"EVALUATION OF CENTRAL NERVOUS SYSTEM ACTIVITIES OF SOME INDIAN MEDICINAL PLANTS"

Thesis submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY, CHENNAI-32

As partial fulfillment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

PHARMACY

Submitted by

C. KALAIYARASI

Ex.II(1)21953/2009

Under the Guidance of

Dr. V. S. SARAVANAN, M. Pharm., Ph.D.,

Professor & Head

Dept. of Pharmaceutical Analysis

at

THE ERODE COLLEGE OF PHARMACY AND RESEARCH

ERODE - 638112.

February 2015

DECLARATION

The research work embodied in this thesis entitled "**EVALUATION OF CENTRAL NERVOUS SYSTEM ACTIVITIES OF SOME INDIAN MEDICINAL PLANTS",** was carried out by me in the Department of Pharmaceutics, The Erode College of Pharmacy, Erode, under the direct supervision of **Dr. V. S. SARAVANAN, M.Pharm., Professor & Head**, **Dept. of Pharmaceutical Analysis,** The Erode College of Pharmacy, Erode – 638112.

This thesis submitted to **The Tamilnadu Dr. M.G.R Medical University, Chennai,** as a partial fulfillment for the award of **Doctor of Philosophy in Pharmacy** during the academic year 2010 – 2014.

The work is original and has not been submitted in part or full for the award of any other Degree or Diploma of this or any other University.

Place: Erode

Date: **Reg. No. Ex.II(1)21953/2009**

The Erode College of Pharmacy

Dr. V. S. Saravanan, M. Pharm., Ph.D.,
Vice - Principal
Head, Dept. of. Pharmaceutical Analysis
E-mail: saravecp@yahoo.co.in
Mobile: +91 – 9443838566

Perundurai Main Road,
Veppampalayam.
Erode – 638 112.
Tamilnadu
India.

CERTIFICATE

This is to certify that the work embodied in this thesis entitled **"EVALUATION OF CENTRAL NERVOUS SYSTEM ACTIVITIES OF SOME INDIAN MEDICINAL PLANTS",** submitted to The Tamilnadu Dr.M.G.R. Medical University, Chennai for the partial fulfillment of the award of **DOCTOR OF PHILOSOPHY in PHARMACY** in Department of Pharmaceutics, at The Erode College of Pharmacy, Erode-638112, is a bonafied work was carried out by **Mrs. C. Kalaiyarasi (Reg. No. Ex. II (1) 21953 / 2009)** under my guidance and supervision.

This work is original and has not been submitted in part or full to any other degree or diploma of this or any other university.

Place : Erode
Date :

Dr.V.S.Saravanan
Research Guide

ACKNOWLEDGEMENT

"With the blessings of Almighty"

It is an ushered honor and privilege to nurture the intelligent guidance and skilful supervision of my respected guide **Dr. V.S. Saravanan.** Sir, I am grateful and highly obliged for your encouragement, prowess, constructive criticisms, care and the invaluable time you bestowed me from your busy schedule that provided me with the needed morale and confidence during the work. Your dedication and discipline, versatility, and rapport will always remain a source of inspiration for me for the rest of my life. I remain indebted to you forever.

I am thankful to **Dr. M. Ramanahan,** Principal, PSG College of Pharmacy, Coimbatore and **Dr. V. Ganesan,** Principal, The Erode College of Pharmacy, Erode for making necessary facilities available.

I owe a special world of thanks to my friends and colleagues- **Mrs. S.E. Maida Engels, Mrs. Gayathri, Mr. B. Premkumar sir, Dr. K.G. Prashanth sir, Dr. B. Balaji, Dr. S. Manokaran sir, Dr. S.P. Senthil sir, Dr. Dinesh** etc… who at every hard turn filled me with real enthusiasm to work further.

I owe my gratitude to my beloved students **K. Karthika, G. Ragupathy and P. Lalithkumar** for their support in completion of my work.

I will fail in my duty if I do not thank my **Guru Dr. C.T. Chopde,** my M. Pharm thesis guide, who remained not only as my research guide, also in most of the important milestones in my personal and professional life his guidance will be the pathfinder.

As words have inherent limitations of restricted expression, I fail to thank adequately my **husband- Mr. K. Kamaraj, lovable kids - Thirupavai & Kiruthickvel**, **beloved parents, my respected in laws and my lovable sisters** for their love, support and sacrifices. I gratefully remember and pay respect to my family, the impetus behind all my good deeds.

Kalaiyarasi C.

CONTENTS

S.No.	Topics	Page no.
	LIST OF TABLES	
	LIST OF FIGURES	
	LIST OF ABBREVIATIONS	
1	INTRODUCTION	1
2	AIM AND OBJECTIVE	18
3	LITERATURE REVIEW	19
4	PLAN OF WORK	39
5	PLANT PROFILE	42
6	METHODOLOGY	45
7	RESULTS AND ANALYSIS	72
8	DISCUSSION	107
9	SUMMARY AND CONCLUSION	128
10	REFERENCE	132
	APPENDIX	

LIST OF TABLES

Table No.	Title of the Table	Page No.
1.	Distribution, characteristics and therapeutic potential of various adenosine receptors	34
2.	Grouping of animals for evaluation of anti-convulsant activity of EAFCF and NHFCA in s.c.PTZ model.	60
3.	Table Grouping of animals for evaluation of anxiolytic activity of EAFCF and NHFCA in EPM, OFT and marble burying behavior.	61
4.	Grouping of animals for evaluation of antidepressant activity of EAFCF and NHFCA in FST and TST.	63
5.	Grouping of animals for evaluation of antidepressant activity of EAFCF in CUS induced anhedonia model	65
6.	Grouping of animals for evaluation of anti-nociceptive activity of EAFCF and NHFCA in hotplate, tail immersion and formalin tests.	67
7.	Grouping of animals for penobarbitone induced sleep time and rota rod behavior test of EAFCF and NHFCA.	69
8.	Grouping of animals for interaction studies	71
9.	Ash values of *Cassia fistula L.* and *Cassia auriculata* L. pods in %w/w	72
10.	Extractive value of *Cassia fistula* L. and *Cassia auriculata* L pods in % w/w	73
11.	Results of the metal contents from EAFCF and NHFCA (ppm)	73
12.	Preliminary phytochemical analysis of hydroalcoholic extract and fractions of *Cassia fistula* L. and *Cassia auriculata* L.	74
13.	HPTLC profile of the EAFCF	78

14.	HPTLC profile of the NHFCA	78
15.	^{H} NMR data of the isolated compound from EAFCF	83
16.	Superoxide scavenging activity of EAFCF and NHFCA	85
17.	Reductive ability of EAFCF and NHFCA	85
18.	Dose dependent anticonvulsant effect of EAFCF on PTZ induced seizure in mice	87
19.	Effect of EAFCF and NHFCA on behavioral parameters in EPM.	89
20.	Effect of EAFCF and NHFCA on open field behavior in mice	90
21.	Analgesic activity of EAFCF in acetic acid induced writhing in mice	97
22.	Anti-nociceptive effect of EAFCF and NHFCA in Eddy's hot plate model using mice	98
23.	Anti-nociceptive activity of EAFCF in mouse tail immersion test	99
24.	Reduction of formalin induced paw licking by EAFCF and EAFCF and morphine treatment in rats	100
25.	Effect of EAFCF on time permanence on rotarod	101
26.	Effect of EAFCF on phenobarbitone induced sleeping time in mice	102
27.	Changes in neurochemical level upon administration of EAFCF	103
28.	Modulatory effect of adenosinergic agents on the anti-seizure effect of EAFCF in s.c. PTZ model	104
29.	Modulatory effect of adenosinergic agents on the anti-seizure effect of EAFCF in s.c. PTZ model.	105

LIST OF FIGURES

Figure No.	Title of the Figure	Page No.
1.	Image of *Cassia fistula* L.	42
2.	Image of *Cassia auriculata* L.	44
3.	Standard curve of gallic acid	75
4.	Standard curve of quercetin	76
5.	HPTLC profile of EAFCF and NHFCA fraction at 254 nm	77
6.	HPTLC profile of EAFCF and NHFCA at 366nm	77
7.	HPTLC chromatogram of EAFCF– Peak densitogram display (Scanned at 500 nm)	79
8.	HPTLC chromatogram of NHFCA–Peak densitogram display (Scanned at 500 nm)	79
9.	3D display of HPTLC chromatogram of EAFCF and at 254 nm	80
10.	HPLC profile of compound isolated from EAFCF	81
11.	FTIR spectrum of isolated compound EAFCF	82
12.	GCMS analysis of the isolated compound from EAFCF	82
13.	^{H}NMR Spectrum of isolated compound from EAFCF	83
14.	Chemical structure of the isolated compound from EAFCF	
15.	Sub chronic treatment of EAFCF suppresses the seizure generation in PTZ kindling model	88
16.	Effect of EAFCF and NHFCA on marble burying behavior in rats	91
17.	EAFCF and NHFCA reduced the duration of immobility in FST	92
18.	Hyper mobility effect of EAFCF and NHFCA in TST	93

19.	Effect of sub chronic treatment of EAFCF on CUS induced changes in sucrose intake	
20.	Sub-chronic treatment of EAFCF reverses the CUS induced hyper-immobility	
21.	Changes erythrocyte SOD activity by sub-chronic EAFCF treatment	
22.	Pretreatment of adenosinergic agents changes the anti-immobility effect of EAFCF	

ABBREVIATION

CNS	**-**	**Central Nervous System**
HPTLC	**-**	**High Performance Thin Layer Chromatography**
NMR	**-**	**Nuclear Magnetic Resonance**
IR	**-**	**Infra Red**
GC-MS	**-**	**Gas Chromatography- Mass spectroscopy**
PTZ	**-**	**Pentylenetetrazole**
FST	**-**	**Forced Swim Test**
TST	**-**	**Tail Suspension Test**
CUS	**-**	**Chronic Unpredictable Stress**
OFT	**-**	**Open Field Test**
SOD	**-**	**Super Oxide Dismutase**
h	**-**	**hours**
s	**-**	**seconds**
mg	**-**	**milligram**
Kg	**-**	**kilogram**
RPM	**-**	**Rotation per minute**
DA	**-**	**Dopamine**
5-HT	**-**	**5- Hydroxy tryptamine**
GABA	**-**	**Gama Amino Butyric Acid**
EAFCF	**-**	**Ethyl acetate fraction of *Cassia fistula***
NHFCA	**-**	**n- Hexane fraction of *Cassia auriculata***
i.p.	**-**	**Intraperitoneal**
s.c.	**-**	**subcutaneous**

p.o.	**-**	**per oral**
%	**-**	**percentage**
ml	**-**	**millilitre**
µg	**-**	**microgram**
min	**-**	**minutes**
^{o}C	**-**	**degree Celsius**
CHA	**-**	**N^6-cyclohexyl adenosine**
DPX	**-**	**1,3-diehtyl-8-phenyl xanthine**
MCS	**-**	**Minimal clonic seizure**
TLC Plate	**-**	**Thin layer Chromatography plate**
GTCS	**-**	**Generalized tonic clonic seizure**
IC	**-**	**Inhibitory concentration**
AED	**-**	**Anti epileptic drugs**

A_1AR, A_{2A}AR, A_{2B}AR , A_3AR – adenosine receptor subtypes

INTRODUCTION

The central nervous system (CNS) is comprised of the brain and spinal cord that receives sensory information from the nervous system and controls the body's responses through chemical messengers such as neurotransmitters, neuromodulators, neurohormones, neuropeptides etc... The significant neurotransmitters include noradrenaline, dopamine, serotonin (5HT), Gama Amino Butyric Acid (GABA) and glutamate. The most widely studied neuromodulators are purines such as adenosine, prostaglandins and nitric oxide. These neurochemicals generally interact with the target sites i.e. receptor and exert their actions[1].

The use of plants to influence brain function has long been essential to medical practice even before some 3500 years. However, very little attention was given by the scientific community to the benefits, as accepted by folk medicine, of the therapeutic usefulness of plants endowed with CNS activity due to the challenge in elucidation of mechanism of action to support the traditional uses[2]. Recently, the usage of herbal medicine for treating central nervous system has been increasing owing to the greater undesirable effects produced by the synthetic counterpart[3].

Comorbidity of CNS disorders

Anxiety, depression and epilepsy are considered as the most common central nervous system ailments (CNS) affecting large number of human population worldwide and pain is believed to be a common symptom for all these conditions [4].

Comorbidity of anxiety in epilepsy

Anxiety is the one of the most common psychiatric comorbidities in epilepsy affecting the quality of life of epileptic patients[5]. Multiple studies have reported increased rates of panic attacks, panic disorder, obsessive-compulsive disorder (OCD), social anxiety disorder and generalized anxiety disorder (GAD) in adult patients with epilepsy as compared with the general population[6-8]. Several factors have been found to be associated with anxiety symptoms and anxiety disorders in patients with epilepsy as follows.

Certain areas of the brain particularly amygdala believed to cause paroxysmal anxiety directly mainly in the form of panic attacks which is also considered as the area responsible for generation of temporal lobe epilepsy[9]. This, intern supported by the observation that patients with temporal lobe epilepsy and ictal anxiety symptoms have been found to have a reduced volume of amydala[10].

Neurotransmitter systems are also play a role in linking anxiety disorders with epilepsy. The role of GABA receptors and neurotransmitters including serotonin, dopamine and noradrenaline in both epilepsy and anxiety disorders indicate another pathophysiological similarity between the two disorders[11, 12].

Psychological factors, such as the unpredictability of seizures, the fear of death, feeling of poor control over seizures, and perceived stigma likely predispose some epilepsy patients to anxiety[13, 14].

Higher seizure frequency has been found to be associated with anxiety disorders in some adult studies[15] as well as in children and adolescents[16, 17]. In adults,

the risk of anxiety disorders appears to be higher in focal (especially temporal lobe) than in generalized epilepsies[18].

Apart from these factors, age of an epileptic patient and the usage of antiepileptic drugs also play a major role in developing anxiety in epilepsy.

Comorbidity of depression in epilepsy

Depression is the most frequent comorbid condition among the psychiatric disorders in patients with epilepsy[1], yet it remains under recognized and under-treated. Depression in persons with epilepsy may be related to psychosocial factors or may have a biologic explanation. Although previous studies have consistently shown an increased risk of depression after epilepsy onset, some data also show that depressed individuals have an increased risk of developing epilepsy. A population-based control studies indicate that people with a history of depression have a 4- to 7-fold higher risk of developing epilepsy[19-21] and a prior history of suicidality intern was associated with a 5-fold increased risk of developing epilepsy[21]. The bidirectional relationship may suggest that common pathogenic mechanisms are operant in both conditions, with the presence of one disorder potentially facilitating the development of the other.

The common pathogenic mechanisms shared by depression and epilepsy are as follows (for review) [22]:

- Abnormal CNS activity of several neurotransmitters, particularly serotonin (5-hydroxytryptamine, 5-HT), norepinephrine, dopamine, GABA, and glutamate.

- Structural changes, presenting as atrophy of temporaland frontal-lobe structures (identified by high resolution MRI and volumetric measurements), in the amygdala, hippocampus, entorhinal cortex, temporal lateral neocortex, as well as in the prefrontal, orbitofrontal, and mesial-frontal cortex, and to a lesser degree, of the thalamic nuclei and basal ganglia.

- Functional abnormalities (identified by positron emission tomography [PET] and single-photon emission computed tomography [SPECT]) in temporal and frontal lobes, consisting of decreased 5-HT1A binding in the mesial structures, raphe nuclei, thalamus, and cingulate gyrus.

- Abnormal function of the hypothalamic–pituitary–adrenal axis.

The epileptic patients with depression pose more severe consequences such as increased suicidal tendency which may affect the quality of life and also has considerable impact on cost management due to the requirement of multi drug therapy[23]. Studies described on an animal model of absence epilepsy suggest that preemptive treatment before the onset of the epileptic syndrome may protect against both the evolution of the epilepsy and the comorbid depressive symptoms[24].

Pain in CNS disorders

Chronic pain and fibromyalgia may be related to physical inactivity, which is more often seen adults with a history of epilepsy than the non epileptic counterparts [25]. Conversely, pain disorders could be related to a higher incidence of bone injuries caused by seizures. Migraine and epilepsy are associated independent of seizure type, etiology, age at onset, or family history of epilepsy[26]. These two conditions might

share a common genetic susceptibility[27] or a common environmental risk factor, such as traumatic head injury[28]. It has been suggested that depression and migraine with aura act synergistically in increasing the risk of incident unprovoked seizure and hence, these three disorders may result from a shared underlying mechanism[29]. Increased frequency of migraine in the relatives of individuals with benign rolandic epilepsy has been reported which provide the evidence for shared genetic susceptibility to migraine and some forms of epilepsy[27].

OVERVIEW OF EPILEPSY

Definitions

Epilepsy is one of the most prevalent neurological disorders, characterized by the recurrent appearance of spontaneous seizures due to neuronal hyperactivity in the brain and has a number of subtypes[30]. A seizure is a symptomatic, behavioral manifestation due to abnormal disorder, spontaneous but synchronizes, high frequency firing of neuron populations in the central nervous system[31].

Types of seizures

Epileptic seizures are generally classified into focal or partial and generalized. The clinical manifestation of focal seizures varies depending upon the origin of epileptic discharges (the epileptic focus) and includes motor, sensory, autonomic and psychic symptoms. When the seizure discharge becomes sufficiently widespread and includes a strong participation of motor system circuitry, the result is occurrence of generalized seizure. Even focal seizures, however can become generalized (secondary generalized seizures)[30].

Etiology of seizures

Variety of etiologies are known in symptomatic epilepsies[30] which can involve birth accidents, abnormal neurodevelopment[32], infection, vascular diseases, head trauma, brain tumors, and neurodegeneration. However, there are also many examples of epilepsy without such clear epileptogenic lesions (cryptogenic epilepsy).

Pathophyology of seizures

The exact pathophysilogy of seizure is not yet clear but some commonly accepted hypotheses include changes in ionic micro-environment, alteration in ionic perturbations and synchronization of neurons.

Alteration in ionic microenvironment

The nervous system functions through its ionic milieu, i.e. the chemical and electrical gradients that create the setting for electrical activity. Resting potential is set normally in the neurons in such a way that inside a nerve cell the membrane potential is very negative relative to the external milieu. This transmembrane potential about -60mV is been created by the differential distribution of ions such as high potassium concentration inside, high calcium and sodium potassium outside and also by various other ions[33]. If the balance is disturbed this can promote depolarization that leads to abnormal activity in many ways[34]: depolarizing the nerve terminal leads to transmitter release finally leading to action potential discharge. Thus, the control of the ionic environment provides many potential targets for novel anticonvulsants. Abnormalities in the sodium channel might lead to a decrease in threshold for an action potential if the method by which sodium channel activation is through changes in the control mechanisms that maintain the ionic equilibrium[35]. Imbalance in electrolytes can

involve not just sodium but others like calcium, potassium, chloride may lead to seizure (for review)[36].

Synaptic transmission

Glutamatergic and γ-aminobutyric acid (GABA)-ergic transmission, are major excitatory and inhibitory transmitters of the nervous system respectively. It has long been proposed that increased seizure susceptibility may be caused by an abnormality in the transmitter systems of the brain such as impaired inhibitory transmission (through GABA), excessive excitatory transmission (through Glutamate) or an imbalance between neuronal excitatory and inhibitory systems[37]. Having hyper excitable population of neurons among the healthy CNS tissue with a functional balance between excitation and inhibition is called epileptogenesis[38].

It is important to point out, however, that both glutamate and GABA may not have a simple, direct relationship to seizures. One reason is that glutamatergic synapses innervate both glutamatergic neurons and GABA-ergic neurons in many neuronal systems. Exposure to glutamate could have little net effect as a result, or glutamate may paradoxically increase inhibition of principal cells because the GABA-ergic neurons typically require less depolarization by glutamate to reach threshold[38].

Synchronization

Excessive discharge alone does not necessarily cause a seizure but synchronization of a group of neurons is involved in the genesis of seizure. Glutamatergic interconnections are one example of a mechanism that can lead to synchronization it cause a giant excitatory postsynaptic potential[39]. Gap junctions on

cortical neurons are another mechanism for synchronization which allow a low-resistance pathway of current flow from one cell to another, so that connected neurons are rapidly and effectively synchronized[40]. Another mechanism of synchronization involves inhibition through GABAergic neurons. When the GABA-ergic inter neurons that innervate cortical pyramidal cells discharge that leads to hyperpolarization of a population of pyramidal cells. As GABA-ergic inhibition wanes, voltage-dependent currents of pyramidal cells become activated. These currents, such as T-type calcium channels and others, are relatively inactive at resting potential, but hyperpolarization relieves this inhibition. The result is a depolarization that is synchronous in a group of pyramidal cells[41]. Some of the changes that develop within the brain of individuals with epilepsy also promote synchronization. These changes include growth of axon collaterals of excitatory neurons, typically those that use glutamate as a neurotransmitter and are principal cells[42].

The occurrence of seizure in epileptic patients is controlled by using available antiepileptic drugs including the new anti epileptic drugs like vigabatrine, topiramate, zonisamide etc… for chronic period of time.

OVERVIEW OF ANXIETY DISORDERS

Anxiety is a complex psychological disorder generally affecting the mood and causing irrational fear as one of the most prominent symptom. The most common sub types are phobia, panic disorders with or without agoraphobia, obsessive compulsive disorder (OCD), post-traumatic stress disorder (PTSD) and generalized anxiety disorder (GAD).

Neuroanatomic circuits involved in processing fear and anxiety

Fear and anxiety normally comprise adaptive responses to threat or stress. These emotional-behavioral sets may arise in response to exteroceptive visual, auditory, olfactory, or somatosensory stimuli or to interoceptive input through the viscera and the endocrine and autonomic nervous systems. Anxiety may also be produced by cognitive processes mediating the anticipation, interpretation, or recollection of perceived stressors and threats. Emotional processing in general can be divided into evaluative, expressive, and experiential components. Evaluation of the emotional salience of a stimulus involves appraisal of its valence its relationship with previous conditioning and behavioral reinforcement experiences, and the context in which it arises. Emotional expression conveys the range of behavioral, endocrine, and autonomic manifestations of the emotional response, whereas emotional experience describes the subjective feeling accompanying the response. To optimize their capacity for guiding behavior, all these aspects of emotional processing are modulated by complex neurobiological systems that prevent them from becoming persistent, excessive, inappropriate to reinforcement contingencies, or otherwise maladaptive. The structures that function in concert with the amygdala during fear learning include other mesiotemporal cortical structures, the sensory thalamus and cortices, the orbital and medial prefrontal cortex (mPFC), the anterior insula, the hypothalamus, and multiple brainstem nuclei. Much of this network appears to participate in the general process of associating a conditioned stimulus (CS) or operant behavior with an emotionally salient unconditioned stimulus (US) (for review)[43]

Neurochemicals modulating fear and anxiety

The neuroanatomic circuits that support fear and anxiety behavior are modulated by a variety of chemical neurotransmitter systems. These include the peptidergic neurotransmitters, CRH, neuropeptide Y (NPY), and substance P, the monoaminergic transmitters, NE, serotonin (5-hydroxytryptamine or 5-HT), and dopamine (DA), and the amino acid transmitters, GABA and glutamate. The neurotransmitter systems that have been best studied in association with responses to stress or threat involve the HPA axis and the central noradrenergic system. These neurochemical systems subserve important adaptive functions in preparing the organism for responding to threat or stress, by increasing vigilance, modulating memory, mobilizing energy stores, and elevating cardiovascular function. Nevertheless, these biological responses to threat and stress can become maladaptive if they are chronically or inappropriately activated. Additional neurochemical systems that play important roles in modulating stress responses and emotional behavior include the central GABAergic, serotonergic, dopaminergic, opiate, and NPY systems (for review)[43].

OVERVIEW OF DEPRESSION

Depression is a heterogeneous syndrome comprised of numerous diseases of distinct causes and pathophysiologies. Various symptoms of depression includes depressed mood, irritability, low self esteem, feelings of hopelessness, worthlessness, guilt, decreased ability to concentrate and think, decreased or increased appetite, weight loss or weight gain, insomnia or hypersomnia, low energy, fatigue or increased agitation, decreased interest in pleasurable stimuli and recurrent thoughts of death and suicide.

The etiology of depression is not clear may be spontaneous but often follows a traumatic emotional experience or can be a symptom of other diseases or also be triggered or precipitated by pharmacological agents or drug abuse[44] . The prevalence is higher in woman (in the range of 1.5 to 2.5) and nearly 50% of the risk for depression is due to genetic factors [45].

Neurotransmitter abnormalities in depression

Monoamine depletion

The observation that pharmacological manipulation of monoamine-influenced depressive symptoms led to the hypothesis that depression results from reduced availability or functional deficiency of serotonin or norepinephrine[46]. This view was supported by the pharmacological action of both tri cyclic antidepressants (TCAs) and mono amino oxidase inhibitors (MAOIs), namely, the resultant increase in synaptic levels of monoamines. However, the effects are specific to the type of antidepressant that produced the initial clinical response. Patients who responded to an selective serotonin reuptake inhibitor (SSRI) relapsed with tryptophan depletion. These data do support abnormalities in monaminergic sensitivity but suggest that antidepressant response involves more than just monoamines [47].

Increased cholinergic sensitivity

Cholinergic agonists, cholinesterase inhibitors, and acetylcholine precursors have all been shown to worsen mood in depression[48]. Furthermore, abnormalities in levels of cortical choline, an acetylcholine precursor, have been reported in several brain imaging studies[49]. It should be noted, however, that the increased levels of

choline may be localized in the frontal cortex with decreased levels of choline evident in other brain regions[50]. Imbalance in aminergic and cholinergic imbalance may play a role in depression[51]. Inherent in this view is the assumption that no single neurotransmitter abnormality underlies depression, a position that has become more appealing [52].

Neuronal plasticity, information processing, neurogenesis and depression

One emerging hypothesis suggests that problems in information processing within specific neural networks, rather than changes in chemical balance, might bring out depression. Depression may arise when some neuronal systems do not exhibit appropriate, adaptive plasticity in response to external stimuli. This possibility is supported by the fact that most antidepressant drugs induce plastic changes in neuronal connectivity, which gradually lead to improvements in neuronal information processing and recovery of mood[53]. Regulations of intracellular messenger cascades mediate the ability of neuronal systems to adapt in response to pharmacological and environmental stimuli[54]. A broad division classifies the intracellular signal transduction pathways into two categories, those that are regulated by G-protein receptors coupled second messengers and those that are regulated by receptors coupled directly or having a close interaction with protein tyrosin kinases[55]. The first category is controlled by neurotransmitters (monoamines and neuropeptides), the second by cytokines and growth factors (including the neurotrophin family, e.g. brain-derived neurotrophic factor (BDNF)). Hence, regaining the plasticity within these molecular cascades is likely to contribute to the effects of the antidepressants. BDNF is the most widespread growth factor in the brain, responsible, among the other functions, of neuronal survival. To activate transcription of BDNF a phosphorylation

of calcium/cyclic-AMP responsive binding protein (CREB) at its transcriptional regulatory residue Serine-133 is necessary[56]. Levels of serum BDNF are decreased in major depressive patients[57] and are associated with vulnerability to develop mood disorders in healthy subjects[58]. A common finding in animal studies is that, when administrated chronically, almost every antidepressant elevates CREB and its activated form, phosphorylated-CREB, in the hippocampus and cerebral cortex[59]. In line with this finding, increased CREB levels have been reported in postmortem brains of antidepressant-treated subjects[60]. Contrary to this, an increase in CREB and p-CREB is found in drug-free depressed suicide victims[61]. In rats, both chronic antidepressant treatments and electroconvulsive shock (ECS) increase BDNF levels[62]. Administration of BDNF displays antidepressant effects in two animal models of depression; the forced swim test (FST) and the learned helplessness model[63]. It has been suggested that a reduced hippocampal cell number may be involved in the pathophysiology of depression[64] and treatment with antidepressants has been shown to increase hippocampal neurogenesis[65]. Clinically, there is evidence of reduced hippocampal volume in patients with MDD or other affective disorders[66].

Stress and affective disorders

Hans Selye coined the term 'stressor' in 1950 to differentiate the condition of stress from the stimulus which evokes it. In the beginning of the '30s, Selye initiated a pioneering program of studies on the adaptive physiology of stress which set the standard for modern biological research in this area[67, 68]. Current theories distinguish four different aspects of the term 'stress'; stress stimulus, stress experience, stress response and the experience of the stress response[69]. The occurrence of stress is described by basic phenomena that are the same across cultures and species[70]. A stress

response starts with the central nervous system processing sensory information related to external stimuli. When a particular situation is interpreted as potentially harmful, a complex cascade of neural, hormonal and behavioral responses is initiated to cope with the situation. Stress related alterations in neuronal and neuroendocrine processes generate an alarm response and general arousal. Briefly, this response includes increased attention to the surroundings, accelerated heart rate, increases in blood pressures, increase in respiration rate and alterations in metabolic processes. Stress as a functional response is necessary for successful coping with environmental challenges. It enables an organism to deal with the wide variety of aversive and undesirable situations present in everyday life facilitating survival in a continuously changing environment[71]. However, responses vary according to individual stress sensitivity (e.g. genetic or due to previous experience) and the type of stressors, whether it is acutely or chronically presented, whether it its controllable or uncontrollable. Stress experience has been suggested to have a direct or indirect causal association in the development of depression[72]. A manifestation or worsening of depression in adulthood is often related to acute life events or ongoing stress[73]. Depressed individuals often report that they feel helpless, hopeless, or unable to control events, and consequently exposure to uncontrollable stressful events has been hypothesized to be significant in bringing about depression[74]. Depression is often associated with physiological changes characteristic of a normal stress response. There has been a major focus on the role of the hypothalamicpituitary- adrenal (HPA) axis as a marker of the stress response. Stress response activates the HPA axis and the release of glucocorticoids, which increases the heart rate, blood pressure, and metabolism. A consistent finding in depressed patients is hyperactivity and

dysregulation of the HPA-axis, demonstrated by increased cortisol levels, enlargement of the pituitary and adrenal glands and decreased glucocorticoid receptor sensitivity[75]. Also, the HPA axis is found to be a mediator of changes in brain monoamines, e.g. locus coeruleus noradrenaline[76] and raphe serotonin[77]. Antidepressants, both individually and in conjunction with anti-glucocorticoid agents, reverse a number of the HPA-axis abnormalities[78]. Other stress-regulated brain chemicals, e.g. certain cytokines that affect the HPA axis, may induce depressive-like syndromes in individuals without a history of mood disorders[79]. Childbirth provokes secretion of cytokines, which have been postulated to contribute to postnatal depression and cytokines also affect monoaminergic transmission[80].

OVERVIEW OF PAIN

The International Association for the Study of Pain defines pain as 'An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage'. It is considered as a major symptom of various diseases that persists to produce severe physical and psychological distress for many patients by disrupting their quality of life[81]

Various types of pain are classified as follows[82]

- Acute physiological nociceptive pain-Pain elicited by application of an acute noxious stimulus to normal tissue.
- Pathophysiological nociceptive pain-occurs when the tissue is inflamed or injured.

- Spontaneous pain-It is pain in the absence of any intentional stimulation or as hyperalgesia and/or allodynia

  Hyperalgesia is extreme pain intensity felt upon noxious stimulation, and allodynia is the sensation of pain elicited by stimuli that are normally below pain threshold.

- Neuropathic pain-results from injury or disease of neurons in the peripheral or central nervous system.

Nociception an overview

Nociception is the encoding and processing of noxious stimuli in the nervous system that can be measured with electrophysiological techniques. Neurons involved in nociception form the nociceptive system. Noxious stimuli activate primary nociceptive neurons with "free nerve endings" (Aδ and C fibres, nociceptors) in the peripheral nerve.Most of the nociceptors respond to noxious mechanical (e.g. squeezing the tissue), thermal (heat or cold), and chemical stimuli and are thus polymodal. Nociceptors can also exert efferent functions in the tissue by releasing neuropeptides [substance P (SP), calcitonin gene-related peptide (CGRP)] from their sensory endings. Thereby they induce vasodilatation, plasma extravasation, attraction of macrophages or degranulation of mast cells, etc. This inflammation is called neurogenic inflammation. Nociceptors project to the spinal cord and formsynapses with second order neurons in the grey matter of the dorsal horn. A proportion of second-order neurons have ascending axons and project to the brain stem or to the thalamocortical system that produces the conscious pain response upon noxious stimulation. Other spinal cord neurons are involved in nociceptive motor reflexes,

more complex motor behaviour such as avoidance of movements, and the generation of autonomic reflexes that are elicited by noxious stimuli. Descending tracts reduce or facilitate the spinal nociceptive processing. The descending tracts are formed by pathways that originate from brainstem nuclei and descend in the dorsolateral funiculus of the spinal cord. Descending inhibition is part of an intrinsic antinociceptive system [83].

AIM AND OBJECTIVE

Epilepsy, anxiety and depression are most common CNS disorders with interlinking etiology and common causative factors. Pain seemed to be most common symptom in all these CNS disorders. Use of herbal remedies for the treatment of central nervous system (CNS) ailments such as anxiety, depression, epilepsy and sleep disorders becomes emerging trend as herbal remedies are believed to produce less undesirable effects than the synthetic one[2].

The phytoconstituents, polyphenols and flavonoids have well attributed for the pharmacological activities of many plants. Plant species from Leguminosea family reported to contain flavonoids and isoflavonoids as major phytoconstituents. Hence, *Cassia fistula* L and *Cassia auriculata* L belonging to the family Caesalpiniaceae, a sub family of Leguminosea have been selected for the present study.

