EVALUATION OF ANTI-ANXIETY AND SEDATIVE EFFECTS OF VARIOUS EXTRACTS OF *Amomum subulatum* SEEDS IN SWISS ALBINO MICE

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MASTER OF PHARMACY

IN

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Submitted by

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CERTIFICATE

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LIST OF ABBREVIATIONS

MEASS	Methanol extracts of *Amomum subulatum* seeds
PEASS	Petroleum ether extracts of *Amomum subulatum* seeds
EPM	Elevated plus maze
GABA	Gamma amino butyric acid
BZDs	Benzodiazepines
TCA_S	Tricyclic antidepressants
MAO	Monoamine oxidase
SSRI	Selective serotonin reuptake inhibitor
SNRI	Serotonin-norepinephrine reuptake inhibitor
SNP	Sodium nitropruside
NO	Nitric oxide
BHA	Butylated hydroxyanisole
GAD	Generalized anxiety disorder
SAD	Social anxiety disorder
OCD	Obsessive compulsive disorder
COPD	Chronic obstructive pulmonary disease

DSM	Diagnostic and statistical manual of mental Disorders
ICD	International classification of diseases
MDD	Major depressive disorder
PTSD	Post-traumatic stress disorder
DSPS	Delayed sleep-phase syndrome
PMDD	Premenstrual dysphoric disorder
5HT	5-hydroxy tryptamine
NK	Neurokinin
CRF	Corticotrophin releasing factor
NPY	Neuropeptide Y
BB	Bombesin
GRF	Gastrin releasing factor
NMB	Neuromedin B
CCK	Cholecystokinin
BDNF	Brain-derived neurotrophic factor
CBT	Cognitive behavior therapy
RAS	Reticular Activating System
REM	Rapid eye movement

FDA	Food and drug administration
US	united States
TLC	Thin layer chromatography
MIC	Minimum inhibitory concentration

1. INTRODUCTION

The complexity of daily life in modern society frequently leads to varying degrees of anxiety. Anxiety disorders have been found to be associated with chronic pain among hospitalized patients in both developed and developing countries.[1] Anxiety disorders, the most prevalent psychiatric illnesses in the general community, are present in 15-20% of hospitalized patients.

Anxiety it is an emotional state, unpleasant in nature, associated with uneasiness, discomfort and concern or fear about some defined or undefined future thread. Some degree of anxiety is a part of normal life. Treatment is needed when it disproportionate to the situation and excessive.[2]

Anxiety disorders occur in approximately 30% of mood cases[3]. Lifetime prevalence rates for total anxiety disorders are 16.6%. Women are more likely to suffer from anxiety disorders because women experience a wider range of life events including events happening to their close as well as distant relatives and friends, in comparison to men, who react to events limited to themselves or close family members.[4] Anxiety disorders are common during the perinatal period, with reported rates of obsessive- compulsive disorder and generalized anxiety disorder being higher in postpartum women than in the general population.[5] Social anxiety disorder (SAD) is among the most common of all psychiatric disorders with lifetime prevalence estimates ranging from 7% to 13%.[3] Co-morbidity of anxiety and depression is highly prevalent. About 47.5% patients of major depressive disorder also meet criteria for anxiety disorders, whereas 26.1% patients of anxiety disorders meet criteria for major

depressive disorder too.[6] About 8% of patients consulting primary care professionals have generalized anxiety disorder. Initial manifestations of anxiety appear at age of 20-35 years and there is predominance in women. Panic disorder commonly coexists with essential hypertension and the postural tachycardia syndrome.[7]

Anxiety states are controlled by both inhibitory and facilitatory mechanisms that either counter or favor anxiety states. These neurochemical and neuropeptide systems have been shown to have effects on distinct cortical and sub cortical brain areas that are relevant to the mediation of the symptoms associated with anxiety disorders.[8] Regional brain networks involved in such stress, anxiety, and anxious behaviors may be appropriate targets for actions of anxiolytics. Drug development in this direction also aims to generate new pharmacological agents with action at specific neurotransmitters and neuropeptides, their reuptake and metabolism. The ultimate objective is to develop substances that are as effective as benzodiazepines, which have been the traditional treatment for anxiety for over 40 years. This search has led to development of unconventional agents, which are either partial benzodiazepine-GABA receptor agonists or target specific subunits of the GABA receptor or manipulate GABA levels, agents that affect the serotonin and nor-epinephrine systems, antagonists of neurotransmitter systems such as corticotropin-releasing factor and Substance P, agents that decrease glutamate neurotransmission, such as metabotropic glutamate receptor agonists, stimulation of neurotrophic factors, such as brain-derived neurotrophic factor, which appears to enhance neurogenesis.[9]

A broad range of pharmacologic agents are available to treat anxiety disorders namely Selective Serotonin Reuptake Inhibitors, Selective Nor epinephrine Reuptake

Inhibitors, Tricyclic Antidepressants, MonoAmineOxidase Inhibitors, Buspirone, Benzodiazepines, Hydroxyzine, Antipsychotic, Anticonvulsants and Adrenergic agents.[2]

Sleep disturbance is amongst the most frequent health complaints, which the Physicians encounter. It is popularly known as insomnia. It is defined as persistent difficulty in falling or staying asleep.[10] Sleep is a physiologic recuperative state that can be disturbed by many factors such as illness, stress and noise. Chronic sleep disorder leads to some health repercussions such as slower reactions, poor memorizing, emotional disturbances, and changes in the immune response.[11, 12] Today, sleep disorders have a relatively high prevalence and are a growing public health problem. It is estimated that more than 27% of people worldwide suffer from sleep disorders with difficulty in initiating or maintaining sleep. In addition, it is expected that by the middle of the 21 century, about 31% of all people will be chronic and frequent users of sleep medications.[12, 14] Currently, the most widely used medications for sleep disorders are the benzodiazepines.

However, the regular uses of the above synthetic drugs results in unpleasant side effects such as drug dependence, tolerance, rebound insomnia, amnesia, psychomotor impairment and potentiating of other central depressant drugs.[15] Thus, researchers, are now exploring natural resources to find out more efficacious and safer drugs.

Traditional and folk remedies have provided us with important drugs in the treatment of many diseases and are being increasingly subjected to scientific study.

In recent years, traditional system of medicine has become a topic of global importance. Many of the plant species that provide medicinal herbs have been

scientifically evaluated for their possible medicinal applications.[16] Even today, this area holds much more hidden treasure as almost 80% of human population in developing countries is dependent on plant resources for healthcare.[17] Herbal medicines offer conventional treatments, providing safe and well-tolerated remedies for chronic illness which typically resulted from the combinations of secondary plant metabolites that are synthesized and deposited in specific parts or in all part of the plant. Since, many of the synthetic drugs cause various side effects, drugs synthesized from the higher plants continue to occupy an important niche in modern medicine and play an important role in modern medicine and introduction of new therapeutic agents.

These are many medicinal plants that have stimulating or calming effects on the central nervous system, and the plant kingdom provides hundreds of CNS active substances covering the whole spectrum of activity such as psychoanaleptic, psycholeptic and psychodysleptic (hallucinogenic) effects.[18, 19] There is tremendous hope that drugs of plant origin will have significantly lesser side effects than that observed with synthetic drugs while having comparable efficacy.[20]

Some of the plants that have been tested had shown to posses antianxiety & sedative effect includes *Matricaria recutita, Tilia europaea, valeriana officeinalis, passiflora caeulea, Valeriana officeinalis, Stachys lavanaulifolia,calotropis gigantean, Passiflora caeulea, pachyrrhizus erosus ,Salvia reterana and Nepeta cataria* [21] etc…

One such search for plant with antianxiety and sedative effect Amomum subulatum was chosen for the study on the basis of its traditional use. Amomum subulatum is commonly known as large cardamom .*Amomum subulatum* seed is used

traditionally in stomachis, indigestion, abdominal pain and rectal disease. The seeds of the plants have good taste and are a tonic for liver and heart. They are astringent to the bowels, appetizing, hypnotic and aid digestion. The current study was carried out in an attempt to investigate potential sedative and anxiolytic effect of various extracts *of Amomum subulatum* seeds in mice using elevated plus maze(EPM) and spontaneous motor activity tests.

2. AIM AND OBJECTIVE

AIM

The aim of the present study is to evaluate the anti-anxiety and sedative effects of various extracts of *Amomum subulatum* seeds in Swiss albino mice.

OBJECTIVE

- To analyse the phytochemical profile of various extracts of *Amomum subulatum* seeds.
- *In-vitro* antioxidant activity is used to determine the presence of active constituents in various extracts.
- *In-vivo* spontaneous locomotor activity is to be done by actophotometer& open field for sedative activity.
- *In-vivo* antianxiety activity is to be done by elevated plus maze method.

3. REVIEW OF LITERATURE

3.1 ANXIETY

Anxiety is a state of excessive fear and is characterized by motor sympathetic hyperactivity, apprehension and vigilance syndromes. The most common observation is an acute stress response characterized by a state of abnormal or exaggerated arousal or fear. Generally, anxiety is an adaptive response to supposedly dangerous stimuli, which may perturb homeostasis. However, when it become disproportional in intensity, chronic and/or irreversible, or not genuine, it manifest as debilitating anxious state presenting itself in form of phobia, panic attacks, post-traumatic stress disorder, social anxiety disorder or generalized anxiety disorder.[8]

3.1.1 Symptoms [22]

Common anxiety signs and symptoms include:

- Feeling nervous, restless or tense
- Having a sense of impending danger, panic or doom
- Having an increased heart rate
- Breathing rapidly (hyperventilation)
- Sweating
- Trembling
- Feeling weak or tired
- Trouble concentrating or thinking about anything other than the present worry
- Having trouble sleeping
- Experiencing gastrointestinal (GI) problems

- Having difficulty controlling worry
- Having the urge to avoid things that trigger anxiety

3.1.2 Etiology of Anxiety Disorders [23]

Table 1: Etiology of anxiety disorders

Biological factor	Psychological factor	Social causes
Heredity	Personality traits	Adverse Life Experiences
Neurotransmitter imbalance	Low self-esteem	Lack of social support
Illness	Cognitive dissonance	Work stress
Medications	Negative emotions	Lack of social skills
Nutritional factors	Inter and/or intra-personal conflicts	Conflict of societal norms
	Perception of situational factors	Natural calamities

Psychological factors

Anxiety can result when a combination of increased internal and external stresses overwhelm one's normal coping abilities or when one's ability to cope normally is lessened for some reason.

The psychological factors are summarized below:

Psychodynamic: When internal competing mental processes, instincts and impulses conflict, causing distress.

Behavioral: Anxiety is a maladaptive learned response to specific past experiences and situations that become generalized to future similar situations.

Spiritual: When people experience a profound, unquenchable emptiness and nothingness to their lives, often leading to distress concerning their mortality and eventual death.

Social factors

Life experiences like death in the family, divorce, job loss, financial loss, accident or major illness affect a person's attitude and response to life situations. Long term exposure to abuse, violence, terrorism and poverty may affect an individual's susceptibility to anxiety disorders.

Oxidative stress

High O_2 consumption, modest antioxidant defenses and a lipid-rich constitution make the brain highly vulnerable to redox imbalances. Oxidative damage in the brain causes nervous system impairment. Recently, oxidative stress has also been implicated in depression, anxiety disorders and high anxiety levels.[24]

Anxiety due to a general medical condition: [22]

For some people, anxiety may be linked to an underlying health issue. In some cases, anxiety signs and symptoms are the first indicators of a medical illness. If your doctor suspects your anxiety may have a medical cause, he or she may order tests to look for signs of a problem.

Examples of medical problems that can be linked to anxiety include:

- Heart disease
- Diabetes
- Thyroid problems, such as hyperthyroidism
- Respiratory disorders, such as chronic obstructive pulmonary disease (COPD) and asthma
- Drug abuse or withdrawal

- Withdrawal from alcohol, anti-anxiety medications (benzodiazepines) or other medications
- Chronic pain or irritable bowel syndrome
- Rare tumors that produce certain "fight-or-flight" hormones

3.1.3 Risk factors [22]

These factors may increase the risk of developing an anxiety disorder:

- **Trauma.** Children who endured abuse or trauma or witnessed traumatic events are at higher risk of developing an anxiety disorder at some point in life. Adults who experience a traumatic event also can develop anxiety disorders.
- **Stress due to an illness.** Having a health condition or serious illness can cause significant worry about issues such as the treatment and the future.
- **Stress buildup.** A big event or a buildup of smaller stressful life situations may trigger excessive anxiety — for example, a death in the family, work stress or ongoing worry about finances.
- **Personality.** People with certain personality types are more prone to anxiety disorders than others are.
- **Other mental health disorders.** People with other mental health disorders, such as depression, often also have an anxiety disorder.
- **Having blood relatives with an anxiety disorder.** Anxiety disorders can run in families.
- **Drugs or alcohol.** Drug or alcohol use or abuse or withdrawal can cause or worsen anxiety

3.1.4 Complications [22]

Having an anxiety disorder does more than make you worry. It can also lead to, or worsen, other mental and physical conditions, such as:

- Depression (which often occurs with an anxiety disorder) or other mental health disorders
- Substance abuse
- Trouble sleeping (insomnia)
- Digestive or bowel problems
- Headaches and chronic pain
- Social isolation
- Problems functioning at school or work
- Poor quality of life
- Suicide

3.1.5 Diagnostic and Statistical Manual of Mental Disorders, 4th edition text revision (DSM IV) Anxiety Disorder include

1. Generalized anxiety disorder
2. Social anxiety disorder
3. Agoraphobia without panic
4. Obsessive compulsive disorder(OCD)
5. Acute stress disorder
6. Panic disorder with or without agoraphobia
7. Post-traumatic stress disorder
8. Anxiety disorder otherwise specified
9. Specific phobia
10. anxious depression

11. substance induced anxiety disorder

12. selective mutism

DSM-IV also list anxiety occurring as an adjustment disorder, or secondary to substance abuse or a general medical condition.[25]

Key phenomenological features of major anxiety disorders as defined by DSM-V-TM [23, 26- 28]

I. Separation anxiety disorder

The individual with separation anxiety disorder is fearful or anxious about separation from attachment figures to a degree that is developmentally inappropriate. There is persistent fear or anxiety about harm coming to attachment figures and events that could lead to loss of or separation from attachment figures and reluctance to go away from attachment figures, as well as nightmares and physical symptoms of distress. Although the symptoms often develop in childhood, they can be expressed throughout adulthood as well.

II. Selective mutism

Selective mutism is characterized by a consistent failure to speak in social situations in which there is an expectation to speak (e.g., school) even though the individual speaks in other situations. The failure to speak has significant consequences on achievement in academic or occupational settings or otherwise interferes with normal social communication.

III. Specific phobia

Individuals with specific phobia are fearful or anxious about or avoidant of circumscribed objects or situations. A specific cognitive ideation is not featured in this disorder, as it is in other anxiety disorders. The fear, anxiety, or avoidance is

almost always immediately induced by the phobic situation, to a degree that is persistent and out of proportion to the actual risk posed.

There are various types of specific phobias:

Animal (e.g. snakes and dogs)

Natural environment (e.g. height)

Blood-injection-injury

Situational (e.g. flying)

IV. Social anxiety disorder

In social anxiety disorder (social phobia), the individual is fearful or anxious about or avoidant of social interactions and situations that involve the possibility of being scrutinized. These include social interactions such as meeting unfamiliar people, situations in which the individual may be observed eating or drinking, and situations in which the individual performs in front of others. The cognitive ideation is of being negatively evaluated by others, by being embarrassed, humiliated, or rejected, or offending others.

V. Panic disorder

Recurrent unexpected panic attacks characterized by four or more of the following

- Sweating and shaking
- Tremor
- Shortness of breath
- Hyperventilation
- A choking sensation
- Chest discomfort and palpitation
- Nausea
- Dyspepsia

- Paresthesia
- Derealisation and depersonalization,
- Sense of impending doom or death

VI. Agoraphobia

Individuals with agoraphobia are fearful and anxious about two or more of the following situations: using public transportation; being in open spaces; being in enclosed places; standing in line or being in a crowd; or being outside of the home alone in other situations. The individual fears these situations because of thoughts that escape might be difficult or help might not be available in the event of developing panic-like symptoms or other incapacitating or embarrassing symptoms. These situations almost always induce fear or anxiety and are often avoided and require the presence of a companion.

VII. Generalized anxiety disorder

The key features of generalized anxiety disorder are persistent and excessive anxiety and worry about various domains, including work and school performance, which the individual finds difficult to control. In addition, the individual experiences physical symptoms, including restlessness or feeling keyed up or on edge; being easily fatigued; difficulty concentrating or mind going blank; irritability; muscle tension; and sleep disturbance.

