AN EVALUATION OF THE CENTRAL AND SEXUAL BEHAVIOURAL EFFECTS AND TOXICITY OF THE ROOT EXTRACT OF *SPHENOCENTRUM JOLLYANUM* PIERRE (MENISPERMACEAE)

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology and Department of Molecular Medicine, KNUST. This work has not been submitted for any other degree.

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ABSTRACT

Roots of Sphenocentrum jollyanum are used as CNS stimulant and aphrodisiac in Ghanaian traditional medicine. The present study is aimed at evaluating the central and sexual behavioural effects and toxicity of the root extract of S. jollyanum Pierre. Extracts of S. jollyanum (SJE) (100, 300 and 1000 mg kg^{-1} *p.o) enhanced mounting and mating behaviour by increasing mounting frequency, intromission frequency and prolonged ejaculation latency. In addition, a decrease in mounting latency, intromission latency and post-ejaculatory interval were observed. These observations indicate increased libido and enhanced sexual behaviour. The dose-response curves of some of the parameters measured (attempted mounts, mounting frequency, anogenital sniffing and penile licking) were U-shaped. Also, the effect of the extract on FSH, LH, prolactin and testosterone levels in rats were determined at weekly intervals for three weeks. Levels of testosterone were increased 4-fold by the third week and there was about 30% increase in FSH levels by the second week which dropped by the third week. Surprisingly, LH levels were reduced by the second week with no significant change in levels of prolactin. These results suggest that there may be more than one mechanism of action of the extract. The immediate increase in sexual behaviour by SJE may be due to a central stimulatory effect whilst long-term effect might be due to increased testosterone levels.*

To evaluate the effect of the alcoholic root extract of S. jollyanum on smooth muscle, anococcygeus smooth muscle and corpus cavernosum were employed. The cumulative application of the extract relaxed the contraction maintained in the isolated anococcygeus of a rat, induced by high potassium. The extract (0.1-10.0 mg ml^{-1}*) also reduced in a concentration-dependent way the maximum response of concentration-response curve to carbachol, phenylephrine and calcium in the anococcygeus muscle. Apart from that, the extract dose-dependently relaxed phenylephrine-precontracted corpus cavernosum smooth muscle. In the tissues investigated, the extract caused a non-specific reduction in both the natural tone and agonist-induced contractions. The extract was found to have constituents (flavonoids and alkaloid corresponding to berberine) which have recorded smooth muscle relaxation via* Ca^{2+} *mobilization effect. It is therefore possible that the observed effect of the extract is through membrane stabilization or inhibition of a common pathway in the signal transduction mechanism possibly* Ca^{2+} *mobilization. This direct effect exerted on the penile tissue by SJE could also contribute to its copulatory performance-enhancing actions.*

The effect of the root extract on anxiety was assessed using multiple behavioural paradigms - hole-board, elevated plus-maze (EPM), light/dark box and open field. Adult male mice were treated with SJE (30 - 300 mg kg^{-1}*, p.o.), caffeine (10 - 100 mg* kg^{-1}*i.p.), diazepam (0.1 - 1.0 mg* kg^{-1}*, i.p) or saline (10 ml* kg^{-1}*) 30 min before subjected to the behavioural*

paradigms. Caffeine and the extract decreased percentage open-arm time and entry as well as locomotor activity in EPM indicative of anxiogenicity. The number and duration of head dip also decreased (31.7 - 68.1 % and 60 – 84% respectively) for SJE. Furthermore, there was a dose-dependent increase in the time spent at the corners and decreases in locomotor activity in open field (1 7.3 - 30.65% and 40.25 - 70.39% respectively) as compared to the control. In contrast, diazepam (1.0 mg kg^{-1}, i.p.) exhibited the typical profile of an anxiolytic drug by, increasing the time spent and entries into open arms as well as locomotor activity by 70%, 83.33% and 25.52% respectively. Diazepam also decreased the time spent at the corners and increased locomotor activity in open field. These results indicate that SJE has CNS stimulant activity, which correlates well with the traditional uses of the plant.

The effect of SJE on depression was evaluated in the forced swimming test (FST) and the tail suspension test (TST). SJE significantly decreased immobility periods of mice in both the FST and the TST. These indicate that, the extract possesses anti-depressant-like property. The extract did not have a significant effect on motor co-ordination, confirming the assumption that the anti-depressant-like effect is specific. The present study also investigated the contributions of monoamines to the acute behavioural effects of SJE by depleting serotonin (5-HT), or norepinephrine (NE) and dopamine (DA). Catecholamine synthesis was inhibited by α-methyldopa (MeDOPA), whereas reserpine was used for the disruption of vesicular storage of catecholamine. Neither treatment completely prevented responses to SJE, or fluoxetine except that of imipramine in the TST. Depleting both newly synthesized and vesicular components of NE and DA transmission with a combination of reserpine and α-methyldopa (MeDOPA) completely prevented the behavioural effects of SJE and fluoxetine and attenuated those of imipramine. Thus, SJE elicited a significant antidepressant-like effect in mice and may possibly exert it effects by modifying monoamine transport and/or metabolism.

In the toxicological study, there were no significant differences found in most of the hematological, serum biochemical parameters and organ/body weight ratio. No abnormality was found in any organ during histopathological examination and no evidence of mutagenicity using Salmonella typhimurium TA$_{97}$, TA$_{98}$, TA$_{100}$ and TA$_{102}$ tester strains. The extract, however, caused a significant increase in cytochrome P450 which correlated with the decreased pentobarbitone induced sleeping times. The results showed that the no-observed adverse- effect level (NOAEL) of SJE was >1000 mg kg^{-1} body weight per day in rats, which can be regarded as virtually non-toxic. In conclusion, SJE had no overt organ specific toxicity but demonstrates a potential for drug interactions via induction of cytochrome P450-mediated metabolism in the rat.

Collectively, these findings support the use of S. jollyanum in traditional medicine.

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ABBREVIATION

3,5-DHBS	3, 5-dichloro-2-hydroxybenzen
4-AAP	4-aminoantipyrine
5-HT	5-hydroxytryptamine
ACH	Acetylcholine
ACTH	Adrenocorticotropic Hormone
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AR	Adrenoceptor
AST	Aspartate Aminotransferase
BCG	Bromocresol Green
BUN	Blood Urea Nitrogen
CCSM	Corpus Cavernosum Smooth Muscle
CGRP	Calcitonin Gene-Related Peptide
CNS	Central Nervous System
CRH	Corticotrophin-Releasing-Hormone
CYP	Cytochrome P450s

DA Dopamine

DAR Dopamine Receptor

D-BIL Direct Bilirubin

DHDL High Density Lipoprotein

ED_{50} Dose of drug which elicits 50% of the maximum response

ED Erectile Dysfunction

EDTA Ethylene Diamine Tetraacetic Acid

EL Ejaculation Latency

EPM Elevated Plus-Maze

ET1 Endothelin-1

FSH Follicle-Stimulating Hormone

FST Forced Swimming Test

GABA γ-Aminobutyric Acid

GC Guanylyl Cyclase

GGT γ-glutamyltranspeptidase

GLDH Glutamate Dehydrogenase

GnRH Gonadotrophin-Releasing-Hormone

GPO Glycerophosphate Oxidase

GRAN Granulocyte

HB Hole Board

HRP Horseradish Peroxidase

HSD Hypoactive Sexual Desire

i.c.v. Intracerebroventricular

i.t. Intrathecal

I-BIL Indirect Bilirubin

ICP Intracavernous Pressure

IF Intromission Frequency

IFCC International Federation of Clinical Chemistry

IL Intromission Latency

LDH Lactate Dehydrogenase

LDH Lactate Dehydrogenase

LDL Low Density Llipoprotein

LH Luteinizing Hormone

LYM Lymphocyte

MAO Monoamine Oxidase

MDH Malate Dehydrogenase

MeDOPA Methyldopa

MF Mount Frequency

MID Mid Cell Count

ML Mount Latency

MPOA Medial Preoptic Area

NANC Non-Adrenergic Non-Cholinergic

NE Norepinephrine

NIH National Institute of Health

NMDA N-methyl-D-aspartate

NOAEL No-Observable- Adverse-Effect Level

NO Nitric Oxide

NOS Nitric Oxide Synthase

NPT Nocturnal Penile Tumescence

NPY Neuropeptide Y

OBI Organ-to-Body Index

OF Open Field

OX Oxytocin

PDE Phosphodiesterase

PEI Post-Ejaculatory Interval

PHC Primary Healthcare

PHT Phentolamine

PRL Prolactin

PSS Physiological Salt Solution

PVN Paraventricular Nucleus

RBC Red Blood Cell Count

SEM Standard Error of the Mean

SIA Sandwich Eenzyme Immunoassay

SJE *Sphenocentrum Jollyanum* Extract

SNP Sodium Nitroprusside

TAG Triglycerides

T-BIL Total Bilirubin

T-CHOL Total Cholesterol

T-PROT Total-Protein

TST Tail Suspension test

VIP Vasoactive Intestinal Polypeptide

VLDL Very Low Density Lipoprotein

WBC White Blood Cell Count

WHO World Health Organization

α-MSH α-melanocyte-Stimulating Hormones

βEP β-endorphin

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

There is yet to be known a condition more devastating to man's ego than impotence. It annihilates his very essence of masculinity. Besides that, next to thirst, starvation and sleep, the sexual urge is the most powerful biological drive (Tharakan and Manyam, 2005). This physiological instinct, so essential to the survival of the species, is one of the mainsprings of human motivation, and its achievement or disappointment is closely related to happiness or misery. It is also easy to discover that a patient enjoying an active sex life with minimal stress generally maintains a better emotional outlook, tends to remain healthier and heal other illnesses more readily than a patient who is sexually inactive (Preckshot, 1999). Impotence is common among men of all ages, ethnicities, and cultural backgrounds.

Impotence originates from the Latin word *impotencia*, which literally means lack of power. This word was first used by Thomas Hoccleve (1370–1454) in 1420, in his 5500-verse poem, 'The Government of Princes' (De Regimine Principum): '*Hir impotence, Strecchith naght so fer as his influence*' (Wright, 1860). Impotence was used by then to mean want of strength. In 1655, Thomas Fuller (1608–1661) used impotence to mean loss of sexual power when he wrote in 'The Church History of Britain': '*Whilest Papists crie up this, his incredible incontinency: others uneasily unwonder the same by imputing it partly to impotence afflicted, by an infirmitie*' (Fuller, 1837). Although the term has been used for ages, it generates much confusion and has been replaced by "erectile dysfunction" since 1992 (NIH, 1992). This then differentiates the problem from the many other processes that are involved in male sexual function.

1.2 ERECTILE DYSFUNCTION (ED)

Erectile dysfunction (ED) is defined as the persistent inability of the male to attain and/or maintain erection of the penis sufficient to permit satisfactory sexual intercourse (NIH, 1993b). However, sexual complaints not included in this definition frequently include a loss of firmness after vaginal intromission and hasty or premature ejaculation. These alterations of sexual behaviour may pave the way for fully developed ED and should be effectively considered for medical treatment. Reduced sexual desire (libido), ejaculatory disorder, reduced orgasmic feeling and sterility may accompany ED (Korenman, 1995). ED represents a social problem occurring in approximately 50% of the general male population aged between 40 and 70 years (Feldman *et al.*, 1994b). It has been projected that more than 152 million men worldwide experienced erectile dysfunction in 1995, and that this number will rise by 170 million, to about 322 million by the year 2025 (Ayta *et al.*, 1999). The etiology of ED involves multiple organic and psychogenic factors that often coexist. Being the most common causes of intermittent erectile malfunction in younger populations, psychogenic factors are usually secondary to or they may coexist with organic factors in older populations (Melman and Gingell, 1999; Rosen, 1998).

ED incidence increases with age (75% >70 years old) (Feldman *et al.*, 1994a; Laumann *et al.*, 1999b), and it frequently coexist with organic diseases such as chronic renal (20–100%) or hepatic failure (50–70%), diabetes (27.5–60%), hypertension (46%), Peyronie's disease (35%), atherosclerosis (33%), ischaemic heart disease (16%) and depression (Schiavi and Rehman, 1995). Approximately 30% of ED is due to the existence of systemic disease which affects the blood delivery to the penis. Cigarette smoking does not characterize a direct cause but may be a risk factor for ED (Feldman *et al.*, 1994b). Even though endocrine disorders have been claimed not to be a cause of ED, low testosterone levels have been linked with > 15% of patients complaining of erectile failure (Govier *et al.*,

1996) and hyperprolactinaemia has been shown to be the cause of ED in 2–3% of men presenting with sexual dysfunction (Baskin, 1989). Neurogenic impotence is not unusual (3–10%) and is observed concomitant with multiple sclerosis, discopathies of lumbosacral tract, after prostatectomy and following spinal cord, pelvic, perineal or penile traumas (Berger *et al.*, 1993). Psychological causes are frequent (30–40%) and include interactive–experiential problems (depressive–anxious behaviour, religious pressure, lifestyle changes, psychological trauma, child abuse etc.) and/or relationship disorders (performance anxiety, sexual incompatibility, loss of attraction, fears of intimacy etc.) (Cole, 1993). These point out that proper erectile dysfunction has both an "above-the-belt" and a "below-the-belt" aspect.

ED is an under reported medical condition the world over (NIH, 1993a). Only about 1 in 10 men with erectile dysfunction between 18 and 59 years of age seek medical advice about their problem (Laumann *et al.*, 1999a). In another study, 44% of 500 patients who were consulting their urologists for reasons other than erectile dysfunction were found to have a history of erectile dysfunction but failed to inform their physicians about their problem. The reason given by 74% of them was embarrassment (Baldwin *et al.*, 2003).

'If a woman's hand, which is the best of all remedies, is not good enough to cure the flabbiness of a man's penis, the other remedies will do little' (Vennette, 1984). Unfortunately, this quote by the Frenchman Nicholas Vennette (1633–1698) summarized the general view of both the public and the medical profession towards impotence and its treatments, until knowledge of anatomy, physiology and the pathology of impotence were discovered.

1.3 PHYSIOLOGY AND PATHOPHYSIOLOGY OF MALE SEXUAL FUNCTION

1.3.1 Central nervous system control of sexual activity

In man, sexual activity is carefully orchestrated by a series of events controlled by the central nervous system. In the presence of an adequate androgen milieu, the brain drives the sexual response by integrating external erotic stimuli (received through the five senses or erotic fantasies), and processes them into the medial-pre-optic area (MPOA) of the hypothalamus (psychogenic erection) (Figure 1.1). Many central nervous transmitters and transmitter systems participate in the regulation with the balance between contractant and relaxant factors controlling the degree of tone of the penile vasculature and of the smooth muscle of the corpora cavernosa and also determines the functional state of the penis: detumescence and flaccidity, tumescence and erection. Of the brain regions, the medial preoptic area (MPOA) and paraventricular nucleus (PVN) of the hypothalamus appear to play a critical role in the integration of sexual functions (Argiolas and Melis, 2005; Giuliano and Rampin, 2000; Rampin and Giuliano, 2001).