AIM

The present study aimed to evaluate the anxiolytic, anticonvulsant, antidepressant and analgesic potentials of *Cassia fistula* L. and *Cassia auriculata* L., to evaluate the mechanism underlying as well as the phytoconstiuents responsible for above pharmacological actions of selected plants.

OBJECTIVES

- To carry out the pharmacological screening of selected plants for anxiolytic, anticonvulsant, antidepressant, analgesic and anti-nociceptive activities.
- To elucidate the mechanism involved in the above activities
- To elucidate the phytoconstituent responsible for the above pharmacological activity of selected plants.

LITERATURE REVIEW

Epidemiology of CNS disorders

Epilepsy recent community-based surveys have shown that epidemiological indices of epilepsy in India are comparable to those from developed countries, with a prevalence rate of ~5 per 1000 and incidence rate of ~50 per 100,000[84]. Burden of mental disorders such as anxiety and depression had also risen over last few decades. WHO estimated that globally over 450 million people suffer from mental disorders. Currently mental and behavioural disorders account for about 12 percent of the global burden of diseases and this is likely to increase to 15 percent by 2020. Major proportions of mental disorders come from low and middle income countries[85]. There seems to be lacunae in psychiatric epidemiology due to complexity related to defining a case, sampling methodology, under reporting, stigma, lack of adequate funding and trained manpower and low priority of mental health in the health policy[86].

An epidemiological study reports that the overall prevalence of chronic pain was 13%. Respondents with chronic moderate and chronic severe pain were 37% and 63%, respectively. Pain in knees (32%), legs (28%), and joints (22%) was most prevalent. Respondents with chronic pain were no longer able to exercise, sleep, maintain relationships with friends and family, and maintain an independent lifestyle[87].

Co-morbidity depression, anxiety and pain with epilepsy

The results of an epidemiological study conducted on the co-morbidity of psychiatric conditions in epilepsy reveals that the depression is the most existing co-

morbidity in epilepsy with 32.5% reported cases and anxiety stood next to depression with the incidence of 22.4%. Though, the studies reported the same strategy of increased risk of depression and anxiety after the occurrence seizure attack, few studies also reported that the depressive patients are more anxious likely to have more chance of developing epilepsy. Furthermore, a review study reported by Sankar *et al* gives the detailed account of epilepsy and associated mood disorders, neurobiological basis, therapeutic approaches and animal models of epilepsy with related mood disorders. Pain, a common symptom in all these conditions intern considered as the etiological factor of psychological disorders[4].

Drug therapy and limitations of epilepsy, anxiety and depression

The following antiepileptic drugs (AEDs) have been approved by regulatory agencies in the United States and Europe: acetazolamide, carbamazepine, clonazepam, clorazepate, ethosuximide, ethotoin, felbamate, gabapentin, lacosamide, lamotrigine, levetiracetam, mephenytoin, methsuximide, oxcarbazepine, phenobarbital, phenytoin, pregabalin, primidone, tiagabine, topiramate, trimethadione, valproate, vigabatrin, and zonisamide. The following additional agents are used mainly for the acute therapyof status epilepticus: diazepam, fosphenytoin, lorazepam, midazolam, and propofol[24]. The major drawback associated with the usage of currently available AEDs include[88]

- Prophylactic treatment. Not much effective for long time followed by head injury or stroke
- Idiopathic generalized epilepsies are particularly prone to pharmacodynamic aggravation by the usage of AEDs

- Development of tolerance (i.e., reduction in response to a drug after repeated administration) is an adaptive response of the body to prolonged exposure to the drug, and tolerance to AEDs is no exception.

- Drug treatment of epilepsy is characterized by unpredictability of efficacy, adverse drug reactions, and optimal doses in individual patients, which, at least in part, is a consequence of genetic variation.

For anxiety, benzodiazepines, azapirones, some antidepressants and psychotherapy are most wildly accepted treatment options. Benzodiazepines are effective anxiolytics for short-term use and the antidepressants, paroxetine and venlafaxine, have demonstrated efficacy in patients with generalized anxiety disorders. Long-term efficacy has been shown with venlafaxine in the treatment of this chronic condition. The major drawback with these anxiolytics include adverse effect, tolerance, drug dependence etc[89,90].

For depression, one of the two initial classes used was the tricyclic antidepressants (TCAs), a family of structurally related compounds with reuptake inhibitory properties on brain monoamine metabolism. The second original class of drugs, the monoamine oxidase inhibitors (MAOIs), have never been widely prescribed because of real (and sometimes exaggerated) concerns about safety, despite their established efficacy in certain subtypes, especially atypical and bipolar depression. A majority of the newer compounds are considered to be SSRIs. .However, currently available antidepressants have notable limitations, relating to their only moderate efficacy relative to placebo, relatively slow onset of action, Suicidality due to antidepressants, possible withdrawal symptoms, and problems of

compliance. Sleep disturbances are often used to identify newly presenting depressive patients, and may be part of a more general alteration of bodily rhythms[91].

Use of plant drugs for CNS disorders

The use of plants to influence brain function has long been essential to medical practice even before some 3500 years. However, very little attention was given by the scientific community to the benefits, as accepted by folk medicine, of the therapeutic usefulness of plants endowed with CNS activity due to the challenge in elucidation of mechanism of action to support the traditional uses[2]. Recently, the usage of herbal medicine for treating central nervous system has been increasing owing to the greater undesirable effects produced by the synthetic counterpart. Central nervous system (CNS) disorders are difficult to treat and have highly complicated patophysiology and cause high costs for clinical therapy and basic research due to unknown and puzzling mechanisms. Moreover, complementary and alternative medicine (CAM) has recently become highly recognized as therapeutic medicine and recommended by the World Health Organization (WHO) and herbal treatment is one among this[92].

Effect of polyphenols and flavonoids in CNS

Phenolics, and flavanoids in particular, are ubiquitous in plants reported to be influence the mammalian brain. A wide range of phenolic compounds interact directly with neurotransmitter systems. As an example, in animal models, a diverse range of individual and combined flavonoids that occur in traditional medicinal extracts exert sedative/anxiolytic effects via direct binding to $GABA_A$ receptors, cognitive enhancement via antagonistic $GABA_A$ receptor binding and resultant cholinergic

upregulation, and antidepressant effects via monoamine oxidase inhibition and resultant increases in levels of serotonin, dopamine, noradrenaline and also certain neuromodulators such as adenosine, nitric oxide in selected brain areas. Plants also synthesize a range of phenolic phytoestrogens, including isoflavonones, flavones, stilbenes, and lignans, which function as defense chemicals against herbivory by disrupting the endocrine functions of the insect and modifying their life course. In the same way, in mammals and other vertebrates, phytoestrogens modulate hormonal systems, and therefore brain function, via a variety of mechanisms (for review)[93].

Overview of *Cassia auriculata* L.

Cassia auriculata L. belonging to the family Leguminosea and sub family Ceaslpiniacea commonly known as tanners cassia is habitat India and Ceylon. This plant has been used in Indian traditional medicine system for treating wide varieties of ailments. The leaves were and fruits were used for fever, worm infestation, scorpion bite, diabetes, CNS disorders, leucorrhoea and for various skin diseases. Flowers are used for spermatorrhoea, white discharge and for heat diseases. The roots and bark powders are used for urinary discharges white discharges, tumor and ophthalmological applications[94].

Various phytochemical studies revealed the presence of alkaloids, flovonoids, saponins, tannins, carbohydrates, phenolic substances, steroids, terpenoids, anthrocyanins etc... The phytochemicals isolated and characterized were quercetin o-glycoside, rutin, Di-(2 ethyl)-hexyl phthalate, emodin, goratensidine, aurricalacadin, alkane nonacosane o-one, Kampferol, rhein, emodin and fisetinolde[95].

Pharmacological activities reported in earlier studies include anti-diabetic, antiviral, antispasmodic, antipyretic, antioxidant, analgesic and anti-nociceptive, anticancer, anthelmintic, nephroprotective, anti-bacteria and immunomodulatory activity. Among these, anti-hyperlipedemic and the anti-diabetic activities have been studied extensively. Toxicity profile studies of *Cassia auriculata* L. reported earlier showed that the aqueous leaf extract was non toxic at 5000mg/kg dose, ethanol extract of root was non toxic at 3000mg/kg and ethanol and aqueous extract of flowers were non toxic at 300mg/kg on acute administrations. In a sub-chronic toxicity study of aqueous leaf extract, 1000, 2000mg/kg doses once daily for three weeks were found to be non toxic (for review)[96].

Overview of *Cassia fistula* L.

Cassia fistual L. also belonging to the family Leguminosea and sub family Ceaslpiniacea and most popularly known as Indian laburnum is habitat throughout India. In the Indian traditional medicine system, this plant has been used for treating various disorders of human and cattle. The bark is used for treating leprosy, wound washing and for painless delivery. Leaves were used for treating epilepsy, worm infestations, indigestion and various skin diseases. Seeds are used in fermentation process and diarrhea. Pods are used for treating cough, indigestion and typhoid. Roots are used in the treatment of hematemias, pruritis, diarrhea, diabetes, antipyretic, analgesic and laxative. The root of *Cassia fistula* is used by the Malamalsar tribes for revival of alcohol induced paralysis of nervous system[94].

Cassia fistula plant parts are known to be an important source of secondary metabolites, notably phenolic compounds. Fistucacidin as first extracted from the

heartwood. Kaempferol and a proanthocyanidin have been isolated from theacetone extract of the flower. A bianthraquinone glycoside, fistulin together with kaempferol and rhein have been isolated from ethanol extracts of *Cassia fistula* flowers. Besides phenolics and their derivatives, a certain amount of alkaloids have also been reported in the flowers; traces of triterpenes have been observed in both flowers and fruits. A compound, 3B-hydroxy-17-norpimar-8(9)-en-15-one was isolated from the pods of *Cassia fistula*[96].

A flavonoid, catechin was isolated from methanolic extract of stem of *Cassia fistula* L. by bioassay guided fractionation[97]. Two new flavonoids known as fistula flavonoid 1 and 2 and five known flavonoids were isolated from bark and stems and structurally characterized[98].

The antioxidant properties of 90% ethanol extracts of leaves, and 90% methanol extracts of stem bark, pulp and flowers from *Cassia fistula* L. were investigated and the antioxidant potential was in the decreasing order of stem bark, leaves, flowers and pulp and was well correlated with the total polyphenolic content of the extracts[99]. The anti-oxidant activity of *Cassia fistula* L. seed powder has also been reported[100].

Senthilkumar *et al.,* reported the wound healing potential[101]and Duraipandiyan & Ignanimuthu assessed the antifungal and antibacterial activities of hexane, chloroform, ethyl acetate, methanol and water extracts from the flower of *Cassia fistula*[102].

Methanolic extract of *Cassia fistula* L. seed have shown to posses antitumor activity against Ehrlich ascites carcinoma as reported by and another study evaluated

the anticancer activity of a phytoconstituent rhein isolated from *Cassia fistula* flower against colon cancer cell lines[103, 104].

In addition, various other pharmacological activities like anti-fertility[105], hepatoprotective[106] and anti-diabetic[96] anti-inflammatory, anti-leishmanial have been reported and well reviewed by Kainsa *et al.,*[95]. Though, number of scientific studies have been reported the other pharmacological activities of *Cassis fistula* L, only one preliminary study reported the central nervous system depressant activity of methanolic extract of *Cassia fistula* L. fruit pulps[107].

Aqueous and methanolic extract of *Cassia fistula* bark did not show any toxic symptom up to the dose of 2000mg/kg administered orally to the albino rats[108].

ANIMAL MODELS

Animal models for screening anti-convulsant activity

A number of animal models have demonstrated in the search for more efficacious and more tolerable antiepileptic drugs. Models like maximal electro shock seizure (MES), pentylenetetrzole induced seizure, picrotoxin induced convulsion, strychnine induced convulsion. In the discovery and development of new AEDs, animal models of seizures or epilepsy serve a variety of purposes described as follows (for review)[109].

- Discovery of new AEDs
- Characterization of spectrum of anticonvulsant activity of new AEDs
- Specific models for pharmacoresistant seizures

- Evaluation whether efficacy of new AEDs changes during chronic treatment
- Comparison of adverse effects of new AEDs in epileptic against nonepileptic animals
- Estimation of effective plasma concentrations of new AEDs for first clinical trials
- Discovery of antiepileptogenic or disease-modifyingtreatments

Not all animal models of seizures and/or epilepsy can be used for all of the above described purposes. Most animal models used in epilepsy research are models of epileptic seizures rather than models of epilepsy.

In the s.c. PTZ (pentylenetrtrazole) seizure test, the convulsive dose of PTZ inducing a clonic seizure of at least 5 s duration in 97% of the animals. PTZ is subcutaneously injected and animals are observed for a post-injection period of usually 30 min for the occurrence of such a "threshold" seizure. The test is thought to be predictive of anticonvulsant drug activity against nonconvulsive (absence or myoclonic) seizures[110]. However, various AEDs that protect against nonconvulsive seizures in epilepsy patients failed in the PTZ test, so that other models of non convulsive seizures, including genetic rat mutants with spontaneous nonconvulsive seizures, are needed for correct prediction of AED efficacy against the particular type of seizure[111].

In most seizure models, investigational drugs are tested after administration of a single dose and the drug effect is then determined at one fixed time point following drug administration. However, treatment of patients with epilepsy is typically by

chronic, daily drug administration, which may change drug efficacy. Kindling model is the chronic model used to find out the efficacy of AEDs after chronic treatment. In PTZ kindling model, the sub convulsive dose of PTZ is administered until the occurrence of stage 5 seizure and the ability of the drug to prevent the seizure development or severity is predicted by this model[111].

Animal models for screening anxiolytic activity

The EPM is etiologically validated and most widely accepted model for screening novel anxiolytic agents in mice[112] as well as in rat[113]. Increase in the number of open arm entries and consequent increase in time spend in open arm are the two parameters generally considered as index of anxiolytic activity of a drug[114].

However, the interpretation of parameters in EPM is always complex. Studies also report increase in time spent in open compartment, open arm entries and no change in closed arm entries as criteria for drugs to have anxiolytic activity[115]. On the other hand, the total number of arm entries is more reliable measure for locomotor activity[116].

The open-field test is based on rodents intrinsic behaviour to stay near the periphery of a novel environment (i.e., thigmotaxis), which may serve the animal a sense of security. Increase in time spent in the central arena of the open field without affecting general motor activity is the indication of anxiolytic activity[117].

Burying behavior consists in forward shoving the diggable material over the source of aversion using the snout and forepaws in order to avoid and protect from the localized threat. Marble burying test has been suggested as a useful model for

evaluating anti- anxiety activity as no change in tendency of marble burying behavior occurred due to repeating the test[118]. Indeed, commonly used anxiolytic benzodiazepines such as diazepam and chlordiazepoxide decreased the marble burying tendency of rodents[119].

Animal models for screening antidepressant activity

Depression is a heterogenic disorder with numerous symptoms. In fact, some symptoms like guilt, suicidality and sad mood are expected to be purely human features, other aspects of the depressive syndrome have been replicated in laboratory animals, and in several instances ameliorated with antidepressant treatment. These include measures of helplessness, anhedonia, behavioral despair and other neurovegetative changes such as alterations in sleep and appetite patterns (for review)[120].

The forced swim test (FST) and tail suspension test (TST) are the most widely used tests of antidepressant action and are also used to infer "depression-like" behavior. In the Porsolt test[121] also known as the FST test, a mouse or rat is placed in an inescapable cylinder of water and, following an initial period of struggling, swimming and climbing, the animal eventually displays a floating or immobile posture. In the TST, immobility is scored while mice are suspended by their tails. Since water is not required, the TST is not confounded by challenges to thermoregulation[122]. FST or TST immobility has been interpreted as an expression of behavioral despair or entrapment[123] and is reversed by the acute administration of almost all available antidepressants. However, antidepressants restore mood in depressed humans only after many weeks of administration. Numerous agents that act

independently of monoamine signaling have also been shown to reduce immobility time, such as recombinant ghrelin[124], ketamine[125], and estradiol[126]. In spite of above mentioned demerits, these models is their ability to rapidly screen novel agents and phenotype genetically manipulated mice, and both paradigms have been successfully automated to reduce errors in subjective scoring.

Chronic mild stress (CMS), better described as chronic unpredictable stress (CUS), paradigms involve the application of varied intermittent physical stresses applied over a relatively prolonged time period. Sucrose drinking is the most commonly utilized assay to assess the impact of CUS and CUS-exposed rats or mice show deficits in their motivation to consume a dilute (1–2%) solution of sucrose measured either as total sucrose intake or as a preference against water[127]. CUS has also been shown to result in a number of other "emotional" changes that are difficult to objectively quantify, such as grooming deficits and changes in aggressive and sexual behavior. Many of these phenotypes are reversed by chronic antidepressants applied either during the stress or as a post stress treatment[128].

Animal models for screening antinociceptive activity

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[129]. NSAIDs are known to inhibit the COX enzyme in the peripheral tissues which is responsible for the production of pain mediators.

In the tail immersion test, animal's tail is immersed in hot water which provokes an abrupt movement of the tail and sometimes the recoiling of the whole body and the reaction time is monitored[130]. Immersion of the tail in a hot liquid increases its temperature very quickly and in a more or less linear fashion, which is different from radiant heat.

Hot plate test consists of introducing a rat or mouse into an open-ended cylindrical space with a floor consisting of a metallic plate that is heated by a thermode or a boiling liquid[131]. A plate heated to a constant temperature produces two behavioral components that can be measured in terms of their reaction times, namely paw licking and jumping. Both are considered to be supraspinally integrated responses. As far as analgesic substances are concerned, the paw licking behavior is affected only by opioids. On the other hand, the jumping reaction time is increased equally by less powerful analgesics such as acetylsalicylic acid or paracetamol, especially when the temperature of the plate is 50°C or less[132] or if the temperature is increased in a progressive and linear fashion, e.g., from 43 to 52°C at 2.5°C/min[133]. The specificity and sensitivity of the test can be increased by measuring the reaction time of the first evoked behavior regardless of whether it is paw-licking or jumping or by lowering the temperature[134].

Formalin test model is useful in evaluating the anti-nociceptive activity in two different phases. In the initial phase, direct chemical stimulation of the sensory afferent nerve ending 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[135].

Adenosine as a neuromodulator

The term neuromodulator was employed to describe the substances that can influences neuronal activity in a manner different from that of neurotransmitters. The distinctive feature of modulator is that it originates from cellular and non-synaptic sites yet influences the excitability of nerve cells.

Adenosine is a potent modulator of neuronal functions in the central and peripheral nervous system[136]. Previous works in this field have established a role for adenosine in a diverse array of neural phenomena, which include regulation of sleep and the level of arousal, neuroprotection, regulation of seizure susceptibility, locomotor effects, analgesia, depression, anxiety, psychosis, mediation of the effects of ethanol and in chronic drug use.

It is mainly derived from the hydrolysis of nucleotides such as adenosine triphosphate (ATP), under normal physiological condition adenosine is derived continuously and the level of adenosine is regulated by several enzymes. ATP is co-released with other neurotransmission on electrical stimulant such as acetylcholine, dopamine, serotonin & nor epinephrine. Released ATP is very rapidly hydrolyzed to adenosine and other metabolites by group of enzyme called as ecto & endo nucleotides[136]. Interstitial levels of adenosine are maintained in Nano molar to micromolar range via the activity of adenosine deaminase or adenosine kinase as well as distinct families of nucleoside transporters.

Adenosine mediates multiple physiological effects by acting on specific groups of G-protein coupled receptors. Till the date four adenosine receptor subtypes have been identified, A_1AR, $A_{2A}AR$, $A_{2B}AR$ and A_3AR[136]. They are distinctly distributed throughout the body and control multiple physiological actions. Specific ligands of adenosine receptor subtypes have potential therapeutic activity.

Table 1. Distribution, characteristics and therapeutic potential of various adenosine receptors

Receptor Type	G Protein	High abundance	Medium/ low abundance	Effects	Major therapeutic potential
A_1	$G_{i/o}$	Hippocampus, Neocortex,	Amygdala, striatum,	Adenylcyclase and Ca^{2+} Channel inhibition,	Activation: seizure suppression,
		Cerebellum, spinal cord	olfactory bulb thalamus, substantia nigra	Activation of PLC and GIRKS	neuroprotection, spinal analgesia
A_{2A}	$G_{s/olf}$	Striatum, olfactory bulb	Hippocampus thalamus, neocortex	Adenylcyclase activation. Inhibition of Ca^{2+} channels	Activation: anti-inflammatory action. Inhibition: Parkinson's disease
A_{2B}	G_s		Uniform	Adenylcyclase and PLC activation	Inhibition: antiasthmatic
A_3	$G_{i3,Gq}$		Hippocampus, cerebellum,	Adenylcyclase inhibition and PLC activation	Inhibition: anti-inflammatory action

Adenosine and epilepsy

The potential of adenosine as an anti-epileptic substance[137-140] has emerged on the basis of following observations: first, A_1R are enriched in excitatory synapses, where they inhibit glutamate release, decrease glutamatergic responsiveness and hyperpolarise neurons, all desirable actions to decrease the hyper excitability associated with epilepsy; second, the levels of endogenous extracellular adenosine rise upon seizure activity[141,142], which could be taken as an indication that adenosine would play a key role as an endogenous anti-epileptic compound. Accordingly, a wealth of studies have confirmed that the acute administration of either agents enhancing the extracellular levels of adenosine (inhibitors of adenosine transporters or of adenosine metabolism) or agonists of A_1R attenuated seizure and/or convulsive activity in different animal models; conversely, the acute administration of either non-selective antagonists of adenosine receptors (such as caffeine or theophylline) or selective A_1R antagonists enhance the duration and severity of seizures and/or convulsions (for review)[137-140]. Thus, it seems evident that A_1R effectively constitute a hurdle curtailing seizure activity, which is further confirmed by the ability of A_1R to control the spreading of seizure activity[143] and the greater susceptibility of A_1R knockout mice to epilepsy[144,145]. Interestingly, this A_1R-mediated control of epilepsy has recently been proposed to be a possible link for the antiepileptic effect of ketogenic diets[146]. However, several studies have now identified a decreased density and efficiency of synaptic A_1R in models of epilepsy[146-148]. Thus, the A_1R-operated inhibitory system seems to act as a continuously active gate-keeper or hurdle to avoid initiating a seizure-like event; once this hurdle is overtaken, then there seems desensitization of this A_1R system.

Adenosine and anxiety

A strong link between the adenosine modulation system and the control of fear processing and anxiety is suggested by several types of studies, namely: i) animal models designed to mimic anxiety traits; ii) epidemiological studies with caffeine, both in normal subjects and in patients in anxiety disorders; iii) gene linkage studies in humans (for review)[149,150]. However, the role of adenosine receptors in specific anxiety disorders, like phobia, is still to be defined. Studies with caffeine consumption by phobic patients are rare, especially in comparison to research on caffeine consumption among patients with other anxiety disorders like panic disorder. A study from Boulenger and Uhde[151] showed that panic disorder patients have increased sensitivity to caffeine and its consumption appears to be more strongly linked to generalized anxiety symptoms than to 'phobic-anxiety' symptoms. The work from Uhde[152] also showed that anxiety symptoms are not made more severe by caffeine consumption in social phobics and that caffeine consumption in social phobic individuals does not differ from the general population. In addition, no differences in electroencephalographic activity of patients with panic disorder versus control subjects were observed after oral administration of caffeine[153]. On the other hand, another study reported that caffeine can induce panic attacks in both panic disorder and social phobic patients following consumption of a dose of 480 mg[154]. It was also demonstrated that caffeine ingestion per se does not appear to reduce fear extinction at least in some specific phobias, whereas changes in caffeine consumption, during learning or recall tests, can enhance the return of fear in spider phobia[155].

Adenosine and depression

The effect of the adenosine neuromodulation system in depression is complex, especially due to its ability to modulate several other neurotransmission systems, such as dopaminergic, glutamatergic and serotoninergic as well as the corticotrophin system (for review)[156.] Adenosine and its analogues were shown to cause a depressant-like response in behavioral despair models[157-159], an effect that was prevented by the administration of classical antidepressants[160]. In contrast, other studies showed an antidepressant effect associated with adenosine administrated both systemically and centrally[161-163]. The strongest evidence supporting the relation between adenosine and depression in preclinical models came from manipulation of A_2AR. The main evidence sustaining this hypothesis relies on the observation that the genetic depletion of A_2AR results in an antidepressant-like phenotype in animal models[164,165]. In addition, A_2AR blockade relieves the early hippocampal modifications induced by stress[166], one of the major environmental factors favoring the implementation of depressive states[167]. It is important to highlight that different therapeutic strategies currently used to manage depressive disorders also have effects related to the adenosine system. Tricyclic antidepressants such as nortriptiline, chlorimipramine or desipramine can bind to adenosine receptors[168] and dose-dependently reduce the activity of ecto-nucleotidases and, expectedly, the levels of extracellular adenosine in cortical synapses[169]. In addition, both electroconvulsive therapy and sleep deprivation cause an increase of adenosine concentration and of A1R activation[170]. In conclusion, although at this stage the relation between adenosine and depression is still circumstantial, it seems evident that the adenosinergic system is able to modulate mood states by functioning as a normalizing system, and both too

high or too low levels of activation can cause a failure in the organism to adapt and a predisposition to disease[170]

Adenosine and pain

Adenosine mediated analgesia and interaction of adenosine with analgesic effect of opioids have been reported in several research studies. In animals, adenosine and adenosine agonists inhibit nociceptive reflexes in a variety of animal models of acute tactile, mechanical or heat pain as well as in models of persistent visceral pain induced by noxious chemicals. In addition, treatment of adenosine agonist enhances morphine-induced analgesia. While, methylxanthines such as caffeine and theophylline (potent adenosine receptor antagonists) have been reported to antagonize opioid effects effectively supporting a role for adenosine in the actions of opioids. Several investigations also suggest that the mechanism of action for opioids involves opioid induced release of adenosine. Recently, adenosine modulation has been described as a novel approach for analgesia and inflammation (for review)[171].

PLAN OF WORK

I. Pharmacognostic study and phytochemical evaluation

1. Collection and authentication of plants material

2. Physiochemical characterization of selected plants material

   a. Morphology.

   b. Ash value

   c. Extractive values

   d. Heavy metal analysis

3. Phytochemical evaluations and standardization of extract fractions

   a. Preliminary phytochemical analysis

   b. Total flavonoid content estimation

   c. Polyphenolic estimation

   d. HPTLC studies

4. Isolation of active principle and characterization

   a. Isolation by column chromatography

   b. Structural elucidation of compound by NMR, IR and Mass Spectroscopy

II. Pharmacological studies

1. *In vitro* antioxidant studies

1.1. Superoxide free radical scavenging activity

1.2. Reducing power assay

2. *In vivo* pharmacological studies

2.1. Oral toxicity studies

2.2. Anti- convulsant activity

2.2.1. Sub cutaneous pentylene terazole (s.c.PTZ) model

2.2.2. PTZ kindling model

2.3. Anxiolytic activity

2.3.1. Elevated Plus Maze

2.3.2. Open field analysis

2.3.3. Marble burying behavior

2.4. Antidepressant activity

2.4.1. Forced swim test (FST)

2.4.2. Tail suspension test (TST)

2.4.3. Chronic unpredictable stress induced anhedonia

2.5. Analgesic and anti-nociceptive activity

2.5.1. Hot plate model

2.5.2. Tail immersion model

2.5.3. Acetic acid induced writhing model

2.5.4. Formalin test

2.6. Evaluation of motor toxicity and sedative property

2.6.1. Rota rod test

2.6.2. Phenobarbitone induced sleeping time

2.7. Neurochemical estimation

2.7.1. GABA estimation

2.7.2. Glutamate estimation

2.7.3. Serotonin estimation

2.7.4. Adenosine estimation

2.8. Interaction studies

2.8.1. Adenosine agonists and sub effective dose of EAFCF

2.8.2. Adenosine antagonists and effective dose of EAFCF

PROFILE OF PLANTS

Plant 1

Name of the plant	:	*Cassia fistula*
Botanical name	:	*Cassia fistula* Linn.
Family	:	Leguminoseae
Sub family	:	Caesalpiniaceae

Name in different languages

Tamil	:	Sarakkondrai
English	:	Golden shower
Malayalam	:	Konna
Hindi	:	Amalthus

Fig 1. Image of *Cassia fistula* L.

Plant description

Medium-sized trees, with 4-8 pairs of leaflets and yellow flowers in long axillary pendulous racemes. Pods cylindrical, long, blackish-brown when ripen.

Medicinal Uses: Bark and fruits are used in traditional systems of medicine Plant is used in snakebite and scorpion sting. Bark has tonic and antidysenteric properties, and leaves possess antiperiodic and laxative properties.

Plant 2

Name of the plant	:	*Cassia auriculata*
Botanical name	:	*Cassia auriculata* L.
Family	:	Leguminoseae
Sub family	:	Caesalpiniaceae

Name in different languages

Tamil	:	Avarai
English	:	Tanner's cassia
Malayalam	:	Avirai
Hindi	:	Tarwar

Fig 2. Image of *Cassia auriculata* L.

Plant description

Bushy shrubs, with 8-12 pairs of leaflets and glands between all pairs. Flowers yellow with orange veins, in axillary and terminal racemes. Pods flat. Flowering and fruiting period-August – November

Traditional uses

The Garasia tribals wash the eyes with the decoction of seeds in ophthalmia and conjunctivitis. Bhils take orally the fresh flowers with sugar to cure diabetes and urinary disorders. They also give leaves as an anthelmintic to the children. The Saharia tribals in Kota district make a paste of root-powder with mustard-oil to apply against skin disesses and on the abdomen against problems in urinary discharge. Kathodias cure cough and cold by taking orally the root-powder in Udaipur district and Bhils of Banswara cure diarrhea. The twigs are used as tooth-brush.

MATERIALS AND METHODS

Pharmacognostic study

Plant material

Cassia fistula L. and *Cassia auriculata* L. fruit pods were collected from surroundings of Coimbatore during the month of September and the specimens were authenticated by Dr.G.V.S. Moorthy, Botanical Survey of India, Agricultural University, Coimbatore. The sample voucher specimens BSI/SRC/5/23/2011-12/Tech 781& 782 were deposited for future use.

The pods were washed thoroughly in purified water to make it free from dirt followed by 10% potassium permanganate solution to remove the microbial contamination. The clean pods were dried in shade to remove moisture content and powdered.

Ash values[172]

The amount of inorganic compounds present in the crude drugs was determined by ash value. The inorganic compounds such as carbonates, phosphates, silicates, potassium, calcium and magnesium are present as residues after incineration of a crude drug.

Determination of total ash value

The total ash value is to measure the total amount of material remaining after ignition that comprises both physiological (derived from plant tissue) and non-physiological ash (extraneous matter adhering plant such as sand and soil). 2 g of

dried and powdered materials from both the plants were incinerated in a pre-ignited and tared crucible by gradually increasing heat up to 500-600ºC until getting white residue which was free from carbon. The residues remained after incineration was cooled in a desiccator and weighed. The total ash content was calculated and expressed as in mg/g of dried material.

Determination of acid insoluble ash

Acid-insoluble ash is the residue that remains after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This is the measure of silica present in the material specifically as sand and siliceous earth. 25 ml of hydrochloric acid was added to the each crucible containing total ash and boiled for 5 min by covering with watch glass. The insoluble residues were collected by using ash less filter paper then were ignited in the same crucibles until getting constant weights. The residues were cooled in suitable desiccators for 30 min and then weighed immediately. The acid-insoluble ash content in mg/gm of each air-dried material was calculated.

Determination of water soluble ash

Water-soluble ash is determined by calculating the difference in weight between the total ash and the weight residue after treatment of the total ash with water. 25 ml of water was added to each crucible containing the total ash of the selected plant materials and boiled for 5 min. The water insoluble matter was collected in ash less filter paper and washed with hot water. Then the residues were ignited in crucibles for 15 min, at a temperature not exceeding 450^0 C and weighed.

The difference in weight of total ash and water insoluble residue represents the water soluble ash.

Extractive value[172]

This value is to determine the quantity of active constituents that can be extracted with the solvent from a weighed quantity of plant drug.

Determination of alcohol soluble extractive value

2g of air dried powder drug material of both the plants were taken in separate flasks and 100 ml of alcohol was added to the flasks and macerated for 24 h with frequent shaking for initial 6 h and allowed to stand for next 18 h. The contents were filtered immediately to avoid the loss of solvent, 25 ml of filtrate was evaporated and dried at 105ºC to constant weight and the weights of the residues were calculated. Percentage of alcohol soluble extract with reference to the air dried crude drug was calculated for each plant materials.

Determination of water soluble extractive value

2g of the air dried drugs were macerated with 100ml of water in separate closed flasks for 24 h, shaken frequently during the first 6 h and allowed to stand for next 18 h then filtered. 25 ml of each filtrate was heated to get solid mass and dried at 105°C to get constant weights. Dried at 105°C, to constant weight and weighed. The percentage of water soluble extract value for both the material was calculated with reference to the air dried drugs.

Preparation of extracts and fractionation[172]

Fresh samples were air-dried and ground, yielded 1500g of powder. From which, (500g) powder of dry pods from both plants were separately defatted with petroleum ether (40–60˚C) for 6 h. Then they were filtered and marc dried and extracted with a mixture of ethanol (700 ml): water (300 ml) (70: 30) as solvent for 24 h at room temperature and placed in rotary shaker for 2 days to get hydro-alcoholic extracts by maceration method. The extracts were filtered by using Whatmann No.1 filter paper and filtrates were taken. The extraction was repeated two times and the filtered hydro-ethanolic extracts were mixed and evaporated under reduced pressure.

