. An aluminium plate (20 × 10 cm) precoated with silica gel 60F254 (E Merck) was used as the adsorbent. The plates were developed using n-hexane: ethyl acetate (5:4) as the mobile phase for CPME in a Camag twin trough chamber and scanned at 254, 366 and 425 nm. The Rf values of the CPME were determined using WINCATS 4 software. The developed plates were photo-documented at 254 nm, 366 nm and 425 nm using a Camag 3 Reprostar. The Rf values of CPME were compared with the Rf value of standard Linoleic acid and confirmed by the overlay of spectra.

Animals

Adult Wister rats (7-8 weeks) were used in the present study. The animals were housed under standard laboratory conditions, maintained on a 12 h light: 12 h dark cycle and had free access to food and water. Animals were acclimatized to laboratory conditions before the test. The experimental protocols were approved by the Institutional Animal Ethics Committee (DSU/PhD/IAEC/12/2017-18). All the procedures performed were in accordance with Control and Supervision of Experiments on Animals (CPCSEA) guidelines, under Ministry of Animal Welfare Division, Government of India, New Delhi.

Measurement of paw volumes

After LPS challenge the volume of hind paw edema was measured at 0, 1, 2, 3, 4, 5 and 6 h. by using a digital plethismometer (Dhadde 2019). The percentage inhibition in paw volume was calculated using the formula given below.

% paw volume inhibition

$$= \frac{\text{Paw volume in LPS alone group} - \text{Paw volume in test group}}{\text{Paw volume in LPS alone group}} \times 100$$

Nociceptive bioassays (Randall-Selitto test)

After paw volume measurement animals were evaluated for pain threshold at the site of inflamed paw by Randall-Selitto using digital paw pressure analgesia meter (Orchid Scientific). The Randall-Selitto test was used to assess the analgesic effect of CPME on the response thresholds to mechanical pressure stimulation in inflammation-induced pain in the hind paw of the rat. In this test, the force was applied to the inflamed paw mechanically. When the animal responds to the pain and struggles then pressure was released and the pressure at which the animal felt pain was displayed on the TFT display was recorded. To minimize the error in the experiment, three readings were recorded and average pressure was considered as pain threshold and reported in gram force **(Wagner *et al.*, 2013)**.

Estimation of 5-HETE in serum

After recording paw volumes and pain threshold, a blood sample was collected from each animal by retro-orbital plexus under light ether anaesthesia. The serum was separated by centrifugation 2000 × g for 10 min. The serum was used for estimation of 5-HETE by ELISA plate reader – BIORAD with kit (Fine test).

In-vitro Soluble Epoxide Hydrolase (sEH) Inhibition Activity

Dried plant extracts were solubilized at 10 mg/ml in DMSO and inhibitory potencies were measured against the mouse, rat and human sEH using a fluorescent reporting system as per the method prescribed by Jones *et al*., (2005). Shortly, IC_{50} values were determined using cyano (2 methoxynaphthalen-6-yl) methyl trans-(3-phenyl-oxyran-2-yl) methyl carbonate (CMNPC) as a fluorescent substrate. Recombinant sEH (1 nM) was incubated with inhibitors for 5 min in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1 mg/mL of BSA at 30° C prior to substrate introduction ([S] = 5 µM). The activity was measured by determining the appearance of the 6-methoxy-2-naphthaldehyde with an excitation wavelength of 330 nm and an emission wavelength of 465 nm for 10 min.

RESULTS

Preliminary phytochemical screening of CPME

Preliminary phytochemical screening indicates that CPME contains components such as alkaloids, carbohydrates, glycosides, proteins, free amino acids and flavonoids, whereas phytosterols, fixed oils, fats, saponins, gums and mucilage were absent.

The quantitative test results indicate that CPME contains 10.6913 QE/g (quercetin equivalent) of total flavonoids, 2.25 µg/ml (equivalent to gallic acid) total phenolic content and 13.24% of alkaloidal content.

