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# Research Article

# Anticonvulsant and anxiolyic activities of ethyl acetate fraction of *Cassia fistula* Linn. pods in mice

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## ABSTRACT

**Context:** *Cassia fistula* L. (Leguminacea) is used by Indian and Tanzanian traditional healers for treating various ailments related to the central nervous system. **Aims:** The present study aimed to evaluate the anticonvulsant and anxiolytic activity of the ethyl acetate fraction obtained from a hydroalcoholic extraction of *Cassia fistula* pods (EAFCF) and thereby to provide scientific validation for its traditional use. **Methods:** Preliminary phytochemical analysis and estimation of the flavonoid content of the ethyl acetate fraction was performed. Anticonvulsant activity was assessed by the subcutaneous pentylenetetrazole test (s.c. PTZ test). Anxiolytic activity was assessed by elevated plus maze (EPM) and open field tests (OFT). The sedative and motor toxicity was evaluated by a phenobarbitone induced sleep test and rotarod behavior respectively. **Results:** Photochemical analysis revealed the presence of a high flavanoid content in the ethyl acetate fraction. Treatment of EAFCF (50 and 100 mg/kg) significantly increased the latency to the onset of minimal clonic seizure and generalized tonic clonic seizure and animals were completely protected from death due to PTZ administration. Administration of EAFCF at the dose of 100 mg/kg markedly increased the open arm entries and time spent in open arm in EPM. In OFT, EAFCF increased the number of central squares crossed and time spent in the central compartment. EAFCF in the doses used in this experiment did not produce sedation or motor toxicity. **Conclusion:** The results obtained herein clearly indicate the anticonvulsant and anxiolytic activity of EAFCF which may be due to the high flavonoid content. These findings give the scientific support for common use of this plant for treating epilepsy and anxiety.

**Key words:** Pentylenetetrazole, elevated plus maze, thigmotaxic behaviour, flavonoid content estimation, *Cassia fistula*, neurobehavioral profile.

## INTRODUCTION

Epilepsy and anxiety are the two most common co-morbid conditions. Epilepsy is a neurological disorder characterized by frequent occurrence of seizure due to abnormal discharge of group of cortical neurons.[1] Anxiety, on the other hand, is a psychological disorder generally affecting the mood and causing irrational fear as one of the most prominent symptoms.[2] The demand for traditional and complementary medicine has been increasing worldwide. Moreover, native healers remain the sole or main health providers for millions of people living in rural areas of some developing countries. Use of herbal remedies for the treatment of central nervous system (CNS) ailments such as anxiety, depression, epilepsy and sleep disorders has long been practiced.[3] Plant extracts that can suppress the occurrence of seizure and that possess anxiolytic activity would provide an alternative and complementary therapy for treating epilepsy and associated anxiety.

*Cassia fistula* L. (commonly known as Indian laburnum) belongs to the family Leguminacea. It has been used in folk medicine to cure burns, constipation, convulsion, depression, dysurea, worm infestation etc.[4] Several pharmacological activities of *cassia fistula L.* including antioxi-

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dant,[5-7] CNS depressant,[8] wound healing,[9] antifungal and antibacterial,[10] antitumor,[11,12] anti-fertility,[13] hepatoprotective[14] and anti-diabetic[15] have been reported.

Previous phytochemical studies on *Cassia fistula* pods showed the presence of flavonoids, phenolic compounds, proanthocyanidins such as epiafzelechin, epicatechin, catechin, and procyanidine B-2 as secondary metabolites.[16-18] Although a number of scientific studies have reported other pharmacological activities of *Cassis fistula* L, only one preliminary study has reported the central nervous system depressant activity of methanolic extract of *Cassia fistula* L. fruit pulps.[8] In a preliminary study conducted on the various fractions in our laboratory, the ethyl acetate fraction of a hydro-alcoholic extract showed a notable CNS depressant potential and was consequently selected for further evaluation. The present study aimed to investigate the anxiolytic and anticonvulsant activity of the ethyl acetate fraction of *Cassia fistula* pods in mice.