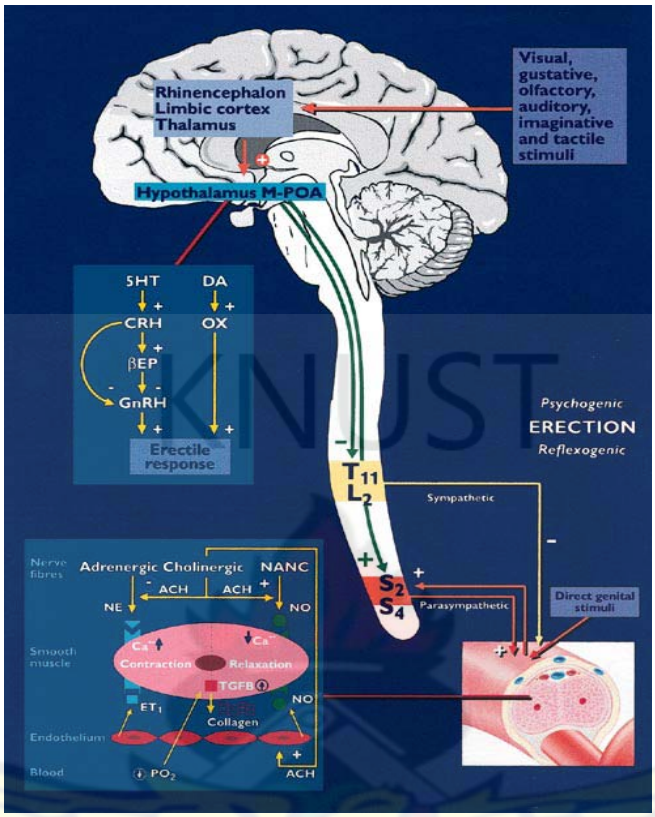

Figure 1.1 Schematic representation of central and intracavernosal mechanisms involved in the control of erection. External visual, gustative, auditory, imaginative and tactile stimuli are transmitted to the rhinencephalon, the limbic cortex and thalamic nuclei, and are integrated into the hypothalamic medial preoptic area (M-POA) where they stimulate supraspinal erections (psychogenic erection). Left box: serotonin (5HT), corticotrophin-releasing-hormone (CRH) and β-endorphin (βEP) exert inhibitory effects, while gonadotrophin-releasing-hormone (GnRH), dopamine (DA) and oxytocin (OX) are the main stimulators of psychogenic erections. Hypothalamic projections (possibly oxytocinergic) to the spinal cord control thoracolumbar sympathetic (T11-L2) and sacral parasympathetic (S2-S4) fibres which inhibit and stimulate erection respectively. Direct genital stimuli initiate a local neural loop leading to parasympathetic stimulation from S2 to S4 and reflexogenic erection. Right box: intracavernosal mechanisms of erection involve cholinergic, adrenergic and non-adrenergic non-cholinergic (NANC) nervous fibres. The main stimulators of smooth muscle cell contraction (detumescence) are norepinephrine (NE) and endothelin-1 (ET1), while acetylcholine (ACH) and nitric oxide (NO) are the main mediators of smooth muscle cell relaxation and erection via reduction of intracellular calcium (Ca^{2+}) content (Argiolas and Melis, 2005).

1.3.2 Central Regulation

Though the spinal and supraspinal pathways and mechanisms are involved in the central nervous regulation of erectile function, the central neurotransmission of penile erection is complex and only partly known. Much of the knowledge gained in this area relates to morphological and pharmacological studies in experimental animal models (e.g., rodents, primates). In these models, neurochemical perturbations can be performed and responses monitored in a reasonably meaningful way.

1.3.2.1 5-Hydroxytryptamine

It is well known that 5-hydroxytryptamine (5-HT; serotonin) neurons participate in the control of sexual behaviour, both in humans and in animals. This amine has been implicated both in the supraspinal and the spinal pharmacology of erectile function. 5-HT involves a sympathetic, parasympathetic, and somatic outflow mechanism which is considered to exert a general inhibitory effect on male sexual behaviour (Bitran and Hull, 1987). However, these pathways may be inhibitory or facilitatory depending upon the action of the amine at different subtypes of 5-HT receptors located at different sites in the central nervous system (Jimenez-Trejo *et al.*, 2007). The effects also seem to be species specific (Paredes *et al.*, 2000).

Serotonin-positive nerve terminals are present throughout the central nervous system, and its neurons can be found in the medullary raphe nuclei and ventral medullary reticular formation, including the rostral nucleus paragigantocellularis, as well as the lumbosacral spinal cord in association with mainly somatic and autonomic outflow projections to the pelvis (Bancila *et al.*, 1999; Marson and McKenna, 1992; Tang *et al.*, 1998). A decreased amount of 5-HT in these structures, with the inhibition of serotonin synthesis (parachlorophenylalanine), destruction of 5-HT-containing axons (5,7-dihydroxytryptamine), or electrolytic destruction of the dorsal raphe nucleus, enhances sexual activity (Kondo *et al.*, 1993). On the other hand, sexual activity is attenuated following the intracerebroventricular (i.c.v.) or

intrathecal (i.t.) administration of 5-HT and drugs that increase central release or synthesis of amine (Ahlenius *et al.*, 1981; Larsson and Ahlenius, 1999).

Thus, 5-HT appears to serve various functions in male sexual function and is likely to act as a major modulator of the central neuroregulatory control of penile erection. As indicated above, the predominant role of 5-HT in the central neuromediation of erectile function appears to be associated with inhibitory control of spinal sexual reflexes involving the brain stem level (Marson and McKenna, 1992). Intrathecal injection of 5-HT in the spinally anesthetized male rat blocked the appearance of the coitus reflex, suggesting that endogenous 5-HT may act in the descending input to the lumbar spinal cord that inhibits sexual reflexes (Marson and McKenna, 1992). A similar procedure in other experiments also inhibited ejaculation as well as penile intromission in rats, suggesting an alternative role of 5-HT in the transmission of sensory feedback information necessary for sexual responses (Svensson and Hansen, 1984).

1.3.2.2 Dopamine

Dopamine facilitates the motivational, appetitive and motor aspects of sexual behaviour in three essential sites. Dopamine in the nigrostriatal tract enables the motor components of sexual behaviour (Hull and Dominguez, 2006; Hull and Dominguez, 2007). In the mesolimbic tract, dopamine enhances appetitive behaviour and reinforcement. Preoptic area dopamine facilitates genital reflexes, sexual motivation and stereotyped sexual behaviours such as mounting and thrusting (Hull and Dominguez, 2006; Hull and Dominguez, 2007). It is possible that the role of dopamine is to prime these systems such that the appropriate sexual stimuli will elicit the proper sexual response. Dopaminergic neurons have also been identified, traveling from the caudal hypothalamus within the diencephalospinal dopamine pathway to innervate the lumbosacral spinal cord (Dominguez and Hull, 2005; Succu *et al.*, 2007). Thus, dopamine may be expected

to participate in the central regulation of both the autonomic and somatic components of the penile reflexes.

Supporting this view, the dopamine receptor agonist apomorphine, administered systemically to male rats, was found to induce penile erection (Benassi-Benelli *et al.*, 1979). The effect of apomorphine was biphasic in the freely moving rat, with low doses facilitating and high doses inhibiting erection (Pehek *et al.*, 1989). These observations were subsequently extended to investigations involving low dose systemic administration of other dopamine agonists such as piribedil, lisuride, and quinelorane to rats and other animals (Andersson, 2000). The effects of these agonists were attenuated by centrally, but not peripherally, acting dopamine receptor antagonists. Dopamine-receptor agonist-induced erections were abolished by castration in rodents, and testosterone replacement restored erectile function (Melis and Argiolas, 1995; Scaletta and Hull, 1990). Interestingly, rhesus monkeys did not respond to apomorphine, suggesting that there are basic differences between rats and rhesus monkeys in the systems mediating sexual behaviour (Chambers and Phoenix, 1989). Whether the proerectile effects of apomorphine in humans are dependent on the androgenic state has not been clarified.

Dopamine receptors are distributed to various regions in the brain, with a high density particularly in the basal ganglia. Dopamine exerts its effects via at least five dopamine receptor (DAR) isoforms that are divided into two families: D1-like (D1 and D5) receptors and D2-like (D2, D3, and D4) receptors (Kudwa *et al.*, 2005). The receptors have been associated with central erectile functions. The D2 receptor seems to be responsible for most of the behavioural effects of dopamine, whereas the effects of D1 receptors are more difficult to define. The dopamine-induced stretching, yawning, and penile erection syndrome seem to involve particularly the D2 receptor subtype.

1.3.2.3 Noradrenaline

Evidence for noradrenergic mechanisms involved in the supraspinal mediation of penile erection is sparse. Noradrenergic neurons from the A5 region and from the locus coeruleus project to the nuclei in the spinal cord involved in erection (Giuliano and Rampin, 2000). Available data suggest that increased noradrenergic activity stimulates, whereas decreased noradrenergic activity inhibits, sexual function (Bitran and Hull, 1987). Insights have almost exclusively been drawn from experimental work involving the administration of agents that interact through α-adrenoceptor (AR) pathways. Furthermore, accurate conclusions can only be drawn from work that suggests that central adrenergic receptors have been selectively stimulated. In rats given the α_2-AR agonist, clonidine, by direct injection into the MPOA, male sexual behaviour was suppressed (Clark and Smith, 1990). The suppression was inhibited by pretreatment with selective α_2-AR antagonists (Clark *et al.*, 1985), consistent with established facilitatory effects of these agents on erectile responses in rats (Clark *et al.*, 1985). However, although several α_2-AR antagonists, most notably yohimbine, have been shown to increase sexual responses in rats, the relatively poor therapeutic efficacy of yohimbine in clinical use among men with ED, casts doubt on the significance of central noradrenergic mechanisms in erectile function.

1.3.2.4 Excitatory Amino Acids

Excitatory amino acids appear to exert a role in penile erection. Thus, microinjections of L-glutamate into the MPOA elicited an increase in intracavernous pressure (Giuliano *et al.*, 1996). Behavioural studies have shown that N-methyl-D-aspartate (NMDA) increases the number of penile erections when injected in the PVN (Melis *et al.*, 2000; Zahran *et al.*, 2000). NMDA, amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid, or trans-1-amino-1,3-cyclo-pentadicarboxylic acid, increased intracavernous pressures when injected into the PVN (Zahran *et al.*, 2000). The effect of NMDA was prevented by i.c.v. administration of an oxytocin antagonist. The NO synthase signal transduction

pathway is considered to mediate the effect of NMDA, since the administration of NOS inhibitors into the PVN and i.c.v. blocked the NMDA effect (Melis *et al.*, 2000).

1.3.2.5 γ-Aminobutyric Acid

Cumulative data resulting from investigations on the role of γ-aminobutyric acid (GABA) in penile erection indicate that this neurotransmitter may function as an inhibitory modulator in the autonomic and somatic reflex pathways involved in penile erection (Catalano *et al.*, 2005). In male rats, high concentrations of GABA have been measured in the MPOA (Elekes *et al.*, 1986), and GABAergic fibers and receptor sites have been localized to the sacral parasympathetic nucleus and bulbocavernosus motor nucleus (Bowery *et al.*, 1987; Magoul *et al.*, 1987). The injection of $GABA_A$ agonists into the MPOA decreases (Fernandez-Guasti *et al.*, 1986), whereas the injection of $GABA_A$ antagonists into this region increases copulatory behaviour of male rats (Fernandez-Guasti *et al.*, 1985). Systemic administration or i.t. injection at the lumbosacral level of the $GABA_B$ receptor agonist, baclofen, decreased the frequency of erections in rats (Bitran and Hull, 1987). Recent investigations showed that activation of $GABA_A$ receptors in the PVN reduced apomorphine-, NMDA-, and oxytocin-induced penile erection and yawning in male rats (Rosaria Melis *et al.*, 2000).

1.3.2.6 Oxytocin

Experiments using retrograde labeling have shown that oxytocin-containing neurons in the PVN project to spinal autonomic nuclei (Sawchenko and Swanson, 1982; Swanson and Kuypers, 1980). This was confirmed by Tang *et al.,* (1998) using retrograde transneuronal tracing with rabies virus. They found that oxytocinergic spinal projections from the PVN are more likely to influence the sacral autonomic rather than the somatic outflow. Plasma oxytocin concentrations are known to be elevated in humans following sexual stimulation (Carmichael *et al.*, 1987; Murphy *et al.*, 1987). Oxytocin was found to be a potent inducer of penile erection when injected into the lateral cerebral ventricle, the PVN, or hippocampus in laboratory

animals (Argiolas and Melis, 2004; Melis *et al.*, 2007). The erectile response was blocked by oxytocin antagonists and by electrolytic lesion of the PVN (Argiolas and Melis, 2004). The oxytocin-induced erections were also abolished by castration, and testosterone replacement restored erectile function (Melis *et al.*, 2007).

1.3.2.7 Adrenocorticotropin and Related Peptides.

Proteolytic cleavage of the precursor, pro-opiomelanocortin, gives rise to several peptides including adrenocorticotropic (ACTH) and the α-melanocyte-stimulating hormones (α-MSH), which both have been associated with erectile responses. After i.c.v. or hypothalamic periventricular injection into various animal models, ACTH and α-MSH induce penile erection and ejaculation, grooming, stretching and yawning (Argiolas, 1999; Poggioli *et al.*, 1998). These effects were shown to be androgen-dependent, since they were abolished by castration and could be fully restored by treating castrated animals with testosterone (Bertolini and Gessa, 1981). Interestingly, ACTH and the ACTH-like peptides do not enhance social interaction, since during periods of sexual stimulation the animals did not seek to copulate with partners (Bertolini and Gessa, 1981).

1.3.2.8 Opioid Peptides.

Endogenous opioid peptides have long been assumed to be involved in the regulation of male sexual responses, since sexual dysfunction has been observed clinically in men chronically using opiates (Crowley and Simpson, 1978). Copulatory behaviour in male rats is depressed experimentally with the systemic administration of morphine or other opioids (McIntosh *et al.*, 1980). β-Endorphin injection into the cerebral ventricles or MPOA of male rats attenuates copulatory behaviour (Hughes *et al.*, 1987; McIntosh *et al.*, 1980). Morphine, injected systemically or into the PVN of male rats, prevents penile erection induced by i.c.v. administration of oxytocin or subcutaneous dopamine (Melis *et al.*, 2000) or NMDA injected into the PVN (Melis and Argiolas, 2003; Melis *et al.*, 2000). However, similar application of a selective agonist of the κ-opioid receptor does not alter

apomorphine- or oxytocin- induced erectile responses (Melis and Argiolas, 2003; Melis *et al.*, 2000). This evidence and the demonstration that the opiate antagonist naloxone administered systemically abolishes the central morphine preventative effect on erections in rats, have supported the belief that μ receptors in the PVN account for the morphine effect (Melis and Argiolas, 2003; Melis *et al.*, 2000). NO metabolite concentrations that are increased in the PVN following apomorphine, oxytocin, or NMDA local administration, become reduced following morphine administration into the PVN, indicating that the morphine effect depresses an NO-mediated erection induction mechanism at this level (Melis and Argiolas, 2003; Melis *et al.*, 2000). Current data support the hypothesis that κ-opioid receptor stimulation centrally prevents penile erection by inhibiting mechanisms that converge upon central oxytocinergic neurotransmission.

1.3.2.9 Acetylcholine

The role of acetylcholine (ACh) at central levels in the regulation of penile erection is mostly inferred from limited neuropharmacologic studies involving systemically and/or intracerebrally administered muscarinic agonists and antagonists and lesioning studies in the brain (Hull and Dominguez, 2007; Maeda *et al.*, 1994a; Maeda *et al.*, 1994b). These studies have suggested that cholinergic mechanisms operating seemingly at the hippocampus and MPOA may have a regulatory role in erectile function.

1.3.2.10 Nitric Oxide

The role of NO in the central neuromediation of penile erection followed observations that the injection of NOS inhibitors i.c.v. or into the PVN prevented penile erectile responses induced by dopamine agonists, oxytocin, ACTH, 5-HT_{2C} agonists, or NMDA in rats (Melis and Argiolas, 2003; Melis *et al.*, 2000) (Figure 1.2). The inhibitory effect of NOS inhibitors was not observed when these compounds were injected concomitantly with L-arginine, the substrate for NO production.

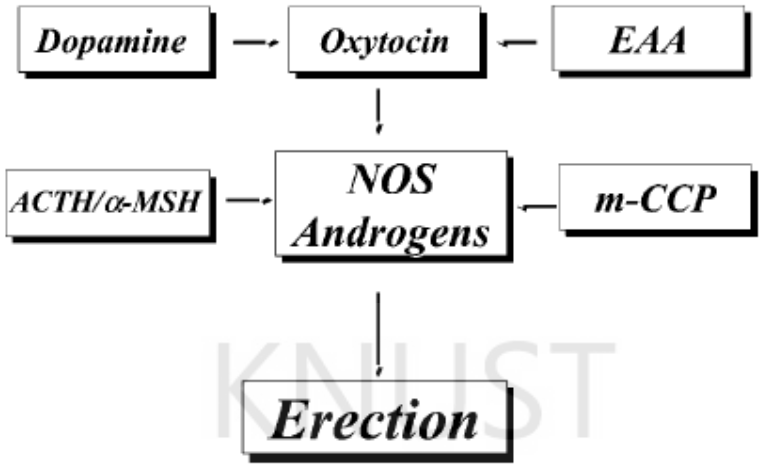

Figure 1.2 In the rat, erectile responses evoked by various centrally acting transmitters/agents appear to be dependent on nitric oxide as well as androgens (Melis and Argiolas, 2003).

