CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

1.1 Background of Study

Herbal medicines are an important part of the culture and traditions of African people. Today, most of the population in urban Nigeria, as well as rural communities, rely on herbal medicines for their health care needs. Epidemiological data suggest that lower incidences of certain chronic diseases such as atherosclerosis, arthritis, diabetes, acquired immune deficiency syndrome (AIDS) mediated by the human immunovirus, asthma, neoplasia, neurodegenerative and cardiovascular diseases are associated with frequent intake of fruits and vegetables (Ames *et al.,* 1993; Chu *et al.,* 2002).

Various factors influence the use of traditional medicine by 80% of developing nation's population which include; accessibility, confidence in treatment, convenience, cost of treatment, socioeconomic status, education and adverse effects of orthodox medicine. As a consequence, there is an increasing trend, worldwide, to integrate traditional medicine with primary health care. Renewed interest in traditional pharmacopoeias has meant that researchers are concerned not only with determining the scientific rationale for the plant's usage, but also with the discovery of novel compounds of pharmaceutical value. Instead of relying on trial and error, as in random screening procedures, traditional knowledge helps scientists to target plants that may be medicinally useful (Cox and Balick, 1994). Already an estimated 122 drugs from 94 plant species have been discovered through ethnobotanical leads (Fabricant and Farnsworth, 2001).

The prescription and use of traditional medicine in Nigeria is currently not regulated, with the result that there is always the danger of misadministration, especially of toxic plants. The potential genotoxic effects that follow prolonged use of some of the more popular herbal remedies, are also cause for alarm. People who use traditional remedies may not understand the scientific rationale behind their medicines, but they know from personal experience that some medicinal plants can be highly effective if used in certain amounts (doses). Since we have a better understanding today of how the body functions, we are thus in a better position to understand the healing powers of plants and their potential as multi-functional chemical entities for treating complicated health conditions.

The positive contribution of plants to medicine, however, has not been without side effects at normal doses, or toxic effects at toxic doses. Although, the literature has documented several toxicity resulting from the use of herbs on many occasions, still the potential toxicity of herbs has not been recognized by the general public or by professional groups of traditional medicine practitioners (Jou-fang, 1994; O'Hara *et al.,* 1998).

Cnestis ferruginea Vahl ex DC (Connaraceae) is a short ornamental shrub, sometimes a climber, which is about 2.5 meters high and is usually covered by dense, brown velvety hairs (Hutchinson and Dalziel, 1958; Nielson, 1965). The plant abounds throughout West Tropical Africa particularly in Gambia, Sierra Leone, Liberia, Ivory Coast, Ghana and Nigeria. In Africa, forty-one species of *Cnestis* have been recorded (Olugbade *et al.,* 1982). The plant is widely employed in the treatment of various ailments in traditional medicine throughout West Africa. The bitter fruit is used to clean teeth in Sierra Leone and in Nigeria (Burkill, 1985). In West Africa, healers use this plant for various infantile illness (Olugbade *et al.,* 1982). The extract of the fruit is used to treat sore throat as a tonic, and boiled fruits or fresh fruits crushed with rum or palm wine (a sweet alcoholic sap obtained from palm trees used as a common beverage) are usually taken systemically but may also be applied topically for the treatment of snake bite in Nigeria and Ghana (Dalziel, 1955). In Ivory Coast, the roots and leaves are employed in the treatment of dysmenorrhoea, epilepsy, mental illnesses (Garon *et al.,* 2007), whereas in Nigeria the leaves are employed as a laxative. A decoction of the bark is used in treating infected gum and a decoction of various parts of the plant is also believed to assist weak children to walk (Dalziel, 1955). The aqueous extract of the roots (Malcolm and Sofowora, 1969) and the methanolic extract of the root, stem and leaves of *C. ferruginea* (Bakye-Yiadom and Konning, 1975) were shown to have antimicrobial properties.

This study was undertaken to investigate the toxicity profile, and pharmacologic effects of the methanolic root extract of *C.ferruginea* with a view to isolating and characterizing the active constituents responsible for these effects.

1.2 Statement of Problem

Inflammation has long been known as a localized protective reaction of tissue to irritation, injury, or infection, characterized by pain, redness, swelling, and sometimes loss of function, but there has been a new realization about its role in a wide variety of diseases. These include; chronic arterial and venous disease (Ross, 1999), myocardial ischeamia (Entman, 1991), acute cerebral stroke and Alzheimer's chronic disease (Koistinaho and Koistinaho, 2005), arterial hypertension (Suematsu *et al.,* 2002) and cancer (Li *et al.,* 2005). Neuroinflammation is integral part of neurodegenerative diseases. A severe form of inflammation is observed in shock and multi-organ failure (Schmid-Schonbein and Hugli, 2005), a condition with one of the highest mortalities. The list of diseases associated with molecular markers of inflammation is large and growing. Today, inflammation has become the Holy Grail for studies of human disease. Anti-inflammatory treatments that have been shown to be effective in one disease may turn out to be effective in another, thereby opening a wider range of opportunities for intervention. Various classes of synthetic analgesic and anti-inflammatory drugs in use today are effective but not without adverse effects like gastrointestinal ulceration accompanied by anaemia from the resultant blood loss, fluid retention, bronchospasm and prolongation of bleeding time (Theophile *et al.,* 2006). Therefore, it is necessary to search for new drugs with little or no side effects. Plant extracts can be an important source of natural and safer drugs for the treatment of pain, inflammation and psychiatric disorders.

Depressive disorders, including major depression and dysthymia, are significant and disabling illnesses. It is estimated that one in five individuals is affected by a mood disorder in his or her lifetime. The World Health Organization estimated that major depression is the fourth most important cause worldwide of loss in disability-adjusted life years (DALY), and will be the second most important cause by 2020 (Murray and Lopez, 1997). Depression affects an estimated 121 million people worldwide. In spite of its prevalence and severe impact, the efficacy of currently available antidepressants is often inconsistent and many of them produce undesirable side effects. With a growing number of herbal medicines being introduced to psychiatric practice, many of them have been chosen as alternative therapies for the management of severe depression and dementia (Kessler *et al.,* 2001; Thachil *et al.,* 2007).

Thus, developing safe and effective agents from traditional herbs may provide us a good way to lessen the side effects as well as improve the efficacy.

In many regions of the world where modern healthcare is not readily available or affordable, the public continues to rely on traditional medicines which are based on locally available natural resources and cultural knowledge. In a public health context, availability, accessibility, affordability, utility, quality, efficiency and equity have relevance in respective order in promotion of traditional medicine (Tan, 2008). The continuous surge in the popularity and patronage of herbal remedies, and the fact that reports of efficacy far outnumber those of toxicity, necessitate concern. This is mainly due to the adverse effects of potentially toxic constituents in plants (e.g. aristolochic acids, pyrrolizidine alkaloids, viscotoxins, saponins, benzophenanthrine alkaloids, lectins, diterpenes, cyanogenetic glycosides, and furonocoumarins (Fennell *et al.,* 2004) which can be fatal. Based on these facts, it has been said that pharmacological and toxicological evaluations of medicinal plants are essential for drug development (Ibarrola *et al.,* 2000; Ahmed *et al.,* 2005).

Different parts and preparations of *Cnestis ferruginea* Vahl ex DC (Connaraceae) have been reported to be used in traditional African medicine for the treatment of various conditions. These include painful and inflammatory conditions like periodontitis (powdered bark); headache (root-bark paste); bronchitis (decoction of leaves); eye defects (leaf sap); dysmenorrhoea (leaves or roots); all manner of pains (pulped plant); migraine; sinusitis (root sap or powder); toothache, mental illness (root); and conjunctivitis (fruit juice) (Burkill, 1985). The high treatment cost and incidences of adverse effects of most allopathic medicine used for pain, inflammation, depression, anxiety, dementia and convulsion, has necessitated the search for cheaper and safer sources of treatment for these ailments. Hence, *Cnestis ferruginea* could be a potential source of remedy for central nervous system disorders and inflammatory conditions. To the best of our knowledge no study has been carried out to investigate neither the pharmacological profile of the folkloric uses of this plant nor its toxicological effects in chronic use.

1.3. Objectives of the study

The main aim of this study was to evaluate the pharmacological and toxicological activities of the methanolic root extract of *Cnestis ferruginea.* The specific objectives are to:

I. determine the acute and sub-chronic toxic effects of the methanolic root extract of *Cnestis ferruginea.*
II. evaluate the analgesic and anti-inflammatory effects of the methanolic root extract of *Cnestis ferruginea.*
III. investigate the effects of methanolic root extract of *Cnestis ferruginea* on central nervous system disorders.
IV. evaluate the effect of the bioactive components isolated from methanolic root extract of *Cnestis ferruginea* through bioactivity-guided assay on painful inflammatory conditions and central nervous system disorders; and
V. investigate the putative mechanisms of action(s) of the methanolic root extract in central nervous system disorders.

1.4. Significance of study

The study sought to:

I. provide information on the toxicity profile of *Cnestis ferruginea*
II. investigate the efficacy of *Cnestis ferruginea* in the treatment of various ailments, based on its ethnomedicinal uses in traditional African medicine, towards the development of standardized phytomedicines; and
III. identify active constituents responsible for the pharmacological effects towards discovering a new drug.

1.5 Operational Definition of Terms

Acute toxicity: an estimate of the intrinsic toxicity of a substance, often times expressed the median lethal dose (e.g., LD_{50}), it also provides information on target organs and other clinical manifestations of toxicity.

Alanine Amino Transferase: an enzyme which promotes transfer of amino group from glutamic acid to pyruvic acid and which if present in high amount in serum could indicate liver disease.

Alkaline Phosphatase: An enzyme present in bone, liver, kidney and placenta and which if present in high amount in blood could indicate disease of any organ.

Analgesic: a drug used to relieve pain.

Anxiolytic: a drug used to treat and prevent anxiety disorders

Anti-inflammatory: property of a substance or treatment that reduces inflammation.

Anxiety: is a psychological and physiological state characterized by somatic, emotional, cognitive, and behavioural components.

Aspartate aminotransferase: a metabolic enzyme that is elevated in heart and liver disorders

Bicuculline: plant alkaloid with Gamma aminobutyric acid (GABA) antagonist properties, used as a probe for GABA function in the CNS.

Complimentary and alternative medicine: any healing practice, "that does not fall within the realm of conventional medicine''.

Dementia: is a serious loss of cognitive ability in a previously unimpaired person, beyond what might be expected from normal aging.

Depression: is a state of low mood and aversion to activity that can affect a person's thoughts, behaviour, feelings and physical well-being.

Glycine: a non essential amino acid which also an inhibitory neurotransmitter in the CNS, especially the spinal cord, and its inhibition is implicated in seizure.

Hyperbilirubinemia: the presence of excess of bilirubin in the blood.

Inflammation: a basic way in which the body reacts to infection, irritation or other injury, the key feature being redness, warmth, swelling and pain.

Pain: is an unpleasant sensation often associated with damage to the body.

Sedative: drug that decreases activity, moderates excitement, and calms the recipient.

Seizure: transient symptom of "abnormal excessive or synchronous neuronal activity in the brain".

Subchronic toxicity: The principal goals of the subchronic study are to establish a NOAEL (the apparent threshold, or "no observable adverse effect level") and to further identify and characterize the specific organ or organs affected by the test compound after repeated administration. At least three doses are employed (a high dose that produces toxicity but does not cause more than 10% fatalities, a low dose that produces no apparent toxic effects, and an intermediate dose).

Traditional medicine: the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral-based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being.

Vehicle: an inert medium in which a medicinally active agent is administered.

1.6 Abbreviations

5-HT: Serotonin

AA: Acetic acid

ALP: Alkaline phosphatase

ALT: Alanine transaminase

AST: Aspartate transaminases

AQ: Aqueous fraction

BuOH: Butanol fraction

CAT: Catalase

CF-2: Amentoflavone

CF-5: Amino acid-like compound

$CHCl_3$: Chloroform

CNS: Central Nervous System

COX: Cyclooxygenase

CTOP: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2

DAMGO: [D-Ala^2, $MePhe^4$, Gly $(ol)^5$] enkephalin

Dic: Diclofenac

DMSO: Dimethyl Sulfoxide

DPDPE: [D-Pen^2, D-Pen^5] enkephalin

EDTA: Ethylenediamine-tetra acetate

ELT: Escape Latency Time

EPM: Elevated Plus Maze

ESMS: Electrospray Ionization Mass Spectrometry

EtOAc: Ethylacetate fraction

FST: Forced swimming test

GABA: Gamma Aminobutyric Acid

GPx: Glutathione peroxidise

GSH: Reduced glutathione

H_2SO4: Sulphuric Acid

Hb: Haemoglobin

HDL: High density lipoprotein

HMBC: Heteronuclear Multiple Bond Correlation

I.P.: Intraperitoneal

LD_{50}: Median lethal dose

LDL: Low density lipoprotein

MCH: Mean cell haemoglobin

MCHC: Mean cell haemoglobin concentration

MCV: Mean red cell volume

MDA: Malondialdehyde

MeOH: Methanol

NMR: Nuclear Magnetic Resonance

NSAID: Non-steroidal Anti-Inflammatory Drugs

N/OFQ: Nociceptin/Orphanin FQ

OECD: Organization of Economic Cooperation and Development

P.O.: *Per os* or Oral

PCV: Packed cell volume

RBC: Red blood cell

S.C.: Subcutaneous

SOD: Superoxide dismutase

TG: Triglycerides

TLC: Thin Layer Chromatography

TLT: Transfer latency Time

TST: Tail suspension test

Veh: Vehicle

WBC: White blood cell

WHO: World Health Organization

CHAPTER 2

LITERATURE

REVIEW

2.0 LITERATURE REVIEW

2.1 Overview of Inflammation

The survival of all organisms requires that they eliminate foreign invaders, such as infectious pathogens, and damaged tissues. These functions are mediated by a complex host response called inflammation (Kumar *et al.,* 2007). The inflammatory process is the response to an injurious stimulus evoked by a wide variety of noxious agents (*e.g.*, infections, antibodies, or physical injuries). The ability to mount an inflammatory response is essential for survival in the face of environmental pathogens and injury; in some situations and diseases, the inflammatory response may be exaggerated and sustained without apparent benefit and even with severe adverse consequences (Kumar *et al.,* 2007).

There has been a new realization about the role of inflammation in a wide variety of diseases, these include; chronic arterial and venous disease (Ross, 1999), myocardial ischeamia (Entman, 1991), acute cerebral stroke and Alzheimer's chronic disease (Koistinaho and Koistinaho, 2005), arterial hypertension (Suematsu, *et al.,* 2002) and cancer (Li *et al.,* 2005). A severe form of inflammation is observed in shock and multiorgan failure (Waxman 1996), a condition with one of the highest mortalities. There are signs of inflammation in patients with depression (Kop *et al.,* 2002., Toker *et al.,* 2005). Even without overt symptoms for inflammation or just exposure to environmental risks (Pitzer *et al.,* 1996). The list of diseases associated with molecular markers of inflammation is large and growing. Today, inflammation has become the Holy Grail for studies of human disease. Anti-inflammatory treatments that have been shown to be effective in one disease may turn out to be effective in another, thereby opening a wider range of opportunities for intervention.

Inflammatory responses occur in three distinct temporal phases, each apparently mediated by different mechanisms: (1) an acute phase characterized by transient local vasodilation and increased capillary permeability; (2) a delayed, subacute phase characterized by infiltration of leukocytes and phagocytic cells; and (3) a chronic proliferative phase, in which tissue degeneration and fibrosis occur. Many mechanisms are involved in the promotion and resolution of the inflammatory process (Kyriakis and Avruch, 2001).

Recruitment of inflammatory cells to sites of injury involves the concerted interactions of several types of soluble mediators. These include the complement factor C5a, platelet-activating factor, and the eicosanoid LTB_4. All can act as chemotactic agonists. Several cytokines also play essential roles in orchestrating the inflammatory process, especially interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) (Dempsey *et al.,* 2003). IL-1 and TNF-α are considered principal mediators of the biological responses to bacterial lipopolysaccharide (LPS, also called endotoxin). They are secreted by monocytes and macrophages, adipocytes, and other cells. Working in concert with each other and various cytokines and growth factors (including IL-8 and granulocyte-macrophage colony-stimulating factor), they induce gene expression and protein synthesis in a variety of cells to mediate and promote inflammation.

Prostanoids biosynthesis is significantly increased in inflamed tissue. Inhibitors of the cyclooxygenases (COXs), which depress prostanoids formation, are effective and widely used anti-inflammatory agents. The rapid induction of COX-2 in inflamed tissue and infiltrating cells provided a rationale for the development of selective COX-2 inhibitors for the treatment of inflammation. Although COX-2 is the major source of pro-inflammatory prostanoids, COX-1 also contributes (McAdam *et al.,* 2000). Both COX isoenzymes are expressed in circulating inflammatory cells *ex vivo,* and COX-1 accounts for approximately 10-15% of the prostaglandins (PG) formation induced by lipopolysaccharide (LPS) in volunteers (Grosser *et al.,* 2011). Impaired inflammatory responses have been reported in both COX-1 and COX-2 deficient mouse models, although they diverge in time course and intensity (Ballou *et al.,* 2000; Yu *et al.,* 2005). Human data is compatible with the concept that COX-1 derived products play a dominant role in the initial phase of an acute inflammatory response, while COX-2 is upregulated within several hours (Grosser *et al.,* 2011). Prostaglandins E_2 and I_2 are the primary prostanoids that mediate inflammation. They increase local blood flow, vascular permeability and leukocyte infiltration through activation of their respective receptors, EP_2 and IP (Grosser *et al.,* 2011). Models for investigating acute inflammation include: carrageenan-, egg albumin-, serotonin-, histamine-, and xylene-induced oedema while adjuvant-, and formaldehyde-induced arthritis are used as chronic inflammation.

Carrageenan-induced inflammation consists of three distinct phases including an initial release of histamine and serotonin; a second phase mediated by kinins; and a third phase is due primarily to the formation of pro-inflammatory prostanoids (Di Rosa *et al.,* 1971; Okpo *et al.,* 2001). The ability of bradykinin to induce inflammation is related to its vasodilatory effect accompanied by increase in vascular permeability and further enhances inflammatory reactions through the release of prostaglandins, cytokines, histamine and free radicals from a variety of cells. These mediators further potentiate and perpetuate the ongoing inflammatory conditions through a variety of mechanisms. On the other hand, egg albumin-induced oedema results from the release of histamine and serotonin (Pearce, 1986; Akindele and Adeyemi, 2007). The ear oedema test is useful for the evaluation of anti-inflammatory steroids and is said to be less sensitive to non-steroidal anti-inflammatory agents (Zaninir *et al.,* 1992; Akindele and Adeyemi, 2007). The model is thus linked to the activity of phospholipase A_2, PLA_2 (Nunez Guillen *et al.,* 1997) while the formaldehyde-induced arthritis inflammation test, involves the proliferative phase of inflammatory reaction (Gupta *et al.,* 1971).

2.2 Evidence for the contribution of inflammation to CNS disease

Inflammation is a key component of host defence responses to peripheral inflammation and injury, but it is now also recognized as a major contributor to diverse, acute and chronic central nervous system (CNS) disorders. Expression of inflammatory mediators including complement, adhesion molecules, cyclooxygenase enzymes and their products and cytokines is increased in experimental and clinical neurodegenerative disease, and intervention studies in experimental animals suggest that several of these factors contribute directly to neuronal injury. Most notably, specific cytokines, such as interleukin-1 (IL-1), have been implicated heavily in acute neurodegeneration, such as stroke and head injury. Inflammation may have beneficial as well as detrimental actions in the CNS, particularly in repair and recovery. Nevertheless, several anti-inflammatory targets have been identified as putative treatments for CNS disorders, initially in acute conditions, but which may also be appropriate to chronic neurodegenerative conditions (Allan and Rothwell, 2003).

2.2.1 Epilepsy

It is a common neurological disorder which affects about 50 million people worldwide. Seizures are caused by abnormal, high-frequency discharge of groups of neurons. The underlying neurochemical mechanisms are unknown, although increasing evidence implicates proinflammatory cytokines. Seizures and epilepsy can develop following events which induce a CNS inflammatory response, and expression of interleukin-1 (IL-1), tumour necrotic factor-alpha (TNF-α), interleukin-1 receptor (IL-1rα) and interleukin-6 (IL-6) are increased by seizure activity. Cytokines affect neuronal excitability directly by acting on ionic currents, and indirectly by inducing gene transcription in glia and neurones.

Exogenous IL-1β enhances chemically induced seizures in rats and IL-1rα inhibits motor seizures and delays their onset (Vezzani *et al.,* 2002). Transgenic mice which over express IL-1rα selectively in astrocytes are less susceptible to seizure induction than wild-type animals, and seizures in these mice have a reduced duration and delayed onset compared to their wild-type counterparts. Furthermore, mice that lack IL-1RI are less susceptible to heat-induced febrile seizures than wildtype mice. Evidence for a proconvulsive role of IL-1*β* is compelling, but it has also been shown to have anticonvulsive properties. In a rodent amygdala kindling model of epilepsy, fully kindled seizures are inhibited by IL-1*β*, and daily injection of IL-1*β* during kindling slows the rate of kindling (Sayyah *et al.*, 2005).

2.2.2 Alzheimer's disease (AD)

Both TNF-α and IL-1 can increase expression of amyloid precursor protein and amyloid β- peptide (Aβ). This suggests that CNS inflammation at least participates in amplification of the disease state. Furthermore, proteins of the complement system are associated with AD lesions, and one risk factor for AD is acute brain injury, leading to long-term inflammation, with increased expression of major histocompatibility complex (MHC) class II, IL-1 and TNF-α (Lucas *et al.,* 2006). The most convincing evidence however for a causal role of inflammation in AD comes from many studies demonstrating associations between polymorphisms in the genes encoding members of the IL-1 family and AD (Rainero *et al.,* 2004), particularly IL-1rα, Statins may have protective effects in AD and other types of dementia. Cross-sectional analysis of three hospital

databases suggests that prevalence of AD in patients taking statins is 60% lower than in patients taking other medications used in the treatment of cerebrovascular diseases (Liao and Laufs, 2005). Based on this significant body of evidence, a plausible hypothesis was that anti-inflammatory agents might reduce the probability of developing AD, or slow its progression. In particular, COX-2 inhibitors protect neuronal cells from amyloid toxicity *in vitro*, and promote neuronal survival in animal models of ischaemic and excitotoxic neurodegeneration.

Retrospective epidemiological studies showed that patients taking anti-inflammatory drugs for long periods of time have a reduced incidence of AD. Although other epidemiological studies also suggest a beneficial role for anti-inflammatory agents in AD (McGeer *et al.*, 1996), the general view from prospective trials of nonsteroidal anti-inflammatory drugs (NSAIDs) is that they are disappointingly variable. In one trial, patients with mild to moderate AD were treated with a selective COX-2 inhibitor, a traditional NSAID or placebo for a period of 1 year. Patients receiving the COX-2 inhibitor or NSAID showed no slowing of cognitive decline compared with patients receiving placebo (Aisen *et al.*, 2003). A larger trial involving the COX-2 inhibitor Vioxx® was then terminated following a dramatic increase in the incidence of cardiovascular side effects (Couzin, 2004), this is not too good for ageing AD patients who are already at high risk of heart disease. The mechanism of action of these drugs is not totally clear, since NSAIDs generally are COX inhibitors, many (e.g. ibuprofen) also reduce generation of Aβ. Dosage may also be part of the problem in the clinical trials, since the degree of inflammation in specific brain areas during AD may be significantly greater than in arthritic joints.

2.3 Pain

Understanding the anatomical pathways and neurochemical mediators involved in noxious transmission and pain perception is key to optimizing the management of acute and chronic pain. The International Association for the Study of Pain (IASP) defines pain as "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*" (Grosser *et al.*, 2011). Sensory systems have the role of informing the brain about the state of the external environment and the internal milieu of the organism. Pain is a perception, and as such, it is one of the outputs of a system in more highly evolved animals, the nociceptive system, which itself is a component of the overall set of controls responsible for homeostasis. In

this context, pain constitutes an alarm that ultimately has the role of helping to protect the organism: it both triggers reactions and induces learned avoidance behaviours, which may decrease whatever is causing the pain and, as a result, may limit the (potentially) damaging consequences.

At the beginning of the twentieth century, Sherrington (1910) developed this concept and introduced the term *nociception* (from the Latin *nocere*, "to harm"). It seems appropriate to take the view of Dennis and Melzack (1983) that pain/nociception has at least three functions: **1**) to warn the individual of the existence of real tissue damage; **2**) to warn the individual of the probability that tissue damage is about to occur by realizing that a stimulus has the potential to cause such damage; and 3) to warn a social group of danger as soon as it exists for anyone of its members. Behaviours resulting from pain can facilitate other fundamental biological functions, such as the maintenance of tissue "trophicity" and regeneration (notably in the processes of inflammation and healing).

Experimental models of pain can be divided into three basic categories: acute, persistent, and neuropathic. Acute pain is modelled by the hot-plate, tail flick, tail clip tests etc.; persistent pain by the formalin test, and neuropathic pain is modelled by nerve ligation. The writhing test is simple, reliable, and affords rapid evaluation of analgesic activity (Singh and Majumdar, 1995). The induction of writhings by chemical substances injected intraperitoneally (*i.p.)* results from the sensitization of nociceptors by prostaglandins (Nunez Guillen *et al.,* 1997) and the test is useful for evaluation of mild analgesic and non-steroidal anti-inflammatory drugs (Berkenkopf and Weichmann, 1988).

The tail clip and hot plate tests are used based on the fact that centrally acting analgesic drugs elevate the pain threshold of rodents towards pressure and heat (Singh and Majumdar, 1995). The hot plate test involves the spinal reflex (Pini *et al.,* 1997) and measures the complex response to a non-inflammatory, acute nociceptive input (Zakaria *et al.,* 2008). The neurotransmitters and neuromodulators that mediate the pain response in each of these models can differ. As a result, different drugs may be effective in attenuating pain for each model.

The formalin test is used to determine the potential analgesic effects of compounds for states of persistent pain in which tissue damage occurs (Hunskaar *et al.*, 1985). Formalin test has been reported to produce distinct biphasic nociceptive response (Zakaria *et al.*, 2008). The early phase (0–5 min) has been associated with direct effect of formalin on nociceptors while the late phase (15–30 min) is said to involve inflammatory processes (Tjolsen *et al.*, 1992). According to Chan *et al.* (1995), centrally acting drugs (e.g. morphine) inhibit both phases of the formalin test while peripherally acting drugs (e.g. ASA) inhibit the late phase only. A basic understanding of the mechanisms involved in the pain response can help investigators choose the model that would be most appropriate for testing the analgesic properties of specific compounds or classes of compounds.

2.4 Therapeutic management of inflammation and pain

The non-steroidal anti-inflammatory drugs (NSAIDs) and narcotics analgesics are the two major classes of drugs used in the treatment of pain. However, the NSAIDs possess both analgesic and anti-inflammatory properties (Sostres *et al.*, 2010). NSAIDs produce their therapeutic activities through inhibition of cyclooxygenase (COX) (Vane and Botting, 1998), the enzyme that produces prostaglandins. They share, to a greater or lesser degree, the same side effects, including gastric and renal toxicity (Rahme and Bernatsky, 2010). Among patients using NSAIDs, up to 4% per year suffer serious gastrointestinal complications (Sostres *et al.*, 2010).

In 1998, the number of deaths in the United States from NSAID-induced gastrointestinal complications was 16,550, virtually identical to that from AIDS (16,685) (Singh and Triadafilopoulos, 1999). NSAIDs also cause a wide range of tubular, interstitial, glomerular and vascular renal lesions and probably chronic renal failure. Several additional side effects, some serious, have been reported (Grosser *et al.*, 2011). The rational design of pharmacological agents includes, amongst others, modification of known compounds in order to optimize their pharmacological properties, primarily by increasing their safety profile while maintaining efficacy. Several efforts have been made to modify NSAIDs to reduce their gastrointestinal toxicity, while maintaining their anti-inflammatory effect (Cicala *et al.*, 2000; Rigas, 2007; Nemmani *et al.*, 2009).

Previous studies have shown that there are at least two COX isoenzymes; COX-1 is constitutive and produces prostaglandins that protect the stomach and kidney from damage. COX-2 is induced by inflammatory stimuli, such as cytokines, and forms Prostaglandins that contribute to the pain and swelling of inflammation (Grosser *et al.,* 2011). Thus, selective COX-2 inhibitors should be anti-inflammatory without side effects on the kidney and stomach. Of course, selective COX-2 inhibitors may have other side effects and perhaps other therapeutic potential. For instance, COX-2 (and not COX-1) is thought to be involved in ovulation and in labour.

In addition, the well-known protective action of aspirin on colon cancer may be through an action on COX-2, which is expressed in this disease. Moreover, NSAIDs delay the progress of Alzheimer's disease; thus, selective COX-2 inhibitors may demonstrate new important therapeutic benefits as anticancer agents, as well as in preventing premature labour and possibly even retarding the progression of Alzheimer's disease (Grosser *et al.,* 2011). Despite the fact that they are often chemically unrelated, they have certain therapeutic actions and adverse effects in common. This class of drugs includes:

Non selective COX Inhibitors:

Salicylic acid derivatives: aspirin, Salsalate, diflunisal, sulfasalazine

Para-aminophenol derivatives: Paracetamol

Indole and indene acetic acids: Indomethacin, sulindac

Heteroaryl acetic acids: tolmetin, diclofenac, ketorolac

Arylpropionic acids: ibuprofen, naproxen, oxaprozin

Enolic acids: oxicams

Selective COX-2 Inhibitors

Diaryl-substituted furanones: Rofecoxib

Diaryl-substituted pyrazoles: celecoxib

Indole acetic acids: etodolac

Sulphonamides: nimesulide

Most 'traditional' NSAIDs are inhibitors of both isoenzymes, although they vary in the degree to which they inhibit each isoform. It is believed that the anti-inflammatory action (and probably

most analgesic actions) of the NSAIDs is related to their inhibition of COX-2, while their unwanted effects-particularly those affecting the gastrointestinal tract-are largely a result of their inhibition of COX-1 (Grosser *et al.*, 2011). Compounds with a selective inhibitory action on COX-2 are now in clinical use, but expectations that these inhibitors would transform the treatment of inflammatory conditions have received a setback because of an increase in cardiovascular risk (Grosser *et al.,* 2011).

2.4.1 Therapeutic uses of NSAIDs

All NSAIDs, including selective COX-2 inhibitors are antipyretic, analgesic, and anti-inflammatory with the exception of paracetamol which is devoid of anti-inflammatory activity. Paracetamol effectively inhibits COXs in the brain but not at the sites of inflammation in peripheral tissues (Grosser *et al.,* 2011). When employed as analgesics, these drugs usually are effective only against pain of low-to moderate intensity, such as dental pain. Although their efficacy is less than that of opioids, NSAIDs lack the respiratory depression and the development of physical dependence seen with opiates. NSAIDs find their chief clinical application as anti-inflammatory agents in the treatment of musculoskeletal disorders, such as rheumatoid arthritis and osteoarthritis. NSAIDs offer mainly symptomatic relief in rheumatoid arthritis, but they have little effect on the progression of bone and cartilage destruction. Interest has therefore centered on finding treatments that might arrest or at least slow this progression by modifying the disease itself. Chronic treatment of arthritic patients with rofecoxib and celecoxib are effective without gastric toxicity (FitzGerald and Patrono, 2001).

Prostaglandins also have been implicated in the maintenance of patency of the ductus arteriosus, and indomethacin and other traditional NSAIDs (tNSAIDs) have been used in neonates to close the inappropriately patent ductus (Grosser *et al.,* 2011). Epidemiological studies suggested that frequent use of aspirin is associated with as much as 50% decrease in the risk of colon cancer and similar observations have been made with other cancers (Burke *et al*., 2006).

2.4.1.1 Adverse effects of NSAIDs

In addition to the similar therapeutic effects, NSAIDs share several unwanted side effects (Borda and Koff, 1992). The adverse effects include the following:

I. Gastrointestinal: anorexia, nausea, dyspepsia, abdominal pain, and diarrhoea. These symptoms may be related to the induction of gastric or intestinal ulcers, which is estimated to occur in 15–30% of regular users. Ulceration may range from small superficial erosions to full-thickness perforation of the muscularis mucosa. There may be single or multiple ulcers and ulceration can be accompanied by gradual blood loss leading to anaemia or by life-threatening haemorrhage.

II. Renal: salt and water retention, oedema, worsening of renal function in renal/cardiac and cirrhotic patients, decreased effectiveness of antihypertensive medications, decreased effectiveness of diuretic medications, decreased urate excretion (especially with aspirin), hyperkalemia.

III. Central nervous system: headache, vertigo, dizziness, confusion, depression, lowering of seizure threshold, hyperventilation (salicylates).

IV. Platelets: inhibited platelet activation, propensity for bruising, increased risk of haemorrhage.

V. Uterus: prolongation of gestation and possible prolongation of labour.

VI. Hypersensitivity: vasomotor rhinitis, angioneurotic oedema, asthma, urticaria, flushing, hypotension, and shock.

VII. Vascular: closure of ductus arteriosus

2.4.2 Opioid Analgesics

The term opioid refers broadly to all compounds related to opium, a natural product derived from the poppy. Opiates are drugs derived from opium, an extract of the juice of the poppy (*Papaver somniferum* (Papaveraceae) and include the natural products morphine, codeine, and thebaine, and many semisynthetic derivatives. Endogenous opioid peptides, or endorphins, are the naturally occurring ligands for opioid receptors. Opiates exert their effects by mimicking these peptides. The term narcotic is derived from the Greek word for “stupor”; it originally referred to any drug that induced sleep, but it is now associated with opioids.

The diverse functions of the endogenous opioid system include the best known sensory role, prominent in inhibiting responses to painful stimuli; a modulatory role in gastrointestinal (GI), endocrine, and autonomic functions; an emotional role, evident in the powerful rewarding and addicting properties of opioids; and a cognitive role in the modulation of learning and memory. The endogenous opioid system has considerable diversity in endogenous ligand (>12) but only 4 major receptor types.

2.4.2.1 OPIOID RECEPTORS

Three classical opioid receptor types; μ, δ and κ, have been studied extensively; the N/OFQ receptor system is still being defined. Highly selective ligands that allowed for type-specific labelling of the three classical opioid receptors (e.g., DAMGO for μ, DPDPE for δ, and U-50,488 and U-69,593for κ) made possible the definition of ligand-binding characteristics of each of the receptor types and the determination of anatomical distribution of the receptors using autoradiographic techniques (Gustein and Akil, 2006). Each major opioid receptor has a unique anatomical distribution in brain, spinal cord, and the periphery. Receptor-selective antagonists and agonists have aided the study of the biological functions of opioid receptors. Commonly used antagonists are cyclic analogs of somatostatin such as CTOP as μ-receptor antagonists, a derivative of naloxone, called naltrindole, as a δ-receptor antagonist, and a bivalent derivative of naltrexone called nor-binaltorphimine (nor-BNI), as a κ-receptor antagonist. In general, functional studies using selective agonists and antagonists have revealed substantial parallels between μ and δ receptors and dramatic contrasts between μ/ δ- and κ- receptors. *In vivo* infusions of selective antagonists and agonists also were used to establish the receptor types involved in mediating various opioid effects.

Narcotics are considered more superior to NSAIDs in the treatment of pain. However, the propensity to induce dependence has limited the uses of opioids in the treatment of chronic pains (Spiller, 2002). By virtue of their mode of action, these drugs abolish both peripheral and central pains especially in severe painful conditions that are often associated with anxiety (Gustein and Akil, 2006). The analgesic actions of opioids have been linked to their ability to interfere with the two main components of pain, namely pain threshold and perception (Gustein and Akil, 2006). Generally, elevation of pain threshold to noxious stimulus results in the diminution of pain.

However, ability of opioids to alter the patients' perception of pain appears to be the major denominator responsible for their effectiveness in the control of chronic pain states that are often associated with anxiety (Spiller, 2002). It is well known that these drugs induce a state of euphoria and increase the capacity of the patient to tolerate pain (Gustein and Akil, 2006). Examples of morphine-like drugs: morphine analogues. These are compounds closely related in structure to morphine and often synthesised from it. They may be agonists (e.g. morphine, diamorphine (heroin) and codeine), partial agonists (e.g. nalorphine and levallorphan) or antagonists (e.g. naloxone). Synthetic derivatives with structures unrelated to morphine: phenylpiperidine series (e.g. pethidine and fentanyl);methadone series (e.g. methadone and dextropropoxyphene); benzomorphan series (e.g. pentazocine and cyclazocine); semisynthetic thebaine derivatives (e.g. etorphine and buprenorphine).

2.4.2.2 Adverse effects of opioid analgesics

The side effects of opioids and strategies for their management are stated below:

Excessive sedation: reduce dose by 25%

Constipation: use of agents like casanthranol-docusate, senna, or bisacodyl + docusate

Nausea and vomiting: use of drugs like hydroxyzine, diphenhydramine, or ondansetron

Gastroparesis: administration of metoclopramide

Vertigo: use of meclizine

Urticaria/itching: administer hydroxyzine or diphenhydramine

Respiratory depression: administer naloxone

CNS irritability: discontinue opioid intake and administer benzodiazepine

2.4.3 Glucocorticoids: anti-inflammatory and immunosuppressive agent

Glucocorticoids potently suppress inflammation, and their usefulness in a variety of inflammatory and autoimmune diseases make them among the most frequently prescribed classes of drugs. Glucocorticoids can prevent or suppress inflammation in response to multiple inciting events, including radiant, mechanical, chemical, infectious, and immunological stimuli. Although the use of glucocorticoids as anti-inflammatory agents does not address the underlying cause of the disease, the suppression of inflammation is of enormous clinical utility and has made these drugs among the most frequently prescribed agents. Similarly, glucocorticoids are of immense value in

treating diseases that result from undesirable immune reactions. These diseases range from conditions that predominantly result from humoral immunity, such as urticaria, to those that are mediated by cellular immune mechanisms, such as transplant rejection. The immunosuppressive and anti-inflammatory actions of glucocorticoids are inextricably linked, perhaps because they both involve inhibition of leukocyte functions (Schimmer and Parker, 2006).

Multiple mechanisms are involved in the suppression of inflammation by glucocorticoids. There is decreased release of vasoactive and chemo attractive factors, diminished secretion of lipolytic and proteolytic enzymes, decreased extravasation of leukocytes to areas of injury, and ultimately, decreased fibrosis. Glucocorticoids can also reduce expression of proinflammatory enzymes, such as COX-2 and NOS (Schimmer and Parker, 2006).

2.4.3.1 Toxicity of adrenocortical steroids

Two categories of toxic effects result from the therapeutic use of corticosteroids: those resulting from withdrawal of steroid therapy and those resulting from continued use at supraphysiological doses. The side effects from both categories are potentially life-threatening and mandate a careful assessment of the risks and benefits in each patient.

2.4.3.2 Withdrawal of therapy

The most frequent problem in steroid withdrawal is flare-up of the underlying disease for which steroids were prescribed. There are several other complications associated with steroid withdrawal. The most severe complication of steroid cessation, acute adrenal insufficiency, results from overly rapid withdrawal of corticosteroids after prolonged therapy has suppressed the hypothalamo-pituitary axis (HPA). Treatment with supraphysiological doses of glucocorticoids for 2–4 weeks may cause some degree of HPA impairment. There is significant variation among patients with respect to the degree and duration of adrenal suppression after glucocorticoid therapy, making it difficult to establish the relative risk in any given patient. Many patients recover from glucocorticoids-induced HPA suppression within several weeks to months; however, in some individuals the time to recovery can be one year or longer (Schimmer and Parker, 2006).

2.4.3.3 Continued use of supraphysiological glucocorticoids doses

Since the pharmacological and physiological actions of corticosteroids are mediated by the same receptors, supraphysiological doses of the various glucocorticoid derivatives generally cause side effects that are exaggerated manifestations of their physiological effects. Besides the consequences that result from HPA suppression, other complications of prolonged therapy include fluid and electrolyte abnormalities, hypertension, hyperglycaemia, increased susceptibility to infection, osteoporosis, myopathy, behavioural disturbances, cataracts, growth arrest, and the characteristic habitus of steroid overdose, including fat redistribution, striae, and ecchymoses (Schimmer and Parker, 2006).

2.5 Depression and Anxiety

Mood and anxiety disorders are generally classified as separate types of syndromes according to the Diagnostic Manual of Mental Disorder, 4th edition (DSM-IV). However, depression and anxiety share many overlapping symptoms including fatigue, impaired concentration, irritability, sleep disturbance, and somatization in addition to subjective experiences of nervousness, worry, and restlessness (Baldessarini, 2006). They may also share a common pathophysiology (Baldessarini, 2006). In fact, the key difference between depression and generalized anxiety disorder is whether the patient subjectively has a primarily depressed or anxious mood, with many other symptoms being shared. The National Comorbidity Survey reported that 58% of patients with major depression also fulfilled criteria for an anxiety disorder; primary anxiety disorders also experience major depressive episodes. The finding that 68% of individuals with co-morbid depression and anxiety were anxious for over 10 years before the eventual development of depression suggests overlapping mechanisms. The co-occurrence of anxiety and affective syndromes, anxiety symptoms in patients with syndromal mood disorders, depressive symptoms in patients with syndromal anxiety disorders, subsyndromal affective and anxiety symptoms, and so-called mixed depression and anxiety have received considerable attention (Gulley and Nemeroff, 1993).

2.5.1 Depression

Depression is a common psychiatric disorder that presents with depressed mood, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep, low energy and poor concentration. It is a common psychiatric disorder, with a lifetime prevalence estimated at 17%, out of which 25% of patients develop chronic depression (Kaplan *et al.,* 1995). The leading mental disorders that cause chronic disabilities include depression, mental retardation, epilepsy, dementia and schizophrenia. Since 2002, depression has been estimated to be second only to heart disease as a burden of disease in the world (Amoran *et al.,* 2007).

The World Health Organization estimates that major depression is the fourth most important cause worldwide of loss in disability-adjusted life years (DALYs), and will be the second most important cause by 2020 (Murray and Lopez, 1997). Approximately one in five of the world's youth, 15 years and younger, suffer from mild to severe mental disorders (Amoran *et al.,* 2007). A large number of these children remain undetected and untreated. In Nigeria, 28.5% of those attending a primary care setting in an urban area were found to have psychiatric morbidity (Omokhodion *et al.,* 2003). A World Health Organization (WHO) publication to mark the World Health Day in June 2001 revealed that 10% of school children in Alexandria, Egypt, suffered from depression, and that 44% of adults in a rural Pakistan village were affected by a depressive disorder (Mental Health Division, WHO, 2001). One out of seven adults in the USA has a mood disorder during a single year, and 5% of children aged 9–17 years were found to have depression. In Brazil, Germany and Turkey, respectively, 7%, 10% and 4.2% of the population were reported to have depression. In an urban health care setting in Nigeria, 7.3% were found to have symptoms related to affective disorders. Depression and schizophrenia are responsible for 60% of all suicides, and no population was found to be free of schizophrenia and depression in a landmark WHO study conducted in developing and developed countries (Mental Health Division, WHO, 2001).

The burden of depression is likely to increase so much so that in 2030, it will be the single biggest burden out of all health conditions. However, a lot of misery could be avoided if depression is given the same attention and resources as AIDS or cancer. In spite of its prevalence and severe

impact, the efficacy of currently available antidepressants is often inconsistent and many of them cause undesirable side effects. With a growing number of herbal medicines being introduced to psychiatric practice, many of them have been chosen as alternative therapies for depression (Kessler *et al.,* 2001; Thachil *et al.,* 2007). Thus, developing safe and effective agent from traditional herbs may provide us a good way to lessen side effects as well as improve efficacy.

2.5.1.1 Experimental models used in the evaluation of potential antidepressants

Depression is defined clinically as a pathological complex of psychological, neuroendocrine and somatic symptoms that cannot be reproduced in animals and especially in mice, only specific measurable behaviours (endophenotypes) can be assayed to be relevant in human depression (Holmes, 2003a). Numerous animal models of depression have been designed, tested and assessed (Willner, 1984; Lucki, 1997; Dalvi and Lucki, 1999; Holmes, 2003b; Cryan and Mombereau, 2004). The reserpine effects reversal test, designed by Costa *et al.* (1960), was the first attempt to screen imipramine-like drugs, which led to the isolation of desipramine. To date, few models are commonly used for screening antidepressant effects or studying the mechanisms of action of these molecules.

The forced swimming test (FST) is often used to determine if pharmacological compounds exhibit antidepressant activity (Porsolt *et al*. 1977, 1978). Animal placed into a chamber of water for an extended period of time display a range of behaviours, typically becoming immobile several minutes into the test period. Immobility can be facilitated by prior exposure to stress (Borsini *et al*. 1989), including a 15-min pretest swim given 24 h prior to the test period. Treatment for varying periods of time with a broad range of antidepressant drugs and treatments results in a shorter duration of immobility (Borsini and Meli, 1988). The FST is sensitive to a wide range of antidepressants, including most tricyclics, monoamine oxidase inhibitors and atypical antidepressants, but varies in response to selective serotonin reuptake inhibitors (SSRIs) (Borsini 1995; Detke *et al*. 1995). A sampling technique was developed for scoring active behaviours, such as swimming and climbing, in addition to immobility (Detke *et al*. 1995; Lucki 1997) in the FST. Results with this technique have shown that pharmacologically diverse antidepressant drugs produce different patterns of behaviour in the FST. For example, SSRIs such as fluoxetine, paroxetine and sertraline, reduce immobility and increase swimming without affecting climbing.

In contrast, selective NE reuptake inhibitors, such as desipramine or maprotiline, reduce immobility and increase climbing without altering swimming. Finally, drugs with effects on both catecholamines and 5-HT can increase both active behaviours simultaneously in certain instances (Rénéric and Lucki 1998). Thus, the FST not only detects antidepressant drugs with a common effect on a core behaviour (immobility), but patterns of active behaviours in the FST reveal multiple components of antidepressant responses that are responsive to specific drug classes (Lucki, 1997).

The tail suspension test (TST) is a behavioural test widely used for measuring antidepressant drug-like activity in drug discovery research with mice (Steru *et al.,* 1985; Cryan *et al.,* 2005). In addition, a variety of genetic studies involving mice have used the TST to measure antidepressant responses or responses to stress (El Yacoubi *et al.,* 2003; Cryan and Mombereau, 2004; Bechtholt and Lucki, 2006; Crowley *et al.,* 2006). Mice suspended from a high support by their tail display immediate episodes of escape-oriented behaviours that subsequently transition to passive immobility. Acute administration of antidepressant drugs typically reduces the time spent immobile, as mice persist in attempting to escape from the bar for longer periods of time. The TST is sensitive to a broad spectrum of antidepressant treatments regardless of their mechanism of action, and the effects of antidepressants are not due to simple changes in locomotor activity. The TST has been evaluated using a broad range of pharmacological and somatic treatments, including: tricyclic antidepressants, monoamine oxidase inhibitors, SSRIs, NRIs, atypical antidepressants such as bupropion, and electroconvulsive therapy (Steru *et al.,* 1985, 1987; Teste *et al.,* 1990; Cryan *et al.,* 2004, 2005). Consistent with views from the aminergic theories of depression, studies have employed monoamine depletion strategies in animals to show that acute and/or adaptive changes in either serotonergic or noradrenergic transmission mediate the attenuation by antidepressants of much depressive-like behaviour in animals (Lucki and O'Leary, 2004).

2.5.1.2 Antidepressants

Most antidepressants exert important actions on the metabolism of monoamine neurotransmitters and their receptors, particularly norepinephrine (NE) and serotonin (5-HT). All drugs commonly used to treat depression share, at some level, primary effects on serotonergic or noradrenergic

neurotransmitter system (Shelton and Lester, 2006). In general, antidepressants enhance serotonergic or noradrenergic neurotransmission, although the nature of this effect may change with chronic treatment (Shelton, 2000). Many antidepressants have established records of efficacy for treating major depression (Millan, 2006). However, they all suffer some limitations in efficacy, since at least 20% of all depressed patients are refractory to multiple different antidepressants at adequate doses (Rush *et al.,* 2006). Following initiation of antidepressant drug treatment there is generally a ‘’therapeutic lag’’ lasting 3-4 weeks before a measurable therapeutic response becomes evident. The most used medications, often referred to as second generation antidepressants, are the selective serotonin reuptake inhibitors (SSRIs) e.g. fluoxetine, citalopram, fluvoxamine, and the serotonin-norepinephrine reuptake inhibitors (SNRIs) e.g. imipramine, clomipramine, which have greater efficacy and safety compared to older drugs (i.e. tricyclic antidepressants (TCAs) e.g. amitriptylline, maproptyline. In monoamine systems, reuptake of the transmitters is the main mechanism by which neurotransmission is terminated; thus, inhibition of reuptake can enhance neurotransmission, presumably by slowing clearance of the transmitters in the synapse.

Like tricyclic antidepressants, which block NE reuptake, the SSRIs block neuronal transport of 5-HT both immediately and chronically, leading to complex secondary responses. Increased synaptic availability of 5-HT results in stimulation of a large number of postsynaptic 5-HT receptor types (Zhao *et al.*, 2009), which may contribute to adverse effects characteristic of this class of drugs, including gastrointestinal effects (nausea and vomiting) and sexual effects (delayed or impaired orgasm). Stimulation of 5-HT_{2C} receptors may contribute to the agitation or restlessness sometimes induced by SSRIs.

2.5.1.3 Anxiety

Everyone feels anxious from time to time. Stressful situations such as meeting, tight deadlines or important social obligations often make us nervous or fearful. Experiencing mild anxiety may help a person become more alert and focused on facing challenging or threatening circumstances. But individuals who experience extreme fear and worry that do not subside may be suffering from an anxiety disorder. The frequency and intensity of anxiety can be overwhelming and interfere with daily functioning. Fortunately, the majority of people with an anxiety disorder improve considerably by getting effective psychological treatment. Symptoms such as extreme fear,

shortness of breath, racing heartbeat, insomnia, nausea, trembling and dizziness are common in these anxiety disorders. Anxiety disorders may be acute and transient, or more commonly, recurrent or persistent. Symptoms may include mood changes (fear, panic, or dysphoria) or limited abnormalities of thought (obsessions, irrational fears, or phobias) or of behaviour (avoidance, rituals or compulsions,"hysterical" conversion signs, or fixation on imagined or exaggerated physical symptoms).