50.0 g of hydroalcoholic extracrs were dissolved in 200 ml of methanol/water (7:3). N-Hexane, ethyl acetate and aqueous fractions were made from the solution obtained through liquid/liquid solvent partition of increasing polarity. From each fraction, the solvent was evaporated in rotary evaporator and dried. Fractions were designated as follows – hexane fraction (NHF), ethyl acetate fraction (EAF) – aqueous fraction (AQF), dried to constant weight and stored at −10 ◦C until used for experiments.

Heavy metal analysis[172]

Heavy metal contents in spices and medicinal plants depend on climatic factors, plant species, air pollution, and other environmental factors. The fractions of both plant materials were subjected to the elemental analysis to find out the presence of heavy metals such as iron, cadmium, lead zinc and copper. The analysis was carried out using atomic absorption spectroscopy (AAS).

Preliminary phytochemical analysis[172]

Hydoalcoholic extract of both the plant materials were evaluated for the presence of various phytoconstituents.

Test for alkaloids:

About 0.2 g of the each extracts or fractions were warmed with 2% H_2SO_4 for two min. It was filtered and few drops of Dragendroff's reagent were added to both the test tubes. Orange red precipitate indicated the presence of alkaloids.

Test for tannins:

Small quantity of extracts in separate test tubes were mixed with water and heated on water bath. The mixtures were filtered and ferric chloride was added to the filtrates. A dark green solution indicates the presence of tannins.

Test for anthraquinones (Borntragers test):

About 0.5 g of the each extracts or fractions were boiled with 10% HCl for few min in a water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% NH_3 were added to the mixture and heat. Formation of rose-pink colour indicates the presence of anthraquinones.

Test for glycosides:

The extracts were hydrolysed with HCl solution and neutralized with NaOH solution. A few drops of Fehling's solution A and B were added. Red precipitate indicates the presence of glycosides.

Test for reducing sugars:

The extracts were shaken with distilled water and filtered. The filtrate was boiled with drops of Fehling's solution A and B for min. An orange red precipitate indicates the presence of reducing sugars.

Test for saponins:

About 0.2 g of the extracts were shaken with 5ml of distilled water and then heated to boil. Frothing (appearance of creamy miss of small bubbles) shows the presence of saponins.

Test for flavonoids:

0.2 g of each extracts or fractions were dissolved in diluted NaOH and HCl was added. A yellow solution that turns colourless, indicates the presence of flavonoids.

Test for steroids:

2ml of acetic anhydride was added to 0.5 g of the extracts of each with 2ml of H_2SO_4. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

Test for terpenoids (Salkowski test):

0.2 g of the each extracts of the fruit pod samples were mixed with 2ml of chloroform ($CHCl_3$) and concentrated H_2SO_4 (3ml) was carefully added to form a layer. A reddish brown coloration of the interface was formed to indicate positive results for the presence of terpenoids.

Polyphenolic content estimation[173]**.**

The total phenolic content of all fractions was estimated by using Folin catechu reagent and measured at 765 nm using gallic acid as standard. Aliquots of fractions or gallic acid were mixed with 0.5 ml of Folin catechu reagent (diluted 1:1 with water) and 2.5 ml of 20% aqueous sodium carbonate and allowed to stand for 45 min in dark room and absorbance was measured by spectrophotometer. The total phenolic content in each fraction was calculated as gallic acid equivalent from the calibration curve.

Flavonoid content estimation[174]

Flavonoid content in all the fractions was determined by aluminium chloride colorimetric method. Aliquot quantity of fractions was diluted with 1.50 ml of distilled water and 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 the resulting reaction mixture was measured at 415 nm UV spectrophotometer. Quantification of flavonoids was done 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**.**

HPTLC studies of selected fractions

Chromatographic condition

Stationary phase	:	Silica Gel GF254
Mobile phase	:	Toluene: Ethylacetate: formic acid (7:2:1 v/v)
Chamber saturation time	:	3 h
Instrument	:	HPTLC (CAMAG-version 1.3.4,USA)
Applicator	:	Linomat V
Scanner	:	CAMAG TLC Scanner
Developing chamber	:	Twin trough glass chamber (20×10)
Developing mode	:	Ascending mode (multiple development)
Detection reagent	:	Vanillin hydrochloride
Scanning wavelength	:	254 & 366nm
Experimental condition	:	25±2˚C Temp/RH: 55-65%

HPTLC instrumentation

The samples were spotted in the form of bands of width 6mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60 F–254, (20 cm × 10 cm with 250 m thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat IV (Switzerland). The plates were prewashed by methanol and activated at 60˚C for 5 min prior to chromatography. A constant application rate of 0.1 µl/s was employed and space between two bands was

5 mm. The slit dimension was kept at 5mm × 0.45mm and 10 mm/s scanning speed was employed. The monochromatic bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of Toluene: ethylacetate: formic acid (7:2:1) and 15 ml of mobile phase was used per chromatography. Linear ascending development was carried out in (20 cm × 10 cm) twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase and the chromatoplate development was carried out for two times with the same mobile phase to get good resolution. The optimized chamber saturation time for mobile phase was 3 hr at room temperature (25±2◦C) at relative humidity of 60 ± 5%. The length of chromatogram run was 8 cm. Subsequent to the development; TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode at 486 nm and operated by CATS software (V 3.15, Camag). The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 254 and 366 nm. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was via peak areas with linear regression.

Isolation and characterization of compounds

Based on the results obtained from preliminary phytochemical analysis, HPTLC studies, quantification of polyphenols and flavonoids, the ethyl acetate fraction of *Cassia fistula* was taken for isolation of active principle by column chromatography and characterization by UV, IR, GC-MS and NMR analysis.

Column chromatography

Based on the phytochemical, HPTLC and polyphenol estimation the ethyl acetate fraction of *Cassia fistula* was taken for the column chromatography studies. About 1.3 g of the ethyl acetate fraction was thoroughly mixed with silica gel (60-120 mesh) and fractionated by using tightly packed silica gel column. Chloroform was used as a solvent for packing the silica gel in the column. Then ratio of fractions of 30:0, 25:5, 20:10, 15:15, 10:20, 5:25 (chloroform: methanol in ml) was collected. Among this, the fourth fraction was seemed to be denser and possessed the maximum phytoconstituents. Hence, the fourth fraction was taken for further fractionation by column chromatography and packed in a column and the ratio of 9.50:0.50, 9.0:1.0, 8.5:0.5, 8:2, 7.5:2.5, 7:3, 6.5:3.5, 6:4, 5.5:4.5 (Chloroform: methanol in ml) was collected. In the column chromatography, the fraction obtained with 90% and 80% of chloroform in methanol mixture showed positive test with folin-ciocalteu reagent.

HPTLC study of isolated fractions from ethyl acetate

Method of preparing chromatographic fingerprint

Stationary phase	:	HPTLC plate (10cm× 10cm; Merck)
Mobile phase	:	Chloroform: methanol (11.8:0.2)
Sample Application	:	Applied 2µl of sample and bands onto TLC Plates.
Development	:	Developing distance 8 cm from the lower edge of the plate. The plates were dried below 50° C.
Detection	:	Observed at UV 254 & 366 nm

HPTLC (CAMAG-version 1.4.4) chromatographic conditions

Pre coated aluminum plate (10 cm x10 cm) with silica gel 60 F254 (Merck, Darmstadt, Germany) was used as stationary phase to perform planer chromatography. Samples were applied to the plates that are 4mm wide and 10 mm from the bottom, by means of pressurized nitrogen gas (150kg/cm2) through CAMAG Linomat V fitted with a 100µl syringe. The rate of application and the distance between the tracks were set according to the type of analysis. The bands were visualized in Camag UV cabinet at 254 nm and 366 nm. Ascending development, with the mobile phase consisting of solvent chloroform: methanol (11.8:0.2) was performed in a twin-trough glass chamber (10cm x 10cm) obtained from CAMAG, with tightly fitting lids and previously saturated with the mobile phases for 20 min at room temperature (25±0.5◦C) and relative humidity (50 ± 5%). After the administration of 2ml of nine fractions of ethyl acetate fraction of *Cassia fistula* obtained by the column chromatography, the TLC plate was developed for a distance of 8mm (migration time of 30 min). After development, the plate was dried at 50 ± 0.5 for 15 min and then it was scanned using Scanner 3 (CAMAG, Switzerland) at 366nm using Win CATS 4 software.

Separation of compound by preparative HPLC

HPLC separation of single band fraction (F2, F3 and F4) of ethyl acetate fraction of *Cassia fistula* was performed in Waters 515 pump, with manual rheodyne injector, (volume of injection is 10µl run time 10 min) using acetonitrile and water as the mobile phase in the ratio of 70:30. The column used was C18 waters sun fire column, (15X5µmX4.6mm) and the detector was UV(2489) with the detection

wavelength of 214nm. The software used is waters Empower 2. A peak was eluted at the retention time of 5.2 min. The eluted fractions were collected in an eppendrof tube, directly from the column outlet**.**

FTIR sample processing method

Sample preparation:

Pressed pellet technique:

The given powder sample was mixed with potassium bromide, which was kept in hot air oven for 1 hour at 90 degree Celsius, in the ratio of about 100 times its weight of powdered potassium bromide. The finely ground mixture was then passed through KBR pellet press under a pressure of about 25,000 Psi, maintained for about 10 s. The resulting transparent pellet was subjected to FTIR analysis.

Instrumental Procedure:

The instrument used was FTIR 8400s (CE**)** Shimadzu; it was calibrated by measuring power spectrum, polystyrene film and blank KBR pellet. This takes about 2 min and the obtained spectrum along with the instrument generated report was monitored for pass result. The made sample pellets were subjected to FTIR analysis, resulting in IR spectra of the compound.

GCMS analysis

The GCMS used was a Thermo Scientific DSQ II series single quadrupole GC-MS. The GC was operated in the splitless injection mode with a flow of 1 ml/min through a DB-35ms capillary standard non-polar column (30 m length x 0.25 mm i.d.

x 0.25μm film thickness). Mass spectrometry was used in selected ion monitoring (SIM) scan mode and operated in the electron impact (EI) mode at 40eV. Oven temperature programme: 50°C (1 min. isothermal) to 260°C (at 10°C / min.). Carrier gas: helium at 1ml/min. Xcalibur version 2, software was used to analysis the data.

NMR analysis of ethyl acetate extract of *Cassia fistula*

NMR spectra was obtained using Bruker Avance 400 equipped with BBI Inverse Probe ,equipped with an automatic tuning and matching (ATM) as well as Z-gradients. 2 Channel amplifier system (20-420) MHz each) Communication control unit with high speed RISC processor, 16 MB memory, dedicated enternet connection ports for communication link to workstation. The spectra were acquired and processed using PC/NT workstation with NMR suite, NT software. The spectra were recorded in $DMSOd^6$ solvent systems at 300 K and the chemical shift calibration was carried out either on the TSP signal or residual solvent peak. The size of all 1D spectra was 65 K and the number of transients varied for different types of spectra. The standard 1D H NMR spectra were acquired with 30 pulse length and a relaxation delay of 2 s, while the 1 H NMR45 spectra were acquired with 45 pulse length and a relaxation delay of 60 s to enable an accurate quantification of peaks .

PHARMACOLOGY STUDIES

***In vitro* pharmacological studies**

Superoxide radical scavenging activity[98-100]

Measurement of superoxide anion scavenging activity of fractions was based on the method described previously. Phenazine methosulphate (PMS)–nicotinamide

adenine dinucleotide (NADH) system generates superoxide radicals by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radicals were generated in 3ml of Tris–HCl buffer (16 mM, pH 8.0) containing 1ml of NBT (50 µM) solution, 1ml NADH (78 µM) solution and 0, 50, 100, 150, 200, 250 µg/ml of fractions were mixed. The reaction was started by adding 1ml of PMS solution (10µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm in a UV-spectrophotometer was measured against blank samples. Decrease in absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula.

$$\text{Percentage inhibition} = [A_0 - A1/A_0] \text{ X } 100$$

Where A_0 was the absorbance of control (blank), and A_1 was the absorbance of EAF/ NHF/ AQF or standard.

Reductive ability[98-100]

Reducing power ability was measured by mixing 50, 100, 150, 200 and 250 µg/ml of EAFCF and NHFCA prepared with distilled water and mixed with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 500°C for 30 min. Trichloroacetic acid (2.5 ml, 10%) (TCA) were added to the mixture and centrifuged for 10 min at 3000 RPM and 2.5 ml from the upper portions were diluted with 2.5 ml water and shaken with 0.5 ml fresh 0.1%, ferric chloride. The absorbance was measured at 700 nm using UV-spectrophotometer. The blank was prepared as above, but contained water instead of the samples. Increase in

the absorbance by reaction mixture indicates increased reducing power. All experiments were done in triplicate using butylated hydroxy toluene as standard.

In vivo pharmacological studies

Based on the preliminary pharmacognostical studies and *In vitro* pharmacological evaluations the ethyl acetate fraction of *Cassia fistula* L. pods (EAFCF) and n-hexane fraction of *Cassia auriculata* L. pods (NHFCA) were selected for further pharmacological evaluations. Prior approval from Institutional Animal Ethical Committee (IEAC), PSG Institute of Medical Sciences and Research were obtained for all the protocols in this study.

Oral toxicity studies[175]

The acute toxicity test was performed as per the guidelines of OECD for the testing of chemicals (OECD, 2001). There were no death observed as well as the animal did not show any change in food consumption and body weight up to the dose of 2000 mg/kg. However, behavioral alterations were noted in animals received higher doses of fractions. The doses for pharmacological evaluations were fixed based on the acute oral toxicity studies.

Anti- convulsant activity

Subcutaneous pentylenetetrazole seizure test (s.c.PTZ test)[109-111]

Mice were administered 60mg /kg dose of PTZ subcutaneously into a loose fold skin of the neck between shoulder blades and were placed in a Plexiglas arena and observed for 60 mins after PTZ administration. PTZ (60mg/kg, s.c.) has been

standardized as 100% convulsant with minimal mortality in mice. Parameters noted were latency to first minimal clonic seizure (MCS) which persist for at least a 5 s period, latency to first generalized tonic clonic seizure (GTCS), protection percentage against GTCS.

Table 2. Grouping of animals for evaluation of anti-convulsant activity of EAFCF and NHFCA in s.c.PTZ model

S.No	Group	Number of animals	Treatment
1	Group I	6	Control (Vehicle 10 ml/kg)
2	Group II	6	Standard (Diazepam 2 mg/kg i.p.)
3	Group III	6	EAFCF (50 mg/kg, p.o.)
4	Group IV	6	EAFCF (100 mg/kg, p.o.)
5	Group V	6	EAFCF (200 mg/kg, p.o.)
6	Group VI	6	NHFCA (100mg/kg, p.o.)
7	Group VII	6	NHFCA (200 mg/kg, p.o.)
8	Group VIII	6	NHFCA (400 mg/kg, p.o.)

PTZ kindling model[109-111]

Rats received a sub-convulsant dose of PTZ (40 mg/kg, i.p) every alternate day until the occurrence minimum three consutive stage 5 seizures. Rats were treated with standard drug/ vehicle/ EAFCF (200 mg/kg) 60 min prior to the administration of PTZ and convulsive behavior was scored as follows stage 0; straub tail stage 1;

facial or jaw movements, stage 2; addition of head nodding, stage 3; unilateral forelimb clonus, stage 4; and rearing with bilateral forelimb clonus, with hind limb extension stage 5.

Anxiolytic activity

The following table shows the grouping pattern animals used for the evaluation of anxiolytic activity in EPM, OFT and marble burying behavior.

Table 3. Grouping of animals for evaluation of anxiolytic activity of EAFCF and NHFCA in EPM, OFT and marble burying behavior

S.No	Group	Number of animals	Treatment
1	Group I	6	Control (Vehicle 10 ml/kg)
2	Group II	6	Standard (Diazepam 1 mg/kg i.p.)
3	Group III	6	EAFCF (25 mg/kg, p.o.)
4	Group IV	6	EAFCF (50 mg/kg, p.o.)
5	Group V	6	EAFCF (100 mg/kg, p.o.)
6	Group VI	6	NHFCA (50mg/kg, p.o.)
7	Group VII	6	NHFCA (100 mg/kg, p.o.)
8	Group VIII	6	NHFCA (200 mg/kg, p.o.)

Elevated plus maze test[112]

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). The maze was elevated 38.5 cm from the room's floor. Each animal was placed at the center of the maze, facing one of the enclosed arms. The number of entries and the time spent in enclosed and open arms were recorded for 5 min. Entry into an arm was defined as the animal placing all four paws onto the arm. Total exploratory activity (number of entries) and other ethologically derived measures (grooming, rearing, stretched attend postures and head dipping) were also registered. After each test, the maze was carefully cleaned up with wet cotton dipped in 10% ethanol solution.

Open field test[116]

This method is used to evaluate exploratory activity and emotionality of animals. The open field test apparatus consist of a square arena (60cm×60cm× 60cm) divided into 9 segments. The mice were placed in the center of arena facing the wall and allowed to explore freely in the apparatus for 5 min with experimenter out of animal sight. Parameters like ambulation (number of partitions crossed with all four paws), rearings (number of times mouse stood on its hind limbs), time spent in central compartment and number of crossings in central compartment were recorded. Mice were carried to the test room in their cages and were handled by the base of their tails at all times. After the 5min, test mice were returned to their home cages. The open field was cleaned with 70% ethyl alcohol and permitted to dry between tests.

Marble burying behavior[117]

This experimental procedure is based on the self-protective burying behavior of rodents in response to aversive stimuli such as glass marbles. Mice were preselected based on their stable burying behavior and were treated with fractions or standard drug. 60 min later they were placed individually in cages containing 25 glass marbles placed in uniform distance for 30 min the mice were removed and number of marbles completely buried in sawdust were counted[119].

Antidepressant activity

The following table represents the grouping pattern of animals followed for the evaluation of anti-depressant like activity of EAFCF and NHFCA in FST and TST.

Table 4. Grouping of animals for evaluation of antidepressant activity of EAFCF and NHFCA in FST and TST

S.No	Group	Number of animals	Treatment
1	Group I	6	Control (Vehicle 10 ml/kg)
2	Group II	6	Standard (Fluoxetine 20 mg/kg, i.p.)
3	Group III	6	EAFCF (25 mg/kg, p.o.)
4	Group IV	6	EAFCF (50 mg/kg, p.o.)
5	Group V	6	EAFCF (100 mg/kg, p.o.)
6	Group VI	6	NHFCA (50mg/kg, p.o.)
7	Group VII	6	NHFCA (100 mg/kg, p.o.)
8	Group VIII	6	NHFCA (200 mg/kg, p.o.)

Mouse Forced Swim Test (FST)[121]:

The method described by Porsolt and coworkers was used. Unlike Porsolt's method for mice which consists of direct immersion of animals after injecting drugs, we subjected the animals to "pre-test session" to avoid variations and for maintaining consistency in the immobility time between different groups. Briefly, mouse was forced to swim individually for 15 min in glass cylinder (21x12 cm) containing fresh water up to the height of 9 cm at a temperature of 25 ± 2ºC. This constituted the "pre test session", Twenty four h latter, the animals were treated either with a drug (test group) or vehicle (control group) and each animal was again forced to swim in similar environment for a period 6 min in "test session" and the duration of immobility was recorded. In this experimental paradigm after a phase of vigorous swimming in water, mice show an immobile floating posture. This immobility is postulated to represent a depression like state and is reduced or eliminated by clinically effective antidepressant drugs. Mouse was judged to be immobile if it ceased struggling and remained floating motionless in water making only those movements necessary to keep its head above water. Reduction in the duration of immobility by a drug was considered, as it possesses antidespair or antidepressant like effect. Each experimental group consists 5 mice and was chosen by means of completely randomized method. Illumination of 100Lux was maintained above the FST model throughout the experiment.

Tail suspension test[123]

Tail suspension test was derived from the Porsolt's forced swim test and was based on the observation that the mouse suspended by its tail shows alternate period

of agitation and immobility similar to that of FST. Briefly, mice were suspended from the edge of the shelves 50 cm above the floor by its tail. The duration of immobility was recorded for 6 min. The reduction in the duration of immobility by a drug was considered as it possess antidepressant activity.

Chronic unpredictable stress induced anhedonia[127]

Stress procedure

Behavioral depression was induced to the animals by chronic unpredictable for the period of five weeks. The stressors used are as follows: 24 h food deprivation; 2 h immobilization; 24 h water deprivation; 5 min cold swim at 4ºC; 1 min tail pinch, wet bedding, placing novel items in the cage. The stressors were applied randomly so that the animal cannot have clue about the following stressors. Sucrose consumption test was performed before stress, two weeks after stress and five weeks after stress session. Forced swim test and Super oxide dismutase (SOD) activity assay were performed at the end of 5 weeks stress procedure.

Table 5. Grouping of animals for evaluation of antidepressant activity of EAFCF in CUS induced anhedonia model

S.No	Group	Number of animals	Treatment
1	Group I	6	Control (Vehicle 10 ml/kg)
2	Group II	6	Model (Stressed)
3	Group III	6	Standard (Fluoxetine 20 mg/kg, i.p.)
4	Group IV	6	EAFCF (50 mg/kg, p.o.)
5	Group V	6	EAFCF (100 mg/kg, p.o.)

Sucrose consumption test

Sucrose consumption is considered as hedonic measurement and animals received chronic unpredictable stress for 5 weeks show decrease in sucrose consumption. Animals were habituated to consume 1% sucrose solution before the experiment. Sucrose consumption was measured by placing one bottle of drinking water and one bottle of 1% sucrose solution for one hour period and reweighing the pre-weighed bottles.

SOD assay[176]

Animals were withheld from drug treatment for twenty four h after the five weeks stress protocol. Blood samples were collected and erythrocytes were separated and hemolyzed in cold double distilled water then it was centrifuged with ice cold extractive reagent at 1500 RPM for 10 min. SOD activity in upper aqueous supernatant phase was assayed by addition of 0.3 ml of phosphate buffer, 0.025 ml of 186µM PMS and 0.075 ml of 300 µM NBT and glacial acetic acid 0.025 ml. The reaction mixture was incubated for 90S at 30ºC and the color intensity was measured at 560nm in spectrophotometer.

Analgesic and anti-nociceptive activity

Analgesic and antinociceptive actions of EAFCF and NHFCA were assessed by acetic acid induced writhing, hot plate, tail immersion and formalin test. The below table (table 6) shows the grouping pattern of animals for the evaluation of analgesic and antinociceptive effect.

Table 6. Grouping of animals for evaluation of anti-nociceptive activity of EAFCF and NHFCA in hotplate, tail immersion and formalin tests

S.No	Group	Number of animals	Treatment
2	Group II	6	Standard (Morphine 2mg/kg i.p.)
3	Group III	6	EAFCF (25 mg/kg, p.o.)
4	Group IV	6	EAFCF (50 mg/kg, p.o.)
5	Group V	6	EAFCF (100 mg/kg, p.o.)
6	Group VI	6	NHFCA (50mg/kg, p.o.)
7	Group VII	6	NHFCA (100 mg/kg, p.o.)
8	Group VIII	6	NHFCA (200 mg/kg, p.o.)

Acetic acid induced writhing[129]

Administration of chemical irritants such as acetic acid causes pain by releasing endogenous pain mediators. The mice were treated with standard drug or fractions, 60 min 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. Complete 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[45].

Hotplate test[131]

Other side effects of herbal supplements have come from anecdotal reports. The sensitizing capacity of many herbal remedies resulted in allergic contact dermatitis, including

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 animals were treated with morphine or selected fractions and were placed on Eddy's hotplate maintained at a temperature of 55± 1°C. The reaction times were noted again at 30, 60, 90, and 120 min interval. A cutoff period of 20 s was set to avoid tissue damage in foot.

Tail immersion test[130]

Mice were treated with standard or selected fractions 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 min after the treatment. To prevent the excessive tail tissue damage, cut off latency period of 20 s was maintained.

Formalin induced nociception[133]

Rats were treated with standard or fractions, 60 min 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 min after the administration of formalin comprises the first phase and the second phase comprises the 15 to 30 min after the formalin administration.

Assessment of motor toxicity and sedative property of EAFCF and NHFCA

Table 7. Grouping of animals for penobarbitone induced sleep time and rota rod behavior test of EAFCF and NHFCA

S.No	Group	Number of animals	Treatment
1	Group I	6	Control (Vehicle 10 ml/kg)
2	Group II	6	Diazepam (2mg/kg, i.p.)
4	Group IV	6	EAFCF (100 mg/kg, p.o.)
5	Group V	6	EAFCF (200 mg/kg, p.o.)
7	Group VII	6	NHFCA (200 mg/kg, p.o.)
8	Group VIII	6	NHFCA (400 mg/kg, p.o.)

Phenobarbitone sodium induced sleeping time[177]

Different groups of mice received phenobarbitone sodium (45 mg/kg i.p.) thirty min prior to the administration of extract fractions, 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.

Rotarod test[178]

Mice were preselected based on their ability to withstand on a horizontal bar (2.5cm diameter) revolving at a speed of 15 rpm for 120 s. 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.

Neurochemical estimation[179]

Animals were sacrificed at the end of chronic treatment with EAFCF and stress by decapitation and the brains were rapidly dissected out in ice-cold conditions and frozen in ice-cold 0.9% (w/v) saline at−20◦C until taken for neurochemical analysis. The tissue was homogenized with 2 volumes of 0.l M HCl and 0.15M NaOH solution prepared in 60% (v/v) methanol and later the pH was neutralized with l volume of 0.l5 M NaOH solution, also prepared in 60% (v/v) methanol. The homogenate was centrifuged 13000 rpm at 4^0C for 45 min and the supernatant was collected and stored at 4^0C for further analysis. Neurochemicals such as GABA, serotonin, glutamate and adenosine were estimated using HPTLC (CAMAG — version 1.4.4, USA).

Interaction studies

Sub-effective and effective doses of ethyl acetate fraction of *Cassia fistula* L. was selected from the previous experiments. To evaluate the synergistic interaction with adenosine agonists sub-effective was chosen and for antagonistic interaction with adenosine antagonists the effective dose was chosen. After the treatment procedure the animals were exposed to forced swim test, elevated plus maze, s.c. PTZ test and formalin test. Separate sets of animals were used for each experiment.

Table 8. Grouping of animals for interaction studies

S.No	Group	Number of animals	Treatment
1	Group I	6	Vehicle + EAFCF 25(Vehicle 10 ml/k)
2	Group II	6	Adenosine(mg /kg, i.p.)+ EAFCF (25 mg/kg, i.p.)
3	Group III	6	CSC mg/kg, i.p.+EAFCF (25 mg/kg, p.o.)
4	Group IV	6	Dipyridamole mg/kg + EAFCF (25 mg/kg, p.o.)
5	Group V	6	Vehicle (10 ml/kg, i.p.) + EAFCF (100 mg/kg, p.o.)
6	Group VI	6	Caffeine (mg/kg, i.p.) + EAFCF (100 mg/kg, p.o.)

Statistics

The data obtained were evaluated by using ANOVA followed by Dunnet's post hoc. The seizure development in PTZ kindling and the drug effects were analysed by repeated measures. The seizure score were analysed by the Kruskal-Wallis H test followed by the Mann-Whitney U test.

RESULTS AND ANALYSIS

Pharmacognostic studies

Ash value

Total ash value, water soluble ash and acid soluble ash value were determined according the standard procedure and results are given in table 9.

Table 9: Ash values of *Cassia fistula L.* and *Cassia auriculata* L. pods in %w/w

S.No	Name of the plant and part used	Type of ash	% Ash in w/w
1.	*Cassia fistula* L. Pods	Total ash	9.25
2.		Acid soluble ash	4.21
3.		Water soluble ash	2.25
4.	*Cassia auriculata* L. Pods	Total ash	10.1
5.		Acid soluble ash	5.21
6.		Water soluble ash	3.21

Extractive value

Extractive values of *Cassia fistula* L. and *Cassia auriculata* L. are given in table 10.

Table 10: Extractive value of *Cassia fistula* L. and *Cassia auriculata* L pods in % w/w

S.No	Name of the plant and part used	Type of extract	Extractive value w/w
1.	*Cassia fistula* L. Pods	Alcohol soluble extract	10.25
2.		Water soluble extract	25.36
3.		Hydro alcohol soluble extract	35.23
4.	*Cassia auriculata* L. Pods	Alcohol soluble extract	14.32
5.		Water soluble extract	16.32
6.		Hydro alcohol soluble extract	30.25

Heavy metal analysis

Table 11 shows that among the five heavy metals, iron (Fe) has been found to contain in large quantities and all other elements are within the permissible range fractions.

Table 11. Results of the metal contents from EAFCF and NHFCA (ppm)

S.No	Fractions	Cd (ppm)	Cu (ppm)	Pb (ppm)	Zn (ppm)	Fe (ppm)
1	EAFCF	0.0016	0.0012	0.2599	0.0087	0.559
2	NHFCA	0.0013	0.1904	0.5657	0.0162	0.4019

EAFCF- Ethyl acetate fraction of *Cassia fistula* L. pods, NHFCA, n-hexane fraction of *Cassia auriculata* L. pods, ppm- parts per million.

Preliminary phytochemical analysis

The results of preliminary phytochemical analysis are given in table 12. It shows hydroalcoholic extract of *Cassia fistula* L. pods (HAECF) contains alkaloids, flavonoids, anthraquinones, tannins, glycosides, reducing sugars, steroids and terpenoids. The ethyl acetate fraction of *Cassia fistula* L. pods (EAFCF) showed the presence of alkaloids, flavonoids, anthroquinones. Hydroalcohoic extract of *Cassia auriculata* L. pods (HAECA) showed the presence of alkaloids, saponins, tannins, flavonoids, anthraquinones, steroids and terpenoids. n-Hexane fraction of *Cassia auriculata* L. pods showed the presence of alkaloids, saponins, tannins, favonoids, steroids.

Table 12. Preliminary phytochemical analysis of hydroalcoholic extract and fractions of *Cassia fistula* L and *Cassia auriculata* L

S.No	Tests	HAECF	EAFCF	HAECA	NHFCA
1	Alkaloid	+	+	+	+
2	Saponins	-	-	+	+
3	Flavonoids	+	+	+	+
4	Anthraquinones	+	+	+	+
5	Tannins	+	-	+	+
6	Glycosides	+	-	-	-
7	Steroids	-	-	+	+
8	Reducing sugars	+	-	-	-
9	Terpenoids	+	+	+	+

+ positive, - negative HAECF- Hydroalcoholic extract of *Cassia fistula* L

EAFCF- Ethylacetate fraction of *Cassia fistula* L

HAECA- Hydroalcoholic extract of *Cassia auriculata* L

NHFCA- n-Hexane fraction of *Cassia auriculata* L

Polyphenolic content estimation

EAFCF and NHFCA were found to contain more polyphenols than other fractions. The amount of phenolic constituent present in EAFCA was found to61.5 µg/mg in terms of gallic acid equivalent and in NHFCA it was found to53.5 µg/mg.

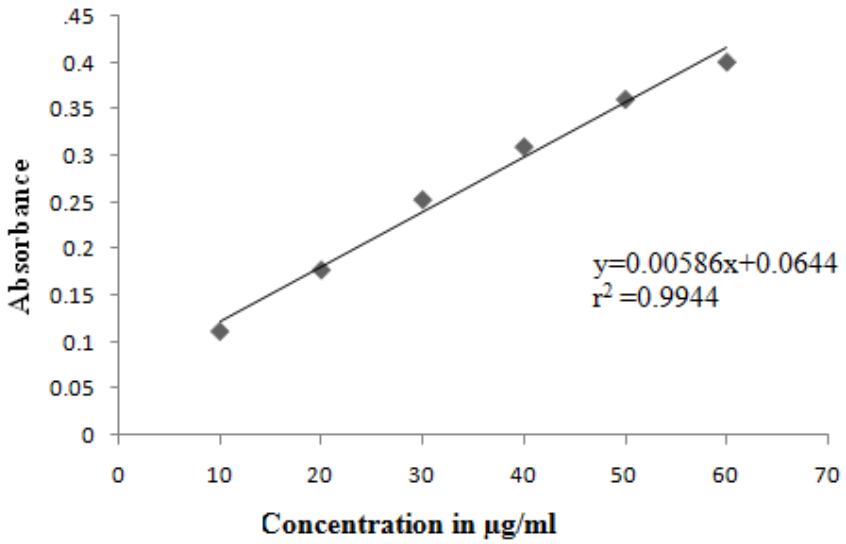


Fig 3. Standard curve of gallic acid

Flavonoid content estimation

The total flavonoids content of EAFCF and NHFCA was estimated and the total flavonoids present in the EAFCF was found to be 30.5 and in NHFCA it was found to be 25.5 mg/mg in terms of quercetin equivalent. .

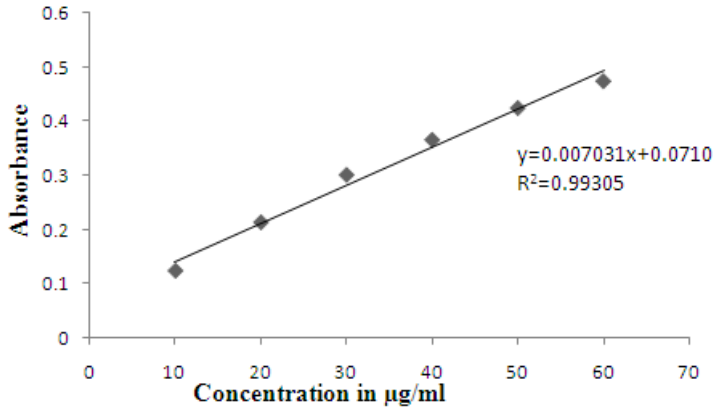


Fig 4. Standard curve of quercetin

Standardization with HPTLC

Standardization of two selected fractions that is EAFCF and NHFCA was done using HPTLC. During standardization the bands were visualized at 254 nm and 366 nm. The solvents used are toluene: ethyl acetate: formic acid (7:2:1).