VIII. Substance/medication induced anxiety

Substance/medication-induced anxiety disorder involves anxiety due to substance intoxication or withdrawal or to a medication treatment. In anxiety disorder due to another medical condition, anxiety symptoms are the physiological consequence of another medical condition.

IX. Anxious depression

In anxious depression, anxiety, tension or agitation accompanies overt depressive affect. Over 60% of anxious patients eventually have symptoms of depression. Other patients are chronically depressed with intermittent exacerbations of anxiety symptoms.

X. Obsessive and compulsive disorder

Patient usually has obsessions and compulsions:

Obsessions:

• Recurrent and persistent thoughts, impulses, or images

• Viewed by patient as intrusive and inappropriate and cause marked anxiety or distress.

• Recognized as a product of his or her own mind.

Compulsions:

• Repetitive behaviors or mental acts

• Performed in response to an obsession, or according to rules that must be applied rigidly.

• Generally not connected in a realistic way with what they are designed to neutralize or prevent or are clearly excessive.

3.1.6 Pathophysiology of anxiety disorder[29-34]

Several preclinical evidence now point to the amygdala as the major mediator of stress response, fear and anxiety. The major mediators of anxiety disorder appear to be Norepinephrine, Serotonin and GABA.

GABA is one of the most widely distributed neurotransmitters in the mammalian brain, as it is expressed in about 30% of all synapse. GABA is an

inhibitory transmitter and therefore reduced the firing rate of excitatory neurons with which it is in contact. In various animal models of anxiety, the facilitation of GABAergic activity is associated with a reduction in anxiety.

Noradrenaline is the neurotransmitter most closely associated with peripheral and central stress response. Drugs that stimulate alpha 2 receptors such as clonidine diminishes the anxiety state by reducing the release of noradrenaline. Patients with panic disorders have increased sensitivity to challenge with isoproterenol because of increased peripheral beta receptor sensitivity.

Several experimental studies have suggested that a reduction in serotonin in the brain results in anxiolysis. Serotonin pathway originating in the dorsal raphe nucleus and innervating the amygdala and frontal cortex facilitate avoidance behavior in response to distal threat. This pathway involves 5-$HT_{2A/2C}$ and 5-HT_3 post synaptic receptors and may be relevant to generalized anxiety disorder. A separate pathway from the dorsal raphe nucleus and innervating the periventricular and periaqueductal grey region inhibit inborn fight or flight reactions in $5HT_{1A}$ receptors and may be relevant to panic attacks. With chronic stress, the serotonin pathway connecting the median raphe nucleus to the hippocampus, likely mediated by postsynaptic $5HT_{1A}$ receptor may be relevant to avoiding and numbing found in post traumatic stress disorders. Several neuropeptides have been shown to play a role in anxiety but so far none has been developed as a drug largely because of their poor pharmacokinetic properties and difficulty in penetrating the blood brain barrier.

Angiotensin peptides – Angiotensin Converting Enzyme inhibitors like captopril, has anxiolytic activity in both experimental and clinical studies. It has recently been shown that the angiotensin 1 receptor antagonist, Losartan has anxiolytic properties whereas the angiotensin 2 antagonists are inactive.

Cholecystokinin ligands – agonists of the central cholecystokinin receptors cause anxiety and precipitate panic attacks in predisposed individuals. Two types of Cholecystokinin receptors have been identified, CCK-A and CCK-B, both of which occur in mammalian brain. CCK-B agonists initiate anxiety while the antagonists are anxiolytic in both experimental and clinical situations. Neurokinin receptor ligands – There are two types of Neurokinin receptors in the brain namely NK1 and NK2. NK 2 agonists have been found to be anxiogenic while the antagonists are anxiolytic at least in animal studies. Some NK 1 antagonists have also been shown to be anxiolytic in experimental studies.

Corticotrophin releasing factor ligands – alpha helical CRF has been shown to block the anxiogenic effects of alcohol withdrawal in rats. It is possible that CRF interacts with neuropeptide Y receptors; NPY1 receptor agonists to have anticonflict effects in animal studies.

Adenosine receptor ligands – the adenosine receptor antagonist, caffeine, induces anxiety in both animals and humans while agonists have anxiolytic effects.

The results of studies investigating neuroactive steroid levels in patients with anxiety disorders are conflicting. Brain-derived pregnane steroids can potently and specifically enhance $GABA_A$ receptor functions. In addition, further studies are needed to determine the precise role of neuroactive steroids in the treatment of anxiety symptoms; pharmacological agents used to treat the symptoms of anxiety disorders often alter brain steroid levels, and understanding the role of these changes in steroid levels in the future lead to more specific and effective drug treatments.

Several lines of investigators support the involvement of the opioid receptor system in the regulation of anxiety. The most compelling evidence for the involvement of the delta opioid receptor system in anxiety comes from a study on

delta opioid receptor knockout mice. Specifically, delta opioid receptor deficient mice exhibit anxiogenic – like phenotype. The modulation of anxiety-like behavior by delta opioid receptor agonists may prove to be a useful clinical alternative to treat anxiety disorders that are resistant to typical anxiolytics.

Bombesin (BB), an amphibian peptide and its mammalian counterparts [various forms of neuromedin B (NMB)] and Gastrin Releasing Peptide (GRP), elicit their effects through various BB receptor subtypes. Neuromedin B binds preferentially to BB 1 subtype and GRP binds to BB2 receptor. BB and NMB increased the firing rate of serotonin cells in the dorsal raphe nucleus. Because reduced Serotonin release has been linked to reduced anxiety. Antagonists of the excitatory actions of BB like peptides on dorsal raphe nucleus serotonin neurons might be expected to decrease anxiety.

3.1.7 Management of Anxiety[23, 35, 29]

Anxiety disorders are the most prevalent of psychiatric disorders, yet less than 30% of individuals who suffer from anxiety disorders seek treatment. People with anxiety disorders can benefit from a variety of treatments and services. Following an accurate diagnosis, possible treatments include psychological treatments and mediation.

Psychological treatments

Psychotherapy is almost always the treatment of choice except in cases where anxiety is so severe that immediate relief is necessary to restore functioning and to prevent immediate and severe consequences. This includes the following:

Behavioral therapies:

These focus on using techniques such as guided imagery, relaxation training, biofeedback (to control stress and muscle tension); progressive desensitization, flooding as means to reduce anxiety responses or eliminate specific phobias. The person is gradually exposed to the object or situation that is feared. At first, the exposure may be only through pictures or audiotapes. Later, if possible, the person actually confronts the feared object or situation. Often the therapist will accompany him or her to provide support and guidance.

Cognitive-behavioral therapy (CBT):

In this therapy, people learn to deal with fears by modifying the ways they think and behave. A major aim of CBT and behavioral therapy is to reduce anxiety by eliminating beliefs or behaviors that help to maintain the anxiety disorder. Research has shown that CBT is effective for several anxiety disorders, particularly panic disorder and social phobia. It has two components. The cognitive component helps people change thinking patterns that keep them from overcoming their fears. The behavioral component of CBT seeks to change people's reactions to anxiety-provoking situations. A key element of this component is exposure, in which people confront the things they fear, i.e., CBT addresses underlying "automatic" thoughts and feelings that result from fear, as well as specific techniques to reduce or replace maladaptive behavior patterns.

Psychotherapy:

Psychotherapy centers on resolution of conflicts and stresses, as well as the developmental aspects of anxiety disorders solely through talk therapy. Psychotherapy involves talking with a trained mental health professional, such as a psychiatrist,

psychologist, social worker, or counselor to learn how to deal with problems like anxiety disorders.

Psychodynamic therapy:

This therapy**,** first suggested by Freud, is based on the premise that primary sources of abnormal behavior are unresolved past conflicts and the possibility that unacceptable unconscious impulses will enter consciousness.

Family therapy and parent training:

Here the focus is on the family and its dynamics. This is based on the assumption that the individuals of a family cannot improve without understanding the conflicts that are to be found in the interactions of the family members. Thus, each member is expected to contribute.

Pharmacotherapy[2, 23, 29, 36, 37, 39]

II.ANTIANXIETY DRUG

1. Benzodiazepines

- Diazepam
- Chlordiazepoxide
- Oxazepam
- Lorazepam
- Alprazolam

2. Azapirones

- Buspirone
- Gepirone
- Ispapirone

3. β blocker

- Proponalol

4. Sedative antihistaminic

- Hydroxyzine

5. Antidepressants

- Selective serotonin reuptake inhibitor($SSRI_S$)
- Tricyclic antidepressants
- Serotonin and nor adrenaline reuptake inhibitors($SNRI_{S)}$

Benzodiazepines

Chlordiazepoxide and diazepam were introduced around 1960 as antianxiety drugs.

Some members have a slow and prolonged action; relieve anxiety at low dose without producing significant CNS depression. They have a selective taming effect on aggressive animals and suppress induced aggression. they also suppress the performance impairing effect of punishment. In contrast to barbiturates, they are more selective for the limbic system and have proven clinically better in both quality of improvement in anxiety and stress related symptoms.

At anti-anxiety doses, cardiovascular and respiratory depression is minor. Benzodiazepines primarily inhibitory GABAergic transmission but other additional mechanism of action has been suggested. Higher dose induce sleep and impair performance.

Mechanism of action

Affect neurons that have receptors for the neurotransmitter GABA

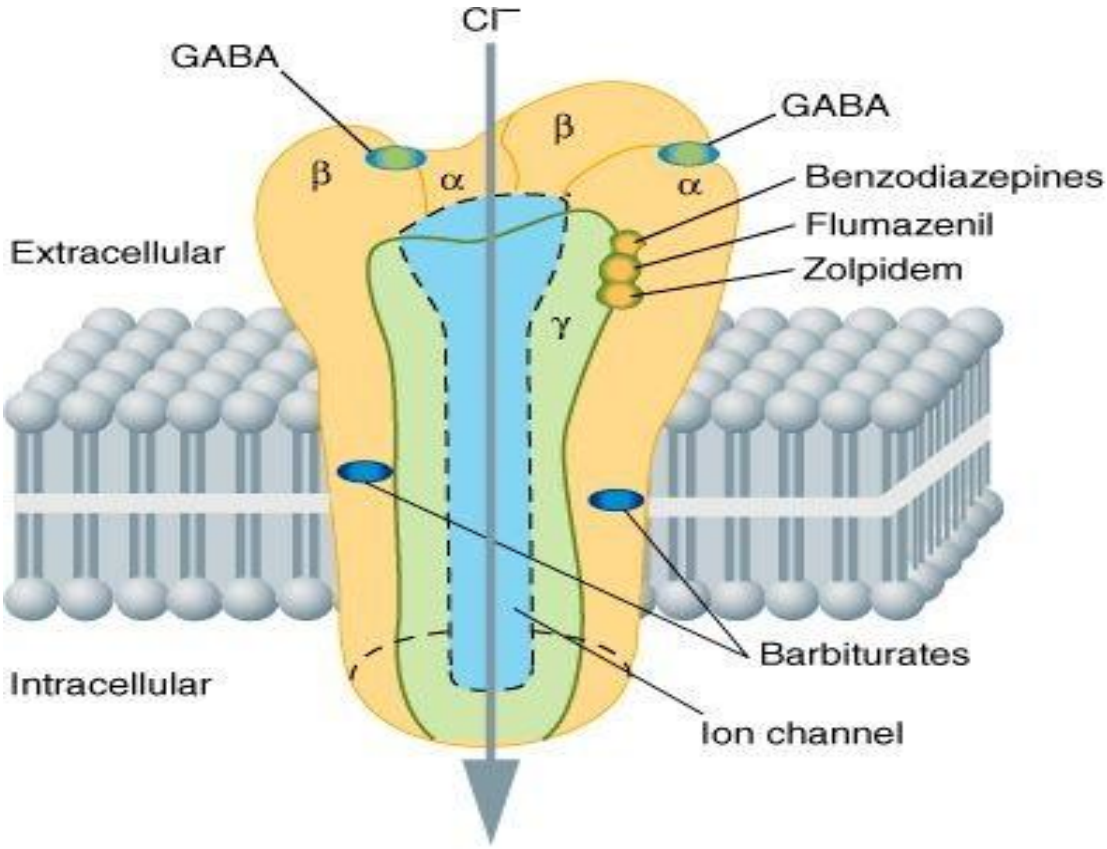


Fig.1. Mechanism of action of benzodiazepines

Benzodiazepines potentiate GABA→ increase frequency of ch.loride ion channel opening→ causes hyper polarization→ raise firing threshold→ and thus inhibits the formation of action potential→ inhibitory effects on different sites on brain especially motor cortex and limbic system.

GABA- inhibitory transmitter in brain regions

- Limbic system(alter mood)
- RAS (cause drowsiness)
- Motor cortex(relax muscle)

Adverse effect

Side effect occurs in the use to relieve anxiety are

- Sedation
- Psychomotor and cognitive impairment
- Increased appetite and weight gain
- Confusional state

Use:

1. Anxiety disorders:

- Short term relief of severe anxiety
- General anxiety disorder
- Obsessive compulsive disorder
- Panic attack with depression Alprazolam (Antidepressant effect)

2. Sleep disorders (Insomnia).

- Triazolam, Lorazepam, Flurazepam

3. Treatment of epilepsy

- Diazepam – Lorazepam

4. in anesthesia

- Preanesthetic medication (diazepam).
- Induction of anesthesia (Midazolam, IV)

β adrenergic blocking agent

No overt central effects are produced by propranolol. However, subtle behavioral changes, forgetfulness, increased dreaming and nightmares have been reported with long term use of relatively high doses. Proponalol suppresses anxiety in short-term stressful situations, but this is due to peripheral rather than a specific central action

Many symptomps of anxiety (palpitation, rise in BP, shaking, tremor, gastrointestinal hurrying, etc.) are due to sympathetic over activity, and these symptoms reinforce anxiety. Propanalol and other nonselective β blockers help anxious patients troubled by these symptoms, by cutting the vicious cycle and provide symptomatic relief. They do not affect the psychological symptoms such as worry, tension and fear, but are valuable in acutely stressful situations (examination fear, unaccustomed public appearance, etc...). They may be used for performance/ situational anxiety or as adjuvant to BZDs .the role of β blockers in anxiety disorders is quite limited.

Hydroxyzine

An H_1 antihistaminic with sedative, antiemetic, antimuscarinic and spasmolytic properties. It is claimed to have selective anxiolytic action, but the accompanying sedation is quite marked. Hydroxyzine may be used in reactive anxiety or that associated with marked autonomic symptoms. Due to antihistaminic and sedative property, it is useful in pruritus and urticaria.

Antidepressants

Antidepressants were developed to treat depression but are also effective for anxiety disorders. Although these medications begin to alter brain chemistry after the very first dose, their full effect requires a series of changes to occur; it is usually about 4 to 6 weeks before symptoms start to fade. It is important to continue taking these medications long enough to let them work

Selective Serotonin Reuptake Inhibitors

The selective serotonin reuptake inhibitors (SSRIs) represent a chemically diverse class of agent that have as their primary action the inhibition of the serotonin transporter (SERT)

Fluoxetine was introduced in the US in 1988 and quickly became one of the most commonly prescribed medications in medical practice. The development of fluoxetine emerged out of the search for chemicals that had high affinity for monoamine receptors but lacked the affinity for histamine, acetyl choline, and alpha adrenoceptors that is seen with the tricyclic antidepressants. There are currently six available SSRIs, and they are the most common antidepressants in clinical use. In addition to their use in major depression, SSRIs have indications in GAD, PTSD, OCD, panic disorder, PMDD, and bulimia. SSRIs have fewer side effects than older antidepressants, but they sometimes produce slight nausea or jitters when people first start to take them. Other adverse effect related to the serotonergic effects of SSRIs includes an increase in headaches and insomnia or hypersomnia.

Selective Serotonin–Norepinephrine Reuptake Inhibitors

The SNRIs include venlafaxine, its metabolite desvenlafaxine, and duloxetine. Another SNRI, milnacipran, has been approved for the approved for the treatment of fibromyalgia in the USA but has been studied extensively as an antidepressant. In addition to their use in major depression, other applications of the SNRIs include the treatment of pain disorders including neuropathies and fibromyalgia and in the treatment of generalized anxiety, stress urinary incontinence, and vasomotor symptoms of menopause. SNRIs are chemically unrelated to each other. Venlafaxine's in vivo effects are similar to those of imipramine but with a more favorable adverse effect profile. All SNRIs bind the serotonin (SERT) and

norepinephrine (NET) transporters, as do the TCAs. However, unlike the TCAs, the SNRIs do not have much affinity for the receptors.