HPTLC analysis of CPME

The HPTLC fingerprint studies were carried out for establishing the presence of the biomarker compound linoleic acid in CPME. The Rf value of standard linoleic acid was found to be 0.87 at 366 nm. The CPME revealed a spot having Rf 0.87 corresponding to that of standard linoleic acid at 366 nm (Figure 1). The presence of linoleic acid in CPME was confirmed by overlay spectrum study of standard linoleic acid at 366 nm. The standard linoleic acid, CPME exhibited blue fluorescence under 254 nm, bright blue fluorescence under 366 nm and no fluorescence under 425 nm. The CPME was also evaluated for presence of linolenic acid but could not be confirmed by overlay spectrum study of standard linolenic acid at 254 nm.
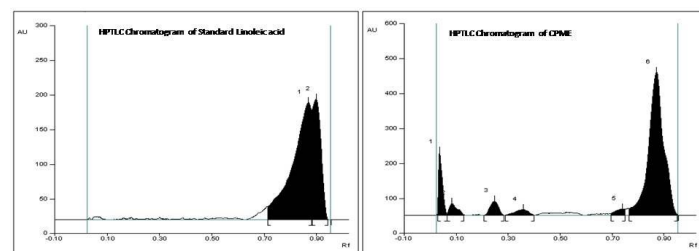

Figure 1 HPTLC Analysis of linoleic acid and *Celastrus paniculatus* seeds methanolic extract

In-vivo Antiinflammatory Activity

Effect of CPME on LPS induced paw edema in rat

Table 1 depicts the result of effect of methanolic extract of *Celastrus paniculatus* on LPS induced paw edema in rats. The paw edema was found to be inhibited in the animals treated with all the doses of CPME at all the time points of measurements. This report suggests that CPME inhibits edema in LPS injected paw in a dose-dependent manner. Treatment with CPME at the dose of 400 mg/kg

showed the highest degree of edema inhibition (22.85 ± 0.62) at 6 h post LPS injection interval. Inhibition of paw edema by CPME is comparable with actions produced by standard drugs ibuprofen (30 mg/kg) and TPPU (0.1 mg/kg). Treatment with CPME at the dose of 400 mg/kg is 87-89% of inhibition as that of standard drugs at all the time points. This result indicates the potent anti-inflammatory actions of CPME. The results were comparable with a plant derived sEH inhibitor TPPU and Ibuprofen.

Table 1 Effect of methanolic extract of *Celastrus paniculatus* on LPS induced paw edema in rats

Time	Paw volume in ml (% inhibition)					
	LPS alone treated group	CPME 100 mg/kg	CPME 200 mg/kg	CPME 400 mg/kg	Ibuprofen 30 mg/kg	TPPU 0.1 mg/kg
0 h	1.25 ± 0.01	1.25 ± 0.02	1.24 ± 0.01	1.26 ± 0.02	1.25 ± 0.03	1.26 ± 0.02
1 h	1.81 ± 0.03	1.57 ± 0.03*** (13.03%)	1.55 ± 0.01*** (14.15%)	1.52 ± 0.03*** (15.58%)	1.49 ± 0.02*** (17.46%)	1.49 ± 0.02*** (17.58%)
2 h	2.11 ± 0.02	1.80 ± 0.03*** (14.45%)	1.78 ± 0.02*** (15.25%)	1.77 ± 0.01*** (16.23%)	1.72 ± 0.03*** (18.65%)	1.71 ± 0.02*** (18.89%)
3 h	2.38 ± 0.03	2.02 ± 0.04*** (15.03%)	1.99 ± 0.02*** (16.18%)	1.97 ± 0.01*** (17.18%)	1.90 ± 0.03*** (19.87%)	1.89 ± 0.02*** (20.58%)
4 h	2.49 ± 0.02	2.08 ± 0.02*** (16.30%)	2.07 ± 0.04*** (17.01%)	2.04 ± 0.02*** (17.79%)	1.96 ± 0.03*** (21.41%)	1.94 ± 0.04*** (22.12%)
5 h	2.61 ± 0.02	2.16 ± 0.03*** (17.21%)	2.14 ± 0.04*** (18.13%)	2.10 ± 0.02*** (19.39%)	1.99 ± 0.05*** (23.44%)	1.99 ± 0.04*** (23.87%)
6 h	2.79 ± 0.01	2.30 ± 0.04*** (17.63%)	2.24 ± 0.05*** (19.68%)	2.15 ± 0.05*** (22.85%)	2.06 ± 0.04*** (26.06%)	2.05 ± 0.03*** (26.52%)

Values are expressed as mean ± SEM (n = 6), ***P < 0.001 Vs LPS alone treated rats. Two-way ANOVA followed by Bonferroni post hock test. The figures in parenthesis indicates the % inhibition of inflammation compared to LPS alone treated animals.

