Anti-nociceptive activity of ethyl acetate fraction of *Cassia fistula* L. pods in experimental animal models

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Abstract

The objective of the present study was to evaluate the anti-nociceptive activity of ethyl acetate fraction of *Cassia fistula* L. pods (EAFCF). Various groups of animals received EAFCF at the doses of 50, 100 & 200 mg/kg through oral route and anti-nociceptive activity was evaluated using acetic acid induced writhing model, hotplate test, tail immersion test and formalin induced paw licking model. EAFCF reduced the number of acetic acid induced abdominal contractions and both early and late phases of formalin induced paw licking. Moreover, it has increased the reaction latency in hotplate and tail immersion test. These findings indicate both central and peripheral anti-nociceptive effects of EAFCF. The study concluded the presence of central and peripheral anti-nociceptive potential of *Cassia fistula* L. pods and justified the traditional use of this plant for treating various painful conditions.

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Key words- *Cassia fistula*, Acetic acid induced writhing, Formalin test, Hotplate model, Anti-nociceptive activity

1. Introduction

According to an estimate by World Health Organization (WHO), about 70 to 80% of the people around the world use herbal medicine for primary health care. Synthetic drugs used currently for the management of pain cause several unwanted effects [1]. Plants, in spite of extensive research, represent huge number of unidentified source of structurally new compounds that might serve as lead for the development of novel drugs [2]. *Cassia fistula* L. (Leguminosea) is commonly is commonly known as Indian laburnum and is native to South Asia. Traditionally, parts of the plant were used for burns, diabetes, pain, epilepsy, worm infestation, fever and stomach disorders [3]. Studies reported on the phytochemical analysis of *Cassia fistula* pods showed the presence of flavonoids, phenolic compounds, proanthocyanidins, alkaloids, tannins and saponins as secondary metabolites [4, 5, 6]. Various pharmacological activities of *Cassia fistula* L. such as Central Nervous System (CNS) depressant activity [7], antioxidant [8], wound healing [9], antifungal and antibacterial [10] antitumor [11], anti-fertility [12], hepatoprotective [13], anti-diabetic [14] and analgesic activity [15,16] have been reported. Though the other pharmacological activities studied extensively, the anti-nociceptive activity has of *Cassia fistula* L. pods has not been reported adequately. The results of preliminary study conducted in our laboratory revealed the presence of high flavonoids in ethyl acetate fraction of *Cassia fistula* L. pods than the other plant parts and extract fractions. Hence, the present study seeks to evaluate anti-nociceptive activity of ethyl acetate fraction of *Cassia fistula* L. pods.

2. Materials and methods

2.1. Plant material

Fruit of *Cassia fistula* pods were collected from surroundings of Coimbatore and voucher specimen authentication was done by scientist ‘F’, Botanical Survey of India, Agricultural University, Coimbatore. The sample voucher specimen BSI/SRC/5/23/2011-12/Tech78 was deposited future use.

2.2. Animals

Healthy albino Sprague Dawley rats of 100-150g and Swiss albino mice weighing 20-25 g were obtained from the animal house of PSG institute of Medical Sciences and Research, Coimbatore, India after obtaining ethical approval for protocol and animal usage from Institutional Animal Ethical Committee (IEAC). The animals were housed under standard environmental condition.
(Temperature 20-22°C, humidity 65-70%, 12 h light/dark cycle) with free access for food and water. The experimental protocols were conducted as per guidelines of Committee for the Purpose of Supervision and Control of Experiments on Animal (CPCSEA).

2.3. Extraction and fractionation of pods
The fruit pods were air dried in shade and the dry material was powdered. The hydroalcoholic extract of powdered pods was prepared using 70% ethanol and 30% distilled water by maceration method for two days followed by evaporation in oven at 60°C for 24 h to get dried waxy brown to black solid hydroalcoholic extract (46 g). From this, 25 g was taken for further fractionation and was dissolved in water and extraction with chloroform was done in separating funnel. Chloroform layer was partitioned with ethylacetate and solvent was evaporated to get ethylacetate fraction of Cassia fistula L. pods (EAFCF) (4.7 g) [17].

2.4. Phytochemical investigations
EAFCF was qualitatively analyzed for the presence of various phytoconstituents such as flavonoids, saponins, carbohydrates, tannins, alkaloids, glycosides, reducing sugars, proteins and steroids by using standard procedures [18].

2.5. Pharmacological evaluations
2.5.1. Dosing schedule
Animals were randomly divided into 5 groups of 6 animals each. Group I served as control, group II, III, IV received EAFCF at the doses of 50, 100 & 200 mg/kg, p.o., respectively and group V received diclofenac sodium (10 mg/kg, i.p.) in acetic acid induced writhing model. Morphine sulphate (1.5 mg/kg, i.p.) was given to group V animals in hotplate and tail flick tests and morphine (5 mg/kg, i.p.) in formalin induced pain model. In rotarod experiment, animals received diazepam (2mg/kg, i.p.) served as standard.