HPTLC studies of EAFCF and NHFCA

Fig 5 & 6 shows different bands observed in 254 nm and 366 nm for EAFCF and NHFCA. The bands obtained in fig 5 shows the various components present in EAFCF and NHFCA fractions. The brown coloured bands obtained in chromatogram 6, shows the presence of flavonoids and phenolics in EAFCF and NHFCA fraction respectively. The presence of flavonoids was confirmed by derivatization with vanillin sulphuric acid and the presence of phenolics with Folin-ciocalteu reagent.

Table 13 represents the various Rf values obtained for seven peaks. The Rf value also shows various constituents that is present in ethyl acetate fraction. Table 13 represents the Rf values obtained for 7 peaks in NHFCA fraction. EAFCF and NHFCA had common peaks and Rf values of 0.48, 0.69, 0.46. The standards used were quercetin, rutin, caffeic acid and catechin.

HPTLC fingerprinting of EAFCF and NHFCA

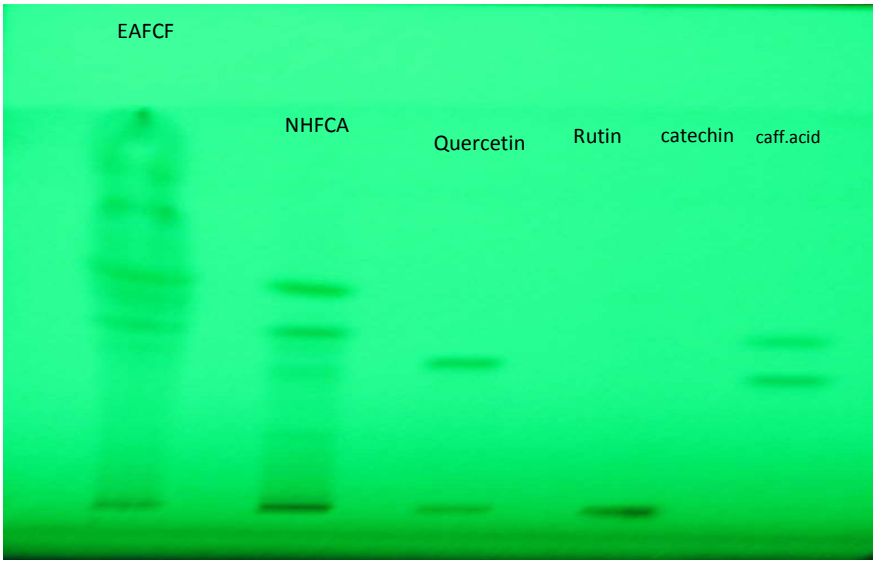


Fig 5. HPTLC profile of EAFCF and NHFCA fraction at 254 nm

Rutin

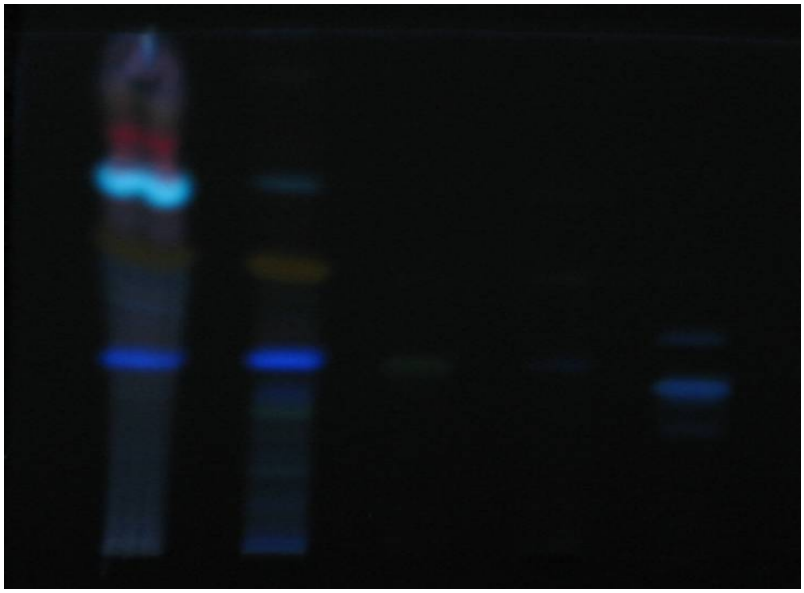


Fig 6. HPTLC profile of EAFCF and NHFCA at 366nm

Rf values of peaks from EAFCF and NHFCA

The below table represents the Rf values of EAFCF (table 13)

Table 13. HPTLC Profile of the EAFCF

Peaks	1	2	3	4	5	6	7
Rf value	0.35	0.39	**0.48**	0.59	0.63	**0.69**	0.70

Profile of NHFCA

The below table represents the Rf values of NHFCA (table 14)

Table 14. HPTLC Profile of the NHFCA

Peaks	1	2	3	4	5	6	7
Rf value	0.35	0.40	**0.46**	0.50	0.57	0.66	0.73

Peak densitiogram

The below diagram showing the peak of various components present in EAFCF (fig 7)

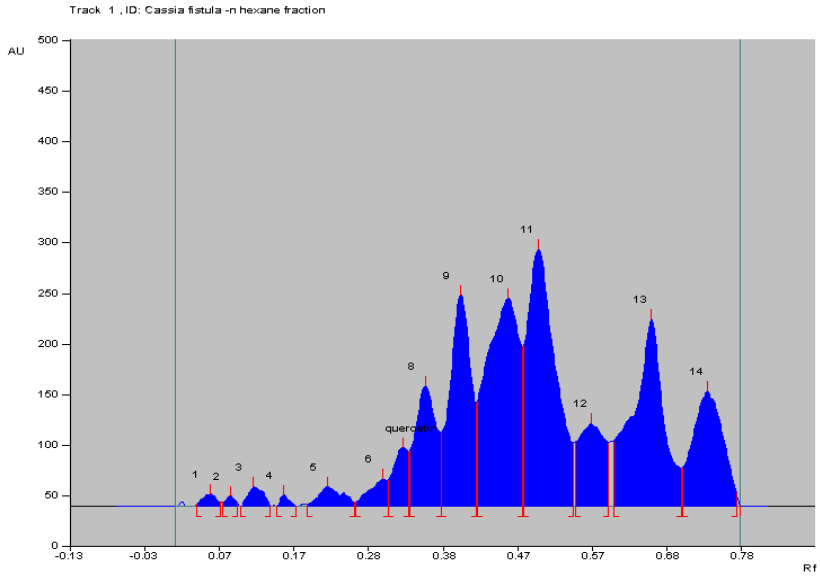


Fig7. HPTLC chromatogram of EAFCF– Peak densitogram display (Scanned at 500 nm)

The below diagram showing the peak of various components present in NHFCA

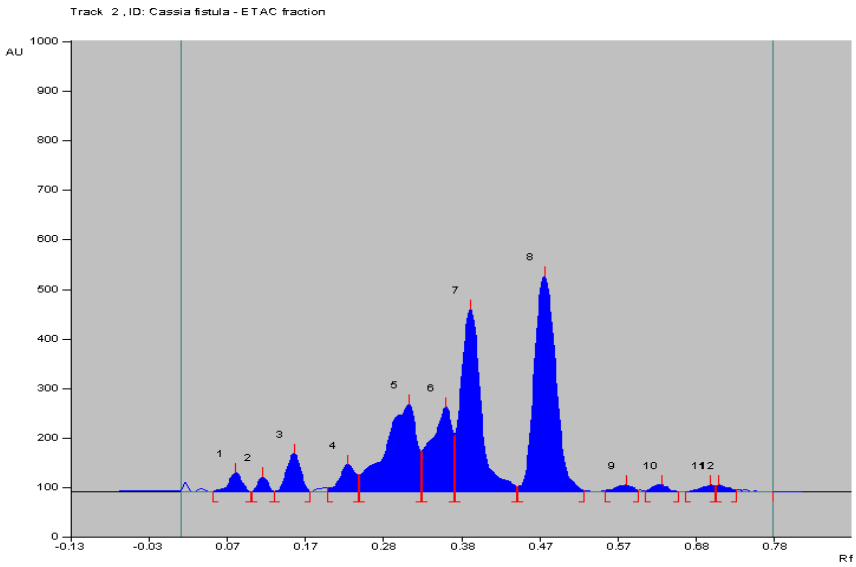


Fig 8. HPTLC chromatogram of NHFCA – Peak densitogram display (Scanned at 500 nm)

Column chromatography

In column chromatography 6 fractions were obtained with 90% and 80% of chloroform in methanol mixture showed positive test with folin-Ciocalte reagent were further subjected to analytical studies by means of HPTLC.

HPTLC techniques, the flavonoids from EAFCF was determined by using solvent system Chloroform: methanol (11.8:0.2) as mobile phase. The fraction obtained by 80% and 70 % of chloroform mixture showed a single band of flavonoids, which gave peak at Rf 0.69 for flavonoid.

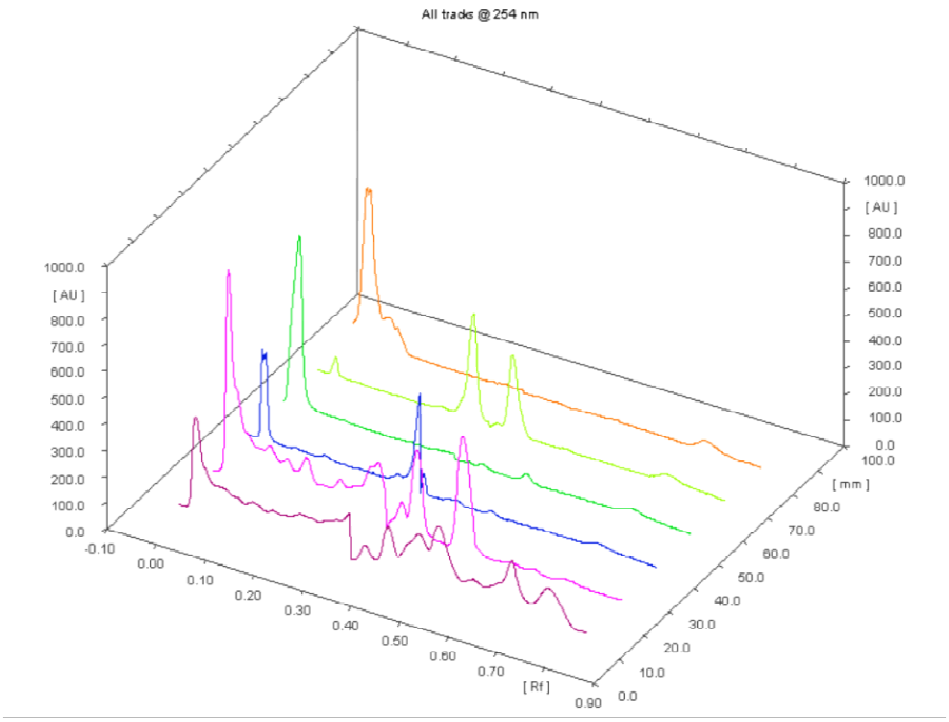


Fig 9. 3D display of HPTLC chromatogram of EAFCF at 254 nm

HPLC separation of single peak from EAFCF

The single peak obtained fractions in HPTLC (F2, F3 and F4) were separated by using HPLC, a single band compound obtained at 6 min, the peek was collected at the column out let, acetonitrate was evaporated in room temperature and the compound was precipitated by freeze drying technique.

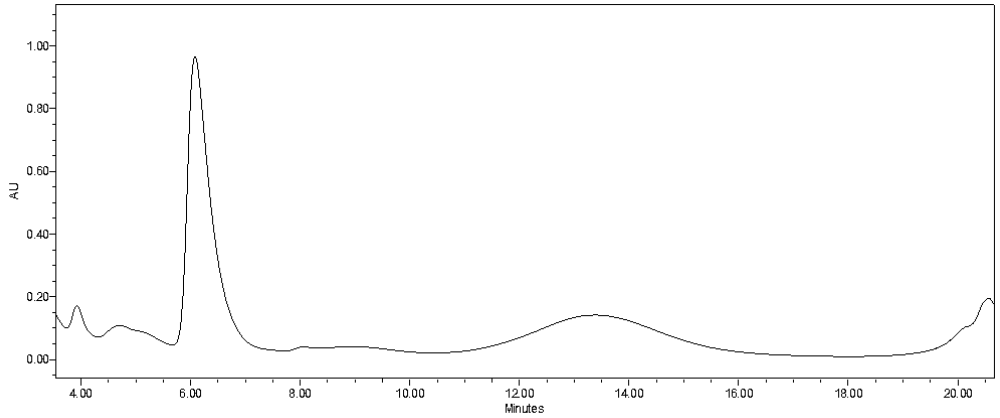
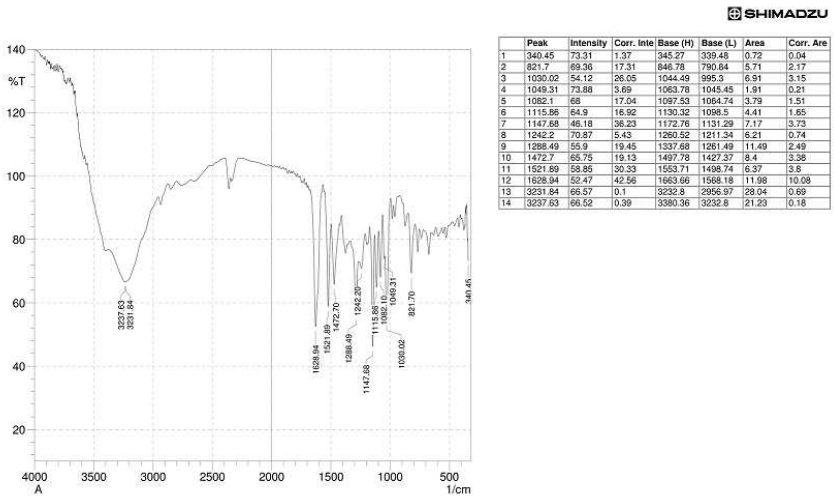


Fig 10 : HPLC profile of compound isolated from EAFCF

FTIR analysis of the isolated compound

A half white crystalline compound was obtained after freeze drying was subjected to FTIR studies to find out the functional groups present in the compound. IR spectra shows following vibrations in the range between 1256 to 800cm^{-1} disubstituted benzene ring, at 1472 cm^{-1}-1288$cm^{-1}$and 1115cm^{-1}-1147 cm^{-1} and 1242 cm^{-1}- 1288cm^{-1}conforms the presence of phenolic (C-OH) hydroxyl group. This conforms the presence various functional groups present in the isolated compound.



	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	340.45	73.31	1.37	345.27	339.48	0.72	0.04
2	821.7	69.36	17.31	846.78	790.84	5.71	2.17
3	1030.02	54.12	26.05	1044.49	995.3	6.91	3.15
4	1049.31	73.88	5.69	1063.78	1045.45	1.91	0.21
5	1082.1	68	17.04	1097.53	1064.74	3.79	1.51
6	1115.86	64.9	16.92	1130.32	1098.5	4.41	1.65
7	1147.88	46.18	36.23	1172.76	1131.29	7.17	3.73
8	1242.2	70.87	5.43	1260.52	1211.34	6.21	0.74
9	1288.49	55.9	19.45	1337.68	1261.49	11.49	2.49
10	1472.7	65.75	19.13	1497.78	1427.37	8.4	3.38
11	1521.89	58.85	30.33	1553.71	1498.74	6.37	3.8
12	1628.94	52.47	42.56	1663.66	1568.18	11.98	10.08
13	3231.84	66.57	0.1	3232.8	2956.97	28.04	0.69
14	3237.63	66.52	0.39	3380.36	3232.8	21.23	0.18

Fig 11 : FTIR spectrum of isolated compound EAFCF

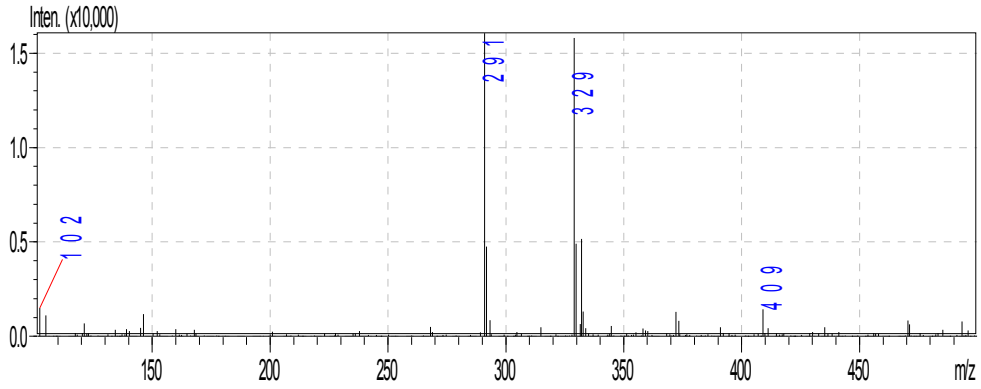


Fig 12 : GCMS analysis of the isolated compound from EAFCF

^{H}NMR spectrum analysis of the iolated compound from EAFCF

The NMR analysis of the isolated compound is shown in fig 13.The H NMR was based on the proton correlation. H coupling data and selective decoupling of H6 and H2',6'.there is an nuclear overhauser from H2',6' to H3 and H8 but not in H6 provied good evidance for 1H assignment the datas were at tabel 15. The NMR data firmly establishe the structure of the isolated compound fig 13.

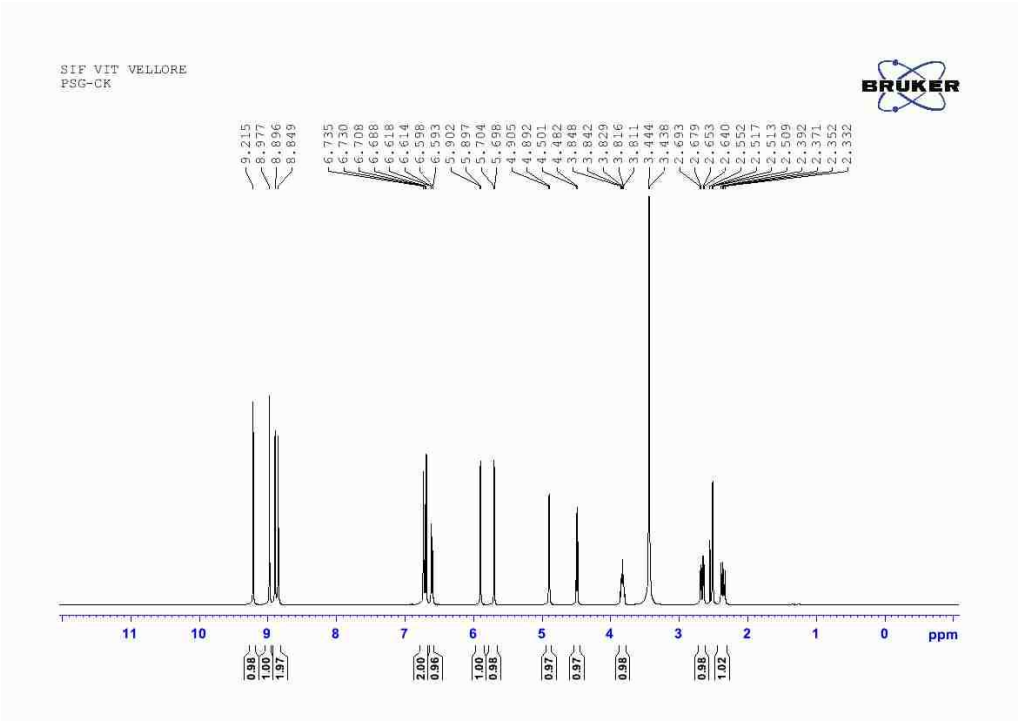


Fig 13: ^{H}NMR Spectrum of isolated compound from EAFCF

Tabel 15: ^{H} NMR data of the isolated compound from EAFCF

S. No	Atom	Exp. Shift (ppm)	Multiplet
1	4	2.50	M09
2	4	2.84	M08
3	3	3.97	M07
4	2	4.56	M06
5	9	5.85	M05
6	7	5.92	M04
7	16	6.71	M03
8	15	6.76	M02
9	12	6.83	M01

^{13}C NMR spectrum of the isolated compound from EAFCF.

From the ^{13}C NMR study of our isolated compound, we found peaks at 60-100 for (CH). the peak obtaied at 100-160 was for aromatic carbon atom and the peak at 185 is for C=O.the 13 C NMR study was based on the carbon CH coretation .

Structure of isolated compound from EAFCF

Based on the strucutrul analysis through FTIR, GC-MS and NMR, the following structrure has been derived for the isolated compound and the chemical name of the compound was identified as 2-(3,4-dihydroxyphenyl)chroman-3,4,7-triol.

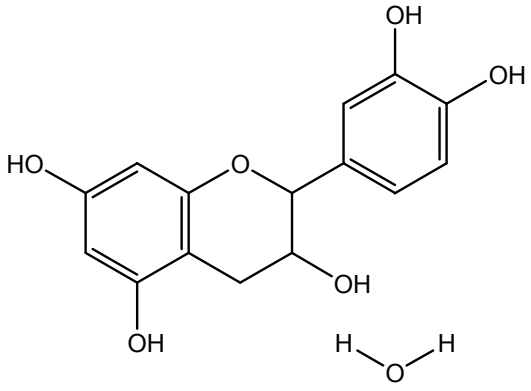


Fig 14. Chemical structure of the isolated compound from EAFCF.

PHARMACOLOGICAL STUDIES

***In vitro* studies**

Superoxide scavenging activity

The super oxide scavenging ability of EAFCF and NHFCA is given in the table 16. The IC_{50} value of EAFCF in scavenging superoxide was found to be 51.38µg/ml and for NHFCA it was found to be 53.23µg/ml

Table 16. Superoxide scavenging activity of EAFCF and NHFCA

	EAFCF			NHFCA		
S.No	**Conc (µg/ml)**	**% Inhibition**	**IC_{50}**	**Conc (µg/ml)**	**% Inhibition**	**IC_{50}**
1	20	17.46	51.38	20	14.25	53.23
2	40	33.46		40	28.92	
3	60	53.46		60	51.33	
4	80	69.46		80	62.53	
5	100	89.19		100	81.46	

Data expressed as average of three observations EAFCA= Ethayl acetate fraction of *Cassia fistula* L., NHFCA= n-hexane fraction of *Cassia auriculata* L. pods, NHFCA, n-hexane fraction of *Cassia auriculata* L. pods

Reducing power ability

The ability with which a natural anti-oxidant to donate electron is designated as reducing power activity. The reducing power ability of fractions was well correlated with their polyphenolic and flavonoid content. The results were shown in table 17.

Table 17. Reductive ability of EAFCF and NHFCA

S. No	Conc (µg/ml)	EAFCF	NHFCA	BHT
1	20	0.183±0.0004	0.178±0.0102	0.193±0.0050
2	40	0.221±0.0030	0.189±0.0180	0.247±0.0071
3	60	0.254±0.0013	0.239±0.0012	0.260±0.00038
4	80	0.278±0.0014	0.260±0.0029	0.386±0.0006
5	100	0.348±0.0017	0.329±0.0017	0.569±0.00189

Data expressed as mean ± SEM of three observations. EAFCA= Ethayl acetate fraction of *Cassia fistula* L., NHFCA= n-hexane fraction of *Cassia auriculata* L. pods BHT= butylated hydroxyl toluene.

In vivo pharmacological studies

Oral toxicity studies

There was no toxicity and no mortality was observed up to a dose of 2000mg/kg of EAFCF and NHFCA treated animals during the 24 h. For pharmacological evaluation, the doses were fixed based on toxicity studies. $1/10^{th}$ of the dose selected and further titrated.

Anticonvulsant activity

Anticonvulsant effect of EAFCF and NHFCA on PTZ induced seizure in mice

Treatment with EAFCF and NHFCA 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, EAFCF (100 mg/kg) protected the animals from death due to PTZ administration observed for 24h (table 18). However, NHFCA did not show 100% protection from death due to PTZ administration. Unlike diazepam, the fractions did not protect the animal from onset of PTZ induced convulsion.

Table 18. Dose dependent anticonvulsant effect of EAFCF on PTZ induced seizure in mice.

Groups	Dose	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	50 mg/kg, p.o.	3.59±0.416	5.52±0.517	33.3
EAFCF	100 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
NHFCA	100 mg/kg, p.o.	03.48±0.654	05.01±1.23	0
NHFCA	200 mg/kg, p.o.	7.58±0.342	15.32±1.36	33.3
NHFCA	400mg/kg, p.o.	15.25±0.52	39.86±0.98	66.5
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

Repeated treatment of EAFCF did not alter the anticonvulsant effect in PTZ kindling model

The course of seizure severity was parallel in control animals (day X group dF= 13, F= 0.53, p=0.9). However, the seizure scores was reduced in EAFCF treated animals compared to control animals (F (1, 14) = 4.20, p=0.05. The obtained results are represented in fig 15. This clearly indicate repeated treatment of EAFCF did not alter the anticonvulsive effectiveness shown in s.c.PTZ treatment.

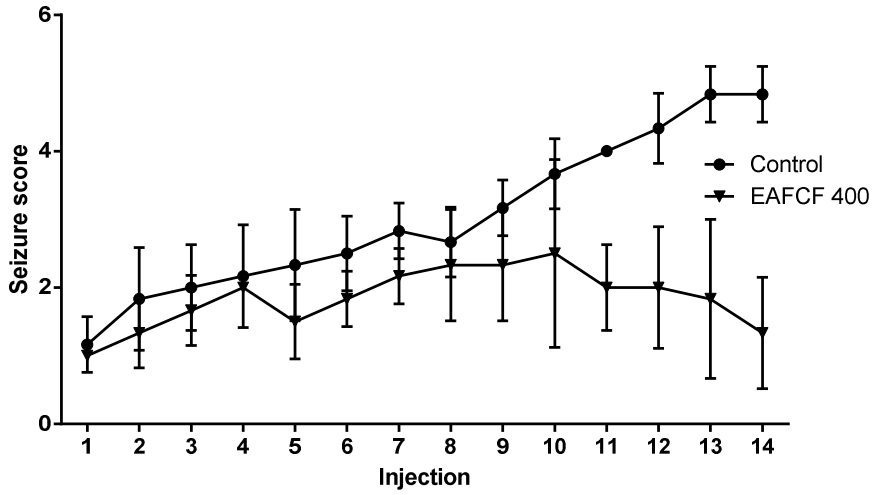


Fig 15. Sub chronic treatment of EAFCF suppresses the seizure generation in PTZ kindling model

PTZ 35 mg/kg, i.p. was given 60 min after the administration of EAFCF. Data expressed as mean±SEM of seizure score. Control animals received saline followed by PTZ.

Anxiolytic effect of EAFCF and NHFCA in elevated plus maze using mice

Acute administration of EAFCF and NHFCA 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 19). EAFCF 25mg/kg and NHFCA 50 mg/kg did not significantly changes the parameters observed in EPM and were sub effective.

Table 19. Effect of EAFCF and NHFCA on behavioral parameters in EPM.

Groups	Dose	% 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
NHFCA	50 mg/kg, p.o.	39.25±2.69	38.23±3.36	14.28±1.22
NHFCA	100 mg/kg, p.o.	49.25±2.56$^{\$}$	51.23±3.14$^{\$}$	13.69±2.33
NHFCA	200 mg/kg, p.o.	60.21±1.23*	58.23±2.12*	7.21±1.25
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 $^{\$}p<0.05$, *$p<0.01$ compared to vehicle treated group One way ANOVA followed by post hoc Dunnett's test were performed.

EAFCF and NHFCA 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) and NHFCA (100 and 200 mg/kg). No significant ($p>0.05$) changes were noted in the total number of ambulation and the number of rearings (table 20).

Table 20. Effect of EAFCF and NHFCA on open field behavior in mice.

Groups	Dose	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*
NHFCA	50 mg/kg, p.o.	15.2±1.2	40.2±1.2	2.9±0.9	3.8±0.6
NHFCA	100 mg/kg, p.o.	14.5±0.9	38.2±2.3	6.2±0.9$^{\$}$	7.5±0.3$^{\$}$
NHFCA	200 mg/kg, p.o.	08.2±0.3	28.2±3.1	9.9±1.1*	11.2±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 $^{\$}p<0.05$, *$p<0.01$ Vs vehicle, One way ANOVA followed by post hoc Dunnett's test

EAFCF and NHFCA reduced the marble burying behavior of rats

Acute administration of EAFCF (50 and 100 mg/g) and NHECA (100 and 200 mg/kg) dose dependently reduced the number of marbles buried by the rats. However, EAFCF 25 mg/kg and NHFCA 50 mg/kg found to be sub-effective (Fig 16)

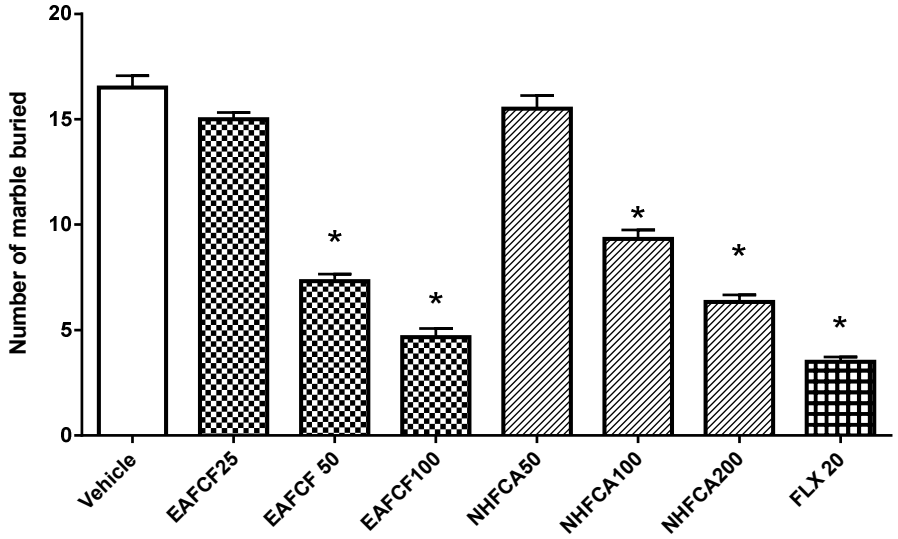


Fig 16. Effect of EAFCF and NHFCA on marble burying behavior in rats

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.

Antidepressant activity

Acute administration of EAFCF and NHFCA reduced the duration of immobility in FST.

One way ANOVA showed significant changes ($p<0.01$) in the duration of immobility upon the acute oral administration of EAFCF and NHFCA in mice. Post hoc analysis revealed treatment of EAFCF (50 and 100 mg/kg) and NHFCA (100 and 200 mg/kg) significantly reduced the duration of immobility in FST, whereas, EAFCF 20 mg/kg and NHFCA 50 mg/kg were found to be sub-effective (fig. 17)

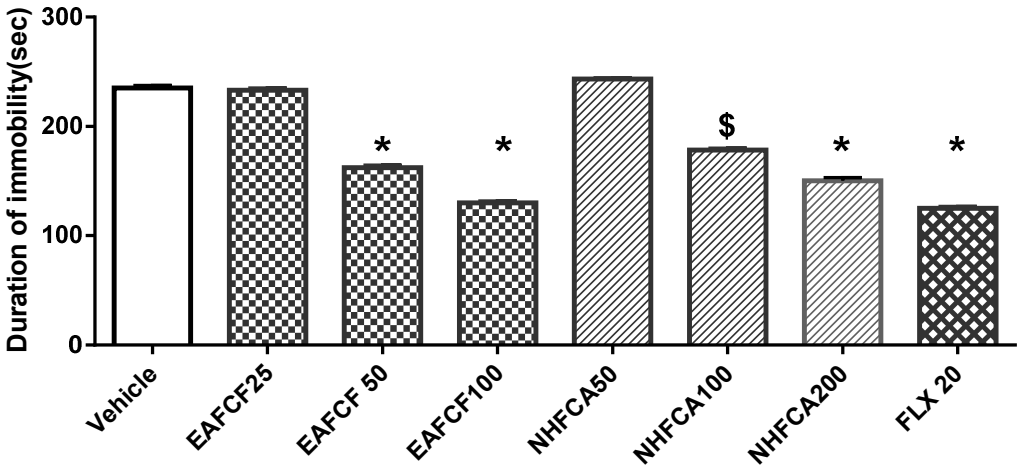


Fig 17. EAFCF and NHFCA reduced the duration of immobility in FST

Data expressed as mean ± SEM n=6 $^{\$}p<0.05$, $*p<0.01$ compared to vehicle treated group One way ANOVA followed by post hoc Dunnett's test were performed.

Anti-immobility effect of EAFCF and NHFCA in TST

One way ANOVA showed significant changes ($p<0.001$) in the duration of immobility in TST upon the drugs treatment. Post hoc test revealed EAFCF (50 & 100 mg/kg) and NHFCA (100 & 200 mg/kg) significantly ($p<0.01$) reduced the immobility period. EAFCF 25 mg/kg and NHFCA 50 mg/kg did not significantly ($p>0.05$) altered the duration of immobility in TST. Results are given in fig 18.