The PVN has been implicated as a prime site for NO interacting with the oxytocinergic mechanisms of penile erection (Melis and Argiolas, 2003; Melis *et al.*, 2000). This brain nucleus was earlier identified to contain one of the highest concentrations of NOS in the brain (Bredt *et al.*, 1990). Nitroglycerin, an NO donor, induces penile erection in the rat when injected into the PVN (Melis and Argiolas, 2003; Melis *et al.*, 2000). The MPOA is also purported to liberate NO with sexual activity in rats. Direct measurements of NO in the MPOA showed NO release associated with copulatory behaviour. Local administration of an NOS inhibitor decreased NO release and copulatory behaviour (Sato and Hull, 2006; Sato *et al.*, 1999). NO production increased in the PVN during noncontact erection and copulation (Melis and Argiolas, 2003; Melis *et al.*, 2000).

Interestingly, since guanylyl cyclase (GC) inhibitors (e.g., methylene blue) injected into the PVN fail to prevent drug-induced penile erection, and 8-bromo-cGMP injected into the PVN fails to elicit erections, it has been proposed that the mechanism of NO action is not associated with the activation of GC (Argiolas and Melis, 2005). The additional finding that the NO scavenger, hemoglobin, does not

prevent penile erection in spite of its ability to block NO production in the PVN, suggested that NO acts as an intracellular rather than an intercellular modulator of erectile responses involving the PVN (Argiolas and Melis, 2005).

1.3.3 Penile structure, vasculature, and innervation

The paired corpora cavernosa and the corpus spongiosum are the two functional compartments of the penis (Figure 1.3) (Lue, 2000). The tissue of the corpora cavernosa consists of bundles of smooth muscle fibers entangled in a collagenous extracellular matrix in histological picture. Interspersed within this parenchyma is a multifarious system of endothelial cell-lined sinuses, or lacunae, helicine arteries, and nerve terminals (Kandeel *et al.*, 2001).

Somatic and autonomic nerve fibers innervate the penis. The somatic supplies the penis with sensory fibers and provides the perineal skeletal muscles with motor fibers. Contraction of the perineal skeletal muscles during erection leads to a momentary rise in corporeal body pressure to a level above the mean systolic pressure, and consequently helps to increase penile rigidity.

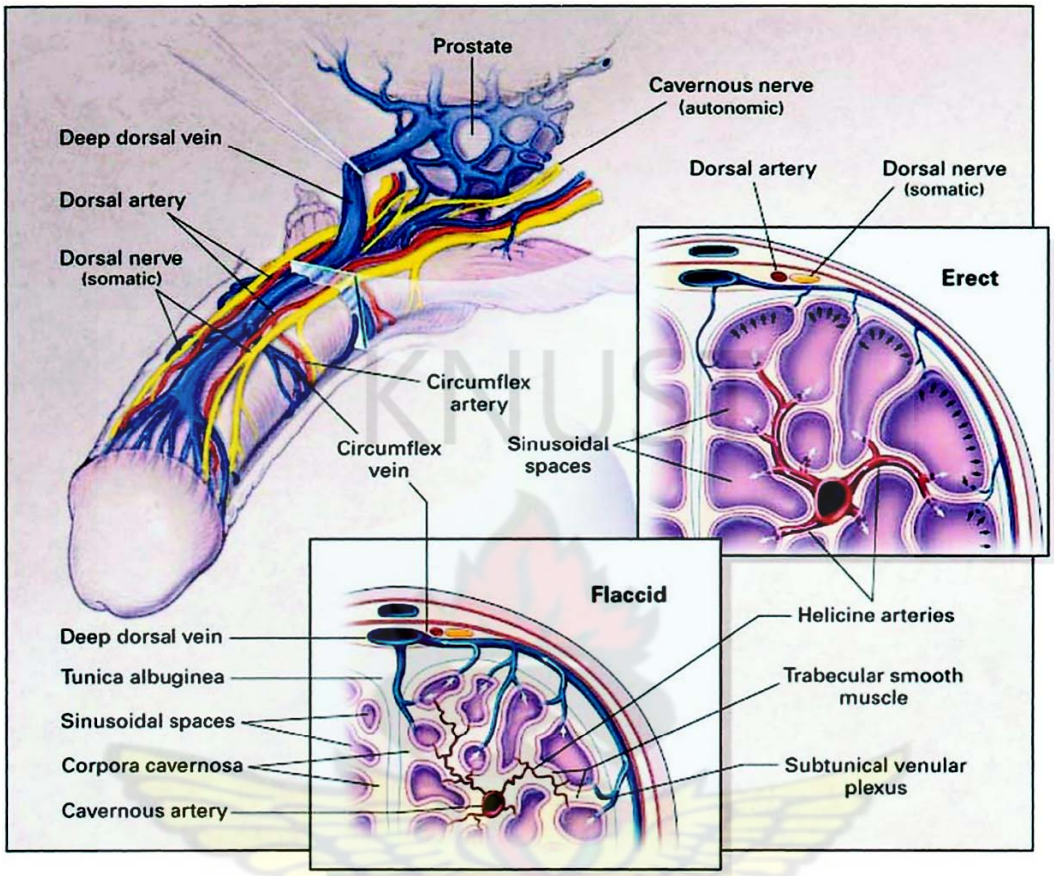

Figure 1.3 Mechanism of Penile erection and flaccidity. The corpora cavernosa and corpus spongiosum received cavernous nerves (autonomic) to control penile blood flow for the period of erection and detumescence. Pudendal nerves branches; dorsal nerves (somatic) are principally responsible for penile sensation. During erection (upper right), relaxation of the trabecular smooth muscle and vasodilatation of the arterioles leads to a number of increases in blood flow, this expands the sinusoidal spaces to elongate and expand the penis. This expansion compresses the subtunical venular plexus against the tunica albuginea. Apart from that, stretching of the tunica compresses the outlet veins, in consequence reducing the outpouring of blood to a minimum. In the flaccid state (lower right), inflow via the constricted and twisted helicine arteries is limited, leading to free outflow through the subtunical venular plexus. (Lue, 2000)

The autonomic innervation of the penis is both parasympathetic and sympathetic (Figure 1.4). The main efferent parasympathetic pathway comes from the intermediolateral portion of the sacral cord (S2–S4) traveling in the Nervi Erigentes (pelvic nerve) to deliver a vasodilating innervation to the corporeal bodies. After exiting the spinal cord, parasympathetic nerve run through the space in the lateral part of the rectum and bladder, before passing inferiorly and laterally in the

direction of the prostate and urogenital diaphragm. Cavernous nerve enters the corporeal body together with the cavernous artery at the crura of the corpora as preganglionic nerve fibers. The most probable neurotransmitter at the synaptic end of these fibers is acetylcholine. Postganglionic nerve fiber bits end either on the vascular smooth muscle of the corporeal arterioles or the nonvascular smooth muscle of trabecular tissue governing the corporeal lacunae (Goldstein, 1988; Goldstein, 2000). Sacral parasympathetic neurons are predominantly liable for the erectile function and are being controlled by a cortical-sacral efferent route. Erection can be started with a single episode of pelvic nerve electrical stimulation. Upholding of erection for an extended period of time without considerable changes in corporeal body blood gases can be accomplished with recurring stimulation for 40–50 sec, given a latency period of 50 sec amid each stimulus (Goldstein, 1988; Goldstein, 2000). Sympathetic innervation of the penis mediates the detumescence after the orgasmic relief, and in the absence of sexual arousal it maintains the penis in the flaccid state.

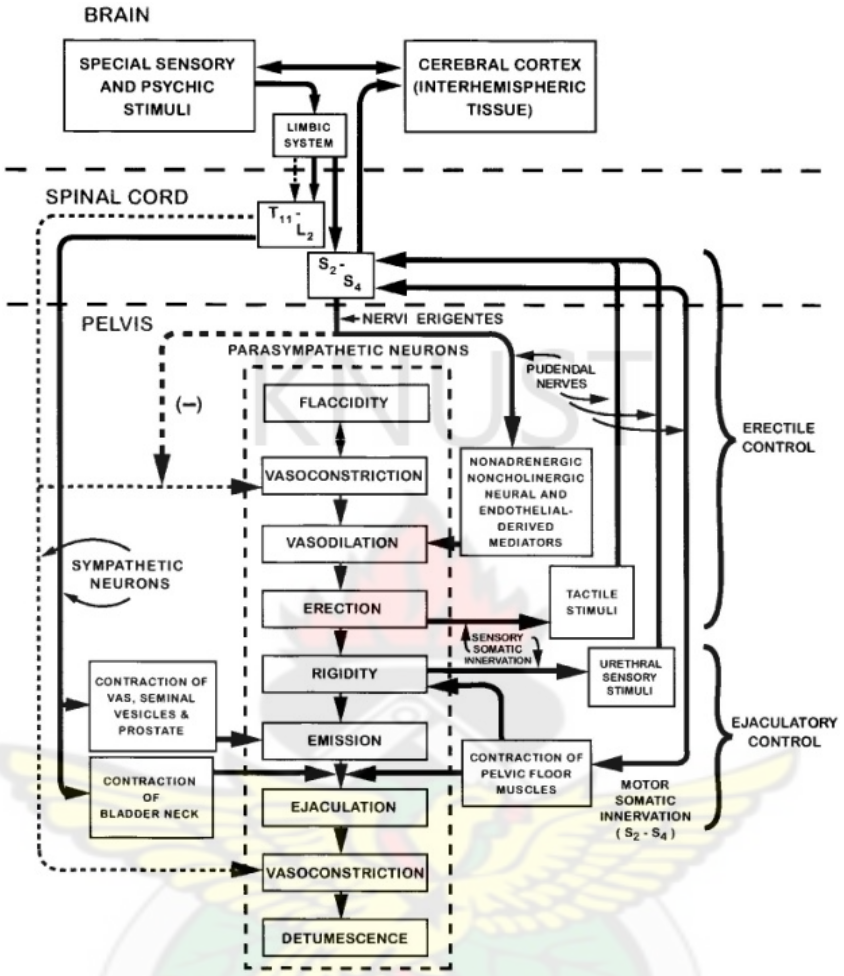

Figure 1.4 **Control of male sexual cycle: connections of autonomic and somatic innervations. Pudendal nerve carried the sensory input from the genital tract to the S2–S4 section of the spinal cord. Ascending sensory fibers synapse in the corticomedullary junction and the thalamus, and then terminate in the contralateral principal sensory area deep in the interhemispheric tissue. Somatic motor fibers start off from the sacral segments S2–S4 and furnish the pelvic floor muscles and the external anal sphincter. Descending parasympathetic innervation leaves spinal cord at the S2–S4 level and get to the penis through pelvic nerve and liable for corporeal vasodilatation and corporeal smooth muscle relaxation. These lead to penile change from the flaccid to the erect state. Penile erection stimuli getting to the spinal cord through pudendal nerve produced more reflex arcs to help set off and/or keep the erection. Sympathetic innervation exits the spinal cord at T11–L2 level, getting to the penis through the lower mesenteric, hypogastric, and pelvic plexuses. Through coordinated contractions of the vas deferens, ampulla, seminal vesicles, prostate, and the bladder neck, it is in charge of emission and ejaculation. Somatic innervation-mediated contraction of the pelvic floor muscles helps in attaining maximum penile firmness with releasing the ejaculatory fluid. Sympathetic innervation mediates corporeal vasoconstriction and corporeal smooth muscle contraction, and thus causes penile detumescence after the orgasmic relief. Activation of one division of autonomous system is associated with inhibition of the other (Litwin *et al.*, 1998).**

1.3.4 Local control of penile erection

Even though acetylcholine seems to be the neurotransmitter of the preganglionic parasympathetic neurons, neurotransmitters for the short postganglionic neurons are yet to be fully defined. Corporeal smooth muscle fibers contractility seems not to be under the direct control of acetylcholine, but via activation of cholinergic receptors on the endothelial cells (Figure 1.5). Endothelial-derived relaxation factor(s) in the corporeal tissue (Bloch *et al.*, 1998) has been recognized to be nitric oxide (NO). NO is produced from its precursor, L-arginine, by the enzyme nitric oxide synthase (NOS). Cavernosal tissues are known to synthesize constitutive and inducible NOS isoforms (Burnett, 1997; Lowenstein *et al.*, 1994). Inducible NOS seems to be synthesized by the corporeal smooth muscle cells only, while constitutive NOS is produced by nerve terminals and endothelial cells.

Noncholinergic parasympathetic neurons and sinusoidal endothelial cells synthesize NO which diffuses into near-by smooth muscle cells, activates guanylate cyclase and increase intracellular cGMP. cGMP is the main intracellular effector of smooth muscle relaxation (Rajfer *et al.*, 1992) by a biochemical sequence of protein kinases. Corporeal smooth muscle relaxation by cGMP involves protein kinase phosphorylation of myosin light chains directly or indirectly by lowering intracellular calcium stores (Burnett, 1997). Type 5 phosphodiesterase was recognized to inactivate cGMP (Boolell *et al.*, 1996a), even though a number of phosphodiesterase (PDE) isoenzymes have been identified in the human corpora cavernosa. Sildenafil citrate inhibits this PDE, which is also found in vascular smooth muscles and platelets (Wallis *et al.*, 1999). Sildenafil, also inhibits PDE type 6 minimally which is responsible for metabolism of the light-stimulated cGMP found in the retinal rod photoreceptors but has little or no effect on calcium/calmodulin-dependent PDE-1 and calcium/ calmodulin-independent PDE-3 isoenzymes in the cardiac muscles (responsible for metabolism of cGMP that is involved in regulation of cardiac contractility) (Wallis *et al.*, 1999). Phosphodiestrase inhibitors are a promising physiological way of initiation and/or

maintenance of erection in man (Boolell *et al.*, 1996b). NO apart from initiation of cGMP synthesis could directly control the contractility of the corporeal smooth muscle fibers by changing the transcellular ion flux by activation of potassium-conductive membrane hyperpolarization (Seftel *et al.*, 1996) and sodium-potassium-ATP pathway (Gupta *et al.*, 1995).

Prostaglandin E-1, bradykinin, galanin, pituitary adenylate cyclase-activating polypeptide, helospectin, peptide histidine methionine, calcitonin gene-related peptide (CGRP), and vasoactive intestinal polypeptide (VIP) are other neurotransmitters from noncholinergic parasympathetic which are able to enhance smooth muscle relaxation (Hedlund *et al.*, 1995; Lerner *et al.*, 1993; Ruffolo Jr *et al.*, 1993; Stief *et al.*, 1991). VIP was considered as the main neuromediator of erectile function until NO was identified in the penile tissue; however, VIP and NOS colocalize penile neurons of rats and humans (Ehmke *et al.*, 1995). VIP effect on corporeal smooth muscle fibers seems to be mediated by the NO-cGMP pathway (Kim *et al.*, 1994) comparable to bradykinin's capability to stimulate the endothelial NOS pathway (Kimoto *et al.*, 1990). The precise mechanisms by which other neuropeptides contribute in the control of the penile erection remain to be resolved.