Drugs can be beneficial in such disorders, particularly by modifying associated anxiety and depression to facilitate a more comprehensive program of treatment and rehabilitation. Antidepressants and sedative-antianxiety agents are commonly used to treat anxiety disorder.

2.5.1.4 Pharmacotherapy of anxiety

Anxiety is a symptom of many psychiatric disorders and an almost inevitable component of many medical and surgical conditions. Symptoms of anxiety commonly are associated with depression and especially with dysthymic disorder (chronic depression of moderate severity), panic disorder, agoraphobia and other specific phobias, obsessive-compulsive disorder, eating disorders, and many personality disorders. Sometimes, no treatable primary illness is found, or if one is found and treated, it may be desirable to deal directly with the anxiety at the same time (O'Donnell and Shelton, 2011). In such situations, antianxiety medications are frequently and appropriately used. Currently, the benzodiazepines and the SSRIs are the most commonly employed pharmacotherapies for common clinical anxiety disorders (O'Donnell and Shelton, 2011). Benzodiazepines (examples; clonazepam, diazepam, alprazolam and lorazepam) sometimes are given to patients presenting with anxiety mixed with symptoms of depression, although their efficacy in altering the core features of severe major depression has not been demonstrated.

The most favourable responses to the benzodiazepines are obtained in situations that involve relatively acute anxiety reactions in medical or psychiatric patients who have either modifiable primary illnesses or primary anxiety disorders. However, this group of anxious patients also has a high response rate to placebo and is likely to undergo spontaneous improvement. Antianxiety drugs also are used in the management of more persistent or recurrent primary anxiety disorders. A particularly controversial aspect of the use of benzodiazepines, especially those of high potency, is

in long term management of patients with sustained or recurring symptoms of anxiety; despite clinical benefit for at least several months, it is unclear if the long-term benefits can be distinguished from nonspecific ("placebo") effects following development of tolerance on the one hand, or prevention of related withdrawal-emergent anxiety on the other. One area of concern regarding the use of benzodiazepines in the treatment of anxiety is the potential for habituation, dependence and abuse. The models used in evaluating anxiolytic drugs are;

1. **Elevated plus maze (EPM)**

The test is based on the natural aversion of rodents for open spaces and uses an EPM with two open and two closed arms. Two indices of anxiety are obtained: the number of entries into open arms expressed as a percentage of the total number of arm entries, and the amount of time spent on the open arms expressed as a percentage of the total time on both open and closed arms. The test is rapid and was found to be sensitive to the effects of both anxiolytic and anxiogenic agents. Anxiolytic agents increase and anxiogenic agents decrease the two measures (Pellow and File, 1984; Kulkarni and Reddy, 1996).

2. **Hole board test (HBT)**

The hole board apparatus was first introduced by Boissier and Simon (1964) and since then has been extensively used to study drug effects (File and Wardill, 1975). The normal mouse of either gender, when confronted with a new environment, will explore holes in the substrate of its environment by plunging its head in and out of the hole a few times, then moving on to the next hole. Boissier and Simon (1964) distinguished curiosity and fear as two factors governing an animal's behaviour in a new situation, with escape reflecting the result of these two factors. They acknowledged that repeated head-dips may reflect greater curiousity or a desire to escape, but the interpretation of single head-dips must also be questioned. They claimed that head-dipping does not reflect basal activity because the situation is new to the animal but it is also necessary to distinguish between locomotor reactivity (File and Wardill, 1975) and exploration, the latter being the behaviour by which an animal gains information about the environment (File and Wardill, 1975).

2.6 Overview of Epilepsy

The prevalence of epilepsy in developing countries is generally higher than in developed countries (Sander and Shorvon, 1996). Studies have reported increased risk of dying and a greater proportion of deaths that are epilepsy-related in Africa – as high as a six-fold increase in mortality in people with epilepsy. This is higher than the two- to three-fold increase reported in developed countries (Christianson *et al.,* 2000; Diop *et al.,* 2005). The reasons for this gap between the developed and the developing countries are not entirely clear but suggestions have been made that it might be due to social deprivation (Sander, 2003). Interestingly, previous studies suggest that people from socio-economically deprived backgrounds in developed countries are more likely to develop epilepsy (Heaney *et al.,* 2002). A study from 2000 on the prevalence of epilepsy in a large rural community situated in the Northern Province in South Africa showed a life time prevalence in children as high as 73/1000 (Christianson *et al.,* 2000). Some infectious diseases might be a part of the explanation. Neurocysticercosis caused by *Taenia solium* (pork tapeworm) infections may trigger epileptic outburst (Garcia *et al.,* 2003; DeGiorgio *et al.,* 2005).

Epilepsy is often viewed with a certain degree of fear and risk of contagious effect due to the cultural attitudes and beliefs in Nigeria. Studies conducted in townships showed that the parents of epileptic children believe that the disorder is caused by various parameters including bewitchment, fear or evil spirits (Eastman, 2005; Akinsulore and Adewuya, 2010). It is viewed as a shameful disorder and has severe social implications in African communities as it carries a stigma. Sufferers are often shunned and discriminated against with respect to education, employment and marriage (Baskind and Birbeck, 2005).

2.6.1 Pathophysiology of Epilepsy

A seizure is a transient alteration of behaviour due to the disordered, synchronous, and rhythmic firing of populations of brain neurons. Epilepsy refers to a disorder of brain function characterized by the periodic and unpredictable occurrence of seizures. Epileptic seizures have been classified into partial seizures, which begin focally in a cortical site, and generalized seizures, which involve both hemispheres widely from the outset. The behavioural manifestations of a seizure are determined by the functions normally served by the cortical site at which the seizure arises. For example, a seizure involving motor cortex is associated with clonic jerking of the body part

controlled by this region of cortex. A simple partial seizure is associated with preservation of consciousness. A complex partial seizure is associated with impairment of consciousness. The majority of complex partial seizures originate from the temporal lobe. Examples of generalized seizures include absence, myoclonic, and tonic-clonic seizure (McNamara, 2011).

Classification of epileptic syndromes guides clinical assessment and management and, in some cases, selection of antiseizure drugs. More than 40 distinct epileptic syndromes have been categorized into partial versus generalized epilepsies. The partial epilepsies may consist of any of the partial seizure types and account for approximately 60% of all epilepsies; the aetiology commonly consists of a lesion in some part of the cortex (e.g., tumour, developmental malformation, damage due to trauma or stroke), but may also be genetic. The generalized epilepsies account for approximately 40% of all epilepsies and usually are genetic (McNamara, 2011).

2.6.2 Nature and mechanisms of seizures

Either reduction of inhibitory synaptic activity or enhancement of excitatory synaptic activity might trigger a seizure (McNamara, 2011). The neurotransmitters mediating the bulk of synaptic transmission in the mammalian brain are amino acids, with γ-aminobutyric acid (GABA) and glutamate being the principal inhibitory and excitatory neurotransmitters, respectively.

Pharmacological studies showed that antagonists of the $GABA_A$ receptor or agonist of different glutamate receptor subtypes (NMDA, AMPA, or kainic acid) trigger seizures in experimental animals. Conversely, drugs that enhance GABA-mediated synaptic inhibition or glutamate-receptor antagonists inhibit seizures. Such studies support the concept that pharmacological modulation of synaptic function can affect the propensity for seizures. Electrophysiological analyses during a partial seizure demonstrate that the individual neurons undergo depolarization and fire action potentials at high frequencies (McNamara, 2011). This pattern of rapid firing is characteristic of a seizure but is uncommon during normal neuronal activity. Thus, selective inhibition of this rapid firing would be expected to reduce seizures with minimal unwanted effects. Inhibition of the high-frequency firing may be mediated by reducing the ability of Na^+ channels to recover from inactivation, thus prolonging the refractory period when another action potential

cannot be evoked. Thus, reducing the rate of recovery of Na^+ channels from inactivation would limit the ability of a neuron to fire at high frequencies, an effect that likely underlies the effects of carbamazepine, lamotrigine, phenytoin, topiramate, valproic acid, and zonisamide against partial seizures.

Enhancing GABA-mediated synaptic inhibition may reduce neuronal excitability and raise the seizure threshold. Several drugs may inhibit seizures by regulating GABA-mediated synaptic inhibition. The principal postsynaptic receptor of synaptically released GABA is the $GABA_A$ receptor. Activation of the $GABA_A$ receptor inhibits the postsynaptic cell by increasing Cl^- inflow into the cell and hyperpolarizing the neuron. Clinically relevant concentrations of benzodiazepines and barbiturates enhance $GABA_A$ receptor–mediated hyperpolarization through distinct actions on the $GABA_A$ receptor; this enhanced inhibition probably underlies their effectiveness against partial and tonic-clonic seizures. At higher concentrations, such as might be used for status epilepticus, these drugs also inhibit high-frequency firing of action potentials. A second mechanism of enhancing GABA-mediated synaptic inhibition is thought to underlie the antiseizure mechanism of tiagabine which inhibits the GABA transporter GAT-1 and reduces neuronal and glial uptake of GABA and thereby enhancing GABA-mediated neurotransmission.

2.6.3 Pharmacological treatment of epilepsy

The ideal antiseizure drug would suppress all seizures without causing any unwanted effects. Drugs used currently not only fail to control seizure activity in some patients, but frequently cause unwanted effects that range in severity from minimal impairment of the central nervous system (CNS) to death from aplastic anaemia or hepatic failure. The task is to select the drug or combination of drugs that best controls seizures in an individual patient at an acceptable level of untoward effects. Complete control of seizures can be achieved in up to 50% of patients, while another 25% can be improved significantly. Success varies as a function of seizure type, cause, and other factors. To minimize toxicity, treatment with a single drug is preferred. If seizures are not controlled with the initial agent at adequate plasma concentrations, substitution of a second drug is preferred to concurrent administration of a second agent. However, multiple-drug therapy may be needed, especially when two or more types of seizure occur in the same patient. Anticonvulsants can be divided into eight groups based on their mechanisms, as follows:

1. Blockers of repetitive activation of sodium channel – phenytoin, carbamazepine, oxcarbazepine
2. GABA enhancers – phenobarbital, benzodiazepines
3. Glutamate modulators – topiramate, lamotrigine, felbamate
4. T-calcium channel blockers – ethosuximide, valproate
5. N- and L- calcium channel blockers – lamotrigine, topiramate, zonisamide, valproate
6. H-current modulators - gabapentin, lamotrigine
7. Blockers of unique binding site – gabapentin, lamotrigine
8. Carbonic anhydrase inhibitors – topiramate, zonisamide.

2.7 Pathophysiol*ogy* of *Alzheimer's* disease

Alzheimer's disease (AD), is the most common form of irreversible dementia, it is placing a considerable and increasing burden on patients, caregivers and society, as more people live long enough to become affected. AD is clinically characterized by a progression from episodic memory problems to a slow global decline of cognitive function that leaves patients with end-stage AD bedridden and dependent on custodial care, with death occurring on average 9 years after diagnosis (Davis and Samuel, 1998).

The current standard of care for mild to moderate AD includes treatment with acetylcholinesterase inhibitors (neostigmine, rivastigmine, galantamine etc) to improve cognitive function. The NMDA (N-methyl-D-aspartate) antagonist, memantine, has also been shown to improve cognitive function in patients with moderate to severe AD. In addition, the common non-cognitive neuropsychiatric symptoms of AD (such as mood disorder, agitation and psychosis) often require the introduction of medication, even though no existing drug is specifically indicated for their management. However, at this point, there is no approved treatment with a proven disease-modifying effect.

2.7.1 Rationale for disease-modifying strategies

Disease-modifying strategies currently being pursued for AD are based on at least one line of evidence that supports the notion that the targeted process is important in AD, which can be grouped into the following categories: pathology, genetics and epidemiology.

Pathology*:* Post-mortem analysis of human AD brains provided the first clues to the mechanisms of disease and potential interventions. It led to the description of the disease by Alzheimer more than a century ago (Alzheimer, 1907), and the identification of the hallmark lesions of AD senile plaques composed of extracellular deposits of amyloid-β (Aβ) and neurofibrillary tangles formed by accumulation of abnormal filaments of tau in brain regions that serve memory and cognition. Besides these hallmarks, prominent activation of inflammatory processes and the innate immune response are observed (McGeer and McGeer, 2007). However, determining whether a given pathological structure drives the disease or, it is a neutral bystander, or just represents an unsuccessful repair attempt remains challenging. Moreover, in an end-stage AD brain there are so many biochemical changes relative to a normal brain that numerous strategies can be rationalized by differences in gene expression or protein concentration between them.

Genetics: Mutations in three genes — amyloid precursor protein (APP), presenilin 1 (PS1; also known as PSEN1) and PS2 (also known as PSEN2) (Cruts and Van Broeckhoven, 1998) and duplication of the APP gene (Rovelet-Lecrux *et al.,* 2006) all lead to early-onset autosomal dominant AD. From a therapeutic perspective, targeting the mechanisms of familial early-onset AD makes the implicit assumption that this disease is fundamentally similar to the common sporadic late-onset form. The genetics of the more common late-onset AD is an active area of investigation. The ε4 allele of the apolipoprotein (APOE) gene has been identified as the major risk factor for late-onset AD (Corder *et al.,* 1993). Exactly how the mutated genes or different isoforms increase the risk of disease risk is not clear, and, at least in the case of $APOE_4$, a consensus mechanism of pathogenesis has not emerged in more than a decade after the discovery of its role in AD.

Epidemiology: No specific environmental toxin has been found to be consistently associated with AD, and there have been no randomized clinical trials as yet to support any specific dietary intervention. Epidemiological studies point to depression, traumatic head injury and cardiovascular and cerebrovascular factors (for example, cigarette smoking, midlife high blood pressure, obesity and diabetes) as increasing disease risk, while anti-inflammatory medications seem to reduce risk. Some studies even suggest a beneficial role of psychosocial factors (for example, higher education, physical exercise and mental activity) (Mayeux, 2008).

2.7.2 Oxidative stress in Alzheimer disease

Within any functional, aerobic cell, the processes involved in respiration inevitably generate reactive oxygen species (ROS) (Petersen *et al.,* 2007). In particular, the oxidation-reduction reactions are necessary for the generation of ATP (via the establishment of a proton gradient in oxidative phosphorylation) produce free radical intermediates as electrons are transferred from one molecule to another. Despite the resident sequestration mechanisms present within the cell that prevent the potentially harmful dispersion of the free radical intermediates, a substantial amount of ROS manage to escape daily, free to wreak havoc on macromolecules. In fact, in a specialized cell with high metabolic activity, such as a neuron, the number of such free radicals produced is estimated by some to be 10^{11} ROS/cell/day (Petersen *et al.,* 2007).

This damaging effect is most notable in AD. That is, oxidative damage marked by lipid peroxidation, nitration, reactive carbonyls, and nucleic acid oxidation is increased in vulnerable neurons in AD, relative to unaffected patients, whether or not they contain any other corresponding pathology (i.e., neurofibrillary tangles (NFTs), etc.) (Castellani *et al.,* 2001; Nunomura *et al.,* 2001). Furthermore, reduced metabolic activity, deemed the result of oxidative damage to vital mitochondrial components, has been demonstrated in AD (Aksenov *et al.,* 1998; Anderson *et al.,* 1994; Aliev *et al.,* 2003). Specifically, cytochrome oxidase, the pyruvate dehydrogenase complex, and the α-ketoglutarate dehydrogenase complex show reduced activity as a result of oxidative damage (Atzori *et al.,* 2001; Butterfield *et al.,* 2001; Aliev *et al.,* 2003).

Notably, these changes precede any other characteristics of AD (Perry and Smith, 1998; Nunomura *et al.,* 2000, 2001; Pratico *et al.,* 2001). The aforementioned presence of oxidation markers (i.e., lipid peroxidation and the like), for example, is evident even in these vulnerable neurons not yet showing other signs of disease (Nunomura *et al.,* 2001). This indicates that oxidative stress in fact precedes these other hallmarks. Moreover, the accumulation of oxidative active modification products, such as 8-hydroxyguanosine (8-OHG) and nitrotyrosine in the cytoplasm of cerebral neurons from Down's syndrome patients, temporally precede amyloid-β (Aβ) deposition - a feature of the majority of these same patients in their teens and twenties (Odetti *et al.,* 1998; Nunomura *et al.,* 2000). These oxidative-stress markers appear decades prior to Aβ deposition in these patients (Odetti *et al.,* 1998; Nunomura *et al.,* 2000).

2.7.2.1 Antioxidants for Prevention

An effective antioxidant treatment regimen could potentially buffer the effects of *in vivo* ROS such that cellular damage remains minimal. Consequently, several methods for antioxidant delivery have been made available (Bonda *et al.*, 2010). One option involves the use of naturally occurring antioxidants, and reports indeed indicate some corresponding potential. RRR-α-tocopherol (vitamin E), for example, has been demonstrated to be a lipid soluble, chain breaking antioxidant, and randomized trials have shown the vitamin to effectively slow the progression of AD (Burton *et al.*, 1982; Perrig *et al.*, 1997; Zandi *et al.*, 2004). Additionally, epidemiological studies have demonstrated that vitamin E and ascorbic acid (Vitamin C) were associated with reduced prevalence and incidence of AD (Zandi *et al.*, 2004). While these compounds are therapeutically appealing, however, their lack of specificity to neuronal mitochondria where ROS production is most significant, leaves room for improvement.

2.7.3 Treatment of Alzheimer's disease

The treatment of AD has involved attempts to augment the cholinergic function of the brain. Precursors of ACh synthesis, such as choline chloride and phosphatidylcholine (lecithin), do not produce clinically significant effects; however, inhibitors of acetylcholinesterase (AChE) have shown efficacy. Four inhibitors of AChE currently are approved by the FDA for treatment of AD: tacrine (1, 2, 3, 4-tetrahydro-9-aminoacridine; COGNEX®), donepezil (ARICEPT®), rivastigmine (EXCELON®), and galantamine (RAZADYNE®). Tacrine is a potent centrally acting inhibitor of AChE. Oral tacrine, in combination with lecithin, produces modest effects on memory performance, and the side effects of tacrine often are significant and dose-limiting; abdominal cramping, anorexia, nausea, vomiting, and diarrhoea are observed in up to one-third of patients receiving therapeutic doses, and elevations of serum transaminases are observed in up to 50% of those treated. Because of significant side effects, tacrine is not used widely clinically.

Donepezil is a selective inhibitor of AChE in the CNS with little effect on peripheral AChE. It produces modest improvements in cognitive scores in AD patients and has a long $t_{1/2}$, allowing once-daily dosing. Rivastigmine and galantamine are given twice daily and produce a similar degree of cognitive improvement. Adverse effects associated with donepezil, rivastigmine, and galantamine are similar in character but generally less frequent and less severe than those observed

with tacrine: nausea, diarrhoea, vomiting, and insomnia. Donepezil, rivastigmine, and galantamine are not associated with the hepatotoxicity that limits the use of tacrine. An alternative strategy for the treatment of AD is the use of the NMDA glutamate-receptor antagonist, memantine (NAMENDA®). Memantine produces a use-dependent blockade of NMDA receptors. In patients with moderate-to-severe AD, use of memantine is associated with a reduced rate of clinical deterioration. Whether this is due to a true disease-modifying effect, possibly reduced excitotoxicity, or is a symptomatic effect of the drug is unclear. Adverse effects of memantine usually are mild and reversible and may include headache or dizziness.

2.8 Plant description

Cnestis ferruginea Vahl ex DC (Connaraceae) is a short ornamental shrub, sometimes a climber, which is about 2.5 meters high and is usually covered by dense, brown velvety hairs (Olugbade *et al.,* 1982). Its branches are densely rusty brown pubescent. Leaves are in deciduous, 4–16-jugate with petiole 2–10 cm, rachis 12–27 cm long and densely rusty brown pubescent (Jongkind and Lemmens, 1989). Inflorescences panicles are 1–10 per leaf axil, 5–20 cm long, up to 100-flowered by little white flowers almost homostylous. Follicles are 1–5 in fruit, often united at base, ovoid, more or less oblique (2–4.5) × (1–2.5) cm with a beak blunt and broad often indistinctly separated. Pericarp has short red hairs outside and long brownish hairs inside. Epicarp is particularly velvety and spongy (Kerharo, 1974). Each follicle contains one seed ovoid (12–20) × (5–10) mm, surrounded by a sarcotesta 3–7mm long. According to Jongkind and Lemmens, (1989), the variability of *C. ferruginea* is little: the flowers are not variable at all, the characteristic rusty brown indumentum of leaves and branchlets is always present (as shown in appendix I).

2.8.1 Botanical profile

Domain: Eukaryota

Kingdom: Plantae

Subkingdom: Viridaeplantae

Phylum: Tracheophyta

Subphylum: Euphyllophytina

Infraphylum: Radiatopses

Class: Magnoliopsida

Subclass: Rosidae

Superorder: Rutanae

Order: Connarales

Family: Connaraceae

Genus: *Cnestis*

Specific epithet: *ferruginea*

Botanical name: *Cnestis ferruginea* DC.

2.8.2 Range

The plant abounds throughout west tropical Africa particularly in Gambia, Sierra Leone, Liberia, Ivory Coast, Ghana and Nigeria.

2.8.3 Local names in Nigeria

ANAANG: útín éwà

EDO: úkpò-ìbìeká, ìbìeká

EFIK: ùsièrè ébuà, útín ébuà, ébuá

HAUSA: fura amarya, otito

IGBO (Owerri): ọ̀kpṵ́ ńkḭ́tā, ámụ̀nkḭ́tā, íkè ńkḭ́tā, ọ́kpū̀kpū̀ ńkḭ́tā, ọ̀kpụ̀ńkḭ́tā, ámụ̀ ṇkḭ́tā, ọ̀kpụ̀ṇkḭ́tā.

IGBO (Umuahia): ọ̀kpụ̀ọ́há ṇkítā

URHOBO: agwọla

YORUBA: àkarà-ajẹ́, ekoro, esise, gbóyingbóyin, ọyàn-àjẹ́.

2.8.4 Phytochemistry

The petroleum ether fraction of *Cnestis ferruginea* fruit has been shown to contain constituents such as octacosanyl stearate and 1-myristo-2-stearo-3-palmitin (Ogbechie *et al.,* 1987) and a novel isoflavone glycoside, afrormosin-7-O-beta-D- galactoside with antimicrobial activity was isolated in the fruit testa (Parvez and Rahman, 1992). Other compounds such as squalene, myricyl alcohol, β-sitosterol, and a higher homolog of methyl linolenate (Olugbade *et al.,* 1982), Ogbeide *et al.* (1986) identified some glycosides of cyanidin, delphinidin and apigenidin from the plant.

2.9 Ethnobotanical uses

Various preparations of the plant are used in traditional medicine to treat diverse conditions. Root decoctions are used to treat headache, migraine, toothache, dysmenorrhoea, skin infections, gynaecological troubles, dysentery, urethral discharge, epilepsy, mental illness and sinusitis (Burkill, 1985; Garon *et al.,* 2007). Decoctions from the root are also employed as appetite stimulant, purgative, skin ointment, aphrodisiac, and as remedy for snake bite (Dalziel, 1955). The leaves are employed as a laxative. A decoction of the bark is used in treating infected gum and a decoction of various parts of the plant is also believed to assist weak children to walk (Olugbade *et al.,* 1982). However, the leaves of this plant have been shown to be poisonous in Casamance, Senegal. A phytochemical approach revealed the presence of methionine sulfoximine (MSX), a neurotoxic amino acid, in the plant extract by gas chromatography – mass spectrometry (GC–MS) (Garon *et al.,* 2007).

2.10 Pharmacological studies

2.10.1 Antimicrobial activity

The aqueous extract of the roots (Malcolm and Sofowora, 1969) and the methanolic extract of the roots, stem and leaves of *C.ferruginea* (Bakye-Yiadom and Konning, 1975) were shown to have antimicrobial properties against *Staphylococcus aureus, Escherichia coli* but did not inhibit the growth of *Aspergillus niger*, but it had antifungal effects against *Candida albicans.*

2.10.2 Antioxidant activity

The antioxidant activity of *C.ferruginea,* and some selected Nigerian medicinal plants, were investigated by Oke and Hamburger (2002). The study shows that *C.ferruginea* is a powerful antioxidant linked to the presence of flavonoids and other phenolic compounds.

2.10.3 Hypoglycaemic activity

The leaves of *Cnestis ferruginea* have been reported to inhibit haemoglobin glycosylation *in vitro* (Adisa *et al.,* 2004), the methanol and ethylacetate extracts of *C. ferruginea* have been shown to significantly produce hypoglycaemic effects in STZ-induced diabetic rats and mice (Adisa *et al.,* 2010).

Literature survey at the commencement of this research work did not show any scientific information on the toxicological, analgesic, anti-inflammatory, anticonvulsant, antidepressant, anxiolytic, antidementic, and neuroprotective activities of *Cnestis ferruginea* root extract.

CHAPTER 3

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 Plant materials

The root of *Cnestis ferruginea* was collected from a farmland in Ikotun (Alimosho Local Government Area of Lagos State, Nigeria). The botanical identification and authentication of the plant was done by Prof. J.D. Olowokudejo of the Department of Botany and Microbiology, Faculty of Science, University of Lagos, Lagos, Nigeria and Mr. Joseph Ariwaodo of the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria. The voucher specimen of the plant was deposited at the herbarium of the Institute with specimen number: FHI108219.

3.2 Preparation of plant extract

Powdered root of *C. ferruginea* (5.2 kg) was loaded into a glass percolator containing methanol (20 L). It was allowed to stand at room temperature (28ºC) for 16 h (overnight). The percolate was collected and the process of extraction was repeated five times. The combined extract was filtered and concentrated on Buchi Rotavapor at 40ºC and was further dried under vacuum pump. The percentage yield was 10.77% (Yield (%) = (Weight of dried extract/Weight of plant starting material) ×100). The brownish solid extract obtained was reconstituted in distilled water to appropriate concentrations before administration to experimental animals.

3.3 Bioactivity guided fractionation

The crude methanolic extract (560 g) was partitioned, which gave chloroform (114 g), ethylacetate (146 g), n-butanol (160 g) and an aqueous fraction (140 g).

Each fraction was subjected to the bioactivity assays using the effective dose obtained from evaluation of the methanolic crude extract (100 mg/kg) in carrageenan- induced paw oedema, hot plate test and mouse writhing reflex. There were mixed activity between aqueous and n-butanol fractions. Hence, both fractions were mixed together; it was subjected to column chromatography (silica gel 60-120 mesh) using step gradient of $CHCl_3$–MeOH as eluents. It was eluted with a gradient of $CHCl_3$–MeOH (100:00) to $CHCl_3$–MeOH (00: 100), sixty fractions were collected (1000 ml each) and their composition was monitored by TLC, those fractions having similar R_f were pooled together to give 7subfractions (F1-F7) with the following yield: 2.2, 13.62, 17.4, 75, 9.44, 4.37 and 0.81 g respectively.

The seven subfractions were evaluated for anti-inflammatory and antinociceptive activities. Fr.4 (75 g) was found to be the most effective. Fr. 4 was rechromatographed using silica gel 60-120 mesh and eluted with a gradient of $CHCl_3$–MeOH (5%H_20) (100:00) to MeOH-H_2O (95:05) to give 86 eluents (500 ml each). Compound CF-2 was obtained at $CHCl_3$- MeOH. H_2O (90:10) as yellow amorphous powder (500 mg) while Compound CF-5 was obtained at CHCl3- MeOH. H_2O (80:20) as brown amorphous powder (yield 2.2 g) (Fig. 1). Other eluents were checked with TLC, those fractions having similar R_f were pooled together to afford 2 fractions. The compounds and the fractions obtained were investigated for their antinociceptive and anti-inflammatory activity (spectra data and structure in appendix III-VIII).

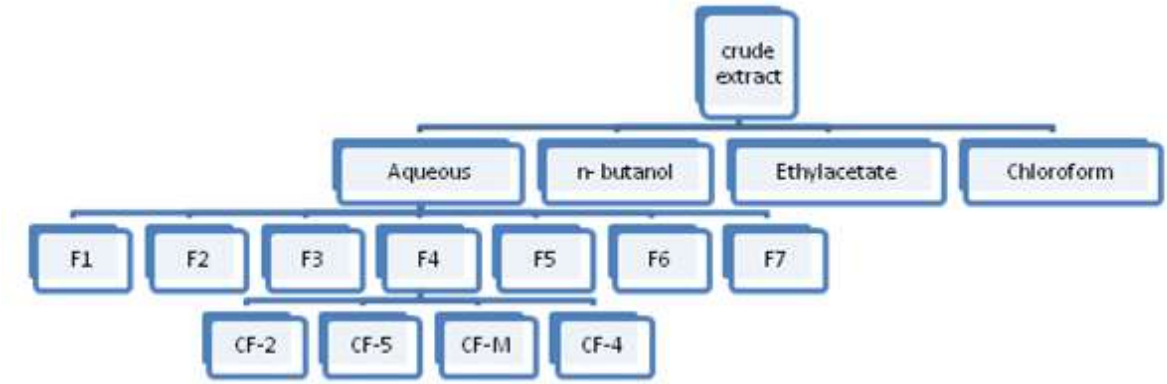

Fig 1. Schematic representation of fractionation procedures

3.3.1 General experimental procedures

^1H and ^{13}C NMR spectra were recorded on a Bruker DRX 300 MHz NMR spectrometer. Molecular weight was determined using ESMS on an advantage Max LCQ Thermo-Finnigan mass spectrometer. Column chromatography was performed using silica gel (60-120 mesh). TLC was carried out on precoated silica gel plates 60 F_{254} (Merck). Spots were visualized by UV light or by spraying with H_2SO_4-MeOH or anisaldehyde-H_2SO_4 and vanillin-H_2SO_4 reagents.

3.4. Laboratory animals

Swiss albino mice (20–30 g) and Sprague-Dawley rats (100–200 g) of either sex used in this study were obtained from the Laboratory Animal Centre of the College of Medicine, University of Lagos, Lagos, Nigeria and Laboratory Animal Services Division of Central Drug Research Institute (CDRI), Lucknow. India.

The animals were kept in well-ventilated and hygienic compartments, maintained under standard environmental conditions and fed with standard rodent pellet (Livestock Feed PLC, Lagos, Nigeria and CDRI, Animal house, India) and water *ad libitum*. The experimental procedures adopted in this study were in accordance with the United States National Institutes of Health Guidelines for Care and Use of Laboratory Animals in Biomedical Research (NIH, 1985). Equal numbers of male and female animals were used in all the groups in this study.

3.5 Toxicological evaluation

3.5.1 Acute toxicity test

Five groups of 5 mice each fasted for 12 h prior to the experiment were administered *Cnestis ferruginea* orally at doses of 1, 2, 4, 8, and 10 g/kg. Animals in the different groups were observed for 2 h post treatment for immediate signs of toxicity. Mortality observed in each group within 24 h was recorded. Animals that survived were observed for signs of delayed toxicity for a further 7 days. The LD_{50} was estimated by the log dose–probit analysis method (Litchfield and Wilcoxon, 1949; Adeyemi *et al.*, 2009).

3.5.2 Sub-chronic toxicity test

A total of 62 albino rats were randomly divided into four groups of 15 or 16 animals each (seven or eight males and eight females). The male and female rats within each group were separately caged. Animals in the different groups were daily treated with distilled water (Group I; control) and *C. ferruginea* at doses of 80 mg/kg (Group II), 400 mg/kg (Group III), and 1000 mg/kg (Group IV) orally. The doses of *C. ferruginea* used in this study represent the sub-therapeutic, therapeutic, and supra-therapeutic doses respectively, based on the modification of the protocol of Yemitan and Adeyemi (2004), Amida *et al.* (2007), and Adeyemi *et al.* (2010).

Toxic manifestations and mortality were monitored daily, and the body weight changes were recorded on days 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, and 90. The animals were generally observed for behavioural and morphological changes. At 90 days, nine or ten rats from each group (four or five males and five females) were anaesthetized by administration of 1 % chloralose in 25 % urethane (w/v) (5 ml/kg, *i.p.*; Adeyemi *et al.*, 2010) and blood samples were collected through the retro-orbital plexus vein of rat eye for haematological and biochemical

analysis. Rats were sacrificed by cervical dislocation. Semen obtained from male rats was assessed for sperm motility, count and morphology. Mortality in each treatment group was recorded in the course of the experiment. Six rats from each group (three per sex) were reserved for reversibility study in which treatment was discontinued and samples collected from animals after 14 days for analysis.

3.5.3 Effect on vital organs

After sacrificing the experimental animals at 90 days, vital organs including the brain, heart, liver, kidney, testis and ovary were harvested. The organs were carefully examined for gross lesions and weighed (using Mettler-Toledo GmbH digital weighing balance (Type BD202, SNR 06653)). Standardization of the weight of organs to 100 g body weight of each animal was done. After weight determination, samples were taken from the vital organs for determination of *in vivo* antioxidants and malondialdehyde (MDA). The remaining parts of the organs were preserved in 10% formol-saline for histopathological assessment.

3.5.4 Measurement of *in vivo* antioxidants and MDA levels

The determination of catalase, superoxide dismutase (SOD), reduced glutathione and MDA was done according to the protocol of Soon and Tan (2002).

3.5.5 Histological assessment

Tissues fixed in 10% formol-saline were dehydrated in graded alcohol, embedded in paraffin, and cut into 4-5 μm thick sections. The sections were stained with haematoxylin-eosin for photomicroscopic assessment using a Model N-400ME photomicroscope (CELTECH Diagnostics, Hamburg, Germany) (Galigher and Kozloff, 1971; Habbu *et al.*, 2008).

3.5.6 Haematological assessment

Blood samples from experimental animals were collected into ethylenediaminetetraacetate (EDTA) bottles and analyzed using standard procedures. Erythrocyte (RBC), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count, mean platelet

volume (MPV), platelet distribution width (PDW), red cell distribution width (RDW), and total and differential leukocyte (WBC), were determined using automated haematology analyzer.

3.5.7 Biochemical assessment

Blood samples collected into plain bottles were allowed to coagulate for 30 min and serum was separated by centrifugation. Serum samples were analyzed for aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), alkaline phosphatise (ALP), total bilirubin, total protein, albumin, total cholesterol, triglyceride, creatinine, urea, and uric acid using Roche and Cobas commercial kits and Roche/Hitachi 904 automated analyzer.

3.5.8 Sperm analysis

Seminal fluid obtained from male animals across the different treatment groups was analyzed to determine sperm motility, count and morphology using the methods of Cheesbrough (2000) and Ogli *et al.* (2009). Seminal fluid was collected according to the method of Ogli *et al.* (2009). After sacrifice, male rats were in turn strapped astride on their back on a dissecting board. A sterile surgical blade was used to make incision on the right scrotum and the right testis was removed with its ipsilateral epididymis into a beaker. The semen was expelled out of the epididymis into another beaker placed in water bath at 36°C.

3.5.9 Sperm motility

A drop (10-15 µl) of semen was placed on a slide such that the spermatozoa were evenly distributed and covered. The specimen was properly focused and several fields were assessed for motility upon examination using the 40 x objective of the microscope. A total of 100 spermatozoa were counted and the number of motile cells was noted (Ogli *et al.,* 2009).

3.5.10 Sperm count

A 1 in 20 dilution of semen was carried out with sodium bicarbonate-formalin diluting fluid and thorough mixing was done. Using a Pasteur pipette, an improved Neubauer ruled chamber was filled with well-mixed diluted semen. After 3-5 min, the number of spermatozoa in an area of 2 sq mm was counted using the 10 x objective of the microscope. The number of spermatozoa in 1 ml of fluid was calculated by multiplication of the number counted by 100 000 (Ogli *et al.,* 2009).

3.5.11 Sperm morphology

A thin smear of the liquefied well-mixed semen was made on a slide. The smear was fixed with 95% v/v ethanol, while still wet, for 5-10 min and allowed to air-dry. The smear was washed with sodium bicarbonate-formalin solution to remove possibly present mucus. The smear was then rinsed severally with water. The smear was covered with dilute (1 in 20) carbon fuchsin and allowed to stain for 3 min. The stain was then washed off with water. Counterstaining was done by covering the smear with dilute (1 in 20) Loeffler's methylene blue for 2 min. The stain was washed off with water, drained and air-dried. The preparation was examined for normal and abnormal spermatozoa using the 40x objective of the microscope. Hundred spermatozoa were counted and the percentages showing normal and abnormal morphology were estimated (Ogli *et al.,* 2009).

3.6 Pharmacological procedure

3.6.1 Analgesic activity

3.5.1.1 Mouse writhing test

Mice fasted overnight were divided into five groups of five animals each. The animals were then treated with vehicle (10 ml/kg, *p.o.*); *C. ferruginea* (100, 200, and 400 mg/kg, *p.o.*); fractions (100 mg/kg), CF-2 and CF-5 (12.5 – 50 mg/kg) and ibuprofen (Reference drug) (100 mg/kg, *p.o.*). Sixty minutes after treatment was carried out, mice were administered with acetic acid (0.6%, v/v in saline, 10 ml/kg, *i.p.*). The number of writhes was counted for 20 min at 5 min interval in writhing test (Singh and Majumdar, 1995; Mbagwu *et al.,* 2007). The percentage inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Number of Writhes (Control)} - \text{Number of Writhes (Treatment)}}{\text{Number of Writhes (Control)}} \text{X100}$$

3.6.1.2 Formalin test

Mice fasted overnight were divided into five groups of five animals each. The different groups of animals were treated with distilled water (10 ml/kg, *p.o.*); *Cnestis ferruginea* (100, 200 and 400 mg/kg, *p.o.*); and morphine (10 mg/kg, s.c.). Sixty minutes after administration for the oral route or 30 min for the subcutaneous route, formalin (20 µl of 1% solution) was injected subcutaneously into the right hind paw of each mouse. The time (in seconds) spent in licking and biting responses

of the injected paw, indicative of pain, was recorded for each animal. The responses of the mice were observed for 5min (early phase) and 15–30 min (late phase) post-formalin injection (Shibata *et al.,* 1989; Mbagwu *et al.,* 2007). The percentage inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Reaction Time (Control)} - \text{Reaction Time (Treated)}}{\text{Reaction Time (Control)}} \times 100$$

3.6.1.3 Haffner's tail clip test

Mice were initially screened by applying a metal artery clip to the root of the tail to induce pain and animals which failed to attempt to dislodge the clip in 10 s were discarded. Eligible mice were divided into five groups of five mice each. The pre-treatment reaction time of all mice to the tail clip was determined after which treatment was done as follows:

Distilled water (10 ml/kg, *p.o.*); *Cnestis ferruginea* (100, 200 and 400 mg/kg, *p.o.*); and morphine (10 mg/kg, s.c.). The reaction time of each mouse was then determined 60 min post-treatment for oral administration and 30 min post-treatment for subcutaneous administration (Adeyemi *et al.,* 2004). A post-treatment cut-off time of 60 s was used. The percentage inhibition was calculated using the formula:

$$\text{\% Inhibition} = \frac{\text{(Post-treatment Latency)} - \text{(Pre-treatment Latency)}}{\text{(Cut-off Time} - \text{Pre-treatment Latency)}} \times 100$$

3.6.1.4 Hot plate test

Mice used in this experiment were initially screened by placing the animals in turn on a hot plate (Electrothermal Eng. Ltd.; catalogue number MH8514B; serial number 10021249) set at 55±1°C and animals which failed to lick the hind paw or jump (nociceptive responses) within 5 s were discarded. Eligible animals were divided into five groups of five mice each and pre-treatment reaction time for each mouse was determined. Mice in the different groups were then treated with vehicle (10 ml/kg, *p.o.*); *C. ferruginea* (100, 200, and 400 mg/kg, *p.o.*); fractions (100 mg/kg), CF-2 and CF-5 (12.5–50 mg/kg) and morphine (10 mg/kg, s.c.). Sixty minutes after oral and 30 min after subcutaneous administration, the reaction time of animals was again recorded. A post-treatment cut-off time of 10 s was used (Omisore *et al.,* 2004; Gupta *et al.,* 2005). The percentage of the maximum possible effect (MPE) was calculated using the formula:

$$\text{\% MPE} = \frac{\text{(Post-treatment Latency drug)} - \text{(Post-treatment Latency control)}}{\text{(Cut-off Time} - \text{Post-treatment Latency control)}} \times 100$$

3.6.1.5 Investigation of possible mechanism(s) of analgesic effect.

The possible mechanism(s) of analgesic activity of the methanolic root extract of *C. ferruginea* was investigated using the mouse writhing and formalin-induced nociceptive tests:

In order to investigate the participation of the opioid system in the antinociceptive action of *C. ferruginea*, mice were pretreated with naloxone (5 mg/kg; *s.c.,* Rajendran *et al.,* 2000) and after 15 min the animals were given *C. ferruginea* (400 mg/kg; *p.o.*). The antinociceptive response was recorded 60 min after *C. ferruginea* treatment using acetic acid assay.

To assess the possible participation of the adrenergic system on the antinociceptive action of *C. ferruginea*, animals were pretreated with yohimbine (1 mg/kg; *i.p.,* Kaur *et al.,* 2005), an α_2 adrenergic antagonist and after 15 min the animals received *C. ferruginea* (400 mg/kg; *p.o.*). Acetic acid challenge was made 60 min after the administration of *C. ferruginea*.

To examine the possible contribution of tryptaminergic or dopaminergic system, mice were pretreated with a 5-HT_3 antagonist ondansetron (0.5 mg/kg *i.p.*, Pietrovski *et al.,* 2006) or a dopaminergic antagonist haloperidol (1 mg/kg *i.p.*, Naidu *et al.,* 2003) 15 min prior to *C. ferruginea* (400 mg/kg; *p.o.*) treatments and were subjected to acetic acid test after 60 min.

To explore the role played by potassium channels in the antinociceptive effect produced by *C. ferruginea*, mice were pretreated with glibenclamide, (10 mg/kg *i.p.*, Venkataramanan *et al.,* 2000) a potassium channel blocker and after 15 min they received *C. ferruginea* (400 mg/kg; *p.o.*) before being subjected to acetic acid assay 60 min later.

The antinociceptive effect in acute neurogenic and inflammatory responses were evaluated in formalin-induced nociception following subcutaneous or intraperitoneal injection of naloxone (5 mg/kg) or glibenclamide (10 mg/kg) respectively 15 min before oral administration of *C. ferruginea* (400 mg/kg) or morphine (10 mg/kg, s.c.) and 1 h or 30 min post-treatment, formalin (1% v/v in saline), injected *s.c*. into the right hind paw using an insulin gauge (29G1/2) needle.

3.6.2 Anti-inflammatory activity

3.6.2.1 Carrageenan-induced paw oedema

Rats used in this experiment were divided into five groups of five animals each and the respective groups were treated with vehicle (10 ml/kg, *p.o.*); *C. ferruginea* (100, 200, and 400 mg/kg, *p.o.*); fractions (100 mg/kg), CF-2 and CF-5 (12.5 – 50 mg/kg) and diclofenac (20 mg/kg, *p.o.*). One hour after administration of the various agents, oedema was induced by injection of carrageenan (0.1 ml, 1%, w/v in saline) into the sub-plantar tissue of the right hind paw (Gupta *et al.*, 2005). The linear paw circumference was then measured using the plethysmometer. Measurements of paw volume were done before injection of the phlogistic agent and at 1 h interval for 6 h and at the 24^{th} h. The percentage inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{change in paw volume (control) - change in paw volume (treated)}}{\text{change in paw volume (control)}} \times 100$$

3.6.2.2. Egg albumin-induced paw oedema

Treatment with distilled water (10 ml/kg, *p.o.*), *C. ferruginea* (100, 200 and 400 mg/kg, *p.o.*), and diclofenac (100 mg/kg, *p.o.*) was carried out in five groups of five rats each. One hour post-treatment, oedema was induced by injection of egg albumin (0.1 ml, 1%, w/v in saline) into the subplantar tissue of the right hind paw (Winter *et al.*, 1962). The linear paw circumference was then measured using the cotton thread method of Bamgbose and Noamesi (1981). Linear paw circumferences of rats were determined just before injection of the phlogistic agent and at 30 min interval for 3 h (Akindele and Adeyemi, 2007; Agbaje *et al.*, 2008). The percentage inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{change in paw volume (control) - change in paw volume (treated)}}{\text{change in paw volume (control)}} \times 100$$

3.6.2.3 Histamine- and serotonin-induced paw oedema

The anti-inflammatory activity of *C. ferruginea* was measured with phlogistic agents (viz. histamine, 5-HT) which act as mediators of inflammation. The paw oedema was induced in mice by subplantar injection of 100µl of freshly prepared histamine (10^{-3} mg/ml) and serotonin (10^{-3} mg/ml) solutions, respectively, 1 h post oral drug treatment; distilled water (10 ml/kg, *p.o.*), *C. ferruginea* (100, 200 and 400 mg/kg, *p.o.*), chlorpheniramine (10 mg/kg; *p.o.*, antihistaminic) and

cyproheptadine (4 mg/kg; *p.o.*, antiserotonergic). Measurements of paw circumference were done 1 h before injection of the phlogistic agent and at 1 h interval for 6 h (Winter *et al.*, 1962; Agbaje *et al.*, 2008). The percentage inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{change in paw volume (control) - change in paw volume (treated)}}{\text{change in paw volume (control)}} \times 100$$

3.6.2.4 Xylene-induced ear oedema.

Mice were allotted to groups of five animals each. Thirty minutes after oral treatment of mice with distilled water (10 ml/kg), dexamethasone (1 mg/kg) and extract (50–400 mg/kg), oedema was induced in each mouse by applying 30 µl of xylene to the inner surface of the right ear. Fifteen minutes later, the animals were sacrificed under ether anaesthesia and both ears cut off, sized and weighed. The mean of the difference between the right and left ears was determined for each group (Nunez Guillen *et al.*, 1997; Akindele and Adeyemi, 2007). The percentage inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Difference in ear weight (control) - Difference in ear weight (treated)}}{\text{Difference in ear weight (control)}} \times 100$$

3.6.2.5 Formaldehyde-induced arthritis.

The method of Seyle (1949) and Perez *et al.*, (1995) was adopted in this study. Animals were allotted to six groups of five rats each. The different groups of rats were treated with distilled water (10 ml/kg, *p.o.*), *Cnestis ferruginea* (100, 200 and 400 mg/kg, *p.o.*), and indomethacin (5 mg/kg, *p.o.*). Treatment was done daily for 10 days except in the case of indomethacin which was administered on alternate days. The animals were injected with 100 µl of 1% formaldehyde solution in the plantar aponeurosis of the left foot on the first and third days of the test. The paw circumference of individual rats was measured daily for 10 days. The percent inhibition of the mean increase in the paw oedema of each group was calculated on the tenth day and compared with the control. The percentage inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{change in paw volume (control) - change in paw volume (treated)}}{\text{change in paw volume (control)}} \times 100$$

3.6.3 Anticonvulsant activity

3.6.3.1 Maximal electroshock-induced seizure

The method described by Swinyard and Kupferberg (1985) as modified by Sayyah *et al.* (2002) was used in the study. Forty mice were randomly divided into eight groups each containing 5 mice. Group I received normal saline (10 ml/kg, *p.o.*), group 2-5 were treated CF (50, 100, 200 and 400 mg/kg, *p.o.*), and group 6-8 were treated with standards; clonazepam (0.5 mg/kg, *p.o.*), carbamazepine (50 mg/kg, *p.o.*), phenytoin (20 mg/kg, *p.o.*). One hour later, maximal electroshock was administered to induce seizure in the mice using Ugo Basile electroconvulsive machine (CAT no 57800-001) connected to ear clip electrodes placed on the pineal of the mice. The shock duration, frequency, current and pulse width were set and maintained at 90 s, 200 pulse/s, 48mA and 1 ms respectively. A current of about 48 mA which produced tonic seizures in 99% of the negative control mice was used throughout the study. Seizures were manifested as tonic hind limb extension (THLE) (Swinyard, 1969). The ability to prevent this feature or prolong the latency and/or onset of the THLE was considered as an indication of anticonvulsant activity (Swinyard, 1969; Sayyah *et al.,* 2002).

3.6.3.2 Strychnine-induced seizure

Mice of either sex were randomly allotted to 5 groups of 8 animals each. Group I received normal saline (10 ml/kg, *p.o.*), group II-IV received CF (50, 100, 200 mg/kg, *p.o.*) and group V received clonazepam (0.5 mg/kg, *p.o.*). 60 min post drug treatment, animal was given strychnine (4 mg/kg, *i.p.*). Onset and duration of seizure were recorded. Mice that did not convulse 30 min after strychnine administration were considered to be protected (Yemitan *et al.,* 2001).

3.6.3.3 Picrotoxin-induced seizure

Albino mice of either sex were randomly allotted to 5 groups (n=8). Group I received normal saline (10 ml/kg, *p.o.*.), group II-IV received CF (50, 100, 200 mg/kg, *p.o.*), and group 5 clonazepam (0.5 mg/kg, *p.o.*.). Picrotoxin (7.5 mg/kg, *i.p.*) was injected 60 min post drug treatments. Onset and duration of seizures were recorded. Mice that did not convulse after 30 min of picrotoxin administration were considered to be protected (Adeyemi *et al.,* 2007; Lehmann *et al.,* 1988).

3.6.3.4 Bicuculline-induced seizure

Albino mice of either sex were randomly allotted to the different control and test groups (n=8). Mice were injected *i.p.* with bicuculline (2.7 mg/kg, *p.o.*) (Masereel *et al.*, 1998; Palmer *et al.*, 1999), 1 h after oral administration of normal saline (10 ml/kg), CF (50-200 mg/kg, *p.o.*), clonazepam (0.5 mg/kg, positive control). The time to onset of clonic or tonic seizure was recorded. Animals that did not have seizures within a 30 min observation period were declared protected.