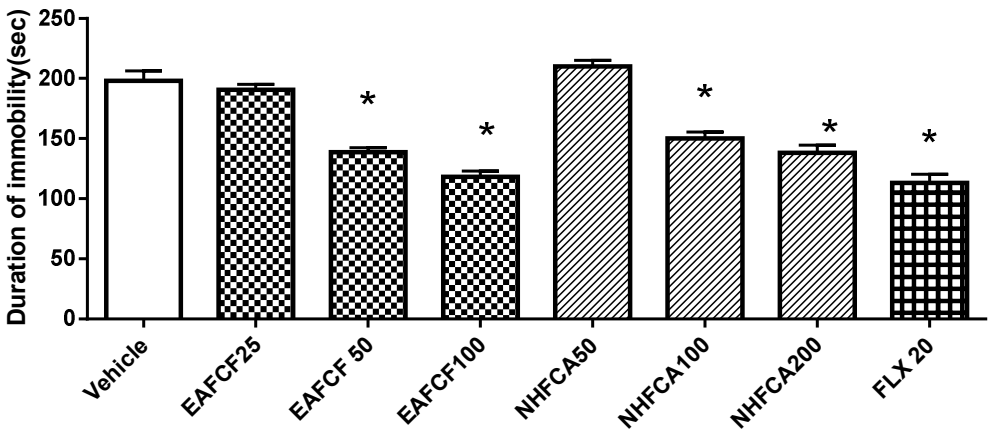


Fig 18. Hyper mobility effect of EAFCF and NHFCA in TST

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.

Anti depressant activity of EAFCF and NHFCA in CUS model.

Repeated treatment of EAFCF reversed the CUS induced decrease in sucrose consumption.

Sucrose consumption was measured at 0 week, 2 weeks and at the end 5 weeks. At 0 week there was no change (p>0.05) in sucrose consumption among the groups. After the 2 weeks of CUS the model group showed reduced sucrose consumption (p<0.05) compared to the normal control group and at the end of 5 weeks the model group showed still reduced (p<0.01) sucrose consumption compared to control group. All treated group except EAFCF 25 mg/kg reversed the reduction in sucrose consumption due to CUS (fig. 19).

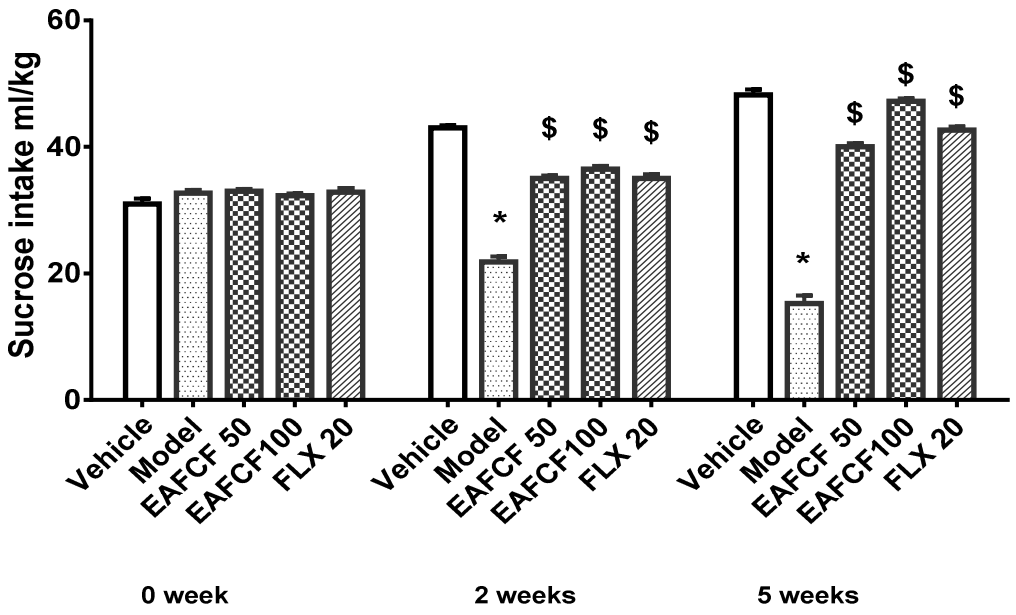


Fig 19. Effect of sub chronic treatment of EAFCF on CUS induced changes in sucrose intake

Data expressed as mean ± SEM n=6 *$p<0.01$ compared to normal control group $^{\$}p<0.01$ comared to model group One way ANOVA followed by post hoc Dunnett's test were performed.

Effect of EAFCF on CUS induced changes duration of immobility in FST

In the model group the duration of immobility was significantly ($p<0.01$) increased at the end of 5 weeks stress procedure when compared to non stressed animals. A significant decrease in duration of immobility ($p<0.01$) compared to the model group was observed in EAFCF and fluoxetine treated groups (fig 20).

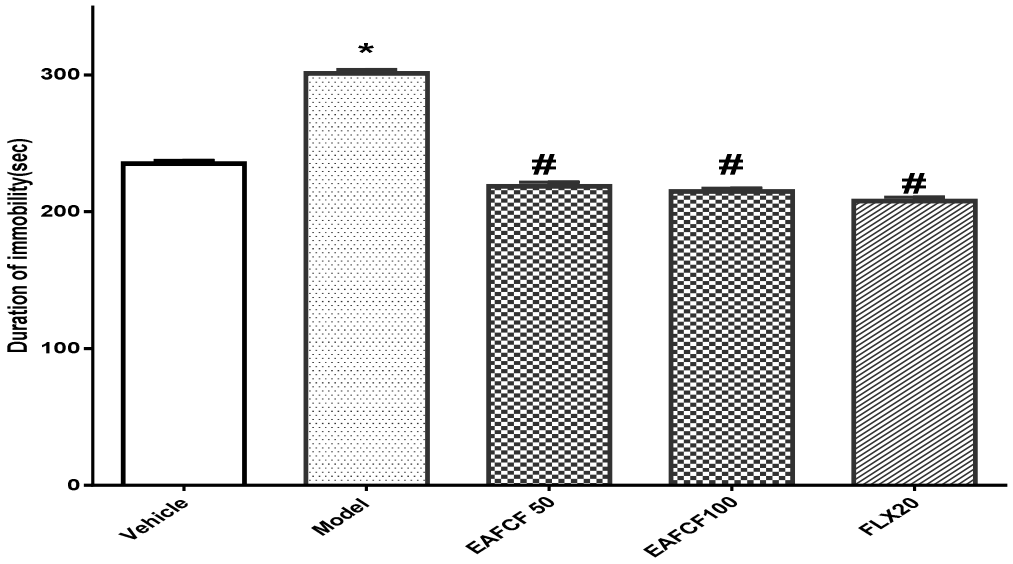


Fig 20. Sub-chronic treatment of EAFCF reverses the CUS induced hyper-immobility

Data expressed as mean ± SEM n=6 *p<0.01 compared to normal control and #p<0.01 compared to model group One way ANOVA followed by post hoc Dunnett's test were performed.

Treatment of EAFCF reversed the CUS induced changes in erythrocyte SOD level

The erythrocyte SOD activity was measured at the end of the experiment. A significant decrease (p<0.01) in the erythrocyte SOD activity was observed after the exposure of various stressors for 5 weeks when compared to the normal control group. Treatment of EAFCF dose dependently increased the SOD activity and the changes were statistically significant (p<0.01) when compared to the model group (fig 21).

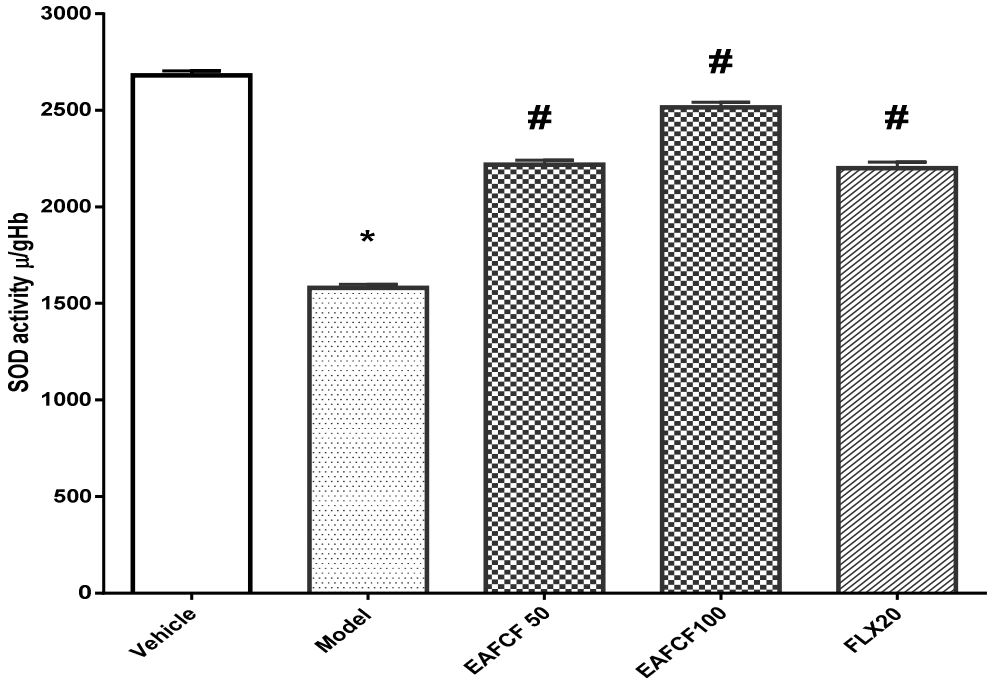


Fig 21. Changes erythrocyte SOD activity by sub-chronic EAFCF treatment

Data expressed as mean ± SEM n=6 *p<0.01 compared to normal control and #p<0.01 compared to model group One way ANOVA followed by post hoc Dunnett's test were performed.

Acetic acid-induced writhing model

Administration of EAFCF (50, 100, and 200 mg/kg, p.o.) and NHFCA (100, 200 and 400 mg/kg, p.o.) reduced the acetic acid induced writhing significantly (p<0.001) compared to control group in dose dependent manner (table 21) 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.

Table 21. Analgesic activity of EAFCF in acetic acid induced writhing in mice

Groups	Dose	Number of Writhing Mean± SEM
Vehicle	10 ml/kg, i.p.	66±1.390
Diclofenac	10 mg/kg, i.p.	5.83±0.477
EAFCF	25 mg/kg, p.o.	39.33±1.520
EAFCF	50 mg/kg, p.o	19.17±0.946*
EAFCF	100 mg/kg, p.o.	05.33±0.613*
NHFCA	100 mg/kg, p.o.	58.13±1.22
NHFCA	200 mg/kg, p.o.	29.22±2.13
NHFCA	400mg/kg, p.o.	07.23±0.9

Values are expressed in terms of 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.=intra peritoneal, NHFCA, n-hexane fraction of *Cassia auriculata* L. pods p.o.= per oral

Eddy's hot plate model in mice

In this model, the reaction latency to thermal stimuli was increased significantly (P<0.01) in EAFCF and NHFCA 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 22).

Table 22. Anti-nociceptive effect of EAFCF and NHFCA in Eddy's hot plate model using mice

Group	Dose	Reaction time in sec				
		Before	30 min	60 min	120 min	180 min
Vehicle	10 ml/kg, i.p.	3.8±0.05	3.6±0.08	4.2±0.03	4.6±0.09	4.8±0.08
Morphine	1.5 mg/kg, i.p.	4.1±0.06	12.6±0.03*	17.8±0.08*	16.2±0.1*	14.8±0.2*
EAFCF	25 mg/kg, p.o.	5.1±0.09	5.1±0.08*	7.4±0.08*	6.7±0.08*	5.2±0.08*
EAFCF	50 mg/kg, p.o	5.2±0.10	10.8±0.05*	13.1±0.1*	12.5±0.3*	10.6±0.09*
EAFCF	100mg/kg, p.o.	4.8±0.12	13.2±0.4*	18.4±0.2*	17.1±0.1*	15.1±0.07*
NHFCA	100mg/kg, p.o.	5.3±0.21	6.2±0.22	6.8±0.12	5.9±0.21	5.3±0.1
NHFCA	200mg/kg, p.o.	3.2±0.09	11.2±0.23*	14.3±0.31*	14.36±0.3*	10.6±0.2*
NHFCA	400mg/kg, p.o.	4.2±1.01	14.23±0.1*	19.5±0.28*	19.8±0.21*	13.6±0.32*

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., NHFCA, n-hexane fraction of *Cassia auriculata* L. pods, i.p.=intra peritoneal, p.o.= per oral

Tail immersion test

The antinociceptive activity exhibited by EAFCF, NHFCA and morphine in tail immersion test is given in table 23. EAFCF (100 & 200 mg/kg, p.o.) and NHFCA (200 & 400 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 effect as that of EAFCF 200mg/kg. EAFCF 50mg/kg was found to sub-effective.

Table 23. Anti-nociceptive activity of EAFCF in mouse tail immersion test

Group	Dose	Reaction time in sec				
		Before	**30 min**	**60 min**	**120 min**	**180 min**
Vehicle	10 ml/kg, i.p.	3.8±0.05	3.6±0.01	4.43±0.04	4.7±0.09	3.58±0.08
Morphine	1.5 mg/kg, i.p.	3.9±0.07	12.6±0.03*	17.8±0.08*	17.2±0.1*	10.8±0.2*
EAFCF	25 mg/kg, p.o.	4.5±0.1	5.1±0.08	6.4±0.08	5.7±0.08	5.2±0.08
EAFCF	50 mg/kg, p.o	4.9±0.20	9.8±0.05*	12.1±0.1*	11.5±0.3*	9.6±0.09*
EAFCF	100 mg/kg, p.o.	5.6±0.22	14.2±0.4*	16.4±0.2*	16.1±0.1*	12.1±0.07*
NHFCA	100 mg/kg, p.o.	4.2±0.32	5.3±0.31	5.0±0.41	4.8±0.21	3.9±0.12
NHFCA	200 mg/kg, p.o.	3.9±0.12	10.2±0.24*	13.6±0.2*	12.6±0.32*	7.8±0.32*
NHFCA	400mg/kg, p.o.	4.8±0.31	13.2±0.23*	16.6±0.36*	15.8±0.23*	10.2±0.4*

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.. NHFCA, n-hexane fraction of *Cassia auriculata* L. pods, i.p.=intra peritoneal, p.o.= per oral

Formalin test

Administration of EAFCF and NHFCA 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 24).

Table 24. Reduction of formalin induced paw licking by EAFCF and EAFCF and morphine treatment in rats

S.No	Treatmentb	Hind paw licking			
		Early phase	% inhibition	Late phase	% inhibition
1	Vehicle 10ml/kg, p.o.	113.6±0.91	----	123.8±0.89	----
2	Morphine 5mg/kg, i.p.	42.6±0.03*	51.60	0	100
3	EAFCF 50mg/kg, p.o.	102.1±1.8	10.12	98.8±2.2	13.02
4	EAFCF 100mg/kg, p.o	59.8±1.25*	47.35	61.5±2.23*	45.8
5	EAFCF 200mg/kg,p.o.	40.6±0.4*	64.2	22.1±3.1*	80.54
6	NHFCA 100 mg/kg, p.o	110.6±2.3	08.2	103.2±2.65	15.3
7	NHFCA 200 mg/kg, p.o	62.23±3.3	53.23	71.5±3.2	46.3
8	NHFCA 400 mg/kg, p.o	41.2±3.2	64.1	25.3±2.1	78.6

Values are given as mean ± SEM and as %, n = 6 in each group, *P <0.01 statistically significant as compared with control group. EAFCF = Ethylacetate fraction of *Cassia fistula* L.. NHFCA, n-hexane fraction of *Cassia auriculata* L. pods, i.p.=intra peritoneal, p.o.= per oral

Effect of EAFCF and NHFCA 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) NHFCF reduced the time permanence (p<0.01) similar to that of standard drug diazepam (2 mg/kg)

Table 25. Effect of EAFCF on time permanence on rotarod.

Groups	Treatment	Time permanence on rotarod (s)
Vehicle	10 ml/kg, i.p.	116.21±0.58
EAFCF	50 mg/kg, p.o.	105.12±0.56
EAFCF	100 mg/kg, p.o	108.16±0.47
EAFCF	200 mg/kg, p.o.	114.16±0.74
NHFCA	100 mg/kg, p.o	112.23±5.62
NHFCA	200 mg/kg, p.o	98.2±5.2
NHFCA	400 mg/kg, p.o.	42.2±2.1
Diazepam	2 mg/kg, i.p.	18.55±1.21*

Each value represent mean±SEM (n=8) *$p<0.01$ compared to control. One way ANOVA and Dunnett's test as post hoc test were

Effect of EAFCF and NHFCA 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 26). However, at higher doses (>400 mg/kg, i.p.) the phenobarbitone induced sleeping time was potentiated significantly ($p<0.01$), similar to diazepam.

Table 26. 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	100 mg/kg, p.o.	7.44±1.66	85.65±3.38
EAFCF	200 mg/kg, p.o	6.92±1.59	83.10±1.96
NHFCA	200 mg/kg, p.o.	8.52±1.21	88.54±3.92
NHFCA	400 mg/kg, p.o	2.23±1.25*	180.24±5.32*
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.

Neurochemical estimation

Effect of EAFCF on GABA, glutamate, adenosine level

CUS induced changes in neurochemicals such as a significant increase glutamate, decrease in GABA, serotonin and adenosine were reversed by 5 weeks treatment of EAFCF and the results are given in table 27

Table 27. Changes in neurochemical level upon administration of EAFCF

Groups	Dose	GABA ηg/ mg	Glutamate ηg/ g	Serotonin ηg/ mg	Adenosine ηg/ g
Vehicle	10 ml/kg, i.p.	782±21.3	2850 ±55.28	750±21.2	79.44± 3.13
Model	10 ml/kg, i.p.	45±08.30	3850±32.36	24±9.20	37.33± 4.08
EAFCF	50 mg/kg, i.p.	325±14.3*	1890±56.36*	390±18.3*	82.36±4.87*
EAFCF	100mg/kg, i.p.	650±25.4*	1520 ±45.23*	685±12.3*	115.19± 2.67*

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

Interaction studies

Effect of adenosinergic agents on EAFCF induced changes in the s.c.PTZ model.

Pretreatment of sub-effective dose adenosinergic facilitators such as exogenous adenosine, adenosine A_1 receptor agonist, CHA and adenosine deaminase inhibitor dypyridamol potentiated the anti-seizure activity of EAFCF as they significantly increased the latency to the MCS and GTCS. Administration of adenosinergic blockers such as non selective adenosine receptor antagonist, caffeine and adenosine A_1 receptor selective antagonist, DPX blocked the anti-convulsant activity of EAFCF in s.c.PTZ model as they reversed the EAFCF induced increase in the latency to MCS and GTCS (table 28).

Table 28. Modulatory effect of adenosinergic agents on the anti-seizure effect of EAFCF in s.c. PTZ model.

Treatment	Latency to MCS (Min)	Latency to GTCS (Min)	% protection from mortality
Veh(10 ml/kg, p.o)+PTZ 60mg/kg, i.p	2.47±0.27	3.44±0.262	0
Adenosine50mg/kg+ EAFCF 50 mg/kg + PTZ 60 mg/kg.	8.59±0.416	35.52±0.517	66.5
CHA 1 mg/kg+ EAFCF 50 mg/kg + PTZ 60 mg/kg.	12.96±0.397*	49.86±0.847*	66.5
Dipyridamole+ EAFCF 50 mg/kg + PTZ 60 mg/kg.	15.39±0.574*	44.66±1.14*	100
EAFCF 200 mg/kg+ PTZ 60 mg/kg	15.25±0.52*	54.66±1.16*	100
Caffeine 40mg/kg+ EAFCF 200 mg/kg + PTZ 60 mg/kg.	5.01±0.63$	6.21±1.316$	0
DPX 1mg/kg+ EAFCF 200 mg/kg + PTZ 60 mg/kg.+	4.36±1.63$	5.81±0.53$	0

Data expressed as mean ± SEM n=6 *p<0.01 compared to normal control and $p<0.01 compared to EAFCF 200 mg/kg treated group One way ANOVA followed by post hoc Dunnett's test were performed.

Effect of adenosinergic agents on anxiolytic activity of EAFCF in EPM.

Pretreatment of sub- effective dose adenosinergic facilitators such as exogenous adenosine, adenosine A_1 receptor agonist, CHA and adenosine deaminase inhibitor dypyridamol potentiated the anxiolytic activity of EAFCF as evidenced from increased time spent in open arm and number of open arm entries. Administration of adenosinergic blockers such as non selective adenosine receptor antagonist, caffeine and adenosine A_1 receptor selective antagonist, DPX blocked the axiolytic activity of EAFCF in EPM (table 29).

Table 29. Modulatory effect of adenosinergic agents on the anxiolytic activity of EAFCF in EPM model

Treatment	% entries into open arm	Time spent in open arm (sec)	Total No. of arm entries
Veh(10 ml/kg, p.o)	37.5±1.5	36.8±0.87	12.17±0.6
Adenosine25mg/kg+ EAFCF 25 mg/kg	68.6±1.29	53.32±1.34*	13.67±0.66
CHA 1 mg/kg+ EAFCF 25 mg/kg	55.23±1.49*	57.2±0.63*	13.33±0.88
Dipyridamole+ EAFCF 25 mg/kg	64.78±1.79*	62.08±0.67*	15.67±0.66
EAFCF 100 mg/kg	39.25±2.69	38.23±3.36	14.28±1.22
Caffeine 2 mg/kg + EAFCF 100 mg/kg	39.25±2.56$^{\$}$	31.23±3.14$^{\$}$	13.69±2.33
DPX 1mg/kg+ EAFCF 100 mg/kg	35.21±1.23$^{\$}$	38.23±2.12$^{\$}$	12.21±1.25

Data expressed as mean ± SEM n=6 *$p<0.01$ compared to normal control and \$$p<0.01$ compared to EAFCF 200 mg/kg treated group One way ANOVA followed by post hoc Dunnett's test were performed.

Effect of adenosinergic agents on anti immobility effect of EAFCF in FST.

Pretreatment of sub- effective dose adenosinergic facilitators such as exogenous adenosine, adenosine A_1 receptor agonist, CHA and adenosine deaminase inhibitor dypyridamol potentiated the anti immobility activity of EAFCF. Administration of adenosinergic blockers such as non selective adenosine receptor antagonist, caffeine and adenosine A_1 receptor selective antagonist, DPX blocked the anti immobility activity of EAFCF in FST (fig 22)

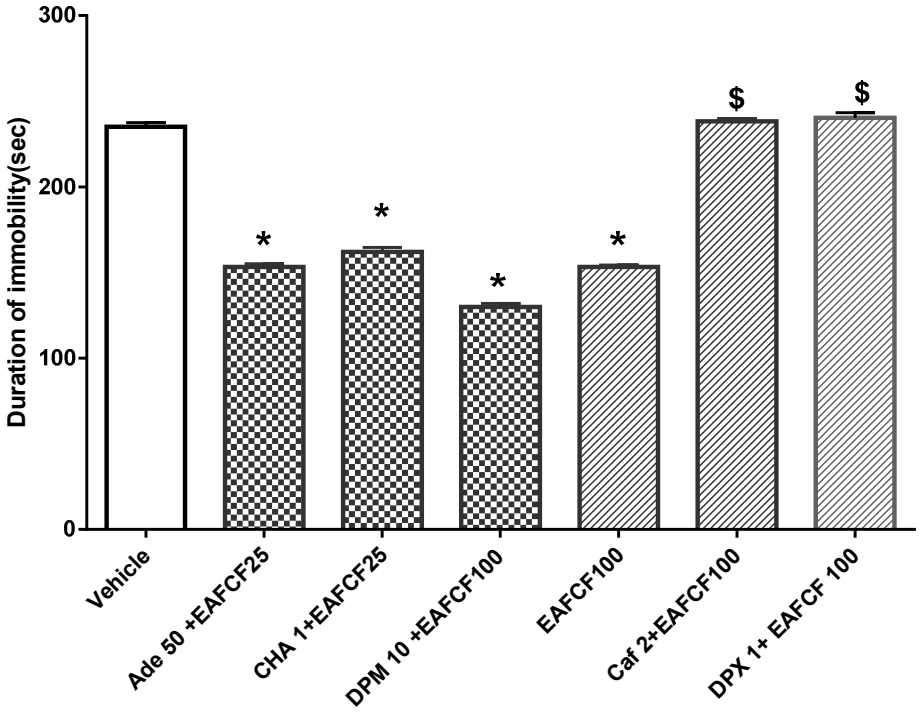


Fig 22. Pretreatment of adenosinergic agents changes the anti-immobility effect of EAFCF

Data expressed as mean ± SEM n=6 *p<0.01 compared to normal control and $p<0.01 compared to EAFCF 200 mg/kg treated group One way ANOVA followed by post hoc Dunnett's test were performed.

DISCUSSION

Epilepsy, anxiety and depression are co-morbid conditions with interlinking etiology requiring chronic drugs treatment[4]. The problems associated with available current therapy with synthetic chemicals are poor response, remission and severe undesirable side effects. Hence, the search for novel drug continues and medicinal plant source became important source for new drug development for these CNS ailments[2].

A large number of plants which were used traditionally exhibit pharmacological properties with great potential for therapeutic applications in the treatment of central nervous system disorders[2]. *Cassia fistula* L. and *Cassia auriculata* L. belonging to the family Leguminoseae have been selected based on their traditional use and their rich flavonoid content nature[95], as flavonoids were reported to have potential CNS effects[93].

The plants were collected form Coimbatore district of Tamilnadu, and authenticated by botanical survey of India, Coimbatore. The ash values such as total ash, water soluble ash and alcohol soluble ash were determined in order to find out the inorganic compound present in the powder of pods. These ash values determined may be served as standard parameters of the original plant and to compare the values for their pharmacognostical standards[172]. Extractive values such as alcohol extractive and aqueous extractive values were also determined and this gives information about the aqueous soluble fraction and the organic soluble fraction present in the root of the plant. Both the plant gave considerable amount of alcohol soluble extracts and water

soluble extracts and therefore hydroalcoholic extract of both the plants were prepared and further fractionation were carried out by using for this study.

The percentage yield obtained by various solvent fractions from hydro-alcoholic extract of *Cassis fistula* L. and *Cassia auriculata* L. pods was calculated to carry out extraction and fractionation.

The preliminary phytochemical studies revealed the presence of flavonoids, anthroquinones, glycosides and terpenoids in *Cassia fistula* L. and *Cassia auriculata* L. From preliminary evaluation we found out that the flavonoids and phenolic compounds were present in the ethyl acetate fraction of *Cassia fistula* L. (EAFCF) and n-Hexane fraction of *Cassia auriculata* L. (NHFCA). Estimation of polyphenols and flavonoids were also carried out and the results indicated that both EAFCF and NHFCA contain more polyphenols and flavonoids content among various fractions. The polyphenol content of EAFCF and NHFCA was found to be 61.5 µg/mg and 53.5 µg/mg galic acid equivalents respectively. The flavonoid content of EAFCF was 30.5 mg/mg and NHFCA contains25.5 µg/mg in terms of quercetin equivalent.

The phytoconstituents, particularly polyphenols and flavonoids have been shown to possess anti-oxidant activity[180]. Phenolics range from simple low-molecular weight compounds, such as the simple phenylpropanoids, coumarins, and benzoic acid derivatives, to more complex structures such as flavanoids, stilbenes and tannins. Of these, flavonoids represent the largest, most diverse group, encompassing some 6000 compounds, all of which share a common underlying structure of two 6-carbon rings, with a 3-carbon bridge, which usually forms a 3rd ring. Flavanoids can then be

subdivided according to modifications of this basic skeleton into chalcones, flavones, flavonols, flavanones, isoflavones, flavan-3-ols and anthocyanins (for review)[93].

Heavy metal analysis by AAS showed that the metals like cadmium, lead, copper, zinc and iron were present within the permissible limits in EAFCF and NHFCA.

HPTLC is a method commonly applied for the identification, the assay and the testing for purity, stability, dissolution or content uniformity of raw materials (herbal and animal extracts, fermentation mixtures, drugs and excipients) and formulated products. Earlier studies on *Cassia fistula* L. and *Cassia auriculata L.* reported the presence of triterpene derivatives, anthraquinone derivatives, polyphenolics, comprising flavonoids, catechins and proanthocyanidins[95]. EAFCF and NHFCA were standardised using quercetin, rutin, caffeic acid and catechin in HPTLC. Results indicate the presence of flavonoids in EAFCF and NHFCA which was confirmed by derivatization with vanillin sulphuric acid spraying reagent.

The number of peaks obtained showed the presence of various phytoconstituents in the two different plant extract fractions. The same Rf values (i.e. 0.48 and 0.69) obtained in both the fractions showed presence of similar phytoconstituents in both the plants. To rationalize the use of botanicals, the novel concept of 'marker' is gaining momentum for evaluation of medicinal plants. The characteristic finger print together with phytochemical evaluation may help to establish the identity and standardize profile of this plant for use as raw material as well as in formulation for effective herbal dosage form.

HPTLC study of the extract also showed the presence of flavonoids in the EAFCF. Hence, it was subjected to column chromatography to isolate the flavonoids present in it. Some six fractions obtained from the column chromatography showed positive test with folin catechu reagent and those fractions were further analysed by HPTLC

The fraction showed a single band at Rf 0.69 for flavonoids. The 2^{nd}, 3^{rd} and 4^{th} fractions showed single band green fluorescent peak. The fractions obtained from the column chromatography (2, 3 and 4^{th}) were taken for HPLC study and found that the fraction showed single peak in the HPLC at 6 min and it was collected from the column outlet.

The IR spectra of the isolated compound from the EAFCF shows following vibration which confirmed certain functional groups for flavonoids. IR spectra shows following vibrations in the range between 1256 to 800cm^{-1} di-substituted benzene ring, at 1472 cm^{-1}-1288$cm^{-1}$and 1115cm^{-1}-1147 cm^{-1} and 1242 cm^{-1}- 1288cm^{-1}conforms the presence of phenolic (C-OH) hydroxyl group. This conforms the presence various functional groups present in the isolated compound.

Gas chromatography /Mass spectral (GCMS) selected ion monitoring scan spectra showed ion with m/z 290, 245 and 153. In comparison with the NIST library which corresponded with a molecular ion composition of $C_{15}H_{12}O_5$ to ions with m/z at 290.0 for the assigned structure of the isolated compound with IUPAC name 2-(3,4-dihydroxyphenyl) chroman-3,5,7-triol.

NMR studies have been done in DMSO-d_6 solvent which gives an optimum viscosity and high resolution. The peaks obtained by the H NMR studies integrate 9

protons. The doublet peaks at 5.92, 6.71, 6.76 and 6.83 conform the following positions for aromatic protons (7', 16', 15', 12') the peak at 6.92(S) confirm the 9th position -H in the structure and the 4.56 and 3.97 confirm the 2nd and 3rd protons in the structure. The peaks at 2.84 and 2.54 confirm the 4th position. The spectral analysis data confirmed the structure of the compound.

Free radicals are frequently generated in our body during normal cellular metabolism as well as under certain environmental conditions, these radicals are more reactive as they lack of an electron and try to become neutral by accepting an electron from or donate an electron to adjacent molecules and create new free radicals. This in turn initiates a chain of reaction that can damage several molecules through accepting or donating an electron leads to degenerative diseases[181]. The phytochemicals such as phenolic acids, polyphenols and flavonoids are generally known as anti-oxidants and their beneficial effects in several chronic ailments have been reported[180]. In addition, the synthetic anti-oxidants available at present are known to produce negative health effects.

The Present study clearly states the significant anti-oxidant activity of EAFCF and NHFCA and among these the EAFCF showed the superior anti-oxidant potential than the NHFCA as evidenced from the reducing power ability and super-oxide scavenging activity.

The reducing property of the plant drug is generally believed to be because of the presence of reductones such as polyphenols and flavonoids. Reductones exert their antioxidant property by breaking the free radical chain by donating hydrogen atoms.

They also react with certain precursor molecules of free radicals and prevent the formation of superoxides [182].

EAFCF and NHFCA, subjected to toxicity studies as per the OECD guidelines 420 and found that there was no mortality up to 2000mg/kg body weight of the animals. So it is clear that both the plant extract fractions were safe and free from any toxicity.

From the preliminary pharmacognostical analysis it was clear that EAFCF and NHFCA were rich in polyphenols and flavonoids. Enormous research reports state the role of flavonoids in normal functioning of brain and the beneficial effect in CNS disorders (for review)[183]. In view of this the present study evaluated the antiepileptic, anxiolytic and antidepressant activity of EAFCF and NHFCA.

Epilepsy is one of the most prevalent neurological disorders, characterized by the recurrent appearance of spontaneous seizures due to neuronal hyperactivity in the brain and has number of subtypes [30]. A seizure is a symptomatic, behavioral manifestation due to abnormal disorder, spontaneous but synchronizes, high frequency firing of neuron populations in the central nervous system [36]. Several studies have clearly indicated the significant role of free radical generation and neuroprotective ability of anti-oxidants isolated from medicinal plants against various types of seizures (for review)[93]. Anti-oxidant activity of *Cassia fistula* L. and *Cassia auriculata* L. has been well documented in previous studies and our present study revealed the potent anti-oxidant activity of EAFCF and NHFCA. However, the CNS activities of both the plants have less well documented.