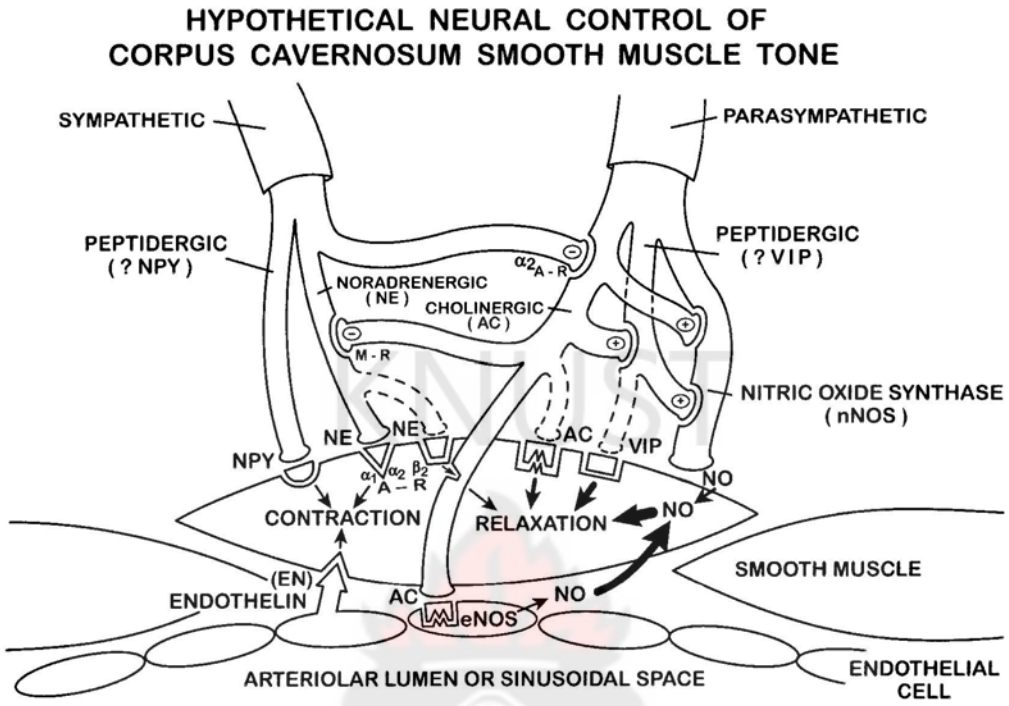

Figure 1.5 **Neuronal regulation of the corporeal smooth muscle. Acetylcholine (AC) which is the parasympathetic neurotransmitter at the endothelial cells initiates the synthesis of constitutive endothelial nitric oxide synthase (NOS) and hence activates nitric oxide (NO) production. Apart from that, innervation of the smooth muscle cells by parasympathetic nerve mainly mediates NOS-containing and to a smaller amount by VIP containing fibers. The chief moderator of smooth muscle relaxation through activation of cGMP production is NO produced locally or those diffuse to it from near-by endothelial cell(s). VIP plays a minor role in direct activation of corporeal smooth muscle relaxation. Sympathetic innervation of smooth muscle cells includes norepinephrine (NE) and nonadrenergic (possible neuropeptide Y fibers). α-1 and α-2 adrenoceptor stimulation, in addition to neuropeptide Y (NPY) and endothelin-1 (EN) actions, are accountable for smooth muscle cell contraction. Interaction between the two sections of the autonomic innervations seems to exist, through an α-2 adrenoceptor (parasympathetic) and a muscarinic receptor (sympathetic) which helps in the inhibition of each division when the other is activated. (Saenz de Tejada *et al.*, 1988).**

Norepinephrine is responsible for controlling corpus cavernosum smooth muscle tone through interaction with α-1 and α-2 adrenergic receptors (Gupta *et al.*, 1998). Apart from that, endothelin-1, substance-P, PGF-2a, thromboxane A-2, angiotensin II, and calcium (Kifor *et al.*, 1997; Lerner *et al.*, 1993; Roy *et al.*, 1984; Saenz de Tejada *et al.*, 2005; Saenz de Tejada *et al.*, 1991; Sparwasser *et al.*, 1998; Stief *et al.*, 1991) are additional neurotransmitters capable of enhancing smooth muscle contraction, and therefore detumescence. A number of these agents exercise their effect via toning

of the presynaptic α-2 adrenergic receptors. Maintenance of full erection in men who undertake bilateral full sympathectomy (Giammusso *et al.*, 2005; Quayle, 1980) has led to the dispute of the notion that psychologically provoked erection is mediated by sympathetic innervation of the penis. Nevertheless, the newer *in vitro* studies indicating the relaxation effect of the β-2 adrenergic receptor agonist isoproterenol on noradrenaline- precontracted human penile smooth muscle cells (Costa *et al.*, 1993) propose that, at least in some instances, β-adrenergic innervation could partake in the intervention of human erection.

Imbalance of smooth muscle to connective tissue could distort venoocclusion and the manifestation of erectile response to occur. In consequence, neuronal disorder or poor intrinsic conformity of the corporeal smooth muscle cells could be a major factor in the pathogenesis of erectile dysfunction (Figure 1.6) (Christ, 1995).

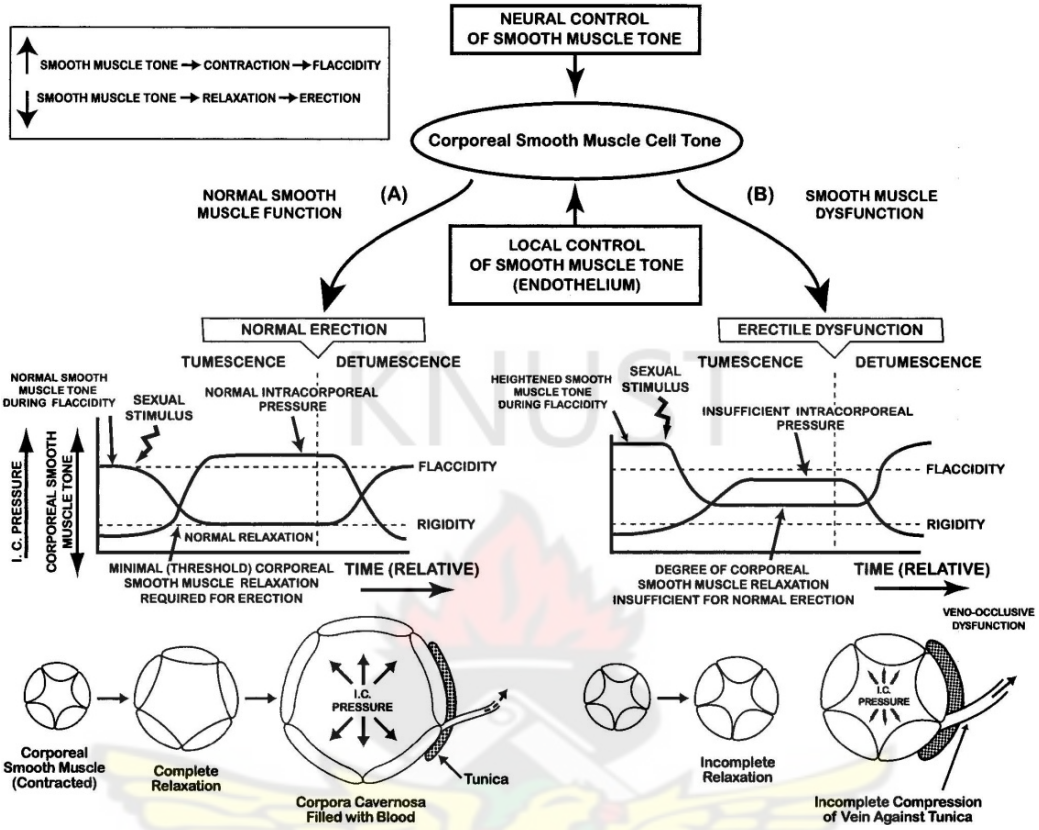

Figure 1.6 Contraction and relaxation of smooth muscle as observed in normal and abnormal erection. Normal smooth muscle response under various conditions gives rise to the increase intracorporeal pressure to the level needed for the full erection to occur. Every physiological disturbance that give rise to heightened contractility for the duration of flaccidity and impaired relaxation of the corporeal smooth muscle during tumescence will threaten balance in support of flaccidity over erection (Christ, 1995)

1.3.5 Control of male sexual response

Male sexual stimulation results in a sequence of psychological, neuronal, vascular, and local genital changes. These changes have been categorized based on psychosexual response cycle that consists of four phases: excitement, plateau, orgasm, and resolution (Kolodny, 2003; Kolodny *et al.*, 1979).

These sequences could also be characterized based on the penodynamic changes during the sexual cycle (Govier *et al.*, 1995). In this category, every psychosexual phase is separated into two interconnected events as follows: excitement into

latency and tumescence; plateau into erection and rigidity; orgasm into emission and ejaculation; and resolution into detumescence and refractoriness.

The final issue worthy of mention is the functional activities during the sexual cycle (Kandeel *et al.*, 2001; Walsh and Wilson, 1987). It sums the initial phase of libido to include the sex-seeking behaviour, adds together excitement and plateau into a single phase of erection, and divides the orgasmic phase into the physical function of ejaculation and psychological sensation of orgasmic satisfaction. So, the normal male sexual response cycle can be divided into five interconnected events that occur in a definite series: libido, erection, ejaculation, orgasm, and detumescence. Because this classification of the male sexual cycle is the measureable one, it will be the center for the subsequent review.

1.3.5.1 Libido or sexual desire.

Libido is the biological need for sexual activity (the sex drive) and normally is considered as sex-seeking behaviour (Kandeel *et al.*, 2001). Between and within individuals, its strength varies over a given time. Even though, the physiological basis of libido is not well defined, sexual experience, psychosocial background, brain and spinal cord dopaminergic receptor stimulation, and gonadal hormones are part of factors that could modulate libido.

Animal and human male studies support the function of central dopaminergic neurotransmission in regulating sexual behaviour and erection (Segraves, 1989; Segraves, 2003). Additionally, testosterone enhancement of copulation seems to be mediated by a rise in dopamine release in the medial preoptic area, perhaps through up-regulation of NO synthesis (Hull and Dominguez, 2007; Hull *et al.*, 1997).

Androgens have long been implicated in the regulation of sexual behaviour in the human male (Mooradian *et al.*, 1987). Elevated serum testosterone seems to be linked with higher sexual activity in healthy adults (Toone *et al.*, 1983) but not

younger (Brown *et al.*, 1978) men. Higher testosterone levels could shorten the latency of erection activated by the introduction to sexual material (Lange *et al.*, 1980), and testosterone substitution in hypogonadal males replaces sexual interest (O'Carroll and Bancroft, 1984), decreases latency, and increases frequency and enormity of nocturnal penile tumescence (NPT) (Kwan *et al.*, 1983). Notwithstanding, androgen deficiency-related disorder, hypogonadism do not seem to compromise the capacity to attain erection in reaction to watching of erotic films (Bancroft and Wu, 1983; Kwan *et al.*, 1983).

Hypoactive sexual desire (HSD) is the persistent or repeated lack (or absent) of sexual fantasy and desire for sexual activity leading to clear distress or interpersonal difficulty (Kandeel *et al.*, 2001). Greater than 15% of adult men have been projected to have HSD with psychogenic etiologies and androgen deficiency being the most common cause (Benet and Melman, 1995; Schiavi and Rehman, 1995).

Psychiatric sicknesses such as depression or psychosis, associated with life predicament or sorrow, motherly change of sexual partners, masculinity identity conflicts, and aging-related psychological issues are some of the psychogenic situations leading to a libido insufficiency state in men (formerly called libido or desire inhibition) (Salmimies *et al.*, 1982; Segraves *et al.*, 1985). Apart from that, secondary libido dysfunction caused by psychological factors is termed "excitement inhibition" and is common in patients who have sexual drive but cannot sustain excitement. This is peculiar in patients with performance anxiety due to the fear of sexual failure and the watchful concern with erection during lovemaking (Salmimies *et al.*, 1982).

1.3.5.2 Erection

Erection is the final reaction to various psychogenic and sensory stimuli from imaginative, visual, auditory, olfactory, gustatory, tactile, and genital reflexogenic sources, which affect several neurological and vascular cascades that lead to penile

tumescence and firmness adequate for vaginal intromission (Kandeel *et al.*, 2001). Erection is also linked with considerable psychological and physical changes, together with increased sexual arousal, full testicular assent and swelling, dilatation of the urethral bulb, enlargement of glans and coronal size, cutaneous flush over the epigastrium, chest, and buttocks, nipple erection, tachycardia and increase in blood pressure, hyperventilation, and widespread myotonia (Kolodny, 2003; Kolodny *et al.*, 1979; Priviero *et al.*, 2007). Vasodilating parasympathetic discharge affects local penile changes subsequent to central nervous system (CNS) inputs or consequence of reflex action in reaction to local afferent activation of the sacral parasympathetic nuclei.

Recent studies suggest that gonadal androgens tone penile erection via local control of NO secretion and/or activity (Kandeel *et al.*, 2001). The return of NOS synthesis and action in androgen replaced castrated rats with low penile tissue NOS content (Mills and Lewis, 1999; Mills *et al.*, 1992) have cast doubts on the former belief that androgens act only centrally to regulate sexual libido. The control of the frequency of nonerotic or "reflex" erection due to androgen, proposes a possible role for peripheral androgen activities in the human (Davidson *et al.*, 1982). On the other hand, because androgens can improve NPT, and not erection in reaction to erotic stimuli (Davidson *et al.*, 1982), there is probably the existence of both androgen-sensitive and androgen-insensitive central pathways for erectile control (Kandeel *et al.*, 2001). This implies that sexual behaviour and erection are androgen dependent and acting both centrally and peripherally (Heaton and Morales, 2003; Mills and Lewis, 1999; Mills *et al.*, 1992).

A number of data have recognized some relationship between sexual dysfunction and psychological disorders. In the Massachusetts Male Aging Study, male erectile dysfunction was established to be linked with depressive symptoms (odds ratio 1.82) (Araujo *et al.*, 1998; Araujo *et al.*, 2004). The organic causes of erectile

dysfunction can be classified into systemic diseases, endocrine, neurological, vascular, or local penile disorders (Burnett, 2006; Kloner, 2007).

1.3.5.3 Ejaculation

Innervation of the genital organs by sympathetic nerve regulates ejaculation. The voluntary inhibitory regulation of ejaculation consists of two sequential processes: First, emission which is coupled with release of seminal fluid into the posterior urethra and concurrent contractions of the ampulla of the vas deferens, the seminal vesicles, and the smooth muscles of the prostate (Porst *et al.*, 2007; Rosenberg and Sadovsky, 2007) mediate emission. Secondly, true ejaculation is followed by ejection of the seminal fluid from the posterior urethra via the penile meatus. Serotonergic neurotransmission has been proposed to have an inhibitory effect on male sexual function and ejaculation which could be mediated by the serotonergic tracts in the medial forebrain bundle (Segraves, 1989; Segraves *et al.*, 2007).

A number of ejaculation disorders ranging from mild premature to severely retarded or absent ejaculation have been reported. 75% of men by 17 or 18 years are able to control their ejaculation (Richardson and Goldmeier, 2005; Richardson, 1993) ideally. Premature ejaculation is the most common male sexual dysfunction (Porst *et al.*, 2007; Quek *et al.*, 2007) with a prevalence of 29%, with a range between 1% and 75% depending on the population and criteria used to define the condition (Metz *et al.*, 1997; Perelman and Rowland, 2006; Quek *et al.*, 2007). An association has been established between premature ejaculation and anxiety (Dunn *et al.*, 1999).

1.3.5.4 Orgasm

Physiologic and psychogenic factors have been found to contribute to the genesis of the orgasmic phase (Barnas *et al.*, 2005; Donatucci *et al.*, 2004; Hartmann, 1998). The following physiological events are the effects of afferent stimuli from the pudendal nerve: smooth muscle contraction of the accomplice sex organs; upsurge and discharge of pressure in the posterior urethra; feeling of the ejaculatory unavoidability; contraction of the urethral bulb and perineum; periodic

contractions of the pelvic floor muscles; semen emission and ejaculation; and lastly, the reversal of the generalized physiological changes and sexual tension. These actions are recognized by sensory cortical neurons as enjoyable. Orgasmic pleasure could be influenced by the degree of sexual pleasure, recency of sexual activity, and the psychosexual composition of the human being. In the absence of the two phases of erection and ejaculation, orgasm can still be achieved. On the other hand, contractions of pelvic musculature and ejaculation could take place in the absence of orgasmic sensations (Kandeel *et al.*, 2001).

Male orgasmic dysfunction is the constant or repeated delay in, or lack of, orgasm after a normal sexual pleasureable phase during sexual activity (Rosen *et al.*, 2007; Rosen and Leiblum, 1995; Rosen *et al.*, 2005) and it occurs in 3–10% of patients presenting with sexual dysfunction (Rosen and Leiblum, 1995).

1.3.5.5 Detumescence

This is the phase where the penis returns to the flaccid state due to vasoconstriction of the arterioles and return of events inside the contractile corporeal units reroute the blood away from the cavernous sinuses and allow a rise in the venous evacuation of their contents (Kandeel *et al.*, 2001). At first, the rate of blood seeping away rises by about 10-fold, followed by a gradual fall until it gets to the pretumescence level (Priviero *et al.*, 2007) and a period of inhibition to the recommencement of erectile and ejaculatory functions. The duration of this refractory phase is reliant upon many factors including age, physical state, and psychological environment (Carrier *et al.*, 1993; Kulmala *et al.*, 1996; Levine *et al.*, 1991). Local penile α-adrenergic receptor activation is the most important neuromediator effecting detumescence. Interference with this function through the α-1 receptor blockade may lead to the development of priapism (Horowitz and Goble, 1979).

