NEUROPHARMACOLOGICAL PROPERTIES OF ETHANOL EXTRACT OF *Adenopus breviflorus* (ROBERTY) FRUIT IN MICE

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ABSTRACT

Adenopus breviflorus is a perennial climber used locally as an anti-convulsant, sedative and pain-killer in West Africa. Several studies have reported gastrointestinal, reproductive and anti-microbial effects of extracts of *Adenopus breviflorus*, but there is dearth of information on its neurological effect. This study was therefore designed to investigate effect of Ethanol Extract of *Adenopus breviflorus* (EEAB) on central nervous system in mice.

Three hundred gram of air-dried *Adenopus breviflorus* fruits were cold macerated in 70% ethanol and concentrated using rotary evaporator. One hundred and ninety-two Swiss male albino mice (20-25 g) were divided into control (distilled water), EEAB-treated (62.5, 125, 250, 500, 1000, 2000 mg/kg, p.o.) and diazepam-treated (2.0 mg/kg) groups (8 per group) for neurobehavioural studies; fifty-six mice(20-25 g) (8 per group) were used to evaluate mechanisms of action using different antagonists (0.5 mg/kg atropine, 0.5 mg/kg cyproheptadine, 0.2 mg/kg haloperidol, 2.0 mg/kg naloxone, 0.2 mg/kg propranolol and 1.0 mg/kg yohimbine). Sixty-four mice (20-25 g) were divided into control and EEAB-treated groups (62.5, 125, 250, 500, 1000, 2000 mg/kg, p.o.) (8 per group) for Y-maize test. One hundred and sixty mice (20-25 g) (8 per group) were divided into control and EEAB-treated groups (250, 500, 1000, 2000 mg/kg, p.o.) for analgesic study; 32 mice (8 per group) were used to evaluate mechanism of action using naloxone (2 mg/kg). Neurobehavioural studies were carried out using novelty-induced rearing, grooming and locomotor activity in open-field. Head dips rate was determined using hole-board. Effect on memory was performed using Y-maze test. Analgesic activity was carried out using hot plate, tail immersion, formalin and acetic acid-induced writhing tests. Data were analysed using descriptive statistics and ANOVA at $p=0.05$.

The EEAB (250-2000 mg/kg) significantly decreased rearing (86.6±2.1, 84.6±2.7, 62.8±2.4, 23.6±2.8, relative to control 131.2±2.9). Diazepam also significantly decreased rearing (11.0±2.6 relative to control 131.2±2.9). The EEAB (62.5-2000 mg/kg) significantly decreased grooming, locomotor activity and head dips relative to controls (32.8-8.0 versus 36.2±2.6, 77.8-29.8 versus 121.0±3.4 and 15.0-6.8 versus 32.6±1.8 respectively). Diazepam also significantly decreased grooming, locomotor activity and head dips relative to controls (14.4±1.7 versus 36.2±2.6, 49.6±1.3 versus 121.0±3.4 and 6.2±1.1 versus 32.6±1.8

respectively). Three antagooists (2 mg/kg naloxone, 0.2 mg/kg propranolol, 1.0 mg/kg yohimbine) reversed effect of EEAB (2000 mg/kg) on rearing relative to controls (112.4±2.9 versus 131.2±2.9, 113.8±2.8 versus 131.2±2.7 and 110.4±1.3 versus 131.2±2.7 respectively). The EEAB (62.5-2000 mg/kg) significantly increased memory (65.4±1.8, 66.0±2.9, 66.6±1.6, 68.4±2.3, 74.2±2.1, 77.6±2.9 relative to control 58.2±2.7). The EEAB (250-2000 mg/kg) significantly increased reaction time (min) to thermal stimulus of hot plate (2.2±0.2, 2.8±0.4, 2.80±0.4, 3.6±0.3 relative to control 1.0±0.0) and hot water (2.8±0.3, 2.8±0.3, 3.4±0.4, 20.0±0.1 relative to control 1.0±0.0). The EEAB (250-2000 mg/kg) also significantly reduced acetic acid-induced writhes (33.6±1.1, 15.8±1.1, 13.8±0.9, 4.0±0.5 relative to control 41.4±1.8) and decreased paw-licking time (sec) in formalin-induced neurogeoic pain (44.0±2.6, 38.2±2.8, 27.6±2.8, 4.6±0.6 relative to coorol 76.0±3.7) which were all reversed by naloxooe (2 mg/kg).

Adenopus breviflorus had central nervous system depressant and analgesic effects which could be mediated via μ-receptor, β and α_2- adrenergic receptors.

Keywords: *Adenopus breviflorus*, Neurobehavioural effects, Naloxone, Analgesia, Mice.

Word counts: 497

DEDICATION

This work is dedicated to my Creator.

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CERTIFICATION

I certify that Kazeem Olusina Oyedeji carried out this work titled: "Neuropharmacological properties of ethanol extract of *Adenopus breviflorus* (Roberty) fruit in mice" under my supervision in the Department of Pharmacology and Therapeutics, College of Medicine, University of Ibadan.

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LIST OF ABBREVIATIONS

EEAB:	Ethanol extract of *Adenopus breviflorus* fruit
IV:	Intravenous
IP:	Intraperitoneum
PO:	*Per os*
MM:	Millimeter
CM:	Centimeter
KG:	Kilogramme
MG:	Milligramme
ML:	Millilitre

CHAPTER ONE

1.0 INTRODUCTION

An estimated 400 million inhabitants of the world, that is about 80% of world's population, are thought to rely chiefly on traditional medicine, which is largely of plant origin, for their primary healthcare needs (Norman *et al.*, 1985). However, it is widely believed that these valuable medicinal resources in plants are largely untapped because of inadequate scientific technical and commercial infrastructures in developing countries (Olayiwola, 1993).

In recent years, there is a growing interest in herbal therapy. Data on scientific screening of plant extracts, whether crude or purified, appears to be accumulating gradually but steadily. Literatures on antidiarrhoeal (Meite *et al.*, 2009), antimalaria (Nwazue *et al.*, 2013), antidiabetic (Shetti *et al.*, 2012) and contraceptive (Oyedeji *et al.*, 2013) activities of plant – based products support this claim. The major contributory factors to this growing interest include: rising costs of orthodox medications, low therapeutic index of synthetic compounds and the growth incidence of drug resistance (Seed, 2000) among the pathogens especially in developing countries with very weak economic indices. It is thought that the use of plant – derived active principles will offer people access to safe and effective products for the prevention and treatment of diseases through self – medication.

Medicinal plants are of great importance to the health of individuals' and communities. The advent of science into the search for antibiotics largely depends on some of these medicinal plants as raw materials. The medicinal value of these plants lies in some chemical active substances that produce a definite physiological action on the human body. The most important of these chemically active constituents of plants are alkaloid, tannin, flavonoid and phenolic compounds. (Edeoga, 2005).

For many years, medicine had depended exclusively on leaves, flowers and barks of plants; only recently have synthetic drugs come into use. At present, nearly 30% or more of the modern pharmacological drugs are derived directly or indirectly from plants and in homeopathic or ayurvedic medicines, medicinal plants, their parts and extracts dominate the scenes (Hasson *et al.*, 2013).

Drugs acting on the Central Nervous System (CNS) were among the first to be discovered by the primitive human and are still the most widely used group of

pharmacological agents. The CNS acting drugs are invaluable therapeutically, because they can produce specific physiological and psychological effects. From the vast array of *materia medica* of the indigenous system, so many plants have been reported to have activity against CNS disorders and thus act as very useful remedies for the alleviation of human suffering (Suba *et al.*, 2002).

Among the medicinal plants that are in current use in Nigeria is the plant called *Adenopus breviflorus*. The plant is used medicinally as a purgative in Tanganyika and as a vermifuge in Nigeria (Ainslie, 1937). A decoction from the plant is said to be used in Nigeria for headache (Ainslie, 1937). It is used in West Africa for a wide range of gastrointestinal disorders and measles in man. It is also used as an anti – convulsant, sedative and pain killer (Burkill, 1985).

The objectives of this study were to evaluate the phytochemical components, neurobehavioural effect, analgesic and anticonvulsant activities of ethanolic extract of *Adenopus breviflorus* fruit in mice.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Herbalism

Herbalism ("Herbology" or "Herbal Medicine") is use of plants for medicinal purposes, and the study of such use. Plants have been the basis for medical treatments through much of human history, and such traditional medicine is still widely practiced today. Modern medicine recognizes herbalism as a form of alternative medicine, as the practice of herbalism is not strictly based on evidence gathered using the scientific method. Modern medicine, does, however, make use of many plant-derived compounds as the basis for evidence-tested pharmaceutical drugs, and phytotherapy works to apply modern standards of effectiveness testing to herbs and medicines that are derived from natural sources. The scope of herbal medicine is sometimes extended to include fungal and bee products, as well as minerals, shells and certain animal parts (Anonymous, 2013).

2.1.1 History

Archaeological evidence indicates that the use of medicinal plants dates at least to the Paleolithic, approximately 60,000 years ago. Written evidence of herbal remedies dates back over 5,000 years, to the Sumerians, who created lists of plants. A number of ancient cultures wrote on plants and their medical uses. In ancient Egypt, herbs are mentioned in Egyptian medical papyri, depicted in tomb illustrations, or on rare occasions found in medical jars containing trace amounts of herbs (Nunn, 2002). The earliest known Greek herbals were those of Diocles of Carystus, written during the 3rd century B.C, and one by Krateuas from the 1st century B.C. Only a few fragments of these works have survived intact, but from what remains scholars have noted that there is a large amount of overlap with the Egyptian herbals (Robson and Baek, 2009). Seeds likely used for herbalism have been found in the archaeological sites of Bronze Age China dating from the Shang Dynasty (Francis, 2004). Over a hundred of the 224 drugs mentioned in the *Huangdi Neijing*, an early Chinese medical text, are herbs (Unschuld, 2003). Herbs were also common in the medicine of ancient India, where the principal treatment for diseases was diet (Ackerknecht a, 1982). De Materia Medica by Pedanius Dioscorides, a Roman physician, is a particularly important example of such writings. The documentation of herbs and their uses was a central part of both Western and

Eastern medical scholarship through to the 1600s, and these works played an important role in the development of the science of botany.

2.1.2 Modern herbal medicine

The World Health Organization (WHO) estimates that 80 percent of the population of some Asian and African countries presently use herbal medicine for some aspect of primary health care. Pharmaceuticals are prohibitively expensive for most of the world's population, half of which lives on less than $2 U.S. per day (Edgar *et al.*, 2002). In comparison, herbal medicines can be grown from seed or gathered from nature for little or no cost.

Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including opium, aspirin, digitalis, and quinine. According to the WHO, approximately 25% of modern drugs used in the United States have been derived from plants. At least 7,000 medical compounds in the modern pharmacopoeia are derived from plants Among the 120 active compounds currently isolated from the higher plants and widely used in modern medicine today, 80 percent show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived (Fabricant and Farnsworth, 2001).

2.1.3 Clinical tests

In a 2010 survey of the most common 1000 plant-derived compounds, only 156 had clinical trials published. Preclinical studies (tissue-culture and animal studies) were reported for about one-half of the plant products, while 12% of the plants, although available in the Western market, had "no substantial studies" of their properties. Strong evidence was found that five were toxic or allergenic, so that their use ought to be discouraged or forbidden. Nine plants had considerable evidence of therapeutic effect (Cravotto *et al.*, 2010). According to Cancer Research UK, "there is currently no strong evidence from studies in people that herbal remedies can treat, prevent or cure cancer".

The U.S. National Center for Complementary and Alternative Medicine of the National Institutes of Health funds clinical trials of the effectiveness of herbal medicines and provides "fact sheets" summarizing the effectiveness and side effects of many plant-derived preparations.

2.2 Herbs affecting the central nervous system

The followings are some of the specific herbs that affect the central nervous system:

2.2.1 Ephedra or Ma Huang (*Ephedra* spp.)

Ephedra is a medicinal preparation from the plant *Ephedra sinica* (Gurley *et al.*, 1998). A wide variety alkaloid and non-alkaloid compounds have been identified in various species of ephedra. Ephedra has been reported to have stimulant and thermogenetic effects due to the presence of its alkaloids (ephedrine and pseudoephedrine) (Abourasheed *et al.*, 2003). It has also been reported to stimulate the brain, increase heart rate, constrict blood vessels, expand bronchial tubes as well as used by athletes as performance enhancing drug (Bents and March, 2006).

2.2.2 Ginkgo (*Ginkgo biloba* L.)

Ginkgo is a unique species of tree with no living relatives and its extracts have been reported to contain flavonoid, glycosides (Myricetin and quercetin) (Oyama *et. al.*, 1994) and terpenoids.

Its extracts are shown to exhibit reversible, nonselective monoamine oxidase inhibition, as well as inhibition of reuptake at the serotonin, dopamine, and norepinephrine transporters, with all but the norepinephrine reuptake inhibition fading in chronic exposure (Winter and Timinceri, 1999). Ginkgo has been reported to be effective in improving cognition in dementia patients (Weinmann *et. al.*, 2010). It is used in the treatment of schizophrenia (Zhang *et. al.*, 2011). It is believed to have nootropic properties, and is mainly used as memory and concentration enhancer as well as an antivertigo agent (Mahadevan and Park, 2007).

2.2.3 St. John's Wort (*Hypericum perforatum* L.)

Hypericum perforation is a flowering plant and n medicinal plant that is sold over – the – counter as a treatment for depression (Nathan, 2001). Its major chemical constituent, hyperforin, may be used for the treatment of alcoholism (Reutera *et al.*, 2008). Hyperforin has been reported to posses antibacterial properties against Gram – positive bacteria (Cecchini,

2007). *Hypericum perforatum* extract is used as tropical remedy for wounds, abrasions, burns and muscle pain (Reutera *et. al.*, 2008).

2.2.4 Kava (*Piper methysticum* G. Forst.)

Kava is a crop of the western pacific whose roots are used to produce a drink with sedative and anaesthetic properties; it is also used in the treatment of short – term social anxiety (Teschke, 2010). It has also been reported to produce mild euphoria and relaxation as well as a deep dreamless sleep (McDonald, 2000).

2.2.5 Valerian (*Valeriana officinalis* L.)

Valerian is a perennial flowering plant whose consumption has been reported to produce sedative and anxiolytic effects which were suspected to be mediated through the GABA receptor. Valerian is most often used in the treatment of insomnia and anxiety disorders (Hadley and Petry, 2003).

2.2.6 Miscellaneous CNS-Depressant Herbs

There are several botanicals with a folkloric reputation for utility in the treatment of restlessness and sleep disturbances whose efficacy is largely unproven by scientific methods. Some of them are frequently used in combination with other CNS depressants in proprietary herbal products.

Hops, the dried strobiles of *Humulus lupulus* L., is one of these. Although very small amounts of methylbutanol, a compound with sedative effects, have been detected in hops, clinical studies have not verified any such activity of the herb in human subjects (Franco *et al.*, 2012)

Passion flower, the dried above-ground parts of *Passiflora incarnata* L., has an ancient reputation as a sleep aid. However, no controlled clinical trials have ever been conducted on single-herb preparations, so preliminary positive results in the few animal studies have not been verified (Nogan and Conduit,2011).

Lavender, the dried flowers of *Lavandula angustifolia* Mill., yield a volatile oil, the calming and relaxing effects of which are better documented by both empirical medicine and experimental studies. Apparently, its actions are mediated by olfactory receptors, but it may

possibly act directly on the CNS following systemic administration. Suitable research in human subjects is required to verify preliminary observations (Chevallier, 2013).

2.3 *Adenopus breviflorus*

2.3.1 Taxonomy

Adenopus breviflorus (Lagenaria breviflora) belongs to the family of Cucurbitaceae. It is commonly called Wild colocynth in English language, in Ibo language: Ogbenwa and in Yoruba language: Tagiri (Ainslie, 1937) as reported by Burkill (1985) .

Its family is moderately large consisting of about one hundred and ten genera and six hundred and forty species (Evans, 2002) and is represented in Nigeria by twenty-one general. Certain genera, such as Telfaira, Cucurbita and Citrullus are cultivated in Southern Nigeria (Okoli, 1984).

Domain: *Eukaryota*
Kingdom: *Plantae*
Subkingdom: *Viridaeplantae*
Phylum: *Tracheophyta*
Subphylum: *Euphyllophytina*
Infraphylum: *Radiatopses*
Class: *Magnoliopsida*
Subclass: *Rosidae*
Superorder: *Violanae*
Order: *Cucurbitales*
Family: *Cucurbitaceae*
Genus: *Adenopus*
Specific epithet: *breviflorus*
Botanical name: *Adenopus breviflorus*

2.3.2 Description

It is a perennial tendril climber. It would usually lie on the ground for want of something to climb and climbs over shrubs and herbs by means of axillary tendrils. The leaves are simple, alternate and palmately veined (Dutta, 1995). The fruit is a pepo and appear green with cream-coloured narrow blotches measuring 1-5 cm in length and its pulp is bitter

(Kar, 2007). The seeds number up to four hundred in an average – size fruit. The flowers are actinomorphic and nearly always unisexual (Burkill, 1985).

2.3.3 Geographic distribution

The family is a diverse family of plants in the temperate zones but also thrives in hot arid regions of the world (Weihrauch and Teter, 1994). It occurs from Senegal to Western Cameroons and generally widespread in tropical Africa (Ainslie, 1937).

Plate 2.1: *Adenopus breviflorus* fruit specimen (Oyedeji, 2014)

2.3.4 Chemical compounds and nutritional value

Some members of the Cucurbitaceae family are grown for their carbohydrate rich fruits, which contain many oil and protein rich seeds that are discarded as waste during the processing of the fruit. A few examples include watermelon *(citrulus vulgaris)*, muskmelon *(Cucumis melo)*, pumpkin (*Cucurbita pepo)*, sponge gourd *(Luffa cylindrica)* (Weihrauch and Teter, 1994). The seed of *Adenopus breviflorus* is a good source of most of the essential amino acid (Oshodi, 1996).

Phytochemical analysis of the plant showed that it contains variety of chemical compounds ranging from saponins, phenolic acids (Elujoba *et al*., 1991) and cucorbitacins (Wakimoto, 2008). There are several types of cucorbitacins that have been discovered in plants of cucurbitaceae family, with profound pharmacological actions. The primary cucurbitacins are types B and E and they have also been found outside the Cucubitaceae family (Momma *et al*., 2008).

2.3.5 Medicinal uses

The plant is used as a purgative in Tanganyika as well as a vermifuge and cathartic in Nigeria (Ainslie, 1937) as described by Burkill (1985). A decoction from the plant is said to be used in Nigeria for headache (Ainslie, 1937) as described by Burkill (1985). It is used in West Africa for a wide range of gastrointestinal disorders and measles in man. In southern Nigeria its seed-decoction is reportedly given to pregnant women but the purpose is not stated (Dalziel, 1937) as described by Burkill (1985). It is used as an anticonvulsant, sedative and pain killer (Burkill, 1985).

They are commonly used in Nigeria for depilating hides. The fruits are cut up, put in water with lye of wood – ashes and in this hides are left to soak for one or two days. Alternatively the hides are stretched and the inner surface scraped clean, and then the fruit pulp is rubbed in followed by a free application of dry wood – ash. Depilating is done after the folded hide has been steeped for a further day in the lye of wood – ash (Dalziel, 1937) as described by Burkill (1985). The seeds are used in some places to stupefy fish, and in Sudan the seeds are said to be chewed while smoking tobacco to induce a sort of intoxication (Dalziel, 1937) as described by Burkill (1985).

It is used with other medicinal plants as concoctions to aid parturition in humans (Sonaiya, 1999). Livestock farmers employ the fruit extract of the plant for the treatment of Newcastle disease and coccidiosis in animals (Sonaiya, 1999).

2.3.6 Pharmacological properties

It has been reported that the methanol extract of its whole fruit has anti-implantation activity (Elujoba *et al.*, 1985) and abortifacient activity (Elujoba and Hymete, 1986). The ethanol extract of its whole fruit has been reported to have a broad spectrum antibacterial activity (Tomori *et al.*, 2007) as well as anti-oxidant and anti-ulcerogenic effects (Onasanwo *et al.*, 2011).

The ethanol extract of its whole fruit has been reported to cause increase in RBC, TWBC, PCV values as well as caused electrolytes imbalances (Saba et al., 2009a) and spermatotoxic effect in rats (Saba *et al.*, 2009b).