## MATERIALS AND METHODS

### Plant material

*Cassia fistula* L. fruit pods were collected from surroundings of Coimbatore by Mr Ragupathy G and the specimen was authenticated by Scientist "F", Botanical Survey of India, Agricultural University, Coimbatore. The sample voucher specimen BSI/SRC/5/23/2011-12/Tech 781 was deposited for future use.

### Preparation of ethyl acetate fraction from hydro alcoholic extract

The plant material was washed with distilled water and dried in the shade at room temperature for 45 days. The dry material was ground to obtain a powder of 2–5mm particles. Milled material (500 g) was defatted with petroleum ether (40–60°C) for 6 hours and the dried marc extracted in 1 liter of hydroalcoholic solution (70% ethanol: 30% distilled water) by placing in a rotary shaker to obtain a hydro-alcoholic extract by maceration method for 2 days. The resultant extract was dehumidified by keeping it in an oven at 60°C for 24 h to yield dried waxy brown to black solid hydroalcoholic extract of *Cassia fistula* (HAECF) with a yield of 46g. An amount of 25 g of the extract was taken for fractionation and was dissolved in water and extracted with chloroform in separating funnel. A NaCl solution (10%) was added drop wise to the aqueous layer in order to precipitate out the tannins. The organic layer was partitioned with ethyl acetate and the solvent was evaporated to yield the ethyl acetate fraction of *Cassia fistula* pods (EAFCF) (4.7 g).[19]

### Animals

Adult male Swiss albino mice (25-30g) were used in this study. The animals were housed in standard environmental conditions (21± 2º C; humidity 60± 5) under 12 hours dark: 12 hours light cycle. Animals had free access to food and water and they were acclimatized to laboratory condition for one week prior to the experiments. The experiments were carried out during 10.00AM – 1.00PM. The experimental protocols were approved by Institutional Animal Ethics Committee of PSG Institute of Medical Sciences and Research and conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

### Drugs

Phenobarbitone sodium, quercetin and pentylenetetrazole were purchased from Hi-media labs, India. Diazepam was from Ranbaxy laboratories, India. Flumazenil and phenobarbitone from Srides Arcolab Ltd. and Abbott Healthcare Pvt. Ltd., India, respectively. All other chemicals used were of analytical grade.

### Phytochemical screening and determination of total flavonoid content

Crude hydro-alcoholic extract and the ethyl acetate fraction were screened for the presence of phytochemicals including flavonoids and tannins by using standard procedures.[20] Flavonoid content in the crude extract and ethyl acetate fraction was determined by the aluminium chloride colorimetric method.[21] Briefly, 0.50 ml of extract sample was diluted with 1.50 ml of distilled water. A volume of 0.50 ml of 10% (w/v) aluminium chloride was added along with 0.10 ml of 1 M potassium acetate and 2.80 ml of distilled water. This mixture was incubated at room temperature for 30 min. The absorbance of resulting reaction mixture was measured at 415 nm using a UV

**Table 1: Preliminary phytochemical analysis of HAECF and EAFCF**

| Tests | HAECF | EAFCF |
|---|---|---|
| Alkaloid | + | + |
| Saponins | - | - |
| Flavonoids | + | + |
| Anthraquinones | + | + |
| Tannins | + | - |
| Glycosides | + | - |
| Steroids | - | - |
| Reducing sugars | + | - |
| Terpenoids | + | + |
| + positive, - negative | | |

spectrophotometer. Quantification of flavonoids was performed on the basis of standard curve of quercetin prepared in 80% methanol and results were expressed in milligram quercetin equivalent (QE) per gm of dry fruits.

### Evaluation of pharmacological activity

EAFCF was suspended in 1% carboxy methyl cellulose (CMC) and administered one hour before the test through oral route at the doses of 25, 50, 100 mg/kg. The animals treated with 1% CMC (10 ml/kg p.o.) served as a control group and the animal received diazepam 2 mg/kg served as standard. PTZ was dissolved in saline and administered at the dose of 80 mg/kg via subcutaneous injection of the animals which received EAFCF one hour prior. To evaluate possible role of the GABAergic system, flumazenil (2 mg/kg, i.p.) was administered 30 minutes prior to the administration of EAFCF (100 mg/kg). Separate groups of animals were used for each experimental protocol.