In PTZ induced convulsions, the EAFCF and NHFCA dose dependently

increased the onset of MICS and GTCS ($p<0.01$) and protected the animal from death. This shows that the treatment of EAFCF and NHFCA delays the seizure generation in mice. Although the extract fraction showed protection from death in PTZ induced convulsion, it could not protect the occurrence of seizure. Further enrichment of flavonoids or treatment with higher dose of EAFCF may produce the seizure protection in PTZ induced convulsion model. Clinical management of seizure involves the usage of medications for chronic period[88]. Hence, the present study also evaluated the anti convulsant activity in PTZ kindling model, where in the sub convulsive dose of PTZ was given for sub chronic period until the occurrence of seizure and the effect of EAFCF and the results clearly indicated that the repeated treatment of EAFCF did not cause decrease in anti seizure activity. 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 (for review)[109]. 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[184]. Anxiety is a complex psychological disorder generally affecting the mood and causing irrational fear as one of the most prominent symptom[112]. Pharmacological treatment of anxiety disorders involves the use of benzodiazepines, buspirone and antidepressant drugs. These drugs shows considerable clinical efficacy but have several demerits such as amnesia, dependence liability and sedation [90]. Use of herbal remedies for treating anxiety disorder has been increasing and mechanism of

action of several anti-anxiety herbs has been elucidated[185]. With this view, the present study was also evaluated the anxiolytic activity of EAFCF and NHFCA in EPM, OFT and marble burying behavior.

The EPM is etiologically validated and most widely accepted model for screening novel anxiolytic agents in mice[112] as well as in rat[113]. Increase in the number of open arm entries and consequent increase in time spend in open arm are the two parameters generally considered as index of anxiolytic activity of a drug[114]. However, the interpretation of parameters in EPM is always complex. Studies also report increase in time spent in open compartment, open arm entries and no change in closed arm entries as criteria for drugs to have anxiolytic activity[115]. On the other hand, the total number of arm entries is more reliable measure for locomotor activity[116]. The present work showed the dose dependent anxiolytic effect as evidenced from the significant increase ($p<0.01$) in the frequency and time spent in open arms by the animals treated with 50 and 100 mg/kg of EAFCF and 100 and 200 mg/kg of NHFCA orally. Moreover, these drugs did not alter the total number of arm entries indicating that the drugs produce anxiolytic activity without influencing the locomotor activity.

The open-field test is based on rodents intrinsic behaviour to stay near the periphery of a novel environment (i.e., thigmotaxis), which may serve the animal a sense of security. Increase in time spent in the central arena of the open field without affecting general motor activity is the indication of anxiolytic activity[117]. In this study, the treatment of EAFCF and NHFCA did not alter number of rearings and ambulations whereas, significantly ($p<0.01$) reduced the thigmotaxis indicating their

anxiolytic activity. The drugs which were previously reported to show anxiolytic activity in OFT include midazolam[116], buspirone[186] and chordiazepoxide [187].

Burying behavior consists in forward shoving the diggable material over the source of aversion using the snout and forepaws in order to avoid and protect from the localized threat[117]. Marble burying test has been suggested as a useful model for evaluating anti- anxiety activity as no change in tendency of marble burying behavior occurred due to repeating the test[118]. Indeed, commonly used anxiolytic benzodiazepines such as diazepam and chlordiazepoxide decreased the marble burying tendency of rodents[119]. In the present study, acute administration of EAFCF and NHFCA dose dependently decreased the number of marbles ($p<0.01$) indicating their anti-anxiety activity.

Depression is a heterogeneous syndrome comprised of numerous diseases with distinct causes and pathophysiologies. The symptoms of depression include depressed mood, irritability, low self esteem, feelings of helplessness, worthlessness, guilt, decreased ability to concentrate and think decreased or increased appetite, weight loss or gain, insomnia or hypersomnia, low energy, fatigue or increased agitation, decreased interest in pleasurable stimuli and recurrent thoughts of death and suicide. Outcomes from the epidemiological studies indicate that behavioral depression is the most common co-morbid condition associated with epilepsy[4]. A number of anti-convulsant drugs are investigated both clinically and pre-clinically for the treatment of depression. Intern, some psychiatrists have advocated the use of anticonvulsants for the management of depression[188]. Hence, the present study also aimed to evaluate the anti-depressant activity of EAFCF and NHFCA in animal models of depression.

In the present study, acute administrations of EAFCF and NHFCA showed dose dependent ($p<0.01$) anti-immobility in FST and TST. The immobility which is exhibited in the FST and TST represents the behavioral despair in animals that is claimed to reproduce the situation similar to depression in clinical conditions. Reduction in the duration of immobility by a drug was considered as it posses anti despair or antidepressant effect.

Generally, the drugs which enhance the psychostimulant effect show false positive results in behavioral despair models and open field test is used to discriminate the psycotimulant action from anti despair effect[122]. In the present study EAFCF and NHFCA did not altered the ambulatory and rearing behavior in OFT at the doses effective in TST and FST indicating that the antidepressant effect showed by EAFCF and NHFCA is due to escape directed behavior and not by central excitation. However, the NHFCA at higher dose used here (400 mg/kg) showed sedative property in phenobarbitone induced sleeping time and motor in-coordination in rota rod analysis.

Another major drawback of these behavioral despair models is that most of the clinically used antidepressants show positive effect after acute administration, but these drugs when used clinically, takes at least three weeks to produce the desired therapeutic effect (for review)[120]. Hence, the present study also included the study of antidepressant activity of EAFCF in chronic model that can reproduce some aspects of behavioral depression in patients.

Involvement of stress directly or indirectly has been suggested in the development of human depression[76]. In animals, a wide range of behavioral

parameters changes have been noted including changes in locomotor and explorative behavior, impairment of feeding, drinking and sexual behavior and most notably the anhedonia (decreased interest in pleasurable events) due unpredictable stressors[127]. Similar behavioral changes are most often seen in human psychiatric disorders. A regime of uncontrollable stress has been used extensively to model the deficits in motivation and reward. CUS involves exposure to unpredictable mild stressors over several weeks, designed to mimic the daily hassles that reportedly provoke the onset of depression in humans[127]. In the CUS model, the major symptom of human depression, anhedonia, is claimed to be reflected in the animals' decreased consumption of palatable solutions[128]. The intake or preference for sucrose solutions is the hedonic measure that has been most widely adopted.

Therefore, the antidepressant activity of EAFCF was assessed in CUS model, a most reliable animal model reflecting anhedonia which is core symptom of human depression. The interesting finding of the present study is that the chronic unpredictable stress induced significant ($p<0.01$) reduction in the intake of sweet solution was normalized by chronic treatment of EAFCF. In this experiment, mice did not show loss of body weight due to the chronic unpredictable stress indicating that the reduction in sucrose solution intake is not due to the loss of body weight. Numerous studies have shown that the CUS induced sub-sensitivity to reward can be reversed by chronic treatment with commonly used antidepressants including tricyclic antidepressants, atypical antidepressants, selective serotonin reuptake inhibitors (SSRIs) and mono amino oxidase inhibitors (MAOI) as well as repeated electro convulsive shock (for review)[120].

Pain, on the other hand, is considered as a major symptom of various diseases including the CNS disorders that is capable to produce severe physical and psychological distress for many patients and the most predominant symptom affecting their quality of life. The definition given for pain by International Association for the Study of Pain is "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage"[81]. Most of the drugs used at present for analgesic effect are synthetic in nature, prolonged use of which causes several side and toxic effects including respiratory depression, constipation, kidney damage, physical dependence as well as gastric irritation. In addition, the drugs used for treating epilepsy, anxiety and depression are also used for the clinical management of pain[189]. In this context, the present study aimed in evaluation of EAFCF and NHFCA for analgesic activity

EAFCF and NHFCA were evaluated for their nociceptive activity in peripheral as well as central analgesic models. 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 [129]. 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 and NHFCA showed dose dependent analgesic and antinociceptive activity as evident through significant ($p<0.01$) reduction in number of writhing caused by acetic acid. Hence, EAFCF and NHFCA 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 and NHFCA in mice, increased the reaction time significantly ($p<0.01$) 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 ineffective. The reaction to the hotplate demonstrates the supraspinal reflex and tail immersion explains the spinal reflex mediated by various sub-types of opioid receptors[130,131]. Findings of the present study indicate that the EAFCF and NHFCA may act as an anti-nociceptive by central mechanisms.

Administration of EAFCF and NHFCA also significantly ($p<0.01$) 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 the sensory afferent nerve ending 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[133].

In this study, EAFCF and NHFCA showed dose dependent ($p<0.01$) inhibition of paw licking in early neurogenic and late inflammatory pain phases. Reduction of neurogenic pain perception by EAFCF and NHFCA was also confirmed in hotplate and tail immersion tests; while the effect of EAFCF and NHFCA in acetic acid

induced writhing further confirms the anti-nociceptive action in inflammatory pain conditions.

Previous research reports have stated that the drugs having anxiolytic, anticonvulsant activity at low doses such as benzodiazepines produce sedative or myorelaxant activity at high doses[190, 191]. Considering this, the present study also evaluated the effect of EAFCF and NHFCA 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 significantly ($p>0.05$) alter motor co ordination, and only at higher dose of NHFCA (400mg/kg) potentiated the phenobarbitone induced sleeping time ($p<0.01$). Moreover, EAFCF did not alter the locomotor activity as it had not significantly ($p>0.05$) 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 and nor by altering locomotor activity. In contrast, methanolic extract of *Cassia fistula* pods has been shown to produce sedation and motor in co-ordination[107].

Fractionation of crude extract in to flavonoid rich portion may be responsible for this non sedative action at low dose of EAFCF as natural and synthetic flavonoids are believed to have potent anxiolytic activity without producing sedative effect. However, NHFCA may have motor toxic and sedative property at higher doses[192]. Indeed, EAFCF contains apart from flavonoids, several components such as anthraquinones, terpenoids, and tannins. Hence, further studies to be focused 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 crude plant extracts are generally proved to be more

potent medicines than pure isolated compounds probably due to synergistic interactions and several actions of complex mixers of components [193].

Determining the mechanism underlying the pharmacological activities of herbal medicines presents certain challenges distinct from the studying the synthetic drugs. For example, synthetic drugs are studied as single component. Whereas, herbal medicines often contain multiple active substances that act in combination. In terms of CNS function, a wide range of phenolic compounds interact directly with neurotransmitter systems. As an example, in animal models, a diverse range of individual and combined flavonoids that occur in traditional medicinal extracts exert sedative/anxiolytic effects via direct binding to $GABA_A$ receptors, cognitive enhancement via antagonistic $GABA_A$ receptor binding and resultant cholinergic upregulation, and antidepressant effects via monoamine oxidase inhibition and resultant increases in levels of 5-hydroxytryptamine (5-HT), Dopamine (DA), and noradrenaline in select brain areas (for review)[93].

Various neurotransmitters levels were considered to be altered in most of the CNS disorders and the drugs used to alleviate the symptoms of underlying disease in most of the cases found to be act by restoring altered neurotransmitter level. Hence, the present study evaluated the changes in various neurochemicals such as GABA, glutamate, serotonin and adenosine. The results revealed that the treatment of EAFCF for sub chronic period caused significant ($p<0.01$) increase in GABA, serotonin and adenosine level and considerable decrease in glutamate level.

Adenosine is a ubiquitous neuromodulator, which takes part in a variety of process in the central nervous system by acting on distinct membrane bound G-

protein coupled receptor subtypes, namely A_1, A_{2A}, A_{2B} and A_3 receptors[136]. It exhibits different array of behavioral effects, including locomotor depressant effect, catalepsy, antipsychotic, anticonvulsant, anxiolytic, neuroprotection and also in sleep wake rhythms. In the brain, endogenous adenosine exerts a potent depressant effect on neurons probably by reducing neurotransmitter release, presynaptically and increasing potassium conductance; postsynaptically might be especially through A_1 or A_{2A} receptors because of their ubiquitous distribution in brain. The role of A_{2B} and A_3 receptors in the mediating the central effect of adenosine seems to less important as there is a paucity of these receptors in the brain (for review)[171].

Therefore to elucidate the probable mechanism of action of EAFCF, we focused on modulatory role of adenosine in the seizure initiation and propagation. Adenosine is an endogenous neuromodulator which is released during seizure, ischemia and hypoxia[138-142]. The multiple physiological actions of adenosine are mediated by binding of adenosine with four type of receptor belonging to the family G- protein coupled receptors namely, A_1, A_{2A}, A_{2B} and A_3 adenosine receptors. Out of which, A_1 and A_{2A} adenosine receptors are found to be widely expressed in CNS[136].

In central nervous system, adenosine inhibits the release of several neurotransmitters, in particular, the release of excitatory neurotransmitters like acetylcholine, glutamate, noradrenaline, dopamine and 5-hydroxytryptamine and this inhibitory role of adenosine is mainly mediated through A_1 receptors, when linked via $G_{i/o}$ proteins to both Ca^{2+} and K^+ ion channel. Activation of adenosine via post synaptic A_1 receptors leads to stabilization of the membrane potential by modulation of Ca^{2+} and K^+ fluxes. Adenosine A_1 receptors mainly localized in the cerebral cortex,

cerebellum and hippocampus of the central nervous system and believed to mediate seizure suppression and neuroprotection (for review)[171].

With this view the current study evaluate the role of adenosinergic system in the observed effects of EAFCF in epilepsy, anxiety and depression models.

Pretreatment of sub effective dose of adenosine, adenosine A_1 receptor agonist, CHA and adenosine deaminase inhibitor, dipyridamole with sub effective dose of EAFCF showed following changes. In s.c.PTZ model, enhanced the onset of MCS and GTCS and protected the animals from death. In FST, duration of immobility was reduced and in EPM, number of entries to open arm and time spent in open arm enhanced. These results indicate the synergistic interaction of adenosinergic enhancers with EAFCF.

To further confirm the role of adenosinergic system in the observed effects of EAFCF, the adenosine blockers such as non selective adenosine receptor anagonist, caffeine and adenosine A_1 receptor antagonist, DPX were pretreated with effective dose of EAFCF and the results showed the blockade of anticonvulsant effect of EAFCF in s.c.PTZ, anxiolytic activity in EPM and antidepressant activity in FST.

The potential of adenosine as an anti-epileptic substance[137-140] has emerged on the basis of following observations: the first significant observation is that the A_1R are predominately located excitatory synapses, where they inhibit glutamate release, decrease glutamatergic responsiveness and hyperpolarise neurons, all desirable actions to decrease the hyper excitability associated with epilepsy; another important observation is that the levels of endogenous extracellular adenosine level demonstrated to rise upon seizure activity[141,142], which could prove that adenosine

would play a key role as an endogenous anti-epileptic compound. Accordingly, a wealth of studies have confirmed that the acute administration of either agents enhancing the extracellular levels of adenosine (inhibitors of adenosine transporters or of adenosine metabolism) or agonists of A_1R attenuated seizure and/or convulsive activity in different animal models; conversely, the acute administration of either non-selective antagonists of adenosine receptors (such as caffeine or theophylline) or selective A_1R antagonists enhance the duration and severity of seizures and/or convulsions (for review)[137-140]. Thus, it seems evident that A_1R effectively constitute a hurdle curtailing seizure activity, which is further confirmed by the ability of A_1R to control the spreading of seizure activity[143] and the greater susceptibility of A_1R knockout mice to epilepsy[144,145]. Interestingly, this A_1R-mediated control of epilepsy has recently been proposed to be a possible link for the antiepileptic effect of ketogenic diets[146]. However, several studies have now identified a decreased density and efficiency of synaptic A_1R in models of epilepsy[146-148]. Thus, the A_1R-operated inhibitory system seems to act as a continuously active gate-keeper or hurdle to avoid initiating a seizure-like event; once this hurdle is overtaken, then there seems desensitization of this A_1R system.

In the present experiment, pretreatment adenosinergic enhancers potentiated the anxiolytic activity in EPM while the inhibitors of adenosinergic system blocked the anti anxiety activity of EAFCF. These results are in line with the previous reports discussing the potent modulation of anxiety by adenosine.

A strong link between the adenosine modulation system and the control of fear processing and anxiety is suggested by several types of studies, namely: i) animal models designed to mimic anxiety traits; ii) epidemiological studies with caffeine,

both in normal subjects and in patients in anxiety disorders; iii) gene linkage studies in humans (for review)[149,150]. However, the role of adenosine receptors in specific anxiety disorders, like phobia, is still to be defined. Studies with caffeine consumption by phobic patients are rare, especially in comparison to research on caffeine consumption among patients with other anxiety disorders like panic disorder. A study from Boulenger and Uhde[151] showed that panic disorder patients have increased sensitivity to caffeine and its consumption appears to be more strongly linked to generalized anxiety symptoms than to 'phobic-anxiety' symptoms. The work from Uhde[152] also showed that anxiety symptoms are not made more severe by caffeine consumption in social phobics and that caffeine consumption in social phobic individuals does not differ from the general population. In addition, no differences in electroencephalographic activity of patients with panic disorder versus control subjects were observed after oral administration of caffeine[153]. On the other hand, another study reported that caffeine can induce panic attacks in both panic disorder and social phobic patients following consumption of a dose of 480 mg[154]. It was also demonstrated that caffeine ingestion per se does not appear to reduce fear extinction at least in some specific phobias, whereas changes in caffeine consumption, during learning or recall tests, can enhance the return of fear in spider phobia [155].

In the present study, the potent modulatory role of adenosine in antidepressant activity also demonstrated as the pretreatment of adenosine enhancers synergistically increased the antidepressant activity and while, the pretreatment of suppressors adenosinergic system blocked the antidepressant activity in FST.

The effect of the adenosine neuromodulation system in depression is complex, especially due to its ability to modulate several other neurotransmission systems, such

as dopaminergic, glutamatergic and serotoninergic as well as the corticotrophin system (for review)[156.] Adenosine and its analogues were shown to cause a depressant-like response in behavioral despair models[157-159], an effect that was prevented by the administration of classical antidepressants[160]. In contrast, other studies showed an antidepressant effect associated with adenosine administrated both systemically and centrally[161-163]. The strongest evidence supporting the relation between adenosine and depression in preclinical models came from manipulation of A_2AR. The main evidence sustaining this hypothesis relies on the observation that the genetic depletion of A_2AR results in an antidepressant-like phenotype in animal models[164,165]. In addition, A_2AR blockade relieves the early hippocampal modifications induced by stress[166], one of the major environmental factors favoring the implementation of depressive states[167]. It is important to highlight that different therapeutic strategies currently used to manage depressive disorders also have effects related to the adenosine system. Tricyclic antidepressants such as nortriptiline, chlorimipramine or desipramine can bind to adenosine receptors[168] and dose-dependently reduce the activity of ecto-nucleotidases and, expectedly, the levels of extracellular adenosine in cortical synapses[169]. In addition, both electroconvulsive therapy and sleep deprivation cause an increase of adenosine concentration and of A_1R activation[170]. In conclusion, although at this stage the relation between adenosine and depression is still circumstantial, it seems evident that the adenosinergic system is able to modulate mood states by functioning as a normalizing system, and both too high or too low levels of activation can cause a failure in the organism to adapt and a predisposition to disease

These interaction study results clearly indicate that the enhancement of adenosinergic system by the adenosine facilitators potentiated the anti convulsanat, anxiolytic and antidepressant actions of EAFCF and suppression of adenosinergic system by adenosine antagonists block the actions of EAFCF.

In any given case, a sole mechanism may be in effect, or there may be complex interactions among active constituents and multiple neurotransmitter system in brain. Since the effects of EAFCF which we observed in this study are obtained by using crude ethyl acetate fraction of the *Cassia fistula* L. pods and not in isolated compound, it important to understand the effects of active constituents in combination and in isolation and their interactions with other neurochemicals.

SUMMARY AND CONCLUSION

Anxiety, epilepsy and depression are co-morbid conditions with interlinking etiology requiring chronic drugs treatment. The problems associated with available current therapy with synthetic chemicals are poor response, remission and severe undesirable side effects. Hence, the search for novel drug continues and medicinal plant source became important source for new drug development for these CNS ailments.

Cassia fistula L. and *Cassia auriculata* L have been selected based on their traditional use and their rich flavonoid content nature as flavonoids were reported to have potential CNS effects.

Preliminary phytochemical analysis showed the presence of flavonoids, terpenoids, tannins, carbohydrate and proteins in various solvent fractions of *Cassia fistula* L. pods and *Cassia auriculata* L. pods. In the quantitative estimation of phytoconstituents, ethyl acetate fraction of *C. fistula* (EAFCF) and n-hexane fraction of *C. auriculata* (NHFCA) showed to contain more polyphenols and flavonoids. HPTLC studies also carried out to confirm the presence of flavonoids in above said fractions. A flavonoid from EAFCF was isolated through column chromatography and further subjected to NMR, FT-IR and mass analysis.

Results of *In vitro* antioxidant studies showed that EAFCF and NHFCA possessed high antioxidant property than the other fractions as both the fractions showed free radical scavenging activity and reducing power ability similar to that of the standards used. The flavonoid rich EAFCF was subjected to column chromatography and fractions were obtained. The fractions obtained from the column chromatography study were subjected to HPTLC finger print analysis. The fractions

which shows single band in the HPTLC finger print analysis were taken for HPLC study. In the HPLC study a single peak was obtained at 6 minutes, was collected at the column outlet.

Fractions collected from the HPLC outlet were evaporated to remove the organic solvent and aqueous phase was freeze dried and a dirty white powder was obtained. The isolated compound was subjected to various characteristic analyses like IR, MASS and NMR. The chemical structure of the isolated compound was elucidated and identified. The isolated compound was a flavonoid having a molecular weight of 290 and the compound was identified as 2-(3,4-dihydroxyphenyl) chroman-3,5,7-triol.

In acute oral toxicity studies, conducted as per OECD (guideline 420) showed no mortality or toxicity up to the dose of 2000 mg/kg observed for 24 hours period. Doses for the pharmacological evaluation were fixed based on the results of acute toxicity studies.

Evaluation of anticonvulsant activity of EAFCF and NHFCA was carried out in s.c. PTZ and PTZ kindling models. Both the fractions exhibited significant anticonvulsant activity as evidenced from increase in the time latency for MCS and GTCS in s.c.PTZ model and significant decrease in seizure score in EAFCF treated animals PTZ kindling model.

EAFCF and NHFCA were evaluated for anxiolytic activity in EPM, OFT and in marble burying behavior models. A significant increase in open arm entries and time spent in open arm by the animals received EAFCF and NHFCA have been noted in EPM, while decrease in number of marbles buried and thigmotaxis in OFT also were noted indicating the anxiolytic activity of the fractions.

Initially, both EAFCF and NHFCA were evaluated for their antidepressant activity in FST, TST models. A significant decrease in duration of immobility was exhibited by the animals treated with the fractions compared to control animals was observed in both FST and TST which shows the antidepressant potential of fractions on acute treatment. However, the clinical scenario is such that the symptoms of depression can be minimized only after three weeks of drugs treatment. Hence, the present study later evaluated antidepressant activity of the EAFCF in CUS induced anhedonia model. In this, the model group, received only the stressors exhibited depression like behavior as shown by the decrease in the sucrose consumption, increase in the duration of immobility and decrease in erythrocyte SOD activity. Above parameters were reversed by the treatment of fractions indicating the antidepressant potential after chronic treatment.

Most of the currently used antiepiletics and antidepressants are also used for alleviating pain as they possess analgesic and anti-nociceptive abilities. Therefore, in the present study, both EAFCF and NHFCF were screened for analgesic and anti-nociceptive activities in acetic acid induced writhing, tail flick, hot plate and formalin tests. Acetic acid induced writhing model is employed for screening peripheral analgesics and both the fractions significantly reduced the acetic acid induced writhing indicating the peripheral analgesic potentials probably through blockade of release of pain mediators. In hot plate and tail flick models, latency of response to pain stimuli was increased significantly in EAFCF and NHFCA treated animals demonstrating the anti-nociceptive potential. In addition, the fractions reduced the initial neurogenic and later inflammatory pain in formalin test as evidenced from the significant decrease in paw licking responses.

Drugs showing either of anxiolytic, anticonvulsant, antidepressant or analgesic activity or combination of above activities may possess sedative property or motor toxicity as adverse effects. In the present study, EAFCF was not shown either of the adverse affects in the doses used in the present study. However, NHFCA at highest dose used in this study showed mild sedative and skeletal muscle relaxant properties ($p<0.05$).

To find out the mechanism involved in above discussed pharmacological activities, the changes in the neurochemicals level such as GABA, glutamate, serotonin and adenosine upon the treatment EAFCF were estimated in HPTLC. Treatment of EAFCF significantly increased the levels of GABA, serotonin and adenosine while it decreased the glutamate level.

Adenosine is one of the significant neuromodulater believed to modulate the release of most of the neurotransmitters. Hence, the present study further focused on the evaluation role of adenosinergic system on the CNS effects of EAFCF. A synergistic interaction of exogenous adenosine administration and its receptors agonists with EAFCF treatment was noted in EPM, FST, s.c. PTZ and formalin test. Meanwhile, adenosinegic blocking agents showed antagonistic interaction with EAFCF in the above chosen models of anxiety, seizure, depression and pain. These results indicate that the EAFCF possess anxiolytic, anticonvulsant, antidepressant and analgesic activity through modulation of adenosinergic system.

In conclusion, the plants *Cassia fistula* and *Cassia auriculata* have potential anxiolytic, anticonvulsant, antidepressant and analgesic activities and adenosine may play a role in the CNS effects of *Cassia fistula*.

REFERENCE

1. Hall JE. The Nervous System: C. Motor and Integrative Neurophysiology. In: Guyton and Hall Text book of medical physiology, 12th edition India: Saunders Elsevier Ltd; 2010

2. Carlini EA. Plants and the central nervous system. Pharmacol Biochem Behav 2003; 3: 501–12

3. Eissa TA, Palomino OM, Carretero ME, Gómez-Serranillos MP. Ethnopharmacological study of medicinal plants used in the treatment of CNS disorders in Sinai Peninsula, Egypt. J Ethnopharmacol 2014;151(1):317-32

4. Ottman R, Lipton BR, Ettinger AB, Cramer JA, *et al.* Co-morbidities of epilepsy: Results from the Epilepsy Co-morbidities and Health (EPIC) survey. Epilepsia 2001; 52(2):308–15

5. Kanner AM, Barry JJ, Gilliam F, Hermann B, *et al*. Anxiety disorders, subsyndromic depressive episodes, and major depressive episodes: do they differ on their impact on the quality of life of patients with epilepsy? Epilepsia 2010; 51: 1152-8.

6. Piazzini A, Canevini MP, Maggiori G, Canger R. Depression and anxiety in patients with epilepsy. Epilepsy Behav 2001; 2: 481–9.

7. Issacs KL, Philbeck JW, Barr WB, Devinsky O, *et al.* Obsessive–compulsive symptoms in patients with temporal lobe epilepsy. Epilepsy Behav 2004; 5:569–74.

8. Baker GA, Spector S, McGrath Y, Soteriou H. Impact of epilepsy in adolescence. a UK controlled study. Epilepsy Behav2005; 6(4): 556–62.

9. Beyenburg S, Stoffel-Wagner B, Bauer J, Watzka M, *et al.* Neuroactive steroids and seizure susceptibility. Epilepsy Res 2001; 44: 141–53.

10. Cendes F, Andermann F, Gloor P, Gloor P, Gambardella A, *et al*. Relationship between atrophy of the amygdala and ictal fear in temporal lobe epilepsy. Brain 1994; 117(4): 739–46.

11. Charney DS. Neuroanatomical circuits modulating fear and anxiety behaviors. Acta Psychiatr Scand 2003; 417: 38–50.

12. Lydiard RB. The role of GABA in anxiety disorders. J Clin Psychiatr 2003; 64: 21–7.

13. Pellock JM. Defining the problem: psychiatric and behavioral comorbidity in children and adolescents with epilepsy. Epilepsy Behav 2004; 5: 3–9.

14. Jacoby A, Snape D, Baker GA. Epilepsy and social identity: the stigma of a chronic neurological disorder. Lancet Neurol 2005; 4: 171–8.

15. Goldstein MA, Harden CL. Epilepsy and anxiety. Epilepsy Behav 2000; 1: 228–34

16. Adewuya AO, Ola BA. Prevalence of and risk factors for anxiety and depressive disorders in Nigerian adolescents with epilepsy. Epilepsy Behav 2005; 6(3): 342–7.

17. Williams J, Steel C, Sharp GB, DelosReyes E,*et al*.. Anxiety in children with epilepsy. Epilepsy Behav 2003; 4: 729–32.

18. García-Morales I, De la Peña Mayor P, Kanner AM. Psychiatric comorbidities in epilepsy: identification and treatment. Neurologist 2008; 14(6): 15-25.

19. Forsgren L, NystromL. An incident case referent study of epileptic seizures in adults. Epilepsy Res 1999; 6: 66–81.

20. Hesdorffer DC, Hauser WA, Annegers JF, Cascino G. Major depression is a risk factor for seizures in older adults. Ann Neurol 2000; 47: 246–9.

21. Hesdorffer DC, HauserWA, Olafsson E, Ludvigsson P, Kjartansson O. Depression and suicidal attempt as risk factor for incidental unprovoked seizures. Ann Neurol *2*006; 59: 35–41.

22. Kanner AM. Current review in clinical science: depression in epilepsy: a neurobiologic perspective. Epilepsy Curr 2005; 5: 21–7.

23. Kanner AM, Current review in clinical science: depression and epilepsy: A New perspective on two closely related disorders. epilepsy curr 2006; 6(5): 141–6

24. Sankar R, Mazarati A, Jasper S. Neurobiology of depression as a comorbidity of epilepsy (internet): Chapter from Jasper's basic mechanisms of the epilepsies, Fourth edition NCBI bookshelf online book version.

25. Kobau R, Zahran H, Thurman DJ, Zack MM, *et al.* Epilepsy surveillance among adults – 19 States, Behavioral Risk Factor Surveillance System, 2005. MMWR Surveill Summ 2008; 57:1–20.

26. Ottman R, Lipton RB. Comorbidity of migraine and epilepsy. Neurology 1994; 44:2105–10.

27. Clarke T, Baskurt Z, Strug LJ, Pal DK. Evidence of shared genetic risk factors for migraine and rolandic epilepsy. Epilepsia 2009; 50:2428–33.

28. Gaitatzis A, Carroll K, Majeed A, Sander JW. The epidemiology of the comorbidity of epilepsy in the general population. Epilepsia 2004; 45:1613–22.

29. Hesdorffer DC, Ludvigsson P, Hauser WA, Olafsson E, *et al.* Co-occurrence of major depression or suicide attempt with migraine with aura and risk for unprovoked seizure. Epilepsy Res 2007; 75:220–3.

30. Engel J. A proposed diagnostics cheme for people with epileptic seizures and with epilepsy report of the ILAET ask Force on Classification and Terminology. Epilepsia 2001; 42:796–803.

31. McNamara JO. Emerging insights into the genesis of epilepsy. Nature 1999; 399: A15–A22.

32. Sato K, Iwai M., Zhang R. Highly polysialylated neural cell adhesion molecule (PSANCAM) positive cell increased and change localization in rat hippo campus by exposure to repeated kindled seizures. Acta Neurochir 2003; 86: 575–9.

33. McCormick DA, Huguenard JR. Electrophysiology of the Neuron: An Interactive Tutorial. Oxford: Oxford University Press; 1994.

34. Somjen GG. Ion regulation in the brain: implications for pathophysiology. Neuroscientist 2002; 8:254–67.

35. Meisler MH, Kearney J, Ottman R, Escayg A. Identification of epilepsy genes in human and mouse. Annu Rev Genet 2001; 35:567–88.

36. Scharfman HE. The Neurobiology of Epilepsy. Curr Neurol Neurosci Rep2007; 7(4): 348–54.

37. Coulter DA. Epilepsy-associated plasticity in gamma-amino butyric acid receptor rexpression, function, and inhibitory synaptic properties. Int Rev Neurobiol 2001; 45: 237–52.

38. Morimoto K, Fahnestock M, Racine R, Kindling and status epilepticus models in epilepsy: rewiring the brain. Progr Neurobiol 2004; 73: 1–60.

39. Brown TH, Johnston D. The synaptic nature of the paroxysmal depolarization shift in hippocampal neurons. Ann Neurol 1984; 16:S65–S71.

40. Traub RD, Michelson-Law H, Bibbig AE, Eberhard H, *et al*. Gap junctions, fast oscillations and the initiation of seizures. Adv Exp Med Biol 2004; 548:110–22.

41. Cobb SR, Buhl EH, Halasy K, Paulsen O, *et al*. Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. Nature 1996; 378:75–8.

42. Sloviter RS, Zappone CA, Harvey BD, Frotscher M. Kainic acid-induced recurrent mossy fiber innervation of dentate gyrus inhibitory interneurons: possible anatomical substrate of granule cell hyper-inhibition in chronically epileptic rats. J Comp Neurol 2006; 494:944–60.

43. Gray JA, McNaughton N. The neuropsychology of anxiety: reprise. Nebr Symp Motiv 1996; 43:61–134.

44. Kendler KS, Thornton LM, Gardner CO. Stressful life events and previous episodes in the etiology of major depression in women: an evaluation of the ‘‘kindling’’ hypothesis. Am J Psychiatry 2000; 157: 1243–51.

45. Monroe SM, Harkness KL. Life stress, the ‘‘kindling’’ hypothesis, and the recurrence of depression: considerations from a life stress perspective. Psychol Rev 2005; 112: 417–45.

46. Bunney WE, Davis J. Norepinephrine in depressive reactions: a review. Arch Gen Psychiatry1965; 13:483–94.

47. Heninger GR, Delgado PL, Charney DS. The revised monoamine theory of depression: a modulatory role for monoamines, based on new findings from monoamine depletion experiments in humans. Pharmacopsychiatry 1996; 29:2–11.