3.6.3.5 Isoniazid-induced seizure

Animals were injected *i.p.* with isoniazid (INH) 250 mg/kg (Bernasconi *et al.*, 1988). 1 h after oral administration of normal saline (10 ml/kg, *p.o.*), CF (50–400 mg/kg, *p.o.*) and clonazepam (0.5 mg/kg, *p.o.*). The time to onset of clonic or tonic seizures was recorded.

3.6.3.6 Yohimbine-induced clonic seizure in mice

Mice of either sex (n=8) were individually placed in clear plastic cylinders. Yohimbine hydrochloride (45 mg/kg, *s.c.*) was administered one hour after oral administration of distilled water (10 ml/kg), clonazepam (0.5 mg/kg), CF (50–200 mg/kg). Animals were observed for 60 min for the onset of clonic seizures. Animals that did not exhibit at least one clonic seizure within 60 min were considered protected (Dun and Fielding, 1987).

3.6.4 Behavioural observations

Mice were randomly divided into four groups of 8 mice in each group. Control group I (vehicle), group II-IV treated with graded doses of *Cnestis ferruginea* (100, 200 and 400 mg/kg, *p.o.*). The mice were subjected to spontaneous motor activity and neuromuscular coordination.

3.6.4.1 Spontaneous motor activity in mice

Gross open field activity was studied using Digiscan Infrared photocell system (test box model: RXYZCM (16 TAO); Omnitech Electronics, Columbus, Ohio) in 42x42x30cm Plexiglass arenas, fitted into infrared beam containing metallic grid. Activity of animals was observed by the interruptions of infrared beams.

Horizontal activity – the total number of beam interruptions that occurred in the horizontal sensor within 2 mins.

Total distance travelled – distance travelled by the animal was recorded in a given sample period (cm). Total distance travelled is a more accurate indicator of ambulatory activity.

Stereotypy counts – if the animal breaks the same beam (or set of beams) repeatedly then the monitor considers that the animal is exhibiting stereotypy, which typically involves grooming, licking, head bobbing etc.

Prior to the experiment, animals were habituated in the text box for 15min. After the initial habituation process, the activity of the control and treated animals were monitored for 2mins at 15mins interval for 2 h post drug treatment (Chatterjee *et al.,* 2010).

3.6.4.2 Rotarod (Motor coordination)

Mice were subjected to the rotarod test to evaluate the possible non-specific sedative or neuromuscular coordination (Dunham and Miya, 1957) effect of *Cnestis ferruginea.* Rotarod apparatus (Rotamex, Columbus; Ohio, USA) consisted of a rod which was coated with polypropylene foam to provide friction. The distance between rod and floor was kept at 15 cm to prevent intentional jumping of mice. The animals were trained on the rotarod at a fixed speed of 20 rpm for 120 s per trial, with 3 trials per day for two days. On the third day, mice were given trials before and at 1, 2, and 3 h post-treatment with graded doses of *Cnestis ferruginea* (50-200 mg/kg, *p.o.*), diazepam (5 mg/kg, *p.o.*), or distilled water (10 ml/kg, *p.o.*). The latency of falling was measured for 120 s. The average time for the mice to stay on the rotarod in each group was expressed as result (Animal that either falls often or always jumping out of the rod was excluded).

3.6.5 Antidepressant

3.6.5.1 Forced swimming test

Graded doses of *Cnestis ferruginea* (25-200 mg/kg, *p.o.*), amentoflavone (25 and 50 mg/kg, *p.o.*), imipramine (20 mg/kg, *p.o.*) were administered to mice (n=8) 1 h prior to the forced swimming test (FST). The effective dose identified in FST was utilised in tail suspension test model to confirm its antidepressant activity using fluoxetine (20 mg/kg, *p.o.*) as standard antidepressant drug.

Porsolt (1977) proposed that a normal animal forced to swim alternates swimming and immobility periods (behavioural despair test). An antidepressant reduces the immobility periods.

The mice were individually forced to swim for 15 min in glass cylinders (height: 25 cm, diameter:10 cm), with 15 cm depth of water at 25°C, which is a pre-test, and then mice were removed and dried before being returned to cages. Twenty-four hours later, mice were placed in the cylinders again for a 6-min test in the same system depicted above. The duration of immobility was recorded during the last 5min of the 6 min testing period after 1 min of acclimatization. Mice were considered to be immobile when they were floating motionless. The duration of swimming during the 5 min test period was automatically recorded by a camera mounted above the cylinders and stored on a computer equipped with the relevant software (ANY MAZE® software).

3.6.5.2 Tail suspension test (TST)

The TST was carried out essentially as previously described by Steru *et al.,* (1985). Mice were allowed to acclimatize to the holding room for 2 hours before the behavioural procedure. 1 h after administration of vehicle (10 ml/kg, *p.o.*), *C. ferruginea* (100 and 200 mg/kg, *p.o.*) and amentoflavone (12.5 –50 mg/kg, *p.o.*) and fluoxetine (20 mg/kg, *p.o.*). Mice were individually suspended by the tail from an horizontal bar (75 cm above the tabletop) using adhesive tape placed approximately 1 cm from the tip of the tail. Immobility duration was recorded for 5 min after 1 min of acclimatization.

Mice were considered immobile only when they hung passively and completely motionless. The duration of swimming during the 5-min test period was automatically recorded by a camera and stored on a computer equipped with the relevant software (ANY MAZE® software).

3.6.5.3 Elucidation of mechanism of antidepressant activity

To assess the involvement of the serotonergic system in the antidepressant-like effect of *C. ferruginea* and amentoflavone, mice were pretreated with cyproheptadine (3 mg/kg, *i.p.*, a 5-HT_2 receptor antagonist) (Ulak *et al.,* 2010) or metergoline (non-selective 5-HT_2 receptor antagonist) (Stachowicz *et al.,* 2007) and were used at doses effective in blocking the *in vivo* effects induced by 5-HT receptor agonists in rats (Stachowicz *et al.,* 2007).

To investigate the possible involvement of the noradrenergic system in the antidepressant-like effect of *C. ferruginea* and amentoflavone, animals were pretreated with prazosin (62.5 μg/kg, *i.p.*, an α_1-adrenoceptor antagonist), yohimbine (1 mg/kg, *i.p.*, an α_2-adrenoceptor antagonist) (Gu *et al.*, 2012).

To test the possible involvement of the dopaminergic system in the antidepressant-like effect of *C. ferruginea* and amentoflavone, animals were pretreated with sulpiride (50 mg/kg, *i.p.*, a dopamine D_2 receptor antagonist) (Gu *et al.*, 2012).

To investigate the involvement of cholinergic system in the antidepressant-like effect of *C. ferruginea* and amentoflavone, animals were pretreated with atropine (1 mg/kg, *i.p.*) (Lienberg *et al.*, 2010).

To assess the involvement of nitric oxide in the antidepressant effect of *C. ferruginea* and amentoflavone, mice were pretreated with N^G-nitro-L-arginine (L-NNA 10 mg/kg, *i.p.* NOS inhibitors) (Harkins *et al.*, 1999). After 15 min, they received *C. ferruginea* and amentoflavone (100 and 50 mg/kg, *p.o.*; respectively) or vehicle. Sixty min post-treatment they were subjected to FST.

3.6.6 Anxiolytic tests

To examine the anxiolytic effects of *C. ferruginea* and its constituents in animal's models the hole board, elevated plus maze, and light and dark compartment methods were utilised. In these three models, groups of mice were tested: vehicle control treated (n = 8) and graded doses of *Cnestis ferruginea* (25, 50, 100 and 200 mg/kg) CF-2 and CF-5 (25 and 50 mg/kg) (n =8). The same set of animals in each group were tested in the hole board (3 min), elevated plus maze (5 min) and light and dark compartment (5 min) after 2 min of acclimatization.

3.6.6.1 Hole board test

In the hole board test, a paradigm involving novelty and uncertainty is employed. Head dipping is generally considered to provide a measure of exploration (curiosity) that was distinct from motor activity. The board is elevated so that the mouse poking its nose into the hole does not see the

bottom. The apparatus composed of a transparent plexiglass arena (42x42x30 cm) with 16 equidistant holes 2.5 cm in diameter (Moreira *et al.*, 2000). The centre of each hole was 10cm from the nearest wall of the box. The floor of the box was positioned 15 cm above the ground. An animal was placed in the centre of the hole–board and allowed to freely explore the apparatus for 3 min. The number of head-dips was recorded. An head dip was scored if both eyes disappeared into the hole. The results are expressed as mean total number of head dips (Chatterjee *et al.*, 2010).

3.6.6.2 Elevated plus maze test

The plus maze was made of stainless steel and consisted of two open arms (30 cm x 5 cm) and two closed arms (30 cm x 5 cm) with 25 cm wall, arranged so that the two open arm are opposite to each other. The arms extended from a central platform (5 x 5 cm). The maze was elevated to height of 38.50 cm above the table. All the four arms consist of infrared beams fitted at regular intervals. Mice were treated with vehicle, *Cnestis ferruginea* (25, 50, 100 and 200 mg/kg, *p.o.*), amentoflavone (25 and 50 mg/kg, *p.o.*) and diazepam (1.5 mg/kg, *p.o.*) 1 h prior to the experiment. The mice are placed at the centre of the maze facing one of the open arm. The time spent in enclosed and open arms was recorded for 5 min after 2 min of acclimatization. The movement of animals across the arms was calculated by interruption of beams which was analyzed by Maze tracking software (M/s Columbus Instruments, USA). After each test, the maze was carefully cleaned up with a wet tissue paper (10% ethanol solution) (Chatterjee *et al.*, 2010).

3.6.6.3 Light and dark test

The apparatus consisted of a plexiglass box with two compartments (20 cm x 20 cm each), one of which was illuminated with a white light, while the other remained dark. Each animal was placed at the junction of the light / dark, facing the illuminated compartment. The time spent in the light compartment within 5 min was recorded (Young and Johnson, 1991). After each test, the box was carefully cleaned up with a wet tissue paper (10% ethanol solution).

3.6.6.4 Elucidation of mechanism(s) of anxiolytic activity

In order to elucidate the possible mechanism(s) of anxiolytic effect of amentoflavone, mice were pretreated with flumazenil (3 mg/kg, *i.p.*., Nogueira and Vassilieff, 2000). 15 min after

pretreatment, mice were given amentoflavone (25 mg/kg; *p.o.*), diazepam (1.5 mg/kg; *p.o.*) and 60 min post-treatment they were subjected to elevated plus maze test.

3.6.7 Antidementic activity

3.6.7.1 Drugs and treatment regimens

Six mice were used in each group. The crude methanolic extract (CF) (25-200 mg/kg, *p.o.*) and its active constituents, CF-2 (6.25-25 mg/kg, *p.o.*) and CF-5 (6.25-25 mg/kg, *p.o.*) were administered orally for three days. The extract and active constituents were suspended in 1% gum acacia and administered in a constant volume of 10 ml/kg body weight. The standard drug, tacrine (5 mg/kg, *p.o.*), was used as positive control. Another group of mice received vehicle of drug (*p.o.*) and scopolamine (*i.p.*) serves as a control vehicle. Scopolamine group was administered with vehicle of drug for three days.

3.6.7.2 Administration of scopolamine

One hour after last dose of drugs, scopolamine was administered (3 mg/kg, *i.p.*) to induce memory impairment. The animals were subjected to passive avoidance or Morris water maze test five min after scopolamine injection to evaluate memory function.

3.6.7.3 Passive avoidance test

Five min after the administration of scopolamine or vehicle, mice were subjected to the passive avoidance test by placing in a light compartment of computerized shuttle box with a software programme PACS 30 (Columbus Instruments, OH, USA). After an acclimatization period of 30 s, the guillotine door was opened and closed automatically after entry of the mouse into the dark compartment. The subject received a low-intensity foot shock (0.5mA; 10 s) in the dark compartment. Infrared sensors monitored the transfer of the animal from one compartment to another, which was recorded as transfer latency time (TLT) in seconds. The duration of a trial was 270 s. The 1^{st} trial was for acquisition and retention was tested in a 2^{nd} trial given 24 h after the 1^{st} trial. The shock was not delivered in the retention trials to avoid reacquisition. The criterion for learning was taken as an increase in the TLT on retention trials as compared to acquisition trial (Tota *et al.,* 2009; 2010; Awasthi *et al.,* 2010).

3.6.7.4 Morris water maze test

Morris water maze with a video tracking was used to assess learning and memory in experimental mice (Columbus instruments, Ohio, USA). It consists of a circular water tank (120 cm diameter and 50 cm height) located in a darkened test room, filled with water (26±2 ºC) to a depth of 30 cm. Four equally spaced points around the edge of the pool were designed as N (North), E (East), S (South) and W (West). A black coloured round platform of 8 cm diameter was placed 1 cm below the surface of water in a constant position in the middle of the NE quadrant in the pool. The water was coloured with non-toxic black dye to hide the location of the submerged platform. The animal was released into the pool from the SW quadrant in all the trials. The mice were given a maximum time of 60 s (cut-off time) to find the hidden platform and were allowed to stay on it for 30 s. The time taken for the mouse to find the escape platform was measured by the video tracking system. In the event the animal was unable to locate the hidden platform within 60 seconds, it was gently guided to it. Each animal was subjected to a daily session of 3 trials per day for five consecutive days. Escape latency time (ELT) to locate the hidden platform in water maze was noted as an index of learning. Mean ELT of all three trials is shown in the results. A significant decrease in latency time from that of 1st session indicates learning in water maze test (Tota *et al.*, 2011).

3.6.7.5 Estimation of biochemical parameters

Acetylcholinesterase and biochemical parameters of oxidative stress, malondialdehyde (MDA), reduced glutathione (GSH) and nitrite, were measured in the brain after the completion of behavioural studies.

3.6.7.6 Brain tissue preparation

The mice were decapitated under ether anaesthesia. The skull was cut open and the brain was exposed from its dorsal side. The whole brain was quickly removed and cleaned with chilled normal saline on the ice. A 10% (w/v) homogenate of brain samples (0.03 M sodium phosphate buffer, pH 7.4) was prepared by using an Ultra-Turrax T25 (USA) homogenizer at a speed of 9500 rpm.

3.6.7.7 Acetylcholinesterase assay in the brain

The brain homogenate in volume of 500 µl was mixed with 1% Triton X-100 (1% w/v in 0.03 M sodium phosphate buffer, pH-7) and centrifuged at 100,000 *g* at 4°C in a Beckman Ultracentrifuge (LE 80, USA), using a fixed angle rotor (80 ti) for 60 min. Supernatant of was collected and stored at 4 °C for acetylcholinesterase estimation. The kinetic profile of enzyme activity was measured spectrophotometrically (Shimadzu, USA) at 412 nm with an interval of 15 s. The specific activity of acetylcholinesterase is expressed in micromoles/min/mg of protein (Ellman *et al.,* 1986).

3.6.7.8 Measurement of malondialdehyde

Malondialdehyde (MDA) (nmol/mg protein), which is a measure of lipid peroxidation, was measured spectrophotometrically by the method of (Colado *et al.,* 1997), using 1, 1, 3, 3-tetraethoxypropane as standard. To 500 µl of tissue homogenate in phosphate buffer (*p*H 7.4), 300 µl of 30% trichloroacetic acid (TCA), 150 µl of 5 N HCl and 300 µl of 2% w/v 2-thiobarbituric acid (TBA) were added and then the mixture was heated for 15 min at 90 °C. The mixture was centrifuged at 12,000 *g* for 10 min. Pink colored supernatant was obtained, which was measured spectrophotometrically at 532 nm.

3.6.7.9 Measurement of glutathione

Glutathione (GSH) (µg/mg protein) was determined by its reaction with 5, 5′-dithiobis (2-nitrobenzoic acid) to yield a yellow chromophore which was measured spectrophotometrically (Ellman, 1959). The brain homogenate was mixed with an equal amount of 10% Trichloroacetic acid (TCA) and centrifuged (Remi cold centrifuge) at 2000 *g* for 10 min at 4 °C. The supernatant was used for GSH estimation. To 0.1 ml of processed tissue sample, 2 ml of phosphate buffer (*p*H 8.4), 0.5 ml of 5, 5′-dithiobis (2-Nitrobenzoic acid) (DTNB) and 0.4 ml of double-distilled water was added and the mixture was shaken vigorously on vortex. The absorbance was read at 412 nm within 15 min.

3.6.7.10 Nitrite estimation

Nitrite was estimated in the mice brain using the Greiss reagent and served as an indicator of nitric oxide production. 100µl of Greiss reagent (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% napthaylamine diamine dihydrochloric acid in water) was added to 100 µl of post-

mitochondrial supernatant and absorbance was measured at 542 nm (Green *et al.,* 1982). Nitrite concentration was calculated using a standard curve for sodium nitrite. Nitrite levels were expressed as µg/mg protein.

3.6.7.11 Protein estimation

Protein was measured in all brain samples for MDA, GSH and nitrite by the method of Lowry *et al.,* (1951) and for AChE activity by the method of Wang and Smith (1975). Bovine serum albumin (BSA) (1 mg/ml) was used as standard and measured in the range of 0.01–0.1 mg/ml.

3.7 *IN VITRO* STUDY

3.7.1 Cell culture

Rat C6 glioma cell line, obtained from the National Centre for Cell Sciences, Pune, India, and maintained in Central Drug Research Institute (CDRI) tissue culture facility. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. Testing was done in a 96 well plate format with different groups containing; vehicle control group (0.5% DMSO), LPS (10 µg/ml) only, LPS + CF-2 (0.1-3 µg/ml), LPS + CF-5 (0.1-3 µg/ml), CF-2 (3 µg/ml) *per se* and CF-5 (3 µg/ml) *per se* for 24 hours. Measurements for nitrite release, ROS generation, MDA formation, intracellular glutathione (GSH) level were carried out in respective groups.

3.7.2 MTT assay for cytotoxicity assessment

The assay shows the ability of the cells to convert the yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide tetrazolium salt (MTT) into a purple MTT formazon, by mitochondrial dehydrogenase activity of live cells, which was measured spectrophotometrically at 530 nm. MTT assay provides an indication of mitochondrial integrity and activity, which is interpreted as a measure of percent cell viability. The assay was carried out using the method of Mosmann (1983) with slight modifications. In brief, cells (10,000 in number) were seeded in 96-well tissue culture plates and incubated in the CO_2 incubator for 24 h at 37°C prior to experiment for the proper attachment of the cells. Then the medium

was aspirated and replaced with various concentrations of CF-2 (0.1-100 µg/ml), CF-5 (0.1-100 µg/ml). Tetrazolium (10 µl/well containing 100 µl of cell suspension; 5 mg/ml of stock in phosphate buffer saline (PBS)) salt was added 19 h post incubation period and then re-incubated for 5 h. i.e., up to total 24 h. Then, the reaction mixture was carefully aspirated and 200 µl of dimethylsulfoxide (DMSO) was added to each well by pipetting up and down several times until the content was homogenized. After 10 mins, the absorbance was read at 530 nm, using multiwell microplate reader (Bio-tek, USA). The untreated sets were also run simultaneously under the same condition, which served as control.

3.7.3 Estimation of nitrite level

Estimation of nitrite in culture supernatant from different groups was done by the Griess reagent (Martinez *et al*., 2000). C6 cells, at the cell density of 1 x 10^4 cells/well, were seeded in 96 well plates. After the incubation, an equal amount of culture supernatant (100 µl) from treated groups were mixed with equal amount (100 µ l) of Griess reagent (1% p-amino-benzene sulphonamide, 0.01% napthylethylenediamide in 2.5 %v/v phosphoric acid) and were incubated for a period of 20 minutes in dark. Absorbance was read at 570 nm. Nitrite release was expressed as percentage increase from basal level.

3.7.4 Measurement of reactive oxygen species (ROS) generation

Intracellular reactive oxygen species (ROS) generation was evaluated using a cell permeable fluorescent dye dichlorofluorescein diacetate (DCF-DA) (Peng *et al.*, 2005). C6 cells, at the cell density of 1 x 10^4 cells/well in the 96 well, were seeded in plates. After treatment, the medium was aspirated and DCF-DA 100 µl/well (10 µ M final concentrations) in phenol red free Hank's balanced salt solution (HBSS) buffer was added to wells containing cells. An incubation period of 30 minutes in dark at 37°C was added to the cells in CO_2 incubator. Fluorescence was read at wavelength excitation = 485 nm and emission at = 530 nm, by micro plate fluorescence reader (using Cary Eclipse software, VARIAN Optical Spectroscopy Instruments, Australia). ROS generation was expressed as percent increase from control.

3.7.5 Estimation of glutathione level

Reduced glutathione (GSH) content of the cells as an indirect measure of the oxidative stress was measured by the method of Anderson *et al.* (1985). Briefly C6 cells were seeded up to a cell density of 20,000 cells per/ml in petri discs. Cells were left for 24 h to get attached properly. After 24 h, medium was aspirated and then treatment of the cells growing at 90% confluency was treated with the various concentrations for a period of 24 h. Treatment medium was aspirated and the cells were washed with the ice cold phosphate buffer saline (PBS) (twice). After washing, cells were scraped into 100 µl of ice cold PBS and collected in the 2 ml eppendroff tubes. Cell lysis was done by ultrasonication (for 2 min). After lysis of the cells, cell lysate was centrifuged at 10,000 *g* for 5 min. Supernatant was collected and the pellet was discarded. Cell supernatant was de-proteinised by adding pre-cooled 10% trichloroacetic acid (TCA) 100 µl with an incubation period of the 1 h at 4°C. This was further re-centrifuged at 5,000 *g* for 5 min. The pellet was discarded and supernatant was collected, which served as sample for further assay.75 µl of the supernatant was mixed with the 25 µl of distilled water + 100 µl of buffer (0.25 M of tris base + 20 mM EDTA) + 50 µl of DTNB (0.1%). After 10 min of incubation absorbance was measured at 412 nm. Results were expressed as a relative GSH level (% of control).

3.7.6 Estimation of malondialdehyde level

Malondialdehyde (MDA) estimation in C6 cells was done according to the method of Colado *et al.* (1997). C6 cells were seeded at the cell density of 4 x 10^5 cells/well in 6 well plate, and were left for 24 h for proper attachment. After the treatment, cells were scraped into 100 µl of sodium phosphate buffer pH 7.0. Cells were lysed by sonication and centrifuged at 10000 rpm for 5 min. Pellets was discarded and supernatant was collected. 100 µl of the supernatant was added to 60 µl of 30% TCA (trichloroacetic acid) and 30 µl of TBA (thiobarbituric acid) in 0.1N NaOH was added. The sample mixture was incubated at 90°C in water bath for 15 min. Again the sample mixture was centrifuged at 10000 rpm for 10 min. The supernatants were transferred to a microplate and absorbance was read at 532 nm in microplate reader (Bio-tek, USA). Results were expressed as a MDA level (% of control).

3.7.7 Estimation of TNF-α in THP-1 cells

3.7.7.1 Stimulation of cells and collection of supernatants

TNF-α level was estimated in THP-1 (human monocytic) cell line using the method of Singh *et al.,* (2005). The cells were grown in 75-mm^2 flasks in RPMI supplemented with foetal bovine serum (10%) until they attained 70% confluency. On reaching confluency, the cells were plated in 12-well tissue culture plates (5 x 10^5 cells/ml) in serum-free (25, 50, 100, 200 ng) of LPS for 4 h. Pentoxifylline (TNF-α synthesis inhibitor, 250 μM) was used as standard. The supernatants were harvested after incubation and stored frozen at -20 °C until assay. The level of TNF-α was assayed using ELISA Kit (RnD Systems) adopting the procedures recommended by the manufacturer.

3.8 Statistical Analysis

Results obtained are expressed as mean±SEM. The data were analyzed statistically using Graphpad prism 5 software. Statistical significance level were carried out using one way ANOVA followed by Dunnet's / Tukey's multiple comparison test, also two way ANOVA followed by Bonferroni multiple comparison test. Result were considered significant when $P<0.05$.

3.9 Drugs, chemicals and reagents

Acetic acid (May & Baker Ltd., Dagenham, England), formaldehyde (Griffin & George, Leics, England), acetylsalicylic acid (Dispirin®; Reckitt & Coleman Ltd., Pakistan), diclofenac sodium (Novartis India Limited, Thane, India); morphine (Martindale Pharma®, Essex, United Kingdom), naloxone (Hameln Pharma Plus GMBH), normal saline (UNIC Pharmaceuticals, Nigeria), methanol, chloroform, ethylacetate, butanol (Merck, India); nimesulide (Panacea Biotec Ltd, India); glibenclamide, formalin, histamine, serotonin, chlorpheniramine, cyproheptadine, prazosin, yohimbine, diazepam, fluoxetine, L-nitro-NG-arginine (L-NNA), carrageenan, egg albumin, indomethacin, xylene, scopolamine, sodium hydroxide, Triton x-100, acetylthiocholine iodide, sodium chloride (NaCl), sodium nitrate ($NaNO_2$), sulphanilamide, napthaylamine diamine dihydrochloric, bovine serum albumin (BSA), 5, 5- dithiobis (2-nitro-benzoic acid) (DTNB), Folin-reagent, trichloroacetic acid and 2-thiobabituric acid (TBA) (Sigma Aldrich, St. Loiuis, MO, USA); imipramine (Ranbaxy, Laboratories Limited, India), Roswell Park Memorial Institute (RPMI-1640), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), L-glutamine, streptomycin, penicillin G, lipopolysaccharide (LPS, from *Escherichia coli* 0127:B8), *N*-(1-naphthyl) ethylenediamine dihydrochloride, foetal bovine serum (FBS), gentamicin (HiMedia Labouratories Pvt. Ltd, Mumbai, India). Starch, sodium nitroprusside, sulfanilamide, orthophosphoric acid, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Tris, ethylenediamine-tetra acetate (EDTA), bromophenol blue, nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate (BCIP), dimethyl sulfoxide (DMSO) and organic solvents of analytical grade were procured from Sisco Research Laboratories, Mumbai, India.

CHAPTER 3

RESULTS

4.0 RESULTS

4.1 Toxicity studies of *C. ferruginea*

4.1.1 Acute toxicity study

Following oral administration of methanolic root extract of *C. ferruginea* no death was recorded at doses of 1 and 2 g/kg. However, 20, 60 and 100% mortality was observed at doses of 4, 8 and 10 g/kg, respectively. The median lethal dose (LD_{50}) was estimated to be 5.22 g/kg following oral administration in mice. At the lower doses of 1 and 2 g/kg, animals were calm, hypoactive and passed out watery stools. The same observations, but with greater intensity, were made at the higher doses of 4, 8 and 10 g/kg, with the animals also exhibiting increased breath frequency (Figure 2).

In addition, intraperitoneally administration of the methanolic root extract of *C. ferruginea* produced dose-dependent mortalities with estimated LD_{50} of 643.65 mg/kg in mice (Figure 3).

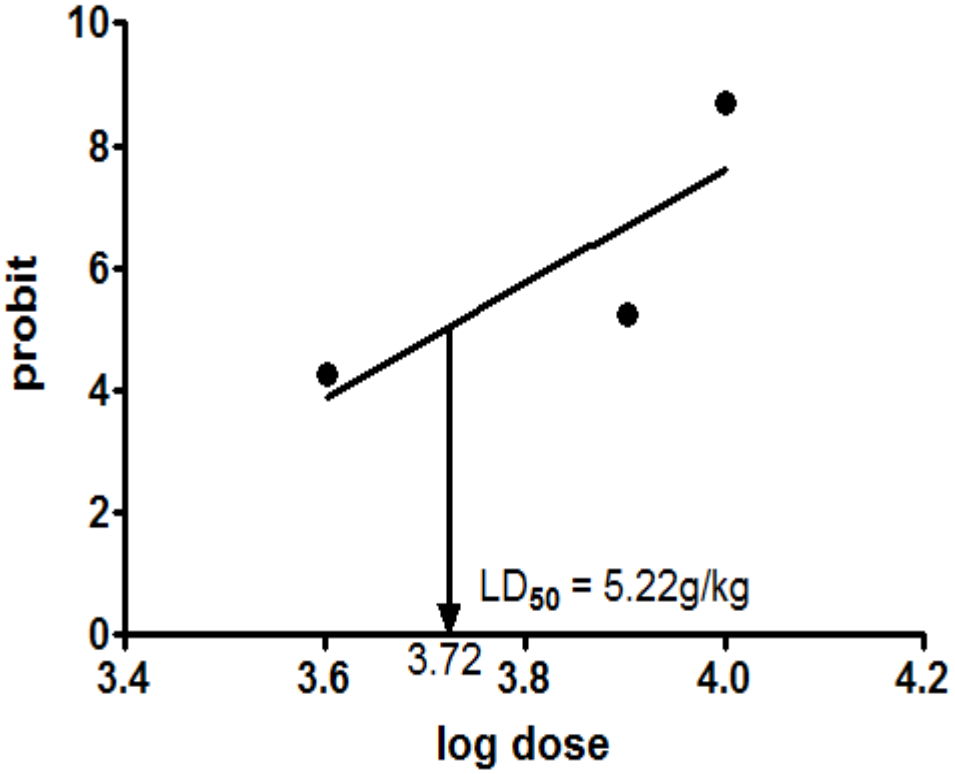

Figure 2. Graph of log dose versus probit value in oral acute toxicity study of methanolic root extract of *C. ferruginea*. The LD_{50} was estimated to be 5.22g/kg.

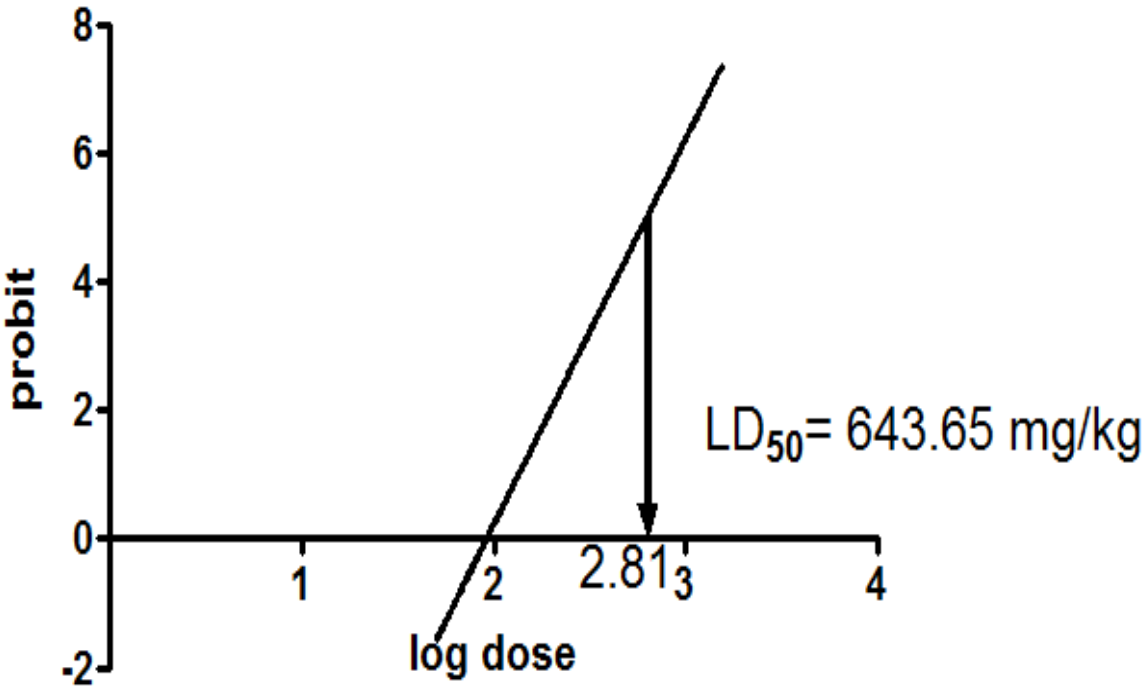

Figure 3. Graph of log dose versus probit value in acute toxicity study for intraperitoneal administration of methanolic root extract of *C. ferruginea*. The LD_{50} was estimated to be 643.65 mg/kg.

4.1.2 Mortality in the sub-chronic toxicity test

No deaths were recorded in the control group throughout the study. However, 2/15, 4/16 and 4/15 deaths were recorded in the 80, 400 and 1000 mg/kg *C. ferruginea* treatment groups, corresponding to 13.33, 25.00 and 26.67% mortality, respectively, in the main study.

4.1.3 Body weight

At doses of 80 and 400 mg/kg, *C. ferruginea* did not produce any significant change in the weight of male and female rats compared to control. However, at the highest dose of 1000 mg/kg, *C. ferruginea* elicited significant ($P<0.05$, 0.01, 0.001) reductions in weight of male rats from the 21st day up to the 90th day but there were no significant changes in the weight of female animals across the treatment intervals. Considering the mean change in weight by the 90th day, *C. ferruginea* produced a significant ($P<0.05$) reduction in the weight of male rats at 1000 mg/kg (16.00 ± 2.80 g) compared to control (40.00 ± 5.40 g). The extract did not produce any significant effect on weight in female rats at all doses used compared to control (Table 1). However, there was a significant ($P<0.001$) difference in the weight of male (16.00 ± 2.80 g) and female rats (47.77 ± 6.26 g).

Table 1: Effects of *C. ferruginea* on weight of rats.

Treatment	Dose (mg/kg)	Male (Change in weight (g)) Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70	Day 77	Day 84	Day 90
DW	-	8.10 ± 0.91	14.00 ± 0.63	19.00 ± 1.50	24.00 ± 1.50	30.00 ± 2.10	36.00 ± 2.90	40.00 ± 2.80	47.00 ± 2.80	51.00 ± 3.70	55.00 ± 2.70	59.00 ± 2.90	63.00 ± 2.80	69.00 ± 3.60
CF	80	12.00 ± 1.00	18.00 ± 1.50	24.00 ± 1.40	29.00 ± 2.00	33.00 ± 2.10	36.00 ± 2.00	41.00 ± 1.70	46.00 ± 1.70	49.00 ± 2.40	52.00 ± 1.80	55.00 ± 2.70	57.00 ± 2.40	58.00 ± 4.00
	400	11.00 ± 2.20	17.00 ± 3.00	23.00 ± 2.10	28.00 ± 2.10	34.00 ± 2.40	40.00 ± 2.30	44.00 ± 3.20	49.00 ± 3.50	58.00 ± 3.10	59.00 ± 3.00	63.00 ± 2.10	63.00 ± 2.50	63.00 ± 3.00
	1000	4.30 ± 1.70	6.40 ± $2.60^{**,\cdot}$	9.30 ± $2.80^{b,***,\cdots}$	8.10 ± $4.00^{c,***,\cdots}$	0.00 ± $4.60^{c,***,\cdots}$	8.60 ± $3.00^{c,***,\cdots}$	19.00 ± $3.60^{c,***,\cdots}$	21.00 ± $3.40^{c,***,\cdots}$	19.00 ± $5.50^{c,***,\cdots}$	22.00 ± $6.70^{c,***,\cdots}$	26.00 ± $6.20^{c,***,\cdots}$	28.00 ± $6.90^{c,***,\cdots}$	33.00 ± $6.90^{c,*,\cdot\cdot}$
Treatment	Dose (mg/kg)	Female (Change in weight (g)) Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70	Day 77	Day 84	Day 90
DW	-	8.10 ± 0.91	14.00 ± 1.50	20.00 ± 1.30	25.00 ± 1.30	30.00 ± 1.90	34.00 ± 2.60	42.00 ± 2.70	48.00 ± 2.50	53.00 ± 2.10	59.00 ± 2.00	64.00 ± 1.50	71.00 ± 1.80	73.00 ± 1.60
CF	80	18.00 ± 2.50^{b}	26.00 ± 2.60^{b}	32.00 ± 2.10^{c}	38.00 ± 1.60^{b}	44.00 ± 2.00^{b}	49.00 ± 2.20^{b}	54.00 ± 2.10	58.00 ± 1.90	63.00 ± 1.30	68.00 ± 1.90	71.00 ± 2.60	76.00 ± 3.10^{α}	81.00 ± 3.90^{α}
	400	13.00 ± 2.50	18.00 ± 2.50	24.00 ± 2.40	32.00 ± 2.80	36.00 ± 3.20	41.00 ± 3.20	47.00 ± 3.10	51.00 ± 3.50	55.00 ± 3.00	58.00 ± 2.70	61.00 ± 3.10	64.00 ± 2.40	67.00 ± 2.70
	1000	7.14 ± 1.01^{**}	17.14 ± 2.41^{α}	28.57 ± 4.46^{γ}	37.14 ± 4.74^{γ}	40.00 ± 6.46^{γ}	42.86 ± 6.53^{γ}	46.43 ± 6.61^{γ}	50.71 ± 7.35^{γ}	57.50 ± 8.24^{γ}	61.67 ± 9.46^{γ}	70.83 ± 9.70^{γ}	76.67 ± 9.72^{γ}	83.33 ± 10.14^{γ}

lues are mean ± SEM (n = 7-8 per sex). $^{a}P<0.01$, $^{b}P<0.001$ vs. control; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. CF 80 mg/kg; $^{\cdot}P<0.05$, $^{\cdot\cdot}P<0.01$, $^{\cdot\cdot\cdot}P<0.001$ vs. CF 400 mg/kg; <0.05, $^{\gamma}P<0.001$ vs. corresponding dose in male rats. Level of significance analyzed by two way ANOVA followed by Bonferonni *post hoc* tests

4.1.4 Sperm parameters

As shown in Table 2A, *C. ferruginea* did not produce any significant effect on sperm motility and morphology (% abnormality). However at the dose of 1000 mg/kg, *C. ferruginea* elicited significant ($P<0.01$) reduction in sperm count ($10.00 \pm 3.10 \times 10^6$) compared to control ($22.00 \pm 3.00 \times 10^6$). In the reversibility study (Table 2B), there was a significant ($P<0.05$) reduction in sperm count in the group that received *C. ferruginea* at the dose of 80 mg/kg ($11.33 \pm 1.64 \times 10^6$ relative to a value of $24.90 \pm 0.67 \times 10^6$ for control).

4.1.5 Effect on vital organs

In male rats, *C. ferruginea* did not produce any significant effect on the weight of the brain, heart, liver and kidney at all doses used. However at the highest dose of 1000 mg/kg, the extract produced significant ($P<0.05$) reduction in the weight of the testes (0.38 ± 0.05 g) compared to control (0.76 ± 0.07 g). In female rats, *C. ferruginea* elicited significant ($P<0.01$, 0.001) effects only on the brain (increase; 1.10 ± 0.06 g at 80 mg/kg vs. 0.77 ± 0.04 g for control), liver (decrease; 3.70 ± 0.09 g at 80 mg/kg vs. 5.00 ± 0.18 g for control) and ovary (increase; 0.10 ± 0.00 at 400 mg/kg vs. 0.04 ± 0.00 for control) (Table 3A). The effects of *C. ferruginea* on the brain, liver and ovary were reversed (Table 3B).

Table 2A: Effects of *C. ferruginea* on sperm parameters and change in body weight of rats over 90 days (main study).

Treatment	Dose (mg/kg)	Sperm motility	Sperm count (x 10^6)	Abnormality (%)		Mean change in weight of rats over 90 days (g)	
						Male	Female
DW	-	65.00±7.50	22.00±3.00	6.00±1.20		40.00±5.40	42.00±6.00
CF	80	51.00±4.30	14.00±0.68	2.80±1.10		39.00±4.20	52.00±5.50
	400	45.00±17.00	20.00±0.31	7.00±1.80		42.00±5.10	44.00±5.00
	1000	31.00±9.70	10.00±3.10[b]	8.20±1.90		16.00±2.80[a,*,¨]	47.77±6.26[γ]

Values are mean ± SEM (n = 4-5). [a]$P<0.05$, [b]$P<0.01$ vs. control; [*]$P<0.05$ vs. CF 80 mg/kg; [¨]$P<0.01$ vs. CF 400 mg/kg. [γ]$P<0.001$ vs. CF 1000 mg/kg male rats.

Table 2B: Effects of *C. ferruginea* on sperm parameters (reversibility study).

Treatment	Dose (mg/kg)	Sperm motility	Sperm count (x 10^6)	Abnormality (%)
DW	-	68.33±10.14	24.90±0.67	0.00±0.00
CF	80	48.33±4.41	11.33±1.64[a]	0.00±0.00
	400	60.00±10.00	19.75±0.25	0.50±0.50
	1000	IND	IND	IND

Values are mean ± SEM (n = 2-3). [a]$P<0.05$, [b]$P<0.01$ vs. control. IND: Insufficient data for analysis. Level of significance analyzed by one way ANOVA followed by Tukey's *post hoc* multiple comparison tests

Table 3A. Effects of *C. ferruginea* on organ weight (main study).

Treatment	Dose (mg/kg)	Male					Female				
		Brain	Heart	Liver	Kidney	Testes	Brain	Heart	Liver	Kidney	Ovary
DW	-	0.70±0.04	0.33±0.01	4.10±0.25	0.33±0.03	0.76±0.07	0.77±0.04	0.30±0.01	5.00±0.18	0.24±0.03	0.04±0.00
CF	80	0.50±0.01	0.34±0.02	3.30±0.37	0.29±0.02	0.79±0.14	1.10±0.06^{b,***}	0.33±0.01	3.70±0.09^{b}	0.33±0.01	0.06±0.01
	400	0.71±0.02	0.32±0.02	3.20±0.20	0.41±0.11	0.69±0.05	0.75±0.08$^{\beta}$	0.44±0.08	3.10±0.28^{c}	0.36±0.06	0.10±0.00$^{c,\beta}$
	1000	0.70±0.03	0.29±0.01	3.80±0.27	0.35±0.10	0.38±0.05$^{a,\alpha,\bullet}$	0.71±0.05$^{\gamma}$	0.36±0.02	3.70±0.16^{b}	0.31±0.02	0.07±0.01$^{\bullet}$

Values are mean ± SEM (n = 4-5 per sex). $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ vs. control; $^{\alpha}P<0.05$, $^{\beta}P<0.01$, $^{\gamma}P<0.001$ vs. CF 80 mg/kg; $^{\bullet}P<0.05$ vs. CF 400 mg/kg; $^{***}P<0.001$ vs. CF 80 mg/kg (Male). Level of significance analyzed by one way ANOVA followed by Tukey's *post hoc* multiple comparison tests.

Table 3B: Effects of *C. ferruginea* on organ weight (reversibility study).

Treatment	Dose (mg/kg)	Male					Female				
		Brain	Heart	Liver	Kidney	Testes	Brain	Heart	Liver	Kidney	Ovary
DW	-	1.03±0.13	0.34±0.02	3.92±0.48	0.37±0.03	0.64±0.01	1.07±0.09	0.30±0.02	3.27±0.34	0.31±0.02	0.05±0.01
CF	80	0.93±0.07	0.34±0.05	3.93±0.74	0.37±0.04	0.48±0.02	1.06±0.06	0.36±0.03	3.82±0.17	0.52±0.23	0.07±0.02
	400	0.83±0.09	0.36±0.02	3.81±0.01	0.37±0.04	0.80±0.19	1.16±0.07	0.35±0.00	3.50±0.02	0.34±0.04	0.10±0.03
	1000	IND	IND	IND	IND	IND	1.00±0.07	0.28±0.02	2.92±0.04	0.32±0.07	0.06±0.01

Values are mean ± SEM (n = 2-3 per sex). $P>0.05$ vs. control. IND: Insufficient data for analysis. Level of significance analyzed by one way ANOVA followed by Tukey's *post hoc* multiple comparison tests.

4.1.6 *In vivo* antioxidant enzymes and MDA

As shown in Table 4, In male rats, *C. ferruginea* did not produce any significant effect on organ levels of GSH, SOD, CAT, and MDA in respect of the kidney and brain. However, there was significant increase ($P<0.001$) in the level of GSH in the liver (337.40 ± 89.91 U/mg protein compared to 62.32 ± 6.23 U/mg protein for control) and reduction ($P<0.01$) in the testes (71.50 ± 17.81 U/mg protein compared to 262.60 ± 23.02 U/mg protein for control) at 1000 mg/kg. At 80 mg/kg, there was a significant increase in testes MDA (25.25 ± 1.69 U/mg protein) compared to control (9.82 ± 1.40 U/mg protein). In female rats, pronounced effects were only produced in respect of the liver and ovaries. For the liver, *C. ferruginea* significantly ($P<0.01$) increased the level of GSH at 1000 mg/kg (259.70 ± 30.98 U/mg protein) compared to control (58.86 ± 15.52 U/mg protein); and significantly ($P<0.05$) reduced the level of SOD at 80 and 400 mg/kg (72.43 ± 14.33, 67.05 ± 14.65 U/mg protein respectively) relative to control (206.80 ± 37.17 U/mg protein). In respect of CAT, there was a significant ($P<0.05$) reduction at 400 mg/kg (276.40 ± 56.67 U/mg protein) compared to control (831.30 ± 149.40 U/mg protein). There was no significant effect on liver MDA level. For the ovary, *C. ferruginea* produced significant ($P<0.05$, 0.01) increase in GSH level at 400 and 1000 mg/kg (374.20 ± 10.01, 326.10 ± 10.59 U/mg protein respectively) relative to control (110.50 ± 54.20 U/mg protein). For SOD, there was a significant ($P<0.05$) increase at 80 mg/kg (217.70 ± 36.64 U/mg protein) compared to control (58.04 ± 26.16 U/mg protein). In respect of CAT, there was also a significant ($P<0.05$) increase at 80 mg/kg and 400 mg/kg (953.80 ± 150.60, 980.00 ± 17.50 U/mg protein, respectively) compared to control (243.30 ± 98.55 U/mg protein). *C. ferruginea* did not produce any significant effect on ovary MDA level. Generally, there were no significant differences between corresponding values in male and female rats (Table 4).

Table 4: Effects of *C. ferruginea* on *in vivo* antioxidant enzymes and MDA level.

Treatment	Dose (mg/kg)	Organ	Male				Female			
			GSH (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (U/mg protein)	GSH (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (U/mg protein)
DW	-	Liver	62.32±6.23	108.80±15.39	437.50±61.87	26.38±4.38	58.86±15.52	206.80±37.17	831.30±149.40	39.93±7.96
CF	80		91.74±1.20	87.55±0.29	351.70±1.67	51.05±0.25	120.60±4.29	72.43±14.33[a]	406.00±90.66	33.24±6.09
	400		59.60±5.78	84.16±55.99	326.70±230.50	41.58±8.52	96.17±11.05	67.05±14.65[a]	276.40±56.67[a]	27.62±5.22
	1000		337.40±89.91[c,γ,***]	132.00±60.60	484.20±207.40	24.48±6.10	259.70±30.98[b,α,**]	112.80±13.19	441.70±50.69	25.90±1.40
DW	-	Kidney	57.24±15.84	55.86±6.45	320.80±77.17	27.86±2.75	31.44±12.40	65.30±21.77	262.50±87.50	34.43±6.89
CF	80		157.60±57.46	43.53±21.77	175.00±87.50	31.12±3.17	60.30±4.07	90.33±25.65	516.30±230.60	34.36±4.55
	400		244.00±95.53	171.20±23.35	729.20±77.17	32.00±7.49	130.50±15.17	129.90±41.15	455.00±76.45	19.53±4.57
	1000		43.12±4.50[*]	33.81±15.89[*]	145.80±60.81	39.06±9.09	99.00±19.37	54.41±10.88	288.80±61.25	31.05±5.94
DW	-	Brain	250.00±73.76	65.30±21.77	262.50±87.50	28.98±8.74	253.50±45.40	70.74±34.84	284.40±140.10	31.02±13.13
CF	80		177.70±27.61	46.00±10.50	184.90±42.22	30.06±4.68	248.10±108.50	67.47±31.62	271.30±127.10	33.85±10.08
	400		125.90±59.50	111.00±61.73	446.30±248.20	30.85±10.44	350.50±27.43	54.41±6.28	218.80±25.62	36.15±2.69
	1000		352.70±36.13	49.30±13.29	198.20±53.43	43.71±11.14	82.34±42.45	32.65±10.88	131.30±43.75	20.76±10.53
DW	-	Testes/ Ovary	262.60±23.02	54.41±6.28	218.80±25.26	9.82±1.40	110.50±54.20	58.04±26.16	243.30±98.55	25.02±12.82
CF	80		299.00±14.22	195.90±0.00	787.50±0.00	25.25±1.69[a]	95.59±11.70	217.70±36.64[a]	953.80±150.60[a]	19.51±7.42
	400		187.30±41.07	133.90±62.79	538.10±252.40	14.75±5.68	374.20±10.01[b,β]	191.70±4.50	980.00±17.50[a]	15.01±3.54
	1000		71.50±17.81[b,γ,*]	160.00±43.83[*]	643.10±176.20	15.36±1.86	326.10±10.59[a,β]	43.53±0.00[α]	218.80±43.75[α,*]	13.97±0.74

Values are mean ± SEM (n = 4-5 per sex). [a]*P<0.05*, [b]*P*<0.01, [c]*P*<0.001 vs. control; [α]*P<0.05*, [β]*P*<0.01, [γ]*P*<0.001 vs. CF 80 mg/kg; [*]*P<0.05*, [**]*P*<0.01, [***]*P*<0.001 vs. CF 400 mg/kg. Generally, there were no significant differences (*P*>0.05) between corresponding values in male and female rats. Level of significance analyzed by one way ANOVA followed by Tukey's *post hoc* multiple comparison tests.

4.1.7 Haematological parameters

As shown in Table 5A in respect of male rats, *C. ferruginea* did not produce any significant effect on all parameters evaluated except on RDW at doses of 400 and 1000 mg/kg in which there were significant ($P<0.05$) reductions (32.73 ± 1.15%, 32.85 ± 0.88%, respectively) compared to control (37.46 ± 1.32%). In female rats, *C. ferruginea* did not elicit any significant effect on all parameters relative to control. In the reversibility study (Table 5B), the effect of *C. ferruginea* on RDW was reversed but there were delayed significant ($P<0.05$, 0.01) effects on MPV (decrease; 7.60 ± 0.10 fl at 400 mg/kg vs. 8.27 ± 0.07 fl in control) in male rats and on Hb (decrease; 12.43 ± 0.30 g/dl at 80 mg/kg vs. 14.67 ± 0.37 in control), PCV (decrease; 41.23 ± 0.49% at 80 mg/kg vs. 48.20 ± 1.37% in control) and MPV (increase; 8.50 ± 0.20 fl at 400 mg/kg vs. 7.80 ± 0.21 fl in control) in female rats.