Tricyclic Antidepressants

Tricyclics are older than SSRIs and work as well as SSRIs for anxiety disorders other than OCD. They are also started at low doses that are gradually increased. They sometimes cause dizziness, drowsiness, dry mouth, and weight gain, which can usually be corrected by changing the dosage or switching to another tricyclic medication. Tricyclics include imipramine which is prescribed for panic disorder and GAD, and clomipramine which is the only tricyclic antidepressant useful for treating OCD.

5-HT_2 Antagonist

Two antidepressant are thought to as act primarily as antagonists at the 5-HT_2receptor: Trazodone and Nefazodone. Trazodone's structure includes a triazolo moiety that is thought to impart antidepressant effect. Its primary metabolite, m-chlorphenylpiperazine (m-cpp), is a potent 5-HT_2 antagonist. Trazodone was among the most commonly prescribed antidepressant until it was supplanted by the SSRIs in the late 1980s. The most commonly use of trazodone in current practice is as an unlabeled hypnotic, since it is highly sedating and not associated with tolerance or dependence.

Monoamine Oxidase Inhibitors

Monoamine oxidase inhibitors (MAOIs) were introduced in the 1950s but are now rarely used in clinical practice because of toxicity and potentially lethal food and drug interactions. Their primary use now is in the treatment of depression unresponsive to other antidepressants. However, MAOIs have also been used

historically to treat anxiety states, including social anxiety and panic disorder. In addition, selegiline is used for the treatment of Parkinson's disease.

Current MAOIs include the hydrazine derivatives phenelzine and isocarboxazid and the non-hydrazines tranylcypromine, selegiline, and moclobemide. The hydrazines and tranylcypromine bind irreversibly and nonselectively with MAO-A and –B, whereas other MAOIs may have more selective or reversible properties. Some of the MAOIs such as tranylcypromine resemble amphetamine in chemical structure, whereas other MAOIs such as selegiline have amphetamine- like metabolites. As a result, these MAOIs tend to have substantial CNS- stimulating effects.

Azapirone

Buspirone is the first azapirone, a new class of antianxiety drugs. Buspirone mimics the antianxiety properties of benzodiazepines but does not interact with $GABA_A$ receptor. Buspirone relieves anxiety without causing marked sedative, hypnotic, or euphoric effects. Unlike benzodiazepines, the drug has no anticonvulsant or muscle relaxant properties. The mechanism of anxiolytic action is not clearly. It may exert its anxiolytic effects by acting as a partial agonist at brain 5-HT_{1A}receptors, but it also has affinity for brain dopamine D_2 receptors. Buspirone treated patient's show no rebound anxiety or withdrawal signs on abrupt discontinuance. The drug is not effective in blocking the acute withdrawal syndrome resulting from abrupt cessation of use of benzodiazepines or other selective-hypnotics. Buspirone has minimal abuse liability. In marked contrast to the benzodiazepines, the anxiolytic effect of buspirone may take more than a week to become established, marking the drug unsuitable for management of acute anxiety states. The drug is used in generalized anxiety states but is less effective in panic disorders.

3.2 SLEEP DISORDERS[26, 39]

International Classification of Diseases (ICD-9-CM) classifications for sleep disorders

The ICSD consists of four categories. The first category comprises the dyssomnias (i.e., the disorders of initiating and maintaining sleep and the disorders of excessive sleepiness). The second category, the parasomnias, comprises the disorders of arousal, partial arousal, or sleep stage transition, which do not cause a primary complaint of insomnia or excessive sleepiness. The third category, sleep disorders associated with mental, neurologic, or other medical disorders, comprises disorders with a prominent sleep complaint that is felt to be secondary to another condition. The fourth category, proposed sleep disorders, includes those disorders for which there is insufficient information available to confirm their acceptance as definitive sleep disorders.

1. Dyssomnias

A. Intrinsic Sleep Disorders

B. Extrinsic Sleep Disorders

C. Circadian Rhythm Sleep Disorders

2. Parasomnias

A. Arousal Disorders

B. Sleep-Wake Transition Disorders

C. Parasomnias Usually Associated with REM sleep

D. Other Parasomnias

3. Sleep Disorders Associated with Mental, Neurologic, or Other Medical Disorders

A. Associated with Mental Disorders

B. Associated with Neurologic Disorders

C. Associated with Other Medical Disorders

4. Proposed Sleep Disorders

1. Dyssomnias

The dyssomnias are the disorders that produce either difficulty initiating or maintaining sleep or excessive sleepiness. This section is divided into three groups of disorders: intrinsic sleep disorders, extrinsic sleep disorders, and circadian rhythm sleep disorders.

1. A. Intrinsic Sleep Disorders

I. Narcolepsy

Narcolepsy is a disorder of unknown etiology that is characterized by excessive sleepiness that typically is associated with cataplexy and other REM sleep phenomena, such as sleep paralysis and hypnagogic hallucinations.

II. Psychophysiologic insomnia

Psychophysiologic insomnia is a disorder of somatized tension and learned sleep-preventing associations that results in a complaint of insomnia and associated decreased functioning during wakefulness

III. Restless legs syndrome:

Restless legs syndrome is characterized by ascending abnormal sensations in the legs when they are at rest (e.g. when the patient watches television, or before falling asleep) accompanied by a urge to move the legs. It is sometimes present as a genetic disorder with autosomal dominant inheritance. Periodic leg movements during sleep are repeated, abrupt twitching movements of the legs that may persist for minutes to hours.

IV. Obstructive sleep apnea:

Obstructive sleep apnea is characterized by daytime somnolence with frequent nocturnal respiratory pauses and loud snoring. Impaired concentration, decreased performance and headaches are also common.

V. periodic limb movement disorder

Periodic limb movement disorder is characterized by periodic episodes of repetitive and highly stereotyped limb movements that occur during sleep

1. B. Extrinsic sleeps disorder

Extrinsic sleep disorders either originate or develop from causes outside of the body. External factors are integral in producing these disorders. Removal of the external factor usually is associated with resolution of the sleep disturbance unless another sleep disorder develops during the course of the sleep disturbance (e.g., psychophysiologic insomnia may follow removal of an external factor responsible for the development of an adjustment sleep disorder)

I. Environmental sleep disorder is a sleep disturbance due to a disturbing environmental factor that causes a complaint of either insomnia or excessive sleepiness

II. Altitude insomnia is an acute insomnia, usually accompanied by headaches, loss of appetite, and fatigue, which occurs following ascent to high altitudes.

This is a common complaint of mountain climbers or other individuals who sleep in high-altitude environments. Symptoms typically occur within 72 hours of exposure. A disturbance of respiration that appears to be directly related to lack of inspired oxygen is associated with the difficulty in initiating and maintaining sleep

III. Insufficient sleep syndrome is a disorder that occurs in an individual who persistently fails to obtain sufficient nocturnal sleep required to support normally alert wakefulness

Complications: Chronic mood disturbance, documented work-performance deficits, disruption of social functioning, and marital discord may be due to this disorder. Traffic accidents or injury at work may result from loss of normal vigilance

IV. **Inadequate sleep hygiene** is a sleep disorder due to the performance of daily living activities that are inconsistent with the maintenance of good quality sleep and full daytime alertness.

1. C. Circadian Rhythm Sleep Disorders

Circadian rhythm sleep disorders are disorders that are related to the timing of sleep within the 24-hour day. Some of these disorders are influenced by the timing of the sleep period that is under the individual's control (e.g., shift work or time-zone change), whereas others are disorders of neurologic mechanisms (e.g., irregular sleep-wake pattern and advanced sleep-phase syndrome). Some of these disorders can be present in both an intrinsic and extrinsic form; however, their common linkage through chronobiologic, pathophysiologic mechanisms dictates their recognition as a homogeneous group of disorders

I. **Time zone change (jet lag) syndrome** consists of varying degrees of difficulties in initiating or maintaining sleep, excessive sleepiness, decrements in subjective daytime alertness and performance, and somatic symptoms (largely related to gastrointestinal function) following rapid travel across multiple time zones.

Complications: Subjective distress about not sleeping well and social embarrassment because of falling asleep at inappropriate times may occur. Self-treatment, especially

involving the use of large amounts of alcohol, may complicate the clinical picture. Menstrual irregularities in female air crew have been attributed to repeated jet lag

II Delayed sleep-phase syndrome is a disorder in which the major sleep episode is delayed in relation to the desired clock time, resulting in symptoms of sleep-onset insomnia or difficulty in awakening at the desired time.

Complications: Occupational, school, and social dysfunctions of varying degrees are a typical accompaniment of DSPS and are often the major complaint that brings the patient to clinical attention. Absenteeism and chronic tardiness are poorly tolerated in the school and day-shift work settings, and many patients with DSPS come to be regarded as lazy, unmotivated, or mentally ill by their families, peers, and superiors in the business or school environment, even in the context of otherwise good social and mental functioning

2. parasomnia

The parasomnias (i.e., the disorders of arousal, partial arousal, and sleep-stage transition) are disorders that intrude into the sleep process and are not primarily disorders of sleep and wake states per se. These disorders are manifestations of central nervous system activation, usually transmitted through skeletal muscle or autonomic nervous system channels. They are divided into four groups: arousal disorders, sleep-wake transition disorders, parasomnias usually associated with rapid eye movement (REM) sleep, and other parasomnias.

2. A. Arousal Disorders

Arousal disorders are manifestations of partial arousal that occur during sleep. These disorders are the “classic” arousal disorders that appear to be primarily disorders of normal arousal mechanisms.

I. Sleep walking consists of a series of complex behaviors that are initiated during slow-wave sleep and result in walking during sleep.

II. Sleep terrors are characterized by a sudden arousal from slow-wave sleep with a piercing scream or cry, accompanied by autonomic and behavioral manifestations of intense fear

2. B. Sleep-Wake Transition Disorders

Sleep-wake transition disorders are those that occur mainly during the transition from wakefulness to sleep or from one sleep stage to another. Although under some circumstances these disorders can occur within specific sleep stages, this is usually the exception rather than the rule.

I. Sleep starts are sudden, brief contractions of the legs, sometimes also involving the arms and head, which occur at sleep onset

II. Sleep talking is the utterance of speech or sounds during sleep without simultaneous subjective detailed awareness of the event.

2. C. Parasomnias Usually Associated with REM sleep

The parasomnias usually associated with REM sleep have their onset during the REM sleep stage; some of these REM sleep parasomnias do occur during other sleep stages, but this occurrence is rare.

I. Nightmares are frightening dreams that usually awaken the sleeper from REM sleep.

II. **Sleep paralysis** consists of a period of inability to perform voluntary movements at sleep onset (hypnagogic or predormital form) or upon awakening, either during the night or in the morning (hypnopompic or postdormital form)

3. Sleep Disorders Associated with Mental, Neurologic, or Other Medical Disorders

This section lists those disorders that are not primarily sleep disorders but are mental, neurologic, or other medical disorders that have either sleep disturbance or excessive sleepiness as a major feature of the disorder. This listing of mental, neurologic, or other medical disorders is not intended to include all mental and medical disorders that affect sleep or wakefulness. It does include, however, those disorders most commonly associated with sleep symptoms.

3. A. Sleep Disorders Associated with Mental Disorders

Although most mental disorders can have an associated sleep disturbance, the psychoses, mood disorders, anxiety disorders, panic disorder, and alcoholism are presented here because they are commonly seen in patients presenting with sleep complaints and need to be considered in differential diagnoses. Panic disorder, one of the anxiety disorders, has a separate text because this disorder can produce only a sleep complaint.

I. The anxiety disorders are mental disorders that are characterized by symptoms of anxiety and avoidance behavior. The sleep disturbance associated with anxiety disorders is characterized by sleep-onset or maintenance insomnia due to excessive anxiety and apprehensive expectation about one or more life circumstances

II. **Panic disorder** is a mental disorder that is characterized by discrete periods of intense fear or discomfort with several somatic symptoms that occur unexpectedly and without organic precipitation. Panic episodes can be associated with sudden awakenings from sleep.

3. B. Sleep Disorders Associated with Neurologic Disorders

Neurologic disorders that are commonly associated with sleep disturbance are listed and described here. Cerebral degenerative disorders, dementia, and Parkinsonism are commonly recognized neurologic disorders that are associated with sleep disturbance. Epilepsy may be exacerbated by sleep disturbance; epileptic phenomena may occur predominantly during sleep.

I. Dementia refers to a loss of memory and other intellectual functions due to a chronic, progressive degenerative disease of the brain. Sleep disturbance in demented patients is characterized by delirium, agitation, combativeness, wandering, and vocalization without ostensible purpose and occurring during early evening or nighttime hours.

II. **Parkinsonism** refers to a group of neurologic disorders characterized by hypokinesia, tremor, and muscular rigidity. Insomnia is the most common sleep-related symptom in patients with Parkinsonism.

4. Proposed Sleep Disorders

This section lists those disorders for which there is insufficient information available to confirm the unequivocal existence of the disorder (e.g., sub wakefulness syndrome). Most newly described sleep disorders fall under this category until replicated data are available in the literature (e.g., sleep choking syndrome). Some sleep disorders that are controversial as to whether they are the extremes of the normal range or represent a definitive disorder of sleep also are included here (e.g., short and long sleepers).

I. A short sleeper is an individual who habitually sleeps substantially less during a 24-hour period than is expected for a person in his or her age group.

II. **Terrifying hypnagogic hallucinations** are terrifying dream experiences that occur at sleep onset and are similar to, or at times indistinguishable from, those dreams that take place within sleep.

III. **Pregnancy-associated sleep disorder** is characterized by the occurrence of either insomnia or excessive sleepiness that develops in the course of pregnancy the sleep disorder associated with pregnancy usually is biphasic. It typically begins with excessive sleepiness and can progress to severe insomnia. In rare instances, nightmares sleep terrors, and postpartum psychosis may occur.

3.2.1 Pathophysiology of sleep disorders: [40]

The physiological mechanisms regulating the sleep-wake rhythm are not completely known. There is evidence that histaminergic, cholinergic, glutamatergic, and adrenergic neurons are more active during waking than during the NREM sleep stage. Via their ascending thalamopetal projections, these neurons excite thalamocortical pathways and inhibit GABA-ergic neurons. During sleep, input from the brainstem decreases, giving rise to diminished thalamocortical activity and disinhibition of the GABA neurons. The shift in balance between excitatory and inhibitory neuron groups underlies a circadian change in sleep propensity, causing it to remain low in the morning, to increase towards early afternoon (middle siesta), then to decline again, and finally to reach its peak before midnight. As the margin between excitatory and inhibitory activity decreases with age, there is an increasing tendency towards shortened daytime sleep periods and more frequent interruption of nocturnal sleep. Imbalance between the excitatory and inhibitory neurotransmission with more shift toward excitatory system underlies many of the sleeping disorders.

3.2.2 Sedative-Hypnotic Drugs[2]

A **Sedative is** a drug that produces a relaxing, calming effect.

1. Barbiturates

Long acting	Short acting	Ultra-short acting
Phenobarbitone	1. Butobarbitone 2. Pentobarbitone	1. Thipentone 2. Methohexitone

2. Benzodiazepines

- Diazepam
- Flurazepam
- Nitrazepam
- Alprazolam
- Temazepam

3. Newer nonbenzodiazipine

- Zopiclone
- Zolpidem
- Zaleplon

Treatment of insomnia: [41]

Management of insomnia is initially based on whether the individual has experienced a short-term, transient, or chronic sleep disturbance. Transient insomnia resolves quickly and should be treated with good sleep hygiene and careful use of sedative hypnotics. For treating short term insomnia that is lasting up to 3 weeks, non pharmacologic treatment is important and if sedative hypnotics are used, care must be taken to prevent the development of tolerance or dependence. Chronic insomnia requires careful assessment for the medical reason for the insomnia, as well as

nonpharmacologic techniques and careful and less frequent use of sedative-hypnotics to prevent tolerance and dependence.

Non pharmacologic therapy:

- Stimulus control therapy
- Sleep restriction
- Relaxation therapy
- Cognitive therapy
- Paradoxical intention
- Sleep hygiene

Pharmacologic therapy: [2, 42]

Benzodiazepines:

The most commonly used treatment for insomnia has been the benzodiazepines. Benzodiazepines reduce sleep latency and increase total sleep time. Benzodiazepines increase stage 2 sleep while decrease REM, stage 3, stage 4 sleep. As REM sleep is interfered with, increased incidence of rebound insomnia and night mares occurs. Prolonged sedation and cognitive and psychomotor impairment are concerns in the elderly. There is an association between falls and hip fractures and use of benzodiazepines with long elimination half-lives. Drug dependency and abuse may pose a problem if used for a longer period. Commonly used benzodiazepines as hypnotic agents include Estazolam, Flurazepam Quazepam, Temazepam, Triazolam, Nitrazepam, Alprazolam.