2.5.1. Acetic acid-induced writhing test
This pharmacological evaluation was performed to find out whether the treatment of EAFCF produces peripheral and central anti-nociceptive activity against chemically induced nociception. The mice were treated with drug or extract, 30 minutes prior to the administration 0.7% acetic acid (10 ml/kg, i.p.). The mice were observed immediately after acetic acid administration and the number of writhing was counted for 30 min [19]. Complete was writhing considered when the animal showed contraction of the abdomen, elongation of the body, twisting of the trunk and/or pelvis ending with the extension of the limbs.

2.5.2. Hotplate test
Mice were placed on hotplate maintained at a temperature of 55± 1°C and basal reaction time of animal (forepaw licking, withdrawal of the paw(s) or jumping response) was recorded. The mice that did not show any response to nociceptive stimuli within 15 s were excluded from this study. The animals were treated with morphine or EAFCF and were placed on Eddy’s hotplate maintained at a temperature of 55± 1°C and the reaction times were noted again at 30, 60, 90, and 120 minutes interval. A cutoff period of 20 s was set to avoid tissue damage in foot [20].

2.5.3. Tail immersion test
This test was performed to evaluate the central analgesic property and this model based on the observation that the central analgesics such as morphine increase the latency to withdraw the tail of a rodent when it is immersed in hot water [21]. Mice were treated with standard or EAFCF and one to two cm of the tail was immersed in hot water kept at the temperature of 55± 1°C. time latency to withdraw the tail was noted at 30, 60, 120 minutes after the treatment. To prevent the excessive tail tissue damage, a latency period of 20 s was maintained.

2.5.4. Formalin induced nociception
Rats were treated with standard or EAFCF 30 minutes prior to the administration of with 0.03 ml of 1% formalin in the sub-planter region of right hind paw. The nociceptive responses (licking or biting of formalin injected site) were noted in two phases. First 5 minutes after the administration of formalin comprises the first phase and the second phase comprises the 15 to 30 minutes after the formalin administration [22].

2.5.5. Rotarod test
Animals were placed on a horizontal bar (2.5 cm diameter) revolving at a speed of 15 rpm. The parameters such as time permanence on the rotating bar and the number of falls for 1 minute were assessed in each animal [17].

2.6. Statistical analysis
The data obtained from all experiments were expressed as mean±SEM. The results were statistically analyzed using one way analysis of variance (ANOVA) followed by Dunnnett’s post hoc test or Bonfer- roni’s test as appropriate and were performed with Prism 4.0. Difference between the groups were considered significant when p<0.05.

3. Results
3.1. Preliminary phytochemical analysis
The results of preliminary phytochemical screening indicated the presence of flavonoids, anthraquinones, terpenoids, phenolic compounds in EAFCF.

3.2. Acetic acid-induced writhing
Administration of EAFCF (50, 100, and 200 mg/kg, p.o.) reduced the acetic acid induced writhing significantly (p<0.001) compared to control group in dose dependent manner (Table 1) and the reduction in writhing was observed as 91.07% in standard analgesic, diclofenac sodium (10 mg/kg, i.p.), treated animals and 40.18%, 71.07% and 92.01% respectively, in 50, 100 and 200 mg/kg of EAFCF treated animals.

3.3. Eddy’s hot plate model in mice
In this model, the reaction latency to thermal stimuli was increased significantly (P<0.01) in EAFCF treated groups compared to the control group. The maximum effect (reaction time of 18.4 s) was observed at the highest dose viz. 200 mg/kg p.o. at 60 min. while the standard drug morphine (1.5 mg/kg i.p.) showed highest reaction time of 17.8 s. The anti-nociceptive effect produced by EAFCF was found to be dose and time dependent (Table 2).