4.1.8 Biochemical parameters

As shown in Table 6A, *C. ferruginea* did not produce any significant effect on all parameters assessed in both sexes except in the case of total bilirubin in female rats in which there was a significant ($P<0.05$) increase (6.45 ± 0.35 mmol/L) compared to control (4.94 ± 0.17 mmol/L). This effect was however reversed in the reversibility study (Table 6B). In both sexes, there were no significant delayed effects in respect of all the parameters evaluated.

Table 5A: Effects of *C. ferruginea* on haematological parameters (main study).

Treatment	Dose (mg/kg)	Sex	RBC (10^6/µl)	Hb (g/dl)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (%)	MPV (fl)	Platelet (10^5/µl)
DW	-	Male	6.51±0.40	11.74±0.60	38.68±1.94	59.72±1.45	17.98±0.43	30.10±0.04	7.80±0.30	510.80±73.02
CF	80		6.95±0.54	12.00±0.40	40.25±2.15	58.05±1.35	17.35±0.75	29.80±0.60	7.75±0.05	471.50±329.50
	400		7.35±0.28	13.75±1.24	41.65±1.94	56.63±0.67	17.03±0.08	30.05±0.27	7.53±0.15	527.00±78.00
	1000		7.14±0.54	12.15±0.76	39.58±1.59	57.33±1.01	17.08±0.21	29.75±0.37	7.53±0.21	239.80±102.40
DW	-	Female	5.87±0.27	10.72±0.83	35.62±1.82	60.64±1.15	18.20±0.91	29.90±1.05	7.80±0.14	419.40±29.32
CF	80		6.42±0.20	11.54±0.25	38.12±1.33	59.34±0.86	18.02±0.52	30.36±0.75	7.80±0.17	404.40±96.82
	400		6.44±0.32	11.15±0.45	36.48±1.56	56.73±1.20	17.33±0.33	23.83±6.81	7.28±0.33	460.40±173.80
	1000		6.67±0.65	11.85±0.99	39.50±3.73	59.33±1.35	17.85±0.44	30.10±0.36	7.75±0.17	510.00±181.90
	Dose (mg/kg)		RDW (%)	PDW (%)	WBC (10^3/µl)	WBC differential (%)				
						L	Monocyte	Neutrophil	Eosinophil	Basophil
DW	-	Male	37.46±1.32	9.44±0.49	11.96±3.20	66.48±6.19	5.96±1.05	24.34±5.41	3.22±0.56	0
CF	80		36.75±1.35	9.80±0.40	9.55±1.25	62.75±2.15	3.85±0.35	32.05±1.55	1.35±0.25	0
	400		32.73±1.15[a]	9.05±0.20	8.83±2.76	53.25±5.17	8.10±1.31	35.53±5.22	3.88±0.52	0
	1000		32.85±0.88[a]	9.08±0.28	9.73±1.15	58.23±4.61	6.25±0.82	32.53±4.25	3.00±0.44	0
DW	-	Female	32.18±0.66	9.90±0.35	9.32±0.73	68.22±1.01	4.92±0.77	24.78±0.98	2.28±0.36	0
CF	80		31.86±0.56	8.34±1.70	6.52±0.44	69.08±0.87	3.88±0.56	26.92±3.18	2.12±0.45	0
	400		31.53±0.90	8.73±0.56	4.90±1.13	64.58±9.60	3.70±0.78[α]	29.88±9.62	3.10±0.80	0
	1000		32.45±1.24	9.78±0.29	11.30±2.62	75.35±3.37	3.40±0.96	20.13±1.98	1.13±0.48	0

Values are mean ± SEM (n = 4-5 per sex). [a]*P<0.05* vs. control value; [α]*P<0.05* vs. corresponding value for male rats. L- leukocyte. Level of significance analyzed by one way ANOVA followed by Tukey's *post hoc* multiple comparison tests.

Table 5B: Effects of *C. ferruginea* on haematological parameters (reversibility study).

Treatment	Dose (mg/kg)	Sex	RBC (10^6/µl)	Hb (g/dl)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (%)	MPV (fl)	Platelet (10^5/µl)
DW	-	Male	7.38±0.25	13.43±0.03	45.07±0.03	60.17±1.70	17.70±0.60	29.80±0.06	8.27±0.07	831.30±23.10
CF	80		7.96±0.02	13.80±0.31	46.20±0.53	58.17±0.55	17.20±0.32	29.63±0.29	8.03±0.03	820.70±40.42
	400		7.82±0.51	14.20±0.60	46.45±1.65	59.50±1.80	18.20±0.40	30.60±0.20	7.60±0.10[a]	798.50±90.50
	1000		IND	IND	IND	IND	IND	IND	IND	IND
DW	-	Female	7.84±0.28	14.67±0.37	48.20±1.37	61.53±1.48	18.70±0.23	30.47±0.41	7.80±0.21	898.30±124.30
CF	80		7.09±0.10	12.43±0.30[b]	41.23±0.49[α,b]	58.27±0.17	17.60±0.15	30.43±0.37	7.53±0.07	836.00±4.62
	400		7.41±0.06	13.05±0.05	44.10±0.70	59.00±1.00	17.60±0.10	29.60±0.40	8.50±0.20[α,a,**]	818.00±32.00
	1000		8.24±0.21	14.35±0.15[*]	47.80±0.00[**]	58.05±1.45	17.45±0.25	30.00±0.30	8.35±0.05[*]	1062.00±102.00
	Dose (mg/kg)		RDW (%)	PDW (%)	WBC (10^3/µl)	WBC differential (%)				
						L	Monocyte	Neutrophil	Eosinophil	Basophil
DW	-	Male	39.70±0.50	10.23±0.09	10.47±1.56	63.27±5.41	5.00±0.55	28.63±5.75	3.13±0.09	0
CF	80		38.23±0.13	9.97±0.12	18.01±0.81	74.20±0.32	5.37±0.20	15.33±0.20	5.00±0.00	0
	400		37.35±0.65	9.15±0.05	15.50±3.30	72.40±1.00	6.20±2.50	46.75±24.25	4.65±2.25	0
	1000		IND	IND	IND	IND	IND	IND	IND	0
DW	-	Female	36.73±4.25	9.57±0.50	6.47±0.34	68.23±3.24	4.90±0.96	26.33±4.94	2.87±0.22	0
CF	80		33.50±0.84	9.20±0.06	9.17±1.16	70.70±0.91	5.00±0.72	21.87±0.20	2.37±0.09	0
	400		34.25±0.55	10.70±0.40	9.25±3.85	69.50±12.20	6.65±0.65	20.25±11.05	3.60±0.50	0
	1000		34.70±0.10	10.90±0.60[*]	7.80±4.40	64.00±7.40	5.50±2.00	5.76±3.45	2.50±0.50	0

Values are mean ± SEM (n = 2-3 per sex). [a]*P<0.05*, [b]*P*<0.01 vs. control value; [α]*P<0.05* vs. corresponding value for male rats. [*]*P<0.05*, [**]*P*<0.01 vs. CF 80 mg/kg. Level of significance analyzed by one way ANOVA followed by Tukey's *post hoc* multiple comparison tests. IND: Insufficient data for analysis; L- leukocyte

Table 6A: Effects of *C. ferruginea* on serum biochemical parameters (main study).

Treatment	Dose (mg/kg)	Male				Female			
		AST (U/L)	ALT (U/L)	ALP (U/L)	Total bilirubin (mmol/L)	AST (U/L)	ALT (U/L)	ALP (U/L)	Total bilirubin (mmol/L)
DW	-	238.50±28.86	102.00±26.00	254.60±36.36	5.98±0.15	216.30±15.74	108.20±8.87	201.90±38.51	4.94±0.17
CF	80	130.00±24.30	56.90±6.90	216.90±13.53	6.30±0.59	240.90±31.43	91.64±5.36	237.10±27.42	5.50±0.35
	400	175.50±13.92	77.20±15.89	279.90±37.88	6.39±0.33	278.10±50.03	192.10±78.41	272.30±51.84	5.78±0.32
	1000	201.60±12.20	88.38±9.24	253.10±39.83	6.47±0.10	266.50±20.39	110.80±12.70	261.00±19.58	6.45±0.35[a]
		Total protein (g/L)	Albumin (g/)	Total cholesterol (mmol/L)	Triglyceride (mmol/L)	Total protein (g/L)	Albumin (g/L)	Total cholesterol (mmol/L)	Triglyceride (mmol/L)
DW	-	63.74±9.44	32.32±1.48	1.52±0.08	0.72±0.07	91.50±2.29	44.50±1.64	1.75±0.10	0.97±0.22
CF	80	80.75±2.05	34.60±1.70	1.55±0.05	0.54±0.03	89.68±2.50	37.32±1.52	2.01±0.09	0.73±0.06
	400	77.25±2.66	32.05±1.85	1.49±0.06	0.78±0.16	97.58±7.18	34.93±4.32	1.86±0.32	0.76±0.15
	1000	68.14±2.83	28.98±1.10	1.21±0.12	0.75±0.05	85.30±3.92	37.03±2.75	1.86±0.19	1.03±0.06
		Creatinine (µmol/L)	Urea (mmol/L)	Uric acid		Creatinine (µmol/L)	Urea (mmol/L)	Uric acid	
DW	-	60.48±1.89	5.94±0.76	116.10±17.83		78.36±1.96	6.20±0.51	65.88±11.47	
CF	80	77.74±3.06	7.30±0.90	100.60±7.59		80.87±4.13	6.42±0.64	83.82±6.22	
	400	74.38±1.65	5.25±0.39	137.20±19.31		74.94±4.83	6.73±0.93	162.10±28.45	
	1000	66.54±3.75	6.80±0.54	185.20±62.50		73.47±6.04	5.78±0.49	152.90±8.35	

Values are mean ± SEM (n = 4-5 per sex). [a]$P<0.05$ vs. control. Generally, there were no significant differences ($P>0.05$) between corresponding values in male and female rats. Level of significance analyzed by one way ANOVA followed by Tukey's *post hoc* multiple comparison tests.

Table 6B: Effects of *C. ferruginea* on serum biochemical parameters (reversibility study).

Treatment	Dose (mg/kg)	Male				Female			
		AST (U/L)	ALT (U/L)	ALP (U/L)	Total bilirubin (mmol/L)	AST (U/L)	ALT (U/L)	ALP (U/L)	Total bilirubin (mmol/L)
DW	-	262.30±14.15	152.40±68.45	244.30±52.38	2.84±1.15	209.10±17.05	106.00±6.55	183.00±1.53	2.58±0.96
CF	80	179.50±7.45	124.40±13.45	356.40±135.80	1.90±0.46	255.40±77.00	125.30±31.20	185.70±6.27	2.39±0.54
	400	221.80±44.80	169.80±58.85	309.90±10.74	2.70±0.39	382.30±105.10	319.90±109.30	279.80±16.70	1.81±0.29
	1000	IND	IND	IND	IND	241.90±26.35	149.00±17.85	343.20±36.30	3.54±0.14
		Total protein (g/L)	Albumin (g/)	Total choles-terol (mmol/L)	Triglyceride (mmol/L)	Total protein (g/L)	Albumin (g/L)	Total choles-terol (mmol/L)	Triglyceride (mmol/L)
DW	-	75.00±0.30	36.10±3.00	1.83±0.02	0.90±0.04	88.80±5.20	43.85±0.25	2.54±0.21	0.67±0.22
CF	80	79.25±5.55	35.75±3.55	1.65±0.22	1.19±0.49	75.55±8.35	36.60±4.50	1.99±0.30	0.87±0.16
	400	81.00±3.80	35.95±1.65	1.92±0.03	0.71±0.25	83.50±1.30	31.47±1.44	2.09±0.25	0.79±0.08
	1000	IND	IND	IND	IND	82.55±0.35	29.60±4.40	1.48±0.38	0.71±0.17
		Creatinine (µmol/L)	Urea (mmol/L)	Uric acid		Creatinine (µmol/L)	Urea (mmol/L)	Uric acid	
DW	-	64.26±0.16	6.75±1.55	74.24±0.38		68.72±3.79	9.15±0.95	73.26±1.06	
CF	80	60.55±4.85	6.65±1.05	133.60±37.18		66.35±10.00	6.85±2.45	141.90±38.14	
	400	62.74±0.30	5.00±0.40	49.19±4.61		62.27±2.51	5.97±0.64	73.94±7.93	
	1000	IND	IND	IND		65.07±0.06	5.60±1.50	91.29±33.87	

Values are mean ± SEM (n = 2-3 per sex). Level of significance analyzed by one way ANOVA followed by Tukey's *post hoc* multiple comparison tests. Data were not significant ($P>0.05$). IND- Insufficient data for analysis.

4.1.9 Histological assessment

4.1.9.1 Heart

There was no adverse histological presentation observed in the distilled water and CF (80, 400 and 1000 mg/kg) treatment groups. The heart appeared normal, composed of interlacing fascicles or syncitium of myocardial cells which are elongated, with spindle nuclei.

4.1.9.2 Kidney

There was no adverse histological presentation observed in the distilled water and CF (400 and 1000 mg/kg) treatment groups. The kidney appeared normal, normocellular glomerular tufts were displayed on a background containing tubules.

4.1.9.3 Liver

Distilled water treated histological presentation appeared normal, normal hepatic architecture with hepatocytes arranged in plates. There was no vascular congestion, no areas of necrosis or haemorrhage. No fatty change or fibrosis was seen. The portal tracts contained the hepatic triads (artery, vein and bile duct), and scant inflammatory cellular infiltrates (Plate 1). However, CF (80 mg/kg) treated showed prominent hepatic sinusoids which were engorged with red blood cells (Plate 2). Similarly, CF (400 and 1000 mg/kg) appeared normal in female treated groups but with intracellular accumulation and fatty change in male treated groups. The hepatocytes stained less with eosin; their cytoplasm appeared glassy and contained inclusions, possibly lipid (Plate 3 and 4 respectively).

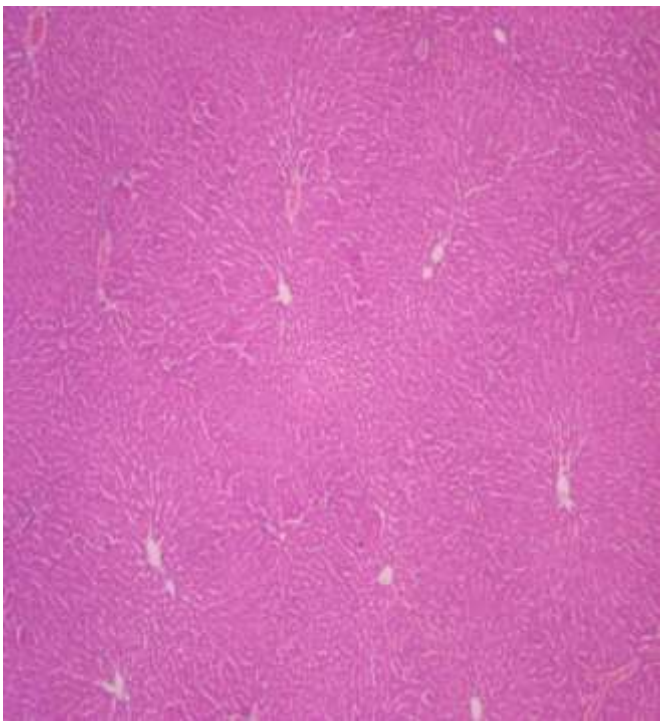

Plate 1: Histologic presentation of distilled water control treated rat liver with normal appearance. x40

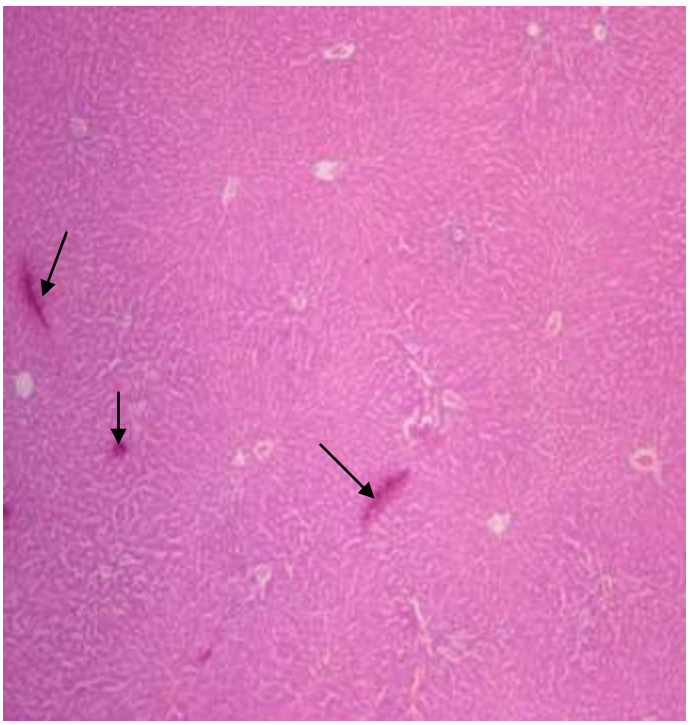

Plate 2: Histologic presentation of CF (80 mg/kg; *p.o*) treated rat liver showing congested sinusoids. x40

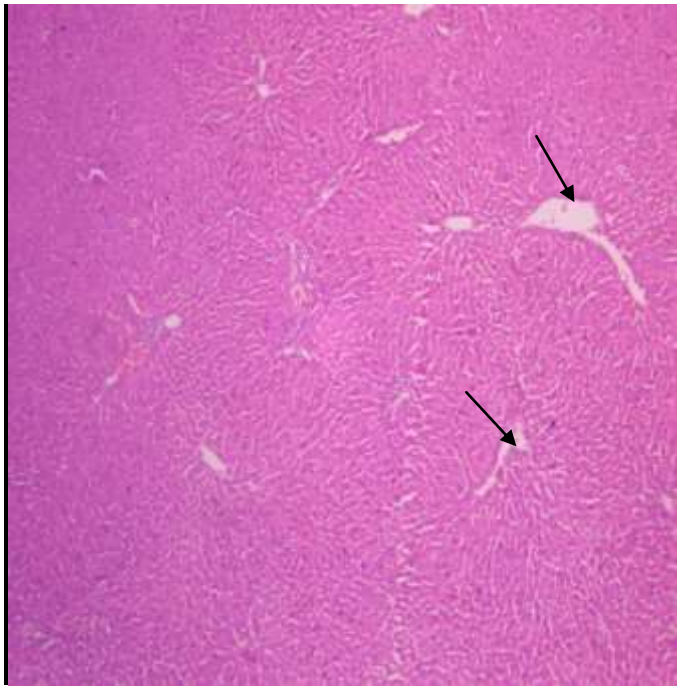

Plate 3: Histologic presentation of CF (400 mg/kg; *p.o.*) treated rat liver showing intralobular fatty accumulation. x40

[Note: the arrows indicates histopathological presentations]

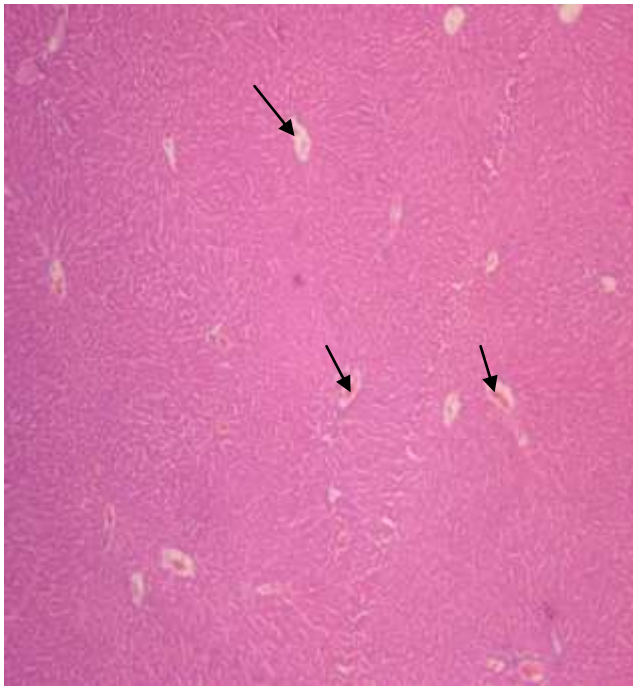

Plate 4: Histologic section of CF (1000 mg/kg; *p.o.)* treated rat liver showing congested sinusoids. x40

4.1.9.4 Testes

There was no adverse histological presentation observed in the distilled water (Plate 5) and CF (80 mg/kg) (Plate 6) and 400 mg/kg treatment groups. The testis appeared normal, the seminiferous tubules were normal sized, lined by 4–5 cell layer thick spermatogenic series cells, and contained numerous spermatids within their lumina. The interstitial cells of Leydig were also normal. However, inflammation/necrosis, dead cells and amorphous debris seen with aggregation of inflammatory cells in CF (1000 mg/kg) treated group (Plate 7).

4.1.9.5 Ovary

There was no adverse histological presentation observed in all the treatment groups. The ovary appaeared normal, it is consisted of spindle-shaped cells, fine collagen fibers and ground substance that together constituted the ovarian stroma. The stromal cells resembled fibroblasts but some contained lipid droplets. Bundles of smooth muscle cells were also scattered throughout the stroma. In the peripheral zone of the stroma, known as the cortex, were numerous follicles that contained female gametes in various stages of development. In addition, there were post-ovulatory follicles of various kinds, namely *corpora lutea*.

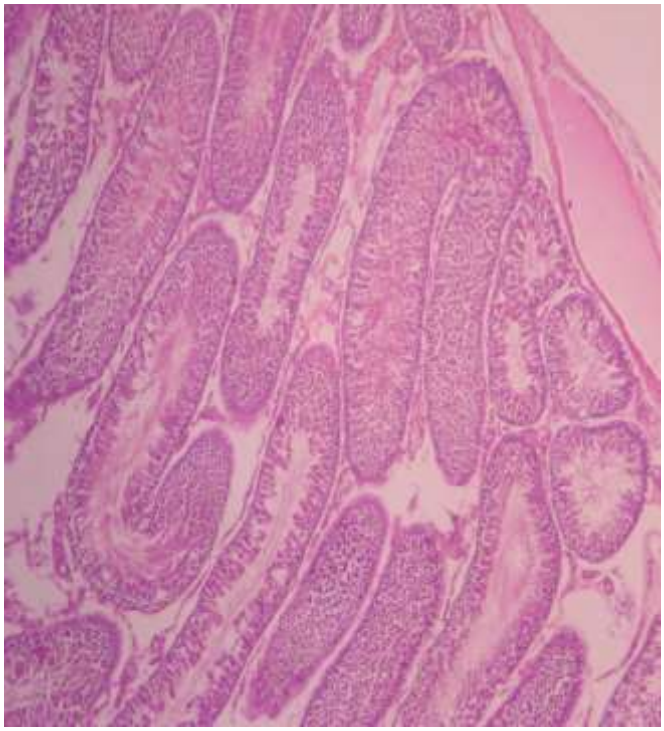

Plate 5: Histologic presentation of normal rat testes of distilled water treated control group. x40

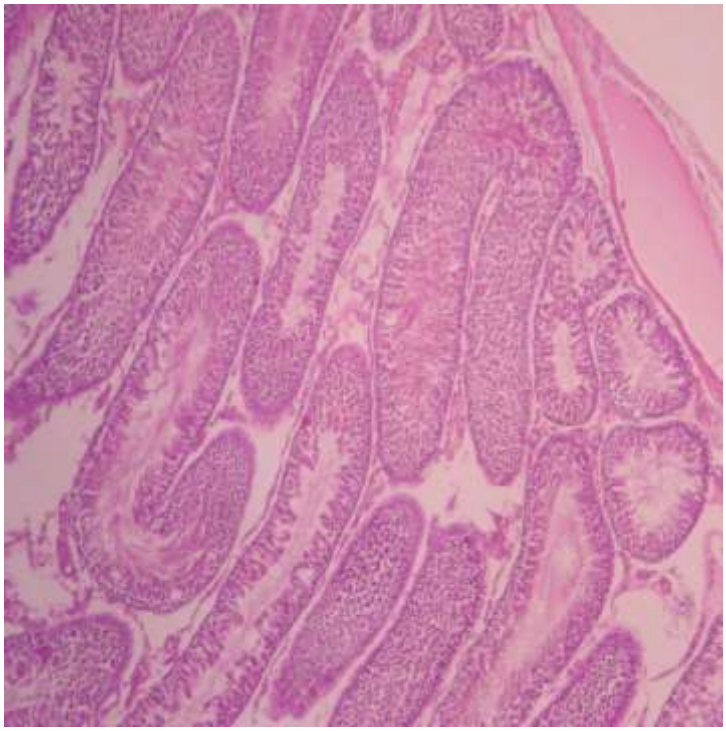

Plate 6: Histologic presentation of normal rat testes of CF (80 mg/kg; *p.o.*) treated group. x40

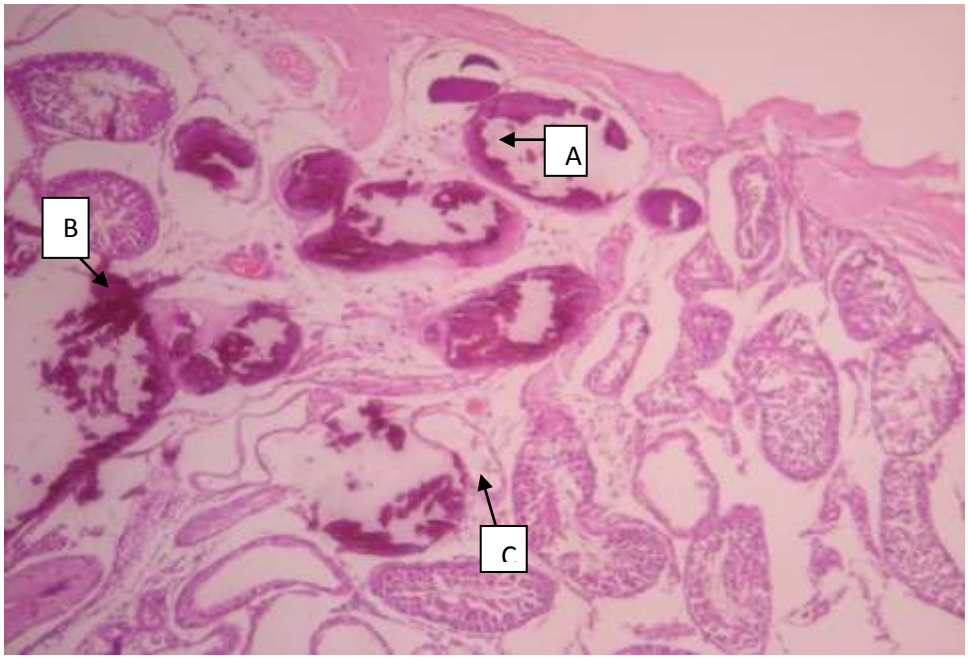

Plate 7: Histologic presentation of rat testes of CF (1000 mg/kg; *p.o.*) treated group showing areas of necrosis (A) and amorphous debris (B) within some seminiferous tubules, with loss of lining germ cell (C) series as well. x40

4.1.9.6 Reversibility study

In respect of the reversibility study, histopathological assessment of the vital organs revealed normal presentations in the heart, kidneys (Plate 10) and ovaries at all doses of *C. ferruginea*. However, in the liver for *C. ferruginea* at the dose of 1000 mg/kg, congestion (histologic section of liver showed prominent hepatic sinusoids which were engorged with red blood cells) was observed (Plate 8). Calcification (areas of necrosis and deposition of calcium salts and amorphous debris within some seminiferous tubules, with loss of lining germ cell series as well) was observed in the testes for *C. ferruginea* at the dose of 80 mg/kg (Plate 9).

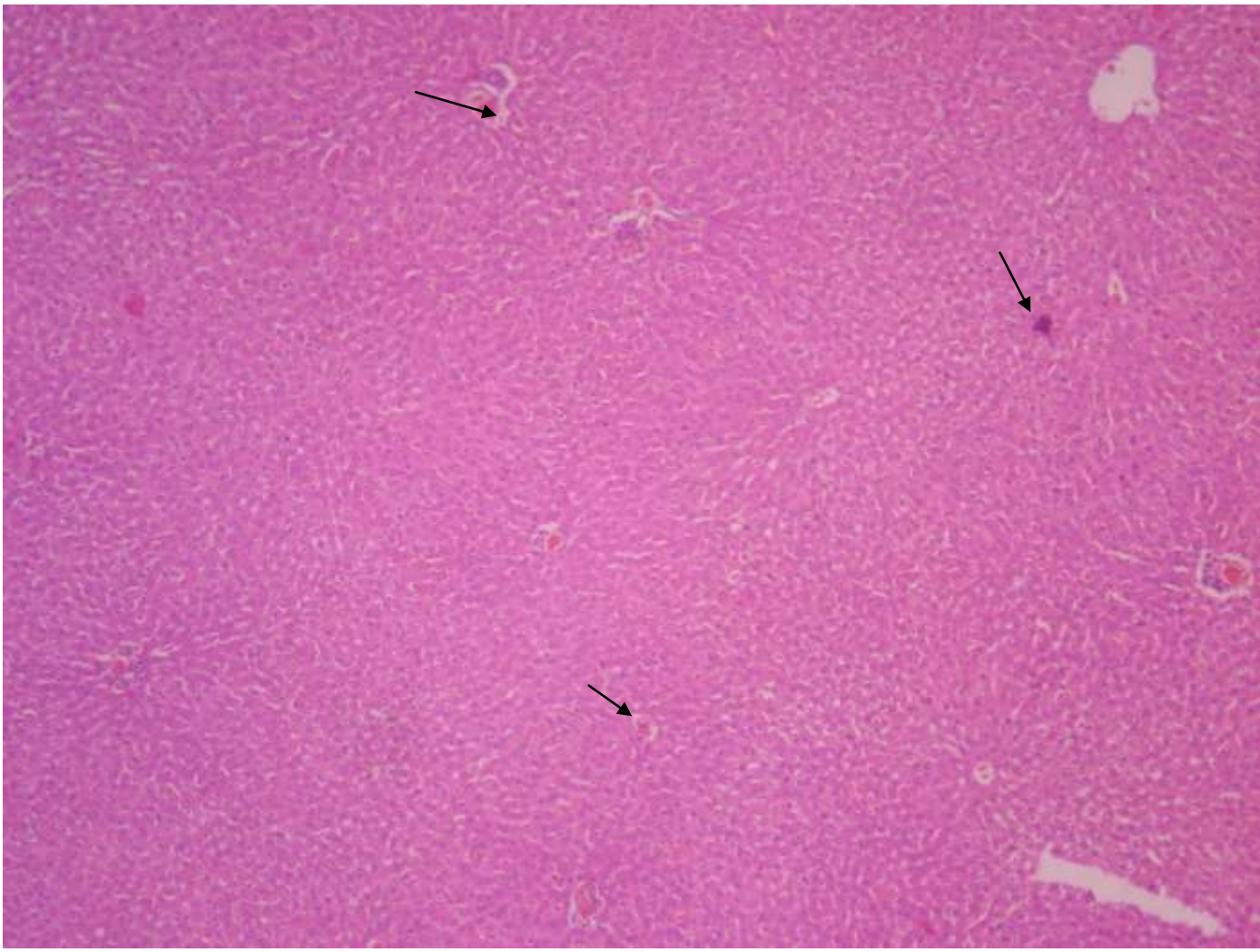

Plate 8: Histologic presentation of rat liver of CF (1000 mg/kg; *p.o.*) treated group in reversibility study showing congested sinusoids / blood vessels. x40

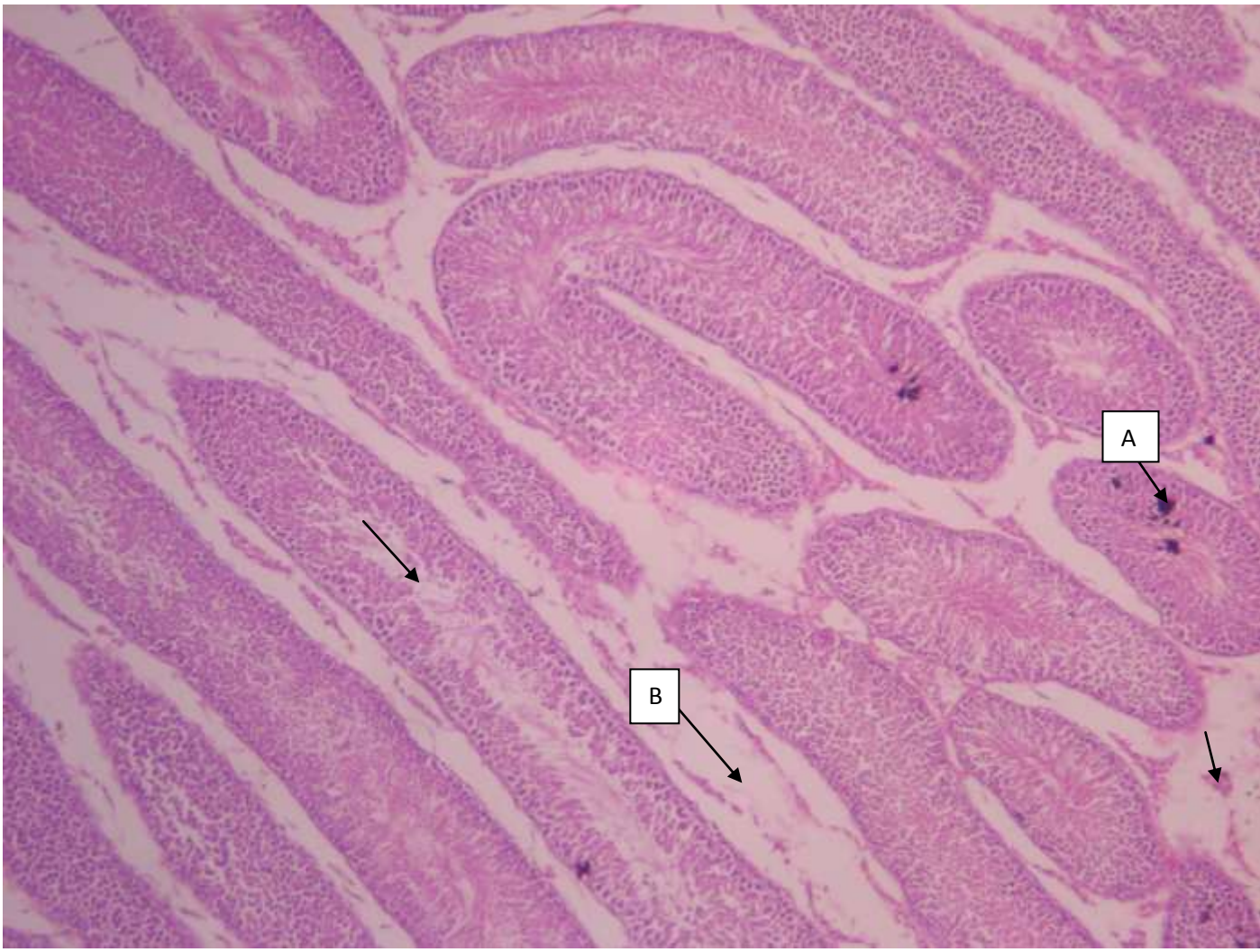

Plate 9: Histologic presentation of rat testes of CF (80 mg/kg; *p.o.*) group reversibility study showing deposition of amorphous debris with loss of lining germ cell. x40

4.2 Analgesic activity

4.2.1 Acetic acid-induced writhing test

As shown in figure 4 A, intraperitoneal injection of acetic acid elicited the writhing syndrome in control mice with 164.00 ± 17.20 writhes counted in 20 min. *C. ferruginea* produced a significant dose-dependent ($P < 0.05, 0.001$) reductions in the number of writhes with peak effect (59.27 % inhibition) produced at the highest dose of 400 mg/kg. This effect was comparable but not significantly different ($P > 0.05$) from that produced by 100 mg/kg ibuprofen (54.15 %). In respect of time-course of writhes elicitation in the control group, the greatest nociceptive effect was produced within the 10 – 15 min time interval (Figure 4). Similarly, the aqueous and butanol fractions (100 mg/kg, *p.o.*) produced significant ($P<0.05$, 0.01, 0.001) reduction in the number of writhes by 71.65 and 53.53 % inhibition respectively (Fig 4 B). The effect was comparable but not significantly different from that produced by 100 mg/kg ibuprofen (85.87%) (Fig.4 B). However, the chloroform and ethylacetate fractions produced mild but not significant ($P>0.05$) peripheral analgesic effect (38.2 and 31.69% inhibition, respectively). Successive fractions obtained from chromatographic separation of aqueous-butanol yielded 7 fractions, the peak reductions in the number of writhes (68.09 % inhibition) was observed in Fr.4 (100 mg/kg, *p.o.*) (Fig. 4 C). Rechromatograhic separation of Fr.4 gave CF-2 and CF-5. CF-2 and CF-5 (6.25 - 50 mg/kg) produce dose dependent significant ($P<0.01$, $P<0.001$) reduction in the number of writhes with peak effect (74.70 and 78.23 % inhibition respectively) produced at the dose of 50 mg/kg. This effect was comparable but not significantly different ($P>0.05$) from that produced by 100 mg/kg ibuprofen (86.62%) (Fig.4 D and E).

However, the significant ($P<0.001$) reduction in the number of abdominal constrictions produced by *C. ferruginea* (400 mg/kg) was reversed by naloxone (5 mg/kg) pretreatment (Table 7). Pretreatment of mice with yohimbine (1 mg/kg), ondansetron (0.5 mg/kg), glibenclamide (10 mg/kg) or haloperidol (1 mg/kg) did not modify the antinociceptive response elicited by *C. ferruginea* (400 mg/kg) (Table 7).

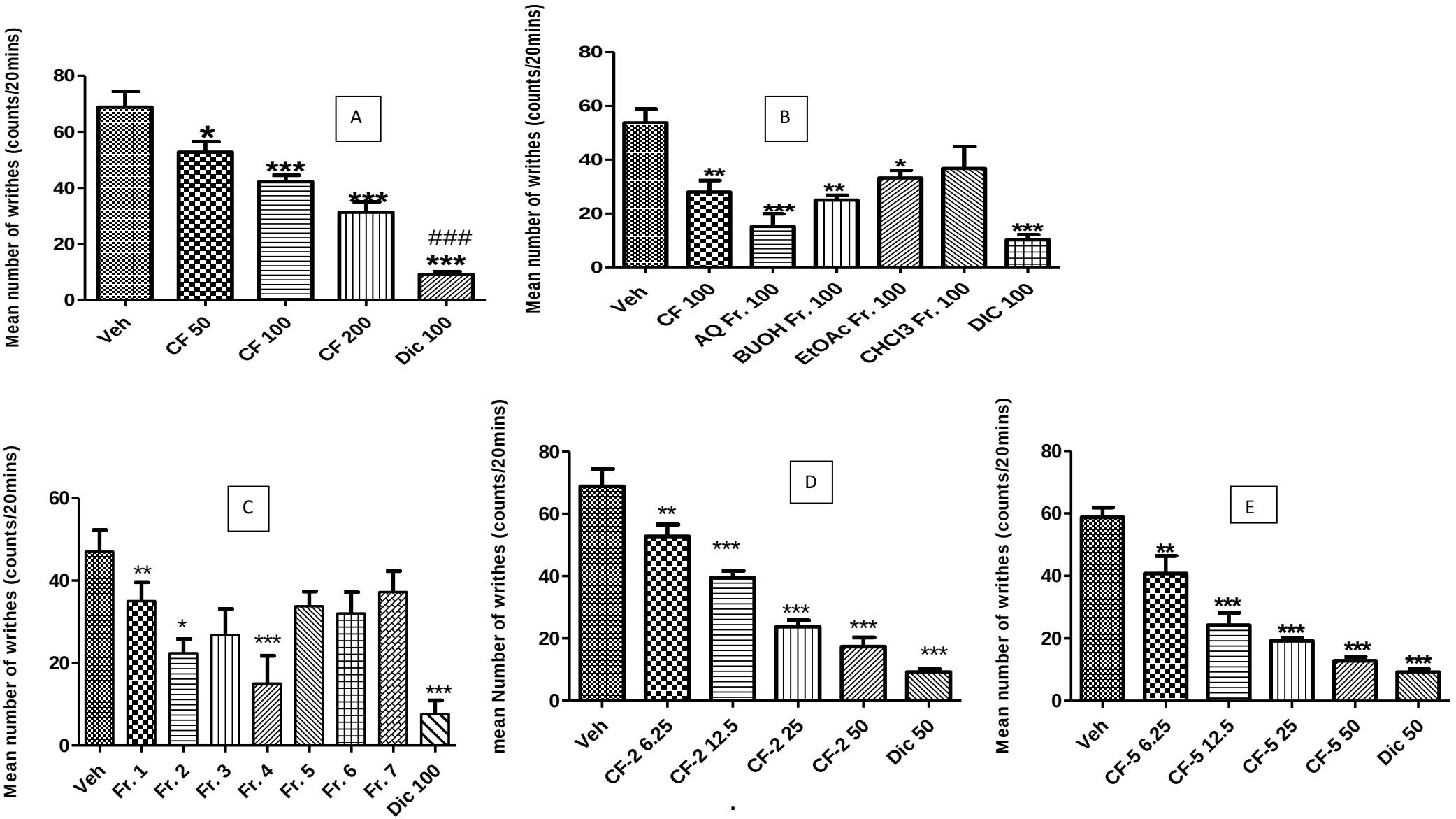

Fig.4 A-E: Effect of oral administration of CF extract, fractions, subfractions, CF-2 and CF-5 against acetic acid- induced writhing in mice. Values are expressed as mean number of writhes in 20 min ± S.E.M (n=5). Significant decrease writhes *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus vehicle treated group. $^{###}P<0.001$ versus CF 200 mg/kg treated group. Note: Veh-vehicle; Dic-diclofenac; Fr.-fraction; AQ-aqueous; BUOH-butanol; EtOAc-ethylacetate

Table 7: Elucidation of the mechanism of analgesic effect

Treatment (mg/kg)	Number of writhes in 20 mins	% inhibition
CF 100	$39.87 \pm 3.14^{***}$	45.53
CF 200	$11.13 \pm 1.04^{***}$	84.8
CF 400	$5.47 \pm 0.91^{***,\alpha}$	92.53
DIC 20	$6.47 \pm 1.13^{***,\alpha}$	91.16
Control 10 ml/kg	73.20 ± 4.95	-
CF 400	$4.80 \pm 0.70^{***}$	89.29
Naloxone 5 + CF 400	$34.60 \pm 1.74^{**,c}$	22.77
Yohimbine 1 + CF 400	$22.80 \pm 3.22^{***,b}$	49.11
Haloperidol 1 + CF 400	$14.90 \pm 0.60^{***,b}$	66.74
Glibenclamide 10 + CF 400	$15.20 \pm 1.51^{***,b}$	66.07
Ondansetron 0.5 + CF 400	$28.20 \pm 1.49^{***,c}$	37.06
Control 10 ml/kg	44.80 ± 2.43	-

Values are expressed as mean ± SEM (n=5), $^{**}P < 0.01$, $^{***}P<0.001$ versus control treated group; $^{\alpha}P<0.05$ versus *C. ferruginea* 100 mg/kg; $^{b}P<0.01$, $^{c}P<0.001$ versus *C. ferruginea* 400 mg/kg. Statistical level of significance analyzed by one way ANOVA followed by Tukey's *post hoc* multiple comparison tests.

4.2.2 Formalin test

In the first phase, injection of formalin into the sub-plantar tissue of the right hind paw of control mice produced nociceptive response of biting and licking of the treated paw with a total duration of 175.00 ± 22.70 s. *C. ferruginea* produced a significant ($P < 0.001$) dose-dependent inhibition of nociceptive reaction with peak effect (68.80 % inhibition) produced at the highest dose of 400 mg/kg. This effect was less but not significantly different ($P > 0.05$) from that produced by 10 mg/kg morphine (98.06 % inhibition). In second phase, the total duration of nociceptive reaction in the control group was 208.00 ± 13.30 s. The effect of CF in inhibiting the biting and licking response was significant (($P < 0.001$) but not dose-dependent. However, the greatest effect (95.67 % inhibition) was produced at the highest dose of 400 mg/kg. The effect of morphine (98.94 % inhibition) was comparable but not significantly different ($P > 0.05$) from that of the most effective dose of CF (Table 8A).

The analgesic property observed in the methanolic root extract of *C. ferruginea* in formalin-induced nociception test were reversed following pretreatment with naloxone (5 mg/kg, s.c., μ-opioid receptor antagonist) and glibenclamide (10 mg/kg, i.p., adenosine triphosphate (ATP) sensitive potassium channel blocker) as shown in Table 8B.

Table 8A: Effect of *C. ferruginea* on formalin-induced pain in mice.

Treatment	Dose (mg/kg)	Response duration (s) 0-5 min	Inhibition (%)	Response duration (s) 15-30 min	Inhibition (%)
CF	100	72.20 ± 4.60***	58.74	14.40 ± 4.35***	93.08
CF	200	57.20 ± 13.80***	67.31	52.60 ± 16.60***	74.71
CF	400	54.60 ± 8.10***	68.80	9.00 ± 4.00$^{***,\alpha}$	95.76
Morphine	10	3.40 ± 0.51$^{***,b,\alpha}$	98.06	2.20 ± 1.11$^{***,\alpha}$	98.94
Distilled water	(10 ml/kg)	175.00 ± 22.70	0.00	208.00 ± 13.30	0.00

Table 8B: Analysis of mechanism of analgesic effect of *C. ferruginea* in formalin test

Treatment (mg/kg)	duration of paw biting (0-5 min)	% inhibition	duration of paw biting (15-30 min)	% inhibition
CF 400	32.60 ± 2.73***	55.59	29.20 ± 1.28***	65.40
Morphine 10	13.80 ± 6.25$^{***,\gamma}$	81.20	13.60 ± 8.70***	83.89
Naloxone 5	85.40 ± 8.29	-16.35	109.80 ± 6.34	-30.09
Glibenclamide 10	65.40 ± 7.88	10.9	94.40 ± 2.86	-11.85
GLI + Diclofenac 20	99.80 ± 7.69	-35.97	122.60 ± 3.30	-45.26
NAL + Morphine 10	49.60 ± 6.01$^{\#\#}$	32.43	60.00 ± 5.36$^{\#\#}$	28.91
NAL + CF 400	76.80 ± 5.49$^{\beta}$	-4.63	69.20 ±5.51$^{\beta}$	18.01
GLI + CF 400	52.80 ± 3.90***	28.07	0.00 ± 0.00***	100
Control 10 ml/kg	73.40 ± 4.01	0.00	84.40 ± 3.08	0.00

Values are expressed as mean ± SEM (n=5), ***P<0.001 versus control ; ^{b}P < 0.01 vs. *C. ferruginea* 100 mg/kg; $^{\alpha}P$ < 0.05 vs. *C. ferruginea* 200 mg/kg treated group; $^{\gamma}P<0.05$ versus *C. ferruginea* 400 mg/kg; $^{\beta}P$<0.05 versus *C. ferruginea* 400 mg/kg; $^{\#\#}P$<0.01 versus morphine 10 mg/kg treated group. Statistical level of significance analyzed by one way ANOVA followed by Tukey's *post hoc* multiple comparison tests. [Note: GLI- glibenclamide 10 mg/kg; NAL – naloxone 5 mg/kg; CF - *C. ferruginea*].

4.2.3 Haffner's tail clip test

Application of the metal artery clip unto the tail of animals in the control group elicited reactions towards clip removal with the post-treatment latency being 1.96 ± 0.19 s compared to a pre-treatment latency of 1.82 ± 0.14 s (Table 9). *C. ferruginea* caused a significant ($P < 0.05$) dose-dependent increase in reaction latency with peak effect (62.46 % inhibition) produced at the highest dose of 400 mg/kg. This effect was less than that elicited by 10 mg/kg morphine (100.00 %).

Table 9: Effect of *C. ferruginea* on tail clip-induced pain in mice.

Treatment	Dose (mg/kg)	Pre-treatment reaction latency (s)	Post-treatment reaction latency (s)	Inhibition (%)
C. ferruginea	100	2.24 ± 0.16	4.96 ± 0.64*	4.71
C. ferruginea	200	2.14 ± 0.24	10.30 ± 3.78	14.10
C. ferruginea	400	2.20 ± 0.15	38.30 ± 12.70*	62.46
Morphine	10	2.74 ± 0.17	180.00 ± 0.00***	100.00
Distilled water	10 ml/kg	1.82 ± 0.14	1.96 ± 0.19	0.24

Values are mean ± SEM (n = 5). $^{*}P < 0.05$, $^{***}P < 0.001$ vs. pre-treatment reaction latency (Student's *t* test).

4.2.4 Hot plate test

The placement of mice on the hot plate elicited nociceptive reaction in the control group with post-treatment latency of 3.10 ± 0.36, 3.20 ± 0.36 and 2.20 ± 0.17s at 60, 90 and 150 min. respectively in comparison to the pre-treatment latency of 2.50 ± 0.22 s (Table 10A). As was the case in the tail clip test, *C.ferruginea* produced a significant (*P<0.05*, 0.01, 0.001) time and dose-dependent increase in pain latency with peak effect (57.35% MPE) at 200 mg/kg 90 min. post treatment in comparison to control. The effect was comparable to that produced by 10 mg/kg morphine (44.12 % MPE) at 90 min. post-treatment (Table 10A).

Similarly, aqueous and butanol fractions (100 mg/kg, *p.o.*) produced a significant (*P<0.05*, 0.01, 0.001) time-dependent increase in pain latency with peak effect (60.92 and 57.96% MPE) at 60 and 90 min. respectively, which was similar to the effect of morphine (56.82% MPE) at 90 min. post-treatment. However, ethylacetate and chloroform failed to produce significant (*P*>0.05) elevation of pain threshold with maximum possible effects (15.91 and 5.70%) at 90 and 120 min respectively (Table 10A).

Subfractionation of aqueous/butanol extract yielded 7 subfractions with fraction 4 producing time-dependent significant (*P<0.05*) increase in pain latency with (47.06% MPE) in comparison to control (Table 10A).