Zolpidem:

Zolpidem is a selective GABA Benzodiazepines – 1 receptor agonist. It reduces sleep latency, nocturnal awakenings increases total sleep time and does not appear to have significant effects on next-day psychomotor performance. Treatment is

initiated with 5 mg and can be increased to 10 mg as a daily dose and optimally should not exceed 4 weeks to minimize tolerance and dependence.

Zaleplon:

Zaleplon has rapid onset of action, short half-life of 1 hour. Effective in decreasing time of onset to sleep onset but not for reducing nighttime awakening or for increasing the total sleep time. It does not interfere with stages of sleep and so rebound insomnia and nightmares are in fewer incidences when compared to Benzodiazepines.[42]

Antihistamines:

Antihistamines are effective in the treatment of mild insomnia and are generally safe. diphenhydramine and doxylamine are preferred agents.

Amino acid L-Tryptophan:

Tryptophan is a precursor of serotonin and was once a popular natural sedative. Cases of Eosinophilia-myalgia syndrome removed this product from the market.

Antidepressants:

Antidepressants are alternatives for patient with non restorative sleep who should not receive benzodiazepines, especially those who have depression, pain or a risk of substance abuse. Sedative antidepressants such as amitriptyline, doxepin and nortriptyline are effective for inducing sleep continuity. Trazodone is used for insomnia patients who are prone to substance abuse. It is frequently used in patients with SSRIs and bupropion-induced insomnia in doses of 25 to 75 mg.[42]

Melatonin:

Melatonin is a hormone released by the pineal gland during the night. It is promoted as a sleep aid. Most studies with melatonin are in children with neurological

impairment and in individuals with jet lag. Ramelteon was designed to be a chemical mimic of the endogenous hormone melatonin and is more potent than melatonin and was recently approved for treatment of insomnia characterized by difficulty with sleep onset. It has the distinction of being the only hypnotic prescription that is not a controlled substance.

Valerian:

Valerian is an herbal sleep remedy. Its mechanism may involve inhibition of an enzyme that breaks down GABA.

3.3 Role of Natural herbal treatment in anxiety & sleep disorders:

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years and have served humans well as valuable components of medicines, seasonings, beverages, cosmetics and dyes. Herbal medicine is based on the premise that plants contain natural substances that can promote health and alleviate illness. Herbal drugs or medicinal plants, their extracts and their isolated compounds have demonstrated spectrum of biological activities. Such have been used and continued to be used as medicine in folklore or food supplement for various disorders. Ethno pharmacological studies on such herbs or medicinally important plants continue to interest investigators throughout the world.[43]

Among the **herbal remedies for anxiety& sleep disorders**, notable are

Valeriana wallichii, Nardostachys jatamansi, Geodorum densiflorum, Lippia nodiflora, Baccharis uncinella, Thuja occidentalis, Casimiroa pringlei, Nerium oleander

There are many plants which have not been subjected to a through scientific evaluation. One such plant is *Amomum subulatum* belongs to the family of

zingiberaceae. The seed of the plant not been so far subjected to sedative and antianxiety activity screening. Hence the present study was carried out to explore the sedative and antianxiety activity of various extract of *Amomum subulatum* seeds.

3.4 PHAMACOLOGICAL INVESTIGATION OF *Amomum subulatum* PLANT EXTRACTS

Jafri MA *et* al., (2001) evaluated the gastric antiulcerogenic effect of large cardamom. A crude methanolic extract and its different fractions, viz. essential oil, petroleum ether (60–80°), ethyl acetate and methanolic fractions, were studied in rats for their ability to inhibit the gastric lesions induced by aspirin, ethanol and pylorus ligature. In addition their effects on wall mucus, output of gastric acid and pepsin concentration were recorded. The crude methanolic extract of *A. subulatum* and its fractions, viz. essential oil, petroleum ether and ethyl acetate, inhibited gastric lesions induced by ethanol significantly, but not those which were induced by pylorus ligation and aspirin. However, ethyl acetate fraction increased the wall mucus in pylorus ligated rats. The results suggest a direct protective effect of ethyl acetate fraction on gastric mucosal barrier. The observation of decrease in gastric motility by essential oil and petroleum ether fractions suggest the gastro protective action of the test drug.[44]

Hiroe Kikuzaki *et al*., (2001) evaluated the ethyl acetate-soluble fraction of the fruits of *Amomum subulatum* showed a high free radical-scavenging activity using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method. Four compounds were isolated from the ethyl acetate-soluble fraction, and their structures were ascribed to protocatechualdehyde (1), protocatechuic acid (2), 1,7 bis(3,4-dihydroxyphenyl)hepta-4E,6E-dien-3-one (3) and 2,3,7-trihydroxy-5-(3,4-dihydroxy-

E - styryl)-6,7,8,9-tetrahydro-5H-benzocycloheptene (4) on the basis of spectroscopic evidence. DPPH radical-scavenging activity of these compounds was measured by colorimetric analysis. Compounds 1 and 3 showed stronger activity than natural antioxidants such as α-tocopherol and L-ascorbic acid. Compounds 2 and 4 were comparable to α-tocopherol and L-ascorbic acid.[45]

Anwar Jamal et al., (2005) evaluated the antiulcerogenic activity of seed of Eletarria cardamom and Amomum subulatum Roxb. Their essential oils and petroleum ether soluble fractions were studied in rats for their ability to inhibit gastric lesions induced by aspirin and ethanol. Both the fractions of drugs inhibited formation of gastric lesions significantly. Fractions of small cardamom were found to be better than large cardamom.[46]

The Modulatory effect of spice extracts on iron-induced lipid peroxidation in rat liver by Yadav AS, Bhatnagar D., (2007) reported the inhibition of lipid peroxidation in rat liver homogenate. The activity can be attributed to their polyphenol content, strong reducing power and superoxide radical scavenging activity.[47]

Kapoor IPS *et al*., (2008) evaluated the antioxidant activity of the essential oil and oleoresins against mustard oil by peroxide, *p*-anisidine, thiobarbituric acid, total carbonyl, ferric thiocyanate and the 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging methods. The essential oil showed signifcant activities in all antioxidant assays and contained a high level of total phenolic content; however, oleoresins have been observed as better antioxidants than butylated hydroxytoluene. Further, the essential oil showed 100% inhibition against *Aspergillus flavus* at a 6 µl dose. For other tested fungi, the essential oil and all oleoresins showed good to moderate

inhibitory effects. Hence, these could be used as natural food preservatives, though the essential oil is more active than the oleoresins.[48]

Mihir Y Parmar *et al*., (2009) evaluated the hepatoprotective activity of methanolic extract of *Amomum subulatum* Roxb (Zingiberaceae) seeds was studied against 20 % ethanol (3.76 g/kg/days, p.o for 18 days) induced liver damage in rats. Ethanol produced significant changes in various liver parameters such as functional (thiopentone-induced sleeping time) and physical (increased liver weight and volume). It also increased the biochemical parameters such as serum glutamate oxaloacetic transaminase and glutamate pyruvic transaminase, alkaline phosphatase, total and direct bilirubin, total cholesterol, and triglyceride and decreased total protein along with changes in histological parameters (damage to hepatocytes). Treatment with methanolic extract of *A. subulatum* (100 and 300 mg/kg/day, p.o. for 18 days) and silymarin significantly prevented the functional, physical, biochemical and histological changes induced by ethanol, indicating the recovery of hepatic cells. These results demonstrate that methanolic extract of *A. subulatum* seeds possessed the hepatoprotective activity.[49]

Aneja KR, Radhika Joshi., (2009) evaluated the *in vitro* antimicrobial activity of *Amomum subulatum* and *Elettaria cardamomum* extracts (acetone, ethanol and methanol) of fruit against *Streptococcus mutans, Staphylococcus aureus, Lactobacillus acidophilus, Candida albicans* and *Saccharomyces cerevisiae*. Minimum inhibitory concentrations (MIC) of the extracts were determined against the four selected microorganisms showing zones of inhibition ≥10mm. This study depicts that ethanol and acetone extracts of fruits of *Amomum subulatum* and *Elettaria cardamomum* can be used as a potential source of novel antimicrobial agents used to cure dental caries.[50]

Shukla SH *et al*., (2010) describe analgesic activity of *Amomum subulatum* Roxb. The methanolic extract at dose 100 mg/kg and 300 mg/kg and ethyl acetate extract at dose 200 mg/kg and 400 mg/kg of seeds of plant were investigated for the analgesic activity using hot plate method and writhing method. The analgesic action of both the extracts can be attributed to the blockage of release of endogenous mediators of pain i.e. prostaglandins. It suggests that the plant has some inhibitory action on cyclooxygenase pathway which is involved in synthesis of prostaglandins biosynthesis. Both methanolic and ethyl acetate extract of seeds of *Amomum subulatum* possessed significant ($p<0.001$) analgesic activity.[51]

Alam K *et al*., (2011) explored the anti-inflammatory activity of ethanolic and aqueous extract of fruit of *Amomum subulatum*. Dose of 100 mg/ml and 200 mg/ml of ethanolic and aqueous extract were evaluated for their anti-inflammatory activity against carrageenan induced paw edema in rats. Both the extracts showed anti-inflammatory activity in dose dependant manner as compared with standard drug Diclofenac sodium 100 mg/ml. The data were found statistically significant by using one way ANOVA ($P< 0.001$).[52]

Khare Divya Prakash *et al*.,(2012) from this study the ethanolic and aqueous extracts of leaves of *Amomum subulatum* is evaluated for antioxidant activity by the 1,1Diphenyl -2 picrylhydrazyle (DPPH) free radical scavenging activity, β-carotene bleaching assay and total phenolic contents methods. The ethanolic extract showed significant antioxidant activity. The IC_{50} of ethanolic extract, total phenolic content, and mean antioxidant activity are 8.25±2.0, 11.04±0.2 and 41.2±1.5 µg/ml respectively and that of ascorbic acid was 2.0±0.14 µg/ml whereas BHA was found to be 50.3±0.6 µg/ml. The study showed that the ethanolic extract consumption could exert beneficial effects due to its antioxidant activity.[53]

Vavaiya RB *et al*., (2012) evaluated Antidiabetic activity of *Amomum subulatum* Roxb. Seed in fructose fed metabolic syndrome in rat. Acetone and methanol extracts were assayed for total phenolic contents by UV method. Presence of protocatechuic acid was estimated by HPTLC method. Oral administration of both *A. subulatum* extracts revealed a significant (P<0.001) increment of serum insulin levels, higher reduction in hyperglycemia when compared to the diabetic control rats (P<0.001). The histological studies of the endocrine region of pancreas of diabetic animals revealed that shrinkage of β cells of islets of langerhans. Animals treated with both extracts of *Amomum subulatum*, revealed restoration of β-cells. This activity of acetone and methanolic extract might be due to presence of phenolics like protocatechuic acid.[54]

Bharat Sharma, Neeru Vasudeva., (2016) studied the in vitro antimicrobial activities of leaf extracts in different solvents viz., aqueous, methanol, petroleum ether, ethyl acetate of selected plant *Amomum subulatum* were accessed by agar well diffusion method against various bacterial and fungal strains. The result revealed that methanolic extract has significant potential to inhibit the growth of pathogenic bacterial and fungal strains. Other extracts of leaves were also found to be effective against microbial strains. On the basis of present finding, they concluded that *Amomum subulatum* leaf extracts might be a good candidate in the search for a natural antimicrobial agent. [55]

Gaurav Garg *et al*., (2016) studied the antibacterial potential of cardamom (*Amomum subulatum)* against the enteropathogenic and food-spoiler bacterial strains *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus pumilus*. Bacterial cell membrane integrity was damaged and an increase in the absorbance at 260nm and 280nm was observed after incubation with cardamom

extract. Cardamom extract inhibited the growth of all the bacterial strains tested with MIC of 6.24mg/ml for *E.coli* and 4.16mg/ml for other bacteria. The zone of inhibition observed by well diffusion method in presence of extract equivalent to 33.3mg/ml cardamom was 15-20mm. Cardamom extract had 10.75mg polyphenol/g dry weight. The results indicate good antibacterial activity was found in polyphenol rich cardamom methanol extract.[56]

ANTIANXIETY AND SEDATIVE EFFECT OF OTHER PLANTS EXTRACTS

The research work deals with the screening of ethanol and chloroform extracts of *Pachyrrhizus erosus* seeds for central nervous system (CNS) depressant activity by Mohd Abid *et al*.,(2006) **.** The different activities studied were potentiation of pentobarbitone-induced sleep, test for locomotor activity, and effect on muscle co-ordination, antiaggressive and antianxiety activities. The result of the study reflected that ethanol extract of the seeds (150 mg/kg, p.o) decreased locomotor activity, produced muscle relaxation and showed antianxiety and antiaggressive activity.[57]

The petroleum ether , chloroform , ethanol and water extracts of *Equisetum arvense* stems were evaluated for anti-anxiety activity in mice using elevated plus maze model, Ketamine induced hypnosis and actophotometer was used to evaluate sedative effect with various extracts in mice by Navdeep Singh *et al*., (2011) the results were compared with standard drug diazepam. The ethanolic extract of *E. arvense* (50 and 100 mg/kg) significantly increased the time-spent and the percentage of the open arm entries in the elevated plus-maze model which was comparable to diazepam. Ethanolic extract (100 mg/kg) prolonged the ketamine-induced total sleeping time and decreased the locomotor activity in mice. The results suggest that the ethanolic extract of *E. arvense* seems to possess anxiolytic effect with lower

sedative activity than that of diazepam. The results could be attributed to the flavonoid content of the ethanolic extract.[58]

Monalisa Jena, Swati Mishra., (2013) evaluated the anti anxiety & sedative effects of Ethanolic extract of leaves of *Eclipta alba*(EEEA) in albino rats using thiopental sodium induced sleeping time (TIS), locomotor activity by actophotometer, elevated plus maze test. EEEA was administered in 50,100,200&400mg/kg doses PO. In the TIS time, the extract in a dose of 200 & 400 mg/kg induced the sleep at an earlier stage & prolonged the duration of sleep. The extract at the dose of 400mg increased % of entries & time spent in the open arms significantly. The EEEA also decreased the locomotor activity in the same dose. The EEEA was found to possess both sedative & anxiolytic activity.[59]

3.5 PLANT PROFILE

Amomum subulatum seeds belong to the family of zingiberaceae.

SYNONYM

Cardamom subulatum (Roxb) kuntze.

BOTANICAL CLASSIFICATION [60]

Kingdom: Plantae

Subkingdom: Tracheobionta

Super division: Spermatophyte

Class: Liliopsida

Subclass: Zinziberidae

Division: Mangnoliophyta

Order: Zingiberals

Family: Zingiberaceae

Genus: Amomum

Species: amomum subulatum or amomum costatum

BOTANICAL NAME [60]

Amomum subulatum Roxb.

CLASSICAL& COMMON NAMES [61]

- Ayurvedic: Prithvikaa, Triputta, Bhadrailaa, Sthula, Elaamahati, Brihad-Elaa, Nishkuti.
- Unani: Heel Kalan(Persian); Heel Zakar(Arabic)

VERNACULAR NAME [60]

- Tamil- kattu yelam
- English-large cardamom or greater cardamom
- Hindi-badi elaichi

- Telugu-peddayelaki
- Malayalam-perelam

PLANT PICTURE

Fig.2. Fruit and seeds of *A. subulatum*

Fig.3. Whole plant of *A. subulatum*

HABITAT:

Amomum subulatum is native to the Eastern Himalayas; the main production regions are Eastern Nepal, India (mostly in the tiny union state Sikkim) and Bhutan. More than 50% of the world's harvests are produced in India.[61]

DESCRIPTION:

Amomum subulatumis a tall perennial herb reaching upto 2.5 m in height with creeping rhizomes, several erect leafy shoots bearing oblong-lanceolate large leaves and short penduncled glabrous spikes. The capsules are upto 2.5 cm long, obcordate, brownish, containing many flattened seeds. A native of Himalayas, this plant is now cultivated widely in northeast India.[62]

PHYTOCHEMISTRY: [49, 51]

From the genus Amomum, a number of derivatives and inaccessible metabolites have been reported. By using standard procedures the presence of various

chemical constituents on the plant extracts, were screened. Various metabolites have been reported such as alkaloids, tannins, terpenoids, flavonoids, saponins, steroid and volatile oil (chiefly composed of 1, 8 cineole).