Effect of CPME on nociceptive response in rat

Nociceptive bioassays (Randall-Selitto test) study report suggests that LPS induces inflammatory pain in the paw and it was evident by pain threshold levels in LPS alone treated animals. Immediately after LPS injection, the pain threshold in all the groups of animals was almost equal there was no statistical difference among the groups. After that response of animals towards pain sensation changes among the groups and it was evident by pain threshold results recorded at different time points. LPS alone treated animals respond quickly to the pressure-induced pain at the inflamed paw. The pain threshold in LPS alone treated animals was recorded between 35-37 gram force where it was 42-44 gram force in normal control animal. Treatment with all the tested doses of CPME decreased the pain sensation in the animals and it was indicated by increased pain threshold in those groups of animals. The highest pain threshold was recorded in CPME 400 mg/kg dose and it was found to be 52-84 gram force at the different time interval of post LPS treatment. The pain threshold recorded in ibuprofen (30 mg/kg) and TPPU (0.1 mg/kg) were comparable with CPME 400 mg/kg dose. The detailed pain threshold at each time point interval is shown in Figure 2.
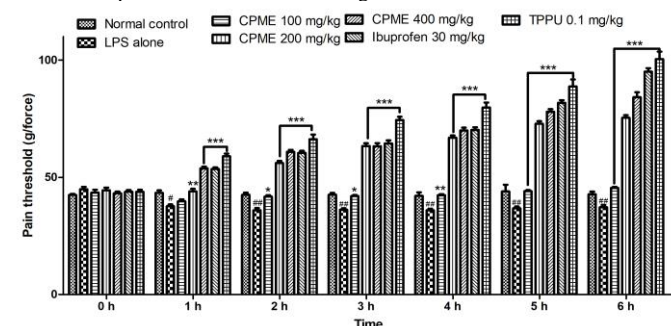

Figure 2 Effect of *Celastrus paniculatus* seeds methanolic extract on nociceptive response in LPS-treated rats. Values are expressed as mean ± SEM (n = 6), #P < 0.05 and ##P < 0.01 Vs normal control and *P < 0.05, **P < 0.01 and ***P < 0.001 Vs LPS alone treated rats. Two-way ANOVA followed by Bonferroni post hock test.

Effect of CPME on serum 5-HETE level in LPS-treated rat

Administration of LPS elevated the levels of 5-HETE in the rat serum by 10.15% and it was found to be statistically significant (P<0.001) when compared to the normal control. Treatment with CPME at all the tested doses restored the 5-HETE levels towards normal compared to the LPS alone treated animals. The levels of 5-HETE in CPME treated animals were significantly (P<0.001) different compared to the LPS alone treated animals. Treatment with ibuprofen (30 mg/kg) and TPPU (0.1 mg/kg) to LPS challenged rats also controlled the elevation of 5-HETE significantly (P<0.05 and P<0.001, respectively). Figure 3 represents the effect of CPME on the levels of 5-HETE in LPS treated rats.
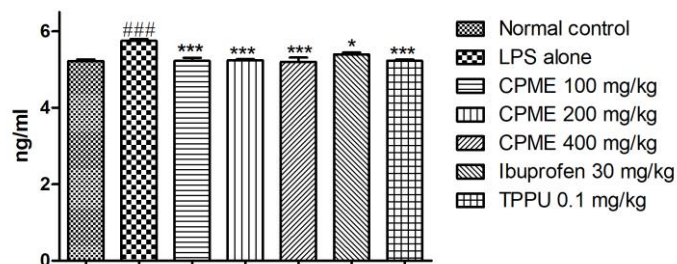

Figure 3 Effect of *Celastrus paniculatus* seeds methanolic extract on serum 5-HETE level in LPS-treated rat. Values are expressed as mean ± SEM (n = 6), ###P < 0.001 Vs normal control rats and *P < 0.05 and ***P < 0.001 Vs LPS alone treated rats. One-way ANOVA followed by Tukey's post hock test.

In-vitro Soluble Epoxide Hydrolase (sEH) Inhibition Activity

The *in-vitro* sEH inhibition test results reveal that CPME inhibits human, mouse and rat sEH enzyme. The IC$_{50}$ values of *in-vitro* sEH inhibition test were found to be 4.60 µg/mL for human sEH, 4.17 µg/mL for mouse sEH and 5.18 µg/mL for rat sEH. Figure 4, represents the detailed results of *in-vitro* sEH inhibition test of CPME.
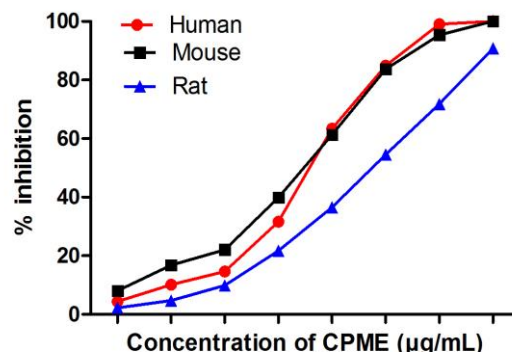

Figure 4 Effect of *Celastrus paniculatus* seeds methanolic extract on soluble epoxide hydrolase (sEH) activity (*In-vitro* test).