Table 2.1: Common Neurotransmitters

Category	Name	Abbreviation	Metabotropic	Ionotropic
Small: Amino acids (Arg)	Agmatine		α_2 adrenergic receptor Imidazoline receptor	NMDA receptor
Small: Amino acids	Aspartate	Asp	-	NMDA receptor
Small: Amino acids	Glutamate (glutamic acid)	Glu	Metabotropic glutamate receptor	NMDA receptor (co-agonist) Kainate receptor, AMPA receptor
Small: Amino acids	Gamma-aminobutyric acid	GABA	$GABA_B$ receptor	$GABA_A$, $GABA_{A-\rho}$ receptor
Small: Amino acids	Glycine	Gly	-	Glycine receptor, NMDA receptor (co-agonist)
Small: Amino acids	D-serine	Ser	-	NMDA receptor (co-agonist)
Small: Acetylcholine	Acetylcholine	Ach	Muscarinic acetylcholine receptor	Nicotinic acetylcholine receptor
Small: Monoamine (Phe/Tyr)	Dopamine	DA	Dopamine receptor	-
Small: Monoamine (Phe/Tyr)	Norepinephrine (noradrenaline)	NE	Adrenergic receptor	-
Small: Monoamine (Phe/Tyr)	Epinephrine (adrenaline)	Epi	Adrenergic receptor	-
Small: Monoamine (Trp)	Serotonin (5-hydroxytryptamine)	5-HT	Serotonin receptor, all but 5-HT_3	5-HT_3
Small: Monoamine (Trp)	Melatonin	Mel	Melatonin receptor	-
Small: Trace amine (Phe)	Phenethylamine	PEA	Trace amine-associated receptors: hTAAR1, hTAAR2	-
Small: Trace amine (Phe)	N-methylphenethylamine	NMPEA	hTAAR1	-
Small: Trace amine (Phe/Tyr)	Tyramine	TYR	hTAAR1, hTAAR2	-
Small: Trace amine (Phe/Tyr)	Octopamine	Oct	hTAAR1	-
Small: Trace amine (Phe/Tyr)	Synephrine	Syn	hTAAR1	-
Small: Trace amine (Phe/Tyr)	3-methoxytyramine	3-MT	hTAAR1	-

Small: Trace amine (Trp)	Tryptamine		hTAAR1, various 5-HT receptors	
Small: Trace amine (Trp)	Dimethyltryptamine	DMT	hTAAR1, various 5-HT receptors.	
Small: Diamine (His)	Histamine	H	Histamine receptor	-
Neuropeptides	N-Acetylaspartylglutamate	NAAO	Metabotropic glutamate receptors; selective agonist of mGluR3	-
PP: Gastrins	Gastrin		-	-
PP: Gastrins	Cholecystokinin	CCK	Cholecystokinin receptor	-
PP: Neurohypophyseals	Vasopressin	AVP	Vasopressin receptor	-
PP: Neurohypophyseals	Oxytocin	OT	Oxytocin receptor	-
PP: Neurohypophyseals	Neurophysin I		-	-
PP: Neurohypophyseals	Neurophysin II		-	-
PP: Neuropeptide Y	Neuropeptide Y	NY	Neuropeptide Y receptor	-
PP: Neuropeptide Y	Pancreatic polypeptide	PP	-	-
PP: Neuropeptide Y	Peptide YY	PYY	-	-
PP: Opioids	Corticotropin (adrenocorticotropic hormone)	ACTH	Corticotropin receptor	-
PP: Opioids	Enkephaline		δ-opioid receptor	-
PP: Opioids	Dynorphin		κ-opioid receptor	-
PP: Opioids	Endorphin		μ-opioid receptor	-
PP: Secretins	Secretin		Secretin receptor	-
PP: Secretins	Motilin		Motilin receptor	-
PP: Secretins	Glucagon		Glucagon receptor	-
PP: Secretins	Vasoactive intestinal peptide	VIP	Vasoactive intestinal peptide receptor	-
PP: Secretins	Grow with hormone-releasing factor	GRF	-	-
PP: Somatostatins	Somatostatin		Somatostatin receptor	-
SS: Tachykinins	Neurokinin A		-	-
SS: Tachykinins	Neurokinin B		-	-

SS: Tachykinins	Substance P		-	-
PP: Other	Cocaine and amphetamine regulated transcript	CART	Unknown G/G$_o$-coupled receptor[dn]	
PP: Other	Bombesin		-	-
PP: Other	Gastrin releasing peptide	GRP	-	-
Gas	Nitric oxide	NO	Soluble guanylyl cyclase	-
Gas	Carbon monoxide	CO	-	Heme bound to potassium channels
Other	Anandamide	AEA	Cannabinoid receptor	-
Other	2-Arachidonoylglycerol	2-AG	Cannabinoid receptor	-
Other	2-Arachidonyl glyceryl ether	2-AGE	Cannabinoid receptor	-
Other	N*-Arachidonoyl dopamine	NADA	Cannabinoid receptor	TRPV1
Other	Virodhamine		Cannabinoid receptor	-
Other	Adenosine triphosphate	ATP	P2Y12	P2X receptor
Other	Adenosine	Ado	Adenosine receptor	-

Adapted from Wikipedia

2.4 Explorative behavior of rodents in novel environment

The movement and behaviour of first colonists in novel environments have important implications for their survival and long term population establishment (Holway and Suarez, 1999; Taylor and Hastings, 2005). Individuals in unfamiliar locations must become familiar with their environment before establishing home ranges, allowing efficient resource utilization and predator avoidance (Bonhamou 1994; Bums 2005). Invasive species continue to colonize new environments (Vitousek *et al.* 1997), and preventing new invasions is a priority (Puth and Post, 2005). Detecting and intercepting the first invaders remains difficult because of behavioural changes at low density such as enhanced neophobia (e.g. Thorsen *et*

al., 2000), and a better understanding of invader behaviour would assist managers in preventing invasions (Holway and Suarez, 1999; Puth and Post, 2005).

Laboratory studies of exploration in novel environments hypothesize that animals will (i) initially prioritize a systematic exploration (avoiding sites previously visited) and (ii) subsequently settle into regular patterns of patrolling (regularly visiting previously used sites) about a home range. Computer simulations hypothesize that released individuals (iii) first explore around and return to their release site, before establishing new den sites about which they forage, and (iv) choose nearly uncorrelated random-walk search strategies in the absence of specific cues in a homogeneous landscape (Zollner and Lima, 1999).

Unfortunately, there is a lack of data outside the laboratory on animal behaviour in novel environments (Birke and Archer, 1983; Gosling, 2001) and on behavioural changes that occur following relocation from high- to low-density populations and familiar to novel environments (Smith and Morrell, 2007). Such data are notoriously difficult to collect in natural systems, almost exclusively requiring an experimental approach (e.g. Burns 2005). Nonetheless, characterization of postarrival movement and behaviour is critical for understanding processes of species dispersal and colonization (Puth and Post, 2005), and results from field experiments would provide a meaningful test of hypotheses developed in the laboratory (i and ii) and by computer simulation (iii and iv).

2.5 Locomotor activity in rodents

The assessment of rodent unconditioned locomotor behaviour has become one of the most widely used behavioral paradigms to determine the effects of various experimental manipulations ranging from genetic changes, e.g. knockout mice, to pharmacological challenges, e.g. amphetamine-induced locomotor activity. This wide range of applications is based on the fact that unconditioned motor activity probes a variety of behaviors, can be recorded automatically, and can quickly generate an effect profile (Geyer, 1990). In rats, locomotor activity has been used to discriminate drug effects, to elucidate the functional roles of specific neurobiological systems, and to screen drugs for potential psychoactivity.

More recently, locomotor activity has been used as a critical assay to establish the phenotype for various genetic manipulations of mice. Nevertheless, as pointed out by others, the increased use of transgenic and null mutation techniques in the development of animal

models of disorders underlines the importance of selecting the appropriate genetic background due to large strain-dependent differences in behavioral measures. For example, significant inter-strain differences have been demonstrated across twelve strains of inbred mice and seven F1 hybrids that were tested in multiple behavioral tasks including open field locomotor activity, Y-maze activity, auditory and tactile startle reactivity, and prepulse inhibition of startle (Logue *et al.*, 1997). Similarly, large individual differences exist among mice in their behavioral responses to drugs of abuse.

Comparing C57BLr6J and 129rSvJ, and their outcrossed F1 offspring using conditioned place preference, mice of the 129rSvJ strain were found to be hypoactive and very sensitive to the locomotor activating effects of cocaine (Miner *et al.*, 1995). Nevertheless, 129/SvJ did not develop cocaine-conditioned place preference under conditions that yielded significant place preference in C57BL/6J mice. Finally, these strain-dependent behavioral characteristics can be inherited in a non-additive manner. Such results emphasize the importance of investigating the underlying behavioral dimensions in different strains of mice and support the notion of using detailed assessment procedures to adequately quantify the strain-dependent behavioral phenotype.

While locomotor activity has been a widely used behavioral assay, its conceptual basis is complex. A variety of different concepts have been applied to the interpretation of aspects of unconditioned motor behavior of rodents in an open field, including arousal, novelty seeking, diversive and inspective exploration, anxiety, stereotypy, and perseveration. Numerous investigators have recognized the necessity for analyses of multivariate profiles and/or spatio-temporal patterns of motor activity and proposed different approaches to quantify the various components of open field behavior (Eilam and Golani, 1988). Some of these approaches were based on observer ratings, while others have attempted to automate the entire measurement process. The measurement approaches developed in studies in rats are now being applied to phenotypic assessments of mice. Multivariate characterizations of rat locomotor activity, typically including measures of crossings, distance traveled, time in the center vs. the periphery, rearings, and sometimes holepokes, have many advantages over univariate assessments limited to measures of the amount of activity (Paulus *et al.*, 1998). Nevertheless, studies in rats also clearly demonstrate the additional utility of assessments of

sequential patterns of locomotor activity in pharmacological and neurobiological studies, insofar as these measures of the organization of the behavior provide further information regarding the differential effects of various manipulations. The present report describes initial efforts to extend these measures of the organization of locomotor behavior, as developed and validated in studies of rats, to studies of the behavior of mice in an open field.

2.6 Pain

Pain is an unpleasant feeling often caused by intense or damaging stimuli, such as stubbing a toe, burning a finger, putting alcohol on a cut, and bumping the "funny bone". The International Association for the Study of Pain's widely used definition states: "Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP, 1979).

Pain motivates the individual to withdraw from damaging situations, to protect a damaged body part while it heals, and to avoid similar experiences in the future (Lynn, 1984). Most pain resolves promptly once the painful stimulus is removed and the body has healed, but sometimes pain persists despite removal of the stimulus and apparent healing of the body; and sometimes pain arises in the absence of any detectable stimulus, damage or disease (Raj, 2007).

Pain is the most common reason for physician consultation in the United States (Turk and Dworkin, 2004). It is a major symptom in many medical conditions, and can significantly interfere with a person's quality of life and general functioning (Breivik *et al.*, 2008). Psychological factors such as social support, hypnotic suggestion, excitement, or distraction can significantly modulate pain's intensity or unpleasantness (Eisenberger and Lieberman, 2005).

2.6.1 Pathophysiology of Pain

Enormous strides have been made in understanding the neurophysiology and neurochemistry of the systems that transmit and modulate information about noxious events (Stein *et al.*, 2009). Much also is known about acute inflammation, which commonly drives these neural processes. In contrast, relatively little is known about the pathophysiology

underlying most persistent pain syndromes notwithstanding increasing knowledge of the long-term changes that can occur in the nervous system in response to injury.

It is now widely accepted that persistent pain may be sustained by different types of mechanisms. Although the various mechanisms associated with specific syndromes probably overlap, it is likely that there are mechanisms that tend to cluster and become associated with a recognizable constellation of symptoms. Based on clinical observations and therapeutic responses, experts have adopted a classification that broadly divides pain syndromes into nociceptive, neuropathic, psychogenic, mixed, or idiopathic. Although this classification is clearly an oversimplification, it has been found useful in assessment and therapeutic decision making.

2.6.1.1 Nociceptive Pain and Its Mechanisms

Clinically, pain can be labeled "nociceptive" if it is inferred that the pain is due to ongoing activation of the nociceptive system by tissue injury. Although neuroplastic changes (such as those underlying tissue sensitization) are clearly involved, nociceptive pain is presumed to occur as a result of the activation of the sensory system by persistent noxious stimuli, a process that involves transduction, transmission, modulation and perception.

Tissue injury activates primary afferent neurons called nociceptors, which are small diameter afferent neurons (A-delta and C-fibers) that respond to noxious stimuli and are found in skin, muscle, joints, and some visceral tissues (Willis, 2007). Cell bodies of primary afferent neurons are located in dorsal root ganglia (DRG) situated outside the central nervous system (CNS) and the spinal sensory nucleus of cranial nerve V. These pseudounipolar neurons have bifurcated axonal processes, one innervating peripheral cells, tissues, and organs for detection of noxious stimuli, and one that enters the spinal cord to transfer information to the CNS. These fibers have specific receptors that may be responsible for noxious mechanical, chemical or thermal stimuli. Functionally, they transduce temperature, chemical, or mechanical forces via voltage-gated Na channels (Nav) and transient receptor potential channels (TRPV1, TRPA1). The TRP receptors have undergone intensive investigation in the hope of ultimately yielding new therapies for pain (Bevan and Andersson, 2009). The TRPV1 receptor, for example, has been found to be the specific site for reaction to capsaicin, a compound that activates C-fiber nociceptors. Presumably, nociceptive processes linked to

noxious events involving somatic or visceral structures begin with activation of these specific receptors, which leads to transduction, the process by which exposure to a sufficient stimulus produces depolarization of the peripheral nerve.

Nociceptive primary afferent neurons are varied. Most are "silent", active only when suprathresbold stimuli impinge. Some are specific to one type of stimulus, such as mechanical or thermal, but most are polymodal. The number and size of the receptive fields served by each fiber may be small or large, respectively. The meaning of this variability in terms of physiology or disease is not yet known, and research linking different types of nociceptors to disease states, or potential therapeutic targets, is still rudimentary.

Depolarization of the primary afferent involves a complex neurochemistry, in which substances produced by tissues, inflammatory cells and the neuron itself influence transduction. The role of prostaglandins, bradykinin, protons, nerve growth factor, and other compounds provide opportunities for the development of new analgesic drugs.

Once depolarization occurs, transmission of information proceeds proximally along the axon to the spinal cord and then on to higher centers. Complex systems that modulate this input occur at all levels of the neuraxis and are best characterized in the spinal cord. The neuroanatomy, neurophysiology and neurochemistry of these processes are very complex (Apkarian *et al.*, 2005). Transmission across the first central synapse may be influenced by activity in the primary afferent itself and modulatory neural pathways that originate segmentally or at supraspinal levels; further modulation results from processes initiated by glial cells (Apkarian *et al.*, 2005). The neurochemistry of these processes involves an extraordinary array of compounds, including endorphins, neurokinins, prostaglandins, biogenic amines, GABA, neurotensin, cannabinoids, purines, and many others.

The endorphinergic pain modulatory pathways are characterized by multiple endogenous ligands and different types of opioid receptors: mu, delta, and kappa. Endorphins and their receptors are present in various tissues (e.g., immune cells and the gastrointestinal tract), on nerve endings, and in multiple areas of the CNS. They are involved in many neuroregulatory processes apart from pain control, including the stress response and motor control systems. Opioid drugs mimic the action of endogenous opioid ligands. Most of the drugs used for pain are full mu receptor agonists.

Other pain modulating systems, such as those that use monoamines (serotonin, norepinephrine and dopamine), histamine, acetylcholine, cannabinoids, growth factors and other compounds, are targets for nontraditional analgesics, such as specific antidepressants and anticonvulsants. It is likely that entirely novel analgesic compounds will become commercially available in the future as drug development programs target these systems.

Characteristics of Nociceptive Pain

Nociceptive pain can be acute (short-lived, remitting) or persistent (long-lived, chronic), and may primarily involve injury to somatic or visceral tissues. Pain that is inferred to be related to ongoing activation of nociceptors that innervate somatic structures, such as bone, joint, muscle and connective tissues, is termed "somatic pain." This pain is recognized by identification of a lesion and characteristics that typically include a well localized site and an experience described as aching, squeezing, stabbing, or throbbing. Acute pain due to tissue injury and chronic pain due to arthritis are commonly used as examples of somatic pain. Nociceptive pain arising from noxious events in the viscera is referred to as visceral pain. Visceral pain caused by obstruction of hollow viscus is poorly localized and is often described as cramping and gnawing, with a daily pattern of varying intensity. When organ capsules or other structures such as myocardium, are involved, however, the pain usually is well localized and described as sharp, stabbing or throbbing, descriptors similar to those associated with somatic pain (Helm, 2014).

Nociceptive pain of any type can be referred and some referral patterns are clinically relevant. For example, injury to the hip joint may be referred to the knee and bile duct blockage may produce pain near the right shoulder blade (Arendt-Nielsen and Svensson, 2001).

Nociceptive pain may involve acute or chronic inflammation. The physiology of inflammation is complex. With tissue injury sufficient to provoke an inflammatory response, various mediators (e.g., cytokines, chemokines, kinins, tumor necrosis factor-α) can directly activate nociceptors and trigger both peripheral sensitization of nociceptors and central sensitization of dorsal horn neurons. With peripheral and central sensitization, low threshold stimuli that are normally innocuous become painful, and noxious stimuli trigger more intense and prolonged pain responses. Heightened pain sensitivity also may develop in adjacent

uninjured areas. These processes involve the production of substances from tissue and immune cells, and retrograde release of substances from C polymodal nociceptors. The latter process, known as "neurogenic inflammation," may lead to increased tissue levels of substance P, serotonin, histamine, acetylcholine, and bradykinin. These substances then activate and sensitize other nociceptors. Prostaglandins produced by injured tissues also may enhance the nociceptive response to inflammation by lowering the threshold to noxious stimulation (Prisk and Huard, 2003).

2.6.1.2 Neuropathic Pain and Its Mechanisms

Neuropathic pain is the label applied to pain syndromes inferred to result from injury to the peripheral or central nervous system. Although some patients with syndromes labeled neuropathic developed pain following injury to non-neural tissues (all of which are, however, innervated with small fiber nerves or nerve terminals), most follow direct injury to peripheral nerves, roots or CNS structures. Although neuropathic pain may be strongly influenced by ongoing tissue injury, there is an assumption that the fundamental mechanisms sustaining the pain have become independent of any ongoing tissue injury (Jarvis and Boyce-Rustay, 2009).

Neuropathic pain has varied characteristics. Some syndromes, such as pain due to nerve entrapment (e.g., carpal tunnel syndrome) or nerve root trauma (e.g., from an acute herniated disk) are characterized by pain that mimics the quality of somatic pain. Others are associated with "dysesthesia," uncomfortable or overtly painful, unfamiliar sensations such as burning, shock-like or tingling. Neuropathic pain syndromes may be associated with referred pain, allodynia (pain induced by non-noxious stimuli, e.g., light touch), hyperalgesia (increased response to a noxious stimuli), or hyperpathia (exaggerated pain responses following a stimulus, often with after sensation and intense emotional reaction).

Although a further simplification of very complex processes, it may be valuable to subclassify neuropathic pain syndromes based on additional inferences about the primary location of the sustaining mechanisms (Truini and Cruccu, 2006). Some neuropathic pain syndromes are presumed to involve a predominating peripheral generator (e.g., entrapment neuropathies, plexopathies, radiculopathies and polyneuropathies). Other syndromes appear to depend on processes that predominantly reside in the spinal cord, brain or both (e.g., pain due to spinal cord injury or post-stroke pain). Peripheral injuries are known to result in profound

CNS changes, and some syndromes that are presumed to have central generators have been initiated by a peripheral injury (e.g., phantom pain). The shifting of the sustaining mechanisms for the pain to a locus in the CNS is sometimes called "centralization." The clinical relevance of an inference concerning a peripheral vs. central location for mechanisms sustaining pain relates primarily to decisions about invasive interventions. If there is a relatively high likelihood that the pain is related to a correctable peripheral process, then an intervention to ameliorate this (e.g., release of entrapment, or injection or resection of a neuroma) should be considered; if there is a high likelihood that the generator is central, further peripheral intervention should be avoided.

Some of the neurophysiologic and neuroanatomic changes that may occur in peripherally-generated neuropathic pain are understood (Dickinson *et al.*, 2010). Injury to a peripheral nerve axon can result in abnormal nerve morphology. The damaged axon may grow multiple nerve sprouts, some of which form neuromas.

These nerve sprouts, including those forming neuromas, can generate spontaneous activity, which peaks in intensity several weeks after injury. These areas of increased sensitivity are associated with a change in sodium receptor concentration and other processes, and also can occur at sites of demyelination or nerve fiber injury not associated with the severing of axons (Cummins *et al.*, 2007). Unlike normal nerve fibers, these injured regions are more sensitive to physical stimuli, which is clinically associated with tenderness and the appearance of Tinel's sign (i.e., pain or tingling when the area over a nerve is tapped). After a period of time, atypical connections may develop between nerve sprouts or demyelinated axons in the region of the nerve damage, permitting "cross-talk" between somatic or sympathetic efferent nerves and nociceptors. Dorsal root fibers may also sprout following injury to peripheral nerves.

Other changes occur in peripheral nerve fibers that are related to pain and remain poorly characterized. Anterograde and retrograde transport of compounds may shift and messages that are received in cell bodies may turn on specific genes. Trans-synaptic communication may result in functional changes in neurons near those directly affected by an injury.

Changes in peripheral nerve morphology and function can result in peripheral sensitization, which manifests as a lower threshold for signaling, an expansion in receptive fields, and spontaneous activity in primary afferents (ectopic activity). Secondary neuroplastic changes in the spinal cord targets of these peripheral afferent nerves may generate central sensitization, which itself can result in a lowered threshold to subsequent afferent inputs in dorsal horn neurons, spontaneous activity, and expansion of receptive fields. One clinical manifestation of central sensitization is heightened pain sensitivity beyond the site of tissue injury, a phenomenon that is known as secondary hyperalgesia.

The pathophysiologic response to neural injury also involves a complex set of potential interactions among neurons, immune cells, and glial cells. With nerve damage, several mechanisms are triggered that affect primary afferent receptors, their axons and cell bodies, components of the inflammatory/immune response, central neurons and their connections, and glial cells (Dickinson *et al.*, 2010). Many of these processes are adaptive, such as removal of cellular debris, neural changes to counteract a loss of input, and mechanisms that promote survival of neurons, synaptic remodeling, and remyelination. However, many responses are clearly maladaptive, including those that worsen peripheral or central sensitization, ectopic impulse generation, phenotypic switching in pain-carrying fibers, neuronal loss, and disinhibition; these processes can alter gene expression and drive long-lasting neuroplastic changes partly determined by apparent structural reorganization in the brain (Costigan *et al.*, 2009).

In contrast to the still rudimentary understanding of the mechanisms in the periphery and spinal cord that may be involved in neuropathic pain states, there is almost no information about the processes in the brain that induce or sustain centrally-generated pain syndromes. Functional neuro-imaging has demonstrated the extraordinary neuroplasticity of the brain in the setting of both peripheral and central neuropathic pains, but the mechanisms that these changes represent are unknown (Bingel and Tracey, 2008).