### Subcutaneous pentylenetetrazole seizure test (s.c. PTZ test)

Seizure was chemically induced in animals using pentylenetetrazole 80 mg/kg dose s.c into a loose fold skin of the neck between shoulder blades. Test animals were placed in a plexiglas arena for 30 minutes for observation. Parameters noted were latency until the first minimal clonic seizure (MCS) which persist for at least a 5 sec period, latency to first generalized tonic clonic seizure (GTCS) and protection percentage against death.[22] The ability of the plant extract fraction to prevent this feature or prolong the latency or onset of the MCS and GTCS was taken as an indication of anticonvulsant activity.

### Elevated plus maze test

The elevated plus maze apparatus was made of plexiglas and consisted of two open arms (30×5 cm) and two closed arms (30×5 cm) with 25 cm walls. The arms extended from a central platform (5×5 cm) and were open at the top. The maze was elevated 40 cm from the room's floor. The animals were placed individually at the center compartment, facing one of the open arms. The number of entries and the time spent in the closed and open arms were recorded for 5 min. Entry into an arm was defined as the animal placing all four paws onto the arm. After each test, the maze was thoroughly cleaned up with wet cotton dipped in 10% ethanol solution followed by dry cloth.[23] An increase in the time spent on open arms (open × 100/300) and percentage of open arm entries (open × 100 (open + closed) by the treatment of drug was considered as the drug possessing anxiolytic activity. Changes in the loco motor activity (total number of arm entries) were also observed in order to differentiate anxiolytic activity from central nervous system stimulatory activity.

### Open field test (OFT)

The effect of EAFCF on exploratory activity of the mice was assessed in OFT.[2] The OFT apparatus consist of a square arena (60cm×60cm× 60cm) divided into 9 segments. The mice were placed at center of arena facing the wall and observed for 5 minutes. Parameters like ambulation (number of partitions crossed with all four paws), rearings (number of times the mouse stood on its hind limbs), time spent in central compartment and number of crossings in central compartment were recorded. The open field was cleaned with 10% ethyl alcohol and permitted to dry between tests.

### Phenobarbitone sodium induced sleeping time

Different groups of mice received phenobarbitone sodium (45 mg/kg i.p.) thirty minutes prior to the administration of the extract fraction, vehicle (control) or diazepam (1mg/kg i.p.). The animals were observed and the latent period (time between the phenobarbitone administration and the onset of sleep) as well as the duration of sleep (time between the loss and recovery of the righting reflex) were recorded.[24]

### Rotarod test

Mice were preselected based on their ability to remain on a horizontal bar (2.5cm diameter) revolving at a speed of 15 rpm for 120 sec. After drug treatment, each animal was evaluated for the time permanence on the rotating bar. Motor toxic drugs generally reduce the time permanence on rotarod.[19]

### Acute toxicity test

The acute toxicity test was performed as per the guidelines of OECD for the testing of chemicals.[25] A total of nine animals were grouped into three groups and EAFCF was administered at the doses of 500 and 2000mg/kg, p.o and 10 ml/1kg of CMC 1% (vehicle, p.o., control group). Thirty minutes thereafter, the animals were observed for several behavioral parameters such as spontaneous motor activity, tremors, grips strength, abnormal behavior, convulsions, abdominal contortions, gait, piloerection, palpebral closure, and constipation. The same procedure was repeated three times a week for 2 weeks. During the test period, animal death, animal weight and food consumption also were noted.

### Statistical analysis

All data were expressed as mean ± SEM. One-way ANOVA followed by Dunnett's test as post hoc test was

used to analyse the data. All statistical analyses were performed with Prism 4.0 and difference between the groups were considered significant when $p<0.05$.

## RESULTS

### Preliminary phytochemical analysis and flavonoid content estimation.

The results of preliminary phytochemical screening indicated the presence of flavonoids, anthraquinones, terpenoids, glycosides, phenolic compounds, reducing sugars and tannins in both crude extract and except tannins, glycosides and reducing sugars other constituents were found to present in ethyl acetate fraction (Table 1). The total flavonoid content analysis was done for hydro-alcoholic extract, ethyl acetate fraction and the results indicated the presence of high content of flavonoid (30.5±0.75 mg/g quercetin equivalent) in ethyl acetate fraction than in crude hydro alcoholic extract (5.5±0.75 mg/g quercetin equivalent).