1.3.6 Penodynamic changes during the male sexual cycle

Decrease in resistance within the corporeal vascular bed and the successive rise in arterial inflow are the chief vascular actions important for erection of the penis (Figure 1.6 and 1.7) (Christ and Wingard, 2005; Christ, 1995). With an increase in penile volume to almost maximum (from, 10 ml in the flaccid state to; 60 ml in the erect state), arterial inflow decreases and plateaus at a level that is adequate to keep the penis in the rigid state.

During the flaccid state, intracorporeal pressure is within 10 to 15 mmHg. The change in pressure is modest within the penis during the early phase of the sexual cycle and remains so until near-maximum changes in circumference and volume are reached. Penile body pressure rises sharply to about 90 mmHg as the penis erects. With the contraction of perineal muscle, the penile body pressure rises to a level higher than 120 mmHg (suprasystolic pressure) leading to full firmness and heightening of the penis to an angle greater than 90 degrees from the plane of lower extremities (Hatzimouratidis and Hatzichristou, 2007; Musacchio *et al.*, 2006). Penile body pressure decreases sharply when the penile volume returns to the flaccid size after orgasm.

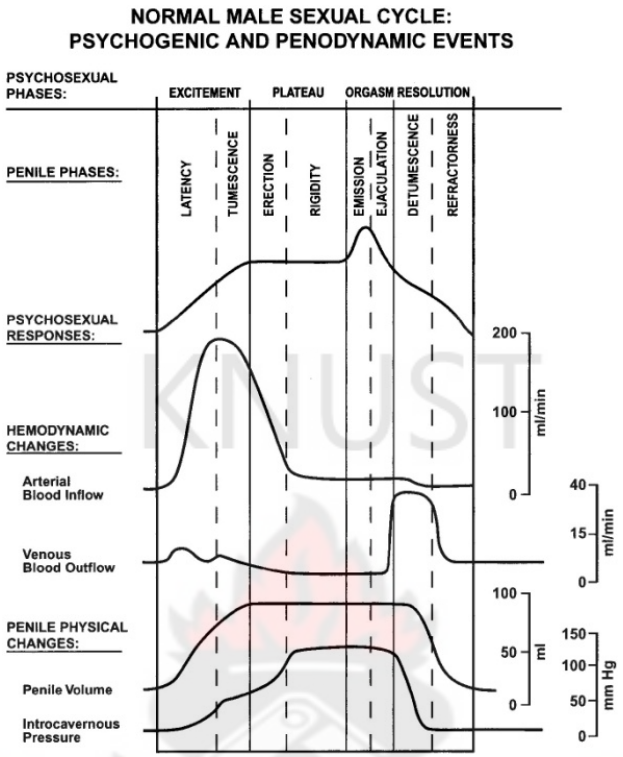

Figure 1.7 The psychogenic and penodynamic events of the normal male sexual cycle. The psychosexual response cycle has 4 major phases: excitement, plateau, orgasm, and resolution. They are represented by the *solid vertical lines* and by the top diagram. Each of the psychosexual phases comprises two interrelated physical events, which are represented by the vertical dashed lines. The penile hemodynamic changes associated with the sexual cycle (arterial and venous flow rates) are depicted in the middle portion, and the penile physical changes (volume and intracorporeal pressure) are depicted in the lower portion of the graph. Arterial blood inflow rate increases dramatically during latency, tumescence and early stages of erection. This increase in arterial inflow is accompanied with an earlier increase in venous return, and results in gradual expansion of the cavernous tissue, increase in intracorporeal pressure, obliteration of emissary veins, and ultimately restriction of the venous return. The rise in intracavernous pressure, in turn, leads to a progressive decline in the arterial inflow to a temporary cessation during the full penile rigidity. Venous drainage also completely ceases with full penile rigidity. As the corporeal smooth muscle cells begin to contract in late ejaculation, venous return increases sharply and remains high during the detumescence phase until the entrapped blood is fully drained and the intracorporeal pressure declines to its baseline level, which is maintained during the flaccid state. Penile volume expands maximally during late erection and intracavernous pressure rises maximally during full rigidity (Kandeel *et al.*, 2001).

1.4 TREATMENT OF ERECTILE DYSFUNCTION

In spite of the growing availability of useful conventional medical treatments, plant-derived and herbal remedies continue to offer a popular alternative for men seeking to improve their sexual life. WHO confirmed in a survey completed in 1983 that developing countries are more interested than ever in making use of traditional, indigenous resources in implementing their primary healthcare (PHC) programs (Abbiw, 1990) and as such have recently adopted a deliberate policy encouraging the development and utilization of traditional medicine in the primary healthcare delivery system particularly in third world countries. This policy is based on the role traditional medicine is already playing in the healthcare programs in most developing countries, especially in Africa, Asia and Latin America (Ghana Herbal Pharmacopoeia, 1992).

Healers in traditional societies use herbal remedy recipes that have been handed down from one generation to another within families (Borins, 1990) and these are mostly plant derived. Plants are chemical factories that directly provide about 25% of currently used drugs: another 25% of drugs are chemically altered natural products (Huxtable, 1992). According to estimates, some 80% of the world's population rely primarily on traditional medicines for their primary health care needs, and a major part of traditional therapy involves the use of plant extracts or their active ingredients (WHO, 1993). Use of herbs for healing is especially important in developing nations, where the cost of drugs is prohibitive, accessibility to drugs in rural areas is virtually nonexistent, and there is a shortage of physicians (Borins, 1990). This is particularly true for Ghana, where it is estimated that there is one (1) traditional doctor to approximately four hundred (400) people as opposed to one allopathic or orthodox doctor to every twelve thousand (12,000) people (Ghana Herbal Pharmacopoeia, 1992).

1.5 APHRODISIAC

Aphrodisiac is a word derived from 'Aphrodite', the Greek goddess of love, beauty and sexuality, and aphrodisiacs are the substances that are used to improve an impaired sexual function (Ramachandran *et al.*, 2004; Shah, 2002). The likelihood of bioactive aphrodisiacs which may be obtained from plants, animals or minerals, has been attractive throughout recorded history. These agents have been used since time immemorial and there is enough evidence showing their use by the ancient Greek and Arab physicians. In ancient Greece, circa 320 B.C., Theophrastus, a philosopher and herbalist, reported that a plant called *satyrion* allows a man to perform 70 consecutive acts of intercourse. Satyrion became extremely popular and was harvested to extinction. In Rome, circa A.D. 50, Roman naturalist Pliny the Elder wrote that ginger was an aphrodisiac when pounded into paste and spread on the stomach, scrotum and anus. In Europe in the 1700s, chemists ground up Spanish fly, a species of the blister beetle, to create a notorious sex stimulant (Preckshot, 1999). Aphrodisiacs can be grouped into 3 categories according to their mode of actions: (1) those that increase libido (i.e. sexual desire), (2) those that increase potency (i.e. effectiveness of erection), and (3) those that increase sexual pleasure (Sandroni, 2001).

1.5.1 Substances that increase libido

These aphrodisiac operate at the level of the central nervous system by changing a particular neurotransmitter or specific sex hormone's concentrations. A number of these are useful in both sexes, while most act via increase in testosterone concentration and as a result are male-specific (Sandroni, 2001). Ambrein a chief constituent of *Ambra grisea* is used in Arab countries and is found in the gut of sperm whales (Taha *et al.*, 1995; Taha *et al.*, 1998). Animal studies have shown that this tricyclic triterpene alcohol increases the concentration of several anterior pituitary hormones and serum testosterone, which in turn stimulate dopamine receptor synthesis and sexual behaviour (Taha *et al.*, 1995).

A nonspecific enhancement in sexually oriented behaviour occurs with the ingestion of central nervous system stimulants, such as amphetamine, cocaine, dopaminergic agents, caffeine, antiserotonin drugs, cannabis, and marijuana. A variety of stimulants are present in beverages and chewable derivatives of the kola nut, guarana, and betel nut, the use of which as leisure and aphrodisiac drugs is widespread in Africa, Asia, and Latin America (Morton, 1992).

1.5.2 Substances that increase potency (allow or sustain erection)

This group of aphrodisiacs acts via initiation of vasodilatation, to allow for erection to occur. Such remedies are mainly for males, even though to a minor extent they could be helpful in women (Sandroni, 2001). Sildenafil citrate, an oral drug for men with erectile disorder, produces acceptable erections and improves sexual satisfaction without affecting sexual desire (Jackson, 1998).

1.5.3 Substances that enhance sensory experience during coitus

These act through irritation of the genital mucosa, therefore enhancing sensation. Not uncommonly, they are ingested by an innocent subject through a drink organized by the (potential) partner (Sandroni, 2001). *Cantharidin* ("Spanish fly") is a chemical with vesicant properties obtained from blister beetles, which have been used for millennia as a sexual stimulant by both sexes (Karras *et al.*, 1996). Its mode of action is by inhibition of phosphodiesterase and protein phosphatase activity and stimulation of β-receptors, inducing vascular congestion and inflammation. The ingestion of live beetles (*Palembus dermestoides*) in Southeast Asia and triatomids in Mexico may have a similar rationale (Chu *et al.*, 1977).

1.6 RESEARCH STRATEGIES FOR EVALUATING SEXUAL FUNCTIONS

Understanding of the function of botanicals on sexual functions is primarily from behavioural and physiological experiments in rodent animal models. For sexual functions experiment, male rats or mice were subjected to sexually primed females. Conclusive parameters here include mount frequency, intromission frequency, mount latency, intromission latency, ejaculation latency and post ejaculation

intervals (Agmo and Ellingsen, 2003; Gauthaman *et al.*, 2003). Estimating the dormant period of erection (LPE) after electrical stimulation of the penis gives a clue on the effect of the test agents on the improvement/revival of sexual potential. In the castrated rat model of sexual disorder, the latent period of erection was used effectively to assess the effect of lipid extracts from the plant *Lepidium meyenii* (Zheng *et al.,* 2000). Here a reduction in the latent period of erection was directly linked to the improvement or revival of sexual function.

Erection is the final effect of the relaxation of the cavernous tissues that includes both the central nervous system and local factors. Measurement of the changes in the penile intracavernous pressure (ICP) in rats gives information on sexual functions and serves as a dependable experimental index (Chen *et al.*, 1992; Davies *et al.*, 2007; Kendirci *et al.*, 2007).

Plasma levels of androgens and luteinizing hormone (LH) offer information on the physiological situation that the treatment mediates. The notion that testosterone production by Leydig cells is primarily under the control of the pituitary gonadotrophin luteinizing hormone (LH) has been documented for several years (Catt *et al.*, 1980; Desjardins, 1981; Huhtaniemi *et al.*, 1982). Besides that, LH secretion is in pulses and the frequency and amplitude of these pulses is crucial for proper activation of gonadal function (Bannister *et al.*, 1986; Bartke *et al.*, 1973; Steiner *et al.*, 1982; White *et al.*, 2007). In the rat, earlier studies have indicated that there is no direct relationship between the trains of LH pulses and the initiation of testosterone secretory episodes, with often an active LH secretory period being dissociated from the testicular response (Ellis and Desjardins, 1982; Sodersten *et al.*, 1983). The lack of correlation between LH and testosterone production in the rat differs from that seen in humans, as more direct testosterone responses were observed in male subjects after trains of LH peaks (Spratt *et al.*, 1988). Nevertheless, in humans, testosterone secretion lags behind LH secretion by, 40 min (Spratt *et al.*, 1988).

1.7 RESEARCH STRATEGIES FOR EVALUATING ANXIETY AND DEPRESSION

Anxiety and depression are exceptionally common, spectacular and devastating multifacetic disorders, and it is now becoming clear that without knowledge of both clinical and biological aspects of anxiety and depression, it is not feasible to offer successful treatment strategies for the patients (Arborelius *et al.*, 1999; Paterson *et al.*, 2001; Willner, 2005). Animal models are used as "experimental provision developed in one species for the purposes of studying phenomena occurring in another species" (McKinney, 1984; McKinney, 2001). Long-ago, there was thorough study of a diversity of neurobiological aspects of depression and anxiety (Nemeroff, 2004; Raison and Miller, 2003). Mice and humans share more then 90% of their genes, and animal models seem to be a useful tool in biomedical sciences, as evidenced by a notable increase in the number of active laboratories working in the field (Belzung, 2001; Borsini *et al.*, 2002). Also, animal models are mainly of help in conditions when the impact of stress cannot be considered in humans because of ethical, social and medical reasons.

Behavioural range of animals has long been used to detect effects on, and the impact of, anxiety and depression (Arborelius *et al.*, 1999; McKinney, 1984; Paterson *et al.*, 2001). A number of models, based on animal emotional reactivity, have been designed and proven to be bidirectionally sensitive to stressful manipulations, including those of anxiety and depression (Fernandez Espejo, 1997). Many of these models have been successfully used to test new anxiolytic or antidepressant drugs on offer and understanding of their underlying neural mechanisms (Arborelius *et al.*, 1999; Paterson *et al.*, 2001; Willner, 2005) by simple, rapid and inexpensive ways of evaluating an animal's condition. Anxiety models can be based on: (i) exploratory; (ii) social; (iii) defensive; (iv) novelty-evoked; (v) conditioned (active/passive avoidance); (vi) anhedonic behaviour; and (vii) conditioned fear-related behaviours (Wall and Messier, 2001).

Moreover, models of anxiety and depression can be "natural", based on measuring natural animal behaviours, or "artificial", utilizing behaviours not normally seen in natural conditions (Kalueff, 2003; King *et al.*, 2002). Natural animal models aim to reproduce behavioural and pathological aspects of the disorder, to investigate the neurobiological mechanisms that are not easily amenable to study in humans, and allow a reliable evaluation of a number of external factors including pharmacological agents (Overall, 2000). Such ethologically based paradigms are more sensitive to stress compared to "artificial" animal conditioned behaviour models which usually use strong and often painful stressors. Extreme stressors suppress general activity and result in non-specific alterations in animal performance (Landgraf and Wigger, 2002; Matar *et al.*, 2006), this research focused on the first, more suitable, group of models of anxiety and depression.

Because it is difficult to interpret subjective anxiety or depression levels based on a single behavioural measure, proper understanding of animal state is only possible through assessment of interaction between behavioural and physiological variables in the multivariate analysis (Calatayud and Belzung, 2001; Calatayud *et al.*, 2004).

1.8 RESEARCH STRATEGIES FOR EVALUATING TOXICITY

1.8.1 Toxicological screening

The human population is exposed to an ever increasing number of chemical substances used to improve the quality of life. With the introduction of new drug, an equally great probability exists for the incidence of adverse effects upon the populace. Subsequently, various legislative bodies are becoming increasingly aware of the need for establishing not only the effectiveness of such drugs but their safety as well. The use of animal experimentation for providing prospective as well as retrospective information profiling chemical safety is of great importance. It should be noted that animal investigations cannot conclusively establish human safety but are conducted to detect the deleterious effects of compounds to establish whether they should be tested further in humans.

Establishing drug safety requires information about the acute toxic effects of such a drug. The results of acute toxicity are used for the planning of chronic toxicity studies. In some cases, it is necessary to determine the toxic effects of drugs over a long period of time. Chronic toxicity or subacute toxicity testing becomes important in such a situation as to determining the dose level at which the animal can live free of side effects over a period of time. Thus, multiple-dose study not only characterizes the dose-response relationship of a test drug but also provides data for more reasonably, predicting the maximum tolerated levels for the species during potential lifetime exposure. The results of chronic toxicity experimentation together with other results from acute toxicity tests and other pharmacological experiments help to evaluate the possible hazardous properties of a new drug.

The duration of administration of a drug under investigation is a subject of controversy. Various workers have given terms such as sub-acute, sub-chronic or chronic toxicity studies. The World Health Organization (WHO) defines a short term study of less than three months as sub-acute and from three to six months as long-term or chronic studies (WHO, 1996). In chronic toxicity studies, the drug is administered daily over a specified period.

1.8.2 Plasma Enzymes in Drug Toxicity

Tissue damage, regardless of whether the damage was caused by a disease process or chemical toxin, often leads to the release of enzymes from the injured cell into the extracellular fluid. However, because of the multiplicity of enzymes occurring in animal tissues, it is rare that a single enzyme with a highly defined source is released and often more than one organ is affected. The measurement of plasma enzyme activity has proved particularly successful in detecting and assessing cell injury caused by drug toxicity (Yao *et al.*, 2006).