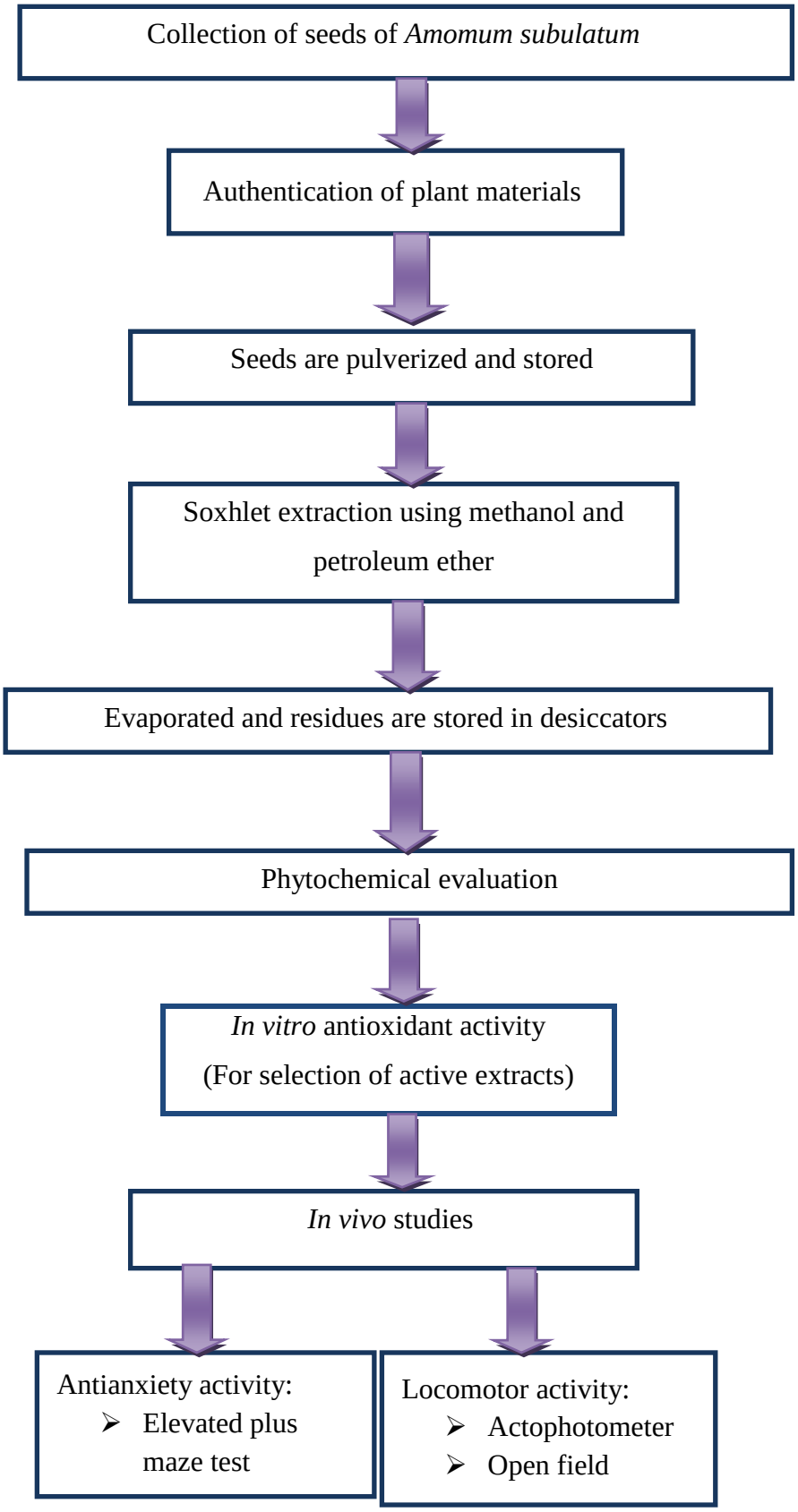
CHEMICAL CONSTITUENTS: [60, 63]

The plant part of *Amomum subulatum* mainly contains the petunidin, glycosides, chalcone, alpinetin, 3, 5-diglucoside, flavonone, subulin, deucocyanidin-3-0-β-D-glucopyranoside and cardamonin. Acid hydrolysis of subulin yield aglycone, subulaurone. Steam distillation of the plant seed yield upto 3% of volatile oil consist of (in%) 1,8 cineole(74.0), limonene(10.0), α-terpineol(2.0), terpin-4-d(2.0), α-pinene(2.0), β-pinene, nerolidol(1.0), myrcene(3.0), sabinene(0.2), γ-lymene(0.2), γ-terpinene(0.2).

TRADITIONAL USE: [60, 61, 64]

The plant is used in Ayurveda, Unani and Siddha. Seeds are astringent, aromatic, hypnotic, cardio tonic, blood purifier, carminative, diuretic, stimulant, stomachi, and are also used in the treatment of neuralgia, gonorrhea, anorexia, eye inflammation,fever,cold,chill,malaria,diarrhea,headache,impotence,dyspepsia and vomiting. It is also serves as antidote to scorpion sting and snakebite. As a gargle used to treat in infection of the teeth and gums; dried powdered seeds given in abdominal pain and in heart and liver disorders; powdered seeds are used in cough, vomiting and rectum diseases. Crushed fruit applied over the area affected by scorpion bite, also given orally.

4. PLAN OF WORK



5. MATERIALS AND METHODS

STUDY CENTRE:

The study was carried out in the Institute of Pharmacology and animal house, Madras Medical College, Chennai-03.

ANIMALS:

The present study was conducted after obtained approval from the Institutional Animal Ethics Committee, Madras Medical College, Chennai-03. The protocol met the requirements of national guidelines of CPCSEA (**PROPOSAL NO: IAEC/MMC/13/2016**) twenty four Swiss albino mice of either sex weight between 20-30g were procured from the animal house of Madras Medical College, Chennai 03.

The animals were maintained under standard laboratory conditions, [temperature (25±1) ºc, relative humidity 55%-65% and normal day/dark circle period [12hr dark/12 hr light]

All the animals included in the study were given standard laboratory feed and water ad libitum. The animals were allowed to acclimatize in the laboratory for one week.

DRUGS AND CHEMICALS

- Diazepam (5mg/kg tablet)
- Methanol and pet. Ether extracts of *Amomum subulatum* seeds.

EQUIPMENTS:

- Actophotometer
- Open field apparatus
- Elevated plus maze
- Mortar and pestle
- Stopwatch

- Orogastric tube and syringe(1 ml)

DIAZEPAM:

3mg/kg body weight for mice was used as standard sedative and antianxiety agents.

5.1 PLANT MATERIALS AND EXTRACTION PROCEDURE:

Seeds of *Amomum subulatum* Roxb. Were collected from local market in Parrys (chennai) and authenticated from Siddha Centre Research Institute, Arumbakkam, Chennai-600106.

CONTINUOUS SOXHLET EXTRACTION OR SOXHELATION: [65]

The preparation of various extracts of *Amomum subulatum* seeds was done in the Department of Pharmacology, Madras Medical College, Chennai-03

The apparatus used for continuous hot percolation process was soxhlet apparatus which consist of three parts:

1. Round bottom flask containing the boiling solvent

2. Soxhlet extractor in which the drug to be extracted is packed. It has a side tube which carries the vapours of the solvent from the flask to be condenser and a siphon tube which siphons over the extract from soxhlet extractor to the flask.

3. A condenser in which the vapour of the solvent are condensed to solvent

PROCEDURE:

The finely divided powder of the *Amomum subulatum* was placed inside a thimble made from thick filter paper, which was loaded into the main chamber of the soxhlet extractor .The soxhlet extractor was placed onto a flask containing the extraction solvent (methanol, petroleum ether).soxhlet was then equipped with a condenser with an inlet and outlet .The fluid was heated to reflux. The vapour travels up the distillation arm and floods into the chamber housing the thimble of solid

material. The condenser ensures that any vapour that cools drips back down into the chamber housing the solid material. The chamber containing the powder slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle was allowed to repeat many times, over 5-6 hours for 4 days. During each cycle, a portion of the non –volatile component dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The non –soluble portion of the extracted solid remains in the thimble, and was discarded.

The seeds of amomum subulatum were dried, powdered and subjected to successive extraction by soxhlet apparatus using methanol and pet. Ether as solvents. After the extraction the products were concentrated by using rotary evaporator by removing solvents. Then the extracts were dried and stored in a suitable air tight container. The extracts can used whenever they required.

Practical yield of Methanol extracts- 3.2%w/w

Practical yield of Petroleum ether extracts- 2.3%w/w

ACUTE TOXICITY STUDY: [49]

Acute toxicity studies have been already performed and it was found to be safe upto 2000mg/kg p.o. Hence, the doses selected for this study were 200 mg/kg p.o.

5.2 PRELIMINARY PHYTOCHEMICAL SCREENING[66, 67]

Phytochemical evaluation is used to determine the nature of Phytoconstituents present in the plant by using suitable chemical tests. It is essential to study the pharmacological activities of the plant. The chemical tests for various

Phytoconstituents in the extracts of *Amomum subulatum* were carried out as described below and the results were recorded.

1] DETECTION OF ALKALOIDS

Dragendorff's reagent:

The substance was dissolved in 5ml of distilled water, to this 5ml of 2M Hcl was added until an acid reactions occurs, then 1ml of Dragendorff's reagent was added and examined for an immediate formation of an orange red precipitate.

Mayer's reagent:

The substance was mixed with little amount of dilute hydrochloric acid and Mayer's reagent and examined for the formation of white precipitate.

Wagner's reagent:

The test solution was mixed with Wagner's reagent and examined for the formation of reddish brown precipitate.

2] DETECTION OF CARBOHYDRATES

Molisch's test:

Filtrate was treated with 2drops of Molisch's reagent in a test tube and 2ml of Conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of a violet ring at the junction indicates the presence of carbohydrates

3] DETECTION OF GLYCOSIDES

Borntrager's test:

A small amount of extract was hydrolysed with hydrochloric acid for few hours on a waterbath and the hydrolysate was extracted with benzene. The benzene layer was treated with dilute solution and observed for formation of reddish pink color.

Legal's Test

The extract was dissolved in pyridine and was made alkaline with few drops of 10% sodium hydroxide and freshly prepared sodium nitroprusside was added and observed for the formation of blue color.

4] DETECTION OF SAPONINS

Foam test

1 ml of sample was taken, to that 2 ml of water was added .The suspension was shaken in a graduated cylinder for 15 minutes. Formation of layer of foam indicates the presence of saponins.

5] DETECTION OF PHYTOSTEROLS

Liebermann-Burchard's Test

1 mg of the extract was dissolved in few drops of chloroform, 3 ml of acedic anhydride and 3 ml of glacial acedic acid. It is warmed and cooled under tap water and drops of concentrated sulphuric acid were added along the side of the test tube. The formation of bluish green colour indicates the presence of steroids.

6] DETECTION OF PHENOLIC ACIDS AND TANNINS

Ferric chloride Test

1ml of sample was taken, to that few drops of 0.1 % ferric chloride was added and allowed to stand for few minutes' formation of Brownish green or blue black coloration indicates the presence of tannins.

1ml of sample was taken; to that 5ml of distilled water and few drops of neutral 5% ferric chloride solution was added. Formation of A dark green colour indicates the presence of phenol.

7] DETECTION OF PROTEINS AND AMINO ACIDS

Ninhydrin Test

To the extract 0.25% of Ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

Biuret Test:

The extracts were treated with 1ml of 10% Sodium hydroxide solution and heated. To this a drop of 0.7% of copper sulphate solution was added. Formation of purplish violet colour indicates the presence of proteins.

8] DETECTION OF FLAVONOIDS

Shinoda Test

To 1 ml of sample, concentrated hydrochloric acid and magnesium chloride was added. Appearance of tomato red colour after few minutes indicates the presence of flavonoids.

9] TEST FOR TERPENOIDS:

Noller's Test

The extracts were warmed with tin and thionly chloride. Pink colouration indicates the presence of terpenoids.

10] DETECTION OF MUCILAGE

The extract is treated with aqueous potassium hydroxide; swelling indicates the presence of mucilage.

5.3 PHARMACOLOGICAL INVESTIGATION

1) ***In-vitro*** **antioxidant activity**

2) ***In-vivo*** **sedative and antianxiety activity**

ANTIOXIDANT

Antioxidant is any substance that, when present at low concentration compared with those of an oxidizing substrate, significantly prevents or delays the oxidation of that substrate. Neutralizes free radicals and prevents cell damage that may lead to cancer. Any nutrient or chemicals that react with and neutralize free radicals to prevent oxidative damage to cells (e.g., oxidation of lipid membranes, DNA damage). A good biological antioxidant is able to accept an unpaired electron to form a free radical intermediate with a relatively long half-life in the normal biological environment. There is a complex intracellular enzymatic antioxidant system, including superoxide dismutase, catalase and enzyme of the glutathione peroxidase family. Nonenzymatic antioxidant includes arginine, vitamins A, C, E, B carotene, glutathione, polyphenols and minerals (selenium and zinc).[69]

5.3.1 *IN-VITRO* ANTIOXIDANT ACTIVITY

Oxidative stress is an imbalance between cellular production of reactive oxygen species and the counteracting antioxidant mechanisms. The brain with its high oxygen consumption and a lipid-rich environment is considered highly susceptible to oxidative stress or redox imbalances. Therefore, the fact that oxidative stress is implicated in several mental disorders including depression, anxiety disorders, schizophrenia and bipolar disorder, is not surprising.[70] Some of the studies suggest that oxidative stress causes anxiety-related behaviors but do not explain the neurobiological pathways underlying the effect of oxidative stress on anxiety

symptoms. Some articles showed the use of antioxidant in the prevention or reduction of high anxiety.[71] This may be due to the reason that GABA receptors activities are enhanced by antioxidants.[72-75] the benzodiazepines act by binding to GABA receptor which is used as both sedative and anti anxiety drug predominantly by patients.

Anti-oxidants like poly phenols and flavonoids are therefore very helpful in reduction of stress factors and free radical formation which inhibits GABA binding activity. It has become evident that flavonoids are able to exert enhancement ofGABA binding activity even at low concentration.[68]

Based on this assumption between the anti-oxidant and the anxiety disorder, the antioxidant activity was conducted for all the extracts using the Nitric oxide radical scavenging activity.

In vitro antioxidant activity was done for the two extracts (methanol and petroleum ether) using nitric oxide scavenging activity.

NITRIC OXIDE RADICAL SCAVENGING ACTIVITY:

Sodium nitroprusside (SNP) was measured according to the method of Marcocci et al. (1994). Briefly, the reaction mixture (5.0 ml) containing SNP (5 mM) in phosphate buffered saline (pH 7.3), with or without the plant extract at different concentrations, was incubated at 25˚C for two hours.The NO. radical thus generated interacted with oxygen to produce the nitrite ion (NO.) which was assayed at 30 min intervals by mixing 1.0 ml of incubation mixture with an equal amount of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediaminedihydrochloride).[76] The absorbance of the chromophore (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was

measured at 546 nm. A control was taken without test compound or standard. Ascorbic acid was taken as a reference antioxidant. All the tests were performed in triplicate and the results averaged. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test samples using following formula.

% Radical scavenging activity = [(Abs) control – (Abs) sample / (Abs) control)] ×100

Where, (Abs) control is the absorbance of the control, and (Abs) sample is the absorbance of the test compound. The IC50 values (concentration of sample required to scavenge 50% of free radicals) were calculated from the regression equation, prepared from the concentration of the samples and percentage inhibition of free radicals.[77-79]

5.3.2 *IN-VIVO* SEDATIVE AND ANTIANXIETY ACTIVITY

I Elevated plus maze in mice were used to evaluate antianxiety activity

II Actophotometer/open field was used to evaluate spontaneous locomotor activity

I EVALUATION OF ANTIANXIETY ACTIVITY IN MICE

1) Elevated plus-maze in mice: [21, 62, 80, 81, 83]

Swiss albino mice divided into 4 groups each group containing 6 animals. The entire animals were freely accessible to water and food.

Drug administration:

Normal saline as vehicle, diazepam as standard and the test compound were administered orally using orogastric tube, 60 minutes prior to introduction of mice into elevated plus-maze.

Group I: control group received normal saline, 10ml/kg per orally

Group II: standard group received diazepam, in the dose of 3mg/kg per orally

Group III: methanol extracts of *Amomum subulatum* seeds, in the dose of 200mg/kg per orally

Group IV: pet. Ether extracts of *Amomum subulatum* seeds, in the dose of 200mg/kg per orally

The antianxiety activity of plant extracts was evaluated using the Elevated plus-maze test. The apparatus consists of two open arms of 18×4cms and two closed arms of 18.4×4×16cms having an open roof, with the plus-maze elevated to 25cms from the floor. Sixty minutes after oral administration of the test and standard drugs, each animal was placed at the center of the maze facing the open arm of the maze. During the 5 min test period, the number of entries and the time spent in open and enclosed arms were recorded. An entry into an arm was defined as the point when the animal places all four paws onto the arm. Following each trial using 70% ethanol thoroughly clean the apparatus. The behavior of the animal scored by using hand operated counters and stop watches. The procedure was carried out in a sound attenuated room; observations were made from an adjacent corner.