CF-2 produced its peak significant (*P*<0.01) time and dose-dependent increase in pain latency with peak effect (39.54% MPE) at 25 mg/kg 90 min post-treatment when compared to vehicle control treated groups (Table 10 B).

Table 10A: Effect of CF extract and its fractions on hot plate-induced pain in mice

Treatment	Dose mg/kg	0 min	30 min	60 min	90 min	120 min	150 min
Vehicle	-	2.50±0.22	3.20±0.50	3.10±0.36	3.20±0.36	2.50±0.24	2.20±0.17
Morphine	10	2.00±0.23	4.60±0.30	6.00±0.67	6.20±0.73	4.50±1.00	2.50±0.39
			32.50	**50.00** [b]	**52.50** [b]	**31.25** [b]	**6.25**
CF	50	2.60±0.21	5.40±0.59	3.70±0.50	4.50±0.46	4.20±0.34	3.30±0.18
			37.83 [a]	**14.86**	**25.67**	**21.62**	**9.45**
CF	100	1.80±0.08	3.90±0.80	5.00±1.10	5.70±0.48	4.90±0.68	2.20±0.21
			25.61	**39.02**	**47.56** [b]	**37.80** [a]	**4.87**
CF	200	1.60±0.16	3.60±0.76	4.80±0.69	7.10±0.82	3.40±0.77	2.80±0.30
			23.81	**38.09**	**65.47** [c]	**21.42**	**14.28**
Fraction							
Vehicle	10 ml/kg	1.30±0.16	1.00±o.13	1.30±0.11	1.20±0.19	0.88±0.09	0.92±0.07
Chloroform fr.	100	1.20±01.5	1.40±0.15	1.40±0.26	1.20±0.10	1.40±0.24	1.70±0.37
			4.44	**1.14**	**0.00**	**5.70**	**8.50**
Ethylacetate fr.	100	1.30±0.15	2.30±0.20	2.50±0.48	2.60±1.10	3.00±1.10	2.00±0.09
			14.44	**13.79**	**15.91**	**23.25**	**11.89**
Aqueous fr.	100	1.30±0.10	5.30±1.00	6.60±1.40	4.50±0.89	3.10±0.53	4.40±1.60
			47.78 [c]	**60.92** [c]	**37.50** [b]	**24.34**	**38.33** [b]
Butanol fr.	100	1.50±0.09	5.00±0.95	4.50±0.82	6.30±1.10	2.50±0.27	3.70±0.33
			44.44 [c]	**36.78** [b]	**57.96** [c]	**17.76**	**30.62** [a]
Subfraction							
Vehicle	-	1.30±0.12	1.40±0.19	1.10±0.07	1.50±0.17	1.40±0.14	1.40±0.13
Fraction 1	100	2.00±0.40	2.60±0.53	2.20±0.51	2.30±0.41	3.30±0.18	3.40±0.71
			7.50	**2.50**	**3.75**	**16.25**	**17.50**
Fraction 2	100	1.70±0.20	2.50±0.86	2.10±0.24	2.70±0.4	3.20±0.35	2.50±0.37
			9.64	**4.82**	**12.05**	**18.07**	**9.64**
Fraction 3	100	1.70±0.18	2.70±0.20	3.10±0.59	3.00±0.24	3.40±0.53	2.50±0.74
			12.05	**16.87**	**15.66**	**20.48**	**9.63**
Fraction 4	100	1.80±0.15	4.80±1.40	3.90±0.6	5.50±1.8	3.20±0.52	5.20±1
			36.58[c]	**25.61**[b]	**45.12** [c]	**17.07**	**41.46** [c]
Fraction 5	100	2.30±0.24	3.30±0.61	4.50±1	3.30±0.52	4.70±0.62	3.40±0.78
			12.98	**28.57** [c]	**12.98**	**31.16** [c]	**14.28**
Fraction 6	100	1.80±0.13	2.40±0.26	2.60±0.42	2.20±0.25	2.70±0.49	2.70±0.71
			7.31	**9.75**	**4.87**	**10.97**	**10.97**
Fraction 7	100	1.90±0.21	3.10±0.96	2.50±0.44	2.70±0.22	2.70±0.78	2.60±0.51
			14.81	**7.40**	**9.87**	**9.87**	**8.64**

Values are expressed as mean±SEM (n = 6). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ versus vehicle (10 ml/kg) control group. Statistical level of significance analyzed using two way ANOVA followed by Bonferroni post hoc multiple comparison test. Values in bold indicates %MPE. Fr-fraction

Table 10B: Effect of amentoflavone on hot plate test in mice

Treatment	Dose (mg/kg)	0 min	30 min	60 min	90 min	120 min	150 min
Vehicle	-	1.30 ± 0.14	1.00 ± 0.13	1.30 ± 0.11	1.20 ± 0.19	0.88 ± 0.09	0.92 ± 0.07
CF-2	12.5	1.20 ± 0.18	1.46 ± 0.19	2.44 ± 0.80	2.86 ± 0.54	2.22 ± 0.58	2.64 ± 1.02
			5.11	**13.1**	**18.86**	**14.69**	**18.94**
CF-2	25	1.02 ± 0.10	2.92 ± 0.66	2.98 ± 1.07	4.68 ± 1.44	3.46 ± 0.84	2.52 ± 0.39
			12.44	**21.69**	**34.09[b]**	**10.52**	**14.54**
CF-2	50	1.08 ± 0.14	1.98 ± 0.51	3.14 ± 0.51	4.24 ± 1.11	1.78 ± 0.27	1.52 ± 0.07
			10.89	**21.15**	**34.54[b]**	**9.87**	**6.61**
Morphine	10	1.09 ± 0.13	3.60 ± 0.20	5.00 ± 0.57	5.20 ± 0.63	3.50 ± 0.55	1.50 ± 0.29
			20.59[a]	**42.02[c,α]**	**44.12[c]**	**26.67[a]**	**3.85**

Values are expressed as mean ±SEM (n=6). [a]$P<0.05$, [b]$P<0.01$ versus vehicle (10 ml/kg, *p.o.)* control group; [α]$P<0.001$ versus CF-2 100 mg/kg treated group Statistical level of significance analyzed using two way ANOVA followed by Bonferroni post hoc multiple comparison test. Values in bold indicates % Maximal Possible Effect.

4.3 Anti-inflammatory activity

4.3.1 Carrageenan-induced paw oedema test

Injection of carrageenan into the sub-plantar tissue of the right hind paw of rats in the control group caused oedema development which peaked (1.05 ± 0.19 cm change in paw circumference) at 4 h post-phlogistic agent injection. The effect of CF was dose-dependent from the 4^{th} to the 6^{th} h with peak effect (91.33 % inhibition) produced at the dose of 400 mg/kg at the 6^{th} hour. This effect was greater than but not significantly different ($P > 0.05$) from that produced by 20 mg/kg diclofenac (65.32 % inhibition). The effect of CF at 400 mg/kg and diclofenac were also time-dependent all through to the 6^{th} h (Table 11 A).

The aqueous and butanol fractions (100 mg/kg, *p.o.*) significantly ($P<0.05$) decreased the carrageenan-induced increase in paw circumference with peak effect (56.14 and 70.21% inhibition) at 1 h and 24 h post treatment respectively. Nimesulide exhibited similar anti-inflammatory properties with peak effect (98.57%) at the 24^{th} hour. Similarly, the chloroform fraction inhibited oedema formation by 57.28% at the 5^{th} hour (Table 11B), while the ethylacetate fraction produced insignificant inhibition of oedema.

Table 11C shows the effect of oral administration of 7 subfractions (100 mg/kg) obtained from column chromatographic separation of aqueous/butanol fractions. Fraction 4 inhibited oedema formation significantly by 72.92 and 68.42% at 1 and 24 h respectively. CF-2 produced significant ($P<0.05$) and dose dependent inhibition of oedema formation with peak effect (75.22 % inhibition) at the dose of 100 mg/kg, 24 hour post-phlogistic injection. Similarly, graded doses of CF-5 showed significant inhibition of oedema with peak effect (63.4 % inhibition) 6 hour after carrageenan subplantar injection which was similar to the effect of nimesulide (50 mg/kg, *p.o.*) 58.50% inhibition 6 hour post carrageenan injection (Table 11 D).

Table 11A: Effect of *C. ferruginea* on carrageenan-induced rat paw oedema.

Treatment	Dose (mg/kg)	Increase in paw circumference (cm) Time					
		1 h	2 h	3 h	4 h	5 h	6 h
Distilled water	-	0.60 ± 0.08 **(0.00)**	0.89 ± 0.10 **(0.00)**	0.91 ± 0.11 **(0.00)**	1.05 ± 0.19 **(0.00)**	0.90 ± 0.19 **(0.00)**	0.69 ± 0.14 **(0.00)**
C. ferruginea	100	0.43 ± 0.04 **(28.33)**	0.63 ± 0.05 **(29.37)**	0.77 ± 0.09 **(15.20)**	0.73 ± 0.15 **(30.48)**	0.51 ± 0.12 **(43.33)**	0.42 ± 0.12 **(39.31)**
C. ferruginea	200	0.41 ± 0.01 (**31.67)**	0.73 ± 0.09 **(18.16)**	0.77 ± 0.09 **(15.20)**	0.63 ± 0.13 **(40.00)**	0.46 ± 0.12 **(48.89)**	0.38 ± 0.13 **(45.09)**
C. ferruginea	400	0.49 ± 0.06 **(18.33)**	0.63 ± 0.10 **(29.37)**	0.33 ± 0.07$^{**,a,\alpha}$ **(63.66)**	0.16 ± 0.08** **(84.76)**	0.12 ± 0.05** **(86.67)**	0.06 ± 0.04** **(91.33)**
Diclofenac	20	0.34 ± 0.07* **(43.33)**	0.49 ± 0.08* **(45.07)**	0.44 ± 0.10* **(51.54)**	0.45 ± 0.11* **(57.14)**	0.33 ± 0.09* **(63.33)**	0.24 ± 0.11 **(65.32)**

Values are expressed as mean change in paw circumference (cm) ± SEM (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control (10 ml/kg); $^{a}P < 0.05$ vs. *C. ferruginea* 100 mg/kg; $^{\alpha}P < 0.05$ vs. *C. ferruginea* 200 mg/kg (two way ANOVA followed by Bonferroni multiple comparison test). Values in parenthesis indicate inhibition (%) of oedema development.

Table11B: Effect of *C.ferruginea* fractions against carrageenan-induced paw oedema in rat.

	Δ in paw size (cm)						
Treatment	1 h	2 h	3 h	4 h	5 h	6 h	24 h
Vehicle 10 ml/kg	0.57 ± 0.05	0.82 ± 0.09	0.77 ± 0.07	0.79 ± 0.06	0.64 ± 0.11	0.49 ± 0.14	0.14 ± 0.05
CF MeOH	0.45 ± 0.07	0.71 ± 0.04	0.67 ± 0.003	0.59 ± 0.10	0.49 ± 0.06	0.41 ± 0.03	0.11 ± 0.05
100 mg/kg	**(20.70)**	**(13.66)**	**(12.99)**	**(25.57)[a]**	**(22.82)[a]**	**(16.53)**	**(24.29)**
CF AQ.Fr	0.25 ± 0.05	0.41 ± 0.04	0.38 ± 0.07	0.40 ± 0.07	0.30 ± 0.05	0.23 ± 0.05	0.07 ± 0.03
100 mg/kg	**(56.14)**	**(50.24)[b]**	**(51.17)[a]**	**(48.86)[a]**	**(52.5)[a]**	**(53.88)**	**(47.14)**
CF BUOH.Fr	0.42 ± 0.01	0.54 ± 0.06	0.39 ± 0.12	0.42 ± 0.08	0.35 ± 0.04	0.17 ± 0.05	0.06 ± 0.03
100 mg/kg	**(12.45)**	**(22.19)**	**(35.33)[a]**	**(30.46)[a]**	**(30.52)**	**(54.05)**	**(70.21)**
CF EtOAc.Fr	0.34 ± 0.06	0.55 ± 0.12	0.50 ± 0.08	0.47 ± 0.09	0.29 ± 0.09	0.32 ± 0.11	0.20 ± 0.05
100 mg/kg	**(29.46)**	**(20.75)**	**(16.67)**	**(22.52)**	**(41.77)[a]**	**(14.59)**	**(0.00)**
$CHCl_3$. Fr	0.31 ± 0.06	0.48 ± 0.12	0.4 ± 0.09	0.3 ± 0.08	0.26 ± 0.10	0.17 ± 0.06	0.14 ± 0.03
100 mg/kg	**(35.68)**	**(31.12)[a]**	**(33.33)[a]**	**(40.16)[a]**	**(57.28)[a]**	**(53.51)**	**(6.60)**
Nimesulide	0.40 ± 0.05	0.40 ± 0.09	0.45 ± 0.08	0.34 ± 0.09	0.26 ± 0.08	0.18 ± 0.09	0.02 ± 0.002
100 mg/kg	**(29.47)**	**(51.22)[b]**	**(41.82)**	**(56.71)[b]**	**(59.38)[a]**	**(63.47)**	**(98.57)**

Values are expressed as mean change in paw circumference (cm) ± SEM (n = 5). Level of significance [a]$P < 0.05$, [b]$P < 0.01$ vs. vehicle (10 ml/kg) treated-control (two way ANOVA followed by Bonferonni multiple comparison posthoc test). Values in parenthesis indicate inhibition (%) of oedema development. MeOH- Methanolic extract., AQ- aqueous., EtOAc- ethylacetate., BUOH- butanol., $CHCl_3$- chloroform., Fr.- fraction.

Table 11C: Effect of *C. ferruginea* subfractions against carrageenan-induced rat paw oedema.

Treatment	Δ in paw size (cm)						
	1 h	2 h	3 h	4 h	5 h	6 h	24 h
Vehicle	0.48±0.04	0.64±0.07	0.68±0.07	0.57±0.08	0.57±0.05	0.45±0.06	0.19±0.05
10 ml/kg	**(0)**	**(0)**	**(0)**	**(0)**	**(0)**	**(0)**	**(0)**
Fraction 1	0.40±0.04	0.70±0.10	0.67±0.08	0.54±0.04	0.43±0.04	0.42±0.04	0.15±0.04
100 mg/kg	**(16.67)**	**(0)**	**(1.47)**	**(5.26)**	**(24.56)**	**(6.67)**	**(21.05)**
Fraction 2	0.23±0.008	0.55±0.12	0.51±0.12	0.36±0.13	0.39±0.07	0.34±0.07	0.14±0.04
100 mg/kg	**(52.08)**	**(14.06)**	**(25)**	**(36.84)**	**(31.58)**	**(24.44)**	**(26.32)**
Fraction 3	0.28±0.03	0.43±0.06	0.48±0.05	0.43±0.03	0.45±0.01	0.44±0.02	0.23±0.06
100 mg/kg	**(41.67)**	**(32.81)**	**(29.41)**	**(24.56)**	**(21.05)**	**(2.22)**	**(0)**
Fraction 4	0.13±0.06	0.30±0.05	0.30±0.07	0.28±0.05	0.28±0.06	0.25±0.07	0.06±0.03
100 mg/kg	**(72.92)[b]**	**(54.68)[b]**	**(55.88)[c]**	**(50.87)[a]**	**(50.07)[a]**	**(44.44)**	**(68.42)**
Fraction 5	0.28±0.06	0.66±0.09	0.60±0.12	0.52±0.09	0.48±0.06	0.50±0.09	0.27±0.06
100 mg/kg	**(41.67)**	**(0)**	**(11.76)**	**(8.77)**	**(15.79)**	**(0)**	**(0)**
Fraction 6	0.30±0.08	0.68±0.04	0.63±0.05	0.69±0.07	0.51±0.03	0.46±0.04	0.18±0.06
100 mg/kg	**(39.58)**	**(0)**	**(11.76)**	**(0)**	**(15.79)**	**(4.44)**	**(33.33)**
Fraction 7	0.26±0.06	0.54±0.12	0.53±0.10	0.52±0.09	0.48±0.09	0.39±0.07	0.18±0.05
100 mg/kg	**(45.83)**	**(15.63)**	**(22.06)**	**(8.77)**	**(17.54)**	**(13.33)**	**(33.33)**
Nimesulide	0.26±0.06	0.48±0.09	0.31±0.09	0.34±0.07	0.31±0.07	0.27±0.07	0.11±0.05
100 mg/kg	**(45.83)**	**(33.33)**	**(54.41)[a]**	**(40.35)**	**(45.61)**	**(40)**	**(59.26)**

Values are expressed as mean change in paw circumference (cm) ± SEM (n = 5). Level of significance [a]$P < 0.05$, [b]$P < 0.01$, [c]$P<0.001$ vs. vehicle treated-control (Two way ANOVA followed by Bonferonni multiple comparison posthoc test). Values in parenthesis indicate inhibition (%) of oedema development.

Table 11D: Effect of CF-2 and CF-5 against carrageenan-induced rat paw oedema

		Δ in paw size (cm)						
Treatment	Dose (mg/kg)	1 h	2 h	3 h	4 h	5 h	6 h	24 h
Vehicle	10 ml/kg	0.33 ± 0.02	0.74 ± 0.11	0.73 ± 0.15	0.64 ± 0.12	0.68 ± 0.11	0.69 ± 0.12	0.39 ± 0.18
Nimesulide	100	0.25 ± 0.02	0.41 ± 0.09	0.38 ± 0.07	0.38 ± 0.05	0.37 ± 0.10	0.29 ± 0.09	0.21 ± 0.12
		(23.49)	**(44.62)[a]**	**(47.68)[a]**	**(40.99)**	**(45.66)**	**(58.50)[a]**	**(44.95)**
CF-2	25	0.25 ± 0.05	0.42 ± 0.05	0.40 ± 0.06	0.38 ± 0.07	0.37 ± 0.04	0.34 ± 0.03	0.25 ± 0.05
		(24.09)	**(43.01)**	**(45.77)**	**(41.61)**	**(45.41)**	**(50.43)[a]**	**(35.48)**
	50	0.23 ± 0.07	0.34 ± 0.07	0.30 ± 0.07	0.39 ± 0.07	0.34 ± 0.06	0.26 ± 0.06	0.14 ± 0.02
		(29.51)	**(54.03)[b]**	**(58.86)[b]**	**(40.06)**	**(49.40)**	**(62.54)[a]**	**(63.35)**
	100	0.40 ± 0.07	0.47 ± 0.07	0.41 ± 0.07	0.41 ± 0.07	0.32 ± 0.06	0.25 ± 0.05	0.10 ± 0.03
		(0.00)	**(37.36)**	**(43.86)[a]**	**(35.71)**	**(52.07)[a]**	**(64.55)[b]**	**(75.22)**
CF-5	25	0.33 ± 0.07	0.69 ± 0.13	0.50 ± 0.09	0.43 ± 0.12	0.45 ± 0.09	0.34 ± 0.06	0.25 ± 0.05
		(0.00)	**(7.53)**	**(31.61)**	**(32.61)**	**(33.43)**	**(50.43)[a]**	**(36.52)**
	50	0.28 ± 0.05	0.37 ± 0.06	0.42 ± 0.06	0.50 ± 0.05	0.49 ± 0.04	0.42 ± 0.07	0.20 ± 0.06
		(14.45)	**(50.80)[b]**	**(42.50)[b]**	**(22.67)**	**(26.92)**	**(39.19)[a]**	**(47.54)**
	100	0.21 ± 0.05	0.36 ± 0.06	0.43 ± 0.08	0.36 ± 0.07	0.33 ± 0.06	0.25 ± 0.06	0.15 ± 0.05
		(36.14)	**(52.15)[b]**	**(41.96)[a]**	**(52.15)[a]**	**(51.18)[a]**	**(63.40)[b]**	**(61.80)**

Values are expressed as mean ± S.E.M (n=5), [a]*P<0.05*, [b]*P*<0.01 versus vehicle (10 mg/kg) treated control group, level of significance analyzed using two way ANOVA followed by Bonferonni *post hoc* multiple comparison test. Values in parenthesis indicates percentage inhibition of oedema formation.

4.3.2 Egg albumin-induced paw oedema

Sub-plantar injection of egg albumin into the right hind paw of rats produced oedema which peaked at 120 min post-injection (0.960 ± 0.081 cm Δ in paw circumference). The effect of CF was dose-dependent at all time intervals with peak effect (71.76 % inhibition) produced at 180 min post-egg albumin injection. This effect was of same magnitude and not significantly ($P > 0.05$) from that produced by 20 mg/kg diclofenac (Table 12). As in the carrageenan model, the effects of CF at 400 mg/kg and diclofenac were also time-dependent and both peaked at the 180 min time interval.

4.3.3 Histamine-induced paw oedema

The oedema induced by histamine was significantly ($P<0.05$) inhibited by CF 200 and 400 mg/kg. The anti-inflammatory effect of CF was dose dependent and remained significant up to 6^{th} h of post drug treatment which was comparable to the effect of chlorpheniramine as shown in Table 13.

4.3.4 Serotonin-induced paw oedema

The oedema induced by serotonin was significantly ($P<0.01$) inhibited by CF 100 - 400 mg/kg. The anti-inflammatory effect of CF was dose dependent and remained significant up to 6^{th} h post drug treatment which was comparable to the effect of cyproheptadine as shown in Table 14.

Table 12: Effect of *C. ferruginea* on egg albumin-induced rat paw oedema.

Treatment	Dose (mg/kg)	Increase in paw circumference (cm) Time					
		0.5 h	1 h	1.5 h	2 h	2.5 h	3 h
Distilled water	-	0.68 ± 0.07 **(0.00)**	0.85 ± 0.06 **(0.00)**	0.95 ± 0.06 **(0.00)**	0.96 ± 0.08 **(0.00)**	0.85 ±0.03 **(0.00)**	0.85 ± 0.08 **(0.00)**
C. ferruginea	100	0.79 ± 0.06 **(0.00)**	0.81 ± 0.06 **(4.71)**	0.77 ± 0.05 **(18.95)**	0.72 ± 0.04 **(25.00)**	0.80 ± 0.03 **(5.88)**	0.78 ± 0.03 **(8.24)**
	200	0.59 ± 0.09 **(13.24)**	0.65 ± 0.05 **(23.53)**	0.66 ± 0.02 **(30.53)**	0.63 ± 0.05 (**34.38)**	0.58 ± 0.04 **(31.76)**	0.52 ± 0.05 **(38.82)**
	400	0.47 ± 0.16 **(30.88)**	0.53 ± 0.13* **(37.65)**	0.56 ± 0.11* **(41.05)**	0.44 ± 0.11** **(54.17)**	0.31 ± 0.11^{***,c} **(63.53)**	0.24 ± 0.08$^{***,c,\alpha}$ **(71.76)**
Diclofenac	20	0.53 ± 0.07 **(22.06)**	0.55 ± 0.05 **(35.29)**	0.50 ± 0.10** **(47.37)**	0.49 ± 0.11** **(48.96)**	0.34 ± 0.07^{***,c} **(60.00)**	0.24 ± 0.06$^{***,c,\alpha}$ **(71.76)**

Values are expressed as mean change in paw circumference (cm) ± SEM (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Distilled water (10 ml/kg) control treated group; $^{c}P < 0.001$ vs. *C. ferruginea* 100 mg/kg; $^{\alpha}P < 0.05$ vs. *C. ferruginea* 200 mg/kg (level of significance using two way ANOVA followed by Bonferonni's *post hoc* multiple comparison test). Values in parenthesis indicate inhibition (%) of oedema development.

Table 13: Effect of *C. ferruginea* against histamine-induced mice paw oedema

		Δ in paw size (cm)					
Treatment	Dose mg/kg	1 h	2 h	3 h	4 h	5 h	6 h
Distilled water	-	0.36 ± 0.05	0.32 ± 0.07	0.28 ± 0.09	0.33 ± 0.06	0.26 ± 0.05	0.22 ± 0.06
C. ferruginea	100	0.20 ± 0.01*	0.14 ± 0.03*	0.12 ± 0.02*	0.14 ± 0.03*	0.08 ± 0.02**	0.06 ± 0.03*
		(44.44)	**(56.23)**	**(57.14)**	**(46.15)**	**(69.23)**	**(72.72)**
	200	0.16 ± 0.03*	0.14 ± 0.03*	0.10 ± 0.05**	0.08 ± 0.04**	0.06 ± 0.04**	0.04 ± 0.02**
		(55.55)	**(56.25)**	**(64.28)**	**(69.23)**	**(76.92)**	**(81.81)**
	400	0.14 ± 0.03**	0.12 ± 0.03**	0.10 ± 0.03**	0.08 ± 0.04**	0.04 ± 0.02***	0.04 ± 0.02**
		(61.11)	**(62.50)**	**(64.28)**	**(69.23)**	**(84.61)**	**(81.81)**
Chlorpheniramine	4	0.16 ± 0.04*	0.10 ± 0.03**	0.08 ± 0.04**	0.06 ± 0.03**	0.06 ± 0.04**	0.06 ± 0.03*
		(55.55)	**(68.82)**	**(71.42)**	**(76.93)**	**(69.27)**	**(72.72)**

Values are expressed as mean±SEM (n=5), $^{*}P < 0.05$, $^{**}P < 0.01$, ***P<0.001 vs. Distilled water (10 ml/kg) control treated group (level of significance using two way ANOVA followed by Bonferroni *post hoc* multiple comparison test). Values in parenthesis indicate inhibition (%) of oedema development.

Table 14: Effect of *C. ferruginea* against serotonin-induced mice paw oedema

		Δ in paw size (cm)					
Treatment	Dose mg/kg	1 h	2 h	3 h	4 h	5 h	6 h
Distilled water	-	0.24 ± 0.06	0.26 ± 0.05	0.24 ± 0.03	0.24 ± 0.03	0.22 ± 0.02	0.20 ± 0.03
C. ferruginea	100	0.18 ± 0.04	0.14 ± 0.05	0.14 ± 0.03	0.14 ± 0.03	0.12 ± 0.04	0.08 ± 0.05
		(25.00)	**(46.20)**	**(41.60)**	**(41.60)**	**(45.50)**	**(60.00)**
	200	0.12 ± 0.05	0.06 ± 0.07**	0.08 ± 0.06*	0.06 ± 0.04**	0.04 ± 0.02**	0.02 ± 0.00**
		(38.00)	**(61.00)**	**(66.60)**	**(87.50)**	**(86.40)**	**(100)**
	400	0.15 ± 0.03	0.10 ± 0.04*	0.08 ± 0.05**	0.03 ± 0.02**	0.03 ± 0.02**	0.03 ± 0.02**
		(58.00)	**(69.00)**	**(66.60)**	**(83.00)**	**(90.90)**	**(85.00)**
Cyproheptadine	3	0.10 ± 0.05	0.08 ± 0.04**	0.08 ± 0.04*	0.04 ± 0.03**	0.02 ± 0.02**	0.00 ± 0.00**
		(50.00)	**(76.00)**	**(66.60)**	**(75.00)**	**(81.80)**	**(100.00)**

Values are expressed as mean±SEM (n=5), $^{*}P < 0.05$, $^{**}P < 0.01$ versus Distilled water (10 ml/kg) control treated group (two way ANOVA followed by Bonferroni multiple comparison *post hoc* test). Values in parenthesis indicate inhibition (%) of oedema development.

4.3.5 Xylene-induced ear oedema

C. ferruginea produced a dose-dependent inhibition of ear oedema development which was significant ($P < 0.05$) and peaked at the highest dose of 400 mg/kg (61.11 % inhibition). The level of inhibition of ear oedema development by dexamethasone (1 mg/kg; *p.o.*) was same as that of the extract at 400 mg/kg (Table 15).

Table 15: Effect of *C. ferruginea* on xylene-induced ear oedema in mice.

Treatment	Dose (mg/kg)	Increase in ear weight (mg)	Inhibition (%)
Distilled water	(10 ml/kg)	36.00 ± 8.72	-
C. ferruginea	100	34.00 ± 6.78	5.56
C. ferruginea	200	26.00 ± 4.00	27.78
C. ferruginea	400	14.00 ± 2.45*	61.11
Dexamethasone	1	14.00 ± 2.45*	61.11

Values are mean ± SEM (n = 5). *P < 0.05 vs. control (one way ANOVA followed by Dunnett's *post hoc* multiple comparison tests).

4.3.6 Formaldehyde-induced arthritis inflammation

Injection of formalin into the sub-plantar tissue of the right hind paw of mice in the control group elicited oedema development with a change in paw circumference of 1.36 ± 0.05 cm over the duration of 10 days. *C. ferruginea* produced significant ($P < 0.05, 0.01$) reductions in oedema development with the greatest effect produced at the lowest dose of 100 mg/kg. The effect of the extract at the different doses (100, 200, and 400 mg/kg) were not significantly different ($P > 0.05$) from each other. The effect of 10 mg/kg (*p.o.*) of indomethacin (57.35% inhibition) was significantly different ($P < 0.001$) from those of the extract at the different doses. Daily treatment of rats with indomethacin at 10 mg/kg (*p.o.*) for 10 days resulted in the death of some animals. This led to the adoption of alternate day treatment for the standard drug at same dose orally (Table 16).

Table 16: Effect of *C. ferruginea* on formaldehyde-induced arthritis inflammation in rats.

Treatment	Dose (mg/kg)	Increase in paw circumference (cm)	Inhibition (%)
Distilled water	(10 ml/kg)	1.36 ± 0.05	-
C. ferruginea	100	1.03 ± 0.09**	24.26
C. ferruginea	200	1.12 ± 0.05*	17.65
C. ferruginea	400	1.10 ± 0.04*	19.12
Indomethacin	10	0.58 ± 0.04$^{***,c,\gamma,3}$	57.53

Values are mean ± SEM (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{c}P < 0.001$ vs. *C. ferruginea* 100 mg/kg; $^{\gamma}P < 0.001$ vs. *C. ferruginea* 200 mg/kg; $^{3}P < 0.001$ vs. *C. ferruginea* 400 mg/kg (one way ANOVA followed by Tukey's *post hoc* multiple comparison test).

4.4 Anticonvulsant activity

4.4.1 Maximal electroshock-induced seizure

Maximal electroshock induced seizure in saline treated mice within 2.60 ± 0.40 s and 105.40 ± 12.79 s duration of unconsciousness. Oral administration of *C.ferruginea* (200 mg/kg) antagonized MES-induced seizures by 20% protection while clonazepam (0.5 mg/kg), phenytoin (20 mg/kg), carbamazepine produced 40, 100 and 100% protection respectively. Similarly, *C.ferruginea* produced graded dose dependent significant ($P<0.001$) decrease in the duration of seizure, the peak effect was observed at 400 mg/kg which was comparable to the effects of phenytoin (20 mg/kg) and carbamazepine (50 mg/kg). However, clonazepam (0.5 mg/kg) produced non-significant ($P>0.05$) decrease in the duration of seizure (Table 17).

4.4.2 Strychnine-induced seizure in mice

Intraperitoneal administration of strychnine (4 mg/kg) produced clonic convulsion at 5.03 ± 1.10 mins followed by tonic extensor of hind limbs at 6.07±1.30 min in vehicle control normal saline treated group. However, oral administration of *C.ferruginea* ameliorated the seizure induced by strychnine with peak effect observed at 50 mg/kg, producing significant ($P<0.05$) increase in onset of tonic convulsion (17.60 ± 5.10 mins) and 40% protection which was comparable to the effect of clonazepam (0.5 mg/kg, *p.o.*), a standard antiepileptic drugs producing 60% protection. While higher doses of *C.ferruginea* exhibited proconvulsant effect by reducing onset of clonic and tonic seizure in mice ($P>0.05$) (Table 18).

Table 17: Effect of *C.ferruginea* on maximal electroconvulsive shock test in mice

Treatment	onset of tonic (secs)	duration of seizure (secs)	% protection
Vehicle 10 ml/kg	2.60 ± 0.40	105.40 ± 12.79	0
Clonazepam 0.5 mg/kg	NC	78.40 ± 17.47	40
Phenytoin 20 mg/kg	NC	23.96 ± 3.78***	100
Carbamazepine 50 mg/kg	NC	33.82 ± 5.70***	100
CF 50 mg/kg	3.60 ± 0.25	31.20 ± 7.69***	0
CF100 mg/kg	3.80 ± 0.20	28.00 ± 3.89***	0
CF 200 mg/kg	3.60 ± 0.98	45.00 ± 12.46***	20
CF 400 mg/kg	5.60 ± 0.40*	16.20 ± 0.97***	0

Values are expressed as mean ± S.E.M (n = 6), *$P<0.05$; $P<0.001$ versus vehicle control group (one way ANOVA followed by Dunnet's *post hoc* multiple comparison test. (NC = no convulsion)

Table 18: Effect of *C.ferruginea* on strychnine-induced seizure in mice

Treatment	onset of clonic (mins)	onset of tonic (mins)	% protection
Vehicle 10 ml/kg	5.03 ±1.10	6.07±1.30	0
CF 50 mg/kg	6.40 ± 0.75	17.60±5.10*	40
CF 100 mg/kg	5.20 ± 0.73	8.4 0± 2 .20	0
CF 200 mg/kg	5.80 ± 0.80	7.20 ± 0.58	0
Clonazepam 0.5 mg/kg	14.20 ± 5.00*	22.60 ± 4.70*	60

Values are expressed as mean ± S.E.M (n = 6), *$P<0.05$ versus vehicle control group (one way ANOVA followed by Dunnet's *post hoc* multiple comparison test.

4.4.3 Picrotoxin-induced seizure in mice

Intraperitoneal administration of picrotoxin (6 mg/kg) induced seizure in mice at 9.14 ± 0.99 min, 1 h post vehicle control treatment. However, oral administration of CF inhibited picrotoxin-induced seizure with 40, 20 and 20% protection respectively at 100, 200 and 400 mg/kg treated groups. Similarly, Clonazepam (0.5 mg/kg) completely inhibited picrotoxin induced seizure with 100% protection (Table 19).

4.4.4 Bicuculline-induced seizure in mice

Bicuculline (2.7 mg/kg, *i.p.*) induced seizure in vehicle treated group (6.00 ± 0.71 min). Oral administration of CF completely inhibited occurrence of bicuculline-induced seizure in mice which was similar to the effect of standard antiepileptic drug (Clonazepam, 0.5 mg/kg; *p.o.*) (Table 20).

Table 19: Effect of *C. ferruginea* on picrotoxin-induced seizure in mice

Treatment (mg/kg)	Onset of seizure (min)	% mortality
Vehicle 10 ml/kg	9.1.4 ± 0.99	100
Clonazepam 0.5	NC	0
C. ferruginea 100	5.17 ± 0.09	80
200	4.10 ± 1.00	20
400	8.14 ± 1.88	40

Values are expressed as mean ± SEM (n=6). Statistical analysis by one way ANOVA followed by Tukey's *post hoc* multiple comparison test (P >0.05). NC- no convulsion

Table 20: Effect of *C.ferruginea* on bicuculline–induced seizure in mice

Treatment (mg/kg)	Onset of Seizure (min)	% Protection
Clonazepam 0.5	NC	100
C.ferruginea 100	NC	100
200	NC	100
400	NC	100
Vehicle 10 ml/kg	6.04 ± 0.71	0

Values are expressed as mean ± S.E.M (n = 6). (NC = no convulsion). (P >0.05)

4.4.5 Isoniazid-induced seizure in mice

Oral administration of *C. ferruginea* produced graded dose dependent significant (*P<0.05*) increase in onset of tonic seizure from 44.40 ± 1.03 min in vehicle control treated to 66.20 ± 9.74 min and 40% protection at 200 mg/kg, the effect was comparable to the anticonvulsant effect of phenytoin and carbamazepine (standard antiepileptic) each producing 60% protection with non-significant (*P*>0.05) increase in onset of clonic and tonic seizure. However, *C.ferruginea* (50 and 100 mg/kg) produced a non-significant (*P*>0.05) increase in latency to tonic seizure and death on the other hand, GABAmimetic drug clonazepam produced 100% protection without seizure (Table 21).

4.4.6 Yohimbine –induced seizure in mice

Subcutaneous injection of yohimbine (45 mg/kg) induced clonic seizure in mice (38.80 ± 3.76 min) with 100% mortality. Initially, mice became motionless, approximately 15-20 min following injection.

Oral administration of *C.ferruginea* produced dose dependent increase in percentage protection, the peak effect was observed at 100 mg/kg (89% protection). However, increasing those to 200 mg/kg produced 100% lethality. Clonazepam (0.5 mg/kg) produced significant (*P*<0.01) increase in onset of clonic convulsion and 100% protection (Table 22).

There were no significant (*P*>0.05) difference between extract treated group (100, 200, 400 mg/kg) and pentobarbitone alone normal saline treated group. However, clonazepam prolonged the sleeping time significantly (*P<0.05*) (Table 22).

Table 21: Effect of *C. ferruginea* on isoniazid-induced seizure in mice

		onset of seizure (minutes)			
Treatment	**Dose (mg/kg)**	**onset of clonic**	**onset of tonic**	**latency to death**	**% Protection**
C. ferruginea	50	42.60 ± 1.44	56.00 ± 0.63	59.40 ± 2.11	0
	100	49.20 ± 6.35	61.20 ± 4.52	62.00 ± 4.34	20
	200	56.40 ± 7.94	66.20 ± 9.74*	68.60 ± 9.71	40
Clonazepam	0.5	NC	NC	-	100
Phenytoin	20	46.80 ± 1.36	56.20 ± 3.412	56.60 ± 3.09	60
Carbamazepine	50	52.80 ± 3.96	61.00 ± 3.08	67.00 ± 3.54	60
Vehicle control	10 ml/kg	40.20 ± 0.86	44.40 ± 1.03	51.80 ± 2.47	0

Values are expressed as mean ± SEM (n=6), $^{*}P$ <0.05 versus vehicle control treated group. Level of significance analyzed using one way ANOVA followed by Dunnet's *post hoc* multiple comparison test [NC- No convulsion].

Table 22: Effect of methanolic root extract of *C.ferruginea* on yohimbine-induced seizure in mice

Treatment	Dose (mg/kg)	Onset of clonic seizure (min)	% Protection	No of deaths
Vehicle control	10 ml/kg	38.80 ± 3.76	0	5/5
C.ferruginea	50	40.20 ± 4.28	60	2/5
C. ferruginea	100	42.00 ± 1.76	80	1/5
C. ferruginea	200	48.40 ± 8.32	0	5/5
Clonazepam	0.5	62.60 ± 1.60**	100	0/5

Values are expressed as mean ± S.E.M, $^{**}P<0.01$ versus vehicle control group. Level of significance was analyzed using one way ANOVA followed by Dunnet's *post hoc* multiple comparison test.

4.5 Spontaneous motor activity in mice

Figures 5 A-D show the effect of graded doses of methanolic root extract of CF on spontaneous motor activity in mice. One way ANOVA revealed no significant treatment effect.

4.6 Rota-rod test (motor coordination)

The methanolic root extract of CF (100-400 mg/kg, *p.o.*) did not alter the motor performance of animals subjected to the rotarod task in comparison to the control group (vehicle, *p.o.*). The mean ± SEM in the test for control, CF (100-400 mg/kg) and diazepam (5 mg/kg, *p.o.*) were 120 ± 0.00, 120 ± 0.00, 120 ± 0.00, 120 ± 0.00 and 11.37 ± 4.97 s respectively.

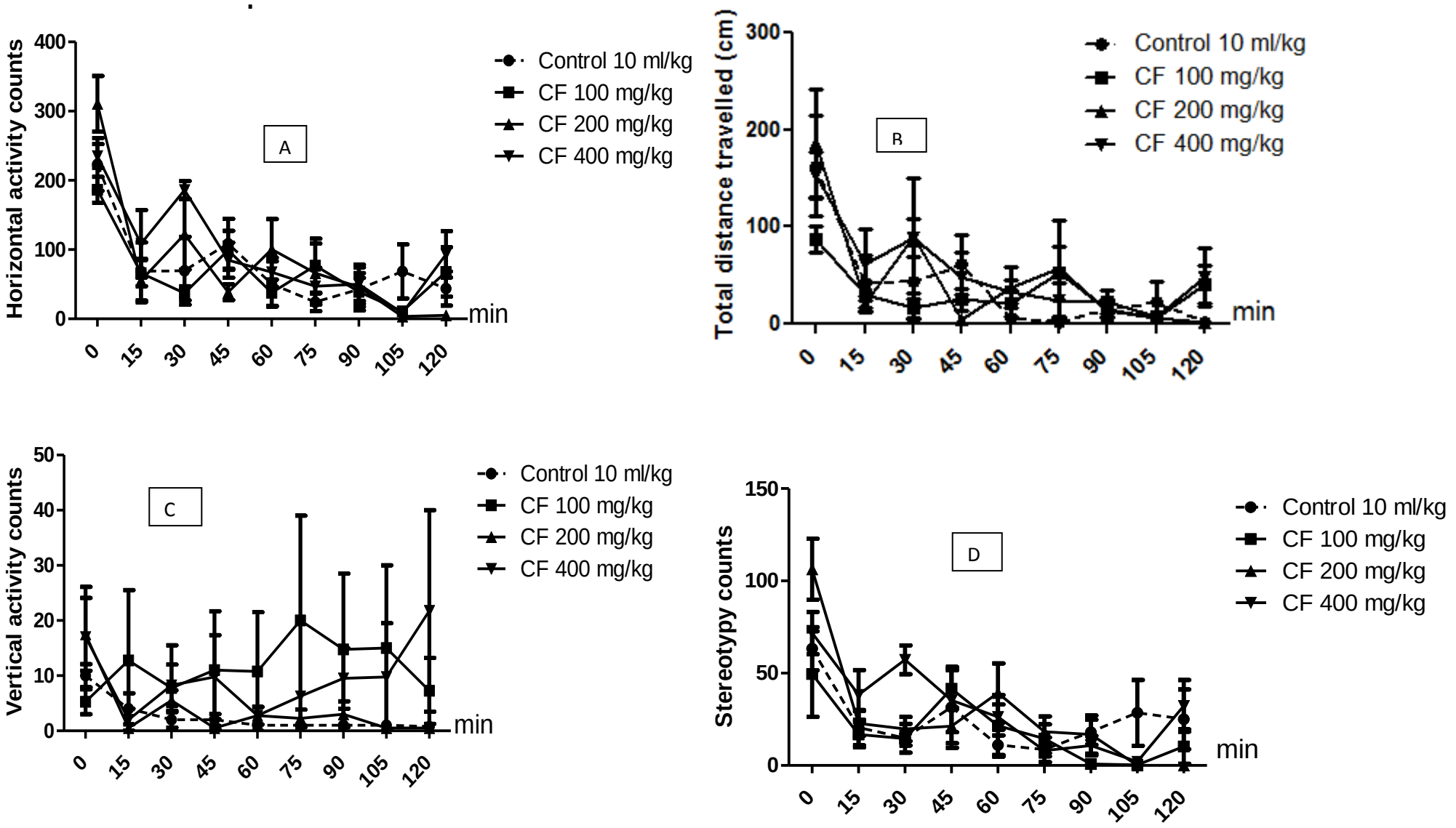

Figure 5 A –D time course effect of oral administration of methanolic root extract of *C. ferruginea* on (A) horizontal (B) total distance (C) vertical activity (D) stereotypy counts in mice. Values are expressed as mean±SEM where n=8 in each group.

4.7 Antidepressant activity
4.7.1 Forced swimming test

The effect of acute administration of *C. ferruginea* in comparison with imipramine in the forced swim test is shown in Figure 6 A. The treatments induced significant changes in the mean time spent immobile ($F(6,53) = 36.85$, $P < 0.0001$), in the duration of swimming ($F(6,56) = 26.07$, $P < 0.0001$). Post hoc analysis revealed that imipramine, a clinically used antidepressant (Belozertseva *et al.*, 2007), significantly induced (30%) reduction in the mean time of immobility. CF (25- 200 mg/kg) dose dependently decreased the occurrence of immobility ($R^2 = 0.8247$) and increased swimming ($R^2 = 0.7652$). Peak effect 58.72% reduction in immobility (when compared to vehicle control group) was achieved in CF (100 mg/kg) whose reduction in immobility and increase in mobility was statistically significant in comparison to positive control (Fig. 6 A).

Animal pretreated with doses of CF-2 (6.25 – 50 mg/kg, *p.o.*) induced significant changes in the mean time spent immobile ($F(5, 46) = 29.26$, $P < 0.0001$), in the duration of swimming ($F(5, 45) = 27.92$, $P < 0.0001$) between groups (Fig. 6 A-B). CF-2 dose dependently decreased the occurrence of immobility ($R^2 = 0.7811$) and increased swimming ($R^2 = 0.7773$). CF-2 pre-treatment produced 27.7, 47.12, 59.72 and 62.26% reduction in immobility at 6.25, 12.5, 25 and 50 mg/kg respectively in comparison to control (Fig. 6 B).

The pretreatment of mice with metergoline (4 mg/kg, *i.p.*, a 5-HT_2 receptor antagonist) and reserpine (2 mg/kg, *i.p.*, a drug known to induce depletion of biogenic amines) 15 mins before the administration of CF (100 mg/kg; *p.o.*) significantly prevented its antidepressant effect in the FST. However, pretreatment with prazosin (62.5 µg/kg, *i.p.*, an α_1-adrenoceptor antagonist), yohimbine (1 mg/kg, *i.p.*, an α_2-adrenoceptor antagonist), sulpiride (50 mg/kg, *i.p.*, a dopamine D_2 receptor antagonist), atropine (1 mg/kg, *i.p.*, a muscarinic receptor antagonist) did not prevent this effect (Fig. 6 C – E).

The pretreatment of mice with metergoline (4 mg/kg, *i.p.*, a 5-HT_2 receptor antagonist), prazosin (62.5 µg/kg, *i.p.*, an α_1-adrenoceptor antagonist), and yohimbine (1 mg/kg, *i.p.*, an α_2-adrenoceptor antagonist), 15 mins before the administration of amentoflavone (50 mg/kg; *p.o.*) significantly prevented its anti-immobility effect in the FST. However, sulpiride (50 mg/kg, *i.p.*, a dopamine D_2 receptor antagonist), atropine (1 mg/kg, *i.p.*, a muscarinic receptor antagonist) 15 mins before amentoflavone (50 mg/kg; *p.o.*) did not prevent this effect (Fig. 7 A – E).

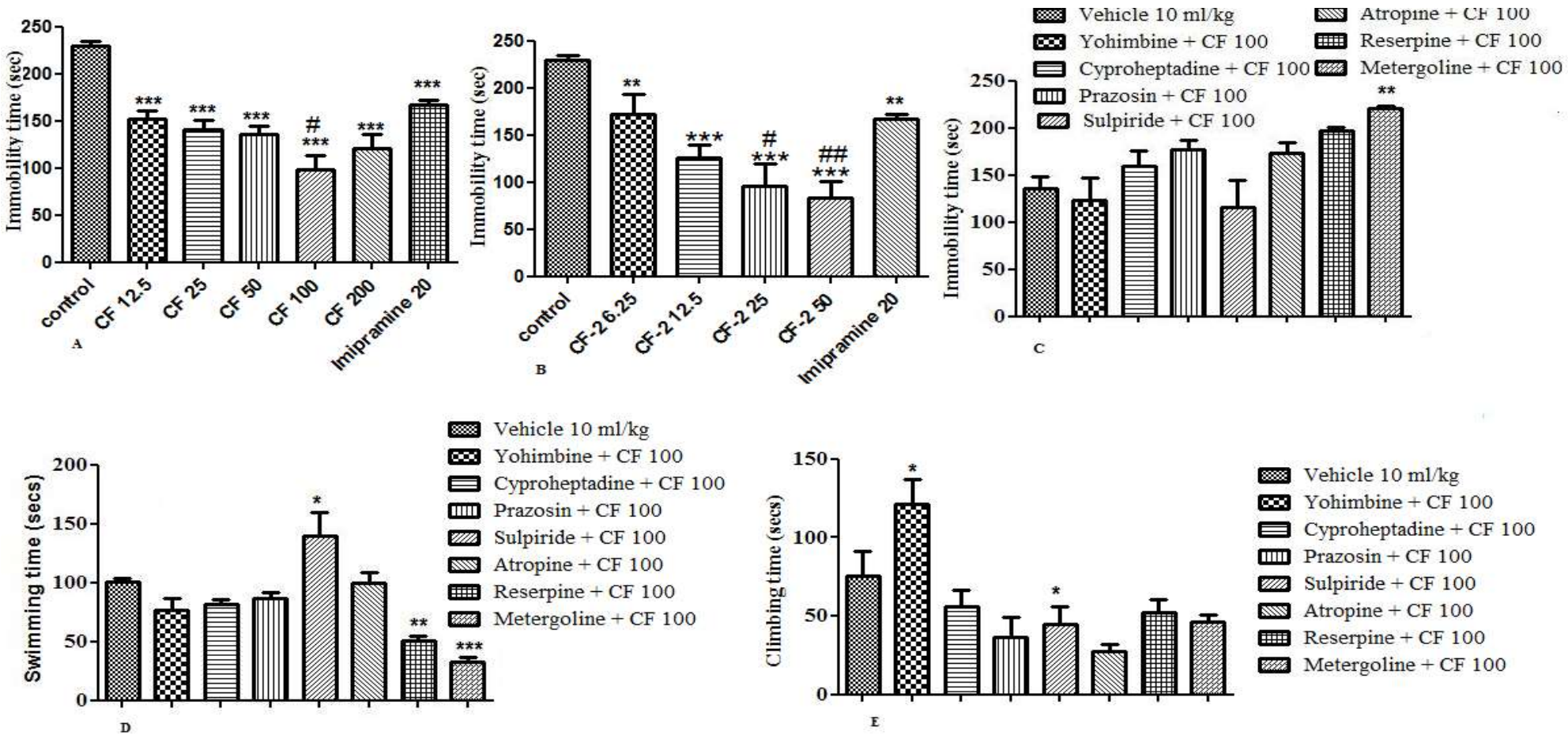

Figure 6 A-E. Effect of oral administration of (A) *C. ferruginea* (B) amentoflavone (CF-2) in forced swimming test. Effect of adrenoceptor, dopaminergic, serotonergic and muscarinic antagonist and vesicular monoamine depleter on antidepressant effect of *C. ferruginea* (C) immobility time, (D) Swimming time, (E) Climbing time. Each column represents the mean ± SEM (n = 8). Significant effect $^{**}P<0.01$, $^{***}P<0.001$ when compared to control vehicle group. Significant effect $^{\#}P<0.05$, $^{\#\#}P<0.01$ when compared with imipramine treated group; One way ANOVA followed by Tukey's multiple comparison test.