Non-functional plasma enzymes are derived from the cell of organs and tissues. High concentrations of these enzymes are present within cells and the low levels of these enzymes in normal plasma are derived from cells destroyed in normal tissue

turnover (Ramirez *et al.*, 2007; Sureshkumar and Mishra, 2006). Non-functional or non-plasma-specific enzymes are clinically important when the serum level is increased above the reference range or control range.

Ideally, non-plasma-specific enzymes are contained within cell membranes, the cell membrane being impermeable to enzymes as long as the cell is functioning normally. However, during periods of stress, such as a period of decreased oxygen or glucose supply, or in the presence of bacterial, viral infections or chemical toxins, the cell membrane becomes permeable or the membrane raptures, causing the release of soluble enzymes into the extracellular fluid. These enzymes eventually reach the blood.

Excessive synthesis or induction of an enzyme which overflows into the plasma is also thought to cause elevated levels. In obstructive liver disease, the increase in liver alkaline phosphatase is thought to be due to this process (Sureshkumar and Mishra, 2006). Increase in systhesis can also occur when cells that normally produce enzymes increase in number, such as when a tumour is present. Neoplastic process may also elevate enzyme levels by causing structural changes that allow intracellular enzymes to leak into circulation (Brandin *et al.*, 2007). Neoplasm may also induce normal cells to alter enzyme-related physiology, leading to increased enzyme production. In some cases, tumour cells are thought to produce enzymes that are normally present in immature cells or foetal cells. It is believed that tumour cells revert to a more primitive cell form and produce enzyme patterns normally found only in foetal or immature cells. The alkaline phosphatase isoenzymes are derived from this process (Habs *et al.*, 1982).

1.9 SPHENOCENTRUM JOLLYANUM

1.9.1 Botanical name:

Sphenocentrum jollyanum (Pierre)

1.9.2 Taxonomical hierarchy of Sphenocentrum jollyanum:

Kingdom	Plantae
Division	Magnoliophyta (Cronquist)
Subdivision	Magnoliophytina (Frohne and Jensen)
Class	Ranunculopsida (Brongn)
Subclass	Ranunculidae (Takht)
Superorder	Ranunculanae (Takht)
Order	Menispermales (Bromhead)
Family	Menispermaceae (Juss.)
Genus	Sphenocentrum (Pierre)
Species	Jollyanum

1.9.3 Common/Vanacular names:

Akan: Aduru kokoo (Red medicine), Okraman kote (Dog's penis)

Asante: Krakoo

Twi: Kraman kote (Dog's penis)

Ewe: Dangbo-Pobè-Niaouli

Yoruba: Ajo or Akerejupon

Benin: Oban abe

Ivory Coast: Ouse-abe

1.9.4 Description:

Sphenocentrum jollyanum Pierre is a small erect sparsely branched shrub, growing up to 1.5 m in height with very few branches. The leaves up to 20 cm long and about 5-12 cm broad are elliptical, margin entire with short and blunt apex, wedge-shaped base and smooth on both sides (Dalziel, 1985; Iwu, 1993). The roots are bright yellow with a sour taste while the ovoid-ellipsoid bright yellow or orange fruits occur in clusters and are edible when ripe (Abbiw, 1990; Neuwinger, 1996). The flowers are auxiliary in cluster and the fruits are ellipsoid, also in clusters on the sterm just below the leaves, fleshy, ripens orange (Abbiw, 1990).

Figure 1.8 Different parts of *Sphenocentrum jollyanum* Pierre.

1.9.5 Traditional Uses:

It is mostly found in the tropical forest zones of West Africa as undergrowth plant it is used in treating various human diseases. *Sphenocentrum jollyanum* bears fruits which are edible (Neuwinger, 1996) and is used as an anti-fatigue snack. In Nigeria the roots are used as chewing sticks, relief for constipation and as a stomachic. It is prepared with *Piper guineense* and mixed with lime juice for use as a cough medicine. All morphological parts are prominent ingredients in several recipes for the management of sickle cell disease (Abbiw, 1990). The root hair is used with other anti-malaria plant as remedies against fevers and body pains and rheumatism while leafy twigs and fruit have been reportedly used for their aphrodisiac activity (Burkill, 1985; Iwu, 1993). It is also claimed that this plant material is effective in the cure of central nervous system (CNS) diseases e.g. psychiatric disorders, inflammation and pains (Oke and Hamburger, 2002). The fruit is charred and used in the treatment of fibroids in traditional Nigerian medicine (Egunyomi *et al.*, 2005). The plant is also reputed as a wound healing agent in traditional medicine (Dalziel, 1985).

However, in Ghana, the root is chewed as a central nervous system (CNS) stimulant and aphrodisiac (Irvine, 1961). Roots are chewed raw or cut up into pieces and infused in gin for 3 days to be taken as "bitters". This is perceived to strengthen the penis and cause the erection to be long-lasting, like that of a dog. Hence, the indigenous name – *okraman* (dog), *kote* (penis) in Twi. This is a very popular aphrodisiac, known by everyone. Anytime men go to the forest they simply pull the plant up by the roots and begin to chew (Abbiw, 1990; Irvine, 1961).

1.9.6 Some Identified Chemical Constituents of Sphenocentrum jollyanum

Earlier studies reported the presence of tannins and saponins. The plant also yields several isoquinoline alkaloids, including palmatine, columbamine and bitter tasting diterpenes (Iwu, 1993).

Methanolic extract of the fruits resulted in the isolation of three furanoditerpenes namely, columbin (**1**), isocolumbin (**2**), fibleucin (**3**) and a flavonoid-rich fraction (FDE) (Moody *et al.*, 2006) (Figure 1.9). It also contains alkaloids including Protoberberine (Bradesi *et al.*, 2005; DeWet, 2005).

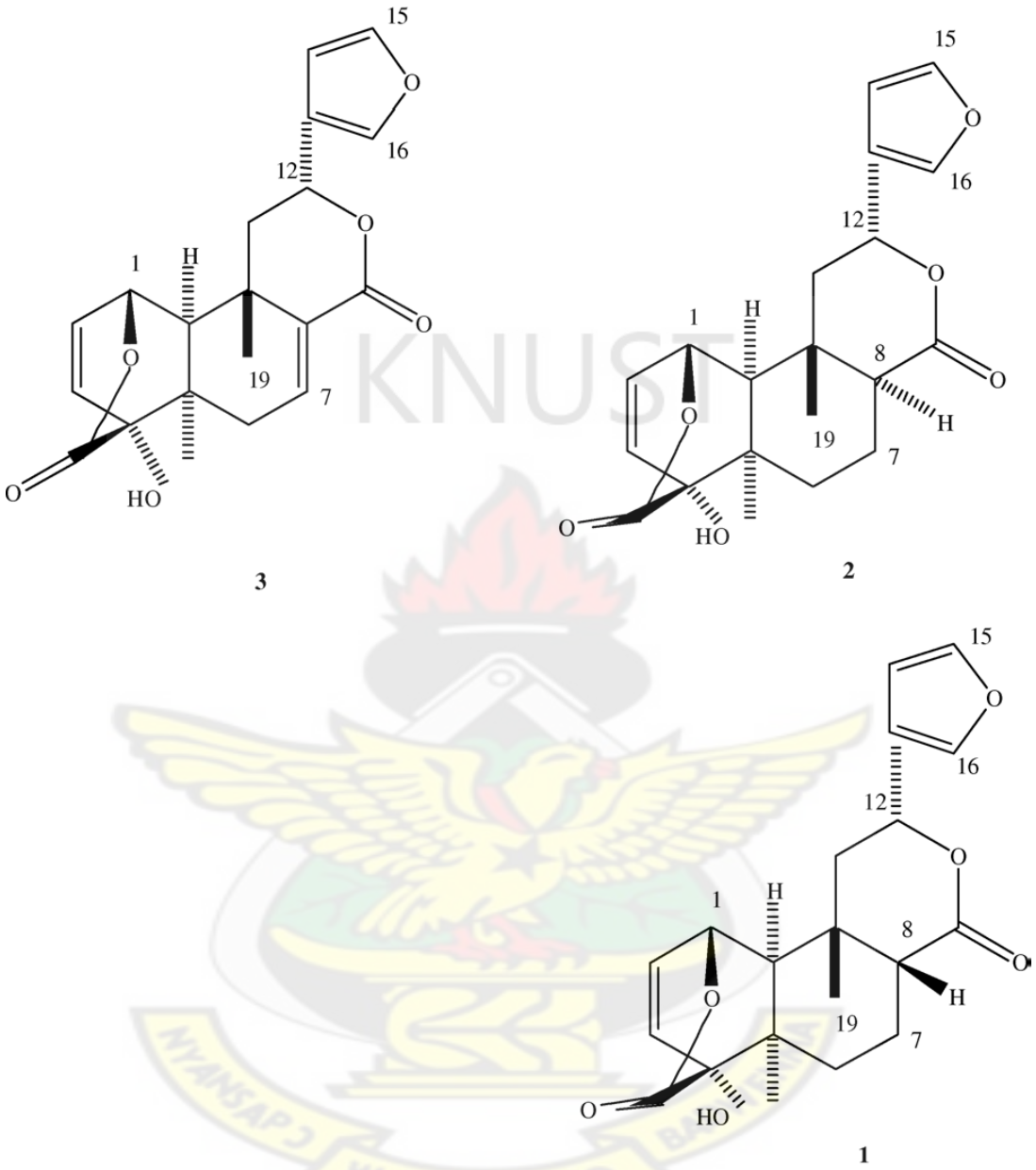

Figure 1.9 **Structures of isolated furanoditerpenes from *Sphenocentrum jollyanum* fruit columbin (1), isocolumbin (2) and fibleucin (3) (Moody *et al.*, 2006).**

1.9.7 A Review of Previous Studies on the Biological Activities of SJE

Although scientific data on *Sphenocentrum jollyanum* is very scanty, Moody *et al.,* reported the anti-viral activity of the chloroform and methanol extracts of this plant on polio virus (Moody *et al.*, 2002a) and cowpea mosaic virus (Moody *et al.*, 2002b). Anti-inflammatory activity of methanolic extracts of this plant has also been reported (Moody *et al.*, 2006). Anti-oxidant and anti-angiogenic property (Nia *et al.*, 2004; Oke and Hamburger, 2002) and male reproductive activity in albino rat was reported by Raji *et al.,* (2006).

1.10 AIMS AND OBJECTIVES

Although a number of treatment regimes became available in the last two decades, problems with costs, efficacy, safety and ease to administer were experienced. These treatments ranged from herbal remedies used by native healers, mostly in the developing countries, to the more sophisticated designer drugs, which are based on a better understanding of the physiological mechanism of erection (Guirguis, 1998).

Sildenafil citrate (Viagra) is a successful drug that modifies the haemodynamics in the penis (Segraves, 2003). However, side effects such as headache, flushing, dyspepsia and nasal congestion are reported with this treatment (Lue, 2000). Sildenafil citrate is contraindicated with cardiac medications such as nitrate therapy (Lue, 2000) and a cautious prescription is recommended in ischaemic heart disease. It is reported that in patients with cardiovascular disease sildenafil is associated with reductions in the mean arterial and pulmonary pressure with little effect on heart rate, cardiac output and systemic or pulmonary vascular resistance (Gillies *et al.*, 2002). Recently introduced drugs such as Tadalafil (Cialis) and Vardenafil (Levitra) are considered effective and well tolerated in the treatment of erectile dysfunction. However, adverse effects such as headache, dyspepsia, back pain and myalgia have been reported with the use of Tadalafil (Carson *et al.*, 2005). Vardenafil which is a selective PDE5 inhibitor is known to have side effects

common to all selective PDE5 inhibitors, including vasodilation, reductions in blood pressure, headache and nasal congestion (Bischoff, 2004).

Even though testosterone may be used for hypoactive sexual desire (Seidman, 2000; Seidman and Roose, 2006), many people in the world still prefer to use natural plants. While testosterone plays a major role in human sexual behaviour, the risks involved with steroid replacement therapy for both men and women can be substantial, especially in more susceptible elderly patients (Tenover, 2000). For instance, testosterone replacement in the male may have undesirable side effects that include testicular atrophy, decreased sperm count, sleep apnea, weight gain, acne, polycythemia, and possibly increased progression of prostate cancer and vascular disease (Steinberger and Smith, 1977; Swerdloff and Wang, 2004; Wang and Swerdloff, 2004).

Therefore agents of natural origin with very little side effects are required as substitute chemical therapeutics. The availability of a large number of sexual function improving drugs in the traditional Ghanaian system of medicine is a unique feature of this system. Besides having many specific drugs for enhancing sexual functions, there are certain most commonly used ones like *Sphenocentrum jollyanum, Mondia whitei, Tunera aphrodisiaca* et cetera. *Sphenocentrum jollyanum* has been catalogued by the center for Research into Plant Medicine at Mampong-Akwapim and is on sale on the Ghanaian market. However, it has not been evaluated in depth for its pharmacological properties, in spite of its traditional use in numerous medical conditions. Traditional use indicates that they probably have several pharmacological properties; however, their efficacy is yet to be scientifically substantiated. The aim of the present study was therefore to scientifically evaluate the central and sexual behavioural effects as well as the toxicity of the root extract of *Sphenocentrum jollyanum* pierre (Menispermaceae). The specific objectives include the following:

- To use multiple parameters to evaluate the aphrodisiac effect of *S. jollyanum* root extract. This will scientifically substantiate its efficacy and potency.

- To evaluate the effect of the extract on libido and reproductive hormones. This will give an informed knowledge about the role of the plant in treating individuals with low libido, hypogonadism and hyperprolactinaemia.

- To assess the central nervous system effect of the alcoholic root extract. This will give an informed knowledge about the role of the plant in treating individuals with psychogenic erectile dysfunction

- To evaluate the smooth muscle relaxation effect of the alcoholic root extract. This will give an informed and consented knowledge about the role of the plant in treating organic erectile problem.

- To assess the hematological parameters after administration of the extract. This will give information about the system toxicity of the plant and how it might help sickle cell anaemia patients with male sexual dysfunction.

- To assess biochemical parameters after administration of the root extract. This will give information about the hepatotoxic and nephrotoxic effects of the plant and its effects in organic erectile dysfunction.

- To determine the effects of the plant extract on drug metabolizing enzyme (total cytochrome P450). This will give information about the interaction of the plant extract with other drugs.

- To assess the histological changes associated with administration of alcoholic root extract of *S. jollyanum*. This will give information on how the plant will affect spermatogenesis, liver and renal function.

- To assess the mutagenicity profile of the plant extract. This will give information about the possibility of the extract inducing mutation in the DNA of the organism.

Chapter 2

MATERIALS AND METHODS

2.1 GENERAL METHODS

2.1.1 Animal care and use

lCR mice of either sex (20-35 g) and male Fischer 344 rats (or male Sprague-Dawley rats) (140-200 g) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Accra and kept in the Animal House Facility of the Department of Pharmacology, Facaulty of Pharmacy, KNUST, Kumasi, Ghana. They were housed in a group of six in stainless steel cages (34 x 47 x 18 cm) with soft wood shavings as bedding and maintained under normal laboratory conditions (temperature 24-28°C, relative humidity 60-70%, and 12 hour light-dark cycle) and given free access to solid pellet diet (GAFCO, Tema-Ghana) and water *ad libitum* throughout the study except during the experiment. All animals used in these studies were treated in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85-23, revised 1985).

2.1.2 Plant material

The sun-dried roots of *Sphenocentrum jollyanum* Pierre (family Menispermaceae), a commonly used medicinal plant, were bought from the Central Market, Kumasi, Ashanti Region-Ghana. It was identified by, Dr. T.C Fleischer of the Department of Pharmacognosy, KNUST, Kumasi, Ghana. A voucher sample was deposited at the Department of Pharmacognosy. The roots were pulverized with a hammer-mill.

2.1.3 Preparation of the root extract

The dried and powdered root (5 kg) was extracted with 70% (v/v) ethanol (i.e. 70 ml of ethanol to 30 ml of water) in a soxhlet apparatus for 24 h. The hydro-alcoholic filtrate was concentrated at a low temperature in a vacuum rotary evaporator, under reduced pressure to obtain a syrupy mass which was air-dried at room temperature (28 °C) for 24 hours. The yield (478 g; 9.56%) which is

yellowish-brown in colour was kept in a dessicator at room temperature and is subsequently referred to as the extract or SJE.