The percentage of time spent and entries in the open arm were considered as index.

1. Percentage of open arm entries= (number of open arm entries /number of open arm entries+ number of closed arm entries) ×100

2. Percentage time spent in open arms= (time spent in open arm/time spent in open arm+ time spent in closed arm) ×100

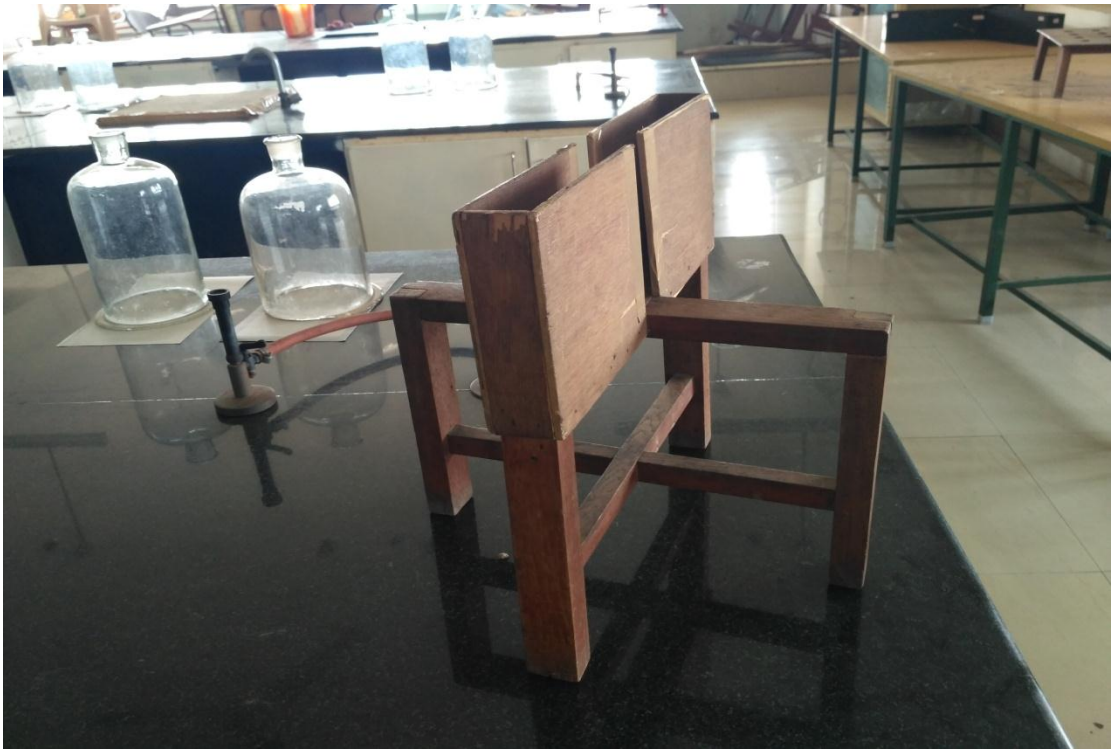


Fig.4. Elevated plus maze apparatus

II EVALUATION OF LOCOMOTOR ACTIVITY IN MICE

Two well established models were used to evaluate the locomotor activity

1. **Actophotometer**
2. **Open field test in mice**

1) Actophotometer: [21, 62]

Albino mice were divided into four groups each containing 6 animals. All the animals were freely accessible to food and water.

Drug administration:

Normal saline as vehicle, diazepam as standard and the test compound were administered orally using orogastric tubes

Group I: control group received normal saline, 10mg/kg per oral

Group II: standard group received diazepam, in the dose of 3mg/kg per oral

Group III: methanol extracts of amomum subulatum seeds, in the dose of 200mg/kg per oral

Group IV: pet. Ether extracts of amomum subulatum seeds, in the dose of 200mg/kg per oral

To evaluate locomotor activity, each mice was placed into an actophotometer and its score of locomotor were measured for a period of 10 minutes.

The locomotor activity was measured using a digital actophotometer which operates on photoelectric cells which are connected in circuit with a counter. When the beam of light falling on the photocell is cut off by the animal, a count is recorded and displayed digitally. Mice were administered with control, standard and test compounds 1 hour prior to the experiment and the response taken for 30 minutes once for 1 hour and were tested for activity score for 10 minutes. Decreased activity score was taken an index of CNS depression.

Fig.5.Actophotometer

2) Open field method [81, 82]

Albino mice were divided into 4 groups each containing 6 animals. All the animals were freely accessible to food and water.

Drug administration

Normal saline as vehicle, diazepam as standard and the test compound were administered orally using orogastric tubes.

Group I: control group received normal saline, 10mg/kg per oral

Group II: standard group received diazepam, in the dose of 3mg/kg per oral

Group III: methanol extracts of amomum subulatum seeds, in the dose of 200mg/kg per oral

Group IV: pet. Ether extracts of amomum subulatum seeds, in the dose of 200mg/kg per oral

The experiment was carried out to determine depressive action of the test drugs on CNS in mice. Open field apparatus is taken for the experiment which consists of 16 squares, each side of square having the length of 24 cm and height 17 cm. the total length is 97 cm on each side of the apparatus. The number of squares visited by the animals was counted for 10 min at 30 and 60 min after oral administration of the test and standard drugs.

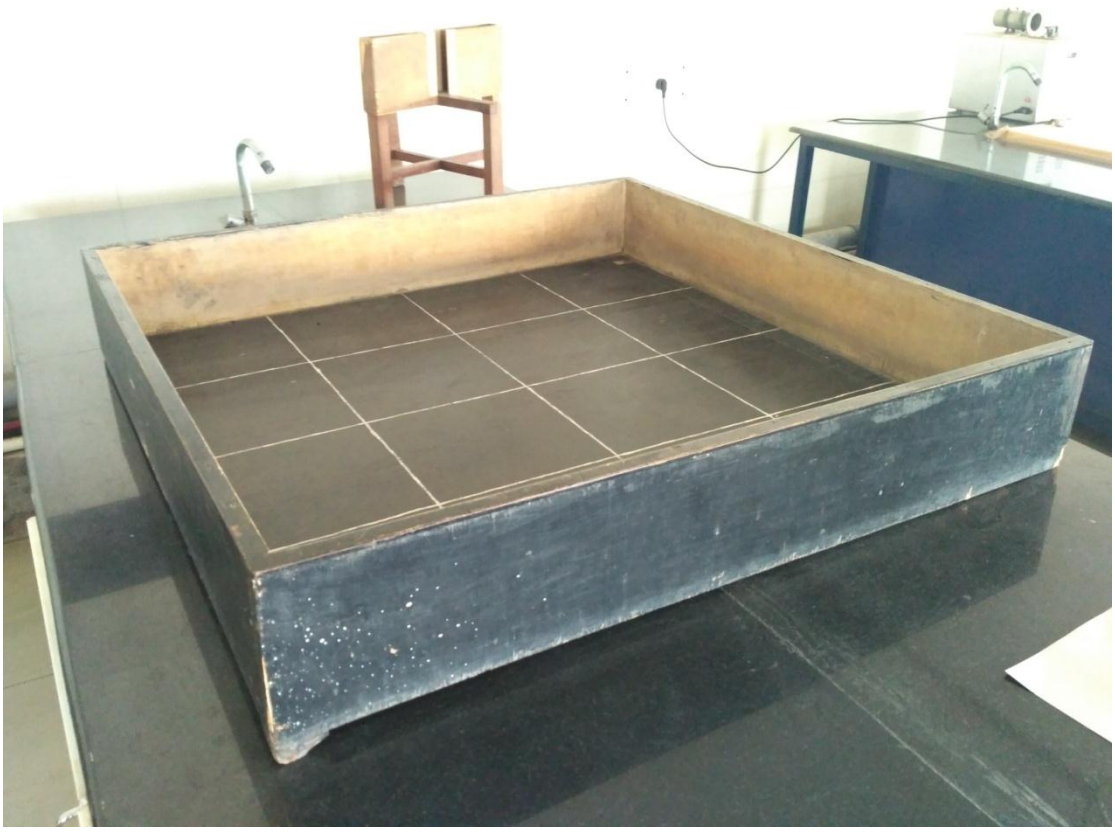


Fig.6. open field apparatus

5.4 STATISTICAL ANALYSIS[84]

All the values were expressed as mean ± SEM. The data was statistically analyzed by one way ANOVA followed by Dunnet's test. One way analysis of variance (ANOVA) was used to correlate the statistical difference between the variables. $P<0.05$ was considered to be significant. Statistical analysis was done using graphpad prism 7 software.

6. RESULTS AND DISCUSSION

6.1 PHYTOCHEMICAL ANALYSIS

The phytochemical analysis of various extracts was performed and presence of flavonoids, phytosterol, carbohydrate, terpenoids, Proteins and amino acids were significant.

Table No. 2: Qualitative Phytochemical analysis

S.No	Tests	Methanolic extracts	Petroleum ether extracts
1	Alkaloids	+	–
2	Carbohydrates	+	+
3	Glycosides	+	–
4	Saponins	–	–
5	Phytosterols	+	+
6	Tannins and phenolic compound	+	–
7	Proteins & amino acids	+	+
8	Flavonoids	+	+
9	Terpenoids	+	+
10	Mucilage	–	+

Note: + indicates presence, - indicates absence

6.2 *INVITRO* ANTIOXIDANT ACTIVITY

Nitric oxide radical scavenging activity

Antioxidant activity was evaluated by using nitric oxide radical scavenging activity method. In this study two extracts are used namely methanol and petroleum ether the % inhibition release of nitric oxide is the parameter considered as their antioxidant activity.

The % inhibition of methanol, petroleum ether and standard ascorbic acid values are mentioned in table 3 and Fig. 7&8. the extracts was able to neutralize nitric oxide in a concentration dependent manner at a concentration range of 200-1000µg/ml and IC_{50} (µg/ml) value has been calculated and was found to be 213µg/ml for ascorbic acid, whereas methanol and petroleum ether extracts showed the IC_{50} – value (µg/ml) of 238.9 and 237 respectively. From the studies the methanol and petroleum ether extracts have significant antioxidant activity in comparison to the standard (ascorbic acid).

Recent studies show that antioxidants are responsible for antianxiety and sedative activity. Hence the extracts are subjected for antioxidant activity and it is found out that all the extracts show antioxidant activity to a certain extent compared to the standard. There forth all the extracts can be further used for in vivo studies.

Table 3: Nitric oxide radical scavenging activity of ascorbic acid, methanol and petroleum ether extracts

S. No.	Concentration(µg/ml)	% of scavenging of NO		
		Ascorbic acid	Methanol extract	Petroleum ether extract
1.	200	49.528	48.58	46.16
2.	400	69.974	57.33	65.41
3.	600	77.857	58.14	70.12
4.	800	93.361	62.58	85.46
5.	1000	96.266	72.27	91.11
IC_{50} (µg/ml)		213	238.9	237

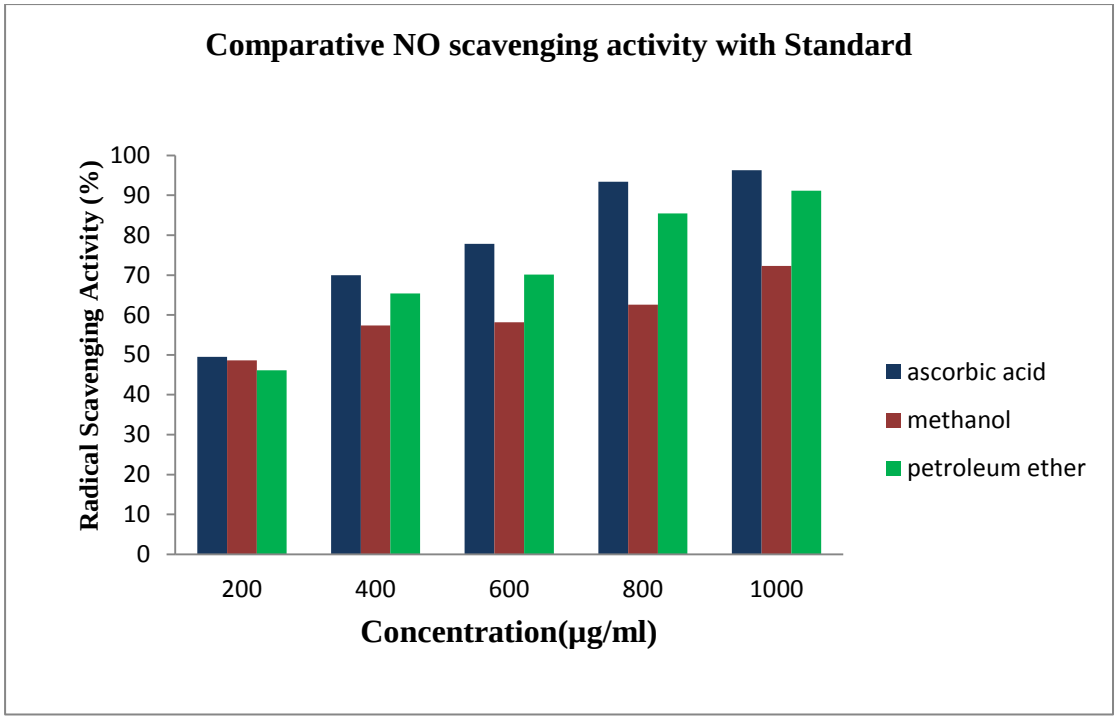


Fig.7. Comparative NO scavenging activity with Standard

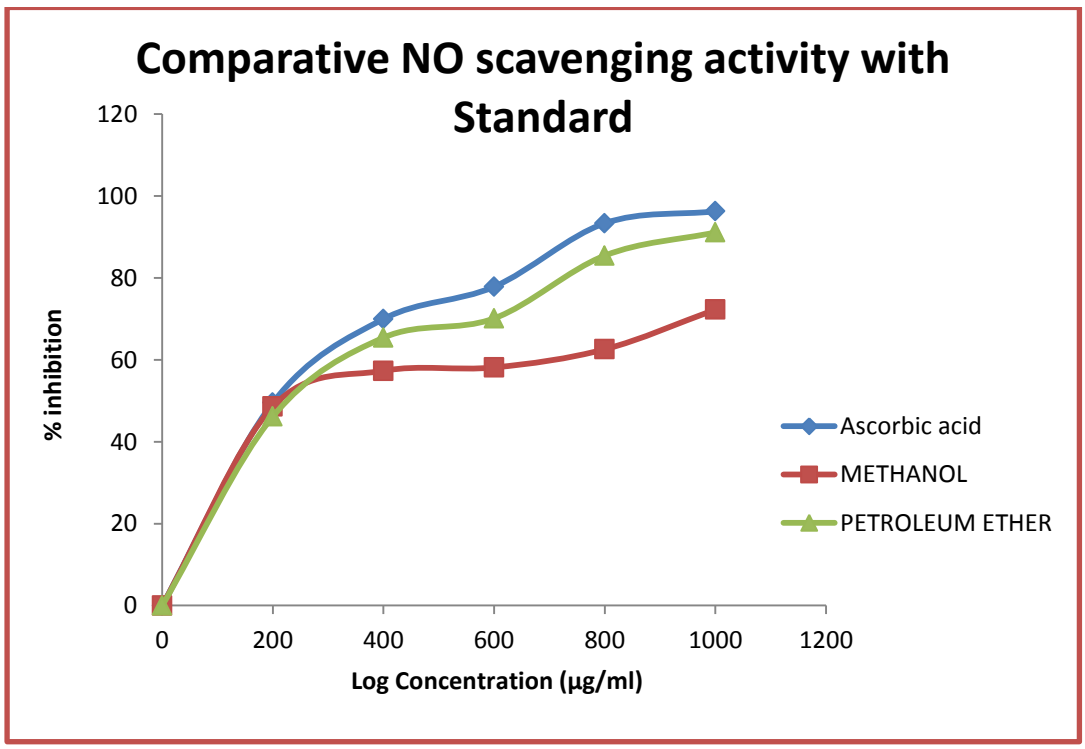


Fig.8. Comparative NO scavenging activity with Standard

6.3 *INVIVO* STUDIES

I ANTIANXIETY ACTIVITY IN MICE USING ELEVATED PLUS MAZE METHOD

The elevated plus maze test is most popular test for evaluation of anxiolytic compounds. The elevated plus maze is highly sensitive to the influence of both anxiolytic and anxiogenic drug acting at the $GABA_A$ benzodiazepine complex. The EPM test is used to evaluate the psychomotor performance and emotional aspects of mice. EPM is considered as one of the well-established model for unconditioned anxiety to detect anxiolytic/anxiogenic like activity by investigating aspects of physiological and pharmacological behavior. In EPM, mice will normally prefer to spend much of their allotted time in enclosed arms. This preference appears to reflect an aversion towards open arms that is generated by the fears of the open spaces. In the EPM test increased number of entries and time spent into the open arm are taken as the index/reliable indicators of decreased anxiety or indicating the anxiolytic-like activity of a compound.[85-87]

The percentage open arms entry with control group was 12.43±1.14 and the percentage time spent in open arms was 7.6±0.67 seconds.