2.6.1.3 Psychological and "Idiopathic" Pain Mechanisms

There is an exceedingly complex relationship between the psyche and pain perception (Gamsa, 1994). In some patients, the experience of persistent pain appears to induce disturbances in mood (reactive depression or anxiety), impaired coping (often with

catastrophization), and other processes, which in turn, appear to worsen pain and pain-related distress. Other patients have premorbid or comorbid psychosocial concerns or psychiatric disorders that are best understood as evolving in parallel to the pain. These disturbances also can contribute to the pain experience and drive pain-related distress. Patients with personality disorders, substance use disorders, or mood disorders often are best served by primary treatment for the psychiatric problem at the same time that pain-related interventions are offered. This array of premorbid, comorbid and reactive psychosocial disturbances is individual, complex and may occur in a shifting mix of primary and secondary concerns.

This complexity highlights the importance of psychosocial and psychiatric evaluation as a fundamental aspect of the pain assessment. All patients with persistent pain and all patients with acute pain that has been challenging to control should be evaluated for mood, status of coping and adaptation, family and social support, and a range of psychiatric disorders that may influence the experience of pain or pose targets for therapy.

On occasion, the psychological evaluation yields evidence that the pain itself is predominantly sustained by psychological factors. This phenomenon is known generically as "psychogenic" pain and is subject to the specific diagnoses codified under the Somatoform Disorders in the Diagnostic and Statistical Manual of the American Psychiatric Association (Frances *et al.*, 2003). The evidence for a somatoform disorder must be more than the mere lack of an identifiable physical etiology for the pain. It is very important that patients who have acute or persistent pain without a known physical source not be inappropriately labeled. This may lead to inadequate assessment in the future and therapeutic decisions that are inappropriately skewed; unfortunately, in many quarters, it also lends to stigmatization of the patient and the potential for greater suffering on this basis. When reasonable inferences about the sustaining pathophysiology of a pain syndrome cannot be made, and there is no positive evidence that the etiology is psychiatric, it is best to label the pain as "idiopathic."

In summary, chronic pain is often a multidimensional experience that, like other chronic conditions such as diabetes, hypertension and asthma, may have multiple contributors, including both pathophysiological mechanisms –both nociceptive and/or neuropathic - as well as psycho-behavioral ones. Effective management often demands a

multidimensional assessment and treatment plan that identifies and addresses all the components of the individual's pain.

2.6.2 Pain and inflammatory mediators

Pain receptors, known as nociceptors, are electrical cells that normally respond to painful stimuli. Following trauma or tissue injury, these stimuli are produced by pain signals and inflammatory mediators released by peripheral sensory fibres. This "pain information" is then transmitted to the central nervous system, which defines the location, nature and intensity of the pain (Kandel *et al.*, 2000). Prolonged or intense release of these mediators may result in "hyperexcitable" neurons, causing an exaggerated pain (hyperalgesic) response, or hypersensitivity to pain (Kandel *et al.*, 2000). This hyperalgesic response is often encountered in patients with significant tissue damage, such as that seen in burn patients (Summer *et al.*, 2007). Aside from the exaggerated pain response that is precipitated by mediators, a host of sequential physiological responses occurs because of mediator release which may have a direct effect on wound healing and scarring.

Damaged endothelial cells and platelets release the pain response mediators, adenosine triphosphate (ATP), acetylcholine and serotonin. Local tissue injury induces the release of chemical factors, such as bradykinin, substance P and prostaglandin E2 (PGE2). Mast cell degranulation with local injury results in the release of histamine, cytokines and serotonin. Additionally, tumour necrosis factor-α (TNF-α) and other cytokines, calcitonin gene-related peptide (CGRP), nitric oxide (NO), platelet-activating factor and neurotrophic growth factor (NGF) are mediators that are all involved in the transmission of pain. This "orchestra" of pain-mediating transmitters has varying effects on angiogenesis, inflammation and fibrogenesis. Exaggeration or prolongation of these effects can result in outcomes that vary from non-healing to fibrotic healing and hyperinflammation. The individual effects of these transmitters are elaborated upon in the Table below.

Normally, when pain fibres are activated by brief high-intensity stimuli in situations of limited tissue damage, pain mediators are released and have little effect or a minor positive impact on wound healing (Kawamoto and Matsuda, 2004). This response is considered to be a protective physiological response, where pain mediators contribute to reflexive activity away

from the pain-eliciting cause, and where mediators such as PGE2, serotonin and CGF contribute to controlling and decreasing inflammation and further mediator release.

Table 2.2: The Actions and Effect of Pain Mediators on Wound Healing

Pain mediator	Short – term physiological action	Effect of exaggerated or protracted release on wound healing
Acetylcholine/ATP	Is the transmitter at the neuromuscular junction that connects motor nerves to the muscles. Induces pain.	No obvious effects have been described
Serotonin (5-HT)	Regulates appetite, sleep, memory and learning, temperature, mood, behaviour, muscle contraction and the functions of the cardiovascular system and endocrine system. Affects pain perception. Counteracts the effects of substance P. Increases type IV collagen and fibroblast proliferation. Induces TGF-β. Upregulates B cells and recruits T-cell lymphocytes	Causes a significant increase in fibroblast proliferation. High serotonin levels, in conjunction with lipid peroxidation in the cell membranes, as well as the release of other inflammatory mediators, disturbs vascular permeability and leads to oedema. It also disturbs collagen synthesis and prolongs inflammation.
Substance P	Transmits pain from the sensory neurons to the central nervous system and converts the message into a sensation of pain, exerts a mitogenic effect on the endothelial cells and fibroblasts. Induces neovascularization and vasodilation by stimulating histamine release from the mast cells, leukocyte release, the release of cytokines by macrophages and NO by endothelial cells.	The enhanced release of substance P levels in the lungs and blood could be a critical factor that leads to the development of systemic inflammation and pathogenesis of local burn injury-induced distant lung organ damage due to overstimulation of the C-fibres. Altered substance P levels may contribute to impaired cutaneous healing responses associated with diabetes mellitus or hypertrophic scar formation.
PGE2	Primarily causes fever and vasodilation. Stimulation occurs during inflammation by COX-2. PGE2 can induce angiogenesis and wound healing by enhancing the expression of basic fibroblast growth factor.	PGE2 has been shown to decrease fibroblast proliferation, inhibit collagen synthesis, and enhance the expression of MMPs. Increased levels decrease tendon contraction and healing.
Bradykinin	Is a potent vasodilator, causes contraction of nonvascular smooth muscle, increases vascular permeability and is involved in the mechanism of pain. Response is mediated by β1 receptor expressed with tissue injury and inflammation, thought to play a role in chronic pain. The β2	Stimulates the release of other mediators. IL-1, TNF-α, PAF and substance P. Collagen contraction is mediated by the β2 receptors. Bradykinin augments fibroblast-mediated collagen contraction. In excess, it is likely that bradykinin may play an important role in

	receptor participates in bradykinin's vasodilatory role.	fibrotic processes.
Histamine	Is released from mast cells in response to substance P. It dilates post-capillary venules, activates the endothelium and increases blood vessel permeability. This leads to local oedema (swelling), warmth, redness and the attraction of other inflammatory cells to the site of release. It also irritates nerve endings, leading to itching or pain.	Large-dose histamine release from the mast cells may play a role in abnormal collagen formation. It enhances the formation of collagen by fibroblasts, elevated in the plasma of patients who develop hypertrophic scars.
TNF-α	Is produced by macrophages, and stimulates angiogenesis and the synthesis of collagen and collagenase. TNA-α is a mitogen for fibroblasts. The primary role of TNF is to regulate the immune cells. TNF is able to induce apoptotic cell death, induce inflammation, and inhibit tumorigenesis and viral replication. It induces overall neuronal hyperexcitability, leading to neuropathic pain.	Elevates levels in nonhealing wound fluids, in comparison to fluids from healing wounds. Affects the deposition of connective tissue. Stimulates the synthesis of MMP, while simultaneously decreasing the synthesis of TIMPs. Levels decrease substantially as the wound heals. There is correlation between nonhealing wounds and increased levels of TNF-α.
Cytokines	Act as intercellular mediators that regulate the functions and differentiation of neighbouring cells. Are produced in response to disease, inflammation, or tissue damage. Include ILs, interferons, TNFs, growth factors and chemokines.	Are mainly pro-inflammatory in excess. Also increase activation of complement, neutrophil chemotactic factors and fibrinopeptides, all inducing proinflammatory responses.
ICGRP	Proposed to contribute to pain transmission and inflammation. Promotes wound healing and shortens healing duration. Induces aggregation of epithelial stem cells towards the wound border or migration to the granulation tissues. CGRP-β form is found in the keratinocyte cells of skin and may cause pain. CGRP is a potent arterial and venous vasodilator.	High levels of substance P or CGRP in scars have been associated with the scar being hypertrophic. Causes blushing, migraines, systemic hypotension, plasma leakage and an overall proinflammatory effect.
NO	NO enhances the transendothelial cell migration of monocytes and improves the proliferation and migration of vascular smooth muscle cells. In the early stages of wound healing, NO is involved in the upregulation of the cytokine cascade, serving as a chemoattractant for immune regulatory cells. Later, it is involved in the transition from the inflammatory phase to the proliferative phase of wound healing.	Large amounts of NO synthesis, as seen in sterile inflammation, can impair wound healing by impairing collagen production. At higher concentrations, NO is cytostatic to the endothelial cells, smooth muscle cells, hepatocytes and fibroblasts.

PAF	PAF is produced in tissues after injury and can increase expression of metalloproteinases-1 and -9 and the urokinase plasminogen activator, as well as proteases involved in the degradation of the extracellular matrix, leading to a facilitation of angiogenesis.	Increased levels of PAF may selectively enhance the response to PAF and cause excessive extracellular matrix degradation
NGF	NGF stimulates degranulation and cytokine release from the skin mast cells and thereby promotes neurogenic inflammation. It also increases vascular permeability, followed by tissue oedema via stimulation of CGRP release from the sensory nerve endings and via induction of mast cell degranulation. In addition to the regulation of these inert immune responses, NGF is involved in the activation, migration and proliferation of keratinocyte, endothelial cells, lymphocytes, fibroblast and macrophages in the skin, indicating that NGF regulates various processes during cutaneous wound repair.	Is found to be elevated in tissue inflammation, e.g. arthritis and pancreatitis, which presumably increase nociception NGF stimulates degranulation and cytokine release from the skin mast cells and thereby promotes neurogenic inflammation. It also increases vascular permeability, followed by tissue oedema

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2.7 Analgesic

An analgesic is any member of the group of drugs used to achieve analgesia, relief from pain. The word analgesic derives from Greek ("without") and ("pain") (Harper, 2001).

Commonly known as painkillers, analgesic drugs act in various ways on the peripheral and central nervous systems. They are distinct from anesthetics, which reversibly eliminate sensation, and include paracetamol (known in the US as acetaminophen or simply APAP), the non-steroidal anti-inflammatory drugs (NSAIDs) such as the salicylates, and opioid drugs such as morphine and opium.

In choosing analgesics, the severity and response to other medication determines the choice of agent; the World Health Organization (WHO) pain ladder (Anonymous, 1990) specifies mild analgesics as its first step.

Analgesic choice is also determined by the type of pain: for neuropathic pain, traditional analgesics are less effective, and there is often benefit from classes of drugs that

are not normally considered analgesics, such as tricyclic antidepressants and anticonvulsants (Dworkin *et al.*, 2003).

2.7.1 Major classes

2.7.1.1 Paracetamol and NSAIDs

The exact mechanism of action of paracetamol/acetaminophen is uncertain but appears to act centrally in the brain rather than peripherally in nerve endings. Aspirin and the other non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenases, leading to a decrease in prostaglandin production. In contrast to paracetamol and the opioids, this not only reduces pain but inflammation as well.

Paracetamol has few side effects and is regarded as generally safe, although excess or sustained use can lead to potentially life-threatening liver damage and occasionally kidney damage. While paracetamol is usually taken orally or rectally, an intravenous preparation introduced in 2002 has been shown to improve pain relief and reduce opioid consumption in the perioperative setting.

NSAIDs predispose to peptic ulcers, renal failure, allergic reactions, and occasionally hearing loss, and they can increase the risk of hemorrhage by affecting platelet function. The use of aspirin in children under 16 suffering from viral illness has been linked to Reye's syndrome, a rare but severe liver disorder.

2.7.1.2 Cox-2 inhibitors

These drugs have been derived from NSAIDs. The cyclooxygenase enzyme inhibited by NSAIDs was discovered to have at least 2 different versions: COX1 and COX2. Research suggested that most of the adverse effects of NSAIDs were mediated by blocking the COX1 (constitutive) enzyme, with the analgesic effects being mediated by the COX2 (inducible) enzyme. The COX2 inhibitors were thus developed to inhibit only the COX2 enzyme (traditional NSAIDs block both versions in general). These drugs (such as rofecoxib, celecoxib and etoricoxib) are equally effective analgesics when compared with NSAIDs, but cause less gastrointestinal haemorrhage in particular (Conaghan, 2012).

After widespread adoption of the COX-2 inhibitors, it was discovered that most of the drugs in this class increased the risk of cardiovascular events by 40 % on average. This led to

the withdrawal of rofecoxib and valdecoxib, and warnings on others. Etoricoxib seems relatively safe, with the risk of thrombotic events similar to that of non-coxib NSAID diclofenac (Conaghan, 2012).

2.7.1.3 Opiates and morphinomimetics

Morphine, the archetypal opioid, and various other substances (e.g codeine, oxycodone, hydrocodone, dihydromorphine, pethidine) all exert a similar influence on the cerebral opioid receptor system. Buprenorphine is thought to be a partial agonist of the opioid receptor, and tramadol is an opiate agonist with serotonin-norepinephrine reuptake inhibitor (SNRI) properties. Tramadol is structurally closer to venlafaxine than to codeine and deliver analgesia by not only delivering "opiate-like" effects (through mild agonism of the mu, μ, receptor) but also by acting as a weak but fast-acting serotonin releasing agent and norepinephrine reuptke inhibitor (Gobbi *et al.*, 2004). Dosing of all opioids may be limited by opioid toxicity (confusion, respiratory depression, myoclonic jerks and pinpoint pupils), seizures (tramadol), but there is no dose ceiling in patients who accumulate tolerance.

Opioids, while very effective analgesics, may have some unpleasant side-effects. Patients starting morphine may experience nausea and vomiting (generally relieved by a short course of antiemetics such as Phenergan). Pruritus (itching) may require switching to a different opioid. Constipation occurs in almost all patients on opioids, and laxatives (lactulose, macrogol-containing or co-danthramer) are typically co-prescribed (Doyle *et al.*, 2004).

When used appropriately, opoids and similar narcotic analgesics are otherwise safe and effective, however risks such as addiction and the body becoming use to the drug (tolerance) can occur. The effect of tolerance means that frequent use of the drug may result in its diminished effect so, when safe to do so, the dosage may need to be increased to maintain effectiveness. This may be of particular concern regarding patients suffering with chronic pain.

2.7.1.4 Flupirtine

Flupirtine is a centrally acting K^+ channel opener with weak NMDA antagonist properties. It is used in Europe for moderate to strong pain and migraine and its muscle

relaxant properties. It has no anticholinergic properties and is believed to be devoid of any activity on dopamine, serotonin or histamine receptors. It is not addictive and tolerance usually does not develop. However, tolerance may develop in single cases (Stoessel *et al*. 2010).

2.7.1.5 Specific agents

In patients with chronic or neuropathic pain, various other substances may have analgesic properties. Tricyclic antidepressants, especially amitriptyline, have been shown to improve pain in what appears to be a central manner. Nefopam is used in Europe for pain relief with concurrent opioids. The exact mechanism of carbamazepine, gabapentin and pregabalin is similarly unclear, but these anticonvulsants are used to treat neuropathic pain with differing degrees of success. Anticonvulsants are most commonly used for neuropathic pain as their mechanism of action tends to inhibit pain sensation.

2.8 Memory

In psychology, memory is the process by which information is encoded, stored, and retrieved. Encoding allows information that is from the outside world to reach our senses in the forms of chemical and physical stimuli. In this first stage we must change the information so that we may put the memory into the encoding process.

Storage is the second memory stage or process. This entails that we maintain information over periods of time. Finally the third process is the retrieval of information that we have stored. We must locate it and return it to our consciousness. Some retrieval attempts may be effortless due to the type of information.

From an information processing perspective there are three main stages in the formation and retrieval of memory:

- *Encoding or registration*: receiving, processing and combining of received information
- *Storage*: creation of a permanent record of the encoded information
- *Retrieval, recall or recollection*: calling back the stored information in response to some cue for use in a process or activity

The loss of memory is described as forgetfulness, or as a medical disorder, amnesia.

2.8.1 Sensory memory

Sensory memory holds sensory information for a few seconds or less after an item is perceived. The ability to look at an item, and remember what it looked like with just a second of observation, or memorisation, is an example of sensory memory. With very short presentations, participants often report that they seem to "see" more than they can actually report.

There are many types of sensory memories. Iconic memory is a fast decaying store of visual information, a type of sensory memory that briefly stores an image which has been perceived for a small duration. Echoic memory is a fast decaying store of auditory information, another type of sensory memory that briefly stores sounds which has been perceived for a small duration (Carlson, 2010).

2.8.2 Short-term memory

Short-term memory allows recall for a period of several seconds to a minute without rehearsal. Its capacity is also very limited, however, memory capacity can be increased through a process called chunking. For example, in recalling a ten-digit telephone number, a person could chunk the digits into three groups: first, the area code (such as 123), then a three-digit chunk (456) and lastly a four-digit chunk (7890). This method of remembering telephone numbers is far more effective than attempting to remember a string of 10 digits; this is because we are able to chunk the information into meaningful groups of numbers. This may be reflected in some countries in the tendency to display telephone numbers as several chunks of three numbers, with the final four-number group generally broken down into two groups of two.

Short-term memory is believed to rely mostly on an acoustic code for storing information, and to a lesser extent a visual code. Conrad (1964) found that test subjects had more difficulty recalling collections of letters that were acoustically similar (e.g. E, P, D). Confusion with recalling acoustically similar letters rather than visually similar letters implies that the letters were encoded acoustically. Conrad's (1964) study however, deals with the encoding of written text, thus while memory of written language may rely on acoustic components, generalisations to all forms of memory cannot be made

2.8.3 Long-term memory

The storage in sensory memory and short-term memory generally have a strictly limited capacity and duration, which means that information is not retained indefinitely By contrast, long-term memory can store much larger quantities of information for potentially unlimited duration (sometimes a whole life span). Its capacity is immeasurably large For example, given a random seven-digit number we may remember it for only a few seconds before forgetting, suggesting it was stored in our short-term memory. On the other hand, we can remember telephone numbers for many years through repetition; this information is said to be stored in long-term memory.

While short-tenn memory encodes infomation acoustically, long-tenn memory encodes it semantically: Baddeley (1966) discovered that after 20 minutes, test subjects had the most difficulty recalling a collection of words that had similar meanings (e.g. big, large, great, huge) long-tenn. Another part of long-tenn memory is episodic memory "which attempts to capture information such as "what", "when" and "where". With episodic memory individuals are able to recall specific events such as birthday parties and weddings.

Short-term memory is supported by transient patterns of neuronal communication, dependent on regions of the frontal lobe (especially dorsolateral prefrontal cortex) and the parietal lobe. Long-term memories, on the other hand, are maintained by more stable and permanent changes in neural connections widely spread throughout the brain. The hippocampus is essential (for learning new information) to the consolidation of information from short-term to long-term memory, although it does not seem to store information itself. Without the hippocampus, new memories are unable.to be stored into long-term memory, as learned from HM after removal of his hippocampus, and there a very short attention span. Furthermore, it may be involved in changing neural connections for a period of three months or more after the initial learning. One of the primary functions of sleep is thought to be improving consolidation of information, as several studies have demonstrated that memory depends on getting sufficient sleep between training and test (Ellenbogen *et al.*, 2006). Additionally, data obtained from neuroimaging studies have shown activation patterns in the sleeping brain which mirror those recorded during the learning of tasks from the previous day, suggesting that new memories may be solidified through such rehearsal.

Research has suggested that long-term memory storage in humans may be maintained by DNA methylation, or prions (Papassotiropoulous *et al.*, 2005).

2.8.4 Working memory

In 1974 Baddeley and Hitch proposed a working memory model which replaced the general concept of short term memory with an active maintenance of information in the short term storage. In this model, working memory consists of three basic stores: the central executive, the phonological loop and the visuo-spatial sketchpad. In 2000 this model was expanded with the multimodal episodic buffer (Baddeley, 2000).

The central executive essentially acts as attention. It channels information to the three component processes: the phonogical loop, the visuo-spatial sketchpad, and the die buffer.

The phonological loop stores auditory information by silently rehearsing sounds or words in a continuous loop: the articulatory process (for example the repetition of a telephone number over and over again). A short list of data is easier to remember.

The visuospatial sketchpad stores visual and spatial information. It is engaged when performing spatial tasks (such as judging distances) or visual ones (such as counting the windows on a house or imagining images).

The episodic buffer is dedicated to linking information across domains to form integrated units of visual, spatial, and verbal information and chronological ordering (e.g., the memory of a story or a movie scene). The episodic buffer is also assumed to have links to long-term memory and semantical meaning.

The working memory model explains many practical observations, such as why it is easier to do two different tasks (one verbal and one visual) than two similar tasks (e.g., two visual), and the aforementioned word-length effect. However, the concept of a central executive as noted here has been criticised as inadequate and vague. Working memory is also the premise for what allows us to do everyday activities involving thought. It is the section of memory where we carry out thought processes and use them to learn and reason about topics.