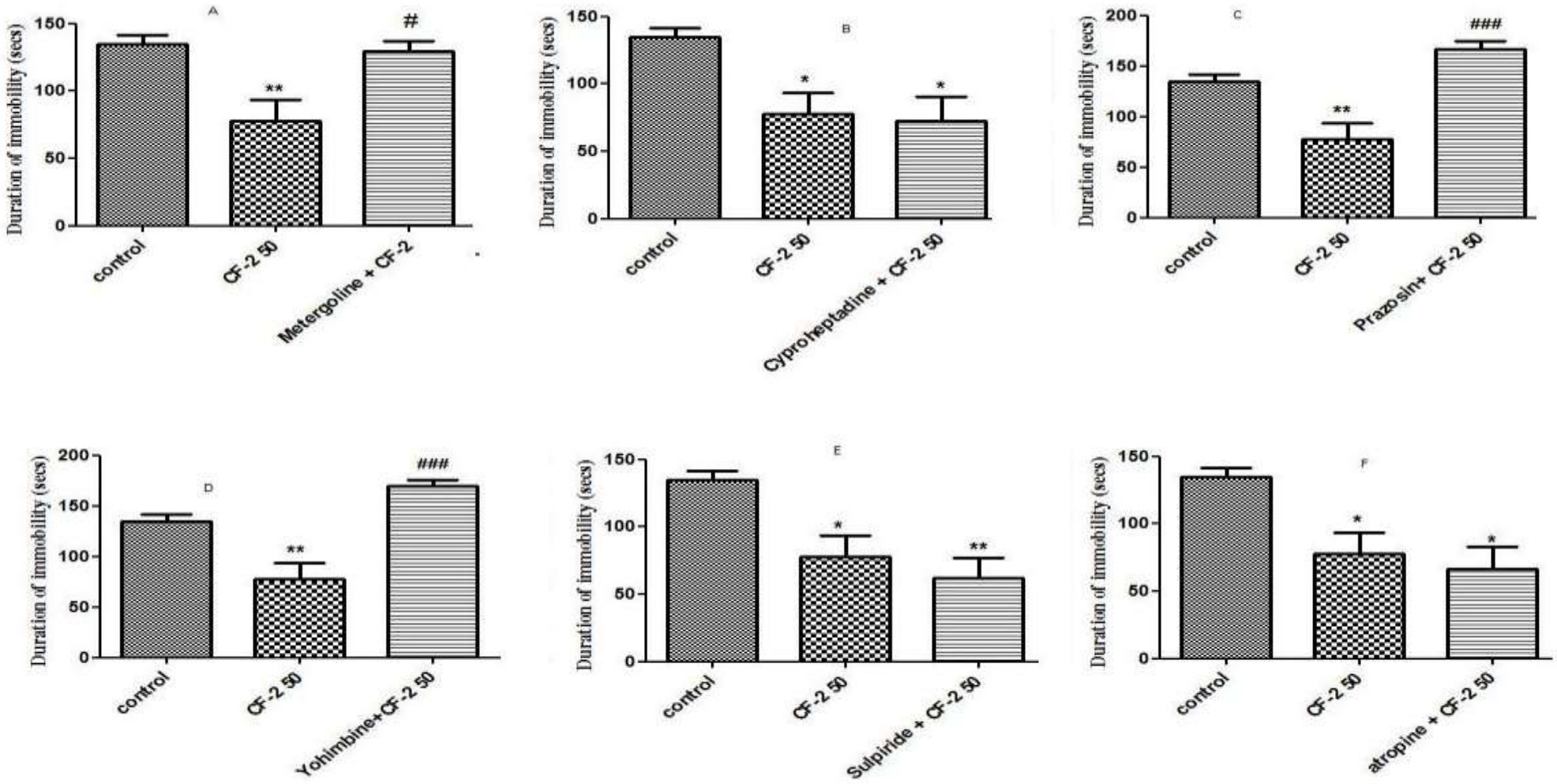

Fig. 7 A–F: Effect of (A & B) 5-HT_2, (C) α_1- adrenoceptor antagonist, (D) α_2- adrenoceptor antagonist, (E) D_2- dopaminergic and (F) muscarinic cholinergic antagonist on anti-immobility effect of amentoflavone. Each column represents the mean ± SEM (n=8). Significant effect $^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$ when compared to control vehicle treated group. Significant effect $^{\#}P<0.05$; $^{\#\#}P<0.01$ when compared with amentoflavone treated group.

4.7.2 Tail suspension test

The influence of CF and CF-2 on TST was also examined in mice based on the result obtained from FST. Observation of the controls suggested that the mice suspended by tail made apparent escape efforts which could be classified into: running movements, forward or backward; body torsion with attempt to catch the suspending body and body jerks. After several attempts, the mice stopped moving and hung motionless.

Figure 8 A - B. Shows that fluoxetine produced a significant reduction of immobility ($F(3, 13) = 4.274$, $P<0.001$) with 42.77% reduction in immobility in comparison with control.
Oral administration of methanolic root extract of CF decreased the duration of immobility (35.98 and 22.23%) ($F(5, 15) = 7.252$, $P<0.001$, and ($F(6, 16) = 4.209$, $P<0.001$) at 100 and 200 mg/kg respectively. Statistical analysis revealed a significant effect of treatment between groups ($F(3, 25) = 8.995$, $P<0.0004$).

Oral administration of CF-2 (12.5 and 25 mg/kg, *p.o.*) dose dependently produced significant decrease in the duration of immobility ($F(6, 16) = 0.6816$, $P>0.05$), ($F(4, 14) = 9.232$, $P<0.001$), with 5.76 and 23.86% decrease in immobility respectively. Statistical analysis revealed a significant effect of CF-2 between groups ($F(3, 23) = 10.60$, $P<0.0002$) (Fig. 8 C-D).

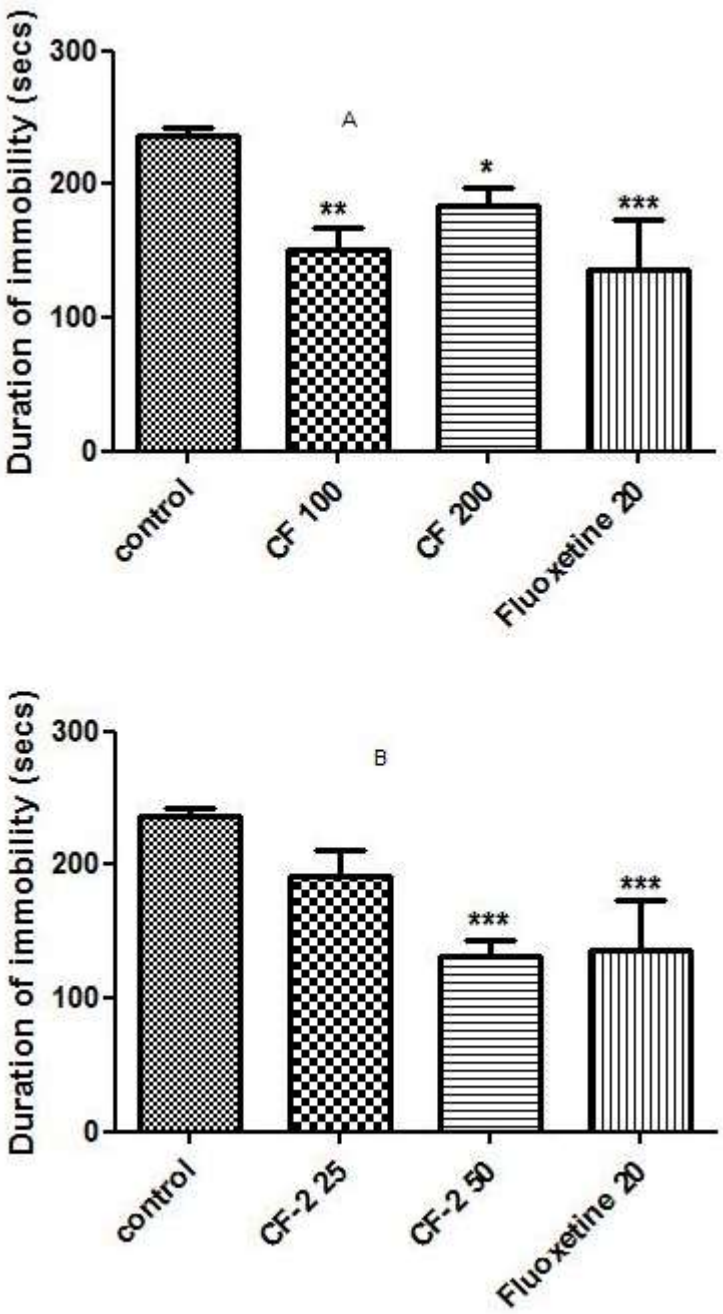

Fig. 8 A-B. Effect of oral administration of *C. ferruginea* and amentoflavone on tail suspension test. Each column represents the mean ± SEM (n = 8). Statistical significance decrease $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ compared with the vehicle treated control group using One way ANOVA followed by Tukey's *post hoc* multiple comparison test.

4.8 Anxiolytic activity

4.8.1 Hole board test

Exposure of 8 mice to the hole board for 3min resulted in a reduction in the number of head – dips in the vehicle control group. An analysis of the exploratory activity in diazepam (1.5 mg/kg) treated group resulted in a significant ($P<0.001$) increase in the number of head – dips in comparison to control. Oral administration of methanolic root extract of CF, 1 h post treatment resulted in significant ($P<0.05$, 0.01, 0.001) increase in number of head-dips at 25, 50 and 100 mg/kg respectively. However, CF (200 mg/kg) produced a marked reduction in the number of head – dips ($P>0.05$) (Fig. 9 A). Statistical analysis revealed that oral administration of CF-2 dose dependently produced a marked and significant ($P<0.05$, $P<0.001$) increase in the number of head – dips (Fig. 9 A).

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4.8.2 Elevated plus maze test.

One way analysis of variance revealed a significant increase in the time spent on the open arm after administration of the positive control (diazepam) with 29.28 % increase in time spent in open arms when compared to control (Fig. 9 B). Oral administration of methanolic root extract of CF showed significant activities in the time spent on open arms ($F (5, 40) = 14.68$, $P<0.0001$), and occurrence of open arm exploration ($R^2 = 0.6771$) at 25, 50 and 100 mg/kg producing 44.95, 34.95 and 28.08 % increase in time spent in open arms respectively. However, 200 mg/kg produced non-significant ($P>0.05$) open arm exploration (Figure 21B). Similarly, oral administration of CF-2 showed significant ($P<0.01$) activities ($F (3, 34) = 11.88$, $P<0.0001$); ($R^2 = 0.5349$) at 12.5 and 25 mg/kg produced 31.01 and 34.37% increase in time spent in open arms respectively, in comparison to vehicle control but no significant difference between diazepam and extract treated group (Figure 9 B). One way ANOVA revealed that pretreatment of animals with flumazenil (3 mg/kg, *i.p.*) and cyproheptadine (3 mg/kg; *i.p.*) significantly ($P <0.05$) reversed the anxiolytic effect of *C. ferruginea* (50 mg/kg) Table 23.

4.8.3 Light - dark compartment test

oral administration of methanolic root extract of CF produced significant activities in the time spent in light compartment ($F (7, 45) = 12.75$, $P< 0.0001$); ($R^2 = 0.7013$) at 25, 50, 100 and 200 mg/kg produced 52.06, 69.88, 48.81 and 46.71 % increase in time spent in light chamber respectively in comparison to vehicle treated group (Figure 9 C). Statistical analysis

showed that diazepam (1.5 mg/kg) produced significantly increase (*P<0.05*) (534%) in the time spent in light compartment when compared to control.

Oral administration of CF -2 produced a significant (*P*<0.001) increase in time spent in light compartment (F (3, 18) = 69.79, *P*<0.0001), (R^2 = 0.9331) at 12.5 and 25 mg/kg respectively, with peak effect (70.32 % increase in time spent in light chamber) at 12.5 mg/kg. One way analysis of variance revealed that CF (50 mg/kg) and CF-2 (12.5 mg/kg) increased the time spent in the light chamber significantly in comparison to the effect of clinically used anxiolytic (diazepam) (*P<0.05*) treated group (Figure 9 C).

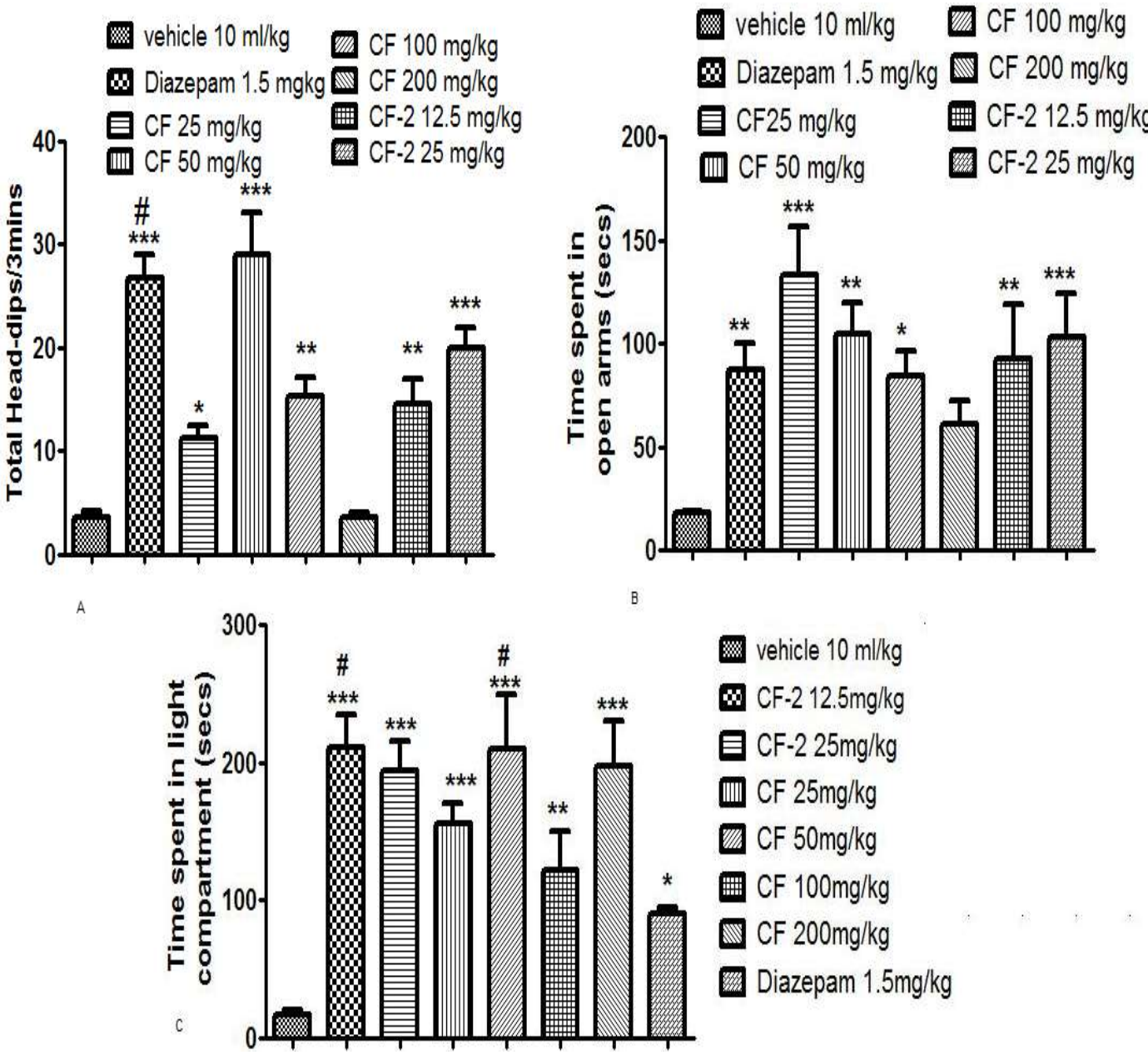


Figure 9 A-C: Anxiolytic effect of *C.ferruginea* and CF-2 in mice. (A) depicts total number of head dips in 3 mins, (B) Time (secs) spent in open arms in the elevated plus maze, (C) Time (secs) spent in light compartment in light and dark test in mice following oral administration of *C.ferruginea* (25, 50, 100 and 200 mg/kg, *p.o.*), amentoflavone (12.5 and 25 mg/kg, *p.o.*) and diazepam (1.5 mg/kg, *p.o.*). Results are expressed as mean ± SEM (n = 8); **P* < 0.05; ***P* < 0.01; ****P* < 0.001 versus control; $^{\#}P$ <0.05 versus diazepam; one way ANOVA followed by Tukey's multiple comparison test.

Table 23: Elucidation of mechanism(s) of anxiolytic effect of *C. ferruginea* in EPM test

Treatment	Dose (mg/kg)	Number of entries	Time spent in open arms
Control	10 ml/kg	6.40±1.03	44.00±6.38
C. ferruginea	12.5	4.20±0.66	47.75±8.39
C. ferruginea	25	4.20±0.74	91.50±7.50$^{***,\alpha}$
C. ferruginea	50	4.60±0.93	44.25±4.70
Diazepam	2	2.40±0.75*	146.30±2.39^{***,a}
Control	10 ml/kg	7.60±0.98	53.60±4.86
Flumazenil	3	5.20±0.74	52.20±5.64
Flumazenil 3 + *C. ferruginea*	50	3.20±0.74	50.00±3.57$^{\beta}$
Flumazenil 3 + diazepam	2	2.75±0.85	34.00±3.83$^{###}$
Cyproheptadine	3	6.33±1.20	46.00±2.12
Cyproheptadine 3 + *C.ferruginea*	50	4.67±1.20	41.68±2.87$^{\beta}$
Cyproheptadine 3 + diazepam	2	3.20±0.49	36.60±5.27$^{##}$

Values are expressed as mean ± SEM (n= 7), $^{*}P<0.05$, $^{***}P<0.001$ versus control treated group; $^{\alpha}P<0.05$ versus *C. ferruginea* 12.5 mg/kg; $^{a}P<0.001$ versus *C. ferruginea* 25 mg/kg; $^{\beta}P<0.05$ versus *C. ferruginea* 25 mg/kg; $^{##}P<0.01$, $^{###}P<0.001$ versus diazepam 2 mg/kg treated group. Statistical level of significance analyzed by one way ANOVA followed by Tukey's *post hoc* multiple comparison tests.

4.9 Antidementic activity

4.9.1 Passive avoidance test

Control mice showed clear retention as indicated by the increase ($P>0.05$) in transfer latency time (TLT) of retention trial in comparison to acquisition trial while administration of scopolamine (*i.p.*) 5 min before acquisition trial caused no significant change in TLT of retention trial when compared to the acquisition trial (Fig 10A). However, CF (25, 50, 100 and 200 mg/kg, *p.o.*) had no significant effect ($P>0.05$) on TLT in acquisition trial but caused a marked and significant increase ($P<0.05$) in TLT in the retention test in a dose-dependent manner suggesting antagonism of scopolamine induced amnesia. Similar effect was observed with tacrine (Fig 10A). Further, oral administration of CF-2 and CF-5 (6.25, 12.5 and 25 mg/kg, *p.o.*) for three days dose dependently ameliorated the scopolamine induced amnesia in mice. Both the compounds exerted maximum effect at a dose 12.5 mg/kg when the retention latencies were comparable to control untreated group. There was no significant difference ($P>0.05$) in the retention latencies of 12.5 and 25 mg/kg treated groups (Fig. 10B).

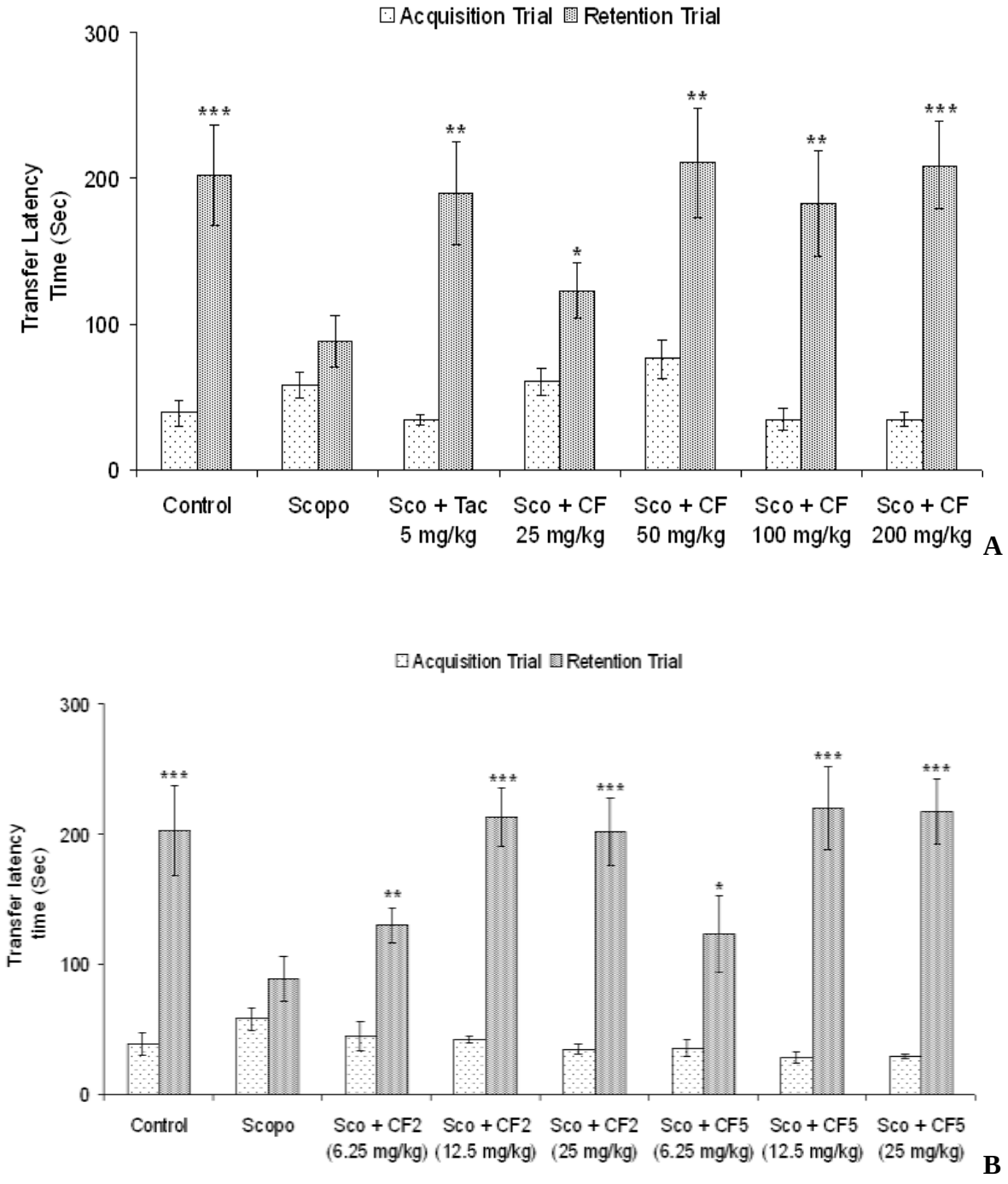
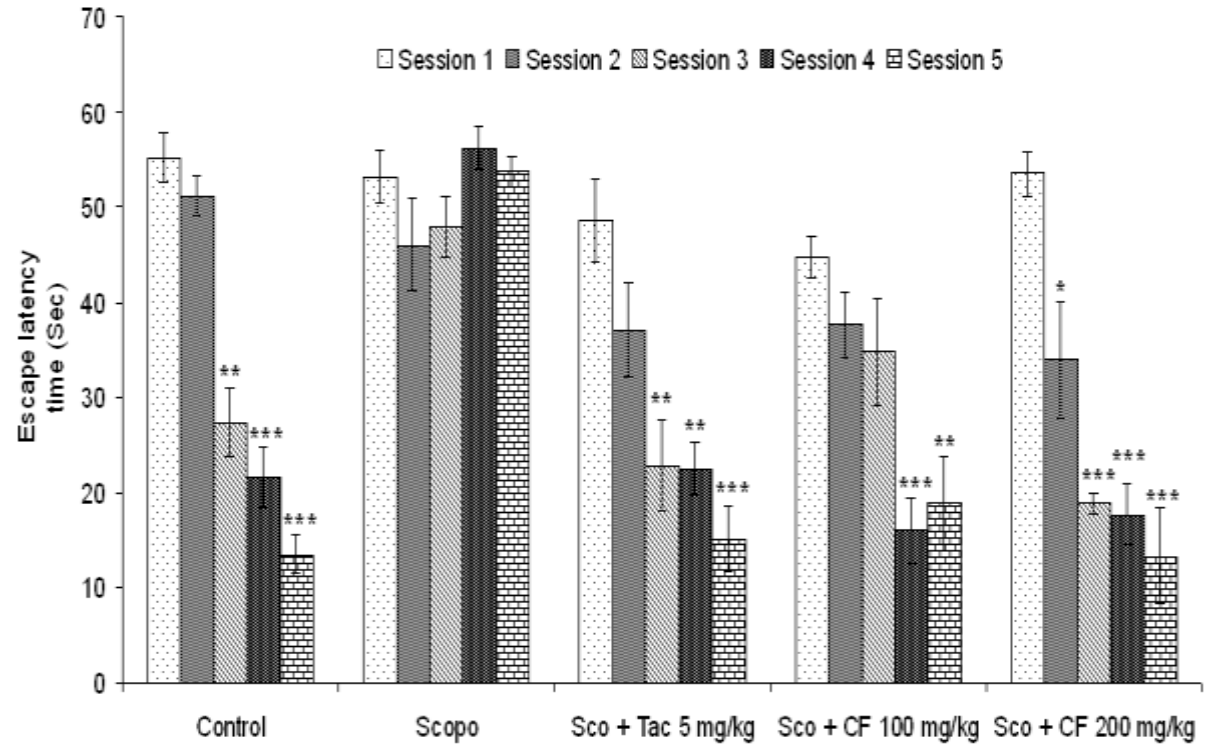


Figure 10 A-B: Effect of (A) CF, (B) CF-2 and CF-5 on scopolamine induced amnesia in mice. Values are expressed as mean TLT (sec) ± S.E.M (n=6). *Significant increase ($^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$) versus acquisition trial. [Sco- Scopolamine; Tac- Tacrine; TLT- Transfer Latency Time].

4.9.2 Morris Water maze test.

Saline-treated mice rapidly acquired the spatial task as indicated by a gradual, session-dependent decrease in escape latency (F (4, 29) = 46.79, $P<0.001$) and pathlength (F (4, 29) = 19.28, $P<0.01$). Administration of scopolamine (3 mg/kg, *i.p.*) 5 min before water maze trial caused spatial memory impairment as indicated by no significant change (F (4, 29) = 1.86, $P>0.05$) in ELT of session 2-5 in comparison to that of session 1. Further, there was no significant change (F (4, 29) = 0.34, $P>0.05$) in pathlength of scopolamine injected group. Treatment with tacrine (5 mg/kg, *p.o.*) followed by scopolamine produced a significant decrease (F (4, 29) =10.59, $P<0.05$) in ELT and pathlength (F (4, 29) =7.3, $P<0.05$) from third session onward when compared to the 1st session. Oral administration of CF produced dose dependent effect on scopolamine induced memory deficit in mice. As shown in Fig.11A. CF 100 mg/kg significantly decreased mean ELT from 4th session (F (4, 29) = 9.35, $P< 0.05$) when compared to 1st session, while 200 mg/kg CF reduced the mean ELT significantly from 2nd session onward (F (4, 29) = 17.26, $P < 0.001$). Investigation of path length revealed a significant decrease in pathlength in CF treated group (100 mg/kg: F (4, 29) = 10.3, $P< 0.01$; 200 mg/kg: F (4, 29) = 20.78, $P < 0.001$) (Fig. 11B). Further, statistical analysis showed a significant correlation (Pearson r = 0.986; R^2 = 0.971 and $P<0.05$) between mean latency time and mean path length of all the groups in all sessions (Fig. 11C). The representative swimming pattern of mice of different groups is shown in Fig. 11D.

Further, the constituents of CF, CF-2 and CF-5, also prevented scopolamine induced memory deficit in mice dose dependently. As shown in Fig.12A, CF-2 significantly reduced mean ELT from 2nd session onward (12.5 mg/kg: F (4, 29) =16.73, $P<0.001$); 25 mg/kg: (F (4, 29) = 27.57, $P<0.001$). Further, CF-5 showed dose dependent amelioration of scopolamine induced amnesia in mice (12.5 mg/kg: F (4, 29) =5.5, $P<0.05$); 25 mg/kg: (F (4, 29) = 19.63, $P<0.01$) as there was a significant decrease in ELT from 4th (12.5 mg/kg) and 2nd (25 mg/kg) session onward. Further investigation of pathlength showed that CF-2 and CF-5 treated groups had significant reduction in pathlength when compared to session 1 (Fig. 12B). There was a significant correlation (Pearson r = 0.985; R^2 = 0.969 and $P<0.01$) between ELT and mean pathlength of all the treated groups in all the sessions (Fig. 12C).



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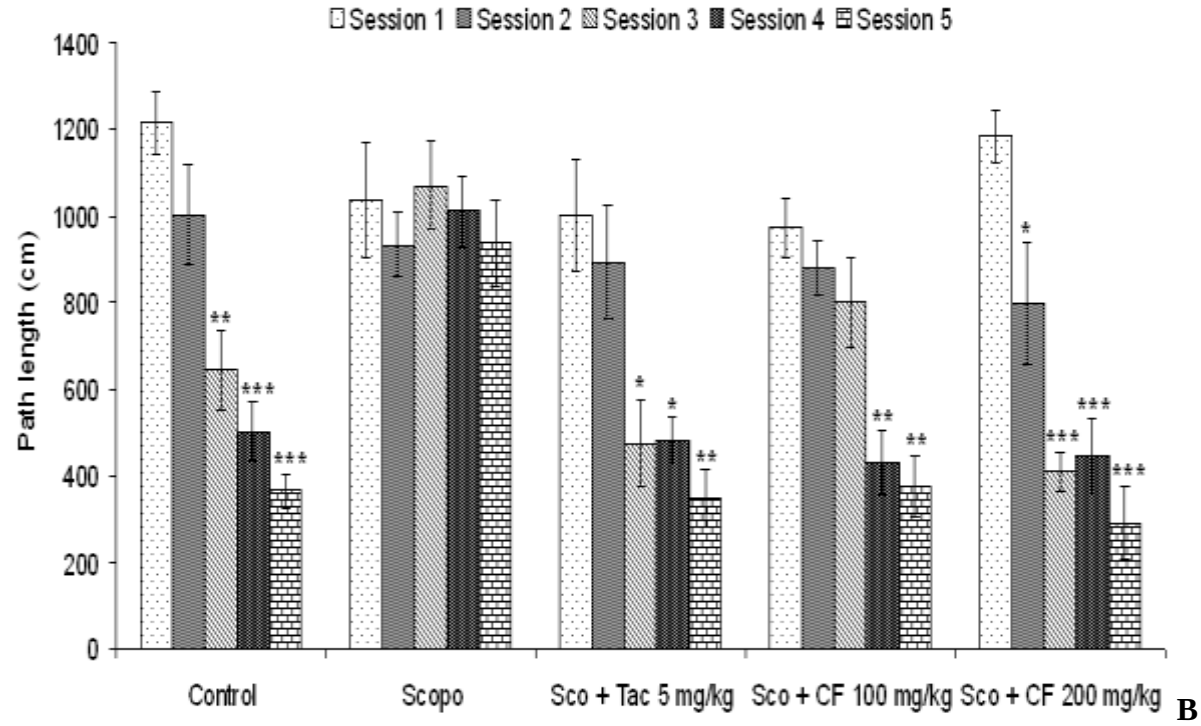
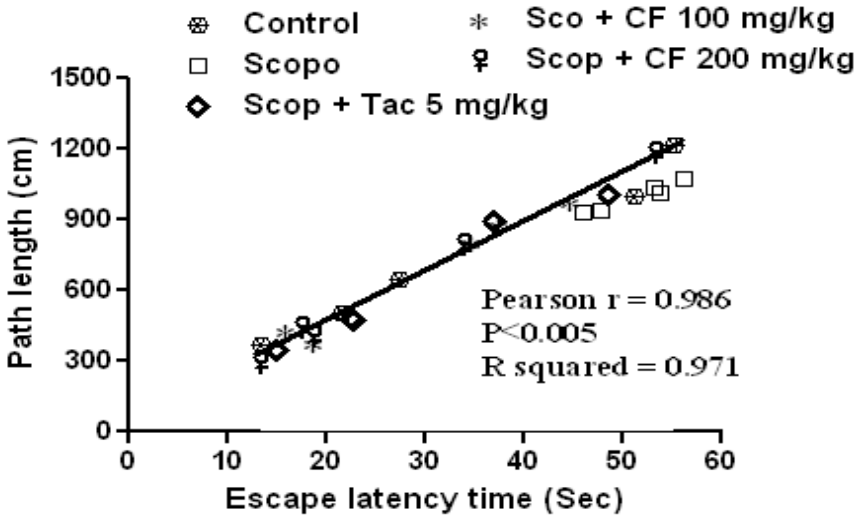


B

Fig. 11 A-B: Effect of methanolic extract of CF root on scopolamine induced amnesia in Morris water maze test. Values are expressed as mean (A) ELT (sec) (B) Path length (cm) ± S.E.M (n=6). *Significant decrease (*$P<0.05$, **$P<0.01$ and ***$P<0.001$) vs session 1. [Sco- scopolamine; Tac- Tacrine; ELT- Escape Latency Time]



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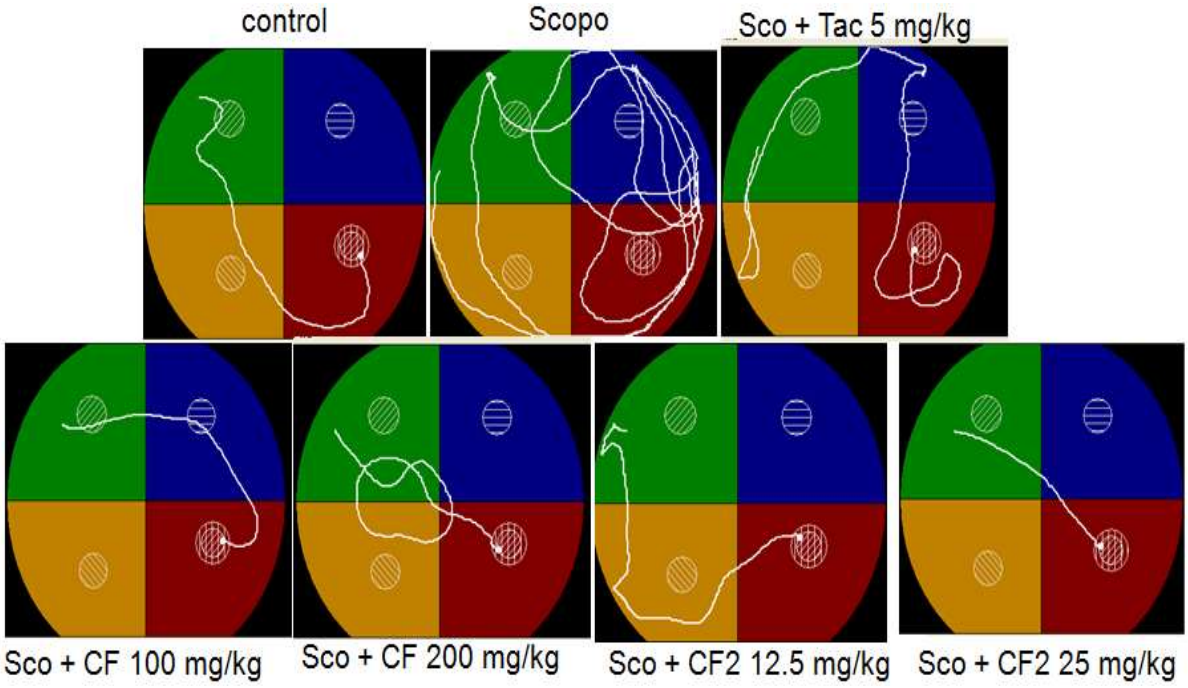


D

Figure 11 C-D: (C) Correlation between latency time and path length (D) Representative Morris water maze tracing of different groups of animals during last trial of session 5.

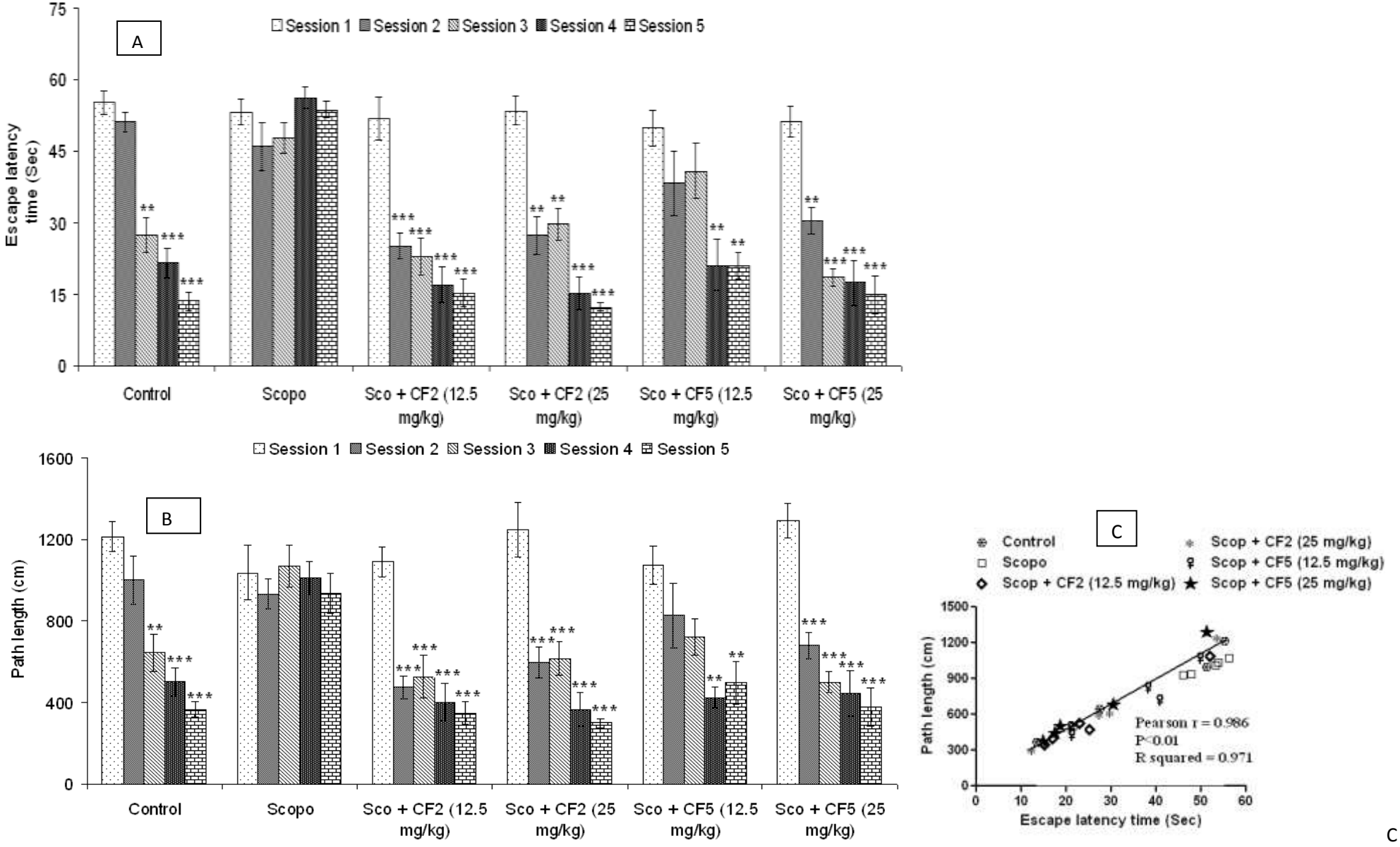
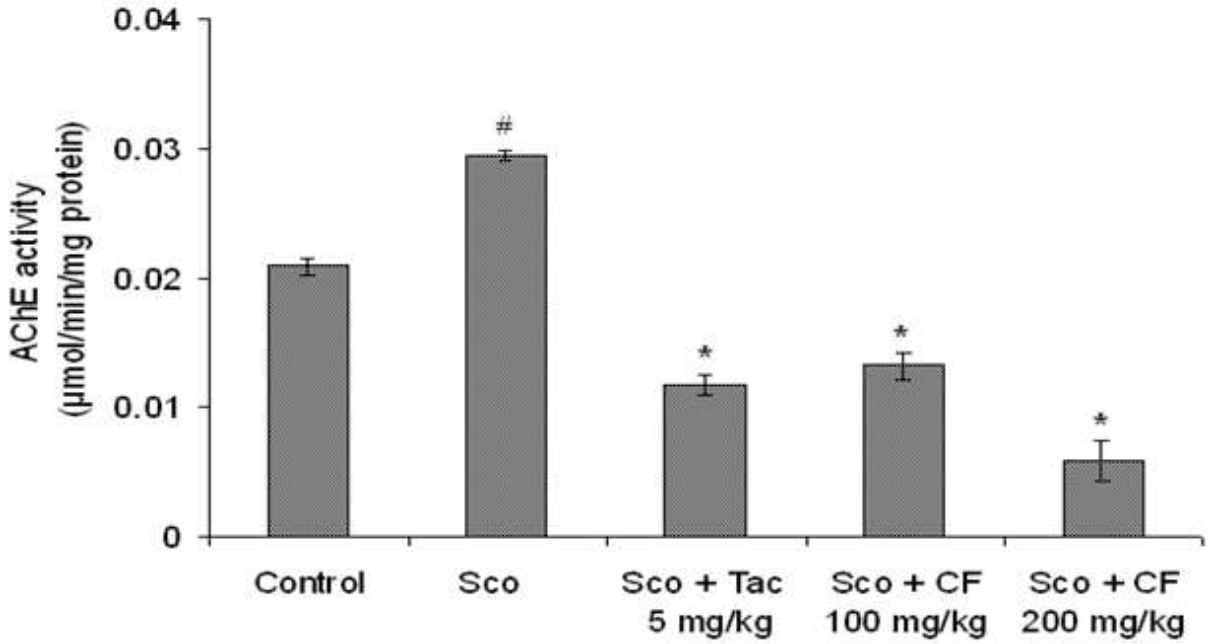


Fig 12 A-C: Effect of CF-2 and CF-5 on scopolamine induced amnesia in Morris water maze test. Values are expressed as mean (A) ELT (sec) (B) path length (cm) ± S.E.M (n=6). *Significant decrease ($^{**}P<0.01$ and $^{***}P<0.001$) versus acquisition trial. (C) Correlation between mean latency time and mean pathlength.

4.9.3 Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity (µmol/min/mg protein) was estimated on day 5 after the first dose of scopolamine. There is significant increase ($P<0.01$) in AChE activity in scopolamine treated group when compared to the control group. The AChE activity was decreased ($P<0.01$) in CF (100 and 200 mg/kg) treated groups when compared to scopolamine group ($F(2, 15) = 27.39$, $P<0.01$)). Tacrine, a known antiamnesic drug, reduced ($P<0.01$) AChE activity in mice brain (Fig. 13A).

Similarly, CF-2 (12.5 and 25 mg/kg) pretreatment significantly reduced AChE activity ($F(2, 15) = 91.56$, $P<0.01$)) in comparison to scopolamine treated group. In addition CF-5 (12.5 and 25 mg/kg) significantly and dose dependently inhibited AChE activity ($F(2, 15) = 78.9$, $P<0.01$)) when compared to scopolamine group (Fig. 13B).



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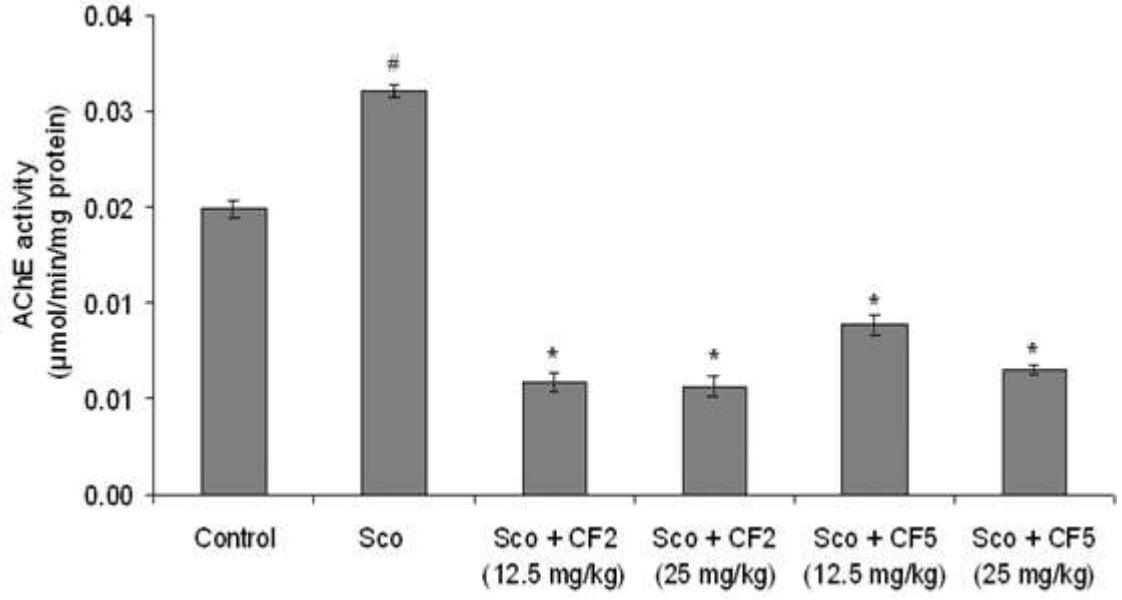


B

Fig. 13 A-B: (A) Effect of methanolic extract of CF root (B) CF-2 and CF-5 on AChE activity. Values are expressed as mean AChE activity (µmol/min/mg protein) ± S.E.M (n=6). $^{\#}$Significant increase ($^{\#}P<0.01$) vs control group and *Significant increase ($^{*}P<0.01$) vs scopolamine group. Level of significance analyzed by one way ANOVA followed by Tukeys *post hoc* multiple comparison tests. [Sco- scopolamine; Tac- Tacrine]

4.9.4 Oxidative stress parameters

4.9.4.1 Malondialdehyde (MDA) level

The MDA level (nmol/mg protein) in the brain was measured first the completion of behavioural tests. The MDA level was significantly higher ($P<0.05$) in scopolamine treated mice as compared to control while in tacrine treated group a significant reduction in the level of MDA was observed. CF (100 and 200 mg/kg) ameliorated scopolamine induced lipid peroxidation as shown by significantly reduced MDA level in mice brain ($F (2, 15) = 47.12$, $P<0.01$)) (Fig. 14A). Similarly, CF-2 ($F (2, 15) = 37.56$, $P<0.01$)) and CF-5 ($F (2, 15) = 21.48$, $P<0.01$)) showed antioxidant effect in scopolamine injected mice as indicated by significant decrease in MDA level (Fig. 14B).

4.9.4.2 Glutathione (GSH) level

A significant fall ($P<0.05$) in the levels of GSH was observed in the scopolamine group as compared to the control. The GSH level in CF (100 and 200 mg/kg) treated mice brain was significantly higher ($F (2, 15) = 24.63$, $P<0.01$)) in comparison to scopolamine group indicating amelioration of scopolamine induced oxidative stress (Fig. 14C). Both active constituents of CF; CF-2 ($F (2, 15) = 29.68$, $P<0.01$)) and CF-5 ($F (2, 15) = 26.31$, $P<0.01$)), also prevented scopolamine induced reduction in GSH level in mice brain (Fig. 14D). The standard drug tacrine also reversed the effect of scopolamine on GSH level.

4.9.4.3 Nitrite levels

As shown in Fig. 15A and B there was no significant change ($P>0.05$) in nitrite level in brain of scopolamine treated mice in comparison to control. Further, CF ($F (2, 15) = 1.2$, $P>0.05$)), CF-2 ($F (2, 15) = 1.66$, $P>0.05$)) and CF-5 ($F (2, 15) = 3.25$, $P>0.05$)) did not affect nitrite level in mice brain.