With diazepam 3mgs/kg in group II, the percentage open arms entries was 66.81±0.58 and the percentage time spent in open arm was 69.93±0.41 seconds when compared to control groups.

With test group III i.e. methanolic extract of *Amomum subulatum* seeds 200mg/kg, the percentage of open arm entry was 40.55±0.52 and percentage time spent in open arm was 46.73±0.63 seconds.

With test group IV i.e. petroleum ether extract of *Amomum subulatum* seeds 200mg/kg, the percentage of open arm entry was 50.28±0.9 and percentage time spent in open arm was 56.50±0.39 seconds.

The result with methanol and petroleum ether extracts of *Amomum subulatum* seeds in the dose of 200mg/kg was highly significant when compared to control as shown in table 4 and Fig.9&10

Table 4: Effect of methanol and petroleum ether extracts of *Amomum subulatum* seeds in elevated plus maze

S. No	Treatment group	% open arm entries	% time spent in open arm
1	Control	12.43±1.14	7.6±0.67
2	Diazepam 3mg/kg	66.81±0.58***	69.93±0.41***
3	MEASS in the dose of 200mg/kg	40.55±0.52***	46.73±0.63***
4	PEASS in the dose of 200mg/kg	50.28±0.9***	56.50±0.39***

All values are expressed as Mean±SEM(n=6). One way ANOVA followed by Dunnet's test. *** P<0.001 when compared to control.

MEASS- methanol extracts of *Amomum subulatum* seeds

PEASS- Petroleum ether extracts of *Amomum subulatum* seeds

In the present study, mice treated with methanol and petroleum ether extracts of *Amomum subulatum* seeds at the doses of 200mg/kg produced significant ($P<0.001$) anxiolytic effects. In the EPM test when compared to control as evidenced by increased percentages of both open arm entries and time spent in open arms when compared to control group of animals.

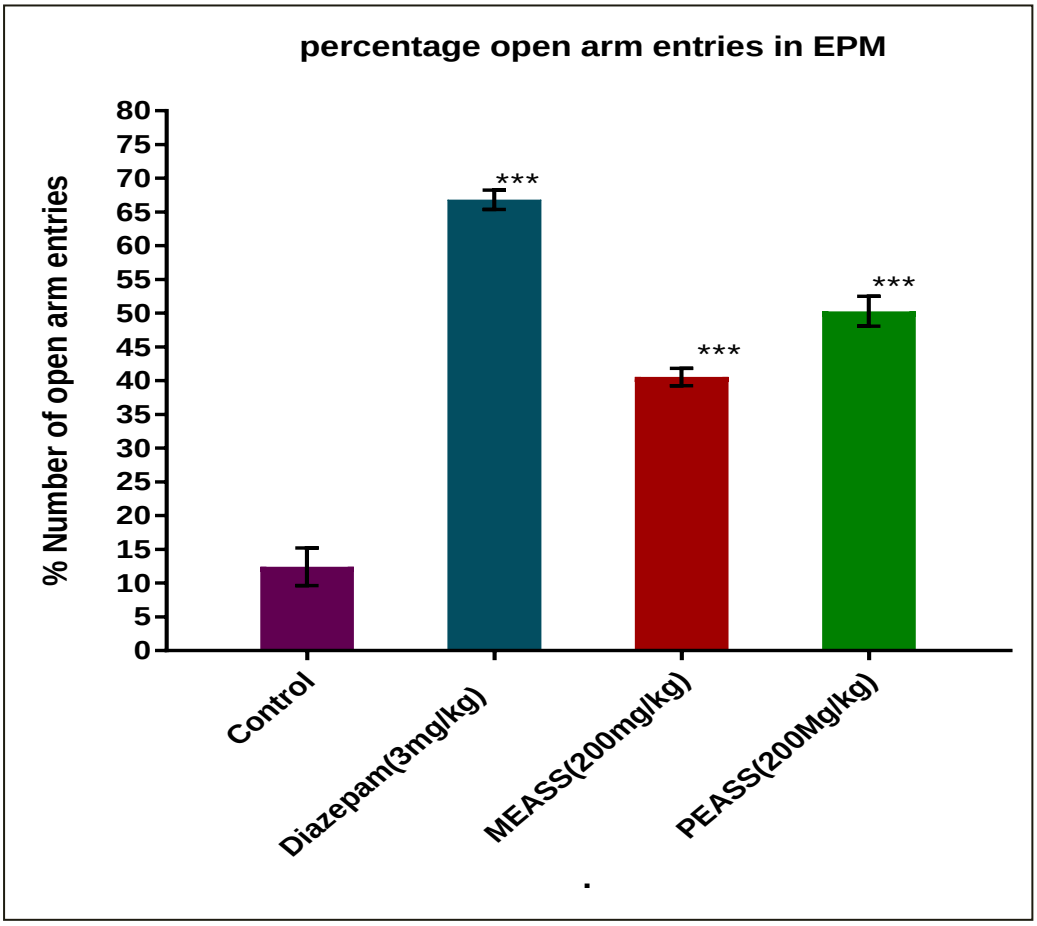


Fig.9.percentage open arm entries in elevated plus maze

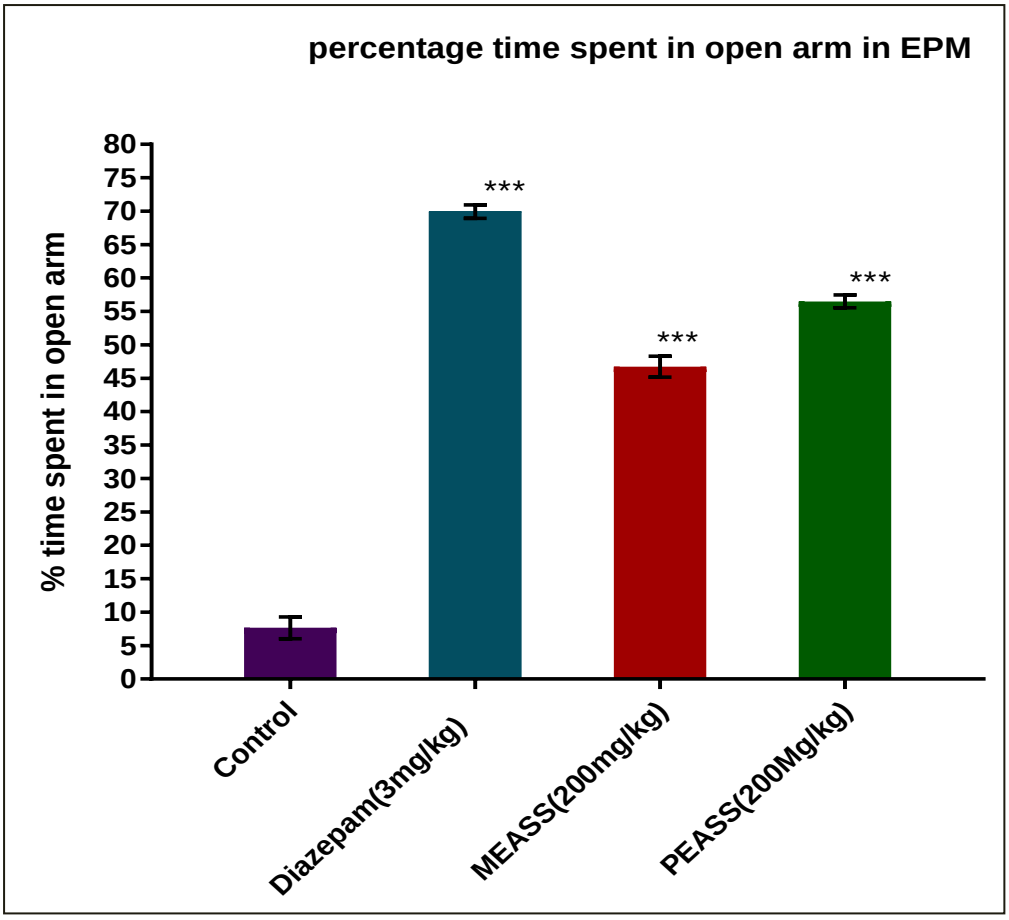


Fig.10.percentage time spent in open arm in elevated plus maze

II SPONTANEOUS LOCOMOTOR ACTIVITY

Spontaneous locomotor activity is considered as an index of alertness and can be helpful to confirm the general depressive activity of any drug. The decrease in motor activity gives an indication of the level of depression of CNS.[88]

Since, an anxiolytic also produces sedation and hypnosis; these activities were evaluated with spontaneous locomotor activity in Actophotometer and open field test.

1) Actophotometer

Actophotometer registers the number of times photo beams of light was broken as the mice moved around inside the cage. Each mice was placed in the centre of the metal cage of actophotometer and its ambulatory activity was measured for 10 minutes.[89]

Locomotor activity is evaluated by using Actophotometer. The spontaneous locomotor activity made by a mouse was noted in control, standard and test group before and 30 and 60 min after the administration of control, standard and test drugs.

The average number of counts at before and 30 and 60 min after the administration of control group of mice was 196.17±2.6, 190.67±2.38 and 189.83±2.5 respectively.

The average number of counts at before and 30 and 60 min after the administration of standard group of mice was 199.17±3.0, 117.5±2.2 and 80.5±2.6 respectively.

The average number of counts at before and 30 and 60 min after the administration of test group (methanol extract) of mice was 192.16±3.0, 162.17±1.6 and 135.83±2.4 respectively.

The average number of counts at before and 30 and 60 min after the administration of test group (pet. Ether extract) of mice was 181.67±2.49, 153±2.9 and 119.3±1.8 respectively.

In actophotometer test, number of cut off (crossing) decreases in test groups compared to control group .The results were shown in table 5 and Fig.11&12

Table 5: Locomotor activity of mice in actophotometer

S.No	Treatment group	Before drug administration	after 30 min	after 60 min
1	Control	196.17±2.6	190.67±2.38	189.83±2.5
2	Diazepam 3mg/kg	199.17±3.03	117.5±2.24***	80.5±2.6***
3	MEASS in the dose of 200mg/kg	192.16±3.07	162.17±1.58*	135.83±2.4***
4	PEASS in the dose of 200mg/kg	181.67±2.49	153±2.9**	119.3±1.8***

All values are expressed as Mean±SEM (n=6). One way ANOVA followed by Dunnet's test. *P<0.05, **P<0.01 and *** P<0.001 when compared to control.

MEASS- methanol extracts of *Amomum subulatum* seeds

PEASS- Petroleum ether extracts of *Amomum subulatum* seeds

In the present study, mice treated with methanol and petroleum ether extracts of *Amomum subulatum* seeds at the dose of 200mg/kg showed A statistically significant (P<0.001) reduction in spontaneous locomotor activity in actophotometer after 60 minutes of administration of standard and test compounds were noted in comparison with control group of animals that signifies sedative activity.

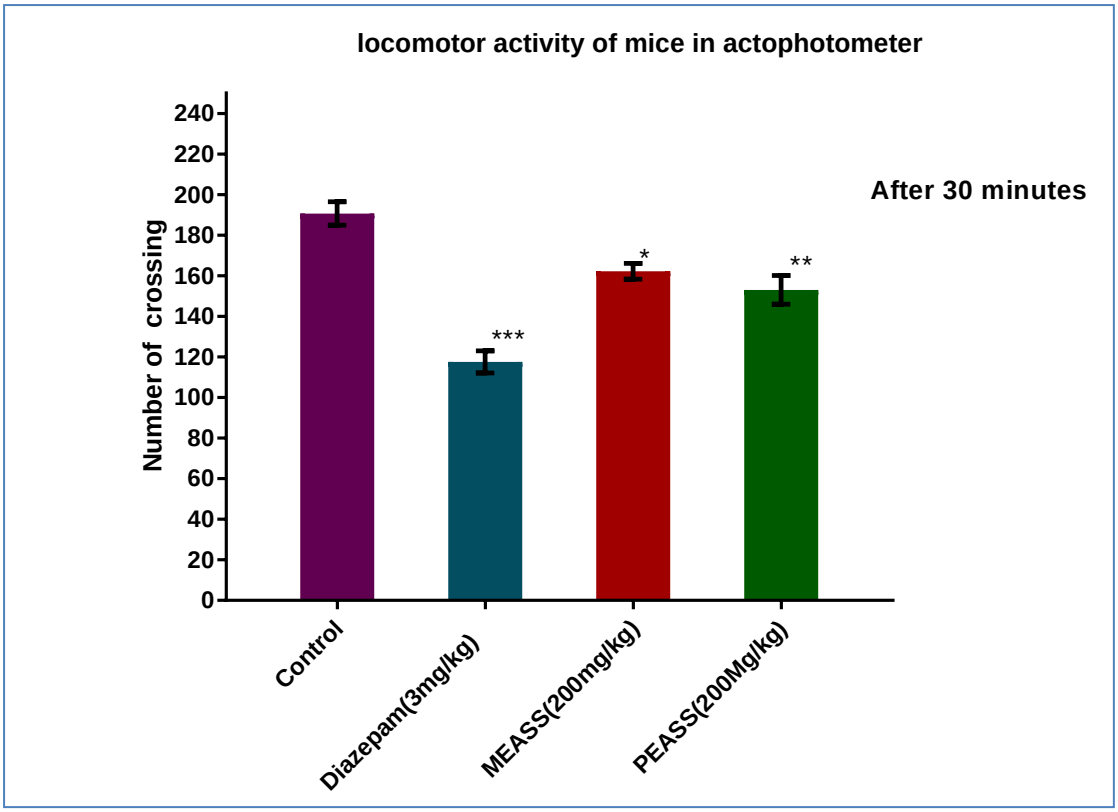


Fig.11.Locomotor activity of mice in actophotometer (After 30 minutes)

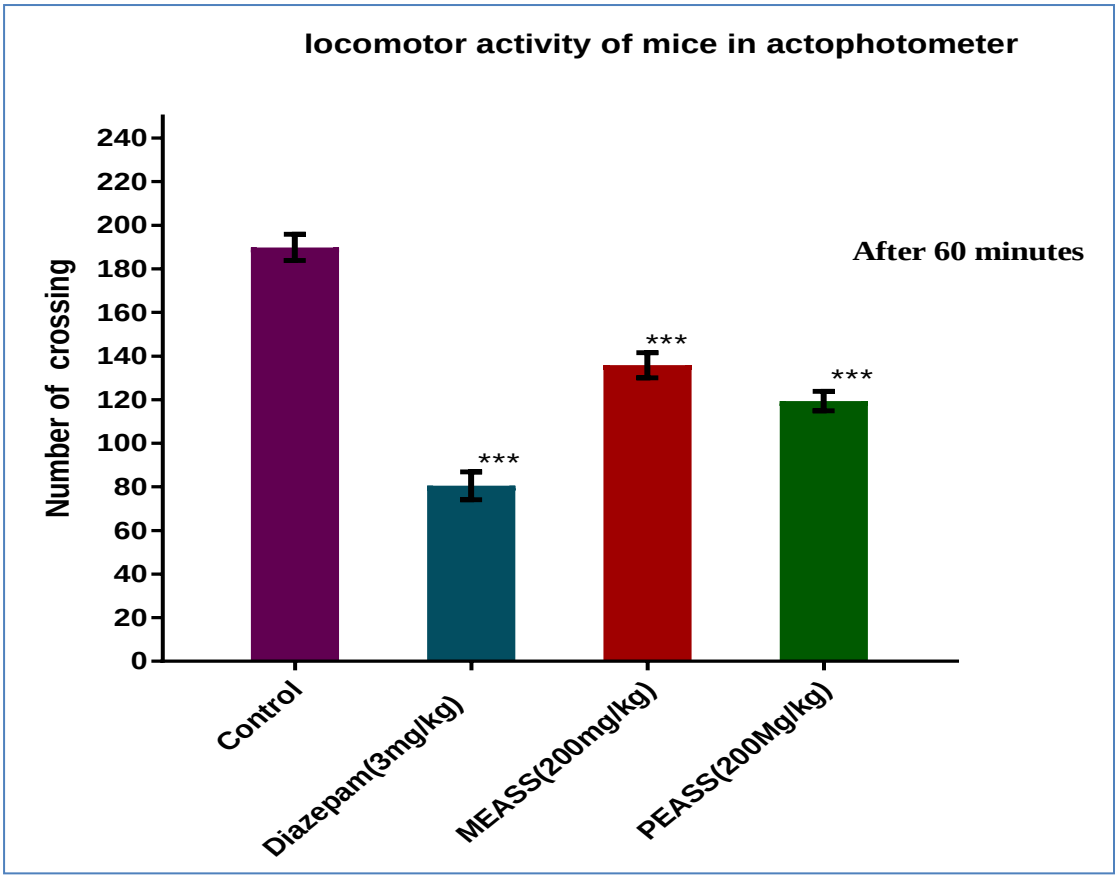


Fig.12.Locomotor activity of mice in actophotometer (After 60 minutes)

2) Open field method

In open field apparatus consists of a square open area which is divided by lines into 16 equal squares. The locomotor activity is determined by manually counting the number of lines crossed by the animal during a fixed time of 10 minutes.