2.8.5 Memory and Stress

Stress has a significant effect on memory formation and learning. In response to stressful situations, the brain releases hormones and neurotransmitters (ex. glucocorticoids and

catecholamines) which affect memory encoding processes in the hippocampus. Behavioural research on animals shows that chronic stress produces adrenal hormones which impact the hippocampal structure in the brains of rats (Conrad, 2010). An experimental study by German cognitive psychologists L. Schwabe and O. Wolf demonstrates how learning under stress also decreases memory recall in humans (Schwabe and Wolf, 2010). In this study, 48 healthy female and male university students participated in either a stress test or a control group. Those randomly assigned to the stress test group had a hand immersed in ice cold water (the reputable SECPT or 'Socially Evaluated Cold Pressor Test') for up to three minutes, while being monitored and videotaped. Both the stress and control groups were then presented with 32 words to memorize. Twenty-four hours later, both groups were tested to see how many words they could remember (free recall) as well as how many they could recognize from a larger list of words (recognition performance). The results showed a clear impairment of memory performance in the stress test group, who recalled 30 % fewer words than the control group. The researchers suggest that stress experienced during learning distracts people by diverting their attention during the memory encoding process.

However, memory performance can be enhanced when material is linked to the learning context, even when learning occurs under stress. A separate study by cognitive psychologists Schwabe and Wolf shows that when retention testing is done in a context similar to or congruent with the original learning task (i.e., in the same room), memory impairment and the detrimental effects of stress on learning can be attenuated. Seventy-two healthy female and male university students, randomly assigned to the SECPT stress test or to a control group, were asked to remember the locations of 15 pairs of picture cards - a computerized version of the card game "Concentration" or "Memory." The room in which the experiment took place was infused with the scent of vanilla, as odour is a strong cue for memory. Retention testing took place the following day, either in the same room with the vanilla scent again present, or in a different room without the fragrance. The memory performance of subjects who experienced stress during the object-location task decreased significantly when they were tested in an unfamiliar room without the vanilla scent (an incongruent context); however, the memory performance of stressed subjects showed no impairment when they were tested in the original room with the vanilla scent (a

congruent context). All participants in the experiment, both stressed and unstressed, performed faster when the learning and retrieval contexts were similar (Schwabe *et al.*, 2009).

This research on the effects of stress on memory may have practical implications for education, for eyewitness testimony and for psychotherapy: students may perform better when tested in their regular classroom rather than an exam room, eyewitnesses may recall details better at the scene of an event than in a courtroom, and persons suffering from post-traumatic stress may improve when helped to situate their memories of a traumatic event in an appropriate context.

2.9 Anxiety

Anxiety (also called angst or worry) is a psychological and physiological state characterized by somatic, emotional, cognitive, and behavioural components. It is the displeasing feeling of fear and concern (Davison, 2008). The root meaning of the word anxiety is 'to vex or trouble'; in either presence or absence of psychological stress, anxiety can create feelings of fear, worry, uneasiness, and dread (Bouras and Holt, 2007). It is also associated with feelings of restlessness, fatigue, concentration problems, and muscle tension. However, anxiety should not be confused with fear, which is more of a dreaded feeling about something which appears intimidating and can overcome an individual. Anxiety is considered to be a normal reaction to a stressor. It may help an individual to deal with a demanding situation by prompting them to cope with it. However, when anxiety becomes overwhelming, it may fall under the classification of an anxiety disorder. Anxiety can be confused with fear. However, fear is concrete, (a real danger) whereas anxiety is the paranoia of something out there that seems menacing but may not be menacing, and, indeed, may not even be out there (Henig, 2012).

2.9.1 Signs and symptoms

Anxiety is a generalized mood that can occur without an identifiable triggering stimulus. As such, it is distinguished from fear, which is an appropriate cognitive and emotional response to a perceived threat. Additionally, fear is related to the specific behaviours of escape and avoidance, whereas anxiety is related to situations perceived as uncontrollable or unavoidable (Ohman, 2000). Another view defines anxiety as "a future-oriented mood state in which one is ready or prepared to attempt to cope with upcoming negative events," suggesting that it is a distinction between future and present dangers which divides anxiety and fear. In a 2011

review of the literature, (Sylvers *et al.*, 2011) fear and anxiety were said to be differentiated in four domains: (1) duration of emotional experience, (2) temporal focus, (3) specificity of the threat, and (4) motivated direction. Fear is defined as short lived, present focused, geared towards a specific threat, and facilitating escape from threat; while anxiety is defined as long acting, future focused, broadly focused towards a diffuse threat, and promoting caution while approaching a potential threat. While most everyone has an experience with anxiety at some point in their lives, as it is a common reaction to real or perceived threats of all kinds, most do not develop long-term problems with anxiety. When someone does develop chronic or severe problems with anxiety, such problems are usually classified as being one or more of the specific types of Anxiety Disorders.

Anxiety takes several forms: phobia, social anxiety, obsessive-compulsive, and post-traumatic stress. The physical effects of anxiety may include heart palpitations, tachycardia, muscle weakness and tension, fatigue, nausea, chest pain, shortness of breath, headache, stomach aches, or tension headaches. As the body prepares to deal with a threat, blood pressure, heart rate, perspiration, blood flow to the major muscle groups are increased, while immune and digestive functions are inhibited (the fight or flight response). External signs of anxiety may include pallor, sweating, trembling, and pupillary dilation. For someone who suffers anxiety this can lead to a panic attack. Sir Aubrey Lewis even suggests that "anxiety" could be defined as agony, dread, terror, or even apprehension.

Although panic attacks are not experienced by every person who suffers from anxiety, they are a common symptom. Panic attacks usually come without warning and although the fear is generally irrational, the subjective perception of danger is very real. A person experiencing a panic attack will often feel as if he or she is about to die or lose consciousness. Between panic attacks, people with panic disorder tend to suffer from anticipated anxiety- a fear of having a panic attack may lead to the development of phobias (Neil and Donald, 2010). Anxiety is the most common mental illness in America as approximately 40 million adults are affected by it. Not only is anxiety common in adults, but it has also been found to be more common in females rather than males.

The emotional effects of anxiety may include "feelings of apprehension or dread, trouble concentrating, feeling tense or jumpy, anticipating the worst, irritability, restlessness, watching

(and waiting) for signs (and occurrences) of danger, and, feeling like your mind's gone blank" (Smith, 2008) as well as "nightmares/bad dreams, obsessions about sensations, deja vu, a trapped in your mind feeling, and feeling like everything is scary.

The cognitive effects of anxiety may include thoughts about suspected dangers, such as fear of dying. "You may... fear that the chest pains are a deadly heart attack or that the shooting pains in your head are the result of a tumor or aneurysm. You feel an intense fear when you think of dying, or you may think of it more often than normal, or can't get it out of your mind."

The behavioural effects of anxiety may include withdrawal from situations which have provoked anxiety in the past (Barker, 2003). Anxiety can also be experienced in ways which include changes in sleeping patterns, nervous habits, and increased motor tension like foot tapping (Barker, 2003).

The symptoms of anxiety include excessive and ongoing worry and tension, an unrealistic view of problems, restlessness or a feeling of being "edgy", irritability, muscle tension, headaches, sweating, difficulty concentrating, nausea, the need to go to the bathroom frequently, tiredness, trouble falling or staying asleep, trembling, and being easily startled.

2.9.2 Causes

An evolutionary psychology explanation is that increased anxiety serves the purpose of increased vigilance regarding potential threats in the environment as well as increased tendency to take proactive actions regarding such possible threats. This may cause false positive reactions but an individual suffering from anxiety may also avoid real threats. This may explain why anxious people are less likely to die due to accidents (Andrews and Thomson, 2009).

The psychologist David H. Barlow of Boston University conducted a study that showed three common characteristics of people suffering from chronic anxiety, which he characterized as "a generalized biological vulnerability," "a generalized psychological vulnerability," and "a specific psychological vulnerability (Barlow and Durand, 2008)." While chemical issues in the brain that result in anxiety (especially resulting from genetics) are well documented, this study highlights an additional environmental factor that may result from being raised parents suffering from chronic anxiety.

Other contextual factors that are thought to contribute to anxiety include gender socialization and learning experiences. In particular, learning mastery (the degree to which people

perceive their lives to be under own control) and instrumentality, which includes such traits as self-confidence, independence, and competitiveness fully mediate the relation between gender and anxiety. That is, though gender difference in anxiety exist, with higher levels of anxiety in women compared to men, gender socialization and learning mastery explain these gender differences. Research has demonstrated the ways in which facial prominence photographic images differs between men and women. More specifically, in official online photographs of politicians around the world, women's faces are less prominent than men's. Interestingly enough, the difference in these images actually tended to be greater in cultures with greater institutional gender equality (Zalta and Chambless, 2012).

Research upon adolescents who as infants had been highly apprehensive, vigilant, and fearful finds that their nucleus accumbens is more sensitive than that in other people when deciding to make an action that determined whether they received a reward. This suggests a link between circuits responsible for fear and also reward in anxious people. As researchers note, "a sense of 'responsibility,' or self agency, in a context of uncertainty (probabilistic outcomes) drives the neural system underlying appetitive motivation (i.e., nucleus accumbens) more strongly in temperamentally inhibited than noninhibited adolescents." Anxiety is also linked and perpetuated by the person's own pessimistic outcome expectancy and how they cope with feedback negativity (Gu *et al.*, 2010).

Neural circuitry involving the amygdala and hippocampus is thought to underlie anxiety. When people are confronted with unpleasant and potentially harmful stimuli such as foul odours or tastes, PET-scans show increased blood flow in the amygdala (Zald *et al.*, 2002). In these studies, the participants also reported moderate anxiety. This might indicate that anxiety is a protective mechanism designed to prevent the organism from engaging in potentially harmful behaviours.

Although single genes have little effect on complex traits and interact heavily both between themselves and with the external factors, research is underway to unravel possible molecular mechanisms underlying anxiety and comorbid conditions. One candidate gene with polymorphisms that influence anxiety is PLXNA2 (Wray et al., 2007).

Caffeine may cause or exacerbate anxiety disorders. A number of clinical studies have shown a positive association between caffeine and anxiogenic effects and/or panic disorder (Vilarim *et al.*, 2011). Anxiety sufferers can have high caffeine sensitivity.

2.10 Sedative

A sedative or tranquilizer (or tranquilliser) is a substance that induces sedation by reducing irritability or excitement.

At higher doses it may result in slurred speech, staggering gait, poor judgment, and slow, uncertain reflexes. Doses of sedatives such as benzodiazepines, when used as a hypnotic to induce sleep, tend to be higher than amounts used to relieve anxiety, whereas only low doses are needed to provide a peaceful and calming sedative effect (Montenegro *et al.*, 2005).

Sedatives can be misused to produce an overly-calming effect (alcohol being the classic and most common sedating drug). In the event of an overdose or if combined with another sedative, many of these drugs can cause unconsciousness (see hypnotic) and even death.

Types of sedatives

- Barbiturates
  - mobarbital (Amytal)
  - Pentobarbital (Nembutal)
  - Secobarbital (Seconal)
  - phenobarbital (Luminal)
- Benzodiazepines (trade names)
  - Clonazepam
  - diazepam (Valium)
  - estazolam (Prosom)
  - flunitrazepam (Rohypnol)
  - Lorazepam (Ativan)
  - midazolam (Versed)
  - nitrazepam (Mogadon)
  - oxazepam (Serax)
  - triazolam (Halcion)
  - temazepam (Restoril, Normison, Planum, Tenox, and Temaze)
  - chlordiazepoxide (Librium)
  - alprazolam (Xanax)
  - (Mogadon)
- Nonbenzodiazepine "Z-drugs" sedatives
  - eszopiclone (Lunesta)
  - zaleplon (Sonata)
  - zolpidem (Ambien)
  - zopiclone (Imovane, Zimovane)
- Antihistamines
  - diphenhydramine
  - dimenhydrinate
  - doxylamine

- mirtazapine
- promethazine

- Herbal sedatives
  - Ashwagandha
  - Duboisia hopwoodii
  - Prostanthera striatiflora
  - Catnip
  - kava (Piper methysticum)
  - valerian
  - cannabis
  - passiflora spp.(passillora incarnata)
- Other
- chloral hydrate
- alcohol
- trazodone
- opiates and opioids
- glutethimide

2.10.1 Therapeutic use

Doctors often administer sedatives to patients in order to dull the patient's anxiety related to painful or anxiety-provoking procedures. Although sedatives do not relieve pain in themselves, they can be a useful adjunct to analgesics in preparing patients for surgery, and are commonly given to patients before they are anaesthetized, or before other highly uncomfortable and invasive procedures like cardiac catheterization, colonoscopy or MRI. They increase tractability and compliance of children or troublesome or demanding patients.

Patients in intensive care units are almost always sedated (unless they are unconscious from their condition anyway).

2.11 Anxiolytic

An anxiolytic (also antipanic or antianxiety agent is a drug used for the treatment of anxiety and its related psychological and physical symptoms. Anxiolytics have been shown to be useful in the treatment of anxiety disoiders.

Beta-receptor blockers such as propranolol and oxprenolol, although not anxiolytics, can be used to combat the somatic symptoms of anxiety.

Anxiolytics are also known as minor tranquilizers. The term is less common in modern texts, and was originally derived from a dichotomy with major tranquilizers, also known as neuroleptics or antipsychotics.

2.11.1 Types of anxiolytics/anti-anxiety drugs

2.11.1.1 Benzodiazepines

Benzodiazepines are prescribed for short-term relief of severe and disabling anxiety. Benzodiazepines may also be indicated to cover the latent periods associated with the medications prescribed to treat an underlying anxiety disorder. They are used to treat a wide variety of conditions and symptoms and are usually a first choice when short-term CNS sedation is needed. Longer-term uses include treatment for severe anxiety. There is a risk of a benzodiazepine withdrawal and rebound syndrome after continuous usage for longer than two weeks, and tolerance and dependence may occur if patients stay under this treatment for longer (Gelder *et al.* 2005). There is also the added problem of the accumulation of drug metabolites and adverse effects. Benzodiazepines include:

- Alprazolam (Xanax)
- Chlordiazepoxide (Librium)
- Clonazepam (Klonopin, Rivotril)
- Diazepam (Valium)
- Etizolam (Etilaam)
- Lorazepam (Ativan)
- Oxazepam (Serax)

Benzodiazepines exert their anxiolytic properties at moderate dosage. At higher dosage hypnotic properties occur (Montenegro *et al.* 2005)

- Tofisopam (Emandaxin and Grandaxin) is a drug that is a benzodiazepine derivative. Like other benzodiazepines, it possesses anxiolytic properties, but, unlike other benzodiazepines, it does not have anticoovulsant, sedative, skeletal muscle relaxant, motor skill-impairing, or amnestic properties.

2.12 Anxiogenic

An anxiogenic substance is one that causes anxiety. Anxiogenic effects can be measured by, for example, the hole-board test in rats and mice (Takeda *et al.*, 1998). A number of agents are used to provoke anxiety (anxiogens) or panic (panicogens) in experimental models. Some of the most common substances are: sodium lactate, carbon dioxide (as carbogen), L-DOPA, caffeine, modafinil, GABA antagonists such as DMCM, FG-7142 and ZK-93426, serotonergic agents such as mCPP and LY-293,284, adrenergic agents such as yohimbine, antipsychotics/dopamine antagonists such as ecopipam and reserpine, and cholecystokinin (CCK) (especially the tetrapeptide and octapeptide fragments CCK-4 and CCK-8). Studies have shown that 10 mL/kg of 0.5 molar sodium lactate infused intravenously over a 20-minute period will provoke a panic attack in most patients with panic disorder but not healthy control subjects (Eric and Daphne, 2003).

Antibiotics drugs such as fluoroquinolones can cause from short-term to long-term anxiety and panic disorders as a side effect. This is due to a possible antagonism of the $GABA_A$ receptor and toxicity of the central nervous system. This effect is potentiated with the combined use of non-steroidal anti-inflammatory drugs.

The $GABA_A$ receptor negative allosteric modulator flumazenil can cause panic attacks in patients with panic disorder.

Anxiolytic substances have the opposite effect, they reduce anxiety. The most common class of anxiolytic drugs are the benzodiazepines.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Animals

Adult male mice weighing between 20-25 g bred in the Pre-Clinical Animal House of the College of Medicine, University of Ibadan were used. They were housed under standard laboratory conditions with a 12 hours daylight cycle and had free access to feed (Ladokun Feeds Limited, Ibadan, Nigeria) and water; they were acclimatized for two weeks to laboratory conditions before the commencement of the experiments. All experiments were carried out in compliance with the recommendations of Helsinki's declaration on guiding principles on care and use of animals.

3.2 Plant Material

Fresh samples of *Adenopus breviflorus* fruit were bought in Bodija Market, Ibadan, and were authenticated in the Taxonomy Unit of the Forestry Research Institute of Nigeria (FRIN), Jericho, Ibadan where a voucher specimen; FHI 108336, was deposited in their Herbarium.

3.3 Preparation of Crude Ethanol Extract

Large quantity (7.5 kg) of fresh specimens of the whole fruit of *Adenopus breviflorus* were washed free of debris and pulverished using mortar and pestle and air-dried for eight weeks. The resultant dried specimens (300 g) were macerated and extracted with 70 % ethanol for 72 hours at room temperature (26 - 28 ^{0}C). The resulting solution was then filtered using a wire-gauze and a sieve with tiny pores (0.25 mm). The 70 % ethanol was later evaporated using steam bath (40 – 45 ^{0}C) to give a percentage yield of 8.6 % of the starting sample. The dried sample was reconstituted in distilled water to make up test solutions of known concentration.

3.4 Phytochemical Screening

Standard phytochemical methods were used to test for the presence of alkaloids, cardenolides, tanins, flavonoids, anthraquinones and saponins (Sofowora, 1993; Evans, 1989; Harbone, 1973).

3.4.1 Alkaloids test

Few drops of Dragendorff's, Mayer's and Wagner's reagent were added separately to the extract in the test tube. A reddish brown, cream and reddish brown precipitate respectively indicates a positive test.

3.4.2 Keller Killiani test

The extract was evaporated to dryness and 3 mL of ferric chloride reagent was added to the cooled residue in a clean test tube. Concentrated sulphuric acid (2 mL) was gently poured down the side of the test tube. A purple or reddish brown ring at the interface and green colour in acetic acid layer indicates a positive test for 2-de-oxy sugar (cardenolides' test).

3.4.3 Tannins test

Few drops of ferric chloride reagent were added to the extract in the test tube. A red colouration indicates a positive test.

3.4.4 Flavonoids test

A small quantity of the extract was dissolved in dilute sodium hydroxide and hydrochloric acid was added to the mixture. A yellow solution that turns colourless on addition of hydrochloric acid indicates the presence of flavonoids.

3.4.5 Anthraquinones (Borntrager's) test

Few drops of chloroform were added to the extract in the test tube, 1mL of dilute (10 %) ammonial was added and the mixture was shaken. A pink-red colour in the ammoniacal (lower) layer shows anthracene derivates.

3.4.6 Saponins (Froth) test

To 0.5 g of the extract in the test tube was added 10 mL of distilled water and this was well shaken and left to stand for 10 minutes. A thick persistent froth indicated the presence of saponins.

3.5 Toxicity test

The method described by Lorke (1983) was used to determine the LD_{50}, which is the index ofacute toxicity. Male albino mice (20.25 g) were used. This method involved an initial dose finding procedure, in which the animals were divided into three groups of three animals per group. Doses of 10 mg/kg, 100 mg/kg and 1000 mg/kg were administered orally, one doses for each group. The treated animals were monitored for twenty-four hours for mortality and general behaviour. After twenty – four hours, based on a record of zero death from the results of the above step, seven different doses (2000 mg/kg, 3000 mg/kg, 4000 mg/kg, 5000 mg/kg, 6000 mg/kg, 7000 mg/kg, 8000 mg/kg) were chosen and administered orally to seven groups of animals of one mouse per group respectively. The treated animals were monitored for twenty-four hours. The LD_{50} was then calculated as the geometric mean of the lowest dose showing death and the highest dose showing no death.

The LD_{50} of the crude extract was found to be 7000 mg/kg. The working dose was half of the LD_{50} i.e 3500 mg/kg, which is now scaled down to 2000 mg/kg, 1000 mg/kg, 500 mg/kg, 250 mg/kg, 125 mg/kg and 62.5 mg/kg.

3.5.1 Preparation of Stock Solution of EEAB

Ten grammes of EEAB were dissolved in 100 mL of distilled water to give a concentration of 0.1 g/mL.

The dosage of the experimental group was calculated using:

$$= \frac{\text{Weight of animal (g)} \times \text{Dose to be administered (mg/kg)}}{1000 \times \text{Concentration of extract (mg/mL)}}.$$

The dosages of EEAB administered in these studies were obtained from the results of the acute toxicity test.

3.6 Behavioural Study

3.6.1 Novelty-induced rearing and grooming

The behavioural profiles of albino mice under the influence of the extract were assessed in the Open Field Box (OFB) (45 cm x 25cm x 25 cm). Sixty-four mice were randomly divided into eight groups (n=8). Group I was given distilled water (0.2 mL/20 g, p.o.), groups

II – VII were given EEAB (62.5 – 2000 mg/kg, p.o.), while group VIII was given diazepam (2.0 mg/kg, i.p.).

Thirty minutes after treatment with the extract behavioural measurements were carried out for a period of thirty minutes. The animals were removed directly from the home cage and placed inside the OFB. Each animal was used only once, with the box cleaned with 70 % ethanol after each assessment to remove olfactory cue from previous animal to the other.

The time of the experiment was kept constant (8.00 a.m.-1.00 p.m.) daily to avoid changes in biologic clock. The behavioural components employed in this observational analysis were rearing and grooming (Ajayi and Ukponmwa, 1994). The frequency of rearing episodes was quantified by using a manual counter and a stop watch. The total frequency was summed up for each animal and totalled for the thirty minutes of observation time.