2.1.4 Phytochemical screening

The ethanolic root extract of *S. jollyanum* was subjected to phytochemical screening for tannins, phlobatanins, glycosides, steroids, terpenoids, flavonoids and alkaloids according to the methods of Trease and Evans, (1989).

2.1.5 Drugs and chemicals

Acetylcholine, phentolamine, caffeine, sodium nitroprusside, formalin, alcohol, xylene, paraffin wax, haematoxylin, eosin, potassium chloride and calcium chloride were obtained from BDH Chemicals Ltd (Poole, England). Ketoconazole and phenobarbitone were purchased from Troge (Hamburg, Germany). Prazosin was purchased from Pfizer (Brooklyn, NY, USA). Progesterone and diazepam were obtained from Krka Pharmaceutical (Novo mesto, Slovenia), Fluoxetine was obtained as Prozac® from Eli Lilly and Company (Indianapolis, USA), imipramine was obtained from Sandoz Pharmaceuticals (Broomfield state, USA), methyldopa was purchased from Merck and Company Inc (West Point state, USA), reserpine was obtained from Phyto-Riker Pharmaceuticals (Accra, Ghana) and oestradiol benzoate was obtained from Medisca Inc (Plattsburgh state, USA). Testosterone, follicle-stimulating hormone (FSH), prolactin (PRL) and luteinizing hormone (LH) assay kits were obtained from HySkill Diagnostics (Bahlingen, Germany).

2.2 EFFECT OF SJE ON SEXUAL BEHAVIOUR TEST

To identify drugs that reliably function as aphrodisiacs, various methodological indices have been employed. However, the criteria used to establish the aphrodisiac nature of a compound remain elusive. Three main components of male sexual behaviour (Hollister, 1975) and their equivalent terms used for man (Clark *et al.*, 1984) have been proposed, i.e., (i) arousal (libido in man); (ii) erectile and ejaculatory responses (potency in man) and (iii) increased sexual pleasure.

2.2.1 Mounting behaviour test

To quantify mounting behaviour, experiments were designed as previously described by Lawler, (1984) to measure the libido of the male mice (Taha *et al.*, 1995; Tajuddin *et al.*, 2005). Mount is operationally defined as the male assuming the copulatory position but failing to intromit and an attempted mount defined as incompetent mounts in which the orientation is wrong, such as mounts of the female's head or side. Male mice were dosed with saline (control group) or with SJE root extract (100-1000 mg kg^{-1}, *p.o.*) and placed individually in a Plexiglas cage (60 × 75 × 20 cm). After 15 minutes of acclimatization, a non-oestrous female was introduced into the arena and the number of mounts recorded during a 15-minute observation period. Then the female was separated for 105 minutes and re-introduced and the number of mounts was observed again for 15 minutes as before. The first observation period was designated as the 1st hour and the second, the 3rd hour. All the experiments were performed between 09.00 to 12.00 hrs at room temperature (26–27°C).

2.2.2 Mating behaviour test

The effect of the extract on mating behaviour was carried out by a modification of methods described by Dewsbury and Davis, (1970) and Szechtman *et al,* (1981). This experiment measures the enhancement of sexual performance by the extract (Taha *et al.*, 1995; Tajuddin *et al.*, 2005). Healthy and sexually-experienced male mice were selected for the study. The animals were divided into four groups each consisting of six mice and placed individually in separate Plexiglas cages during the experiment. A baseline sexual behaviour was carried out in mice from all groups to render them sexually experienced. Group 1 served as control group and received 10 ml kg^{-1} of saline orally. Groups 2-4 received a suspension of the extract orally at the doses of 100, 300 and 1000 mg kg^{-1}, respectively, 30 minutes before the start of the experiment. Female mice were brought to estrous by sequential administration of oestradiol benzoate (10 µg/l00 g) and progesterone (500 µg/100

g), through subcutaneous injections, 48 hours and 4 hours before the copulatory studies respectively (Srilatha *et al.*, 2007; Srilatha *et al.*, 1999).

Sexual behaviour studies were carried out in a room under dim red illumination as previously described (Dewsbury *et al.*, 1972). Each male mouse was placed in a rectangular Plexiglas chamber, 10 minutes before the introduction of a primed female, for it to get acclimatized to the chamber conditions. The primed female was then introduced into the chamber with 1 female to 1 male and the following sexual behaviour parameters were recorded:

a) mount frequency (MF): the number of mounts without intromission from the time of introduction of the female until ejaculation,

b) intromission frequency (IF): the number of intromissions from the time of introduction of the female until ejaculation,

c) mount latency (ML): the time interval between the introduction of the female and the first mount by the male,

d) intromission latency (IL): the interval from the time of introduction of the female to the first intromission by the male (characterized by pelvic thrusting and springing dismount),

e) ejaculation latency (EL): the time interval between the first intromission and ejaculation (characterized by longer, deeper pelvic thrusting and slow dismount followed by a period of inactivity),

f) post-ejaculatory interval (PEl): the time interval between ejaculation and the first intromission of the following series.

Male reproductive behaviour occurs as a sequence of complex motor behaviours. In the rodent the sequence typically starts with anogenital investigation of the stimulus female, followed by mounts and intromissions, and culminates in

ejaculation. The experiment was terminated when a period of inactivity (which normally follows ejaculation) is observed after a mount and intromission.

2.3 HORMONAL ASSAYS

To measure the effect of the extract on the reproductive hormones: testosterone, FSH, LH and prolactin levels, blood samples were collected at weekly intervals for three weeks during the study from vehicle-treated or SJE-treated (100-1000 mg kg^{-1} *p.o.)* animals. The blood was collected into Vacutainer™ tubes from the jugular veins of animals killed by a sharp blow on the head. The blood was centrifuged at 500 *g* for 15 min and serum was collected and stored at -20°C until assayed. Male rats were placed in four groups of 18 animals each. *Group A,* the vehicle-treated control, received 10 ml kg^{-1} of saline daily via an intra-gastric syringe. *Group B, C* and *D* were dosed with SJE at 100, 300 and 1000 mg kg^{-1} (*p.o.*) respectively daily. At the end of each week rats (6 per group) were sacrificed and blood samples collected for the assays

2.3.1 Sandwich enzyme immunoassay (SIA) for prolactin, LH, FSH and testosterone

Serum testosterone, follicle-stimulating hormone (FSH), prolactin (PRL) and luteinizing hormone (LH) were determined by sandwich enzyme immunoassay (SIA) using NoviWell™ assay kits (HySkill Diagnostics, Bahlingen, Germany). Assays were carried out as described by the manufacturer. The assay is based on simultaneous binding of hormone to two monoclonal antibodies; one is immobilized on the microplate, the other is soluble and conjugated with horseradish peroxidase (HRP). Briefly, 2 µl aliquots of standards and samples were dispensed into their respective wells in ready-to-use microtitre plates precoated with anti-hormone IgG antibodies. After the addition of 100 µl anti-hormone HRP conjugate (1:100 dilution) to each well, the plates were incubated for 30 min at room temperature. The contents of the well were then aspirated and the wells washed twice with 200 µl of distilled water. The enzyme reaction was started by

addition of the chromogen (tetramethylbenzidine/hydrogen peroxide system) into each well. Plates were then incubated for 10 min. The reaction was stopped by addition of 100 µl of 0.15 M H_2SO_4. Absorbance was measured at 450 nm in an ELx800™ Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). Within-assay coefficient of variation was 6.1% for PRL, 6.1% for FSH, 5.4% for LH, and 6.2% for testosterone. The analytic sensitivities of the assays were 1.0 mIU/ml for FSH and LH and 1.0 ng/ml and 0.1 ng/ml for PRL and testosterone as provided by the manufacturer.

2.4 *IN VITRO* EXPERIMENT ON ISOLATED TISSUE

2.4.1 Effects of SJE on rat anococcygeus preparation

2.4.1.1Rat anococcygeus muscle preparations

Male Fischer 344 rats (200-250 g) were sacrificed and the anococcygeus muscle was removed as previously described by Gillespie, (1972). The abdomen was opened in the midline and the pelvis split. While the colon was carefully lifted, the connective tissue was cut along the dorsal side until the paired muscles could be seen passing from the vertebral column to the ventral side of the colon. The muscles were exposed, carefully freed of connective tissue and dissected out. Each muscle was mounted in a 10 ml organ bath under a resting tension of 1 g; the organ bath contained Krebs physiological salt solution (PSS) of the following composition (mM): NaCl, 130; $NaHCO_3$, 14.9; dextrose, 5.5; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4.7H_2O$, 1.17 and $CaCl_2.2H_2O$, 1.6. The PSS was gassed constantly with air and maintained at 32°C. Isotonic transducers were used to measure changes in isotonic contraction of the tissues and recorded with a Harvard Universal Student Oscillograph (Harvard Apparatus Ltd. Edenbridge, Kent, England). Isolated muscles were allowed to equilibrate for 1 h, in accordance with the previously published work (de Godoy and de Godoy, 2003; Gibson *et al.*, 2006; Gillespie, 1972) before adding drugs. The organ bath PSS was replaced with fresh PSS every 15

min. After the equilibration period, tissues were stimulated with K^+ (80 mM) to check their responsiveness.

2.4.1.2 Experimental protocols

After the equilibration period, tissues were precontracted with K^+ (80 mM) to give maximal contraction. After reaching maximal contraction, the cumulative relaxation of the K^+-induced precontraction by SJE (1-100 mg ml^{-1}) was determined. Responses were expressed as percentage of maximal relaxation (loss in tone) induced by the addition of 1.0 mM papaverine HCl to the bath at the end of the experiment. In another set of experiment, the cumulative relaxation of the K^+-precontracted anococcygeus muscle by sodium nitroprusside (1-100 µg ml^{-1}) was determined. Responses were expressed as percentage of maximal relaxation (loss in tone) induced by the addition of 1.0 mM papaverine HCl to the chambers at the end of the experiment.

Also, the cumulative relaxation of the K^+-precontracted anococcygeus smooth muscle by phentolamine (0.04-100 µg ml^{-1}) was determined. Responses were expressed as percentage of maximal relaxation (loss in tone) induced by the addition of 1.0 mM papaverine HCl to the chambers at the end of the experiment.

Cumulative contractile response to phenylephrine was obtained, and then the tissue was washed three times with fresh media and equilibrated for 30-40 minutes. After that, the tissue was pre-treated for 10 minutes with prazosin (0.1 µg and 1.0 µg) or the extract (0.1, 1.0 then 10.0 mg ml^{-1}) before another set of contractile response to phenylephrine was obtained. This protocol was repeated for carbachol in the presence of atropine and the extract and for calcium in the presence of the extract.

2.4.2 Effect of SJE on the rabbit corpus cavernosum

2.4.2.1Rabbit corpus cavernosum preparations

Male rabbits (2.0 to 2.5 kg) were decapitated and the penis removed at the level of the attachment of the corporal bodies to the ischium. A slit was made in the proximal end of the tunical and extended distally. The corpus cavernosum was sharply dissected free of the tunica albuginea bilaterally. Corpus cavernosum excised from rabbit penis were cut into longitudinal strips of about 10 mm in length and mounted in organ chamber containing Krebs solution in mM: (NaCl, 130; $NaHCO_3$, 14.9; dextrose, 5.5; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4$ $7H_2O$, 1.17 and $CaCl_2$ $2H_2O$, 1.6). The solutions were gassed constantly with air and maintained at 37°C. The corpus cavernosum tissues were isolated with an intact endothelium, as were assessed by the capacity of acetylcholine to elicit relaxation. Isotonic transducers were used to measure changes in isotonic contraction of the tissues, which was displayed on a Harvard Universal Student Oscillograph (Harvard Apparatus Ltd. Edenbridge, Kent, England). Isolated tissues were allowed to equilibrate for 60 to 90 min, during which time the medium was replaced every 10 to 15 min. 10 μM indomethacin was added to the bath to attenuate spontaneous activity

2.4.2.2 Experimental protocols

After the equilibration period, tissues were contracted with 1 mM phenylephrine to give maximal contraction. After reaching maximal contraction, the cumulative relaxation of the phenylephrine precontracted corpus cavernosum smooth muscle (CCSM) by SJE was determined (1-100 mg ml^{-1}). Responses were expressed as percentage of maximal relaxation (loss in tone) induced by the addition of 1.0 mM papaverine HCl to the chambers at the end of the experiment.

In another set of experiments, the cumulative relaxation of the phenylephrine-precontracted corpus cavernosum smooth muscle (CCSM) by sodium nitroprusside (0.01-1 μg ml^{-1}) and phentolamine (0.1-10 μg ml^{-1}) were determined.

Relaxation responses were expressed as percentage of total relaxation (loss in tone) induced by the addition of 1.0 mM papaverine HCl to the bath at the end of the experiment.

2.5 CENTRAL NERVOUS SYSTEM EFFECT OF SJE

2.5.1 Effects of SJE on anxiety

2.5.1.1 Drugs and dosages

The alcoholic root extract (100, 300 and 1000 mg kg^{-1} *p.o*), diazepam (0.1, 0.3 and 1.0 mg kg^{-1} *i.p.*) and caffeine (10, 30 and 100 mg kg^{-1} *i.p.*) were suspended in normal saline. The drugs were freshly prepared each time. The extract was orally administered; the caffeine and diazepam were administered intraperitoneally. Control animals received the same volume of vehicle (normal saline). In order to determine the CNS effect of the extract, the following tests were carried out 30 min after drug administration.

2.5.1.2 Procedure

All behavioural testing occurred between 08:00 and 14:00 and the mice weighed 20-30 g. The mice were subjected to a series of tests conducted in the following order: activity cage, open field, and hole board on one day. Light/dark box test and elevated plus-maze were performed with different set of animals on the following day. After each mouse had finished a test the apparatus was cleaned with a 10% ethanol solution and dried with paper toweling.

2.5.1.3 Spontaneous locomotor activity

Spontaneous locomotor activity was measured by using an automated activity cage (Model 7401, Ugo Basile, Milan, Italy; 19 x 23 x 35 cm). The movement of a mouse inside the box was detected by 29 stainless steel bars placed 1 cm apart on the floor of the cage. Activity for each mouse was counted automatically for 5 minutes after 30 minutes of drug administration.

2.5.1.4 Open field

Open field test was based on that described by Harro *et al.,* (1990). Testing was conducted in clear Plexiglas boxes (40 x 40 x 30 cm) whose floor was divided into 16 equal squares by black lines. For behavioural analysis, the arena of the open field was designated as (i) corner (one of the four corner squares); (ii) periphery (the squares along the walls); or (iii) center (the four inner squares). The animals were divided into ten groups of six animals each, and received either the extract (100, 300 or 1000 mg kg^{-1}, *p.o.*), the vehicle or the standard reference drugs diazepam (0.1, 0.3 or 1.0 mg kg^{-1}, *i.p.*) or caffeine (10, 30 or 100 mg kg^{-1}, *i.p.*). Thirty minutes after *i.p.* and oral administration of the test compound, mice were placed individually in the center of the open field and allowed to explore freely for 5 minutes. Each session was recorded by a video camera suspended approximately 100 cm above the arena. After each session, the number of fecal pellets was noted and the open-field cleaned with 10% alcohol before introducing the next animal (Phillips, 1982). All animals were regularly handled before individual tests in order to minimize handling-related stress. Videotapes arena and the following variables of motor activity were recorded: locomotor activity, fine movement and rearing. Furthermore, distance traveled, total time, rest time, number of entries and head pokes in individual zones were also recorded. The exploration activity in the open field was determined as distance covered of horizontal movements and vertical (rearings) explorations (Bouwknecht *et al.*, 2007; Diana *et al.*, 2007; Schulz *et al.*, 2007). Thereafter, behaviour in the open field was analyzed for 5 minutes. Mean values (± SEM) were calculated for each and compared to vehicle-treated animals. The test was performed under dim red light in a sound attenuated room.

2.5.1.5 Hole board

This test was based essentially on that described by File and Wardill, (1975b). The hole-board apparatus consisted of a square Plexiglas chamber (30 x 30 x 30 cm) with four holes (2.5 cm diameter) each situated 5 cm in from each of the corners, elevated 5 cm from the ground so that the mice could peep through the holes. The

animals were grouped and treated as described above for the open field experiments. Each mouse was placed individually in the center of the field and videotaped for 5 minutes. The number of times the mouse dipped its head in a hole and the duration were measured. Automated tracking software was used to record the distance traveled. Increased exploration behaviour, characterized by an increase in the number as well as duration of head dips is an indication of anxiolytic activity (File and Wardill, 1975b).