DISCUSSION

The present study results demonstrate that CPME protect the rats from LPS-induced inflammatory response and it was evident by attenuation of paw edema, elevated pain threshold and decreased 5-HETE levels in LPS-treated animals.

These effects may be attributed to inhibition of the sEH enzyme and it was indicated by in-vitro sEH inhibition study results of CPME.

Administration of LPS to rats caused edema and pain and it was evident by increased paw volume (62%) and decreased pain threshold (14%) when compared with animals of normal control group. Treatment with CPME (100, 200 and 400 mg/kg) to LPS-treated rats attenuated the LPS-induced paw edema by 22.85% and increases the pain threshold by 112.19% compared to LPS alone treated animals. These results indicate the anti-inflammatory and nociceptive effects of CPME.

5-HETE are the eicosanoids (leukotrienes) synthesized from free arachidonic acid in presence of enzyme 5-lipooxygenase (5-LOX) after reduction of 5-HPTE and it plays important role in immune response, allergy, asthma and inflammation. 5-HETE is a highly active metabolite that acts as a proinflammatory in various diseases associated with inflammation (**Hanna et al., 2018**). The present study results are in agreement with the above finding as there was significantly increased levels of 5-HETE (10.15%) was observed in serum of LPS-treated animals when compared with normal control animals. Therefore, restricting the production of 5-HETE in the body is one of the targets to attack the inflammatory process. Administration of CPME to LPS-treated rats attenuated the LPS-induced elevated levels of 5-HETE by 9.56% compared to LPS alone treated animals. Hence, attenuation of 5-HETE level may be one of the mechanisms through which CPME protect the animals from LPS triggered inflammation.

In addition to restricting the production of 5-HETE in the body, CPME also has inhibitory action on sEH enzyme activity. This was evidenced by sEH activity inhibition study conducted using human, mouse and rat sEH enzymes. It was observed that CPME inhibits human, mouse and rat sEH activity with IC50 of 4.60 µg/mL, 4.17 µg/mL and 5.18 µg/mL, respectively.

The cytochrome P450 mediated synthesis of eicosanoids yields hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs). The HETEs are proinflammatory on other hand EETs inhibits inflammation and associated pain. Moreover, EETs exert various beneficial effects in the body and helps in reducing blood pressure, generation of reactive oxygen species, apoptosis, pain and platelet aggregation (**Harris & Hammock 2013; Yang et al., 2015a; Yang et al., 2015b**). However, EETs have a very short half-life, enzyme, sEH hydrolyzes them into their corresponding dihydroxyeicosatrienoic acids (DHETs). The hydrolyzed product DHETs have less potent compared to its corresponding EETs. Hence, inhibition of sEH enzyme activity increases the half-life of EETs as results extend its beneficial effects in the body including anti-inflammatory and anti-nociceptive actions (**Wagner et al., 2013**). The present study data well supports the above finding and it is evident by sEH enzyme activity and increased pain threshold in CPME treated animals when compared to the LPS alone treated animals.

Hence, anti-inflammatory and nociceptive action exerted by CPME is the combined effect of restricting the production of 5-HETE and inhibition of sEH enzyme activity by ultimately extending the half-life of anti-inflammatory mediator EETs.

The phytochemical analysis of CPME revealed that it contains the principle active constituents like alkaloids, glycosides and flavonoids and biomarker compound linoleic acid. These active compounds of CPME may be responsible for their inhibitory action on 5-HETE synthesis and sEH enzyme activity and ultimately anti-inflammatory and nociceptive actions.

CONCLUSION

In conclusion, methanolic extract of *Celastrus paniculatus* seeds normalized the altered paw edema, reduced the pain sensation and behaviour in LPS-treated rats, suggesting its anti-inflammatory and anti-nociceptive efficacy. The possible mode of protection may be due to its attenuation action on 5-HETE levels and sEH enzyme inhibition properties. However, further research is warranted to elucidate and isolate the active constituents from *Celastrus paniculatus* seeds.

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