Rearing was taken as the number of times the mouse was standing on its hind limb or with its forelimbs against the wall of the box or in the free air. Grooming was taken as the number of body cleaning with mouth and face washing with forelimbs.

3.6.2 Locomotor activity

Motor activity was measured in an OFB (45 cm x 25 cm x 25 cm) with painted black grids dividing the floor into 16 (7 cm x 7 cm) equal squares. Sixty-four mice were randomly divided into eight groups (n=8). Group I was given distilled water (0.2 mL/20 g, p.o.), groups II – VII were given EEAB (62.5 – 2000 mg/kg, p.o.), while group VIII was given diazepam (2.0 mg/kg, i.p.).

One hour after treatment with the extract, each mouse was placed in one of the corners of the box and the number of squares crossed with all four paws were counted for 5 minutes. The cage was cleaned with 70 % ethanol at intervals when each animal was removed (Brocco *et al.*, 2002).

3.7 Anxiolytic Study

3.7.1 Hole board test

The effect of the extract on the rate of head dipping was determined in the hole board which is made up of a number of holes (usually 16) through which the animal can poke its head. Sixty-four mice were randomly divided into eight groups (n=8). Group I was given

distilled water (0.2 mL/20 g, p.o.), groups II – VII were given EEAB (62.5 – 2000 mg/kg, p.o.), while group VIII was given diazepam (2.0 mg/kg, i.p.).

One hour after treatment with the extract, each mouse was placed on the hole board and the number of times that each animal dipped (poked) its head into the holes in 5 minutes were counted (Dorr *et al.*, 1971). The hole board was cleaned with 70 % ethanol at intervals when each animal was removed.

3.7.2 Elevated Plus Maze test

The elevated plus maze (EPM) test was used to evaluate the animal anxiety (Pillow and File, 1986; Lister, 1987). The EPM for mice consisted of two open arms (30 cm x 5 cm) and two closed arms (30 cm x 5 cm x 15 cm) that extended from a common central platform (5 cm x 5 cm) with an open roof, arranged such that the two arms of each type were opposite to each other. The floor and the walls of each arm were wooden and painted white. The maze was elevated to a height of 38.5cm above the floor level. Testing was conducted in a quiet room that was illuminated by light.

Each animal was placed in the centre of the EPM facing one of the open arms. An entry into an arm was defined as the animal placing all four paws over the line marking that area. The number of entries and time spent in the open and closed arms were recorded during a 5 minutes test period. The percentages of open arm entries (100 x open/total entries) were calculated for each animal. Sixty-four mice were randomly divided into eight groups (n=8). Group I was given distilled water (0.2 mL/20 g, p.o.), groups II – VII were given EEAB (62.5 – 2000 mg/kg, p.o.), while group VIII was given diazepam (2.0 mg/kg, i.p.).

One hour after treatment with the extract, each mouse was placed in the central square of the maze facing an open arm and its behaviours were recorded for 5 minutes. The frequency of each of the following behaviours was scored and the duration of each behaviour was recoded:

i. Open arm entries.
ii. Closed arm entries.
iii. Time spent in open arms.
iv. Time spent in closed arms.

The index of open arm avoidance which is interpreted as level of anxiety (Pellow and File, 1986) is calculated as:

$$100 - \frac{(\%\ \text{time spent in open arms} + \%\ \text{entries into open arms})}{2}$$

3.8 Mechanism of Action

In another set of experiments, mice were pretreated i.p. for 15 minutes with neurotransmitter blockers to evaluate the mode of action of the extract on novelty-induced rearing and grooming behaviours, head dips and locomotor activity. The following receptor blockers were used: atropine (muscarinic blocker, 0.5 mg/kg), cyproheptadine (5-HT blocker, 0.5 mg/kg), haloperidol (dopaminergic blocker, 0.2 mg/kg), naloxone (μ-opoid antagonist, 2 mg/kg), propranolol (β-adrenergic blocker, 0.2 mg/kg) and yohimbine (α_2-adrenergic blocker, 1.0 mg/kg). The doses administered are the doses that have been found not to induce behavioural effects of their own in experimental animals and as such they only block the receptors involved. The mice were then treated for another 30 minutes with maximal dose of the extract (2000 mg/kg). The animals were observed for behavioural responses as previously explained.

3.9 Sedative Study

3.9.1 Pentobarbital-induced Sleeping Time and Sleep latency

The effect of extract on pentobarbital-induced sleeping time and sleep latency in mice was measured as described by Erden *et al.*, (2001). Forty-eight mice were randomly divided into six groups (n=8). Group I was given distilled water (0.2 ml/20 g, p.o.), groups II – V were given EEAB (250 – 2000 mg/kg, p.o.), while group VI was given diazepam (2.0 mg/kg, i.p.).

This was followed one hour later by i.p. administration of pentobarbital sodium (40 mg/kg). The sleep latency and sleeping time were recorded. The sleep latency was measured as time in minutes after treatment with pentobarbital sodium and the loss of right reflex. While the time in minutes between losses and regaining of righting reflex was taken as sleeping time.

3.9.2 Effect on Rectal Body Temperature

The recording of the rectal body temperature was carried out using a thermoprobe inserted 1.5 cm into the rectum of each mouse. Forty-eight mice were randomly divided into six groups (n=8). Group I was given distilled water (0.2 mL/20 g, p.o.), groups II – V were given EEAB (250 – 2000 mg/kg, p.o.), while group VI was given diazepam (2.0 mg/kg, i.p.).

The temperatures of the animals were recorded immediately before treatment (0 minute) and 30, 60, 90, 120 and 180 minutes after treatment. The pre-treatment results served as the reference point for the determination of temperature changes (Parimaladevi *et al.*, 2003).

3.10 Skeletal Muscle Relaxant Activity

3.10.1 Traction Test

The ability of a mouse hanging with its fore paws on a small twisted wire rigidly supported above the bench top and placing at least one hind foot on the wire within 5 seconds was determined (Rudzik *et al.*, 1973). Forty-eight mice were randomly divided into six groups (n=8). Group I was given distilled water (0.2 ml/20 g, p.o.), groups II – V were given EEAB (250 – 2000 mg/kg, p.o.), while group VI was given diazepam (2.0 mg/kg, i.p.)

One hour after treatment, each animal was suspended by means of their fore paws and the time of holding the wire was recorded. The number of animals in each group that could not touch the wire with their hind paws within 5 seconds after placement were also recorded.

3.11 Effect on Memory

3.11.1 Y-Maze test

The Y-maze test can be used as a measure for short term working memory and locomotor activity. Spontaneous alternation is a measure spatial working memory. To alternate among spatial location, a mouse must remember its previous location. Spontaneous alternation performance was assessed using n Y-maze composed of three equal spaced arms (120^0 38 cm x 33 cm x 13 cm). This test was carried out using this apparatus to obtain results for spontaneous alternation performance (memory) and locomotor activity (total arm entries). Sixty-four mice were randomly divided into eight groups (n=8). Group I was given

distilled water (0.2 mL/20 g, p.o.), groups II – VII were given EEAB (62.5 – 2000 mg/kg, p.o.), while group VIII was given diazepam (2.0 mg/kg, i.p.).

One hour after treatment with the extract, each mouse was placed in one of the arm compartments usually arm A for consistency and was allowed to move freely for 5 minutes. An arm entry is defined as the body of a mouse (except for its tail) completely entering into an arm compactment. The sequence of arm entries is manually recorded. An alternation is defined as an entry into all three arms on consecutive devices. The percentage alternation was expressed as the ratio of actual alternations to possible alternations (defined as the total number of arm entries minus two) multiplied by 100. Ethanol (70 %) was used to clean the Y-maze at interval when each animal was removed (Akanmu *et al.*, 2007).

3.12 Anticonvulsant Activity

3.12.1 Effect on pentylenetetrazol (PTZ)-induced convulsions

Pentylenetetrazol (PTZ) at 85 mg/kg (i.p.) was used to induce clonic-tonic convulsions in mice according to Swinyard *et al.* (1983). Forty-eight mice were randomly divided into six six groups (n=8). Group I was given distilled water (0.2 mL/20 g, p.o.), groups II – V were given EEAB (250 – 2000 mg/kg, p.o.), while group VI was given diazepam (2.0 mg/kg, i.p.).

Pentylenetetrazol (85 mg/kg) was administered i.p to the control and extract treated groups after one hour and to the diazepam treated group after 30 minutes. The mice were then observed for latency and duration of convulsions and monitored for mortality for 24 hours.

3.12.2 Effect on strychnine-induced convulsions

The method described by Elisha *et al.* (1988) was used. Forty-eight mice were randomly divided into six groups (n=8). Group I was given distilled water (0.2 mL/20 g, p.o.), groups II – V were given EEAB (250 – 2000 mg/kg, p.o.), while group VI was given diazepam (2.0 mg/kg, i.p.).

Strychnine (2.0 mg/kg) was administered i.p to the control and extract treated groups after one hour and to the diazepam treated group after 30 minutes. The mice were then observed for latency and duration of convulsions and monitored for mortality for 24 hours.

3.12.3 Effect on picrotoxin-induced convulsions

For this model, Stone and Javid (1979) method was employed. Forty-eight mice were randomly divided into six groups (n=8). Group I was given distilled water (0.2 mL/20 g, p.o.), groups II – V were given EEAB (250 – 2000 mg/kg, p.o.), while group VI was given diazepam (2.0 mg/kg, i.p.).

Picrotoxin (7.0 mg/kg) was administered i.p. to the control and extract treated groups after one hour and to the diazepam treated group after 30 minutes. The mice were then observed for latency and duratioo of convulsions and monitored for mortality for 24 hours.

3.13 Analgesic Activity

3.13.1 Hot plate test

The apparatus comprised a water bath filled with hot water containing a metal plate in which each animal was placed for the testing time. The temperature of the water was maintained at 55±0.5^{0}C in order to cause the animal to lick its forelimbs and/or to produce jumping responses. Forty mice were randomly divided into five groups (n=8). Group I was given distilled water (0.2 mL/20 g, p.o.), while groups II – V were given EEAB (250 – 2000 mg/kg, p.o.).

Each mouse was dropped gently on the hot plate and the time (in seconds) taken for the mice to lick its paws and/or produce jumping responses was recorded at time 0 minute (before treatment) and at time 30, 60, 90 and 120 minutes after treatment. The cut-off time was set at 15 seconds to avoid tissue damage.

In another set of experiments, the animals were divided into three groups (n=8). The first group received morphine (10 mg/kg, i.p.), the second group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with morphine (10 mg/kg, i.p.); the third group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with 1000 mg/kg (sub-maximal dose) of the extract. The mice were then tested as earlier described (Vianna *et al.*, 2000).

3.13.2 Tail Immersion test

This test was performed as described by Sewell and Spencer (1979) and as modified by Furst *et al.*, (1993). Forty mice were randomly divided into five groups (n=8). Group I was

given distilled water (0.2 mL/20 g, p.o.), while groups II – V were given EEAB (250 – 2000 mg/kg, p.o.).

The tail of each mouse (up to 5cm) was dipped in a water-bath containing hot water maintained at 55 ± 0.5^{0}C and the time (in seconds) for the mouse to withdraw the tail clearly out of the water was recorded at time 0 minute (before treatment) and at time 30, 60, 90 and 120 minutes after treatment was taken as the reaction time. The cut-off time was set at 10 seconds to avoid tissue damage.

In another set of experiments, the animals were divided into three groups (n=8). The first group received morphine (10 mg/kg, i.p.), the second group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with morphine (10 mg/kg, i.p.); the third group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with 1000 mg/kg (sub-maximal dose) of the extract. The mice were then tested as earlier described.

3.13.3 Acetic acid-induced writhing test

This model was performed as described by Yin *et al.*, (2003). Forty-eight mice were randomly divided into groups (n=8). Group I was given distilled water (0.2 mL/20 g, p.o.), groups II – V were given EEAB (250 – 2000 mg/kg, p.o.), while group VI was given aspirin (150 mg/kg, p.o.).

This was followed one hour later by i.p. administration of 10 ml/kg of 0.6% acetic acid. The animals were allowed a 5 minutes observation period in a plexiglass cage (25 cm x 25 cm x 30 cm) before assessment (counting). Nociception was evaluated by counting the number of writhings (abdominal constrictions) displayed by each mouse for 15 minutes. Antinociceptive activity was expressed as the percentage reduction or inhibition of the number abdominal writhes.

The percentage inhibition of writhing was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Mean no of writhes in control group} - \text{Mean no of writhes in treated groups}}{\text{Mean no of writhes in control group}} \times 100$$

3.13.4 Formalin test

This test was performed as described by Hunskaar and Hole (1997). Forty mice were randomly divided into five groups (n=8). Group I was given distilled water (0.2 mL/20 g, p.o.), while groups II – V were given EEAB (250– 2000 mg/kg, p.o.).

This was followed 30 minutes later by the administration of 50 µL (0.05 mL) of 1% formalin into the sub-planter space of the right hind paw. The duration (in seconds) of paw licking was determined 0-5 minutes (1st phase or neurogenic phase) and 20-30 minutes (2nd phase or inflammatory phase) after formalin administration.

In another set of experiments, the animals were divided into three groups (n=8). The first group received morphine (10 mg/kg, i.p.), the second group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with morphine (10 mg/kg, i.p.), the third group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with 1000 mg/kg (sub-maximal dose) of the extract. The mice were then tested as earlier described.

3.14 Statistical Analysis

The mean and standard error of mean (S.E.M) were calculated for all values. Comparison between the control and experimental groups was done using one – way analysis of variance (ANOVA) with Duncan's multiple range test. Differences were considered statistically significant at $p<0.05$.

CHAPTER FOUR

RESULTS

4.0

4.1 Phytochemical Screening

Phytochemical analyses of the crude extract revealed the presence of alkaloid, cardenolides, tannins and flavonoids, while anthraquinones and saponins were absent (Table 4.1).

Table 4.1: Phytochemical constituents of EEAB

Phytochemicals	EEAB
Alkaloids	+
Cardenolides	+
Tannins	+
Flavonoids	+
Anthraquinones	-
Saponins	-

+ = Present

- = Absent

4.2 Acute Toxicity

The LD_{50} of the crude extract was found to be 7000 mg/kg p.o.

4.3 Effect of EEAB on Behavioral Responses

4.3.1 Effect of EEAB on novelty-induced rearing, grooming and locomotor activity

The effect of EEAB at various doses on novelty-induced rearing, grooming, and locomotor activity are shown in Figure 4.1, Figure 4.2 and Figure 4.3.

The administration oftreatment EEAB (250 mg/kg BW, 500 mg/kg BW, 1000 mg/kg, 2000 mg/kg) and diazepam (2.0 mg/kg) to mice caused significant ($p<0.05$) reductions in novelty-induced rearing relative to the control. Treatment of mice with all the treatment doses of EEAB (62.5 mg/kg, 125 mg/kg, 250 mg/kg, 500 mg/kg,1000 mg/kg, 2000 mg/kg) and diazepam (2.0 mg/kg) caused significant ($p<0.05$) reductions in grooming relative to the control. Also,treatment of mice with all the treatment doses of the extract and diazepam caused significant ($p<0.05$) reductions in locomotor activity relative to the control.

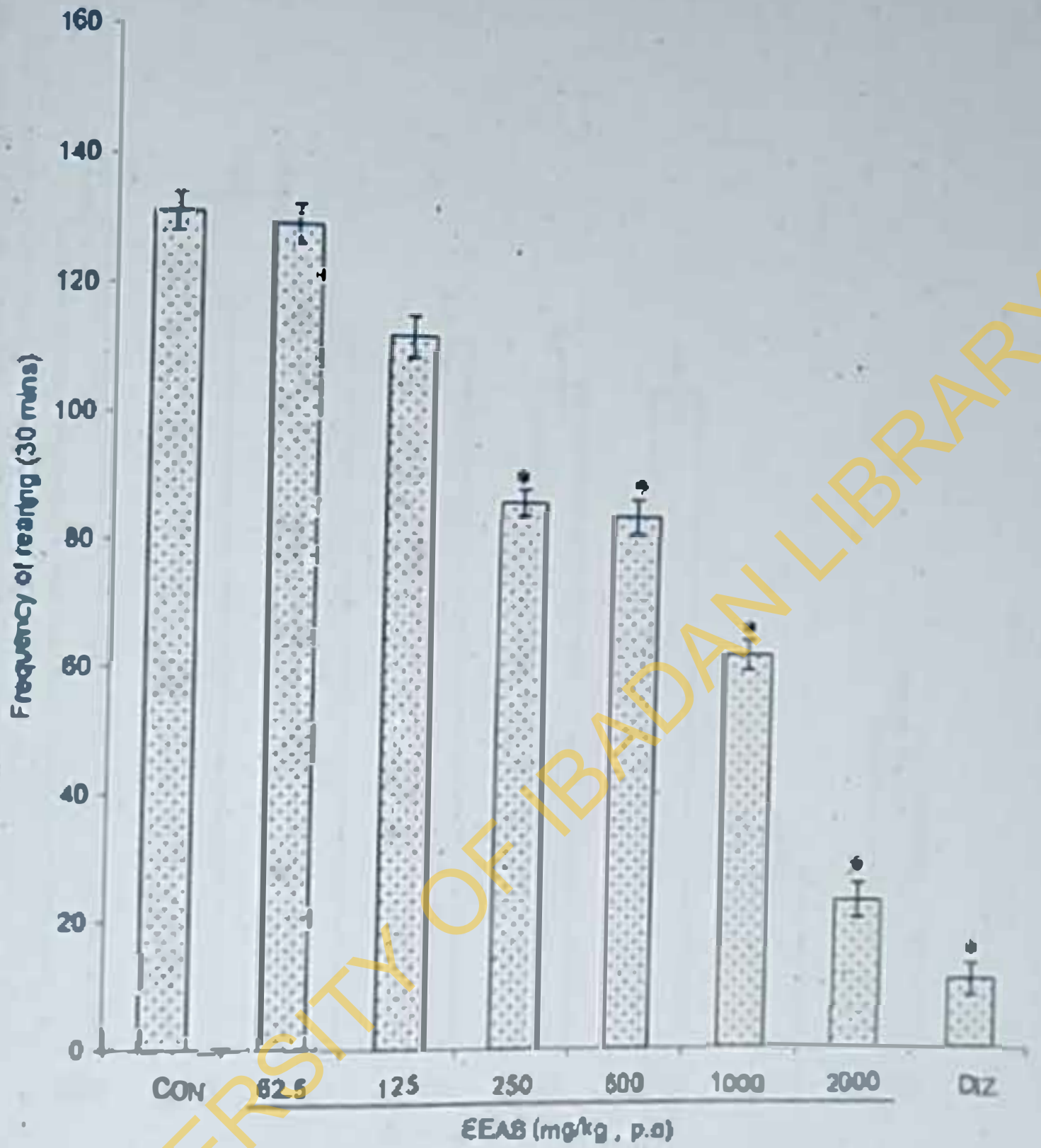

Figure 4.1: Effect of EEAB on novelty-induced rearing in mice

CON: Control, DIZ: Diazepam (2.0 mg/kg, i.p.)

Bars are mean values ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. • Indicates significant difference from control $p<0.05$

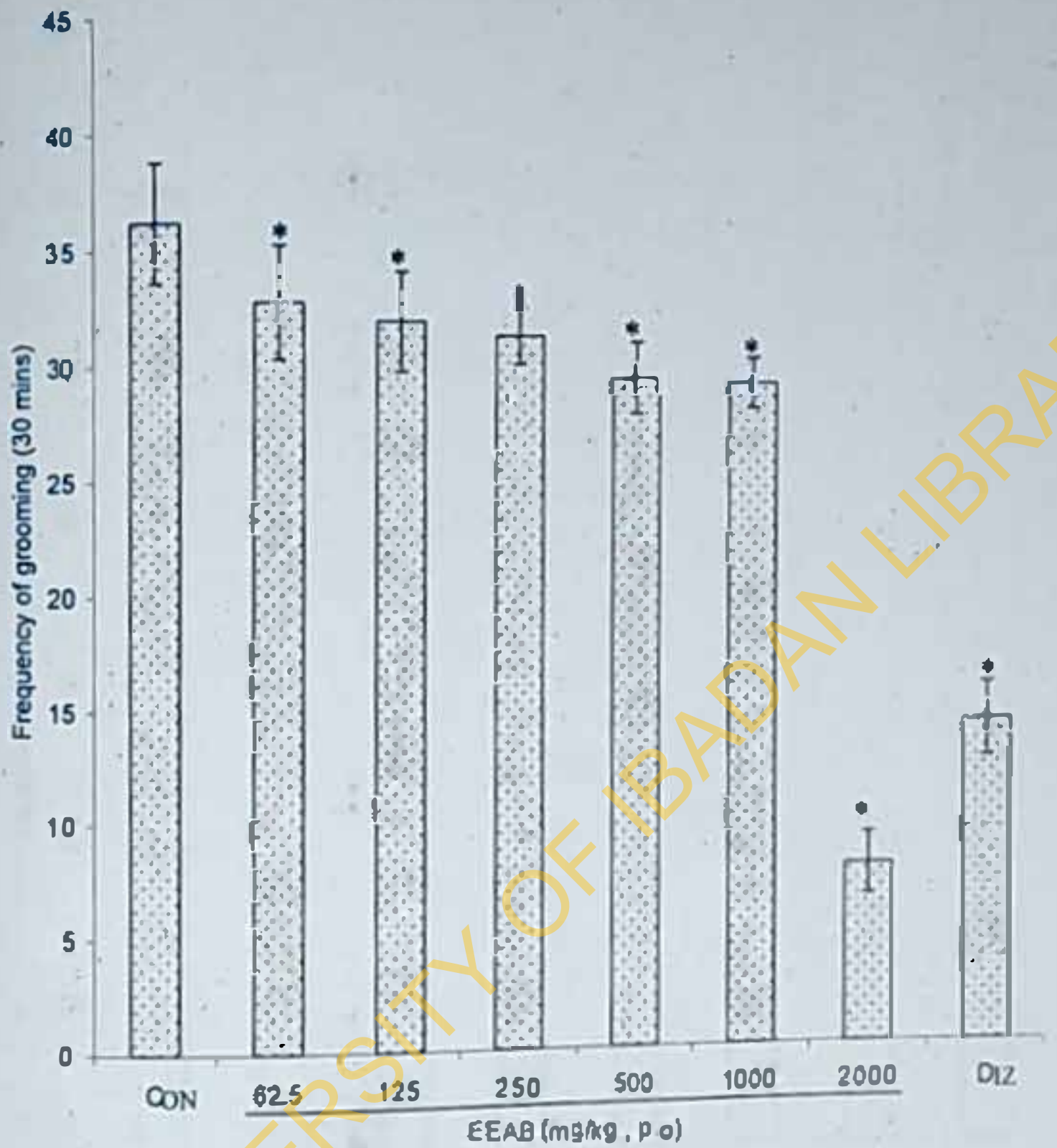

Figure 4.2: Effect of EEAB on novelty-induced grooming in mice

CON: Control, DIZ: Diazepam (2.0 mg/kg, i.p.)