3.4. Tail immersion test
The antinociceptive activity exhibited by EAFCF and morphine in tail immersion test is given in Table 3. EAFCF (100 & 200 mg/kg, p.o.) showed dose dependent increase in the reaction latency to hot-water induced thermal stimuli (p<0.01). Morphine also produced similar
Table 1. Analgesic activity of EAFCF in acetic acid induced writhing in mice

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment</th>
<th>No. of writhing</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle 10ml/kg, p.o.</td>
<td>66±1.07</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Diclofenac 10mg/kg, i.p.</td>
<td>5.89±0.9*</td>
<td>91.07</td>
</tr>
<tr>
<td>3</td>
<td>EAFCF 50mg/kg, p.o.</td>
<td>39.48±0.67*</td>
<td>40.18</td>
</tr>
<tr>
<td>4</td>
<td>EAFCF 100mg/kg, p.o.</td>
<td>19.09±0.57*</td>
<td>71.07</td>
</tr>
<tr>
<td>5</td>
<td>EAFCF 200mg/kg, p.o.</td>
<td>5.27±0.89*</td>
<td>92.10</td>
</tr>
</tbody>
</table>

Values are expressed in terms of mean ± SEM, n = 6 in each group, *P<0.001 statistically significant as compared with control group. EAFCF = Ethylacetate fraction of Cassia fistula L.. i.p.= intraperitoneal, p.o.= per oral

Table 2. Anti-nociceptive effect of EAFCF in Eddy’s hot plate model using mice

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment</th>
<th>Reaction time in seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pretreatment</td>
</tr>
<tr>
<td>1</td>
<td>Vehicle 10ml/kg, p.o.</td>
<td>3.8±0.05</td>
</tr>
<tr>
<td>2</td>
<td>Morphine 1.5mg/kg, i.p.</td>
<td>4.1±0.06</td>
</tr>
<tr>
<td>3</td>
<td>EAFCF 50mg/kg, p.o.</td>
<td>5.1±0.09</td>
</tr>
<tr>
<td>4</td>
<td>EAFCF 100mg/kg, p.o.</td>
<td>5.2±0.10</td>
</tr>
<tr>
<td>5</td>
<td>EAFCF 200mg/kg, p.o.</td>
<td>4.8±0.12</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM, n = 6 in each group, *P<0.01 statistically significant as compared with control group. EAFCF = Ethylacetate fraction of Cassia fistula L.. i.p.= intraperitoneal, p.o.= per oral

Table 3. Anti-nociceptive activity of EAFCF in mouse tail immersion test

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment</th>
<th>Reaction time in seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pretreatment</td>
</tr>
<tr>
<td>1</td>
<td>Vehicle 10ml/kg, p.o.</td>
<td>3.8±0.05</td>
</tr>
<tr>
<td>2</td>
<td>Morphine 1.5mg/kg, i.p.</td>
<td>3.9±0.07</td>
</tr>
<tr>
<td>3</td>
<td>EAFCF 50mg/kg, p.o.</td>
<td>4.5±0.1</td>
</tr>
<tr>
<td>4</td>
<td>EAFCF 100mg/kg, p.o.</td>
<td>4.9±0.20</td>
</tr>
<tr>
<td>5</td>
<td>EAFCF 200mg/kg, p.o.</td>
<td>5.6±0.22</td>
</tr>
</tbody>
</table>

Experimental data given as mean ± SEM, n = 6 in each group, *P<0.01 statistically significant as compared with control group. EAFCF = Ethylacetate fraction of Cassia fistula L.. i.p.= intraperitoneal, p.o.= per oral

Table 4. Reduction of formalin induced paw licking by EAFCF and morphine treatment in rats

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment</th>
<th>Hind paw licking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Early phase</td>
</tr>
<tr>
<td>1</td>
<td>Vehicle 10ml/kg, p.o.</td>
<td>113.6±0.91</td>
</tr>
<tr>
<td>2</td>
<td>Morphine 5mg/kg, i.p.</td>
<td>42.6±0.03*</td>
</tr>
<tr>
<td>3</td>
<td>EAFCF 50mg/kg, p.o.</td>
<td>102.1±1.8</td>
</tr>
<tr>
<td>4</td>
<td>EAFCF 100mg/kg, p.o.</td>
<td>59.8±1.25*</td>
</tr>
<tr>
<td>5</td>
<td>EAFCF 200mg/kg, p.o.</td>
<td>40.6±0.4*</td>
</tr>
</tbody>
</table>

Values are given as mean ± SEM and as %, n = 6 in each group, *P<0.001 statistically significant as compared with control group. EAFCF = Ethylacetate fraction of Cassia fistula L.. i.p.= intraperitoneal, p.o.= per oral

Table 5. Effect of EAFCF on time permanence on rotarod.