Locomotor activity is evaluated by using open field method. The spontaneous locomotor activity made by a mouse was noted in control, standard and test group before and 30 and 60 min after the administration of control, standard and test drugs.

The average number of counts at before and 30 and 60 min after the administration of control group of mice was 165.83±1.5, 170±1.28 and 164.7±1.26 respectively.

The average number of counts at before and 30 and 60 min after the administration of standard group of mice was 159±1.16, 99.33±2.2 and 72.67±1.22 respectively.

The average number of counts at before and 30 and 60 min after the administration of test group (methanol extract) of mice was 153.17±2.5, 136.17±2.03 and 101.83±1.6 respectively.

The average number of counts at before and 30 and 60 min after the administration of test group (pet. Ether extract) of mice was 156.5±2.9, 117.67±2.8 and 98.5±2.0 respectively.

In the open field test, the number of squares traveled by the mice was suppressed significantly in the test group at dose of 200mg/kg. The CNS depressant activity obtained for the extracts was statistically significant and the results were shown in table 6 and Fig.13&14

Table 6: Locomotor activity of mice in open field

S. No	Treatment group	Before drug administration	after 30 min	after 60 min
1.	Control	165.83±1.5	170±1.28	164.7±1.26
2.	Diazepam 3mg/kg	159±1.16	99.33±2.24***	72.67±1.22***
3.	MEASS in the dose of 200mg/kg	153.17±2.5	136.17±2.03**	101.83±1.6***
4.	PEASS in the dose of 200mg/kg	156.5±2.9	117.67±2.8**	98.5±2.0***

All values are expressed as Mean±SEM(n=6). One way ANOVA followed by Dunnet's test. **P<0.01, *** P<0.001 when compared to control.

MEASS- methanol extracts of *Amomum subulatum*

PEASS- Petroleum ether extracts of *Amomum subulatum* seeds

In the present study, mice treated with methanol and petroleum ether extracts of *Amomum subulatum* seeds at the dose of 200mg/kg showed A statistically significant (P<0.001) reduction in spontaneous locomotor activity in open field after 60 minutes of administration of standard and test compounds were noted in comparison with control group of animals that signifies sedative activity.

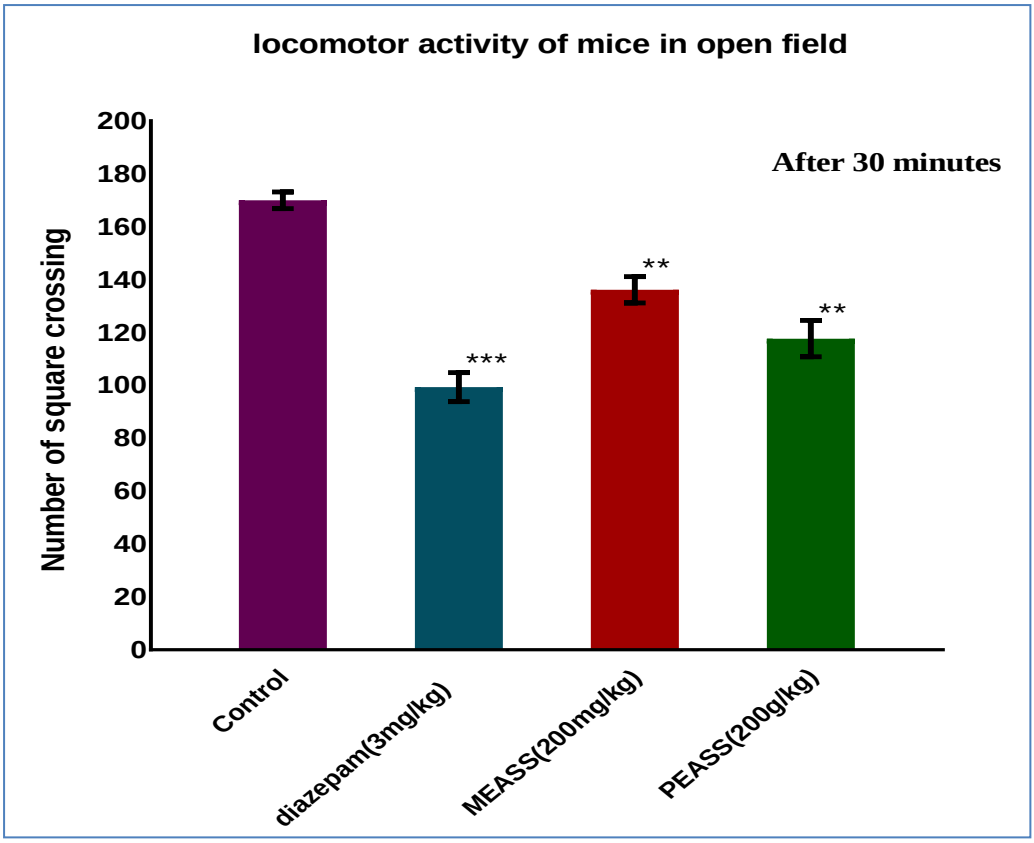


Fig.13.locomotor activity of mice in open field (After 30 minutes)

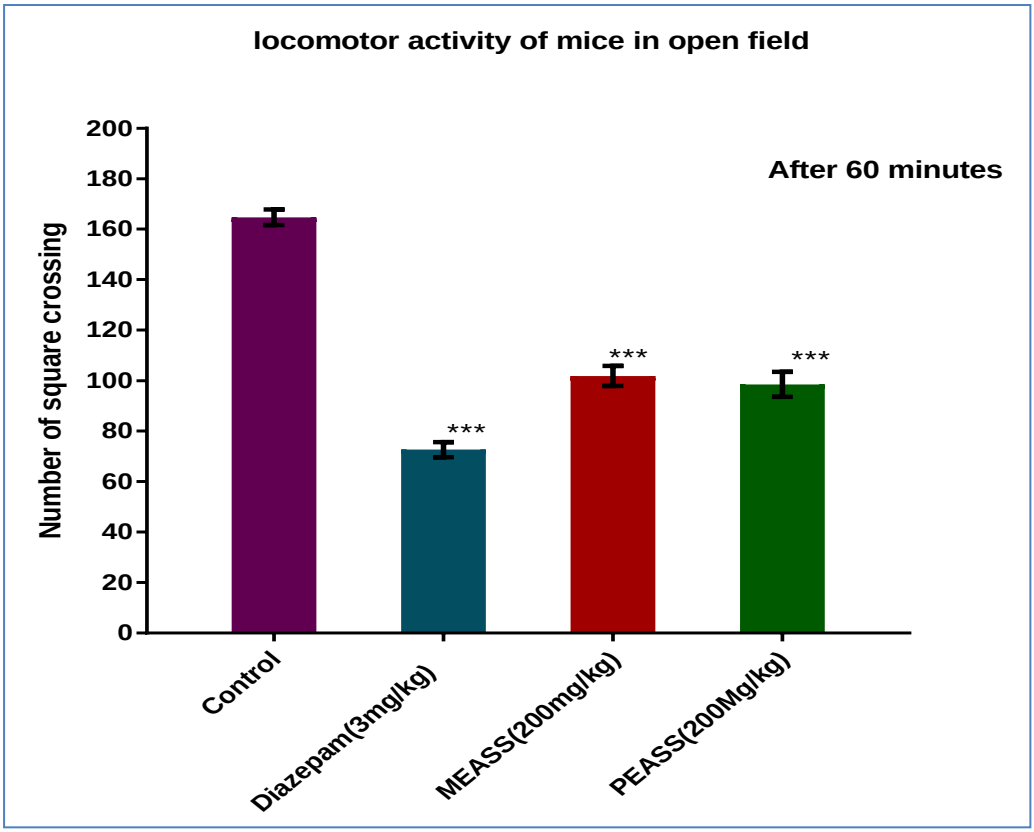


Fig.14.locomotor activity of mice in open field (After 60 minutes)

Despite intensive efforts to develop novel psychiatric drugs for anxiety and depression disorders over the past two decades, all drugs have so far failed to minimize side effects. In this respect, herbal medicines could be an attractive candidate as the therapeutic strategies for this conditions.[90] A major role for plant-derived compounds based on the reported immunomodulatory effects has emerged in recent times and has led to the rigorous scientific examination to determine efficacy and safety.[81]

The result of actophotometer and open field tests showed that the studied plant decreased the frequency as well as the bountifulness of movements. Since the level of excitability of the CNS is measured by locomotor activity, this reduction in spontaneous motor activity that could be considered as the sedative effect of the plant extracts.

The above result showed that crude methanolic and petroleum ether extracts of *Amomum subulatum* plant had strong sedative activity that principally mediated in the CNS by the $GABA_A$ receptor complex. Benzodiazepines and barbiturate drug produce sedative-hypnotic effect at a certain dose due to their interaction with $GABA_A$ receptors which enhances the GABAergic transmission. It potentiates GABA activity, entering chloride into the neuron by prolonging the duration of chloride channel opening. All of these molecular action lead to decrease of neuronal activity that support the following reference substances which possess sedative action.[81]

However, the anxiolytic activity of the methanolic and petroleum ether extracts of *Amomum subulatum* plant was measured by using EPM suggested when the test drug increases open arms entries without altering the total number of arm entries. Diazepam has been used as a standard anxiolytic and also frequently

employed in behavioral pharmacology as a reference compound of potentially anxiolytic-acting substances. But the fractions of plant extract at 200 mg/kg body weight in mice showed significant increase in the percentage of entries into open arms and time spent in the open arms of the maze.

Analyzing the results of present study, it can be inferred that the crude methanolic and petroleum ether extracts of *Amomum subulatum* plant seeds at the dose of 200mg/kg possess strong sedative and anxiolytic activity. Therefore, this extract could be considered for the treatment of anxiety and related neuropsychiatric disorders by conducting further pharmacological studies and mechanism of sedative and anxiolytic action, as well as to identify the active compound(s) responsible for this bioactivity in the animal model

CONCLUSION

Preliminary phytochemical investigation was done for the various extracts of *Amomum subulatum* seeds. It was found to contain flavonoids, phytosterol, carbohydrate, terpenoids and Protein& amino acids, etc…

The present study shows that the methanol and petroleum ether extracts of *Amomum subulatum* seeds have higher antioxidant activity which was determined using the method (Nitric oxide radical scavenging activity).

The study shows that extracts of *Amomum subulatum* seeds at the dose of 200mg/kg has significant antianxiety and sedative activity.

The study also shows that the antianxiety and sedative activity of various extracts of *Amomum subulatum* seeds at the dose of 200mg/kg is less efficacious when compared to the standard drug diazepam at the dose of 3mg/kg.

The results from the experiments confirmed that the methanolic and petroleum ether extract from *Amomum subulatum* seeds posses a strong sedative and anxiolytic potential. However, the further studies in other models and extensive phytochemical analysis are necessary to identify the exact chemical compound and its possible mechanism of action underlying the anxiolytic and sedative effect of *Amomum subulatum* seeds.

SUMMARY

Anxiety related disorders are the most common mental illness and a major cause of disability in the world. Mental disorders have been found to be common, with over a one third of people in most countries reporting them with sufficient criteria to be diagnosed at one point in their life.[91]

Insomnia is a frequent problem that affects people of all the ages around the world. It is a prevalent and potentially serious condition that adversely affects the diurnal functioning, health status and life quality of people of all of age. Stress, anxiety and depression could trigger insomnia.

Despite a phenomenal development of modern drug industry, medicinal plants still constitute an important part of pharmacopoeias in both the developed and developing countries. These plants are important elements of traditional medicine and can be developed as potential drug after scientific validation. However, many of these traditionally used plants have not yet been studied scientifically.[91]

Amomum subulatum is an important medicinal plant of family zingiberaceae commonly known as black cardamom. A number of pharmacological activities have been reported such as antiulcer, antibacterial, antifungal, hepatoprotective, antidiabetic, hypolipidaemic activity, anti-inflammatory and analgesic activity.[92]

The results of Preliminary phytochemical investigation shown the presence of various phytochemical constituents like flavonoids, phytosterol, carbohydrate, terpenoids and Protein& amino acids, etc...

In vitro studies in nitric oxide radical scavenging assay was carried out to select the most active extract. Based on the results both methanol and petroleum ether extract of seeds were selected for further *in vivo* studies.

In-vivo study was done using elevated plus maze test for the evaluation of anti-anxiety activity of the extracts. Anxious mice mostly preferred the closed arm, after administration of the extracts the animal spent more time in open arm due to the anti anxiety activity of the extracts.

Further two well established animal models, actophotometer and open field methods were used to evaluate locomotor activity of mice. The animal treated with methanol and petroleum ether extracts showed decrease in locomotor activity.

The result of the study showed that extracts *Amomum subulatum* seeds at the dose of 200mg/kg has antianxiety and sedative activity that was statistically significant.

Further studies need to be done to elucidate specific mechanism and active principles responsible for its sedative and antianxiety activity.

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9th Aug 2016

CERTIFICATE

Certified that the fruit submitted for identification by S. Deepan Chakkaravarthi, M. Pharm 2nd year, Department of Pharmacology, College of Pharmacy, Madras Medical College, Chennai - 600 003, is identified as *Amomum subulatum* Roxb.

Sasikala Ethirajulu
Sasikala Ethirajulu
Consultant (Pharmacognosy)

P.Sathiyarajeswaran
Assistant Director Incharge

Dr. P. SATHIYARAJESWARAN
Assistant Director (Scientist-2) 1/C
Siddha Central Research Institute (CCRS)
Min. of AYUSH, Govt. Of India
Arumbakkam, Chennai-600 106.

CERTIFICATE

This to certify that Mr.**S.DEEPANCHAKKARAVARTHI**, M.Pharm II year, Institute of Pharmacology, Madras Medical College, Chennai – 600003 had submitted his protocol (Part B Application) IAEC/MMC/13/2016 for the dissertation programme to the Animal Ethical Committee, Madras Medical College, Chennai-600003.

TITLE: EVALUATION OF ANTI-ANXIETY AND SEDATIVE EFFECTS OF VARIOUS EXTRACTS OF *Amomum subulatum* SEEDS IN SWISS ALBINO MICE

The Animal Ethical Clearance Committee experts screened his proposal No: IAEC/MMC/13/2016 have given clearance in the meeting held on 21.11.2016 at Anatomy Demo hall III in Madras Medical College, Chennai – 600003. His study involves only Swiss albino mice.

GOVERNMENT MADRAS MEDICAL COLLEGE * ANIMAL EXPERIMENTAL LABORATORY
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Signature

Dr. S.K. SEENIVELAN, B.V.Sc.,
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SRI RAMACHANDRA UNIVERSITY

(Declared under Section 3 of the UGC Act, 1956)
Accredited by NAAC with 'A' Grade
Porur, Chennai - 600 116.

Certificate

This is to certify that Mr./~~Ms./Prof./Dr.~~ S. Deepan Chakkaravarthi *has participated as a Delegate / Resource person in the CME on* ***"Recent Advances in Research and Therapeutics of Anti Cancer Drugs"*** *held on 20th June, 2015 organized by the Department of Pharmacology, Sri Ramachandra Medical College and Research Institute, Sri Ramachandra University.*

This CME offers SIX Credit Hours.

Dr. Darling Chellathai David
Professor and HOD
Department of Pharmacology

Dr. K.V. Somasundaram
Dean of Faculties

INSTITUTE OF BIOCHEMISTRY

MADRAS MEDICAL COLLEGE

&

RAJIV GANDHI GOVERNMENT GENERAL HOSPITAL, CHENNAI

Certificate

This certificate is awarded to Dr. S. DEEPAN CHAKKARAVARTHI, M.Pharm

of INST. OF PHARMACOLOGY, MMC *for participating in the*

Continuing Medical Education Programme

"NEPHELOMETRY & ITS APPLICATION IN CLINICAL DIAGNOSIS"

as Delegate / Guest Speaker / Faculty

held on 4th June, 2016 at Seminar Hall of Institute of Biochemistry, Madras Medical College, Chennai.

CME Credit points-5, Category-II, awarded by The Tamil Nadu Dr. MGR Medical University , Chennai-32.

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