Bars are mean values ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. $p<0.05$.

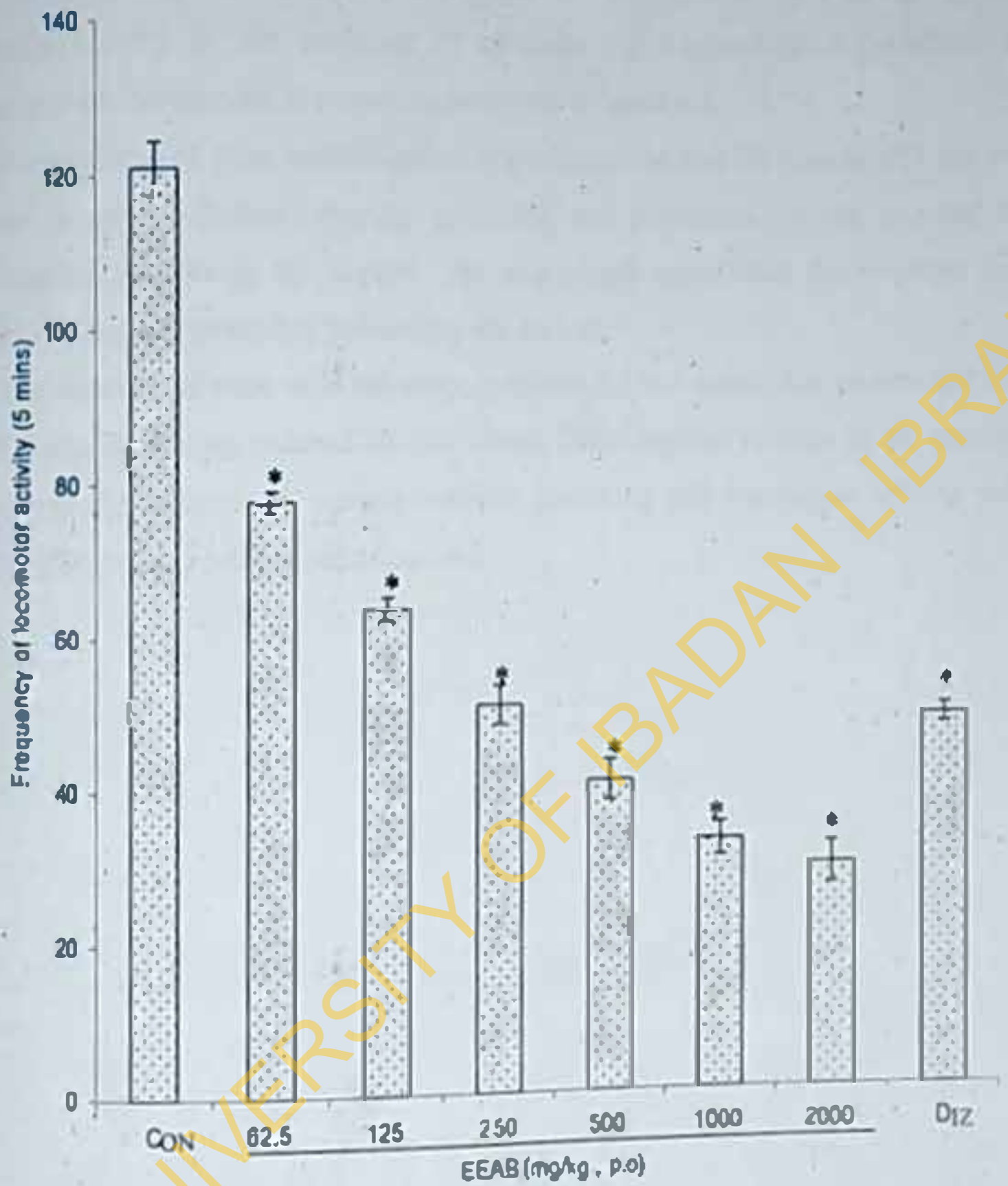

Figure 4.3: Effect of EEAB on locomotor activity in mice

CON: Control, DIZ: Diazepam (2.0 mg/kg, i.p.)

Bars are mean values ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. $p<0.05$.

13.2 Effect of EEAB on novelty-induced rearing, grooming and locomotor activity in the presence of antagonists

The effect of EEAB at various doses on novelty-induced rearing, grooming and locomotor activity in the presence of atropine, cyproheptadine, haloperidol, naloxone, propranolol and yohimbine is shown respectively in Table 4.2.

Pretreatment of mice with atropine, cyproheptadine and haloperidol did not reverse the decrease in novelty-induced rearing, grooming and locomotor activity induced by EEAB (2000 mg/kg) relative to the control. The antagonists potentiated the decrease in novelty-induced rearing and grooming induced by the extract.

Pretreatment of mice with naloxone, propranolol and yohimbine reverse the decrease in novelty-induced rearing induced by the extract (2000 mg/kg) relative to the control but did not reverse the decrease in novelty-induced grooming and locomotor activity induced by EEAB (2000 mg/kg) relative to the control.

Table 4.2: Effect of EEAB on novelty-induced rearing, grooming and locomotor activity in presence of antagonists

Treatment	NIR/30 min	NIG/30 min	LA/5 min
Control (0.2 ml/20 g)	131.20 ± 2.9	36.20 ± 2.6	121.00 ± 3.4
EEAB (2000 mg/kg)	23.60 ± 2.8*	8.00 ± 1.4*	29.80 ± 1.8*
Atropine (0.5 mg/kg)	119.80 ± 2.7	32.60 ± 2.6*	93.20 ± 0.9*
Atropine (0.5 mg/kg) + EEAB (2000 mg/kg)	4.20 ± 0.6*	7.80 ± 1.4*	31.40 ± 1.3*
Cyproheptadine (0.5 mg/kg)	109.80 ± 3.2	24.00 ± 1.1*	94.00 ± 2.5*
Cyproheptadine (0.5 mg/kg) + EEAB (2000 mg/kg)	4.80 ± 0.8*	2.60 ± 04*	27.20 ± 1.7*
Haloperidol (0.2 mg/kg)	101.80 ± 2.5*	24.60 ± 0.7*	66.00 ± 2.9*
Haloperidol (0.2 mg/kg) + EEAB (2000 mg/kg)	19.80 ± 1.7*	6.00 ± 0.8*	30.80 ± 2.0*
Naloxone (0.2 mg/kg)	114.60 ± 2.6	15.60 ± 1.0*	63.60 ± 1.7*
Naloxone (0.2 mg/kg) + EEAB (2000 mg/kg)	112.40 ± 2.9	10.80 ± 0.8*	42.20 ± 2.7*
Propranolol (0.2 mg/kg)	120.00 ± 2.6	26.80 ± 2.0*	60.80 ± 2.3*
Propranolol (0.2 mg/kg) + EEAB (2000 mg/kg)	113.80 ± 2.8	23.80 ± 0.8*	59.00 ± 2.7*
Yohimbine (0.1 mg/kg)	112.8 ± 1.9	29.40 ± 1.4*	67.20 ± 2.7*
Yohimbine (0.1 mg/kg) + EEAB (2000 mg/kg)	110.40 ± 1.3	24.40 ± 1.7*	5900 ± 2.1*

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. *p<0.05.

NIR: Novelty induced rearing, NIG: Novelty induced grooming.

LA: Locomotor activity

4.4 Anxiolytic Effect of EEAB

4.4.1 Effect of EEAB on head dips

Treatment of mice with all the treatment doses of EEAB (62.5 mg/kg, 125 mg/kg, 250 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 mg/kg) and diazepam (2.0 mg/kg) caused significant ($p<0.05$) reductions in head dips relative to the control (Figure 4.4).

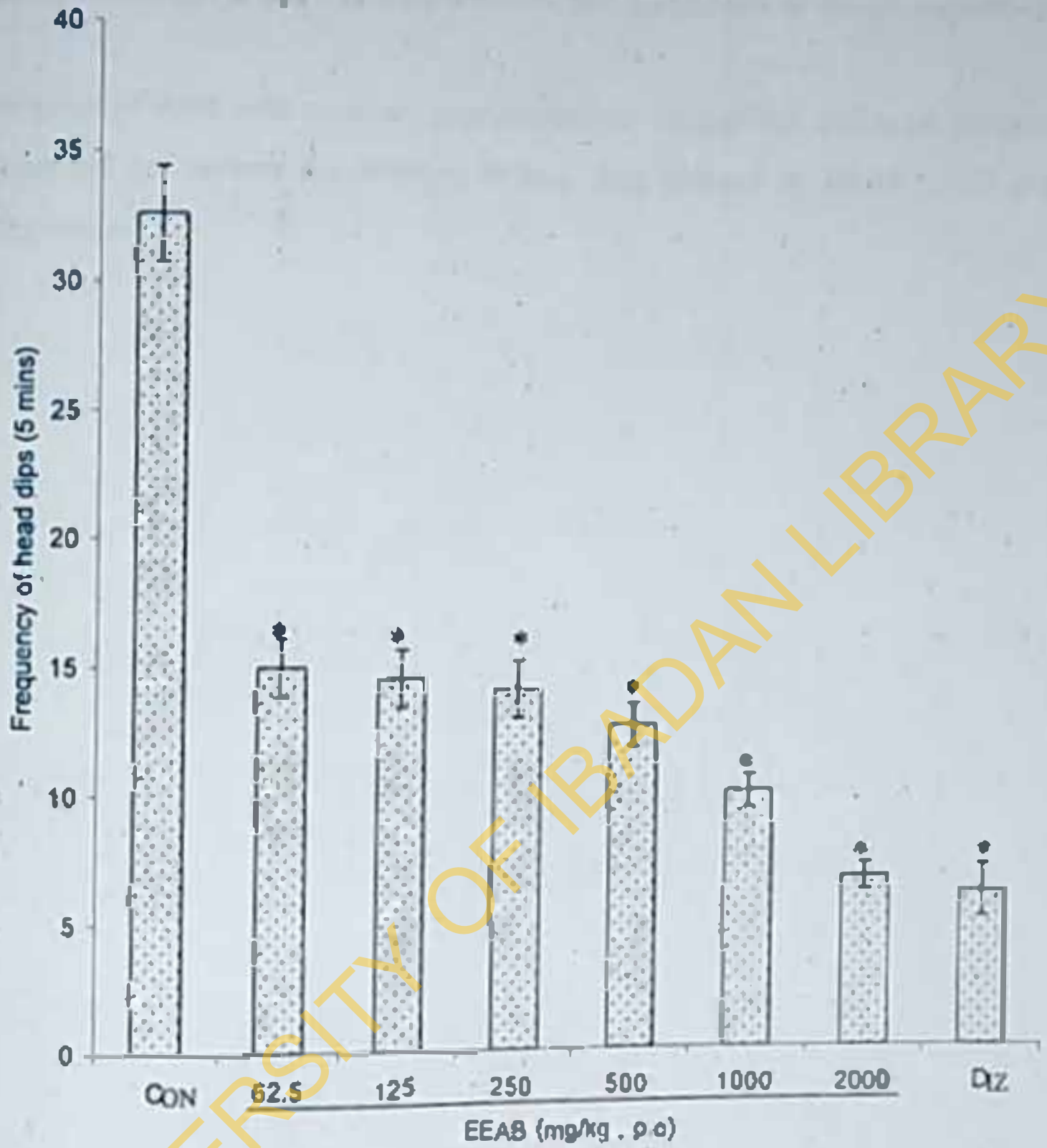

Figure 4.4: Effect of EEAB on head dips in mice

CON: Control, DIZ: Diazepam (2.0 mg/kg, i.p.)

Bars are mean values ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. $p<0.05$.

4.4.2 Effect of EEAB on head dips in the presence of antagonists

The effect of EEAB at various doses on head dips in the presence of atropine, cyproheptadine, haloperidol, naloxone, propranolol and yohimbine is shown respectively in Table 4.3.

pretreatment of mice with atropine, cyproheptadine, haloperidol, naloxone, propranolol and yohimbine did not reverse the decrease in head dips induced by EEAB (2000 mg/kg) relative to the control.

Table 4.3: Effect of EEAB on head dips in presence of antagonists

Treatment	HD/ 5 min
Control (0.2 ml/20 g)	32.60 ± 1.8
EEAB (2000 mg/kg)	6.80 ± 0.5*
Atropine (0.5 mg/kg)	20.20 ± 1.2*
Atropine (0.5 mg/kg) + EEAB (2000 mg/kg)	7.40 ± 0.6*
Cyproheptadine (0.5 mg/kg)	10.80 ± 0.6*
Cyproheptadine (0.5 mg/kg) + EEAB (2000 mg/kg)	3.40 ± 0.6*
Haloperidol (0.2 mg/kg)	10.00 ± 0.8*
Haloperidol (0.2 mg/kg) + EEAB (2000 mg/kg)	4.00 ± 0.5*
Naloxone (0.2 mg/kg)	10.20 ± 0.4*
Naloxone (0.2 mg/kg) + EEAB (2000 mg/kg)	4.20 ± 0.6*
Propranolol (0.2 mg/kg)	9.80 ± 0.4*
Propranolol (0.2 mg/kg) + EEAB (2000 mg/kg)	9.80 ± 0.6*
Yohimbine (1.0 mg/kg)	9.80 ± 0.4*
Yohimbine (1.0 mg/kg) + EEAB (2000 mg/kg)	8.20 ± 0.4*

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control.

*$p<0.05$.

HD: Head dips

4.4.3 Effect of EEAB on elevated plus maze

Treatment of mice with all the treatment doses of EEAB (62.5 - 2000 mg/kg) did not alter the frequency of open arm entries relative to the control, but diazepam caused a significant ($p<0.05$) increase in the frequency of open arm entries relative to the control. The extract at all the treatment doses induced significaat ($p<0.05$) increase in time spent in the closed arms relative to the open arms, while diazepam (1.0 mg/kg) induced a significant ($p<0.05$) increase in time spent in the open arms relative to the closed arms. The extract at all the treatment doses and the control produced significantly higher index of open arm avoidance when compared to diazepam with a significantly lower index of open arm avoidance (Table 4.4)

Table 4.4: Effect of EEAB on frequency of arm entries and time spent in the arms of an elevated plus maze

Treatment	No of entries into arms		Time spent in each arm (sec)		% Time spent in open arms	Index of open arm avoidance
	Open	Close	Open	Close		
Control (0.2 ml/20 g)	4.80 ± 0.2	7.60 ± 0.8	98.40 ± 3.6	223.40 ± 5.8	30.6	65.4
EEAB (62.5 mg/kg)	4.20 ± 0.4	6.60 ± 0.7	76.60 ± 2.7	167.60 ± 4.7*	31.4	64.9
EEAB (125 mg/kg)	4.80 ± 0.3	6.40 ± 0.7	87.80 ± 3.9	160.20 ± 3.2*	35.4	60.9
EEAB (250 mgkg)	4.80 ± 0.4	6.20 ± 0.7	84.80 ± 3.7	152.60 ± 3.8*	35.7	60.3
EEAB (500 mg/kg)	5.00 ± 0.4	6.20 ± 0.5	105.60 ± 3.8	144.80 ± 4.7*	42.0	56.7
EEAB (1000 mg/kg)	5.00 ± 0.5	6.20 ± 0.5	106.60 ± 4.3	126.20 ± 4.7*	45.8	54.8
EEAB (2000 mg/kg)	5.00 ± 0.5	6.20 ± 0.3	121.8 ± 4.8	187.20 ± 4.2*	39.4	58.0
Diazepam (1.0 mg/kg)	7.80 ± 0.7*	3.20 ± 0.3*	183.40 ± 5.8*	97.80 ± 3.2	65.2*	32.0*

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. *p<0.05.

4.5 Sedative Effect of EEAB

4.5.1 Effect of EEAB on pentobarbital-induced sleep latency and sleeping time

Treatment of mice with all the treatment doses of EEAB (250 - 2000 mg/kg) and diazepam (2.0 mg/kg) caused significant ($p<0.05$) reductions in sleep latency relative to the control (Figure 4.5) as well as significant ($p<0.05$) increase in sleeping time relative to the control (Figure 4.6).

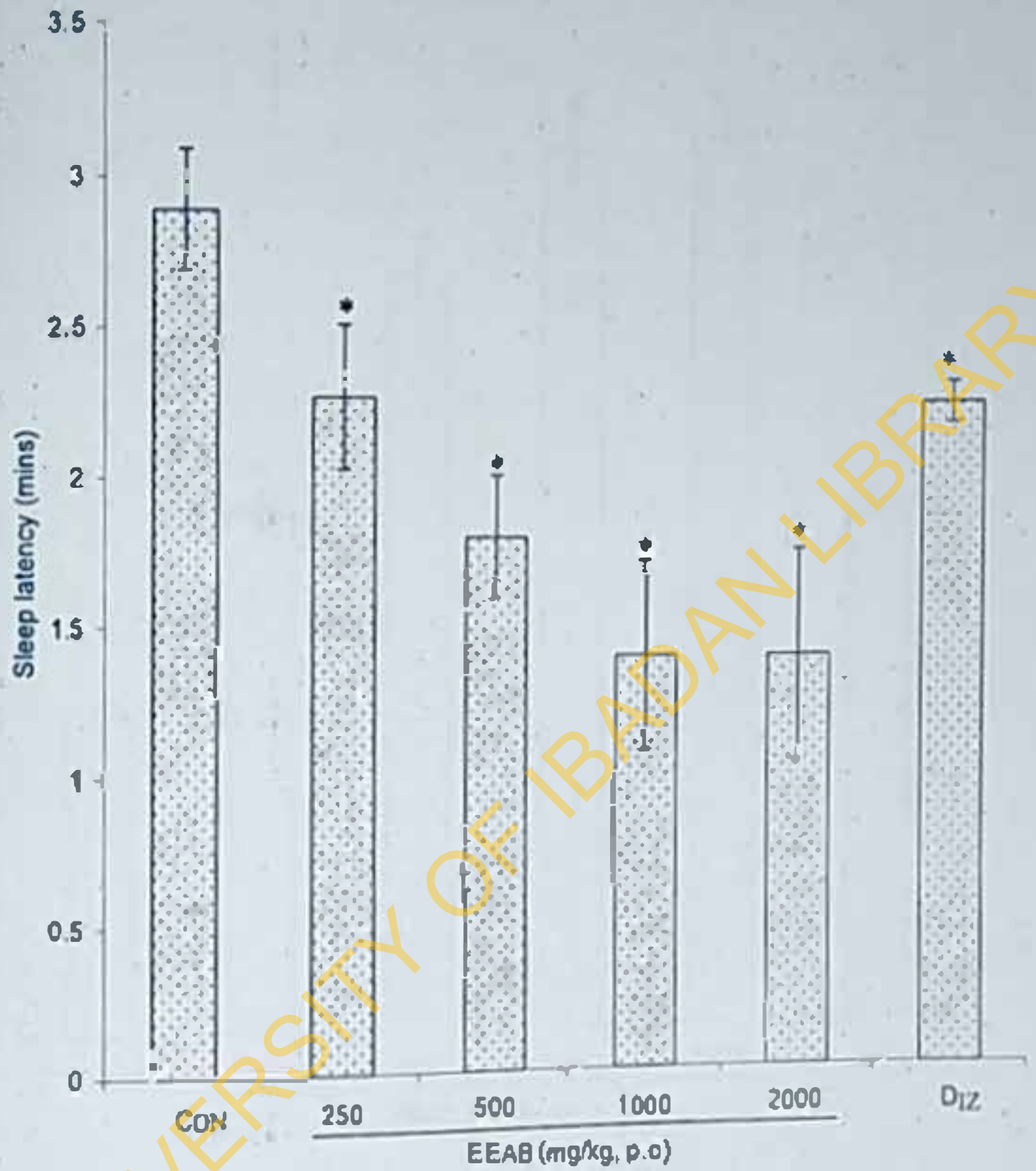

Figure 4.5: Effect of EEAB on pentobarbital – induced sleep latency in mice

CON: Control, DIZ: Diazepam (2.0 mg/kg, i.p.)

Bars are mean values ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. $p<0.05$.