### Anticonvulsant effect of EAFCF on PTZ induced seizure in mice

Treatment with EAFCF resulted in significant ($p<0.01$) increases in the latency to the occurrence of minimal clonic convulsion, as well as tonic clonic convulsions produced by PTZ administration. In addition, the ethyl acetate fraction (100 mg/kg) protected the animals from death due to PTZ administration observed for 24h (Table 1). Unlike diazepam, the fraction did not protect the animal from onset of PTZ induced convulsion.

### Anxiolytic effect of EAFCF in elevated plus maze using mice

Acute administration of EAFCF dose dependently increased the percentage entries into open arm and time spent in the open arm when compared to the control ($p<0.01$), indicating anxiolytic activity. However, it did not significantly ($p>0.05$) change the total number of arm entries (Table 2).

**Table 2: Dose dependent anticonvulsant effect of EAFCF on PTZ induced seizure in mice.**

| Groups | Treatment | Latency to MCS (Min) | Latency to GTCS (Min) | % protection from mortality |
|---|---|---|---|---|
| **Vehicle** | 10 ml/kg, i.p. | 2.47±0.27 | 3.44±0.262 | 0 |
| **EAFCF** | 25 mg/kg, p.o. | 3.59±0.416 | 5.52±0.517 | 33.3 |
| **EAFCF** | 50 mg/kg, p.o | 12.96±0.397* | 39.86±0.847* | 66.5 |
| **EAFCF** | 100 mg/kg, p.o. | 15.39±0.574* | 54.66±1.136* | 100 |
| **Diazepam** | 2 mg/kg, i.p. | 60±0* | 60±0* | 100 |

§ Data represented as mean±SEM with n=6 ; MCS- Minimal clonic seizure, GTCS- Generalised tonic clonic seizure ; Data analysed by one way ANOVA followed by Dunnett's test. ; *p <0.01 compared to vehicle control

**Table 3: Effect of EAFCF on behavioral parameters in EPM.**

| Groups | Treatment | % entries in open arm | % time spent in open arm | Total number of entries (n) |
|---|---|---|---|---|
| **Vehicle** | 10 ml/kg, i.p. | 37.5±1.5 | 36.8±0.87 | 12.17±0.6 |
| **EAFCF** | 25 mg/kg, p.o. | 44.6±1.29 | 43.32±1.34* | 13.67±0.66 |
| **EAFCF** | 50 mg/kg, p.o | 55.23±1.49* | 57.2±0.63* | 13.33±0.88 |
| **EAFCF** | 100 mg/kg, p.o. | 64.78±1.79* | 62.08±0.67* | 15.67±0.66 |
| **Diazepam** | 2 mg/kg, i.p. | 65.79±1.74* | 68.17±0.64* | 10.17±0.95 |

§ Data expressed as mean ± SEM n=6 ; *p<0.01 compared to vehicle treated group ; One way ANOVA followed by post hoc Dunnett's test were performed.

**Table 4: Effect of EAFCF on open field behavior in mice.**

| Groups | Treatment | No. of Rearings | No. of ambulations | No of central squares crossed | Time spent in central compartment (s) |
|---|---|---|---|---|---|
| **Vehicle** | 10 ml/kg, i.p. | 18.8±0.9 | 42.5±2.1 | 3.2±0.5 | 3.2±0.5 |
| **EAFCF** | 25 mg/kg, p.o. | 16.7±0.9 | 37.8±1.1 | 3.7±0.7 | 5.2±0.3 |
| **EAFCF** | 50 mg/kg, p.o | 20.0±0.6 | 39.0±1.4 | 9.0±0.6* | 9.5±0.4* |
| **EAFCF** | 100 mg/kg, p.o. | 16.3±0.6 | 39.5±1.9 | 12.8±0.7* | 16.2±0.5* |
| **Diazepam** | 2 mg/kg, i.p. | 8.20±0.6 | 23.0±1.5 | 13.3±0.9* | 18.8±0.6* |