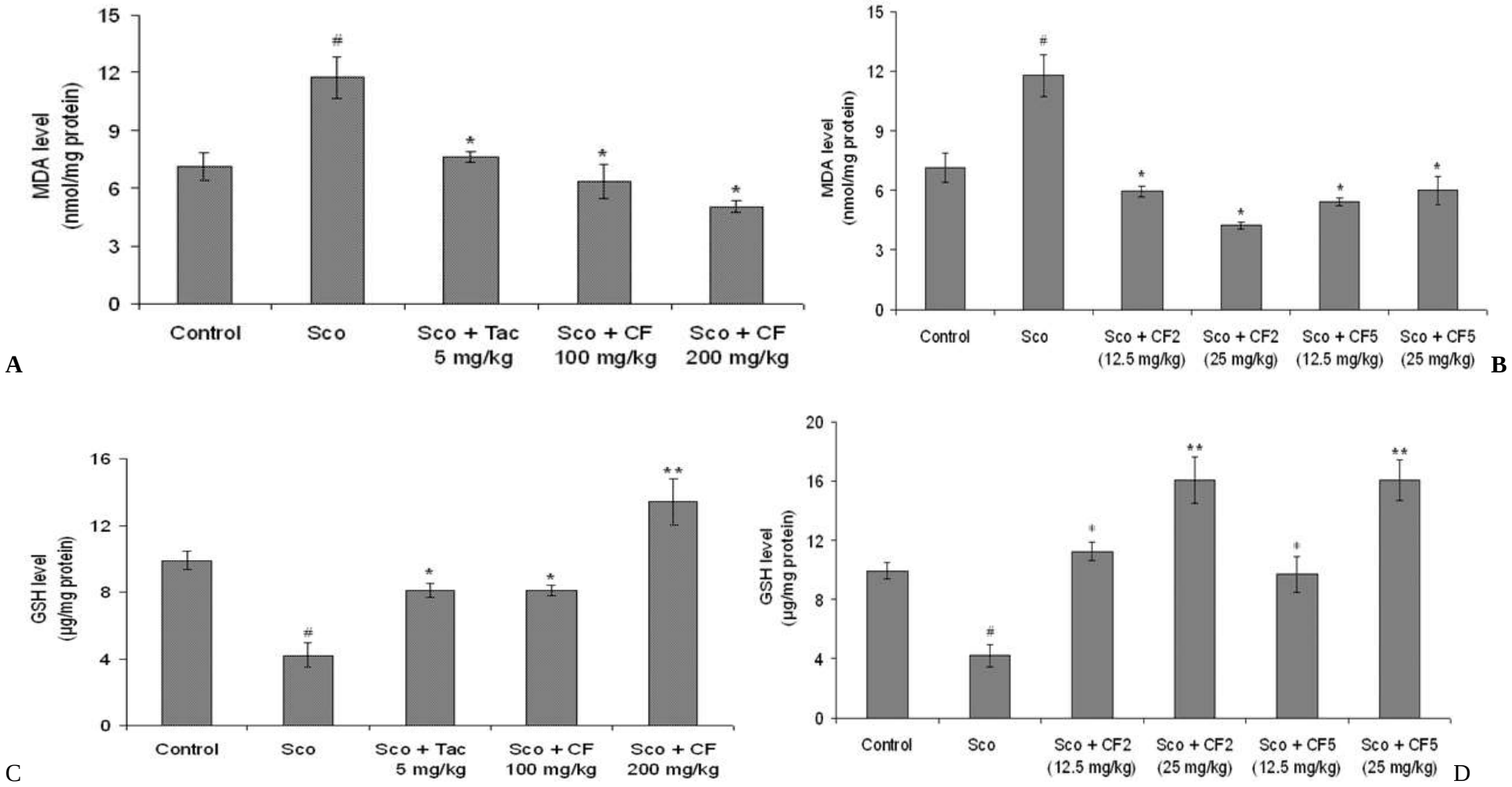
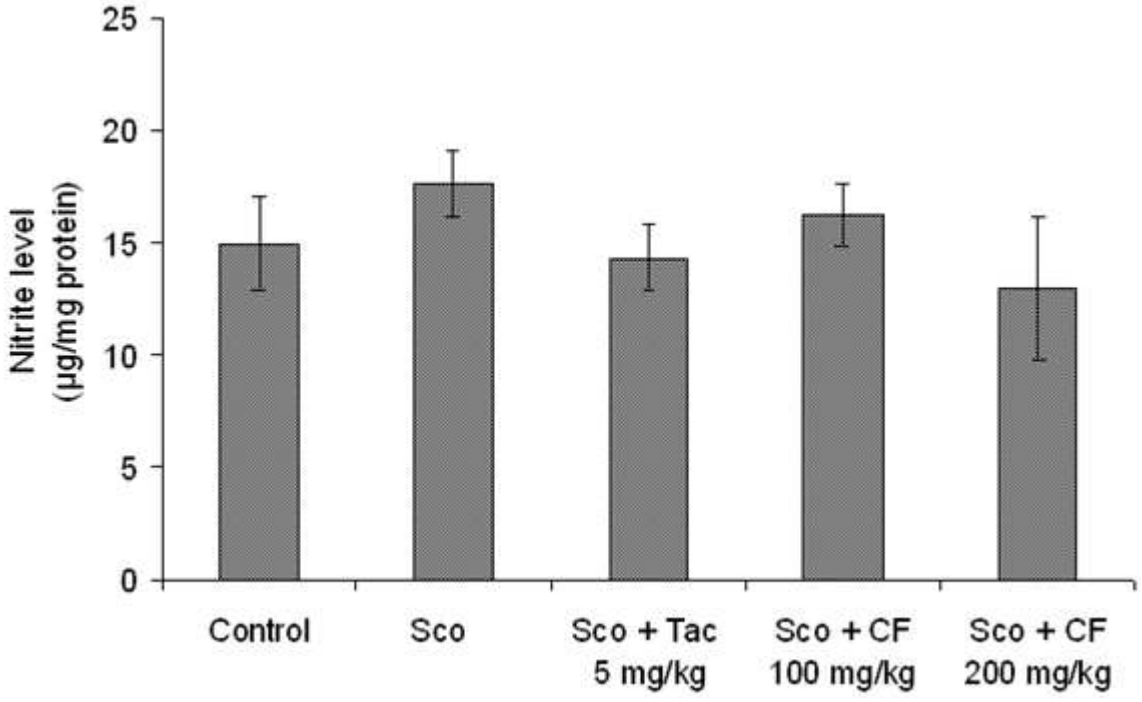


Fig. 14 A-D: Effect of (A) methanolic extract of CF root (B) CF-2 and CF-5 on MDA level, (C) methanolic extract of CF root (D) CF-2 and CF-5 on GSH level. Values are expressed as mean MDA level (nmol/mg protein) ± S.E.M (n=6). $^{\#}$Significant increase ($P<0.05$) vs control group and *Significant decrease ($^{*}P<0.05$; $^{**}P<0.01$) vs scopolamine group in MDA level estimation but vice versa in GSH level estimation. Level of significance analyzed by one way ANOVA followed by Tukeys *post hoc* multiple comparison tests. (Sco- scopolamine; Tac- Tacrine)



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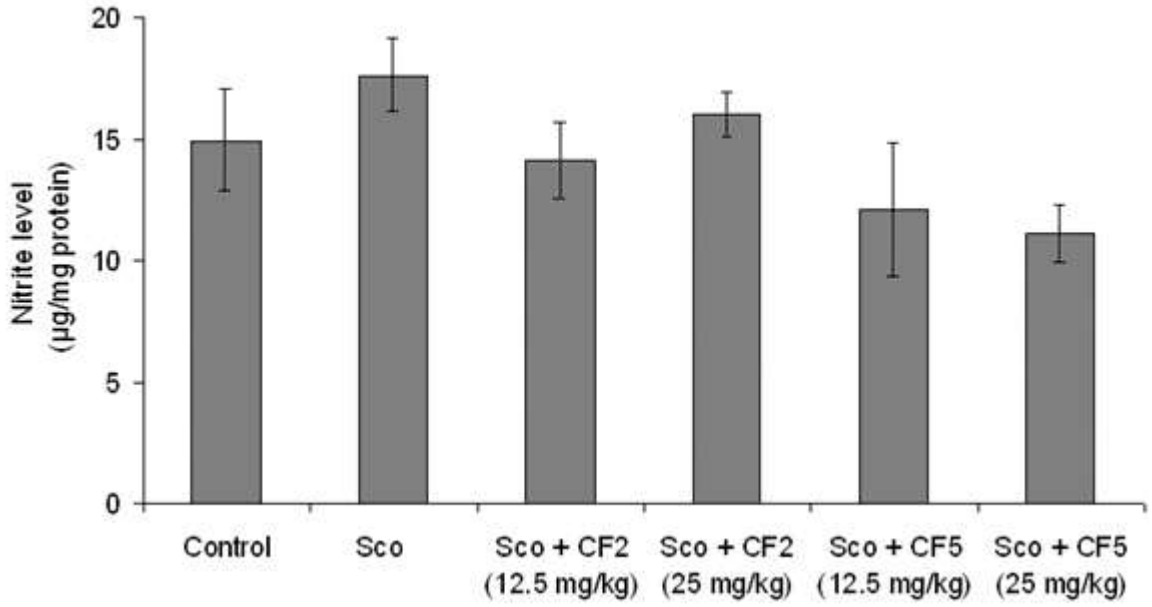


B

Fig. 15 A-B: Effect of (A) methanolic root extract of CF, (B) CF-2 and CF-5 on nitrite level. Values are expressed as mean nitrite level (µg/mg protein) ± S.E.M (n = 6). Level of significance analyzed by one way ANOVA followed by Tukeys' *post hoc* multiple comparison tests.

4.10 *In vitro* anti-inflammatory activity of CF-2 and CF-5 in C6 cell line

4.10.1 MTT cell viability assay

Non-cytotoxicity concentration of CF-2 and CF-5 was measured by MTT reduction assay after 24 h of incubation with various concentrations (0.1-100 µg/ml of CF-2 and CF-5). CF-2 concentrations up to 100 µg/ml and CF-5 up to 10 µg/ml were found non-cytotoxic as they did not cause any significant change in the growth of C6 cells (Fig. 16 A-B).

4.10.2 LPS-induced nitrite release in C6 cells

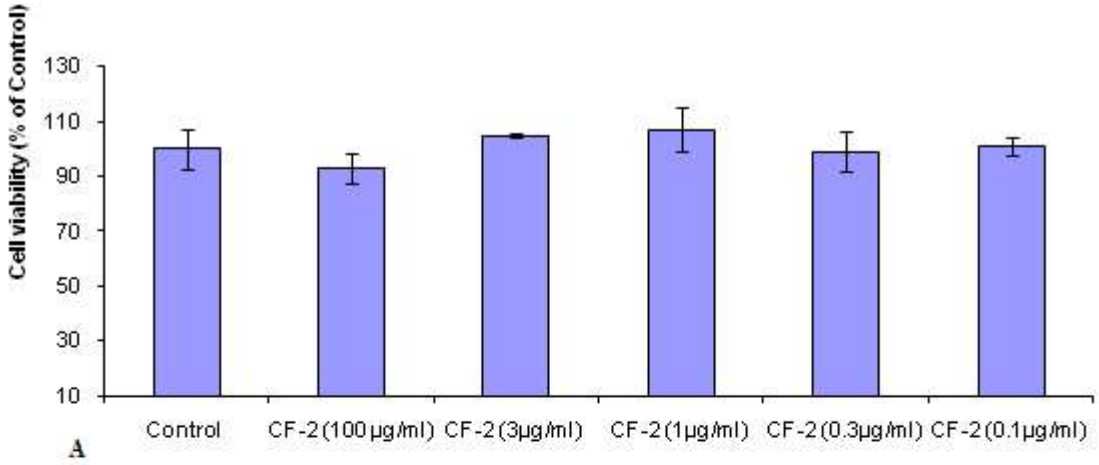
C6 cells treated with LPS (10 µg/ml) alone increased (P <0.001) nitrite release in culture supernatant at 24 h compared with unstimulated cells. However, LPS plus CF-2 and CF-5 (0.1, 0.3,1 and 3 µg/ml) reduced (P <0.001) nitrite release in culture supernatant at 24 h compared with unstimulated cells in a dose dependent manner. In addition, CF-2 and CF-5 alone (3 µg/ml) did not show significant difference (P >0.05) when compared with control group (Fig. 17A-B).

4.10.3 LPS-induced ROS generation in C6 cells

LPS (10 µg/ml) increased (P <0.05) iROS generation in C6 cells at 24 h compared to unstimulated cells. CF-2 (0.1, 0.3,1 and 3 µg/ml) attenuated (P<0.01; P<0.001) LPS (10 µg/ml) induced ROS generation in dose dependent manner. Similarly, CF-5 (0.1, 0.3, and 1 µg/ml) reduced (P <0.001) LPS-induced ROS production. However, CF-2 alone (3 µg/ml) and CF-5 only 3 µg/ml did not show significant difference when compared with control group (Fig. 18 A-B).

4.10.4 LPS-induced MDA formation in C6 cells

LPS (10 µg /ml) generated (P <0.05) MDA formation (about 180%) in C6 cells compared with non stimulated C6 cells (P < 0.001). All concentrations of CF-2 (0.1, 0.3,1 and 3 µg/ml) significantly reduced (P<0.001) LPS (10µg/ml) induced MDA formation in dose dependent manner (P < 0.001). In addition, CF-5 dose dependently (0.1, 0.3, and 1 µg/ml) decreased (P <0.001) LPS (10µg/ml) induced MDA formation (P < 0.001) in C6 cells. However, CF-2 and CF-5 alone did not produce any significant effect when compared with control (non stimulated cells) (Figure 19 A-B).



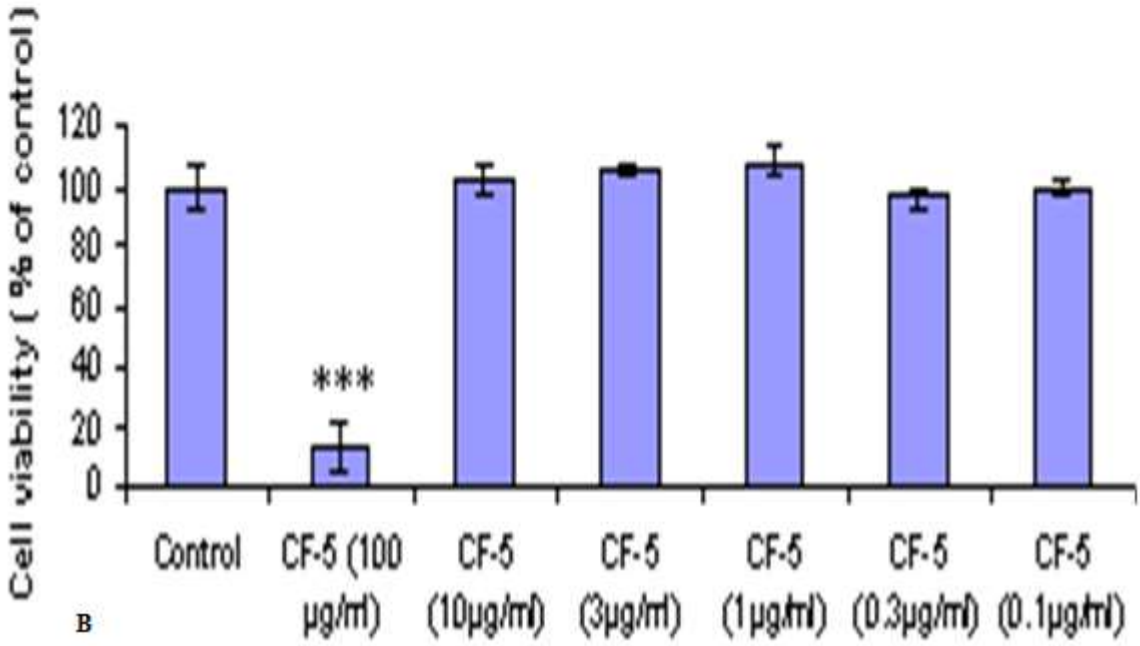


Fig. 16A-B. The Effect of (A) CF-2, (B) CF-5 on cell viability of C6 cells. Values are expressed as mean ± S.E.M. of 3 independent experiments (n=3). *Significant decrease $^{***}P < 0.001$ in comparison to vehicle treated control group. Level of significance analyzed by one way ANOVA followed by Tukeys *post hoc* multiple comparison tests.

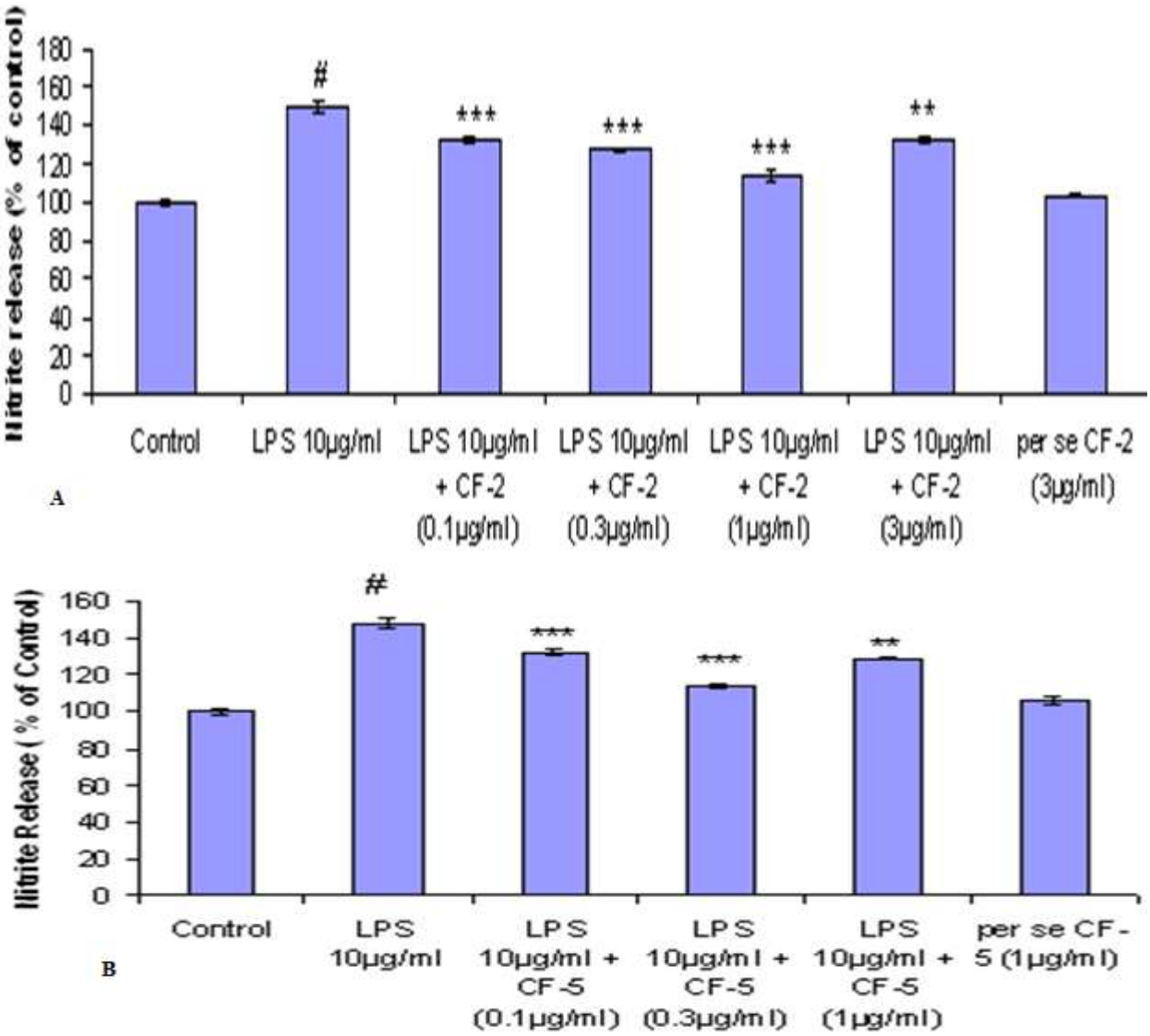


Fig 17A-B. Effect of CF-2 and CF-5 on LPS induced nitrite release by C6 glioma cells. Values are expressed as mean ± S.E.M. of 3 independent experiments (n=3). $^{\#}$Significant increase $^{\#}P < 0.001$ versus vehicle treated control group; *Significant decrease $^{**}P<0.01$; $^{***}P < 0.001$ versus LPS treated only group. Level of significance analyzed by one way ANOVA followed by Tukeys *post hoc* multiple comparison tests.

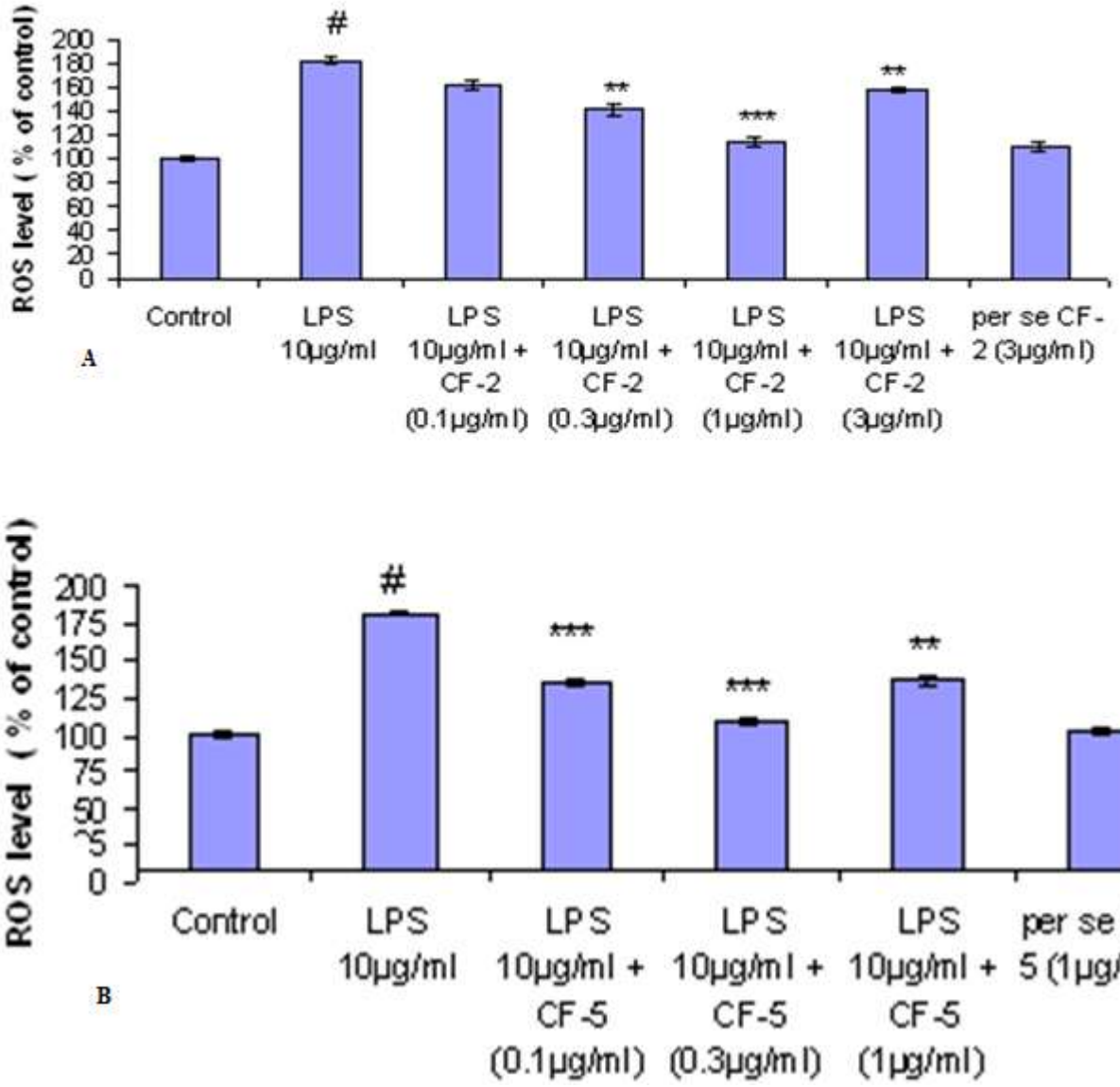


Fig 18 A-B: The antioxidant effect of CF-2 and CF-5 on LPS- induced ROS formation in C6 glioma cells. Values are expressed as mean ± S.E.M. of three independent experiments (n = 3). $^{\#}$Significant increase $^{\#}P< 0.05$ when compared to vehicle treated control; *Significant decrease $^{**}P< 0.01$, $^{***}P<0.001$ in comparison to LPS treated group. Level of significance analyzed by one way ANOVA followed by Tukeys *post hoc* multiple comparison tests.

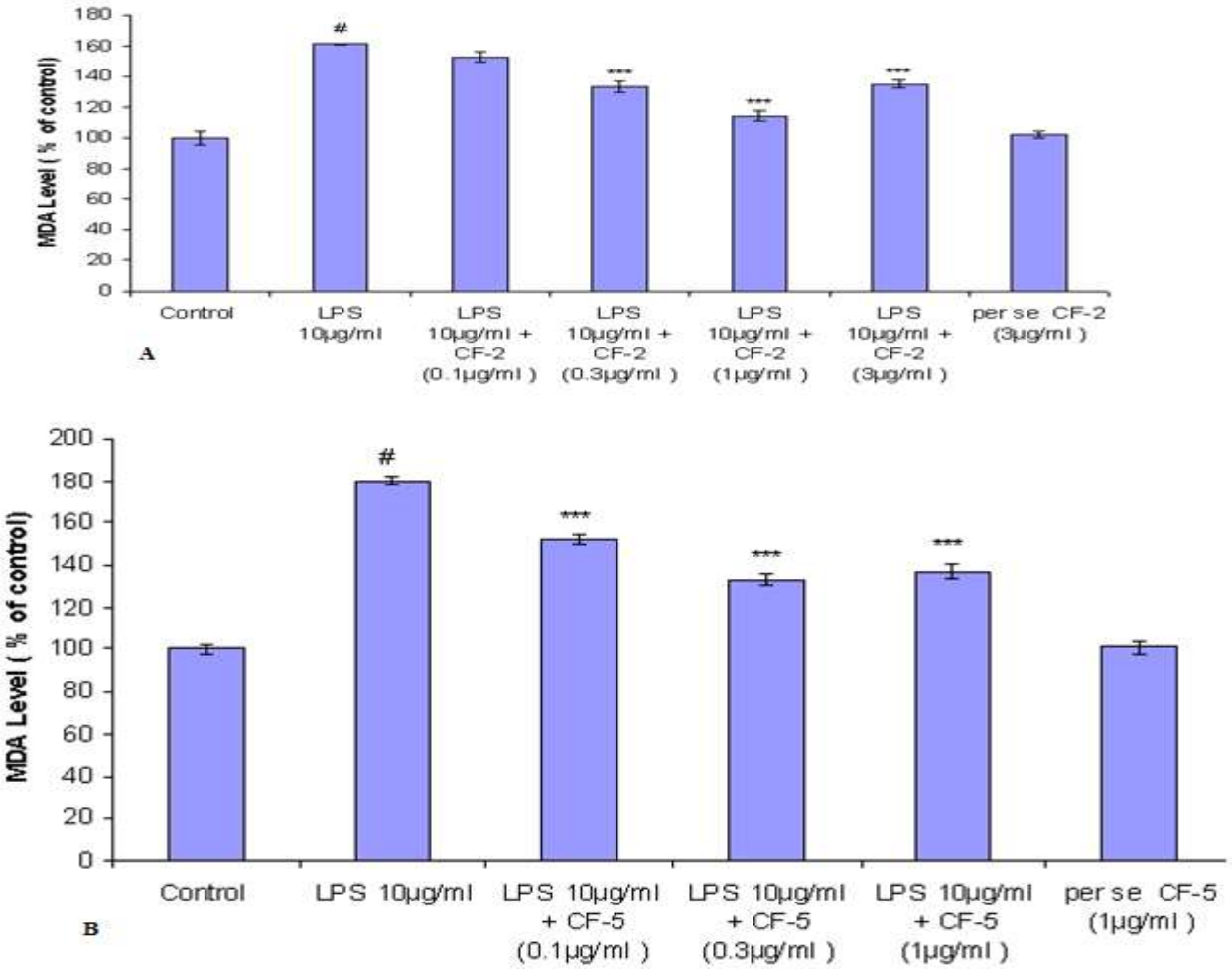


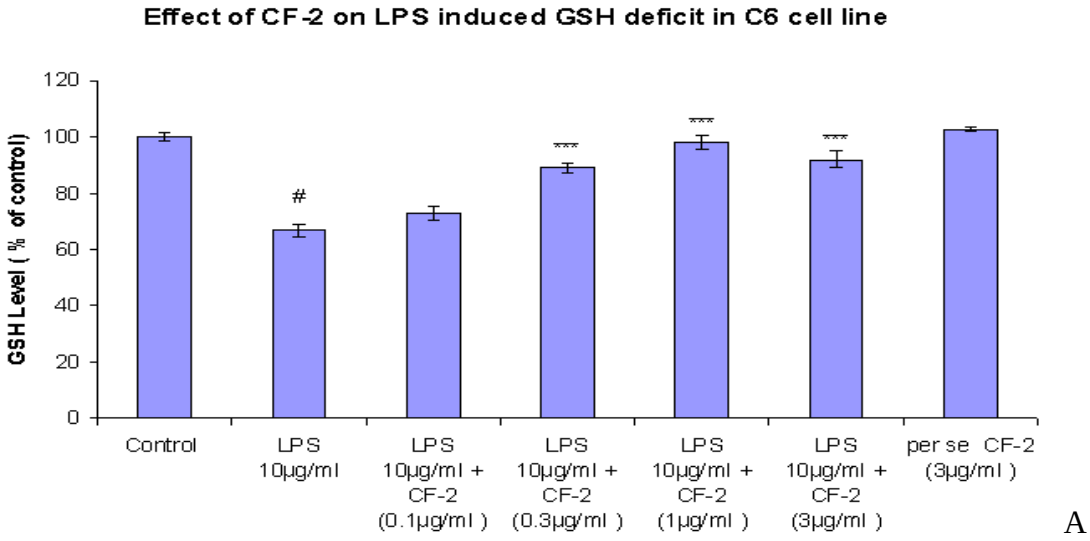
Figure 19 A-B: The antioxidant effect of CF-2 and CF-5 on LPS induced MDA formation in C6 glioma cells. Values are expressed as mean ± S.E.M. of three independent experiments (n = 3). $^{\#}$Significant increase $^{\#}P< 0.05$ when compared to vehicle treated control; *Significant decrease $^{***}P <0.001$ in comparison to LPS treated group. Level of significance analyzed by one way ANOVA followed by Tukeys *post hoc* multiple comparison tests.

4.10.5 LPS-induced GSH deficit in C6 cells

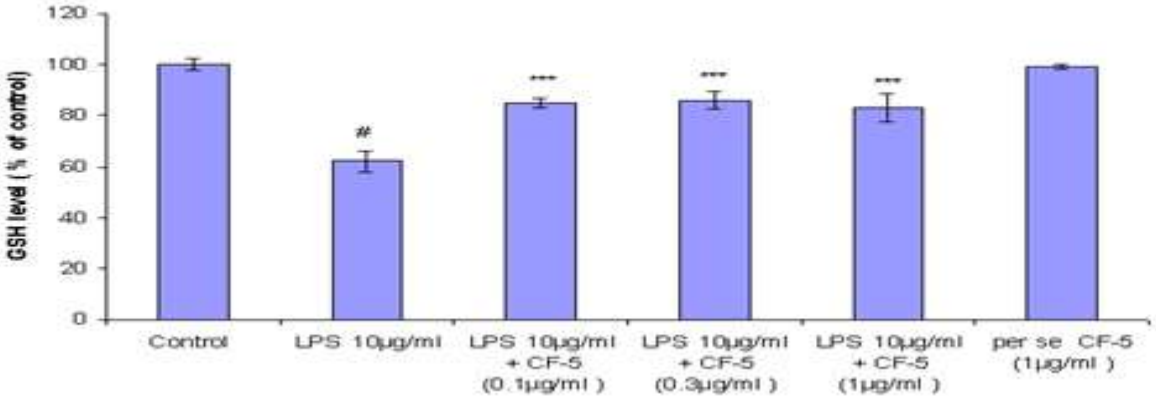
Effect of CF-2 and CF-5 on LPS induced (10µg/ml) intracellular reduced glutathione as an indirect measure of oxidative stress at 24 h was also assessed. To accomplish this C6 cells were treated with LPS (10µg/ml) alone, LPS plus CF-2 (0.1, 0.3,1 and 3 µg/ml), LPS plus CF-5 (0.1, 0.3,1 and 3 µg/ml) and CF-2 3 µg/ml alone. LPS (10 µg/ml) stimulation significantly decreased GSH level (about 50%) at 24 h in C6 cells ($P < 0.001$) (Figure 20 A and B).

4.10.6 LPS-induced TNF-α release in THP-1 cells

LPS significantly increased TNF-α expression in THP-1 cells (Fig. 21) depicting pathophysiological state of atherosclerosis. CF- 2 significantly attenuated LPS induced increased expression of TNF- α in THP-1 cells in a concentration dependent manner with peak inhibitory effect at 100 µg/ml with 27% inhibition in comparison to 54% inhibition by the standard reference drug pentoxifylline.



A



B

Fig 20A-B: The antioxidant effect of CF-2 and CF-5 on LPS induced GSH deficit in C6 glioma cells. Values are expressed as mean ± S.E.M. of three independent experiments (n = 3). $^{\#}$Significant decrease $^{\#}P < 0.05$ when compared to vehicle treated control; *Significant increase $^{***}P < 0.001$ in comparison to LPS treated group. Level of significance analyzed by one way ANOVA followed by Tukeys *post hoc* multiple comparison tests.

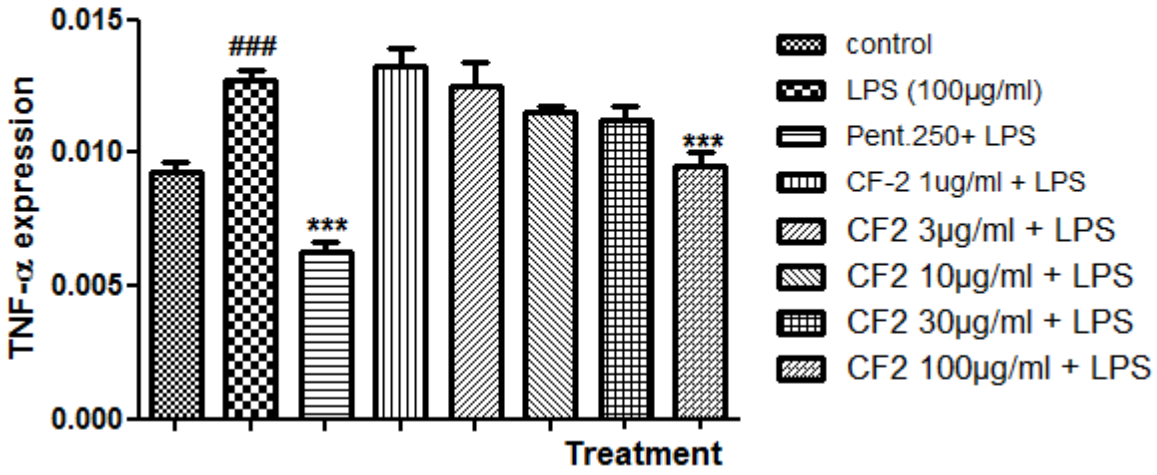


Fig. 21. The effect of CF-2 and Pentoxifylline on LPS induced TNF-α expression in THP-1 cells. $^{###}P$ <0.001 significant in comparison to control; $^{***}P$ <0.001 significant in comparison to LPS group. Values are expressed as Mean ± S.E.M of each group. Level of significance analyzed by one way ANOVA followed by Tukeys *post hoc* multiple comparison tests.

CHAPTER 4

DISCUSSION

5.0 DISCUSSION

5.1 Toxicological evaluation

5.1.1 Acute toxicity test

The methanolic root extract of *C. ferruginea* administered orally caused no mortality at 1 and 2 g/kg but mortality was 20, 60, and 100% respectively, at doses of 4, 8, and 10 g/kg. The LD_{50} was estimated to be 5.22 g/kg. Mice exhibited dose-dependent calmness, hypoactivity, diarrhoea, and increased breath frequency. In accordance with the assertion of Hayes (1989) that no dose-related toxicity should be considered above 5 g/kg body weight, the methanol root extract of *C. ferruginea* can be considered safe when administered acutely via the oral route. The LD_{50} for intraperitoneally administered *Cnestis ferruginea* was estimated to be 643.65 mg/kg, no visible sign of delayed toxicity was observed within 14 days in surviving mice.

5.1.2 Sub-chronic toxicity test

Cnestis ferruginea methanolic root extract was investigated in this study for possible sub-chronic toxicity effects using a 90 day administration schedule at determined sub-therapeutic (80 mg/kg), therapeutic (400 mg/kg), and supra-therapeutic (1000 mg/kg) doses. Administered for 90 days at the therapeutic dose, the methanol root extract of *C. ferruginea* did not produce any significant effect on the weight of rats over the entire duration of administration. According to Raza *et al.,* (2002) and Tan *et al.,* (2008), reductions in body weight gain and internal organ weights are simple and sensitive indices of toxicity after exposure to toxic substances. In general terms, there was also no significant change in the weight of vital organs except in respect of the liver (decrease) and ovary (increase) in female rats. These changes were reversed after cessation of therapy. *In vivo,* Antioxidants and MDA assays showed reductions in liver SOD and CAT but no change in MDA levels in female rats. Mammalian cells contain endogenous antioxidant enzymes, including SOD, glutathione peroxidase and catalase which are able to detoxify free radicals by converting them to more stable molecules within the cell (Saeed *et al.,* 2005). Malondialdehyde, a major breakdown product of lipid peroxides, is an index of lipid peroxidation. The overwhelming of antioxidant enzymes by free radicals results in the depletion of the antioxidant defences and induction of lipid peroxidation evident in elevation of MDA level (Akindele *et al.,* 2010). In respect of the ovary, *C. ferruginea* produced significant increase in GSH and CAT but also no change in

MDA level. Histological assessment of the ovary and liver of female animals showed normal presentations. *C. ferruginea* at the therapeutic dose did not elicit any significant change in biochemical and sperm parameters assayed. In respect of haematological parameters, *C. ferruginea* did not generally produce any significant effect except in respect of red blood cell distribution width (RDW) which was increased in male rats. Red blood cell distribution width is a measure of variation of red blood cell (RBC) width and it is often used with mean corpuscular volume (MCV) to determine the cause of anaemia. A low MCV in combination with low RDW may denote iron deficiency but in this case the MCV value was not significantly different from control. Also the observed effect on RDW was reversed on cessation of treatment. An observation that was made in the reversibility study is the reduction in mean platelet volume (MPV) value for male rats and increase in female rats. These may suggest delayed effects. Mean platelet volume is a measure of the average size of platelets. The MPV value gives an idea of platelet production in the bone marrow and it is interpreted with the platelet count. Extremely low MPV value is associated with thrombocytopenia as is the case with aplastic anaemia and high MPV value correlates with increased peripheral destruction observed in conditions such as immune thrombocytopenic purpura and myeloproliferative diseases.

However, in this study, the delayed effect observed on MPV was not accompanied by a change in platelet count. Findings in this study suggest that the methanolic root extract of *C. ferruginea* administered at the therapeutic dose is relatively safe given over an extended period with the potential to enhance antioxidant activity in the ovary. However, results showed that *C. ferruginea* had a tendency to produce delayed platelet anomaly at this dose.

Considering the sub-therapeutic dose, the methanolic root extract of *C. ferruginea* did not produce any significant effect on the body weight of male rats all through the 90 day treatment period. However, in respect of female rats, there was significant increase in body weight from the 7^{th} to the 42^{nd} day only. In respect of the mean change in weight over the 90 day period, there was no significant change in the body weight of this set of animals compared to control. For the weight of vital organs, there were generally no significant changes except in the case of the brain (increase) and liver (decrease) for female rats. The possible concern from this observation is addressed by the fact that there was no significant change in the level of

antioxidant enzymes and MDA in respect of the brain, ruling out the involvement of lipid peroxidation, while the liver presented with concurrent reduction in SOD level but no change in MDA. In respect of histological assessment, the presentation of the female rat liver was normal while that of the male showed congestion. There was a significant increase in the MDA level in the testes of male rats while there was a significant increase in SOD and CAT levels, without a change in MDA, in the ovary of female animals. However, there was no significant change in the weight of these organs and histopathological assessment showed normal presentations. Assessment of haematological parameters showed no deleterious effect in the 90 day study but reversibility study results showed a reduction in haemoglobin (Hb) and PCV in female rats. These findings possibly suggest induction of anaemia as a delayed effect in female rats. Biochemical and sperm parameters were not significantly affected in the main study at the sub-therapeutic dose of 80 mg/kg. However, reduction in sperm count was observed in the reversibility study. This may be a delayed effect as a consequence of significant lipid peroxidation (increased MDA) observed in the testes. Presentations in the histological report give credence to this assertion. There was calcification showing as areas of necrosis and deposition of calcium salts and amorphous debris within some seminiferous tubules, with loss of lining germ cell series as well. This calls for caution in the use of the root extract of *C. ferruginea* as an aphrodisiac in traditional medicine, as reported by Burkill (1985). Findings in this study suggest that the methanolic root extract of *C. ferruginea* administered at the sub-therapeutic dose is relatively safe administered over an extended period but with the potential to cause delayed anaemia in females and infertility in males. *C. ferruginea* in this study also showed a tendency to enhance antioxidant activity in the ovary.

In respect of the supra-therapeutic dose of the methanol root extract of *C. ferruginea* administered for 90 days, there were significant reductions in weight change in male rats from the 21st to the 90th day and in the overall mean change in weight. In female rats, there were no significant reductions in weight during the period of treatment and in the overall mean change in weight. For vital organs, the weight of the testes in male and liver in female animals were significantly reduced, reversibly in respect of the later. *C. ferruginea* administered at the supra-therapeutic dose increased liver and ovary GSH levels in both sexes with no change in MDA. However in the testes, the GSH level was reduced but also with no change in MDA. Histopathology results showed normal female rat liver and ovary. The male rat liver revealed intracellular accumulation/fatty change while the testes showed inflammation/necrosis (dead

cells and amorphous debris seen with aggregation of inflammatory cells). The finding in the testes is corroborated by the observed reduction in sperm count. As observed at the therapeutic dose, the supra-therapeutic dose also caused significant reduction in RDW in male rats, but there was no significant change in the MCV. For the biochemical parameters, there was a significant increase in total bilirubin in female rats. The destruction of haemoglobin leads to the generation of bilirubin which is conjugated in the liver and excreted in bile. Any overload or blockage of this system results in the elevation of the level of bilirubin which is indicative of possible liver disease or anaemia (Carter, 1989). Navarro and Senior (2006) categorized the patterns of liver injury as hepatocellular (elevated ALT), cholestatic (elevated ALP and total bilirubin) and mixed (elevated ALP and ALT). However, results obtained in this study rule out cholestatic liver injury as there was no significant change in the level of ALP and also excludes anaemia as there was no significant change in Hb, PCV, and other relevant haematological parameters. An important observation is the reversal of the elevated total bilirubin level in female rats upon cessation of treatment with *C. ferruginea*. Findings in this study suggest that the methanol root extract of *C. ferruginea* administered at the supra-therapeutic dose is also relatively safe given over an extended period with a capacity to enhance antioxidant activity in the liver and ovary. However, *C. ferruginea* exhibited a potential to cause weight reduction and sterility in males at this dose. The tendency of the extract to cause sterility calls for caution in its use as aphrodisiac in traditional medicine especially in view of inherent issues concerning dosage in this system of healthcare.

The differences observed in this study in the effects of *C. ferruginea* on body weight changes, organ weight and liver toxicity in male and female rats may be due to sex-related differences in body composition, hormones (Nies and Spielberg, 1995) and pharmacokinetic and pharmacodynamic properties (Wang and Huang, 2007). Mortalities recorded in the course of this study may be a pointer to sub-chronic toxicity. However, in view of the manifestation of dyspnoea by majority of the animals just before death, the mortalities are more likely to be administration related. The absence of adverse behavioural and morphological changes coupled with the results obtained with the parameters evaluated in surviving rats give credence to this assertion.

5.2 Analgesic activity

The intraperitoneal injection of acetic acid elicited writhing (a syndrome characterized by a wave of abdominal musculature contraction followed by extension of the hind limbs). The dose-dependent inhibition of writhings induced by acetic acid in this study by *Cnestis ferruginea* suggest a peripherally mediated analgesic activity based on the association of the model with stimulation of peripheral receptors especially the local peritoneal receptors at the surface of cells lining the peritoneal cavity (Bentley *et al.,* 1983; Zakaria *et al.,* 2008).

The fact that the greatest number of writhes was produced at the 10–15 min time interval provides a basis for the modification of the acetic acid-induced writhing test such that the number of writhes is counted only for 5 min within the mentioned interval. This modification will enhance the rapidity associated with the model. In order to confirm the peripheral mechanism of action and establish any possible central involvement, other models of analgesic activity evaluation were used.

The formalin test, a method commonly used to study the anti- inflammatory, anti-nociceptive properties of drugs (Hunskaar *et al.,* 1985), has been reported to produce distinct biphasic nociceptive response (Zakaria *et al.,* 2008). The early phase (0–5 min) has been associated with direct effect of formalin on nociceptors while the late phase (15–30 min) is said to involve inflammatory processes (Tjolsen *et al.,* 1992). According to Chan *et al.,* (1995), centrally acting drugs (e.g. morphine) inhibit both phases of the formalin test while peripherally acting drugs (e.g. ASA) inhibit the late phase only. From the results obtained in the formalin test, *Cnestis ferruginea* inhibited both phases thus confirming a peripheral mechanism of action while also suggesting the involvement of a central mechanism of analgesic effect.

The fact that the effect of *Cnestis ferruginea* in the second phase was greater than that produced in the first phase suggests greater involvement of peripheral mechanisms in its anti-nociceptive action. Another deduction from the results obtained in the formalin test is that the extract of *Cnestis ferruginea* is effective against nociceptive and inflammatory pains with a more pronounced effect on the later.

To confirm the involvement of central mechanism(s) in the analgesic activity of *Cnestis ferruginea*, the tail clip and hot plate tests were used based on the fact that centrally acting analgesic drugs elevate the pain threshold of rodents towards pressure and heat (Singh and Majumdar, 1995). The hot plate test involves the spinal reflex (Pini *et al.*, 1997) and measures the complex response to a non-inflammatory, acute nociceptive input (Zakaria *et al.,* 2008). Based on the effectiveness of *Cnestis ferruginea* in the tail clip and hot plate tests, a central mechanism of action is confirmed for its observed anti-nociceptive effect.

The results revealed that naloxone was able to significantly attenuate the antinociceptive activity of *C. ferruginea*. This observation confirms the earlier reports and conclusively suggests a role for opioid mechanism in the antinociceptive action of *C. ferruginea.* A role for alpha adrenergic system in opioid action was suggested by Ramaswamy *et al.*, (1981) from the observation that, clonidine treatment could effectively antagonize the development of acute and chronic tolerance to morphine analgesia. The possible role of alpha-1&2 adrenergic system in the antinociceptive action of *C. ferruginea* showed that the pretreatment with prazosin and yohimbine, an alpha-1&2 adrenergic receptor antagonist failed to modify the antinociceptive effect of *C. ferruginea*. This observation suggests that alpha-1&2 adrenergic system may not be involved in the antinociceptive effect of investigated *C. ferruginea*.

Serotonin is an important neurotransmitter in modulating the nociceptive response at many stages in the pain pathway. The descending serotonergic pathways may directly modulate the activity of projection neurons and also via interneuron (Alchaider, 1991). Among the various subtypes of serotonin receptors, $5HT_{1A}$, $5HT_2$, and $5HT_3$ receptors are considered to play a major role in nociceptive modulation (Bardin *et al.,* 2000, Sasaki *et al.,* 2001, Millan, 2002).

In this study ondansetron was employed to investigate the role of serotonergic system in the antinociceptive action of *C. ferruginea*. Pretreatment with ondansetron significantly reversed the antinociceptive effect of *C. ferruginea* revealing that the serotonergic system (especially $5HT_3$ receptor mechanism) is involved in the action of *C. ferruginea*.

An interaction between dopaminergic, adrenergic and opioid systems has been suggested in the modulation of pain perception (Akil and Liebeskind, 1975, Pollard *et al.,* 1978). Dopamine has been suggested to play an important role in the modulation of nociceptive information by basal ganglia (Chudler and Dong, 1995). In this study haloperidol (D_1/D_2 receptor antagonist)

pretreatment failed to alter the antinociceptive effect of *C. ferruginea*. This observation rules out the role of dopaminergic system in the antinociceptive action of *C. ferruginea*.

A role for ATP sensitive potassium channels in opioid induced antinociception has been documented (Ocana *et al.,* 1990; Wild *et al.,* 1991). Various drugs acting on μ opioid receptor (Rodrigues and Duarte, 2000), δ opioid receptor, (Duarte and Pacheco, 2005) as well as an anti-inflammatory analgesic like diclofenac (Alves *et al.,* 2004) and resveratrol (Granados-Soto *et al.,* 2002) have been shown to interact with ATP sensitive potassium channels in mediating their antinociceptive action. The antinociceptive effect of *C. ferruginea* was attenuated by glibenclamide, thus establishing a role for ATP sensitive potassium channels in this action (Venkataramanan *et al.,* 2000).

5.3 Anti-inflammatory activity

The anti-inflammatory activity of *Cnestis ferruginea* was evaluated in this study using the carrageenan, egg albumin, xylene, and formaldehyde-induced oedema tests. Carrageenan-induced inflammation consists of three distinct phases including an initial release of histamine and serotonin; a second phase mediated by kinins; and a third phase involving prostaglandins (Di Rosa *et al.,* 1971; Okpo *et al.,* 2001). On the other hand, egg albumin-induced oedema results from the release of histamine and serotonin (Pearce, 1986; Akindele and Adeyemi, 2007). In this study, *Cnestis ferruginea* showed significant inhibitory effect on rat paw oedema development in the middle phase and more pronouncedly in the later phase of carrageenan and egg albumin-induced inflammation. This suggests that the extract possibly acts by inhibiting the release and/or actions of vasoactive substances (histamine, serotonin and kinins) and prostaglandins.

Oedema induced by histamine and serotonin were significantly attenuated by *Cnestis ferruginea* which is a pointer to the interaction between *Cnestis ferruginea* and these inflammatory mediators. This further confirmed the ability of the extract to significantly inhibit early phase of carrageenan-induced paw oedema.

The ear oedema test is useful for the evaluation of anti-inflammatory steroids and is said to be less sensitive to non-steroidal anti-inflammatory agents (Zaninir *et al.,* 1992; Akindele and Adeyemi, 2007). The model is thus linked to the activity of phospholipase A_2, PLA_2 (Nunez Guillen *et al.,* 1997). The effectiveness of *Cnestis ferruginea* in the xylene-induced oedema

test is therefore suggestive of the possible involvement of PLA_2 inhibition in its mechanism of anti-inflammatory action.