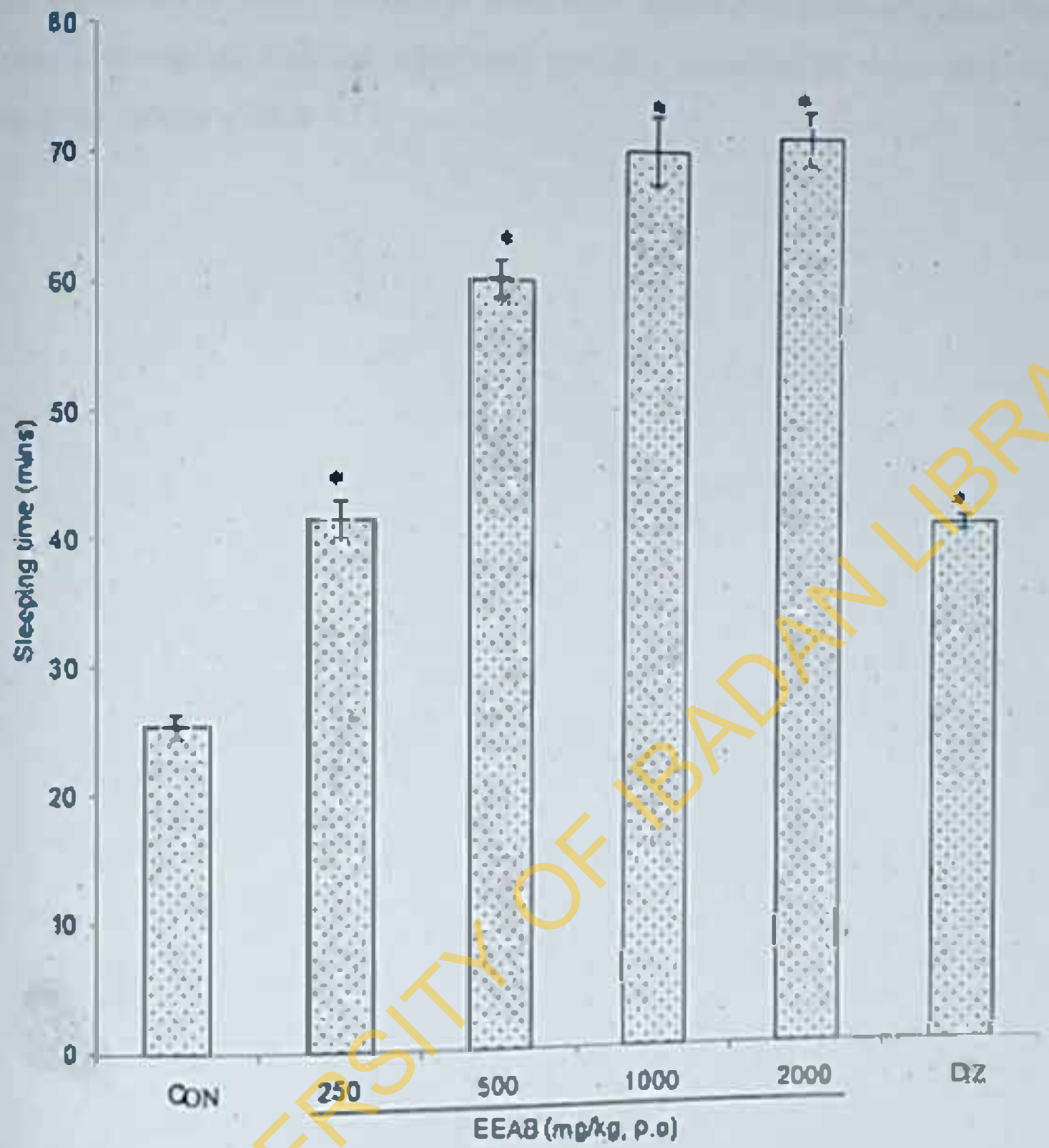

Figure 4.6: Effect of EEAB on pentobarbital – induced sleeping time in mice

CON: Control, DIZ: Diazepam (2.0 mg/kg, I.p.)

Bars are mean values ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. • Indicates significant difference from control. $p<0.05$.

1.5.2 Effect of EEAB on rectal body temperature

Treatment of mice with all the treatment doses of EEAB (250-2000 mg/kg) did not produce significant ($p>0.05$) changes in rectal body temperature relative to the control, while diazepam (2.0 mg/kg) induced significant ($p<0.05$) reduction in rectal boy temperature relative to the control (Table 4.5).

Table 4.5: Effect of EEAB on rectal body temperature in mice

Treatment	0 min	30 min	60 min	90 min	120 min	180 min
Control (0.2 ml/20 g)	37.64 ± 0.2	37.34 ± 0.1	37.24 ± 0.1	37.34 ± 0.2	37.38 ± 0.1	37.32 ± 0.2
EEAB (250 mg/kg)	37.46 ± 0.5	36.82 ± 0.1	37.26 ± 0.2	37.06 ± 0.1	37.18 ± 0.2	37.10 ± 0.1
EEAB (500 mg/kg)	37.12 ± 0.3	37.00 ± 0.8	37.34 ± 0.3	37.08 ± 0.2	37.42 ± 0.1	37.62 ± 0.2
EEAB (1000 mg/kg)	37.36 ± 0.4	37.16 ± 0.1	37.34 ± 0.2	37.38 ± 02	37.42 ± 0.2	37.16 ± 0.1
EEAB (2000 mg/kg)	37.30 ± 0.2	37.34 ± 0.2	37.60 ± 0.3	37.42 ± 0.2	37.42 ± 0.2	37.44 ± 2.0
Diazepam (2.0 mg/kg)	37.62 ± 0.1	35.40 ± 0.6*	35.54 ± 0.2*	35.50 ± 0.1*	35.34 ± 0.1*	35.56 ± 0.2*

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. *$p<0.05$.

4.6 Effect of EEAB on skeletal muscle relaxant activity

Treatment of mice with all the treatment doses of EEAB (250 - 2000 mg/kg) did not produce significant changes ($p>0.05$) in muscle coordination activity relative to the control, while diazepam (4.0 mg/kg) caused significant ($p<0.05$) decrease in muscle coordination activity relative to the control (Figure 4.7).

Figure 4.7: Effect of EEAB on skeletal muscle relaxant activity in mice

CON: Control, DIZ: Diazepam (4.0 mg/kg, i.p.)

Bars are mean values ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. $p<0.05$.

4.7 Effect of EEAB on learning and memory

Treatment of mice with all the treatment doses of EEAB (62.5 – 2000 mg/kg) and diazepam (2.0 mg/kg) caused reductions in total arm entries (locomotor activity) relative to the control. Treatment of mice with 1000 mg/kg and 2000 mg/kg of EEAB caused significant ($p<0.05$) increase in percentage alternation relative to the control, while diazepam (2.0 mg/kg) caused significant decrease in percentage alternation relative to the control (Figure 4.8 and Figure 4.9).

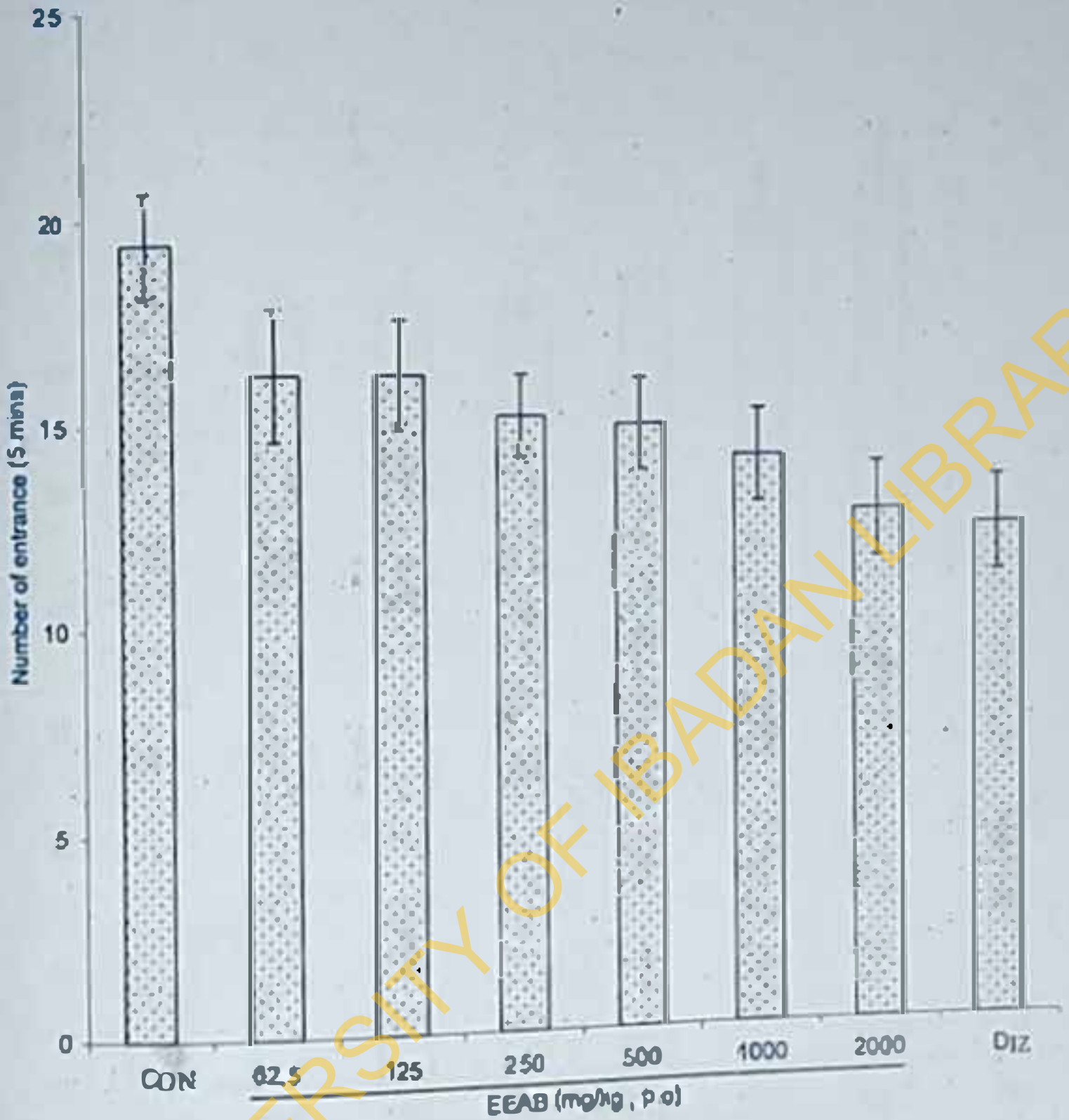

Figure 4.8: Effect of EEAB on locomotor activity in Y-Maze test in mice

CON: Control, DIZ: Diazepam (2.0 mg/kg, i.p.)

Bars are mean values ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. • Indicates significant difference from control. $p<0.05$

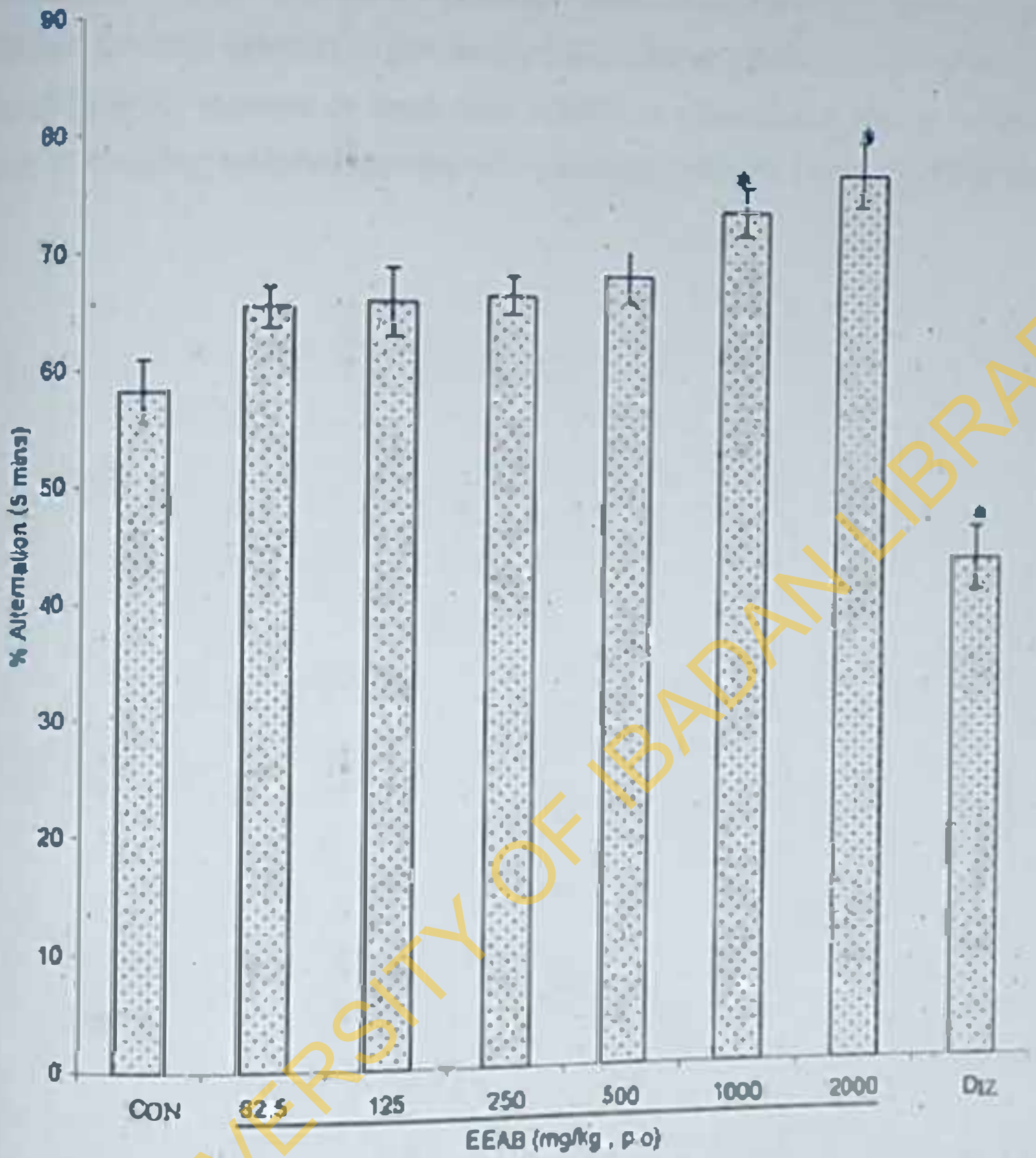

Figure 4.9: Effect of EEAB on memory in mice (% Alternation)

CON: Control, DIZ: Diazepam (2.0 mg/kg, i.p.)

Bars are mean values ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. $p<0.05$.

.8 Anticonvulsant Effect

.8.1 Effect of EEAB on PTZ-induced convulsions

Treatment of mice with all the treatment doses of EEAB (250 - 2000 mg/kg) caused nsignificant ($p>0.05$) increase in the onset of convulsions relative to the control as well as ignificant ($p<0.05$) increase in death time relative to control with 100 % mortality, while liazepam (2.0 mg/kg) inhibited the onset of convulsion with 0 % mortality (Table 4.6).

Table 4.6: Effect of EEAB on Pentylenetetrazol (PTZ)-induced convulsions in mice

Treatment	Onset of convulsion (sec)	Death time (sec)	% Protection	% Mortality
Control (0.2 ml/20 g)	44.80 ± 3.0	124.56 ± 6.5	0.0	100.0
EEAD (250 mg/kg)	48.20 ± 2.2	188.28 ± 8.5*	0.0	100.0
EEAD (500 mg/kg)	49.40 ± 4.0	217.20 ± 8.8*	0.0	100.0
EEAD (1000 mg/kg)	50.60 ± 2.2	261.60 ± 9.3*	0.0	100.0
EEAB (2000 mg/kg)	51.60 ± 1.2	268.92 ± 9.8*	0.0	100.0
Diazepam (2.0 mg/kg)	-	-	100	0.0

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. *p<0.05.

4.8.2 Effect of EEAB on strychnine-induced convulsions

Treatment of mice with all the treatment doses of EEAB (250 - 2000 mg/kg) and diazepam (2.0 mg/kg) caused significant ($p<0.05$) increases in the onset of convulsions and death time relative to the control (Table 4.7).

Table 4.7: Effect of EEAB on strychnine-induced convulsions in mice

Treatment	Onset of convulsion (sec)	Death time(sec)
Control (0.2 ml/20 g)	129.00 ± 5.8	137.40 ± 6.0
EEAB (250 mg/kg)	138.00 ± 6.0*	181.20 ± 7.0*
EEAB (500 mg/kg)	163.80 ± 6.2*	222.60 ± 7.9*
EEAB (1000 mg/kg)	175.20 ± 6.9*	252.00 ± 8.4*
EEAB (2000 mg/kg)	211.20 ± 7.5*	258.60 ± 9.5*
Diazepam (2.0 mg/kg)	320.4±12.6*	334.8±13.4*

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. *p<0.05.

4.8.3 Effect of EEAB on picrotoxin-induced convulsions

Treatment of mice with EEAB (1000 mg/kg, 2000 mg/kg) induced significant ($p<0.05$) increase in the onset of convulsions relative to the control, while all the treatment doses of EEAB (250 - 2000 mg/kg) caused significant ($p<0.05$) increase in death time relative to the control with 100 % mortality. Diazepam (2.0 mg/kg) inhibited the onset of convulsion with 0 % mortality (Table 4.8).

Table 4.8: Effect of EEAB on picrotoxin-induced convulsions in mice

Treatment	Onset of convulsion(sec)	Death time (sec)	% Protection	% Mortality
Control (0.2 ml/20 g)	307.80 ± 12.0	727.20 ± 14.3	0.0	100.0
EEAB (250 mg/kg)	310.20 ± 12.2	775.80 ± 15.9*	0.0	100.0
EEAB (500 mg/kg)	337.20 ± 12.5	867.60 ± 16.6*	0.0	100.0
EEAB (1000 mg/kg)	374.40 ± 13.1*	1005.00 ± 17.6*	0.0	100.0
EEAB (2000 mg/kg)	766.80± 14.7*	1308.60* ± 18.2*	0.0	100.0
Diazepam (2.0 mg/kg)	-	-	100.0	0.0

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. *p<0.05.

4.9 Analgesic Effect

4.9.1 Effect of EEAB on hot plate test

The administration of EEAB (250 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 mg/kg) and morphine (10 mg/kg) to mice significantly ($p<0.05$) reduced the pain response to the thermal stimulus of hot plate as indicated by the increase in reaction time when compared to the control. Pretreatment with naloxone (2 mg/kg) reversed the analgesia induced by morphine (10 mg/kg) and the sub-maximal dose of EEAB (1000 mg/kg) (Table 4.9).

Table 4.9: Analgesic activity of EEAB (Hot plate test)

Treatment	0 min	30 min	60 min	90 min	120 min
Control (0.2 ml/20 g)	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0
EEAB (250 mg/kg)	1.00 ± 0.0	1.00 ± 0.0	1.20 ± 0.2	1.80 ± 0.1	2.20 ± 0.2*
EEAB (500 mg/kg)	1.00 ± 0.0	1.40 ± 0.2*	2.00 ± 0.1*	2.80 ± 0.3*	2.80 ± 0.4
EEAB (1000 mg/kg)	1.00 ± 0.0	2.00 ± 0.5*	2.00 ± 0.1*	2.80 ± 0.5*	2.80 ± 0.4*
EEAB (2000 mg/kg)	1.00 ± 0.0	2.20 ± 0.3*	2.80 ± 0.3*	3.20 ± 0.4*	3.60 ± 0.3
Mor (10 mg/kg)	1.00 ± 0.0	2.20 ± 0.2*	2.72 ± 0.3*	3.00 ± 0.3*	3.20 ± 0.3
NAL + EEAB (1000)	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.4	1.00 ± 0.0	1.00 ± 0.0
NAL + Mor	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.36 ± 0.1	1.62 ± 0.2

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control.

*$p<0.05$.

Mor: Morphine (10 mg/kg), NAL: Naloxone (2 mg/kg),

4.9.2 Effect of EEAB on tail immersion test

The administration of all the treatment doses of EEAB (250 - 2000 mg/kg) and morphine (10 mg/kg) to mice ($p<0.05$) induced analgesia by causing an increase in the reaction time to the thermal stimulus of hot water relative to the control. Pretreatment with naxolone (2 mg/kg) reversed the analgesia induced by morphine (10 mg/kg) and the sub-maximal dose of EEAB (1000 mg/kg) (Table 4.10).

Table 4.10: Analgesic activity of EEAB (Tail immersion test)

Treatment	0 min	30 min	60 min	90 min	120 min
Control (0.2 ml/20 g)	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0
EEAB (250 mg/kg)	1.00 ± 0.0	1.60 ± 0.3	2.00 ± 0.3*	2.00 ± 0.2*	2.80 ± 0.3*
EEAB (500 mg/kg)	1.00 ± 0.0	2.00 ± 0.3*	2.40 ± 0.4*	2.80 ± 0.4*	2.80 ± 0.3*
EEAB (1000 mg/kg)	1.00 ± 0.0	2.00 ± 0.3*	2.40 ± 0.3*	2.80 ± 0.3*	3.40 ± 0.4*
EEAB (2000 mg/kg)	1.00 ± 0.0	2.20 ± 0.4*	5.40 ± 1.5*	10.00 ± 0.6*	10.00 ± 0.1*
Mor (10 mg/kg)	1.00 ± 0.0	2.20 ± 0.2*	4.20 ± 0.5*	10.00 ± 0.5*	10.00 ± 0.4*
NAL + EEAB (1000)	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0
NAL + Mor	1.00 ± 0.0	1.00 ± 0.0	1.00 ± 0.0	1.56 ± 0.2	1.60 ± 0.3

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control.

*$p<0.05$.

Mor: Morphine (10 mg/kg), NAL: Naloxone (2 mg/kg).