48. Dube S. Cholinergic supersensitivity in affective disorders. In: Mann JJ, Kupfer DJ, editors. Biology of depressive disorders, Part A: a systems perspective. New York: Plenum 1993; 51–78.

49. Steingard RJ, Yurgelun-Todd DA, Hennen J, Moore JC, *et al*. Increased orbitofrontal cortex levels of choline in depressed adolescents as detected by in vivo proton magnetic resonance spectroscopy. Biol Psychiatry 2000.48:1053–61.

50. Renshaw PF, Lafer B, Babb SM, Fava M, *et al.* Basal ganglia choline levels in depression and response to fluoxetine treatment: an in vivo proton magnetic resonance spectroscopy study. Biol Psychiatr 1997; 41: 837–43.

51. Janowsky DS, Risch SC, Gillin JC. Adrenergic-cholinergic balance and the treatment of affective disorders. Prog Neuropsychopharmacol Biol Psychiatry 1983; 7:297–307.

52. Siever LJ, Davis KL. Overview: toward a dysregulation hypothesis of depression. Am J Psychiatry 1985; 142:1017–31.

53. Hashimoto K E. Shimizu, Iyo M. Critical role of brain-derived neurotrophic factor in mood disorders. Brain Res Rev 2004; 45(2): 104-14.

54. Duman RS, Heninger GR, Nestler EJ. Molecular psychiatry. Adaptations of receptor-coupled signal transduction pathways underlying stress- and drug-induced neural plasticity. J Nerv Ment Dis 1994; 182(12): 692-700.

55. Manji HK, Potter WZ, Lenox RH. Signal transduction pathways. Molecular targets for lithium's actions. Arch Gen Psychiatry 1995; 52(7): 531-43.

56. Conti AC, Cryan JF, Dalvi A, Lucki I. cAMP response element-binding protein is essential for the upregulation of brain-derived neurotrophic factor transcription, but not the behavioral or endocrine responses to antidepressant drugs. J Neurosci 2002; 22(8): 3262-8.

57. Karege F, Perret G, Bondolfi G, Schwald M. Decreased serum brain-derived neurotrophic factor levels in major depressed patients. Psychiatry Res 2002; 109(2): 143-8.

58. Lang UE, Hellweg R, Gallinat J. BDNF serum concentrations in healthy volunteers are associated with depression-related personality traits. Neuropsychopharmacol 2004; 29(4): 795-8

59. Thome J, Sakai N, Shin K, Steffen C. cAMP response element-mediated gene transcription is upregulated by chronic antidepressant treatment. J Neurosci 2000; 20(11): 4030-6.

60. Dowlatshahi D, MacQueen GM, Wang JF, Young LT. Increased temporal cortex CREB concentrations and antidepressant treatment in major depression. Lancet 1998; 352(9142): 1754-5.

61. Odagaki Y, Garcia-Sevilla JA, Huguelet P, La Harpe R. Cyclic AMP-mediated signaling components are upregulated in the prefrontal cortex of depressed suicide victims. Brain Res 2001; 898(2): 224-31.

62. Russo-Neustadt A, Beard RC, Cotman CW. Exercise, antidepressant medications, and enhanced brain derived neurotrophic factor expression. Neuropsychopharmacol 1999; 21(5): 679-82.

63. Siuciak JA, Lewis DR, Wiegand SJ, Lindsay RM. Antidepressant-like effect of brain-derived neurotrophic factor (BDNF). Pharmacol Biochem Behav 1997; 56(1): 131-7.

64. Malberg JE. Implications of adult hippocampal neurogenesis in antidepressant action. *J* Psychiatry Neurosci 2004; 29(3): 196-205.

65. Malberg JE, Schechter LE. Increasing hippocampal neurogenesis: a novel mechanism for antidepressant drugs. Curr Pharm Des 2005; 11(2): 145-55.

66. Sheline YI. 3D MRI studies of neuroanatomic changes in unipolar major depression: the role of stress and medical comorbidity. Biol Psychiatr 2000; 48(8): 791-800.

67. Selye H. The general adaptation syndrome and the diseases of adaptation. J Clin Endocrinol 1949; 6: 117-230.

68. Selye H. Stress and psychobiology. J Clin Exp Psychopathol 1956; 17(4): 370-5.

69. Ursin H, Eriksen HR. The cognitive activation theory of stress. Psychoneuroendocrinol 2004; 29(5): 567-92.

70. Eriksen HR, Murison R, Pensgaard AM, Ursin H. Cognitive activation theory of stress (CATS): from fish brains to the Olympics. Psychoneuroendocrinol 2005; 30(10): 933-8.

71. Ursin H, Olff M. Psychobiology of coping and defence strategies. Neuropsychobiol 1993; 28(1-2): 66-71.

72. Brown GW. Life events and affective disorder: replications and limitations. Psychosom Med 1993; 55(3): 248-59.

73. Hammen C, Davila J. Brown G, Ellicott A. Psychiatric history and stress: predictors of severity of unipolar depression. J Abnorm Psychol 1992; 101(1): 45-52.

74. Seligman ME, Weiss J, Weinraub M, Schulman A. Coping behavior: learned helplessness, physiological change and learned inactivity. Behav Res Ther 1980; 18(5): 459-512.

75. O'Toole SM, Chiappelli F, Rubin RT. Plasma neopterin in major depression: relationship to basal and stimulated pituitary-adrenal cortical axis function. Psychiatry Res 1998; 79(1): 21-9.

76. Mello Ade A, Mello MF, Carpenter LL, Price LH. Update on stress and depression: the role of the hypothalamic-pituitary-adrenal (HPA) axis. Rev Bras Psiquiatr 2003; 25(4): 231-8.

77. Chaouloff F. Serotonin, stress and corticoids. J Psychopharmacol 2000; 14(2): 139-51.

78. Anand A, Malison R, McDougle CJ, Price LH. Antiglucocorticoid treatment of refractory depression with ketoconazole: a case report. Biol Psychiatry 1995; 37(5): 338-40.

79. Hindmarch I. Expanding the horizons of depression: beyond the monoamine hypothesis. Hum Psychopharmacol 2001; 16(3): 203-18.

80. Maes M, Ombelet W, *et al*. Effects of pregnancy and delivery on the availability of plasma tryptophan to the brain: relationships to delivery-induced immune activation and early postpartum anxiety and depression. Psychol Med 2001; 31(5): 847-58.

81. Merskey H, Albe-Fessard D, Bonica JJ, Carmon A, *et al*. Pain terms: a list with definitions and notes on usage. Recommended by the IASP Subcommittee on Taxonomy. Pain 1979; 6:249.

82. Cervero F, Laird JMA. One pain or many pains? A new look at pain mechanisms.News Physiol Sci 1991; 6:268–73

83. Fields HL, Basbaum AI. Central nervous system mechanisms of pain modulation. In: Wall PD, Melzack R (eds) Textbook of pain 1999. Churchill Livingstone, London ; 309–29

84. Radhakrishnan K, Pandian JD, Santoshkumar T. Prevalence, knowledge, attitude, and practice of epilepsy in Kerala, South India. Epilepsia 2000; 41:1027-35.

85. World Health Organization. The world health report 2001 -Mental Health: New Understanding, New Hope. World Health Organization 2001, Geneva.

86. Kessler RC. Psychiatric epidemiology: selected recent advances and future directions. Bull World Health Organ.2000;78(4):464-74

87. Dureja GP, Jain PN, Shetty N, Mandal SP, et al. Prevalence of chronic pain, impact on daily life, and treatment practices in India. Pain Pract. 2014 14(2):E51-62.

88. Schmidt D. Drug treatment of epilepsy: Options and limitations. Epilepsy Behav 2009; 15: 56–65

89. Meldrum BS, Rogawski MA. Molecular targets for antiepileptic drug development Neurotherapeutics 2007; 4(1): 18–61.

90. Boulenger JP, Capdevielle D. Pharmacological treatment of generalized anxiety disorders: rationale and limitations. Encephale 2007; 33(1):84-94.

91. Lader M. Limitations of current medical treatments for depression: disturbed circadian rhythms as a possible therapeutic target. Eur Neuropsychopharmacol. 2007; 17(12):743-55.

92. Hsieh CL, Lao L, Lin YW, Vo GL, Complementary and alternative medicine for the treatment of central nervous system disorders. Evid Based Complement Alternat Med. 2014; 2014:1-2

93. Kennedy DO, Wightman EL. Herbal Extracts and Phytochemicals: Plant secondary metabolites and the enhancement of human brain function. Adv Nutr 2011; 2: 32–50.

94. Nadkarni A.K. Indian Materia Medica 1996.3rd eds, Popular Prakashan Pvt Ltd; India.

95. Kainsa S, Kumar P, Rani P. Pharmacological potentials of *Cassia auriculata* L. and *Cassia fistula* L. Plants: a review, Pak J Biol Sci 2012; 15(9): 408-41

96. Daisy P, Balasubramanian K, Rajalakshmi M, Eliza J, *et al.* Insulin mimetic impact of Catechin isolated from *Cassia fistula* on the glucose oxidation and molecular mechanisms of glucose uptake on Streptozotocin-induced diabetic Wistar rats. Phytomedicine 2010; 17(1):28-36.

97. Zhao W, Zeng X, Zhang T, Wang L, *et al*. Flavonoids from the bark and stems of *Cassia fistula* and their anti-tobacco mosaic virus activities. Phytochem Lett 2013; 6:179–82.

98. Siddhuraju P, Mohan PS, Becker K. Studies on the antioxidant activity of Indian Laburnum (*Cassia fistula* L.): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. Food Chem 2002; 79: 61-7.

99. Bhatnagar M, Vimal S, Vyas Y, Sharma D, *et al.* Antioxidant activity of fruit pulp powder of *Cassia fistula.* Phcog J 2010; 2: 219-28.

100. Nayan R, Bhalodia RN, Acharya V, Shukla J. Evaluation of *in vitro* antioxidant activity of hydroalcoholic seed extratcs of *Cassia fistula* linn. Free Rad. Antiox 2011; 1: 68-76.

101. Senthilkumar M, Sripriya R, Vijayaraghavan H, Sehgal P. Wound healing potential of *Cassia fistula* on infected albino rat model. J. Neurosci Methods 2006; 131: 283-89

102. Duraipandiyan V, Ignacimuth S. Antibacterial and antifungal activity of *Cassia fistula* L.: An ethnomedicinal plant. J Ethnopharmacol 2007; 112: 590-4.

103. Duraipandiyan V, Albertbaskar A, Ignacimuthu S, Muthukumar C, *et al*. Anticancer activity of rhein isolated from *Cassia fistula* L. flower. Asian Pac J Trop Dis 2012; 2: S517-23.

104. Gupta M, Mazumder UK, Rath N, Mukhopadhyay DK. Antitumor activity of methanolic extract of *Cassia fistula* L. seed against Ehrlich ascites carcinoma. J Ethnopharmacol 2000; 72: 151-6

105. Chauhan A, Agarwal M. Evaluating the anti-fertility potential of an aqueous extract from *Cassia fistula* seeds in male rats. Fertil Steril 2010; 93: 1706-10.

106. Bhakta T, Mukherjee PK, Mukherjee K, Banerjee S, *et al*. Evaluation of hepatoprotective activity of *Cassia fistula* leaf extract. J Ethnopharmacol 1999; 66: 277-82.

107. Mazumdar UK. Gupta M, Rath N. CNS activities of *Cassia fistula* in mice, Phytotherapy Res 1998; 12: 520-522.

108. Ilavarasan R, Malika M, Venkataramam S. Anti-inflammatory and anti-oxidant activities of *Cassia fistula* Linn. bark extracts, Afr J Trad Comp Alt Med 2005; 2:70-85

109. Loscher W. Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. Seizure 2011; 20:359–68

110. Krall RL, Penry JK, White BG, Kupferberg HJ, *et al*. Antiepileptic drug development: II. Anticonvulsant drug screening. Epilepsia 1978; 19:409–28.

111. Lo¨ scher W, Schmidt D. Which animal models should be used in the search for new antiepileptic drugs? A proposal based on experimental and clinical considerations. Epilepsy Res 1988; 2:145–81.

112. Belzung C, Griebel G. Measuring normal and pathological anxiety-like behavior in mice: a review. Behav Brain Res 2001; 125: 141–9.

113. Rodgers RJ, Cao BJ, Dalvi A, Holmes A. Animal model of anxiety: an ethological perspective. Braz J Med Biol Res 1997; 30: 289–304.

114. Pellow S, Chopin P E, File SE, Briley M. Validation of open: closed arm entries in an elevated plus- maze as a measure of anxiety in the rat. J Neurosci Methods 1985; 14: 149-67.

115. Taksande BG[1], Kotagale NR, Patel MR, Shelkar GP, *et al*. Agmatine, an endogenous imidazoline receptor ligand modulates ethanol anxiolysis and withdrawal anxiety in rats. Eur J Pharmacol. 2010; 637(1-3):89-101.

116. Nunes-de-Sousa RL, Canto-de-Sousa A, da-Costa M, Fornari RV, *et al*. Anxiety induced antinociception in mice: effects of systemic and intraamydala administration of 8-OH-DPAT and midazolam. Psychopharmacol 2000: 150, 300-10.

117. Treit D, Fundytus M. Thigmotaxis as a test for anxiolytic activity in rats. Pharmacol Biochem Behav 1988; 31: 959–62.

118. Poling, A., Cleary, J., Monaghan, M., Burying by rats in response to aversive and nonaversive stimuli. J Exp Anal Behav 1981; 35: 31–44.

119. Gyertyan I. Analysis of the marble burying response: marbles serves to measure digging rather than burying. Behav Pharmacol 1995; 6:24-31

120. Krishnan R, Nestler EJ. Animal Models of Depression: Molecular Perspectives. Curr Top Behav Neurosci. 2011; 7: 121–47.

121. Porsolt RD, Le Pichon M, Jalfre M. Depression: a new animal model sensitive to antidepressant treatments. Nature. 1977; 266:730–2.

122. Cryan JF, Mombereau C. In search of a depressed mouse: utility of models for studying depression related behavior in genetically modified mice. Mol Psychiatry 2004; 9:326–57.

123. Steru L, Chermat R, Thierry B, Simon P. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology 1985; 85:367–70.

124. Lutter M, Sakata I, Osborne-Lawrence S, Rovinsky SA, *et al*. The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. Nat Neurosci. 2008; 11:752–3.

125. Maeng S, Zarate CA Jr, Du J, Schloesser RJ, *et al*. Cellular mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-hydroxy-5- methylisoxazole-4-propionic acid receptors. Biol Psychiatry 2008; 63:349–52.

126. Dhir A, Kulkarni SK. Antidepressant-like effect of 17beta-estradiol: involvement of dopaminergic, serotonergic, and (or) sigma-1 receptor systems. Can J Physiol Pharmacol 2008; 86:726–35.

127. Willner P. Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. Neuropsychobiol 2005; 52:90–110

128. Strekalova T, Gorenkova N, Schunk E, Dolgov O, *et al.* Selective effects of citalopram in a mouse model of stress-induced anhedonia with a control for chronic stress. Behav Pharmacol. 2006; 17:271–287.

129. De ouza MM, PereiraMA, Ardenghi JV, Mora TC, *et al.* Filicene obtained from Adiantum cuneatum interacts with cholinergic, dopaminergic,

glutamatergic, GABAergic, and tachykinergic systems to exert antinociceptive effect in mice. Pharmacol Biochem Behav 2009; 93:40–6.

130. Grotto M, Sulman FG. Modified receptacle method for animal analgesimetry. Arch Int Pharmacodyn Ther 1967; 165:152–9.

131. O'Callaghan JP, Holzman SG. Quantification of the analgesic activity of narcotic antagonists by a modified hot plate procedure. J Pharmacol Exp Ther 1975; 192**:**497–505.

132. Ankier SI. New hot plate tests to quantify antinociceptive and narcotic antagonist activities. Eur J Pharmacol 1974; 27:1–4.

133. Hunskaar HS, Fasmer OB, Hole K. Formalin test in mice, a useful technique for evaluating mild analgesics. J Neurosci Methods 1985; 14:69–76.

134. Plone MA, Emerich DF, Lindner MD. Individual differences in the hotplate test and effects of habituation on sensitivity to morphine. Pain 1996; 66:265–70.

135. Tjolsen A, Berge OG, Hunskaar S, Rosland JH, *et al*. The formalin test: an evaluation of the method. Pain 1992; 51: 5–17.

136. Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, *et al*. Nomenclature and classification of purinoreceptors. Pharmacol Rev 1994; 46: 143-56.

137. Dragunow M. Purinergic mechanisms in epilepsy. Prog Neurobiol 1998; 31:85–108.

138. Dunwiddie TV, Adenosine and suppression of seizures. Adv Neurol 1999; 79 1001–10.

139. Malva JO, Silva AP, Cunha RA, Presynaptic modulation controlling neuronal excitability and epileptogenesis: role of kainate, adenosine and neuropeptide Y receptors, Neurochem Res 2003; 28: 1501–15.

140. Boison D. The adenosine kinase hypothesis of epileptogenesis. Prog Neurobiol 2008; 84:249–62.

141. During MJ, Spencer DD. Adenosine: a potential mediator of seizure arrest and postictal refractoriness. Ann Neurol 1992; 32:618–24.

142. Berman RF, Fredholm BB, Aden U, O'Connor WT. Evidence for increased dorsal hippocampal adenosine release and metabolism during pharmacologically inducedseizures in rats. Brain Res 2000; 872: 44–53.

143. Fedele DE, Li T, Lan JQ, Fredholm BB. Adenosine A1 receptors are crucial in keeping an epileptic focus localized. Exp Neurol 2006; 200: 184–90.

144. Kochanek PM, Vagni VA, Janesko KL, Washington CB, *et al.* Adenosine A1 receptor knockout mice develop lethal status epilepticus after experimental traumatic brain injury. J Cereb Blood Flow Metab 2006; 26:565–75.

145. Li T, Steinbeck JA, Lusardi T, Koch P, *et al*. Suppression of kindling epileptogenesis by adenosine releasing stem cell-derived brain implants, Brain 2007; 130: 1276–88.

146. Masino SA, Geiger JD. Are purines mediators of the anticonvulsant / neuroprotective effects of ketogenic diets? Trends Neurosci 2008; 31: 273–78.

147. Glass M, Faull RL, Bullock JY, Jansen K, *et al.,* Loss of A1 adenosine receptors in human temporal lobe epilepsy. Brain Res 1996; 710: 56–68.

148. Ochiishi T, Takita M, Ikemoto M, Nakata H, *et al.,* Immunohistochemical analysis on the role of adenosine A1 receptors in epilepsy, NeuroReport 1999; 10 3535–41.

149. Cunha RA, Ferré S, Vaugeois JM, Chen JF. Potential therapeutic interest of adenosine A2A receptors in psychiatric disorders. Curr Pharm Des 2008; 14: 1512–24.

150. Correa M, Font L. Is there a major role for adenosine A2A receptors in anxiety? Front Biosci 2008; 13 4058–70.

151. Boulenger JP, Uhde TW. Caffeine consumption and anxiety: preliminary results of a survey comparing patients with anxiety disorders and normal controls, Psychopharmacol Bull 1982; 18: 53–7.

152. Uhde TW. Anxiety and growth disturbance: is there a connection? A review of biological studies in social phobia. J Clin Psychiatry 1994; 55: 17–27.

153. Newman F, Stein MB, Trettau JR, Coppola R, *et al.* Quantitative electroencephalographic effects of caffeine in panic disorder. Psychiatry Res 1992; 45: 105–13.

154. Tancer ME, Stein MB, Uhde TW. Lactic acid response to caffeine in panic disorder: comparison with social phobics and normal controls. Anxiety 1994; 1: 138–40.

155. Mystkowski JL, Mineka S, Vernon LL, Zinbarg RE. Changes in caffeine states enhance return of fear in spider phobia. J Consult Clin Psychol 2003; 71:243–50.

156. Gomes CV, Kaster MP, Tomé AR, Agostinho PM. *et al.* Adenosine receptors and brain diseases: Neuroprotection and neurodegeneration. Biochimica et Biophysica Acta 2011; 180: 1380–99

157. Hunter AM, Balleine BW, Minor TR. Helplessness and escape performance: glutamate-adenosine interactions in the frontal cortex. Behav Neurosci 2003; 117:123–35.

158. Minor TR, Winslow JL, Chang WC. Stress and adenosine. II. Adenosine analogs mimic the effect of inescapable shock on shuttle-escape performance in rats. Behav Neurosci 1994; 108:265–76.

159. Woodson JC, Minor TR, Job RF. Inhibition of adenosine deaminase by erythro- 9-(2-hydroxy-3-nonyl)adenine (EHNA) mimics the effect of inescapable shock on escape learning in rats. Behav Neurosci 1998; 112:399–409.

160. Kulkarni SK, Mehta AK. Purine nucleoside-mediated immobility in mice: reversal by antidepressants. Psychopharmacol 1985; 85:460–63.

161. Kaster MP, Rosa AO, Rosso MM, Goulart EC, *et al.* Adenosine administration produces an antidepressant-like effect in mice: evidence for the involvement of A1 and A2A receptors. Neurosci Lett 2004; 355:21–4.

162. Kaster MP, Rosa AO, Santos ARS, Rodrigues ALS. Involvement of nitric oxide-cGMP pathway in the antidepressant-like effects of adenosine in the forced swimming test. Int J Neuropsychopharmacol 2005; 8:601–6.

163. Kaster MP, Budni J, Santos ARS, Rodrigues ALS, Pharmacological evidence for the involvement of the opioid system in the antidepressant-like effect of adenosine in the mouse forced swimming test. Eur J Pharmacol 2007; 576: 91–8.

164. El Yacoubi M, Ledent C, Parmentier M, Bertorelli R, *et al.* Adenosine A2A receptor antagonists are potential antidepressants: evidence based on pharmacology and A2A receptor knockout mice. Br J Pharmacol 2001; 134:68–77.

165. El Yacoubi M, Costentin J, Vaugeois JM. Adenosine A2A receptors and depression. Neurology 2003; 61: S82–7.

166. Cunha GMA, Canas PM, Oliveira CR, Cunha RA. Increased density and synapto-protective effect of adenosine A2A receptors upon sub-chronic restraint stress. Neuroscience 2006; 141:1775–81.

167. de Kloet ER, Joëls M, Holsboer F. Stress and the brain: from adaptation to disease. Nat Rev Neurosci; 6:463–75.

168. Deckert J, Gleiter CH. Adenosinergic psychopharmaceuticals? Trends Pharmacol Sci 1989; 10: 99–100.

169. Barcellos CK, Schetinger MR, Dias RD, Sarkis JJ. In vitro effect of central nervous system active drugs on the ATPase-ADPase activity and acetylcholinesterase activity from cerebral cortex of adult rats. Gen Pharmacol 1998; 31:563–7.

170. Greene RW, Haas HL. The electrophysiology of adenosine in the mammalian central nervous system. Prog Neurobiol 1991; 36:329–41.

171. Dunwiddie TV, Masino SA The role and regulation of adenosine in the central nervous system. Ann Rev Neurosci 2001; 24**:** 31-55.

172. Evans WC. Trease and Evans' Pharmacognosy, 16th edition: Saunders Elsevier Ltd; 2009.

173. Singleton VL, Orthofer R, Lamuela-Ravent´os RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol 1998; 299: 152–78

174. Chang C. Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal 2002; 10: 178-82.

175. OECD/OCDE 423. OECD web. Consulted 22 May 2011.

http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECD GL423.pdf.

176. Wheeler CR, Salzman JA, Elsayed NM, Omaye ST, *et al.,* Automate assays for superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activity. Anal Biochem 1990; 184: 193-9.

177. Wu X, Zhao J, Zhang M, Li F, *et al*. Sedative, hypnotic and anticonvulsant activities of ethanol fraction from *Rhizoma pinelliae* preparatum. J Ethnopharmacol 2011; 135: 325-9.

178. Choudhary N, Bijjem KRV, Kalia AN. Antiepileptic potential of flavonoids fraction from the leaves of *Anisomeles malabarica*. J Ethnopharmacol 2011: 135; 238 -42.

179. Saravana Babu C, Ramanathan M. Pre-ischemic treatment with memantine reversed the neurochemical and behavioural parameters but not energy metabolites in middle cerebral artery occluded rats. Pharmacol Biochem Behav 2009; 92: 424–32

180. Brown JE, Rice-Evans CA. Luteolin-rich artichoke extract protect low density lipoprotein from oxidation *in vitro*. Free Radi Res 1998; 29: 247-55.

181. Joyce DA. Oxygen radicals in disease. Adv Drug React Bull 1987; 127:476-9.

182. Rice E, Miller CA, Paganga G. Free radical scavenging activity of plant polyphenolic. Gen Trends Plant Sci 1997; 2:152-9

184. Jäger AK, Saaby L. Flavonoids and the CNS. Molecules 2011; 16: 1471-85

185. Ameringen MV, Mancini C, Pipe B, Bennett M. Antiepileptic drugs in the treatment of anxiety disorders. Drugs 2004; 64: 2199-220.

186. Herrera-Ruiz M, González-Carranza A, Zamilpa A, Jiménez-Ferrer E, *et al*. The standardized extract of Loeselia mexicana possesses anxiolytic activity through the γ-amino butyric acid mechanism. J Ethnopharmacol 2011; 18:138(2):261-7.

187. Kostowski W, Plaznik A, Stefanski R. Intra-hippocampal buspirone in animal models of anxiety. Eur J Pharmacol 1989; 68: 393–6.

188. McNamara RK, Skelton RW. Effects of intracranial infusions of chlordiazepoxide on spatial learning in the Morris water maze I. Neuroanatomical specificity. Behav Brain Res 1993; 59: 175–91.

189. Dietrich DE, Emrich HM. The use of anticonvulsants to augment antidepressant medication. J Clin Psychiatry. 1998; 59:(5)51-8.

190. Levine JD. New directions in pain research: molecules to maladies. Neuron 1998; 20:649–54.

191. Onaivi ES, Maquire PA, Tsai NF, Davies MF, *et al*. Comparison of behavioral and central BDZ binding profile in three rat lines. Pharmacol Biochem Behav 1992; 43: 825–31.

192. Wolffgramm J, Mikolaiczyk C, Coper H. Acute and subchronic benzodiazepine-barbiturate-interactions on behaviour and physiological responses of the mouse. Naunyn Sch Arc Pharmacol 1994; 349: 279–86.

193. Herberlain H, Tscheirsch KP, Schazer HI. Flavonoids from *Leptospermum scoparium* with affinity to the benzodiazepine receptors characterized by structure activity relationship and in vivo studies of plant extract. Pharmazie 1994; 49: 912-22.

194. Fernández SP., Wasowski C, Paladini A, Marder M, Synergistic interaction between hesperidin, a natural flavonoid, and diazepam. Eur J Pharmacol 2005; 512: 189–98

PUBLICATIONS

List of Publications

- Kalaiyarasi C, Karthika K, Lalithkumar P, Ragupathi G, Saravanan S. *In vitro* antioxidant activity of various fractions of Cassia fistula L. pods. Journal of Pharmacognosy and Phytochemistry 2014; 3(4): 73-76

- Kalaiyarasi C, Lalithkumar P, Ragupathi G, Karthika K, Ramanathan M, Saravanan S. Anti-nociceptive activity of ethyl acetate fraction of Cassia fistula L. pods in experimental animal models. International Journal of Natural Products Research 2014; 4(3): 72-76.

- Kalaiyarasi C, Karthika K, Ragupathi G, Saravanan S. Anticonvulsant and anxiolyic activities of ethyl acetate fraction of *Cassia fistula* Linn. pods in mice. Phcog Commn 2015; 5(1): 48-55

Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8196
JPP 2014; 3(4): 73-76
Received: 09-09-2014
Accepted: 02-10-2014

Chinnasamy Kalaiyarasi
Department of Pharmacology, PSG College of Pharmacy, Coimbatore, Tamil Nadu, India.

Kaliavaradhan Karthika
Department of Pharmacology, PSG College of Pharmacy, Coimbatore, Tamil Nadu, India.

Padi Lalithkumar
Department of Pharmacology, PSG College of Pharmacy, Coimbatore, Tamil Nadu, India.

Govindaraj Ragupathi
Department of Pharmacology, PSG College of Pharmacy, Coimbatore, Tamil Nadu, India.

Sampath Saravanan
Department of Pharmaceutical Analysis, The Erode College of Pharmacy, Erode, Tamil Nadu, India.

Correspondence:
Sampath Saravanan
Department of Pharmaceutical Analysis, The Erode College of Pharmacy, Erode, Tamilnadu, India

In vitro anti-oxidant activity of various solvent fractions of *Cassia fistula* L. pods

Chinnasamy Kalaiyarasi, Kaliavaradhan Karthika, Padi Lalithkumar, Govindaraj Ragupathi, Sampath Saravanan

Abstract
Cassia fistula L. is a common medicinal plant has been used for the treatment of various ailments such as convulsion, inflammation, pain, infectious diseases etc. The Present study was aimed to estimate the total phenolic and flavonoid content and to examine the antioxidant activity of such as n-hexane (NHF), ethyl acetate (EAF) and aqueous fractions (AQF) of *Cassia fistula* L. pods. The antioxidant activity was assessed by superoxide scavenging and reducing power abilities. The antioxidant potency was found to be decreasing order of EAF, NHF and AQF and had good co-relation with their polyphenolic and flavonoid content. This study shows that *Cassia fistula* L pods can serve as a good source of antioxidants and thus used for preventing oxidative stress related disorders.

Keywords: *Cassia fistula*, Polyphenol estimation, Superoxide scavenging activity, Reducing power assay, Flavonoid content estimation

1. Introduction
Natural antioxidants present in the plant parts such as whole grains, fruits and vegetables are important ingredients of food as they scavenge free radicals [1]. Free radicals are frequently generated in our body during normal cellular metabolism as well as under certain environmental conditions, these radicals are more reactive as they lack of an electron and try to become neutral by accepting an electron from or donate an electron to adjacent molecules and create new free radicals. This in turn initiates a chain of reaction that can damage several molecules through accepting or donating an electron leads to degenerative diseases. The phytochemicals such as phenolic acids, polyphenols and flavonoids are generally known as anti-oxidants and their beneficial effects in several chronic ailments have been reported [2]. In addition, the synthetic anti-oxidants available at present are known to produce negative health effects. Thus, the search for antioxidants from plant source has been increased recently. Plants, owing to their healing potential have been greatly by the practitioners of Indian traditional medicinal system [3].

Cassia fistula L. commonly known as Indian laburnum belonging to the family Caesalpiniaceae has been widely used in traditional medicine for worm infestation, wound healing, convulsion, antipyretic, haematemesis, pruritus, intestinal disorders, leucoderma, diabetes, analgesic, antipyretic and as laxative [4]. The various medicinal implications have been mainly attributed due to the presence of alkaloids, triterpene derivatives, anthraquinone derivatives, polyphenolics, comprising flavonoids, catechins and proanthocyanidins [5-7]. The phytoconstituents, particularly polyphenols and flavonoids have been shown to possess anti-oxidant activity [8]. Hence, the present study was aimed to estimate the amount of polyphenols and flavonoids present and to study the antioxidant activity of various fractions of *Cassia fistula* pods.

2. Materials and Methods
2.1 Collection of plant material and authentication
With the support of professional collectors, pods of *Cassia fistula* were collected from the surroundings of Coimbatore and shade dried. The plant material was taxonomically identified by a Scientist F (Dr. G.V.S. Murthy), Botanical survey of India, Coimbatore, Tamil Nadu, India and the voucher specimen BSI/SRC/5/23/2011-12/Tech 782 was retained in our laboratory for future reference.

2.2 Preparation of hydroalcoholic extract

Fresh samples were air-dried and ground, yielding 1500 g of powder. From this, 500 g of powder was defatted with petroleum ether (40–60 ˚C) for 6 hours [9] and filtered. The resultant marc dried and extracted with a mixture of ethanol (700 ml): water (300 ml) (70:30) as solvent for 24 h at room temperature and placed in a rotary shaker to get hydro-alcoholic extract by maceration method for 2 days. The extract was filtered by using Whatman No.1 filter paper and the filtrate was taken. The extraction was repeated two times and the filtered hydro-ethanolic extracts were mixed and evaporated under reduced pressure (56 g) and denoted as HAE.

2.3 Fractionation of hydroalcoholic extract of *Cassia fistula L.*

50.0 g of HAE was dissolved in 200 mL of methanol/water (7:3). N-Hexane, ethyl acetate and aqueous fractions were made from the solution obtained through liquid/liquid solvent partition of increasing polarity. From each fraction, the solvent was evaporated in rotary evaporator and dried. Fractions were designated as follows – hexane fraction (NHF), ethyl acetate fraction (EAF) – aqueous fraction (AQF), dried to constant weight and stored at −10 ◦C until used for experiments.

2.4 Total phenolic content estimation.

The total phenolic content of all fractions was estimated by using Folin catechu reagent and measured at 765 nm using gallic acid as standard [10]. Aliquots of fractions or gallic acid were mixed with 0.5 ml of Folin catechu reagent (diluted 1:1 with water) and 2.5 ml of 20% aqueous sodium carbonate and allowed to stand for 45 min in dark room and absorbance was measured by spectrophotometer. The total phenolic content in each fraction was calculated as gallic acid equivalent from the calibration curve.