§Data shown as mean±SEM ; *p<0.01 Vs vehicle, One way ANOVA followed by post hoc Dunnett's test

| Table 4: Effect of EAFCF on phenobarbitone induced sleeping time in mice. | | | |
|---|---|---|---|
| **Groups** | **Treatment** | **Latency to sleep (min)** | **Duration of Sleep (min)** |
| **Vehicle** | 10 ml/kg, i.p. | 6.21±0.28 | 82.78±1.49 |
| **EAFCF** | 25 mg/kg, p.o. | 7.44±1.66 | 85.65±3.38 |
| **EAFCF** | 50 mg/kg, p.o | 6.92±1.59 | 83.10±1.96 |
| **EAFCF** | 100 mg/kg, p.o. | 5.52±1.21 | 88.54±3.92 |
| **Diazepam** | 2 mg/kg, i.p. | 2.86±0.41* | 189.51±2.66* |
| Each value represented as mean±SEM (n=6) ; *p<0.01 compared to control. One way ANOVA and Dunnett's test as post hoc test were performed. | | | |

| Table 5: Effect of EAFCF on time permanence on rotarod. | | |
|---|---|---|
| **Groups** | **Treatment** | **Time permanence on rotarod (s)** |
| **Vehicle** | 10 ml/kg, i.p. | 120±0 |
| **EAFCF** | 25 mg/kg, p.o. | 120±0 |
| **EAFCF** | 50 mg/kg, p.o | 120±0 |
| **EAFCF** | 100 mg/kg, p.o. | 120±0 |
| **EAFCF** | 400mg/kg, p.o. | 106±0.931 |
| **Diazepam** | 2 mg/kg, i.p. | 18.55±1.21* |
| § Each value represent mean ± SEM (n=6) ; *p<0.01 compared to control. One way ANOVA and Dunnett's test as post hoc test were performed. | | |

### EAFCF reduced the thigmotaxic behavior of mice in OFT

A significant increase ($p<0.01$) in the time spent in central compartment and the number of crossings of the open field were observed in mice which received EAFCF (50 and 100 mg/kg). No significant ($p>0.05$) changes were noted in the total number of ambulation and the number of rearings (Table 3).

### Effect of EAFCF on phenobarbitone induced sleeping time in mice

EAFCF did not exhibit sedative properties at the selected doses examined in this experiment as there was no significant ($p>0.05$) reduction in the latency to loss of righting reflex and the duration of sleep caused by phenobarbitone (Table 4). However, at higher doses (>400 mg/kg, i.p.) the phenobarbitone induced sleeping time was potentiated significantly ($p<0.01$), similar to diazepam.

### 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, the standard drug diazepam (2 mg/kg) significantly ($p<0.01$) reduced time permanence on rotarod.

### Toxicity

Animals which received vehicle alone showed normal behavior whereas behavioral alterations such as decrease in loco motor activity, low grip strength and constipation were noted in EAFCF treated animals. However, no death was observed, nor did the test animals show any change in food consumption and body weight.

## DISCUSSION

The search for alternative and complementary therapy from medicinal plants for CNS ailments has been increasing considerably in recent years.[26] in this study, EAFCF was evaluated for its anticonvulsant and anxiolytic activity. The preliminary phytochemical studies revealed the presence of flavonoids, anthroquinones, glycosides and terpenoids. Quantitative estimation of flavonoid content showed that the fractionation of hydroalcoholic extract to produce an ethyl acetate extract increased the concentration of flavonoids. In addition to flavonoids, the presence of other phytoconstituents may also have contributed to the observed effect of EAFCF on CNS.

In PTZ induced convulsions, the EAFCF dose dependently increased the onset of MICS GTCS and protected the animal from death. This shows that the treatment of EAFCF delays the seizure generation in mice. Although the extract fraction showed protection from death in PTZ induced convulsion, it could not protect against the occurrence of seizure. Further enrichment of flavonoids or treatment with higher dose of EAFCF may produce seizure protection in the PTZ induced convulsion model. The mechanisms postulated for PTZ induced convulsions include blockade of $GABA_A$ receptor activity, antagonizing the adenosine mediated inhibitory action on neuronal firing, opioidergic mediation, glutamineric modulation, through alteration of hormonal activity and by increasing the $Ca^{2+}$ T current.[27] Further investigations are required to find out the exact mechanism by which EAFCF produced anti-seizure activity in PTZ induced convulsive model.