In the formaldehyde-induced arthritis inflammation test, which involves the proliferative phase of inflammatory reaction (Gupta *et al.,* 1971), *Cnestis ferruginea* showed significant inhibitory effect on oedema development thus suggesting potential usefulness in chronic inflammation. However, the effect of the extract in this model was not as pronounced as in the other models of inflammation used. The deaths recorded in the indomethacin group in the formaldehyde model may be linked to the side-effects associated with non-steroidal anti-inflammatory drugs (NSAIDs), especially gastric erosion/ulceration, gastrointestinal haemorrhage and inhibition of platelet activation resulting in propensity for bruising and increased risk of haemorrhage (Burke *et al.,* 2006).

5.4 Bioactivity guided isolation of analgesic and anti-inflammatory constituents

Due to the promise shown by *C.ferruginea* in acute inflammation and nociception, further study was carried out to isolate and characterize the active constituent(s) responsible for the observed effects. From the fractionated methanolic root extract of *C.ferruginea,* fraction 4 was found to possess the main analgesic and anti-inflammatory activities. This was shown to contain an amino acid like compound and amentoflavone, isolated for the first time in *C. ferruginea.* The aqueous and butanol fractions were shown to possess anti-nociceptive activity against acetic acid induced pain at 100 mg/kg, *p.o.*. Similarly, pre-treatment with fraction 4 reduced the number of writhes in mice significantly. Amentoflavone and the amino acid like compound produced significant reduction in the number of writhes.

The aqueous-butanol fractions and fraction 4 produced marked and significant increase in pain latency time response in hot plate test which was similar to the effect of morphine. CF-2 and CF-5 (25 mg/kg) produced significant increase in pain latency confirming the probable involvement of central and peripheral analgesic mechanism.

The aqueous fraction effectively inhibited both phases of carrageenan induced inflammation while the effect of the butanol fraction was most pronounced at the later stages of inflammatory responses, which corresponds to the phase of prostaglandin release. Most reports suggest that NSAID preferentially inhibit the `late' phase response presumably by inhibiting

the inducible COX-2 isoform which is believed to be responsible for the generation of pro-inflammatory prostanoids in the later stages of this and other inflammatory models (Vane and Botting, 1998).

It seems likely that the `late' phase inhibitory effect of aqueous-butanol fraction and fraction 4 depends upon inhibition of prostanoid formation presumably by an effect on COX-2 activity, which is likely to be the predominant isoform at this stage in the response (Seibert *et al.,* 1994). Oral administration of aqueous-butanol fraction and fraction 4 also reduced the `early' phase response. Although some reports suggest that COX-2 may be present as early as 60-90 min after induction of an inflammatory response (Seibert *et al.,* 1994), the possibility that aqueous-butanol fraction and fraction 4 may additionally reduce constitutive COX-1 activity to bring about an anti-inflammatory effect in the `early' phase should be considered.

Previous studies have shown that amentoflavone (biflavone) potently inhibited the activity of group II secretory phospholipase A_2 (Chang *et al.,* 1994) as well as inhibition of TNF-α induced activation of NF-kB (Banerjee *et al.,* 2002). Amentoflavone has also been shown to inhibit cyclooxygenase in guinea pig epidermis without affecting lipoxygenase (Kim *et al.,* 1998). In addition, Amella *et al.,* (1985) have reported the effectiveness of amentoflavone in the inhibition of mast cell histamine release in the micromolar range suggestive of its antiallergic action. One important anti-inflammatory mechanism of biflavonoids is transcription regulation of pro-inflammatory molecules. They suppress inducible nitric oxide synthase (iNOS) and COX-2 expression in LPS- treated RAW 2647 cell (Baek *et al.,* 1999). The observed anti-inflammatory and analgesic activities of the extracts can be attributed to the presence of amentoflavone and the amino acid like compound.

5.5 Anticonvulsant activity

The methanolic root extract of *C.ferruginea* contains at least one component that antagonizes chemically and electrically induced seizures in mice. The methanolic root extract of *C.ferruginea* significantly protected mice against bicuculline (BIC), Yohimbine (YHB), strychnine (STR), isoniazid (INH), and picrotoxin (PIC)-induced seizures. In non-protected mice, the methanolic root extract of *C.ferruginea* significantly delayed the onset of seizures

induced by MES, STR and INH. The methanolic root extract of *C.ferruginea* had very significant antiepileptic effect on BIC-induced seizures and modest effect on PIC-induced seizures. INH and yohimbine has been shown to interact with the GABA neurotransmitter and the GABA receptor complex (Dun and Fielding, 1987; De Deyn *et al.,* 1992), antagonism of yohimbine and INH-induced seizures suggests that the extract of *C.ferruginea* might have effects on GABAergic neurotransmission.

Moreover, these effects seem to be related to the GABA or PIC sites of the GABA receptor complex (De Deyn *et al.,* 1992) since BIC- and PIC-induced seizures were significantly antagonized. The moderate antagonism of the methanolic root extract of *C.ferruginea* in STR-induced seizures suggests that additional mechanisms might be involved. The multiplicity of putative mechanisms of action and the broad spectrum of anticonvulsant activity of the methanolic root extract of *C.ferruginea* might be due to the presence of different active components in the methanolic extract interacting simultaneously. These mechanisms and their contribution to the anticonvulsant properties of the extract will be better understood once the active components in the methanolic root extract of *C.ferruginea* are identified.

5.6 Antidepressant activity

The behavioural despair models such as the forced swim test (FST) and tail suspension test (TST) are widely used to screen new antidepressant drugs (Porsolt *et al.,* 1979; Steru *et al.,* 1985). This study shows that amentoflavone, CF-5 (an amino acid) and methanolic root extract of *Cnestis ferruginea* possess antidepressant activity in the FST and TST models.

This study showed a more consistent antidepressant-like activity. *Cnestis ferruginea,* starting from 12.5 mg/kg reduced immobility time and simultaneously enhanced swimming behaviour in mice in the forced swim test. Reduction of immobility in mice was comparable to that observed after the acute administration of the reference antidepressant drug imipramine. In contrast to the original protocol (Porsolt *et al.,* 1978) that involved triple administration of the compound prior to the forced swim test, in this study, a single oral administration of imipramine (20 mg/·kg) was sufficient, to decrease immobility (Belozertseva *et al.,* 2007).

The observation of detailed behaviours, such as swimming and in addition to the traditional immobility measure, showed that imipramine increased swimming behaviour, suggesting an involvement of noradrenergic neurotransmission (Detke *et al.,* 1995). Similarly, the effect of oral administration of amentoflavone and CF-5 in FST, starting from 6.25 mg/kg was more

pronounced and potent in reducing duration of immobility when compared to the tricyclic antidepressant (imipramine 20 mg/kg). Amentoflavone significantly shortened immobility time in a dose range of 6.25- 50 mg/kg.

This was further confirmed in the tail suspension test, using some of the effective doses of 100 – 200 mg/kg and 25-50 mg/kg of CF, CF-5 and CF-2 respectively, obtained from forced swimming test. Moreover, CF, CF-5 and CF-2 significantly shortened duration of immobility, this effect was comparable to that observed after the acute administration of selective serotonin reuptake inhibitor (fluoxetine 20 mg/kg).

In a separate series of experiments, the involvement of serotonin, noradrenaline, dopamine, and muscarinic cholinergic receptor subtypes in the antidepressant-like activity of *C.ferruginea* and amentoflavone in the FST was studied. To this end, mice were co-treated in turn with the non-selective 5-HT receptor antagonists (metergoline and cyproheptadine), the prefential α_1- adrenoceptor antagonist (prazosin) and α_2- adrenoceptor antagonist (yohimbine), reserpine (a drug known to induce depletion of biogenic amines), dopamine D_1 antagonist (sulpiride), L-N^G-Nitroarginine (nitric oxide synthase inhibitor) and muscarinic acetylcholine receptor antagonist (atropine).

Several lines of evidence indicate that serotonergic, dopaminergic, noradrenergic and cholinergic neurotransmissions are involved in the expression of an antidepressant-like effect in the behavioural despair models of depression (Elhwuegi, 2004). In the mouse forced swimming test, it has been reported that acute treatment with antagonists of dopamine D_2/D_3 receptors (Ferrari and Giuliani, 1997) and α_1-adrenoceptors (Stone and Quartermain, 1999) significantly increase the duration of immobility. These observations indicate the possibility that the function of dopamine D_2/D_3 receptors and α_1-adrenoceptors might be needed for the antidepressant-like effect. Furthermore, Mayorga *et al.,* (2001) reported that the antidepressant-like effects of SSRIs were not exerted in 5-HT_{1A} receptor mutant mice. It has also been reported that the antidepressant-like effect of fluoxetine was antagonized by pretreatment with either a selective 5-HT_{1A} or 5-HT_2 receptor antagonist (Miyata *et al.*, 2004).

Implication of the cholinergic system in the aetiology of major depression, although not as widely accepted as the monoamine hypothesis, was postulated several decades ago. It was suggested that central cholinergic activation caused depressant inhibitory effects, while

anticholinergic drugs or adrenergic stimulation induced behavioural activation and arousal (Dagyte *et al.*, 2010). In the present study, the pretreatment of animals with 5-HT_2 receptor antagonist metergoline, but not cyproheptadine was able to reverse the anti-immobility effect of *C.ferruginea* and amentoflavone in the FST. Therefore, the involvement of 5-HT_2 serotonergic receptors in the antidepressant-like effect. Similarly, reserpine (a presynaptic monoamine depleting agent) significantly attenuated the antidepressant activity of *C.ferruginea.*

Dysfunctions in the noradrenergic system are implicated in depression. In fact, some antidepressants such as mazindol and reboxetine act by increasing the synaptic levels of NA (Scates and Doraiswamy, 2000; Nikiforuk *et al.,* 2010). The actions of NA are carried out via the binding to adrenergic receptors. It has been shown that the anti-immobility effect of desipramine was antagonized by prazosin blocking α_1-adrenoceptor (Danysz *et al.*, 1986).
In this study, prazosin (an α_1-adrenoceptor antagonist), and yohimbine (an α_2-adrenoceptor antagonist) were able to reverse the antidepressant-like effect of amentoflavone in the FST. This result indicates that amentoflavone may exert its effect by interacting with α_1-, and α_2-adrenoceptors as well as 5-HT_2 receptor.

The dopaminergic system is also an important target implicated in the regulation of depression (Klimek et al., 2002). DA has many functions in the brain, including important roles in behaviour and cognition, punishment and reward, sleep, mood, attention and learning (Arias-Carrion and P˘oppel, 2007; Dunlop and Nemeroff, 2007).

In this study, the pretreatment of mice with D_2 receptor antagonist sulpiride could not reversed the anti-immobility effect of *C.ferruginea* and amentoflavone in the FST. Similarly, pretreatment of mice with muscarinic cholinergic antagonist atropine was also ineffective in reversing the antidepressant-like effect of *C.ferruginea* and amentoflavone. In the L-NNA pretreated animal, the anti-immobility effect of *C.ferruginea* was significantly reversed. NO is a regulator of both short- and long-term neuronal adaptive changes and consequently, may play a role in neuronal adaptation to antidepressant drugs. Targets of NO include guanylate cyclase, G proteins, amino acid, amine and neuropeptide release and transport. NO mediated cGMP synthesis also mediates induction of immediate early gene expression which is implicated in long-term synaptic changes and more recently in the mechanism of action of antidepressant drugs (Harvey, 1996; Duman *et al*., 1997).

5.7 Anxiolytic activity

Administration of graded doses of CF, CF-5 and CF-2 in mice were able to induce anxiolytic effects, without significantly modifying the spontaneous motor activity when compared to control. The hole-board, light-dark and elevated plus maze tests were used to access the anxiolytic potentials of CF, CF-5 and CF-2.

The hole-board is a simple way to measure exploratory activity (Durcan and Lister, 1989). In the present study, diazepam at a dose of 1.5 mg/kg caused a significant increase in the number of head-dips. CF extract (25- 100 mg/kg) significantly increased the number of head-dips signifying an anxiolytic-like effect of the extract. However, CF (200 mg/kg) significantly reduced number of head-dips.

It has been reported that anxiolytic possess a biphasic profile, showing a facilitation of exploratory behaviour at low doses and an inhibition at high doses (Treit, 1984). The behaviour's similarity to diazepam once more strengthens the hypothesis of an anxiolytic-like property of *C.ferruginea*. Similarly, amentoflavone and CF-5 (25-50 mg/kg) significantly increased number of head-dips which was comparable to diazepam suggesting an anxiolytic effect. Moreover, Amentoflavone has been shown to bind to brain benzodiazepine receptors with an affinity comparable to diazepam (Nielsen *et al.*, 1988; Baureithel *et al.*, 1997). It also had a remarkable affinity for the δ-opioid receptor subtype (K_i= 36.5 nM). In addition, amentoflavone significantly inhibited binding at 5- HT_{1D}, 5-HT_{2C}, and dopamine D_3- receptors (Butterweck *et al.*, 2002).

To further evaluate the anxiolytic property of CF, CF-5 and CF-2, the elevated plus maze test was carried out. The elevated plus maze is considered to be an etiologically valid animal model of anxiety because it uses natural stimuli (fear of a novel open space and fear of balancing on a relatively narrow, raised platform) that can induce anxiety in humans (Dawson and Tricklebank, 1995). An anxiolytic agent increases the frequency of entries into the open arms and increases the time spent in open arms of the EPM. In the present study, oral administration of *Cnestis ferruginea* induced an anxiolytic-like effect in mice, since it increased the number of entries and the time spent on open arms and decreased the time spent in closed arms in the EPM test. In agreement with previously published reports, diazepam increased the duration of time spent on open arms and the number of entries on open arms

(Eguchi *et al.,* 2001; Helton *et al.,* 1996; Moser, 1989) without altering locomotor activities as indicated by the open field test monitor by digiscan activity meter.

Cnestis ferruginea (25-100 mg/kg) significantly increased time spent in open arms of the elevated plus maze but no statistical difference in the number of entries (result not shown). The anxiolytic effect peaked at 25 mg/kg which was comparable to diazepam. However, CF extract (200 mg/kg) produced non-significant increase in time spent in open arms. We have no adequate explanation for the lack of dose dependence in these assays. Further studies to investigate the effect of *Cnestis ferruginea* at different intervals of time might disclose a dose-dependent effect.

Further evaluation of anxiolytic effect of amentoflavone was shown by the progressive dose-dependent increase of percentage time spent in the open arms of the elevated plus maze. The maximal anxiolytic effect was obtained with a dose of 25 mg/kg and was maintained even by increasing the dosage. The increase in time spent in open arms after amentoflavone treatment was comparable to that seen after treatment with the classical anxiolytic compound, diazepam, but the number of open-arm entries was similar for both drugs, suggesting that amentoflavone possesses a good anxiolytic effect.

In the light-dark test, the peak anxiolytic like effect were observed at 50 and 12.5 mg/kg in CF and CF-2 treated animal, as the time spent by animal in light chamber was more significant when compared to diazepam treated group. This corroborate the suggestion that the time mice spent in the illuminated side of the light-dark chamber is the most useful and consistent parameter of anxiety (Young and Johnson, 1991). It is worth-noting that lack of dose-dependent effect could be explained based on the biological variability and secondary metabolites interaction present in CF extract (Maribel *et al.,* 2006). Previous work with amentoflavone has shown that it acts as a negative modulator of GABA-gated chloride currents at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Hanrahan *et al.,* 2003). Previous studies have shown that amentoflavone selectively binds to $GABA_A$ receptor subtypes containing different α -subunits in a manner resembling diazepam binding (Nielsen *et al.,* 1988). Although amentoflavone was shown to have affinity for $GABA_A$ receptor subtypes containing the α_1 or α_5 subunit, a loss of competitive binding was observed at $GABA_A$ receptor subtypes containing the α_4 or α_6 subunit. Amentoflavone binding at the benzodiazepine site may involve

binding contacts that also play a role in the binding of classical benzodiazepine positive modulators like diazepam (Maribel *et al.*, 2006).

Flumazenil is widely reported in studies in which the purpose was to demonstrate the specific involvement of $GABA_A$ and benzodiazepine receptors in the mechanism of action of substances or of a plant extract with anxiolytic activity (Zhang, 2004). Flumazenil is a $GABA_A$ receptor antagonist in the benzodiazepine binding site that possesses the ability to induce anxiety (File *et al*., 1982). It has been shown that the effect of the administration of 1.5 mg/kg of diazepam is reversed by flumazenil (3 mg/kg, *i.p.*.) (Poleszak, 2008). In this study, anxiolytic effect of *C. ferruginea* and amentoflavone were reversed by intraperitoneal injection of flumazenil through a significant decrease in open arms exploration of the elevated plus maze, indicating an interaction of the amentoflavone with the benzodiazepine receptor.

5.8 Antidementic activity

It is well known that the cholinergic system in the basal forebrain plays an important role in learning and memory (Bartus, 2000). Scopolamine interferes with memory and cognitive function in humans (Riedel *et al.*, 1995) and experimental animals Sharma *et al.*, 2010; Kulkarni *et al.*, 2011) by blocking muscarinic receptors. Therefore, scopolamine is considered as reliable tool to study antiamnesic effects of candidate molecules or extracts. Hence, scopolamine was used in this study to impair memory in passive avoidance and spatial learning in Morris water maze test. In this study *Cnestis ferruginea* (CF) and its constituents (CF-2 and CF-5) were evaluated for memory enhancing effect in scopolamine induced amnesic mice.

Preventive treatment with CF for three days ameliorated scopolamine induced amnesia in mice as indicated by significant decrease in escape latency time (ELT) in Morris water maze test and increase in retention latencies in passive avoidance test. Similarly, 3 days oral administration of amentoflavone (CF-2) or CF-5 also showed antiamnesic effect in passive avoidance and Morris water maze tests. Further, CF and its active constituents caused a significant reduction in pathlength (distance travelled) in Morris water maze test. There was a significant correlation in between mean pathlength and mean ELT of all the groups in all the sessions. This indicates that scopolamine and/or drug administration did not alter the swimming speed or motor performance of the animals. Further, there was no significant difference in locomotors activity among different groups excluding possibility that the

disturbances in the motor function may have contributed in the performance of the animals in behavioural tests. Previous studies have shown the beneficial effect of amentoflavone against cytotoxic insults induced by oxidative stress and amyloid beta, suggesting their therapeutic potential against neurodegenerative diseases (Kang *et al.,* 2005).

Since scopolamine induced memory impairment is associated with cholinergic hypofunction and oxidative stress in the brain and because agents which increase central cholinergic function ameliorates scopolamine induced amnesia (Jones *et al.,* 1991; Jeong *et al.,* 2008; Bartus, 2000), we studied mechanism of antiamnesic effect of CF in the context of cholinergic function and oxidative stress in the brain. Scopolamine caused a significant increase in acetylcholinesterase (AChE) activity in mice brain which was ameliorated by CF in dose dependent manner. Further, both the constituents of CF decreased AChE activity in mice brain. The inhibition of AChE by CF leads to improved acetylcholine (ACh) level in brain which is responsible for its antiamnesic effect in scopolamine model.

Recently, growing lines of evidence have suggested that bioflavonoids have neuroprotective effects due to their strong antioxidant potential (Kang *et al.,* 2005, Tota *et al.,* 2010; Awasthi *et al.,* 2009; Tota *et al.,* 2011). Some amentoflavone type biflavonoids exhibited neuroprotective effects on oxidative stress and amyloid β- peptide-induced cell death in neuronal cells (Kang *et al.,* 2005). Therefore, CF, CF-2 and CF-5 were evaluated for their effects on biochemical markers of oxidative stress in the brain. MDA and GSH were used as indicators of lipid peroxidation and endogenous antioxidant, respectively. An elevated level of MDA suggests neuronal degeneration. GSH is the principal intracellular non-protein thiol and plays a major role in the maintenance of the intracellular redox state. The level of GSH diminishes with an increase in the generation of free radicals (Dringen, 2000). In the scopolamine model of dementia, MDA and GSH were estimated on the 5th day after the 1st injection of scopolamine. Scopolamine treated mice showed a significant increase in MDA and decrease in GSH level in the brain from control values, indicating elevated oxidative stress. Administration of CF, CF-2 and CF-5 showed antioxidant effect as revealed by reduced MDA level and elevated GSH level in scopolamine treated mice brain. In contrast, Nitrite level were insignificant among all the treated groups.

Thus, it may be concluded that beside the predominant role of acetylcholinesterase inhibition, antioxidant activity may also be an important contributory factor to the beneficial effects of

amentoflavone and CF-5 against scopolamine induced amnesia. This result showed that both CF-2 and CF-5 could be a novel drug in the management of dementia.

5.9 Effect on pro-inflammatory markers

To further understand the anti-inflammatory mechanism of action, an *in vitro* study was carried in C6 glioma cells. It is well known that the LPS stimulated NO release mimics the pathophysiological situation of nitrosative stress in neuroinflammatory disorders (Rampe *et al.,* 2004). CF-5 and CF-2 significantly inhibited the LPS stimulated NO release in C6 cells which depicts the beneficial role of these drugs in NO mediated neuroinflammatory disorders. Elevated oxidative stress (ROS generation, MDA formation and GSH deficit) in activated astroglial cells is main event in neuroinflammation and important cause of neuronal cell death in neurodegenerative diseases and CNS disorders (Stepananko *et al.,* 2000). Intracellular ROS triggers a number of signalling pathways leading to activation of numerous transcription factors which promote inflammatory mediators release and subsequent neuronal death. Malondialdehyde (MDA) formation is a key event of oxidative stress (Flemming *et al.,* 1997) and in the process of reactive astrogliosis (Kamendulis *et al.,* 1999). LPS significantly increased intracellular ROS formation, decreased GSH level and significantly increased MDA formation in C6 cells compared with non stimulated cells, resembling the patho-physiological state of oxidative stress in CNS disorders. CF-2 and CF-5 significantly reduced intracellular ROS generation, MDA formation and increased reduced GSH level indicating reduction in intracellular oxidative stress revealing the important antioxidant role of these drugs in maintenance of intracellular oxidative stress and subsequent neuroprotection.

Activated glia-derived proinflammatory cytokines are important pathologic factors for progression of AD and other neurodegenerative disorders, neuropathologic changes and inflammation in the CNS (Hull *et al.,* 2000). Thus the anti-inflammatory drugs can reduce AD risk and delay some forms of neuroinflammatory pathology (Moore and O'Banion, 2002). LPS significantly increased TNF-α expression in C6 cells depicting pathophysiological state of CNS disorders. CF-2 and CF-5 significantly attenuated LPS induced increased expression of TNF- α (responsible for major neuronal cell death) in C6 cells providing anti-inflammatory evidence. CF-5 and amentoflavone, downregulates COX-2 expression in TNFα-activated A549 cells with concomitant inhibition of NF-κB mediated signaling cascades amentoflavone inhibits NF-κB/DNA binding activity potently along with inhibition of degradation of IκBα and NF-κB translocation into nucleus in TNFα-activated A549 cells. This Flavonoid up

regulates PPAR γ, a transcription factor involved in repressing many cytokine-induced gene expressions (Banerjee *et al.*, 2002).

The ability of amentoflavone to inhibit both cyclooxygense and 5-lipooxygenase pathways of the arachidonate metabolism may contribute to the anti-inflammatory properties (Williams, *et al.*, 1995). On the other hand, flavonoids are known to display many antioxidant properties including scavenging free radicals and preventing lipid peroxidation (Torel *et al.,* 1986).

5.10 Phytochemical constituents

In respect of the identification of phytoconstituents, the root methanolic extract of *Cnestis ferruginea* was found in this study to contain glycosidic anthraquinones, sterols, phenols, tannins, alkaloids, saponins, flavonoids, amentoflavone and amino acid-like compound (CF-5). One or a combination of these phytoconstituents may be responsible for the observed activities of *Cnestis ferruginea* in this study.

6.0 CONCLUSION

Findings from this study suggest that methanolic root extract of *Cnestis ferruginea* is relatively safe when consumed over an extended period and possesses analgesic, anti-inflammatory, anticonvulsant, antidepressant, anxiolytic and nootropic effects which could result from the presence of one or combination of its phytoactive components. In addition, bioactivity guided fractionation assay of this extract led to the isolation of amentoflavone and CF-5 (an amino acid like compound), with potent analgesic, antinflammatory, antidepressant, anxiolytic and antiamnesic effects. Hence, could be a potential phytotherapeutic agent in the management of inflammatory and neurological disorders. Thus validate its usefulness in the folklore medicines.

7.0 SUMMARY OF FINDINGS

Objectives	Summary of findings
1. Determine the acute and sub-chronic toxicity effects of the methanolic root extract of *Cnestis ferruginea.*	1. The methanolic root extract of *Cnestis ferruginea* is safe when administered orally. Sub-chronic administration revealed effects which suggest that the extract possesses potential hepatoprotective, nephroprotective, neuroprotective and antifertility properties.
2. Determine the analgesic and anti-inflammatory effects of the methanolic root extract of *Cnestis ferruginea.*	2. **a**. The methanolic root extract of *C.ferruginea* possesses analgesic and anti-inflammatory activities. **b**. The analgesic effect involves an interaction with opioidergic, α_{2}-adrenergic, serotonergic (5-HT_3), and ATP sensitive potassium channel pathways. **c**. The anti-inflammatory action involves inhibition of release and/or actions of vasoactive substances (histamine, serotonin and kinins) and prostaglandins.
3. Investigate the effects of methanolic root extracts of *Cnestis ferruginea* on central nervous system disorders	3. The extract possesses anxiolytic, antidepressant and antidementic properties.
4. Evaluate the effect of the bioactive components isolated from methanolic	4. **a**. Amentoflavone and an amino acid-like compound (CF-5) were isolated

root extracts of *Cnestis ferruginea* through bioactivity-guided isolation on painful inflammatory conditions and central nervous system disorders.	through bioactivity guided fractionation. **b**. Oral administration of amentoflavone and CF-5 produced antinociceptive, anti-inflammatory, antidepressant, anxiolytic and antidementic effects. **c.** The neuroprotective effect of CF-2 and CF-5 involve attenuation of intracellular oxidative stress and TNF-α release in C6 glioma cell line.
5. Investigate the putative mechanisms of action(s) of the methanolic root extract in central nervous system disorders	**5. a.** The anxiolytic effect of *C. ferruginea* involves an interaction with GABAergic and serotonergic system. **b.** The antidepressant effect of *C. ferruginea* is dependent on interaction with the serotonergic (5-HT_2 receptors) and nitric oxide pathway. **c.** The antidementic effect of *C. ferruginea* involves inhibition of acetylcholinesterase activity and attenuation of free radical generation.

8.0 CONTRIBUTION TO KNOWLEDGE

The study established that:

1. Methanolic extract of *C. ferruginea* and its constituents possess analgesic and anti-inflammatory activities devoid of toxic effect associated with traditional non steroidal anti-inflammatory drugs, hence, they could be potential phytotherapeutic agents in the management of painful and inflammatory conditions.
2. The methanolic extract possesses antidepressant, anxiolytic and antidementic effects which may be due to the presence of amentoflavone and (CF-5) an amino acid-like compound thus providing a new therapeutic option in the management of schizophrenia and Alzheimer's of dementia type.
3. The isolation of amentoflavone a novel antidepressant and anxiolytic drug which could be an alternative for the treatment of anxiety, depression and mixed anxiety-depressive disorders.

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APPENDIX I

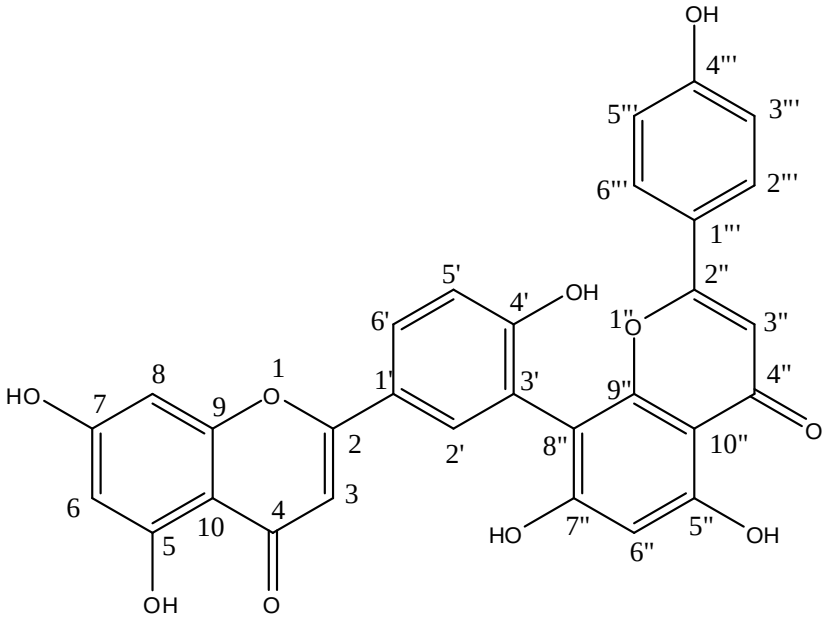
Photograph of *Cnestis ferruginea* Vahl ex DC in its natural habitat

APPENDIX II

Photograph of Herbarium voucher specimen of *Cnestis ferruginea* Vahl ex DC (Connaraceae)

APPENDIX III

Structure and NMR data of CF-2

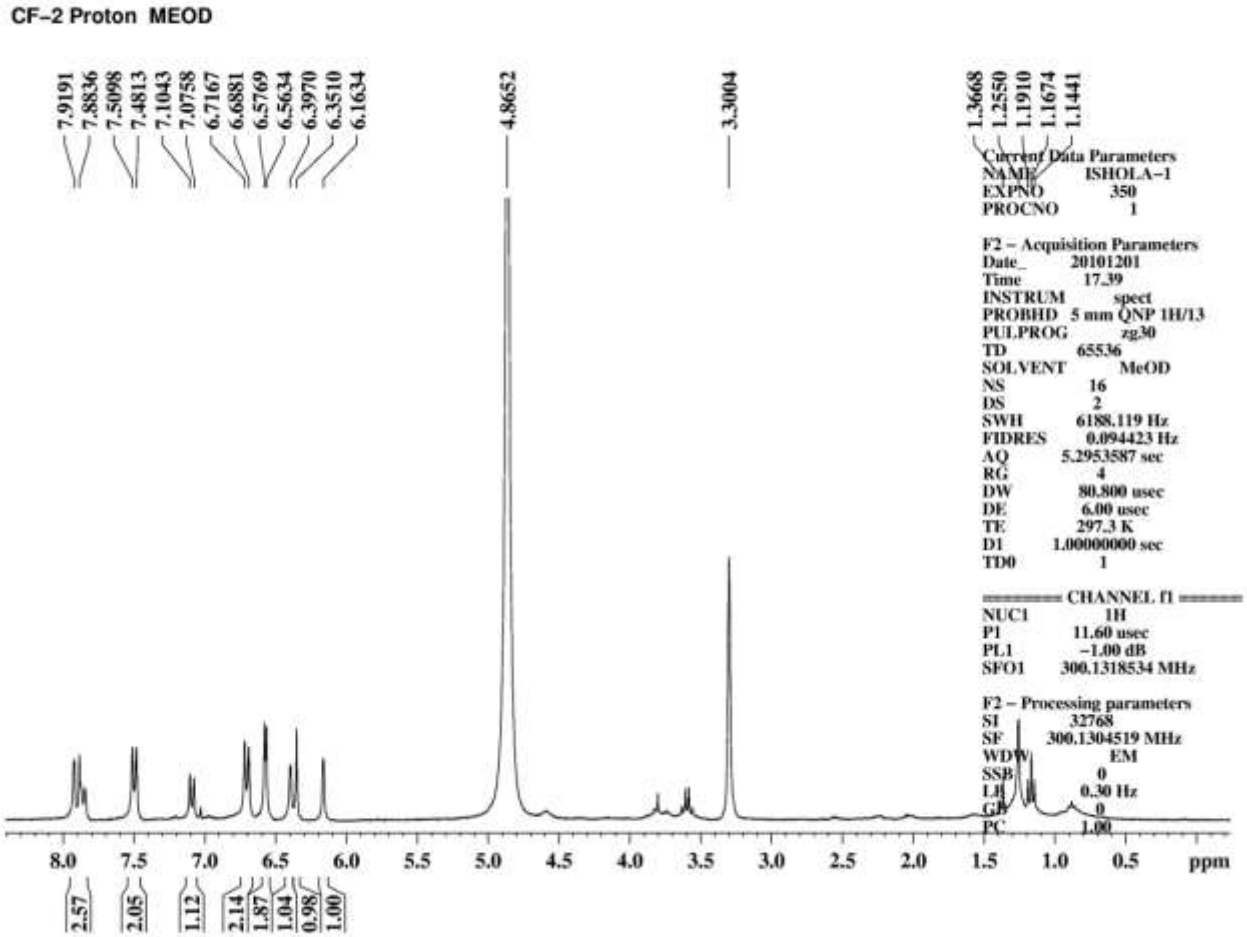


Structure of amentoflavone

NMR data of amentoflavone (Pyridine-D5 at 400MHz)

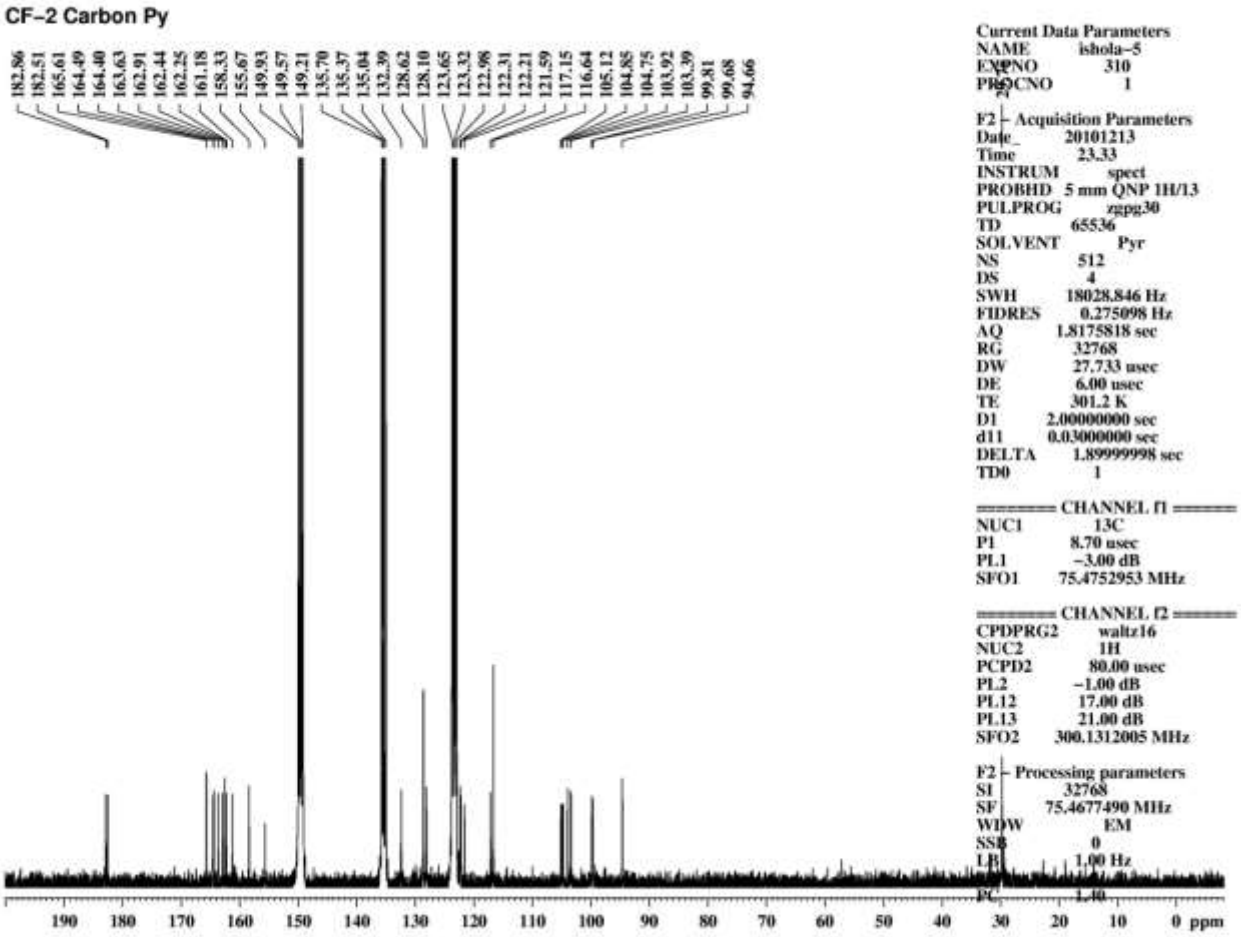
C	**δc**	**δH,multiplicity, J(Hz)**	**HMBC Correlations**
2	164.4	-	H-3;H-2’; H-6’
3	103.3	6.92,s	-
4	182.5	-	H-3
5	162.8	-	H-6
6	99.6	6.19,s	-
7	165.6	-	H-6;H-8
8	94.6	6.33,s	H-6
9	158.3	-	-
10	104.7	-	H-6
1’	117.9	-	H-2’; H-5’;H-6’
2’	132.3	8.51, s	H-6’
3’	121.4	-	H-2’; H-5’
4’	164.3	-	H-2’; H-5’;H-6’
5’	117.0	7.45,d (8.0)	H-6’
6’	128.1	7.92, dd (8.0,1.8)	H-2’; H-5’
2’’	164.5	-	H-3’’;H-2’’’; H-6’’’
3’’	103.9	7.01,s	-
4’’	182.8	-	H-3’’’
5’’	161.0	-	H-6’’’
6’’	99.8	6.06,s	-
7’’	164.4	-	H-6’’’
8’’	106.7	-	H-2’; H-6’’
9’’	155.6	-	-
10’’	101.7	-	H-6’’
1’’’	121.7	-	H-2’’’,H-6’’’, H-3’’’, H-5’’’
2’’’, 6’’’	128.6	7.88, d(8.0)	H-3’’’, H-5’’’
3’’’, 5’’’	116.6	7.16, d (8.0)	H-2’’’, H-6’’’
4’’’	160.6		H-2’’’, H-6’’’, H-3’’’, H-5’’’’
Chelated Hydroxyl		13.7 and 13.8	

Appendix IV



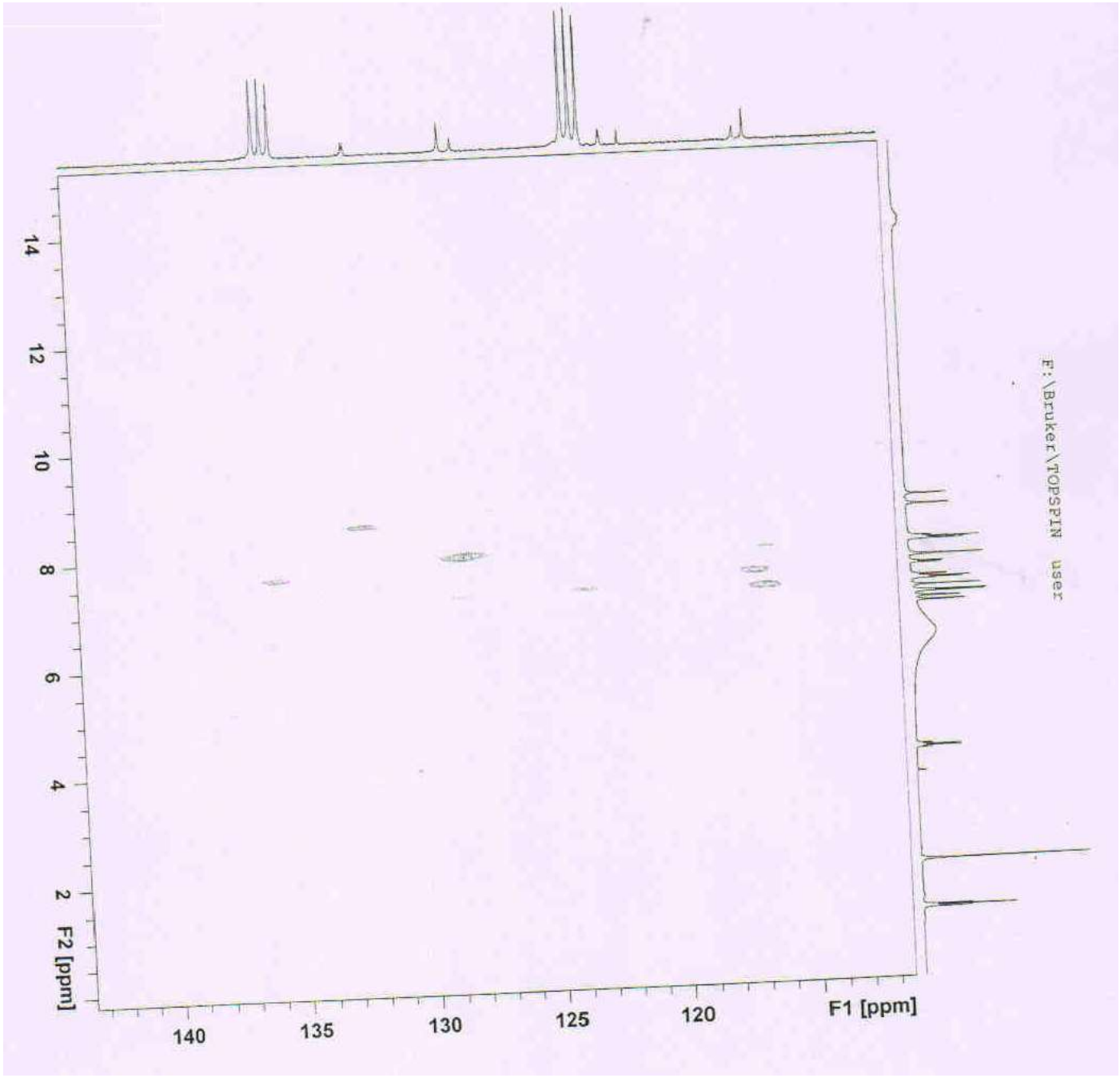
Hydrogen Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy of CF-2

Appendix V



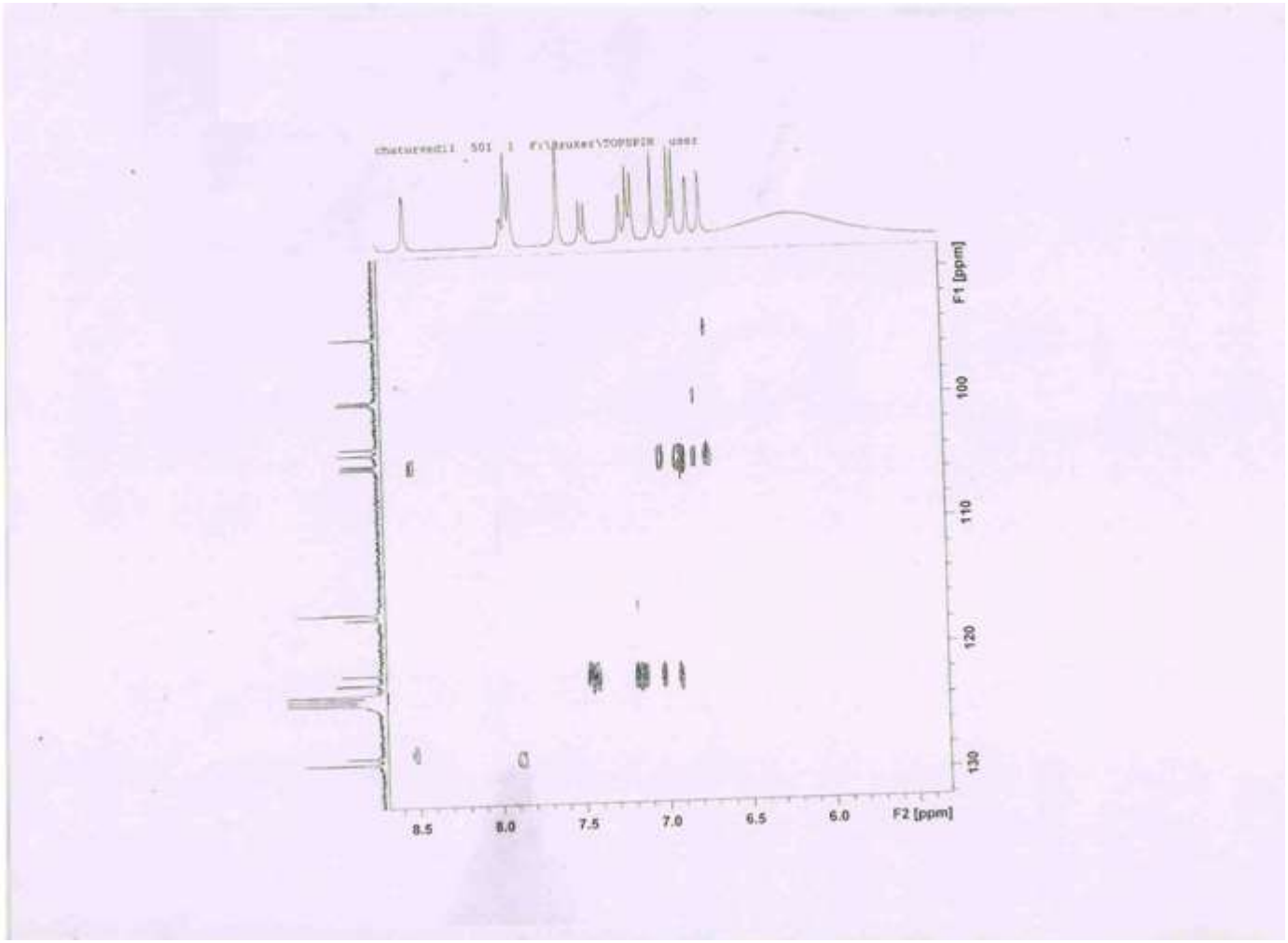
Carbon Nuclear Magnetic Resonance Spectroscopy (^{13}C NMR) spectra of CF-2

Appendix VI



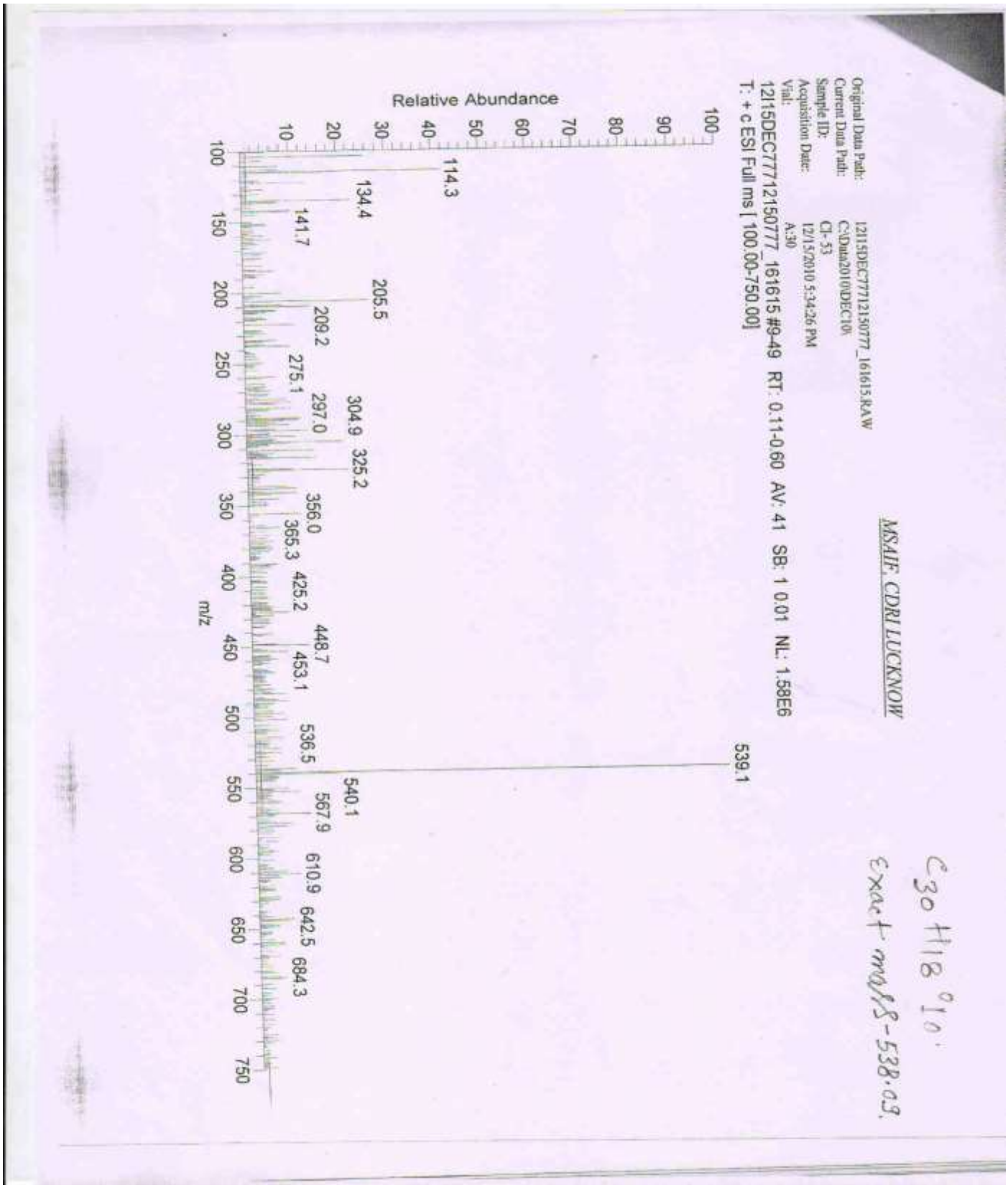
Heteronuclear Single Quantum Correlation (HSQC) 2D NMR spectra of CF-2: It is based on proton-detection, offering high sensitivity when compared with the conventional carbon-detected

Appendix VII



2D Heteronuclear Multiple Bond Correlation (2D HMBC) spectra of CF-2: $^{1}H,^{13}C$-HMBC correlates chemical shifts of protons with carbons separated with two or three bond.

Appendix VIII



Mass spectrometry of CF-2