2.5 Determination of total flavonoid content

Flavonoid content in all the three fractions was determined by aluminium chloride colorimetric method [11]. Aliquot quantity of fractions was diluted with 1.50 ml of distilled water and 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 the resulting reaction mixture was measured at 415 nm UV spectrophotometer. Quantification of flavonoids was done 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**.**

2.6 Superoxide radical scavenging activity

Measurement of superoxide anion scavenging activity of fractions was based on the method described by [12]. Phenazine methosulphate (PMS)–nicotinamide adenine dinucleotide (NADH) system generates superoxide radicals by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radicals were generated in 3 ml of Tris–HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 µM) solution, 1 ml NADH (78 µM) solution and 0, 50, 100, 150, 200, 250 µg/ml of fractions were mixed. The reaction was started by adding 1 ml of PMS solution (10 µM) to the mixture. The reaction mixture was incubated at 25 ˚C for 5 min, and the absorbance at 560 nm in a UV-spectrophotometer was measured against blank samples. Decrease in absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula.

$$\text{Percentage inhibition} = [A_0 - A1/A_0] \text{ X } 100$$

Where, A_0 was the absorbance of control (blank), and A_1 was the absorbance of EAF/ NHF/ AQF or standard.

2.7 Reductive ability

Reducing power ability was measured by mixing 50, 100, 150, 200 and 250 µg/ml of EAF, NHF and AGF prepared with distilled water and mixed with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 500 ˚C for 30 minutes. Trichloroacetic acid (2.5 ml, 10%) (TCA) were added to the mixture and centrifuged for 10 min at 3000 RPM and 2.5 ml from the upper portions were diluted with 2.5 ml water and shaken with 0.5 ml fresh 0.1%, ferric chloride. The absorbance was measured at 700 nm using UV-spectrophotometer. The blank was prepared as above, but contained water instead of the samples. Increase in the absorbance by reaction mixture indicates increased reducing power. All experiments were done in triplicate using butylated hydroxy toluene as standard [13].

3. Results and discussion

3.1 Polyphenolic content estimation

EAF and NHF were found to contain more polyphenols than AQF. The amount of phenolic constituent present in EAF was found to 61.5 µg/mg in terms of gallic acid equivalent and in NHF and AQF it was found to 53.5 µg/mg and 13.5 µg/mg gallic acid equivalents respectively. Phenolic compounds are one of the most significant phytoconstituent because their hydroxyl groups confer scavenging ability [14] and they are known as powerful chain breaking antioxidant [15]. The calibration curve of standard polyphenol, gallic acid is shown in figure 1

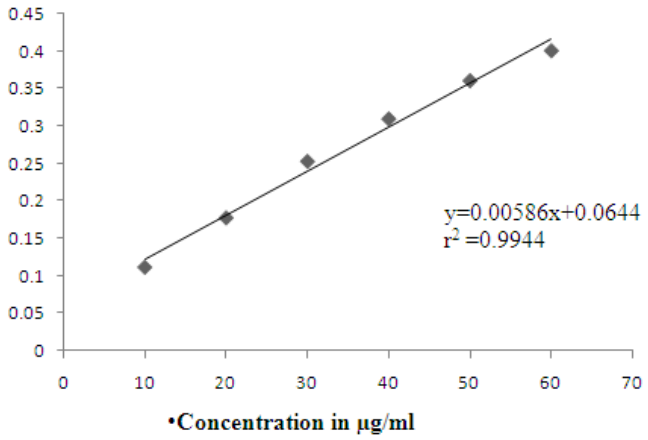


Fig 1: Standard curve of gallic acid

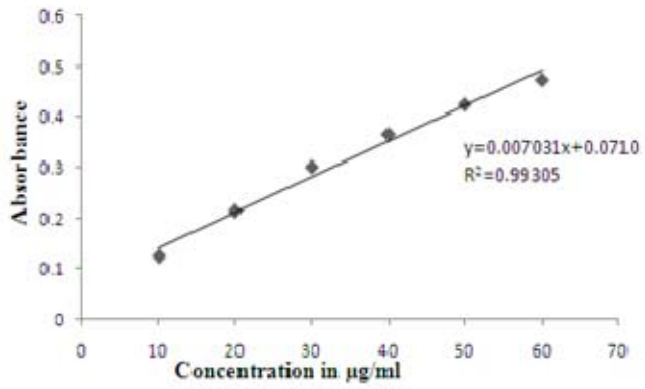


Fig 2: Standard curve of quercetin

3.2 Flavonoid content estimation

Flavonoids are considered to be the most important group of polyphenolic compounds and have been shown to exhibit several pharmacological activities such as neuroprotective, cardioprotective, anti-diabetic, anti-inflammatory, anti-hepatotoxic, anti-ulcer, anti-allergic, anti-viral and anticancer activities [16]. Their phenolic hydroxyl groups effectively scavenge the O_2 free radicals and considered to be most potent antioxidants [17]. The total flavonoids content of CAF, NHF and AQF was estimated to be 30.5, 25.5 and 5.5 mg/mg respectively in terms of quercetin equivalent.

3.3 Reducing power ability

The ability with which a natural anti-oxidant to donate electron is designated as reducing power activity [18-19]. Several research reports stated the direct correlation between the anti-oxidant activity and reducing power ability of plant extracts [1, 18]. The order reducing power ability was found as follows BHT>EAF>NHF>AQF. The reducing power ability of fractions was well correlated with their polyphenolic and flavonoid content. The results were shown in table 1.

Table 1: Reductive ability of various fractions of *Cassia fistula* L. pods

Sl. NO	Conc µg/mg	Absorbance (Mean±SEM)*			
		EAF	NHF	AQF	BHT
1	20	0.183±0.0004	0.178±0.0102	0.083±0.0007	0.193±0.0050
2	40	0.221±0.0030	0.189±0.0180	0.100±0.0069	0.247±0.0071
3	60	0.254±0.0013	0.239±0.0012	0.128±0.0074	0.260±0.00038
4	80	0.278±0.0014	0.260±0.0029	0.150±0.0032	0.386±0.0006
5	100	0.348±0.0017	0.329±0.0017	0.193±0.0074	0.569±0.00189

¥ Data expressed as mean±SEM of three observations EAF= Ethyl acetate fraction, NHF= n-hexane fraction, AQF= aqueous fraction of hydroalcoholic extract of *Cassia fistula* L. pods. BHT= butylated hydroxyl toluene

3.4 Superoxide scavenging activity

The relationship with reducing power and anti-oxidant activity has long been established [13, 20]. Among all the fractions examined in our study, the EAF showed a higher reducing power than the rest of the fractions. The lower anti-oxidant activity of AQ could be attributed to the presence of reducing sugars [21] as reducing sugars have been shown to generate prooxidants and that may nullify the anti-oxidant activity of other phytoconstituents [22].

Table 2: Superoxide scavenging activity of various fractions of *Cassia fistula* L. pods

EAF				NHF			AQF		
S.No	Conc (µg/ml)	% Inhibition	IC_{50}	Conc (µg/ml)	% Inhibition	IC_{50}	Conc (µg/ml)	% Inhibition	IC_{50}
1	20	17.46	51.38	20	14.25	53.23	100	5.99	440.52
2	40	33.46		40	28.92		200	18.79	
3	60	53.46		60	51.33		300	28.93	
4	80	69.46		80	62.53		400	44.93	
5	100	89.19		100	81.46		500	60.39	

¥ Data expressed as average of three observations EAF= Ethyl acetate fraction, NHF= n-hexane fraction, AQF= aqueous fraction of hydroalcoholic extract of *Cassia fistula* L. pods. BHT= butylated hydroxyl toluene

4. Conclusion

The Present study clearly states the significant anti-oxidant activity of EAF and NHF and among these the EAF showed the superior and AQF showed the inferior anti-oxidant potential. This study further reveals that the presence of polyphenols and flavonoids could play a major role in the anti-oxidant activity of the EAF and AQF. The inferior anti-oxidant potential of AQF may be due to the presence of negligible amount of polyphenols and flavonoids or may be due to the presence of pro-oxidants such as reducing sugars. Hence, the

findings of the present study clearly reveal the anti-oxidant activity of *Cassia fistula* L. pods. However, further study focused on the isolation of active anti-oxidant molecule and *in vivo* studies could be useful in understanding the mechanism of action of anti-oxidants.

5. Acknowledgement
The authors are thankful to Dr. Dinesh, St James College of Pharmacy, Kerala, India for his valuable help in technical aspects.

6. Conflicts of interest
The authors declare no conflicts of interest.

7. References
1. Deshpande S, Kewatkar SM, Paithankar VV. *In vitro* antioxidant activity of different fraction of roots of *Cassia auriculata* Linn. Drug Invention Today 2013; 5:164 -168
2. Brown JE, Rice-Evans CA. Luteolin-rich artichoke extract protect low density lipoprotein from oxidation *in vitro*. Free Radicals Research 1998; 29:247-255.
3. Jayaprakash GK, Rao LJ. Phenolic constituents from lichen *Parmotrema stuppeum*. Food Control 2000; 56:1018-1022.
4. Neelam C, Ranjan B, Komal S, Nootan C. Review on *Cassia fistula.* International Journal of Research in Ayurveda and Pharmacy 2011; 2(2):426-430.
5. Agrawal GD, Rizvi SAI, Gupta PC, Tewari JD. Structure of fistulic acid a new colouring matter from the pods of *Cassia fistula*. Planta Medica 1972; 2:150-155.
6. Morimoto S, Nonaka G, Chen R. Tannins and related compounds. LXI. Isolation and structures of novel bi-and triflavonoids from the leaves of *Cassia fistula* L. Chem Pharmacology Bulletin 1988; 36:39-47.
7. Kashiwada Y, Toshika K, Chen R, Nonaka G, Nishioka I. Tannins and related compounds. XCIII. Occurrence of enantiomeric proanthocyanidins in the Leguminosae plants, *Cassia fistula* L. *Cassia Javanica* L. Chemical and Pharmaceutical Bulletin 1996; 38:888-893.
8. Luximon-Ramma A, Bahorun T, Soobrattee MA, Aruoma OI. Antioxidant activities of phenolic, proanthocyanidins, and flavonoid components in extracts of *Cassia fistula*. Journal of Agricultural and Food Chemistry 2002; 50:5042-5047.
9. Choudhary N, Bijjem KRV, Kalia AN. Antiepileptic potential of flavonoids fraction from the leaves of *Anisomeles malabarica*. Journal of Ethnopharmacology 2011; 135:238-242.
10. Singleton VL, Orthofer R, Lamuela-Ravent´os RM, Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods in Enzymology 1998; 299:152-178.
11. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of Food Drug Analysis 2002; 10:178-182.
12. Gulcin I, Kufreyvioglu OI, Oktay M, Buyukokuro. Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.). Journal of Ethnopharmacology 2004; 90:205-215.
13. Yen CC, Duh PD, Chuang DY. Antioxidant activity of anthraquinones and anthrone. Food Chemistry 2000; 70:437-441.
14. Hatano T, Edamatsu R, Mori A. Effect of interaction of tannins and related polyphenols on superoxide anion radical and on DPPH radical. Chemical and Pharmaceutical Bulletin 1989; 37:2016-2021
15. Shahidi F, Wanasundara PKJPD. Phenolic antioxidants. Food Science and Nutrition 1992; 32:67-103
16. Umamaheswari, Chatterjee TK. *In vitro* antioxidant activities of the fractions of *Coccinia grandis* L. leaf extract. African Journal of Traditional Complementary and Alternate Medicine 2008; 5:61-73.
17. Cao G, Sofic E, Prior RL. Antioxidant and pro-oxidative behavior of flavonoids: structure activity relationships. Free Radical Biology and Medicine 1997; 22:749-760.
18. Yildirim A, Mavi A, Oktay M, Kara AA, Algur OF, Bilaloglu V. Comparison of antioxidant and antimicrobial activities of tilia (*Tilia argentea* Desf. Ex. D.C.) sage (*Salvia triloba* L.) and black tea (*Camellia sinensis* L.) extracts. Journal of Agricultural and Food Chemistry. 2000; 48:5030-5034.
19. Dorman HJD, Peltoketo A, Hiltunen R, Tikkanen MJ. Characterisation of the antioxidant properties of deodourisation aqueous extracts from selected Lamiaceae Herbs. Food Chemistry 2003; 83:255-256.
20. Yen CC, Duh PD. Antioxidant properties of methanolic extracts from peanut hull. Journal of the American Oil Chemist's Society 1993; 70:383-386.
21. Barthakur NN, Arnold NP, Alli I. The Indian laburnum (*Cassia fistula* L.) fruit: an analysis of its chemical constituents. Plant Foods for Human Nutrition 1995; 47:55-62.
22. Siddhuraju P, Mohan PS, Becker K. Studies on the antioxidant activity of Indian laburnum (*Cassia fistula* L.): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. Journal of Agricultural and Food Chemistry 2002; 79:61-67.

Available online at http://www.urpjournals.com

International Journal of Natural Products Research

ISSN: 2249-0353

Original Article

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

Chinnasamy Kalaiyarasi[a], Padi Lalithkumar, Govindaraj Ragupathi[a], Kaliavaradhan Karthika[a], Muthiah Ramanahan[a], Sampath Saravanan[b,*]

[a] Department of Pharmacology, PSG College of Pharmacy, Coimbatore, Tamilnadu, India
[b] Department of Pharmaceutical Analysis, The Erode College of Pharmacy, Erode, Tamilnadu, India
E-mail addresses: saravecp@yahoo.co.in (S. Saravanan)

Received 09 July 2014; Accepted 25 July 2014

Abstract

The objective of the present study was to evaluate the anti-nociceptive activity of ethylacetate 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.

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 flavonoides, 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 flavonoides 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 diclofenenac 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 (2.5cm 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 Dunnett'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

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

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.=intra peritoneal, p.o.= per oral

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

S.No	Treatment	Reaction time in seconds				
		pretreatment	30 min	60 min	90 min	120 min
1	Vehicle 10ml/kg, p.o.	3.8±0.05	3.6±0.08	4.2±0.03	4.6±0.09	4.8±0.08
2	Morphine 1.5mg/kg, i.p.	4.1±0.06	12.6±0.03*	17.8±0.08*	16.2±0.1*	14.8±0.2*
3	EAFCF 50mg/kg, p.o.	5.1±0.09	8.1±0.08*	10.4±0.08*	9.7±0.08*1	7.2±0.08*
4	EAFCF 100mg/kg, p.o	5.2±0.10	10.8±0.05*13	13.1±0.1*	2.5±0.3*17.	10.6±0.09*
5	EAFCF 200mg/kg, p.o.	4.8±0.12	.2±0.4*	18.4±0.2*	1±0.1*	15.1±0.07*

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.=intra peritoneal, p.o.= per oral

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

S.No	Treatment	Reaction time in seconds				
		pretreatment	30 min	60 min	90 min	120 min
1	Vehicle 10ml/kg, p.o.	3.8±0.05	3.6±0.01	4.43±0.04	4.7±0.09	3.58±0.08
2	Morphine 1.5mg/kg, i.p.	3.9±0.07	12.6±0.03 *	17.8±0.8 *	17.2±0.1 *	10.8±0.2 *
3	EAFCF 50mg/kg, p.o.	4.5±0.1	5.1±0.08	6.4±0.08	5.7±0.08	5.2±0.08
4	EAFCF 100mg/kg, p.o	4.9±0.20	9.8±0.05 *	12.1±0.1 *	11.5±0.3 *	9.6±0.09 *
5	EAFCF 200mg/kg, p.o.	5.6±0.22	14.2±0.4 *	16.4±0.2 *	16.1±0.1 *	12.1±0.07 *

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.=intra peritoneal, p.o.= per oral

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

S.No	Treatment	Hind paw licking			
		Early phase	% inhibition	Late phase	% inhibition
1	Vehicle 10ml/kg, p.o.	113.6±0.91	----	123.8±0.89	----
2	Morphine 5mg/kg, i.p.	42.6±0.03 *	51.60	0	100
3	EAFCF 50mg/kg, p.o.	102.1±1.8	10.12	98.8±2.2	13.02
4	EAFCF 100mg/kg, p.o	59.8±1.25 *	47.35	61.5±2.23 *	45.8
5	EAFCF 200mg/kg, p.o.	40.6±0.4 *	64.2	22.1±3.1 *	80.54

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

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

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

Each value represent 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.=intra peritoneal, p.o.= per oral

effect as that of EAFCF 200mg/kg. EAFCF 50mg/kg was found to 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

The authors are grateful to Dr. Shailendra Gurav, Professor in Pharmacy, Government College of Pharmacy, Karad for his valuable guidance in analyzing the phytochemical constituents.

Abbreviations

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

References

1. C.C. Barua, J.D. Roy, B. Buragohain , A.G. Barua , P.B. Borah , M. Lahkar, Analgesic and anti-nociceptive activity of hydroethanolic extract of *Drymaria cordata* Willd, Indian J. Pharmacol. 43(2011) 6-12
2. F. Ahmad, R.A. Khan, S, Rasheed, Study of analgesic and anti-inflammatory activity from plant extracts of *Lactuca scarliola* and *Artemsia absinthium*, J Int Acad Sci. 5(1992)111-114
3. S.A. Bhalerao, T.S. Kelkar, Traditional medicinal uses, phytochemical profile and pharmacological activities of *Cassia fistula* Linn, Int. Res. J. Biol. Sci. 1 (2012)79-84
4. M. Leticia, C. Asseleih, O. Hernandez, J.R. Sanchez, Seasonal variations in the content of sennosides in leaves and pods of two *Cassia fistula* populations, Phytochemistry, 29(1990) 3095-3099.
5. M.A. Nagpal, S. Rahar, G .Shah, G. Swami, R. Kapoor, Phytochemical investigation of methanolic extract of *Cassia fistula* leaves, Pharmacog. J. 3(2011) 61-69.
6. W. Zhao, X. Zeng, T.Zhang, L. Wang, G. Yang, Y. Chen, Q. Hu, M. Miao, Flavonoids from the bark and

stems of *Cassia fistula* and their anti-tobacco mosaic virus activities, Phytochem. Lett. 6 (2013) 179-182.
7. U.K. Mazumdar, M. Gupta, N. Rath, CNS activities of *Cassia fistula* in mice, Phytotherapy Res. 12(1998) 520-522.
8. P. Siddhuraju, P.S. Mohan, K. Becker, Studies on the antioxidant activity of Indian Laburnum (*Cassia fistula* L.): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp, Food Chem. 79 (2002) 61-67.
9. M. Senthilkumar, R. Sripriya, H. Vijayaraghavan, P. Sehgal, Wound healing potential of *Cassia fistula* on infected albino rat model, J Surg. Res. 131 (2006) 283-289
10. V. Duraipandiyan, S. Ignacimuthu, Antibacterial and antifungal activity of *Cassia fistula* L.: An ethnomedicinal plant, J. Ethnopharmacol. 112 (2007) 590-594.
11. V. Duraipandiyan, A. Baskar, S. Ignacimuthu, C. Muthukumar, N.A. Al-Harbi, Anticancer activity of Rhein isolated from *Cassia fistula* L. flower, Asian Pac. J. Trop Dis. 2 (2012) S517-S523.
12. A. Chauhan, M. Agarwal, Evaluating the antifertility potential of an aqueous extract from *Cassia fistula* seeds in male rats, Fertil. Steril. 93(2010) 1706-1710.
13. T. Bhakta, P.K. Mukherjee, K. Mukherjee, S. Banerjee, S.C. Mandal, T.K. Maity, M. Pal, B.P. Saha, Evaluation of hepatoprotective activity of *Cassia fistula* leaf extract, J. Ethnopharmacol. 66 (1999) 277-282.
14. P. Daisy, K. Balasubramanian, M. Rajalakshmi, J. Eliza, J. Selvaraj, Insulin mimetic impact of Catechin isolated from *Cassia fistula* on the glucose oxidation and molecular mechanisms of glucose uptake on Streptozotocin-induced diabetic Wistar rats, Phytomedicine. 17(2010) 28-36.
15. S. Patwardhan , G. Sakhare , A. Singhai , K. Jain , R. Somani , Evaluation of analgesic activity of *Cassia fistula* on albino mice, Pharmacologyonline 2(2009) 887-893
16. J.I. Khan, B. Nahar, M.A. Jakaria , S. Rahman, M.H. Chowdhury, M. Rahmatullah, An Evaluation of antihyperglycemic and antinociceptive effects of methanol extract of *Cassia fistula L*. (Fabaceae) leaves in swiss albino mice, Adv. Nat. App. Sci. 4 (2010) 305-310.
17. N. Choudhary, K.R.V. Bijjem, A.N. Kalia, Antiepileptic potential of flavonoids fraction from the leaves of *Anisomeles malabarica*, J. Ethnopharmacol. 135 (2011) 238 -242.
18. M.E. Pascual, M.E. Carretero, K.V. Slowing, A. Villar, Simplified screening by thin layer chromatography (TLC) of plant drugs, Pharm. Biol. 40(2002) 139-143.
19. E. Woode, P. Amoateng, C. Ansah, M. Duwiejua, Anti-nociceptive effects of an ethanolic extract of the whole plant of *Synedrella nodiflora* (L.) Gaertn in Mice: Involvement of adenosinergic mechanisms, J. Pharmacol. Toxicol. 4 (2009) 17-29.
20. E.F. Pietrovski , K.A. Rosa, V.A. Facundo, K. Rios, M.C. Marques, A. Santos, Antinociceptive properties of the ethanolic extract and of the triterpene 3 beta,6 beta,16 beta-trihidroxilup-20(29)-ene obtained from the flowers of *Combretum leprosum* in mice, Pharmacol .Biochem .Behav. 83(2006) 90-9.
21. W. Toma, J.S. Graciosa, C.A. Hiruma-Lima, F.D.P. Andrade, W. Vilegas, A.R.M.S. Brita, Evaluation of the analgesic and antiedematogenic activities of *Quassia amara* bark extract, J. Ethnopharmacol. 85 (2003) 19–23.
22. M. Shibata, T. Ohkubo, H. Takahashi, R. Inoki, Modified formalin test: characteristic biphasic pain response, Pain. 38 (1989) 347-352.
23. M.M. De Souza, M.A. Pereira, J.V. Ardenghi, T.C. Mora, L.F. Bresciani, R.A. Yunes, F.C. Delle Monache, V. Cechinel-Filho, Filicene obtained from *Adiantum cuneatum* interacts with cholinergic, dopaminergic, glutamatergic, GABAergic, and tachykinergic systems to exert antinociceptive effect in mice, Pharmacol. Biochem. Behav. 93 (2009) 40–46.
24. K. Srinivasan, S. Muruganandan, J. Lal, S. Chandra, S.K. Tandan, V. Raviprakash, D. Kumar, Anti-nociceptive and anti-pyretic activities of *Pongamia pinnata* leaves, Phytotherapy Res.17 (2003) 259–264.
25. Y. Jinsmaa, Y. Fujitab, K. Shiotanib, A. Miyazakic, T. Lib, Y. Tsuda, Y. Okada, A. Amboe, Y. Sasakie, S.D. Bryanta, L.H. Lazarus, Differentiation of opioid receptor preference by [Dmt1]endomor-phin-2-mediated antinociception in the mouse, Eur. J. Pharmacol. 509 (2005) 37–42.
26. A. Tjolsen, O.G. Berge, S. Hunskaar, J.H. Rosland, K. Hole, The formalin test: an evaluation of the method, Pain 51 (1992) 5–17.

Source of support: Nil; Conflict of interest: None declared

Pharmacognosy Communications
Volume 5 | Issue 1 | Jan-Mar 2015
www.phcogcommn.org

Research Article

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

Chinnasamy Kalaiyarasi[a], Kalivaradhan Karthika[a], Govindharaj Ragupathi[a], Sampath Saravanan[b*]

[a] *Department of Pharmacology, PSG College of Pharmacy, Coimbatore, Tamilnadu, India.*
[b] *Department of Pharmaceutical Analysis, The Erode College of Pharmacy, Erode, Tamilnadu, India.*

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-

Corresponding Address
Sampath Saravanan
Department of Pharmaceutical Analysis,
The Erode College of Pharmacy,
Erode, Tamilnadu. India., Erode-India
E Mail: saravecp@yahoo.co.in
Dol : 10.5530/pc.2015.1.8

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

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.		

($p<0.01$), indicating anxiolytic activity. However, it did not significantly ($p>0.05$) change the total number of arm entries (Table 2).

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 anti-epileptic 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.

ACKNOWLEDGEMENT

Authors are thankful to Dr. Dineshkumar, St James College of Pharmacy, Kerala for his technical help.

CONFLICT OF INTEREST

Authors declare no conflicts of interest

REFERENCES

1. Engel J. A proposed diagnostics scheme for people with epileptic seizures and with epilepsy report of the ILAET task force on classification and terminology. Epilepsia. 2001; 42(6): 796–803.
2. Belzung C, Griebel G. Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res. 2001; 125(1): 141–9.
3. Carlini EA. Plants and the central nervous system. Pharmacol Biochem Behav. 2003; 75(3): 501–12.
4. Rizvi MMA, Irshad M, El Hassadi G, Younis SB. Bioefficacies of *Cassia fistula*: an Indian labrum. Afr J Pharmd Pharmacol. 2009; 3(6): 287–92.
5. Siddhuraju P, Mohan PS, Becker K. Studies on the antioxidant activity of Indian Laburnum (*Cassia fistula* L.): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. Food Chem. 2002; 79(1): 61-7.
6. Bhatnagar M, Vimal S, Vyas Y, Sharma D, Sharma K. Antioxidant activity of fruit pulp powder of *Cassia fistula*. Phcog J. 2010; 2: 219-28.
7. Nayan R, Bhalodia RN, Acharya V, Shukla J. Evaluation of in vitro antioxidant activity of hydroalcoholic seed extratcs of *Cassia fistula* linn. Free Rad. Antiox. 2011; 1(1): 68-76.
8. Mazumdar UK, Gupta M, Rath N. CNS activities of *Cassia fistula* in mice. Phytotherapy Res. 1998; 12(7): 520-2.
9. Senthilkumar M, Sripriya R, Vijayaraghavan H, Sehgal P. Wound healing potential of *Cassia fistula* on infected albino rat model. J. Neurosci Methods. 2006; 131(2): 283-9
10. Duraipandiyan V, Ignacimuth S. Antibacterial and antifungal activity of *Cassia fistula* L.: An ethnomedicinal plant. J Ethnopharmacol. 2007; 112(3): 590-4.
11. Duraipandiyan V, Baskar AA, Ignacimuthu S, Muthukumar C, Al-Harbi NA. Anticancer activity of rhein isolated from *Cassia fistula* L. flower. Asian Pac J Trop Dis. 2012; 2: S517-23.
12. Gupta M, Mazumder UK, Rath N, Mukhopadhyay DK. Antitumor activity of methanolic extract of *Cassia fistula* L. seed against Ehrlich ascites carcinoma. J Ethnopharmacol. 2000; 72(1): 151-6
13. Chauhan A, Agarwal M. Evaluating the anti-fertility potential of an aqueous extract from *Cassia fistula* seeds in male rats. Fertil Steril. 2010; 93: 1706-10.
14. Bhakta T, Mukherjee PK, Mukherjee K, Banerjee S, Mandal SC, Maity TK, et. al. Evaluation of hepatoprotective activity of *Cassia fistula* leaf extract. J Ethnopharmacol. 1999; 66(3): 277-82.
15. Daisy P, Balasubramanian K, Rajalakshmi M, Eliza J, Selvaraj J. Insulin mimetic impact of catechin isolated from *Cassia fistula* on the glucose oxidation and molecular mechanisms of glucose uptake on Streptozotocin-induced diabetic Wistar rats. Phytomedicine. 2010; 17(1): 28-36.
16. Leticia M, Asseleih, C, Hernandez O. Sanchez JR. Seasonal variations in the content of sennosides in leaves and pods of two *Cassia fistula* populations. Phytochemistry. 1990; 29(10): 3095-99.
17. Nagpal MA, Rahar S, Shah G, Swami G, Kapoor R. Phytochemical investigation of methanolic extract of *Cassia fistula* leaves. Phcog J. 2011; 3: 61-9.
18. Zhao W, Zen X, Zhang T, Wang L, Yang G, Chen Y, Hu Q, Miao M. Flavonoids from the bark and stems of *Cassia fistula* and their anti-tobacco mosaic virus activities. Phytochem Lett. 2013; 6(2): 179-82.
19. Choudhary N, Bijjem KRV, Kalia AN. Antiepileptic potential of flavonoids fraction from the leaves of *Anisomeles malabarica*. J Ethnopharmacol. 2011; 135(2): 238 -42.
20. Pascual ME, Carretero ME, Slowing KV, Villar A. Simplified screening by thin layer chromatography (TLC) of plant drugs. Pharm Biol. 2002; 40(2): 139-43.
21. Chang C, Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal. 2002; 10(3): 178-82.
22. Loscher W, Schmidt D. Which animal models should be used in search for new antiepileptic drugs? A proposal based on experimental and clinical considerations. Epilepsy Res. 1988; 2(3): 145 -81.
23. Herrera-Ruiz, M, Jimenez-Ferrer JE, De-Lima TC, Aviles-Montes D, Perez-Garcia D, Gonzaliz-Cortazar M, Tortoriello J. Anxiolytic and antidepressant-like activity of a standardized extract from *Galphimia glauca*. Phytomedicine. 2006; 13(1): 23–8.
24. Wu X, Zhao J, Zhang M, Li F, Zhao T, Yang L. Sedative, hypnotic and anticonvulsant activities of ethanol fraction from *Rhizoma pinelliae* preparatum. J Ethnopharmacol. 2011; 135: 325-9.
25. OECD guideline for testing of chemicals, OECD publishing, test no 423, Acute oral toxicity – Acute toxic classic model. Available at http://www.oecd-ilibrary.org/environment/test-no-423-acute-oral-toxicity-acute-toxic-class-method_9789264071001-en;jsessionid=4ql70faa8bljj.x-oecd-live-01
26. World Health Organization (WHO) Traditional medicine, fact sheet no. 134. Available at: http://www.who.int/mediacentre/factsheets/fs134/en/index.html. ; 2008.
27. Loscher W. Critical review of current animal models of seizure and epilepsy used in discovery and development of new antiepileptic drugs. Seizure. 2011; 20(5): 359-68.
28. Ameringen MV, Mancini C, Pipe B, Bennett M. Antiepileptic drugs in the treatment of anxiety disorders. Drugs. 2004; 64(19): 2199-20.
29. Rodgers RJ, Cao BJ, Dalvi A, Holmes A. Animal model of anxiety: an ethological perspective. Braz J Med Biol Res. 1997; 30: 289–304.
30. Pellow S, Chopin PE, File SE, Briley M. Validation of open: closed arm entries in an elevated plus- maze as a measure of anxiety in the rat. J Neurosci Methods. 1985; 14(3): 149-67.
31. Hanrahan JR, Chebib M, Johnston GA. Flavonoid modulation of GABA (A) receptors. Br J Pharmacol. 2011; 163(2): 234-45.
32. Herrera-Ruiza M, Gonzalez-Carranza A, Zamilpaa A, Jimenez-Ferrera E, Huerta-Reyesa M, Navarro-Garciaa VM. The standardized extract of *Loeselia mexicana* possesses anxiolytic activity through the gama-amino butyric acid mechanism. J Ethnopharmacol. 2011; 138(2): 261– 7.
33. Singh Y, Singh N. Therapeutic potential of Kava in the treatment of anxiety disorders. CNS Drugs. 2002; 16(11): 731–43.
34. Anuradha H, Srikumar B, Rao B, Lakshmana M. Euphorbia hirta reverses chronic stress-induced anxiety and mediates its action through the GABAA receptor-benzodiazepine receptor–Cl− channel complex. J Neural Trans. 2008; 115(1): 35–42.
35. Costaa CARA, Kohna DO, Limaa VM, Garganoa AC, Flório JC, Costaa M. The GABAergic system contributes to the anxiolytic-like effect of essential oil from *Cymbopogon citratus* (lemongrass). J Ethnopharmacol. 2011; 137(1): 828–36.
36. Treit D, Fundytus M. Thigmotaxis as a test for anxiolytic activity in rats. Pharmacol Biochem Behav. 1988; 31(4): 959–62.
37. Kostowski W, Plaznik A, Stefanski R. Intra-hippocampal buspirone in animal models of anxiety. Eur J Pharmacol. 1989; 168(3): 393–6.
38. McNamara RK, Skelton RW. Effects of intracranial infusions of chlordiazepoxide on spatial learning in the Morris water maze I. Neuroanatomical specificity. Behav Brain Res. 1993; 59(1): 175–91.
39. Stefanski R, Palejko W, Bidzinski A, Kostowski W, Plaznik A. Serotonergic innervations of the hippocampus and nucleus accumbens septi and the anxiolytic-like action of midazolam and 5-HT1A receptor agonists. Neuropharmacol. 1993; 32: 977–85.
40. Onaivi ES, Maquire PA, Tsai NF, Davies MF, Loew GH. Comparison of behavioral and central BDZ binding profile in three rat lines. Pharmacol Biochem Behav. 1992; 43(3): 825–31.
41. Wolffgramm J, Mikolaiczyk C, Coper H. Acute and subchronic benzodiazepine-barbiturate-interactions on behaviour and physiological responses of the mouse. Naunyn Schmiedebergs Arch Pharmacol. 1994; 349(3): 279–86.
42. Herberlain H, Tscheirsch KP, Schazer HI. Flavonoids from *Leptospermum scoparium* with affinity to the benzodiazepine receptors characterized by structure activity relationship and in vivo studies of plant extract. Pharmazie. 1994; 49(12): 912-22.
43. Fernández SP, Wasowski C, Paladini A, Marder M. Synergistic interaction between hesperidin, a natural flavonoid, and diazepam. Eur J Pharmacol. 2005; 512(2): 189–98.