In addition to the anticonvulsant action most of the antiepileptic drugs produce anxiolytic activity.[28] Therefore, the present study also evaluated the anxiolytic activity of EAFCF in EPM and OFT. The EPM is etiologically validated and most widely accepted model for screening novel anxiolytic agents in mice[2] as well as in rats.[29] An increase in the number of open arm entries and a consequent increase in time spent in the open arm are the two parameters generally considered as an index of anxiolytic activity of a drug.[30] The present work showed the dose dependent anxiolytic effect as evidenced from the significant increase in the frequency and time spent in open arms by the animals treated with 50 and 100 mg/kg of EAFCF orally. Since, benzodiazepines are used for validating this animal model[30] and flavonoids are believed to modulate GABAergic system,[31] it is suggested that EAFCF, being rich in flavonoids, may produce its anxiolytic activity by acting on $GABA_A$ receptor mediated GABAergic system. To investigate the role of GABAergic system, flumazenil ($GABA_A$ receptor antagonist) was administered before the treatment of EAFCF and the results showed that the pretreatment of flumazenil blocked the anxiolytic effect of EAFCF in EPM. This clearly indicates the involvement of GABAergic system in mediating the anxiolytic activity of EAFCF. Similarly, other plants (eg. *Loeselia Mexicana,*[32] *Piper methysticum*,[33] *Euphorbia hirta,*[34] and *Cymbopogon citratus*) have also been reported to mediate their anxiolytic activity through GABAergic system.[31,35]

The open-field test is based on the rodents intrinsic behaviour to stay near the periphery of a novel environment (ie. thigmotaxis), which may provide the animal a sense of security. An increase in the time spent in the central arena of the open field without affecting general motor activity is the indication of anxiolytic activity.[36] In this study, treatment with EAFCF did not alter the number of rearings and ambulations, whereas it significantly reduced thigmotaxis, indicating its anxiolytic activity. Other drugs which have previously been reported to show anxiolytic activity in OFT include midazolam,[37] chordiazepoxide,[38] and buspirone.[39]

Previous research reports have stated that the drugs having anxiolytic and anticonvulsant activity at low doses (eg. benzodiazepines) produce sedative or myorelaxant activity at high doses.[40,41] Considering this, the present study also evaluated the effect of EAFCF on phenobarbitone induced sleeping time and time permanence on rota rod and the results showed that, the EAFCF at the doses used in this study did not alter motor co-ordination. Only at higher dose (>400mg/kg) did it potentiate the phenobarbitone induced sleeping time. Moreover, EAFCF did not alter the loco motor activity as it had not influenced the rearing and ambulation in OFT and total number of entries in EPM. This indicates that actions produced by EAFCF in this study is neither through peripheral neuromuscular blockade, nor by altering loco motor activity. In contrast, the methanolic extract of *Cassia fistula* pods has been shown to produce sedation and motor coordination deficits.[8] Fractionation of the crude extract into a flavonoids rich portion may be responsible for this nonsedative action at low dose of EAFCF, as natural and synthetic flavonoids are believed to have potent anxiolytic activity without producing a sedative effect.[42] In contrast, EAFCF also contains several components such as anthraquinones, terpenoids, and tannins. Hence, further studies are needed to identify the components responsible for antiepileptic and anxiolytic potential and the mechanisms underlying the properties. However, one cannot rule out the significant fact about phytochemistry that is crude plant extracts are generally shown to be more potent medicines than pure isolated compounds in all likelihood due to synergistic interactions and various actions of complex mixers of components.[43]

## CONCLUSION

In conclusion, the present study clearly gives the scientific evidence for traditional use of *Cassia fistula* L. for treating epilepsy and other associated behavioural disorders. Further studies are ongoing in our laboratory to investigate its mechanism, the effect on chronic treatment and isolation and the characterization of active principle(s) of this plant.

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## CONFLICT OF INTEREST

Authors declare no conflicts of interest

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