4.9.3 Effect of EEAB on acetic acid-induced writhing test

Treatment of mice with all the treatment doses of EEAB (250 - 2000 mg/kg) and aspirin (150 mg/kg) caused a significant ($p<0.05$) reduction in the number of acetic acid-induced abdominal writhes when compare to the control (Table 4.11).

Table 4.11: Analgesic activity of EEAB (Acetic acid-induced writhing test)

Treatment	No of writhes/ 15 min	% Inhibition
Control (0.2 ml/20 g)	41.40 ± 1.8	0.0
EEAB (250 mg/kg)	33.60 ± 1.1*	18.8
EEAB (500 mg/kg)	15.80 ± 1.1*	61.8
EEAB (1000 mg/kg)	13.80 ± 0.9*	66.7
EEAB (2000 mg/kg)	4.00 ± 0.5*	90.3
Aspirin (150 mg/kg)	5.40 ± 0.3*	87.0

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. *$p<0.05$.

4.9.4 Effect of EEAB on formalin test

Treatment of mice with all the treatment doses of EEAB (250 - 2000 mg/kg) and morphine (10 mg/kg) induced a dose-dependent inhibition in paw-licking time in both phases of the test relative to the control. Pretreatment with naloxone (2 mg/kg) reversed the analgesia induced by morphine (10 mg/kg) and the sub-maximal dose of EEAB (1000 mg/kg) in both phases of the test (Table 4.12).

Table 4.12: Analgesic activity of EEAB and mechanism of action (Formalin test)

Treatment	Licking time (sec)		Percentage inhibition	
	1st Phase	2nd Phase	1st Phase	2nd phase
Control (0.2 ml/20 g)	76.00 ± 3.7	179.60 ± 3.8	-	-
EEAB (250 mg/kg)	44.00 ± 2.6*	69.00 ± 2.4*	42.1	61.6
EEAB (500 mg/kg)	38.20 ± 2.8*	58.00 ± 3.0*	49.7	67.7
EEAB (1000 mg/kg)	27.60 ± 2.8*	49.40 ± 2.1*	63.7	72.5
EEAB (2000 mg/kg)	4.60 ± 0.6*	1.20 ± 0.3*	93.9	99.3
Mor (10 mg/kg)	36.20 ± 0.5*	17.40 ± 0.8*	52.4	90.3
NAL + EEAB (1000)	73.80 ± 2.4	174.60 ± 3.9	2.9	2.8
NAL + Mor	77.20 ± 2.3	188.40 ± 2.9	-	-

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control *$p<0.05$.

Mor: Morphine (10 mg/kg), NAL: Naloxone (2 mg/kg).

CHAPTER FIVE

DISCUSSION

5.0

Phytochemical analysis is an important tool to identify and screen plants for their use (Sadhu *et al.*, 2007). The phytochemical analysis of the extract indicated the presence of tannins, and it has been reported that herbs that have tannins as their major components are astringent in nature and are used for treating intestinal disorders such as diarrhea and dysentery (Dhannanonda 2003). Therefore, these observations could support the use of this plant in West Africa for treating a wide range of gastrointestinal disorders (Burkill, 1985). The analyses also revealed the presence of alkaloids and flavonoids which have been reported to possess various important pharmacological activities including inflammatory, antioxidant and antinociceptive activities (Duke, 1992; Geetha and Varalakshmi, 2001) which justify the use of this plant in herbal cure remedies.

Acute toxicity test gives clues on the range of doses that could be toxic to the animal; it could also be used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs and xenobiotics (Rang *et al*, 2001). LD_{50} is the dose at which mortality occurs in 50 % population of the experimental animals. The higher the value of the LD_{50} for a substance, the relatively safer the substance is assumed to be. The LD_{50} determination for the extract in mice via the oral route was 7000 mg/kg, which was not toxic to the animals, and since the recommended single high dose by OECD guidelines 423 (OECD, 2002) for testing acute toxicity is 2000 mg/kg ; this probably indicates the extract has wide safety margins (low toxicity). Similar result was reported by Ali *et al.*, (2009) in *Eichhornia crassipes* extract treated mice.

The extract was examined for novelty – induced rearing (NIR) in mice. NIR is a behaviour of rodents in novel environments. The behaviour is employed by rodents as one of the survival strategies in assessing the environment for food, protection and possibly escapes (Blanchard *et al.*, 2001). Measurement of the frequency of rearing in rodents and the modification can therefore be employed in assessing test drugs and extracts for both sedative property and central nervous system stimulation (Vogel, 2002). Rearing has been described as the vertical locomotion activity when the animal stands on its hind leg while raising up its forearm in the air or placed on the wall of the cage (Onigbogi *et al.*, 2000). Drugs that stimulate the CNS increase rearing behaviour, while those that depress the CNS inhibit rearing behaviour. The extract

inhibited NIR in mice which probably indicate a sedative or depressant property. Similar result was reported by Akanmu *et al.*, (2011) in Nigeria Honey treated mice.

The crude extract was examined for novelty – induced grooming (NIG) in mice. Grooming is an important behavioural component in animals and is associated with de-arousal of the central nervous system (CNS). De – arousal indicates absence of stimulation. Grooming is described in animals (rat or mice) as face or head washing with forearm or body grooming with mouth (Ukponmwan *et al.*, 1985). Drugs that have depressant effect inhibit grooming behaviour. The extract reduced NIG in mice which suggest that the extract have depressant effect on the CNS. Similar result was reported by de Araujo *et al.*, (2009) in *Alpinia zerumbet* essential oil treated mice.

Locomotor activity is considered as an index of alertness and a reduction is indicative of sedative activity (Lowry *et al.*, 2005). The open field test is a simple assessment used to establish the general activity levels, gross locomotor activity and exploration habits of rodents (Prut and Belzung, 2003). The extract caused reduction in locomotor activity which further confirms the CNS depressant activity of the extract. Similar result was reported by Suganyanam *et al.*, (2012) in *Tecoma stans* flowers extracts treated mice.

The hole board is a method used to measure the animal's response to a novel environment and to assess emotionality, anxiety and/or responses to stress (Han *et al.*, 2009). In this test, head dipping behaviour may change in response to the emotional state of the animal and an increase in this behaviour could reflect the expression of an anxiolytic reaction of the animal (Takeda *et al.*, 1998). On the other hand, a decrease in the number of head dipping reveals a sedative or depressant behaviour (File and Pellow, 1985; Viola *et al.*, 1995). Since, the extract caused decrease in head dips, this probably indicates that the extract posses a sedative or CNS depressant property. Similar result was reported by Martinez – Vazquez *et al.*, (2012) in *Dracocephalum moldavica* extract treated mice.

Novelty – induced rearing and grooming behavioural response is regulated by multiple neurotransmitter system, such transmitters include: gamma – aminobutyric acid (GABA), cholinergic, adrenergic, opioid, serotonin, glutamate and dopamine receptors (Waking, 1998). The administration of atropine, cyproheptadine and haloperidol to mice did not reverse the inhibitory effect of the extract on novelty – induced rearing, grooming, head dips and locomotor

activity, this probably indicates that muscarinic, serotonergic and dopaminergic receptors were not involved in the inhibitory effect of the extract on the aforementioned behavioural response. However, the administration of naloxone, propenolol and yohimbine to mice reverse the inhibitory effect of the extract on novelty – induced rearing, this suggests the involvement of μ - opioid, β-adrenergic and α_2 – adrenergic receptors in the inhibitory effect of the extract on the aforementioned behavioural response.

Anxiety, a state of fear, is characterized by motor tension, sympathetic hyperactivity, apprehension and vigilance syndromes (Sadock and Sadock, 2003). Anxiety may interfere with intelligence, psychomotor function and memory (Pine *et al.*, 1999). Among the models of anxiety disorders that are used in determining anxiolytic or anxiogenic properties of substances is elevated plus maze. The elevated plus maze (EPM) represent one of the most widely used animal models for screening anxiolytic and anxiogenic drugs (Lister, 1987). This test is able to reproduce anxiolytic or anxiogenic effects in rodents such that anxiolytics produce increase in the time spent in the open arm of the elevated plus maze, while anxiogenic substances produce the opposite effect (Pellow and File, 1986). The extract induced reductions in the number of entries into open arm and time spent in the open arm as well as induced significantly higher index of open arm avoidance when compared to diazepam with a significantly lower index of open arm avoidance which probably indicates that the extract is anxiogenic. This anxiogenic property validates the CNS depressant property of the extract. Similar result was reported by Felipe Melo *et al.* (2013) in *Tabernaemontana solanifolia* extracts treated rats.

Pentobarbitone – induced (animals) is used to test centrally acting effect of agents (Carpendo *et al.*, 1994). The interval between loss and recovery of righting reflex was taken as the index of hypnotic effect (Sairaf *et al.*, 2013). Two parameters were measured in this experiment, sleep latency and sleeping time. Sleep latency is defined as the time in minute from injection time to loss of righting reflex (unconsciousness) while sleeping time is defined as the total time in minute from loss of righting reflex (loss of consciousness) to regain of righting reflex (recovery of consciousness) (Ayoka *et al.*, 2006). Studies have shown that the potentiation of barbiturate hypnosis is an index of CNS depression (Delur *et al.*, 2012). The extract could interact with the GABAergic system to induce its hypnotic effect since it has been reported that several neurotransmitters and endogenous molecules are involved in regulation of sleep and wakefulness.

The sleep – promoting neurons located in the anterior hypothalamus release gamma aminobutyric acid (GABA) to suppress activity of wake – inducing areas of the brain (Datta, 2010). Pentobarbital is known to act at GABA receptors ionophore complex and favour the biding of GABA. Also benzodiazepine agonists such as diazepam enhance the affinity of GABA for its receptor and hence prolong pentobarbital – induced sleep duration (Gottesmann, 2002). Similar result was reported by Chu *et al.* (2007) in *Ganoderma lucidum* extract treated mice and rats.

Thermoregulation is a complex physiological process involving both central and peripheral autonomic mechanisms. The primary thermoregulatory centre resides in the preoptic area of the hypothalamus and controls the balance between heat gain and heat loss. GABAergic terminals and $GABA_A$ receptors on the neurons of the preoptic area of the hypothalamus have been reported to be involved in the process of thermoregulation (Griti *et al.*, 1993). In addition, studies have also shown that systemic administration of either GABA or $GABA_A$ agonist usually produce hypothermia (Frosini *et al.*, 2004). The extract did not induce significant changes in rectal body temperature which probably indicates that it has no effect on the thermostat regulatory centre in the brain. Contrary result was reported by Butterweck *et al.* (2007) in hops (*Humulus lupulus*) treated mice.

A muscle relaxant is a drug which affects skeletal muscle function and decreases the muscle tone. It may be used to alleviate symptoms such as muscle spasms, pain, and hyperreflexia. The extract did not produce skeletal muscle relaxant effect in mice which corroborates the CNS activity of this extract. Contrary result was reported by Jha *et al.* (2011) in *Parthenium hysterophorus* extract treated mice.

The Y – maze has been reported to be used as a measure of short term memory, general locomotor activity and stereotypical behaviour (Ilco *et al.*, 2003; Mamiya *et al.*, 2004). Learning and memory is one of the most important functions of the brain, which is associated with complex neurophysiologic and neurochemical changes. Many neurotransmitters including acetylcholine, dopamine, norepinephrine and serotonin play an important role in the learning and memory processes (Irond, 2003). It is well known that spontaneous alternation is a measure of spatial working memory and to alternate among spatial locations, a rodent (rat or mouse) must remember its previous location. The extract induced increase in percentage alternation which probably indicates an enhancement of spatial working memory. Similar result was reported by Yahaya *et*

al. (2013) in *Parkia biglobossa* extract treated rats. The extract also caused decrease in total arm entries which corroborate the sedative effect of this extract. Similar result was reported by Stavani *et al.* (2012) in *Pergularia daemia* extract treated mice.

Currently available anticonvulsant drugs are able to efficiently control epileptic seizures in about 50 % of the patients, another 25 % may show improvement whereas the remainder does not benefit significantly. Furthermore, undesirable side effects from the drugs used clinically often render treatment difficult so that a demand for new types of anticonvulsants exists. One of the approaches to search for new antiepileptic drugs is the investigation of naturally occurring compounds, which may belong to new structural classes (Duraisami *et al.*, 2009). The anticonvulsant potential of a drug is not only determined by its ability to prevent convulsion and mortality, but also by its ability to delay the onset of convulsion, shorten the frequency and duration of tonic – clonic seizur (Kendall *et al.*, 1981). Pentylenetetrazol (PTZ) – induced convulsions represent the petit – mal type of seizures and this has been primarily utilized as animal model to evaluate anticpileptic drugs. PTZ is known to block the postsynaptic $GABA_A$ receptor mediated Cl^- conductance and thus produces seizures (Ramanjaneyulu and Ticku, 1984). The extract caused increase in the onset of convulsions as well as death time in PTZ – induced convulsions; this suggests that its anticonvulsant activity could be mediated through the GABAergic modulation. GABA is an important endogenous inhibitory neurotransmitter widely distributed throughout the central nervous system. A reduction in GABA function in the brain is associated with psychiatric and neurological disorders, including anxiety, depression, insomnia, and epilepsy (Kulkarni and Verma, 1993). Numerous natural and synthetic compounds interact with $GABA_A$ receptor at distinct, yet incompletely defined sites (Dhir *et al.*, 2006). These compounds include barbiturates, benzodiozepines, neurosteroids and picrotoxin (Sieghart, 1992). The postsynaptic $GABA_A$ receptors are implicated in the inbibitory mechanisms. $GABA_A$ receptor agonists as well as drugs, which allosteically modulate the $GABA_A$ receptor channel complex, are therapeutically effective anticonvulsant agents (David, 2001). Similar result was reported by Nouri and Abad (2011) in *Valeriana officinalis* extract treated mice

In the strychnine – induced seizure model, it is known that strychnine a potent spinal cord convulsant, blocks glycine receptor selectively to induce excitatory response in the CNS (Adesemi *et al.*, 2010). The extract caused increase in the onset of convulsion and death time in

strychnine - induced convulsions; this suggests that its anticonvulsant activity could be through suppression of the action of strychnine on glycine inhibitory mechanism or through the enhancement of glycine inhibitory mechanism. Similar result was reported by Nimbal *et al.* (2011) in *Benincasa hispida* fruit extract treated mice.

Picrotoxin, a potent selective $GABA_A$ receptor antagonist produces seizures by blocking the effect of GABA at central $GABA_A$ receptors which have been associated with epilepsy (Nicol, 2007). Postsynaptic $GABA_A$ receptors are functionally linked to benzodiazepine receptors, barbiturate receptors and chloride ion channels to form GABA – chloride ionophore complex, which is intimately involved in the modulation of GABAergic neurotransmission epilepsy (Gale, 1992). The extract induced increase in the onset of convulsion and death time in picrotoxin – induced convulsions which suggests that its anticonvulsant activity could be via the enhancement of GABAergic neurotransmission by increasing chloride ion flux through the chloride ion channels at $GABA_A$ receptor sites. It has been reported that picrotoxin, a $GABA_A$ receptor antagonist, produces seizures by blocking the chloride ion channels linked to $GABA_A$ receptors, thus preventing the entry of chloride ions into the brain (Nicol, 2007). Similar result was reported Kumar *et al.* (2011) in *Vitex negundo* extract treated mice.

Pain as a real compluint in clinical training, has different causing factors. Although there are many analgesic drugs for prescription, but because of many complexities including broad side effects, different origins of pain and weak potency of many coovention al drugs (Eisner, 1990), medicinal plant substitution has been recommended for this purpose (Farnsworth, 1989).

The hot plate method has been found to be very effective for evaluating drugs possessing analgesic property which act centrally (Silva *et al.*, 2003). The extract caused increase in reaction time in the hot plate test which probably indicates its central analgesic effect. Similar result was reported by Chandrashekar *et al.* (2011) in *Phyllanthus fawelli* extract treated mice.

The tail immersion and hot plate models have been used to study centrally acting analgesics (Woolfe and MacDonald, 1994). In these models, sensory nerves sensitise the nociceptors and the involvement of endogenous substances such as prostaglandins are minimized (Buchlov *et al.*, 2009). The extract induced increase in reaction time in the tail immersion test which suggests its centrally mediated antinociceptive activity. Similar result was reported by Agarwal *et al.* (2010) in *Trapa natans* root extract treated mice.

Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting in release of free arachidonic acid from tissue phospholipid (Ahmed *et al.*, 2006) via cyclooxygenase and prostaglandin biosynthesis (Duarte *et al.*, 1988). In other words, the acetic acid induced writhing test has been associated with increased level of PGE_2 and $PGF_{2\alpha}$ in peritoneal fluids as well as lipoxygenase products (Derardt *et al.*, 1980). The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability (Zakaria and Gani, 2008). The acetic acid induced writhing method was found effective to evaluate peripherally active analgesics. The agent reducing the number of writhing will render analgesic effect preferably by inhibition of prostaglanding synthesis, a peripheral mechanism of pain inhibition (Ferdous *et al.*, 2008). The extract inhibited writhing responses induced by acetic acid which suggests that its antinociceptive effect could be peripherally mediated via inhibition of synthesis and/or release of prostaglandins. Similar result was reported by Iniaghe *et al.*, (2012) in *Alstonia boonei* extract treated mice.

The formalin – induced pain model is very useful for elucidating the mechanism of pain and analgesia (Yongna *et al.*, 2005). Drugs that act mainly centrally, such as narcotics, inhibit both phases of the formalin – induced pain, while peripherally acting drugs such as NSAIDs only inhibit the late (second) phase (Alam *et al.*, 2012). This biphasic model is represented by neurogenic and inflammatory pain respectively (Hunskaar and Hole ,1987). The extract inhibited both phases of formalin – induced pain which suggests that it has central antinociceptive action. This probably implies that the extract can be used to manage acute and chronic pain. The mechanism by which formalin triggers C – fiber activation remained unknown for a relatively long time. Recently, however, McNamara *et al.* (2007) demonstrated that formalin activates primary afferent neurons through a specific and direct action of TRPA1, a member of the transient receptor potential family of cation channels, expressed by a subset of C – fiber nociceptors and this effect is accompanied by increased influx of Ca^{2+} ions. TRPA1 channels at primary sensory terminals were also reported to mediate noxious mechanical stimuli (Kerstein *et al.*, 2009). These experiments suggest that Ca^{2+} mobilization through TRPA1 cation channels is concomitant with noxious chemicals and mechanical stimuli as they produce their analgesic action. Hence, the antinociceptive action of the extract could be due to the inhibition of influx of intracellular Ca^{2+}

ions through TRPA1 cation channels. Similar result was reported by Pournourbed *et al.* (2010) in *Teucrium chamaedrys* extract treated rats.

The mechanism of action of three analgesic models used revealed that naloxone, a potent opioid antagonist, reversed the antinociceptive effect of the extract which suggests that the analgesic effect of the extract could be mediated via interaction with the opioid system.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

It can be concluded that *Adenopus breviflorus* fruit may possess central nervous system depressant, anticonvulsant and analgesic effects which provides scientific bases to the folkloric claims of the plant in the management of convulsion and pain. Its central nervous depressant effect could be mediated via μ-opioid, β–adrenergic and α_2–adrenergic receptors; its anticonvulsant activity via GABAergic and glycine systems, and its analgesic effect via μ - opioid receptor and inhibition of prostaglandins synthesis or release.

6.1 RECOMMENDATIONS

The folkloric claim of *Adenopus breviflorus* as an anticonvulsant, sedative and pain killer has been explored scientifically in animal model in this study. Hence, it is hereby recommended that people suffering from convulsion, irritability as well as acute and chronic pains may use the extract of *Adenopus breviflorus* fruit in the nearest future after isolation of the active component(s) and clinical trials.

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APPENDICES

Appendix I

Acute Toxicity Test

a. **Oral Method**

1st Phase

10 mg/kg – 0/3

100 mg/kg – 0/3

1000 mg/kg – 0/3

2nd Phase

2000 mg/kg – 0/1

3000 mg/kg – 0/1

4000 mg/kg – 0/1

5000 mg/kg – 0/1

6000 mg/kg – 0/1

7000 mg/kg – 1/1

8000 mg/kg – 0/1

9000 mg/kg – 1/1

10,000 mg/kg – 1/1

$LD_{50} = \sqrt{7000 \times 8000}$

≈ 7000 mg/kg

The working dose will be half of LD_{50} which is 3500 mg/kg, but according to OECD, 3500 mg/kg is too high, and since 2000 mg/kg is the highest dose acceptable by the OECD, so 2000 mg/kg was chosen as the highest dose.

b. **Intraperitoneal Method**

1st Phase

10 mg/kg – 0/3

100 kg – 3/3

1000 mg/kg – 3/3

2nd Phase

20 mg/kg – 0/1

40 mg/kg – 0/1

40 mg/kg – 1/1

60 mg/kg – 1/1

$$LD_{50} = \sqrt{40 \times 60}$$

$$\approx 49 \text{ mg/kg}$$

The working dose will be half of the LD_{50} which is 25 mg/kg, which was scaled down to 25 mg/kg → 12.5 mg/kg → 6.25 mg/kg.

APPENDIX 2

Calculation of % Alteration from the Y – Maze Test Results

a. BCABABACBACBAB (14 alphabets, 8 circles).

$$\frac{8}{14-2} \times 100 = 57\ \%$$

b. CBABCABCBACB (12 alphabets, 8 circles)

$$\frac{8}{12-2} \times 100 = 73\ \%$$

c. BCABACBACAB (11 alphabets, 7 circles)

$$\frac{7}{11-2} \times 100 = 78\ \%$$

APPENDIX 2

Calculation of % Alteration from the Y – Maze Test Results

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$$\frac{8}{12-2} \times 100 = 73\ \%$$

c. BCABACBACAB (11 alphabets, 7 circles)

$$\frac{7}{11-2} \times 100 = 78\ \%$$