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Groups</th>
<th>Treatment</th>
<th>Time permanence on rotarod (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Vehicle</td>
<td>10 ml/kg, i.p.</td>
<td>116.21±0.58</td>
</tr>
<tr>
<td>2.</td>
<td>EAFCF</td>
<td>50 mg/kg, p.o.</td>
<td>105.12±0.56</td>
</tr>
<tr>
<td>3.</td>
<td>EAFCF</td>
<td>100 mg/kg, p.o.</td>
<td>106.16±0.47</td>
</tr>
<tr>
<td>4.</td>
<td>EAFCF</td>
<td>200 mg/kg, p.o.</td>
<td>94.16±0.74</td>
</tr>
<tr>
<td>5.</td>
<td>Diazepam</td>
<td>2 mg/kg, i.p.</td>
<td>18.55±1.21*</td>
</tr>
</tbody>
</table>

Each value represents mean±SEM (n=8) *P<0.01 compared to control. One way ANOVA and Dunnett’s test as post hoc test were performed. EAFCF = Ethylacetate fraction of Cassia fistula L.. i.p.= intraperitoneal, p.o.= per oral
effect as that of EAFCF 200mg/kg. EAFCF 50mg/kg was found to be sub-effective.

3.5. Formalin test
Administration of EAFCF orally at the doses of 50,100, and 200 mg/kg dose dependently reduced the paw licking induced by formalin in both early and late phases of the experiment (p<0.001). A complete inhibition of licking in late phase was evident from morphine treated animals (Table 4).

3.6. Effect of EAFCF on rotarod behavior in mice
Treatment of EAFCF showed no significant (p<0.05) change in time permanence on the rotating bar when compared to control group. However, at higher dose (>400 mg/kg) EAFCF reduced the time permanence (p<0.01) similar to that of standard drug diazepam (2 mg/kg)

4. Discussion
In the present study, EAFCF was evaluated for its nociceptive activity in peripheral as well as central analgesic models. Earlier studies reported the analgesic and anti-nociceptive activity of Cassia fistula L. leaves [16, 17], while this study, for the first time, investigated the anti-nociceptive potential of Cassia fistula L. pods. Acetic acid induced writhing in mice is simple and most reliable inflammatory pain model widely used for the evaluation of peripheral analgesics. The pain caused by acetic acid is said to be an inflammatory pain due to increase in the capillary permeability and release of endogenous mediators such as PGE1, PGE2, histamine, bradykinin, substance P etc... which sensitize the nociceptive nerve endings [23]. NSAIDs are known to inhibit the COX enzyme in the peripheral tissues which is responsible for the production of pain mediators. In this study, EAFCF showed dose dependent analgesic and antinociceptive activity as evident through reduction in number of writhing caused by acetic acid. Hence, EAFCF may act via blockade of the release or activity of endogenous pain mediators resulted in the interruption of pain stimuli transduction similar to that of the standard drug, diclofenac sodium.

Treatment of EAFCF in mice, increased the reaction time significantly to the thermal stimuli in both hotplate and tail immersion model. These two models are mainly used for centrally acting analgesics, while the peripheral analgesics are found to be ineffective [24]. The reaction to the hotplate demonstrates the supraspinal reflex and tail immersion explains the spinal reflex mediated by various sub-types of opioid receptors [25]. Findings of the present study indicate that the EAFCF may act as an anti-nociceptive by central mechanisms.

Administration of EAFCF also reduced the paw licking caused by sub-plantar formalin injection. This model is useful in evaluating the anti-nociceptive activity in two different phases. In the initial phase, direct chemical stimulation of sensory afferent nerve endings particularly C fibers causes neurogenic pain. In the later phase, induction of inflammatory pain occurs due to the increased production and/or action of various inflammatory mediators. Centrally acting analgesics such as morphine effectively reduce or prevent the paw licking in both the phases whereas, peripheral analgesics such as diclofenac reduce paw licking only in late phase due to inflammatory pain [26]. In this study, EAFCF showed dose dependent inhibition of paw licking in early neurogenic and late inflammatory pain phases. Reduction of neurogenic pain perception by EAFCF was also confirmed in hotplate and tail immersion tests; while the effect of EAFCF in acetic acid induced writhing further confirms the anti-nociceptive action in inflammatory pain conditions.

Treatment of EAFCF at the doses used in this study, did not alter the skeletal muscle tone as it did not cause any significant change in time permanence on rotarod. This clearly indicates that EAFCF particularly reduces the pain perception and not through central nervous system depression or skeletal muscle relaxation.

In conclusion, the results of the present study clearly demonstrated the strong anti-nociceptive activity of cassia fistula pods are in central as well as peripheral pain models. The anti-nociceptive effect of Cassia fistula L. pods may be through inhibition of pain transmission as well as by inhibition of peripheral inflammatory mediators. Current study justifies the traditional use of Cassia fistula L. for treating burns and pain of various origins. However, further phytochemical characterization of fraction and elucidation of molecular mechanism responsible for anti-nociceptive activity of Cassia fistula L. pods is required which could prove identification of lead molecule for the development of new analgesic drugs.

Acknowledgement
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Abbreviations
EAFCF- Ethylacetate fraction of Cassia fistula L. pods
i.p.- intraperitoneal
p.o.- per oral

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