2.5.1.6 Light/Dark Test in Mice

The light-dark exploration test is typically used to more directly assess anxiety-related responses. This apparatus was based on the initial model described by Crawley and colleagues, (1980) and as modified by other workers (Belzung and Le Pape, 1994; Belzung *et al.*, 1987). It consisted of wooden box (45 x 30 x 30 cm), which was divided into two equal compartments by a wooden board with a 7 x 7 cm opening located centrally at the floor level, connecting the compartments. One compartment was painted black and covered with a wooden lid. The other box (not covered) was painted white and lit by a 60-W light bulb set 30 cm above the box. Mice were grouped and treated with drugs as described for the open field experiments. At the beginning of the experiment, mice were placed individually in the center of the illuminated box, facing away from the dark compartment. Behaviours of the animals were recorded for 5 minutes with a digital camera placed 1 m above the box. Videotapes were scored manually with the aid of a computer program, Behaviour Tracker Version 1.5 for following parameters: 1) initial latency to enter dark compartment; 2) frequency of compartment entries; 3) total time spent by mice in each compartment; 4) total number of transitions between compartments.

2.5.1.7 Elevated plus maze

The elevated plus maze was a modification of a previous design (Pellow *et al.*, 1985b). It consisted of two open arms (30 cm x 5 cm x 1 cm) and two closed arms

(30 cm x 5 cm x 15 cm) that extended from a central platform (5 cm x 5 cm). Like arms opposed each other across the central platform. The maze was constructed from opaque Plexiglas and was elevated on a woody stand 60 cm above the floor. The test was performed under dim red light (~750 lux) in a sound attenuated room and the behaviour was recorded on a videotape with a digital camera placed 100 cm above the maze. Mice were grouped and treated with drugs as described for the open field experiments. Each mouse was placed on the center of the platform facing an open arm. Animals were tested individually and only once for 5 min. The maze was cleaned following each trial to remove any residue or odors. Behavioural parameters were scored from the videotapes with the aid of a computer program, Behavioural Tracker Version 1.5, for the following : (i) number of closed and open arm entries-(absolute value and percentage of the total number); (ii) time spent in exploring the open and closed arm of the maze- absolute value and percentage of the total time of testing; (iii) number of head dips- protruding the head over the edge of an open arm and down toward the floor; (iv) number of stretch attend postures- the mice stretche forward and retract to original position; (v) number of nose pokes; (vi) number of grooming; and (vii) number of rearing. An arm entry was counted only when all four limbs of the mouse were within a given arm.

2.5.2 Antidepressant property of SJE

2.5.2.1 Forced Swimming Test (FST)

The FST was based on that described by Porsolt and other workers (Porsolt, 1979; Porsolt *et al.*, 1978). Mice were divided into ten groups of six animals each, and received the extracts (100, 300 or 1000 mg kg^{-1}, *p.o.),* the vehicle or the reference drugs imipramine (3, 10 or 30 mg kg^{-1}, *p.o.*) or fluoxetine (3, 10 or 30 mg kg^{-1}, *p.o.).* Thirty minutes after administration of the test compound, mice were gently dropped individually into transparent cylindrical polyethylene tanks (25 cm high, 10 cm internal diameter) containing water (25°C to 28°C) up to a level of 20 cm and left there for 6 minutes. Four identical polyethylene cylinders were prepared and four animals, separated by opaque screens, were exposed simultaneously and

videotaped. Each session was recorded on a videotape with a digital camera suspended approximately 100 cm above the cylinders. After each session, animals were removed from the cylinders, dried with absorbent towels, placed in cages near to a heater until they were completely dried and then returned to their home cages. Water was changed for each mouse and tanks were cleaned in between studies. An observer scored the behaviour from the videotapes with the aid of a computer program, Behaviour Tracker Version 1.5, for duration of immobility during the last 4 minutes of the 6-minute period.

2.5.2.2Tail Suspension test (TST)

The TST was carried out essentially as previously described (Nomura *et al.*, 1992; Porsolt *et al.*, 1987; Steru *et al.*, 1985). Mice were divided into ten groups of six animals each, and received either the extracts (100, 300 or 1000 mg kg^{-1}, *p.o.*), the vehicle or the standard reference drugs imipramine (3, 10 or 30 mg kg^{-1}, *p.o.*) or fluoxetine (3, 10 or 30 mg kg^{-1}, *p.o.*). Mice were allowed to acclimatize to the holding room for 3.5–4 h before the behavioural procedure. Thirty minutes after injection of saline or the test compounds, mice were individually suspended by the tail from a horizontal bar (distance from floor = 30 cm) using adhesive tape (distance from tip of tail = 1 cm). A 6-min test session was employed, which was videotaped. Videotapes were subsequently analyzed. The behavioural parameter recorded was the number of seconds spent in a completely immobile posture, termed immobility.

Because the TST presents some advantages over the FST in allowing an objective measure of immobility and does not induce hypothermia by immersion in water (Ripoll *et al.*, 2003), this model was chosen for the study of the probable mechanism of action of SJE. Two different strategies were employed to deplete catecholamines. To deplete newly synthesized pools of norepinephrine (NE) and dopamine (DA), mice were treated with a single dose of the α-methyldopa (MeDOPA) (400 mg kg^{-1},

i.p.) 3.5 h before behavioural testing. To deplete vesicular pools of NE and DA, mice were treated with a single dose of reserpine (1 mg kg^{-1}, *s.c.*) 24 h before behavioural testing. In an effort to deplete both the vesicular and cytoplasmic pools of NE and DA, mice were pretreated with a combination of reserpine (1 mg kg^{-1}, *s.c.*, 24 h before behavioural testing) and MeDOPA (200 mg kg^{-1}, *i.p.*, 3.5 h before behavioural testing), respectively. All control animals received 0.9% saline on the same schedule as the treated groups.

2.5.2.3 Motor Co-ordination - Rotarod Test.

The effect on motor co-ordination was assessed using rotarod apparatus. The rota rod apparatus (Model 7600, Ugo Basile, Milan, Italy) which rotated at a speed of 12 rpm, consists of a base platform and a rotating rod of 3 cm diameter with a non-skid surface. The rod, 50 cm in length, is divided into five equal sections by six disks. Five mice were tested simultaneously. The mice were placed individually on the cylinder. Before the start of the experiment, animals were trained to stay on the rotarod for 300 s. Mice that failed to learn the test or did not reach the criterion (300-s endurance) were excluded from the study. On the test day, the length of time each mouse remained on the rod ("endurance time," maximal score 300 s) was measured after administration of the test compounds or vehicle.

The animals were acclimatized to the revolving drum and habituated to handling in order to avoid stress during testing. The integrity of motor coordination was assessed as the performance time on the rod measured from the time acceleration started until falling from the drum. The mice were acclimatized to acceleration by three training runs.

2.6 IN VITRO ASSAYS FOR DRUG-METABOLIZING ENZYMES

Multiple-drug therapy is a common therapeutic practice, particularly for patients with various diseases. Whenever two or more drugs are administered concurrently, there is the possibility of drug interactions (Guengerich, 1992b).

Many drug interactions are clinically caused by inhibition of drug-metabolizing enzymes, cytochrome P450, leading to decreased metabolic clearance and increased exposure to the inhibited drug. This study therefore seeks to examine the potential role of SJE in drug-drug interactions.

2.6.1 Total Cytochrome P450

2.6.1.1Animals and Grouping

The animals were divided into four groups of six animals per group. *Group I* served as control and received the vehicle (normal saline), *Group II* was treated with 80 mg kg^{-1} ketoconazole (*i.p.*), *Group III* was treated with 100 mg kg^{-1} phenobarbitone (*i.p.*) and *Group IV* received 300 mg kg^{-1} of the extract (*p.o.*). They were treated for seven days and on the 8th day, they were killed by decapitation.

2.6.1.2 Preparation of tissue homogenates

Rats were killed by decapitation and their livers rapidly excised and immediately placed in ice-cold 0.25 M sucrose to wash off excess blood and to cool the liver. The liver is then blotted dry, weighed and added to four times its weight of 0.25 M sucrose, i.e. a 20% (w/v) homogenate. The liver was finely chopped with scissors and homogenized with Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged in a refrigerated centrifuge to isolate subcellular fractions.

Post-mitochondrial supernatant were prepared by centrifuging the homogenate in a refrigerated centrifuge at 12 500 g for 15 min to pellet intact cells, cell debris, nuclei and mitochondria. The resultant supernatant (the post-mitochondrial supernatant) was carefully decanted. This contains the microsomal plus soluble (cell sap) fractions of the cell.

Microsomal tissue fractions were prepared from the post-mitochondrial supernatant by calcium precipitation of the microsomes at a low g force (Gibson and Skett, 1994). The calcium precipitation method was based on the calcium

dependent aggregation of endoplasmic reticulum fragments and subsequent 'low speed' centrifugation of the aggregated microsomal particles. The advantage of this method is that it is less time-consuming and does not require an ultracentrifuge.

Aliquots (approximately 10-12 ml) of post-mitochondrial supernatant were mixed with 88 mM $CaCl_2$, such that 0.1 ml, 88 mM $CaCl_2$ is added per ml of supernatant (final $CaCl_2$ concentration is 8 mM) and left to stand on ice for 5 min, with occasional gentle swirling. The mixture was then centrifuged at 27 000 g for 15 min, the supernatant discarded and the pellet resuspended by homogenization in 5 ml of 0.1 M Tris buffer, pH 7.4, containing glycerol (20% v/v) yielding the microsomal suspension.

2.6.1.3 Protein determination

The protein concentration was estimated by the method of Lowry *et al.,* (1951) using bovine serum albumin as a standard. The coloured complex is thought to arise as a result of a complex between the alkaline copper-phenol reagent used and tyrosine and tryptophan residues of the protein (copper reagent was prepared freshly by mixing 2% (w/v) Sodium carbonate in 0.1 M NaOH, 1% (w/v) Copper sulfate (hydrated) in water and 2% (w/v) Sodium potassium tartrate in water, in the ratio of 100:1:1 by volume, respectively). The standard procedure used for the protein determination is as follows:

2.6.1.4 Tissue sample

The tissue sample was diluted 1:100 with 0.5 M NaOH. 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the diluted sample was taken into a test tube and made up to a final volume of 1 ml with 0.5 M NaOH. One ml (1.0 ml) of 0.5 M NaOH was used as blank. Five ml (5 ml) of copper reagent was added to all samples (including the blank), mixed thoroughly by vortexing and allowed to stand for 10 min. Half a ml (0.5 ml) of 1 N Folin reagent was added to each sample, mixed immediately and completely and allowed to stand for 30 min. The absorbance was read at 750 nm on a

spectrophotometer, after zeroing the instrument on the blank. The tissue protein content was directly interpolated from the standard curve below.

2.6.1.5 Bovine serum albumin standard curve

A stock solution of bovine serum albumin (100 µg ml^{-1}) in 0.5 M NaOH was prepared. 0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of the above stock solution (equivalent to 0, 20, 40, 60, 80 and 100 µg protein ml^{-1} respectively) was taken into test tube and made up to a final volume of 1 ml with 0.5 M NaOH. One ml (1.0 ml) of 0.5 M NaOH was used as blank. Five ml (5 ml) of copper reagent was added to all samples (including the blank), mixed thoroughly by vortexing and allowed to stand for 10 min. Half a ml (0.5 ml) of 1 N Folin reagent was added to each sample, mixed immediately and completely and allowed to stand for 30 min. The absorbance was read at 750 nm on a spectrophotometer, after zeroing the instrument on the blank. The standard curve was constructed by plotting absorbance against the concentration (µg) of the bovine serum albumin per assay.

2.6.1.6 Spectral determination of cytochrome P450

Cytochrome P450 is a haemoprotein and when the haem iron is reduced and complexed with carbon monoxide, a characteristic absorption spectrum results. The reduced, carbon monoxide difference spectrum of cytochrome P450 absorbs maximally at around 450 nm (hence the name) and the extinction coefficient for the wavelength (450-490 nm) has been accurately determined to be 91 mM^{-1} cm^{-1} thus allowing quantitative determination of this haemoprotein (Omura and Sato, 1964). Because of the turbidity of tissue homogenates containing cytochrome P450, spectrophotometric determination of the haemoprotein was carried out in a split beam instrument. The spectrophotometric assay method used is described below:

Tissue samples were diluted in 0.1 M Tris buffer, pH 7.4 containing 20% (v/v) glycerol to approximately 2 mg ml^{-1}. Two ml (2 ml) of the diluted samples was then added to both matched sample and reference cuvettes and a baseline recorded between 400 and 500 nm. A few grains of solid sodium dithionite were added to

both sample and reference cuvettes with gentle stirring and the sample cuvette only was gently bubbled with carbon monoxide for approximately 1 min. The spectrum was then re-scanned from 400 to 500 nm and cytochrome P450 content was calculated around 450 nm using Beer's Law and assuming a cuvette path length of 1 cm:

$$\boldsymbol{Cyt.P450\ (nmol\ ml^{-1} dil.\ sample)} = \frac{\boldsymbol{Absorbance\ difference\ (nm) \times 1000}}{\boldsymbol{Extinction\ coeffient\ (mM^{-1}\ cm^{-1})}}$$

The specific content of cytochrome P450 in the original tissue sample was then calculated knowing the dilution factor used and the protein content of the original sample as followed:

$$\boldsymbol{Cyt.P450\ (nmol\ mg^{-1}\ protein)} = \frac{\boldsymbol{Cyt.P450\ (nmol\ ml^{-1})\ \times dilution\ factor}}{\boldsymbol{Tissue\ protein\ (mg\ ml^{-1})}}$$

2.6.1.7 Production of Carbon Monoxide

Carbon monoxide was produced by mixing 20 ml concentrated sulfuric acid and 40 ml formic acid in a test tube. The test tube was plugged with a perforated rubber stopper containing a bent glass tube.

2.6.2 Pentobarbitone Sleeping Time

Another set of animals were grouped and treated as described above for cytochrome P450 determination. Thirty (30) min after the last dose each animal was injected with sodium pentobarbitone (40 mg kg^{-1}, *i.p.*). The time which elapsed from the injection to the loss of the rightness reflex (induction time) and the time from the loss of rightness reflex to awakening (duration of sleeping) was registered for each animal.

2.7 EFFECTS OF SJE ON TOXICOLOGICAL PARAMETERS

To measure the toxicological effect of the extract several blood samples were collected at monthly intervals for three months during the study by exactly the

same protocol: in vehicle-treated or SJE-treated (100-1000 mg kg^{-1}, *p.o.*) animals. Male rats were placed in four groups of 18 animals each. *Group A,* the vehicle-treated control, received 10 ml kg^{-1} of saline daily (*p.o.*). *Group B,* C and D was dosed with SJE at 100, 300 and 1000 mg kg^{-1} (*p.o.*) respectively daily. At the end of each month rats (6 per group) were sacrificed and blood samples collected from the jugular veins of the animals. An amount of 1.5 ml of blood was collected in a vial containing 2.5 µg of ethylene diamine tetraacetic acid (EDTA) as an anticoagulant and 3.5 ml of the blood was collected into plain Vacutainer™ tubes without anticoagulant. The blood was centrifuged at 500 g for 15 min and serum was collected and stored at – 20^0C until assayed.

2.7.1 Animal and organ weight

Appearance and overt behaviour were recorded daily, so that any changes in the skin and fur, eyes and mucous membranes, as well as any disturbances on the respiration, circulation, autonomic or central nervous system and behaviour pattern, were observed. Weights of all the animals were recorded weekly and abnormalities in food and water intake were registered. Each organ (heart, lungs, liver, kidney, spleen and testes) were excised quickly, blotted with filter paper and weighed to determine the organ-to-body index (OBI), which was calculated as the ratio of organ weight x 100 and the animal body weight.

2.7.2 Haematological variables

Various haematological parameters including white blood cell count (WBC), lymphocyte count (LYM), mid cell count (MID), granulocyte count (GRAN), red blood cell count (RBC), haemoglobin concentration (HGB), packed cell volume (PCV), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red cell distribution width (RDW), and platelet concentration (PLT) were determined by automated blood analyzer CELL-DYN 1700®, version 1.08, (Abbott Diagnostics, Abbott Park, Illinois, USA).

CELL-DYN hematology autoanalyzer relies primarily on flow cytometry to determine the WBC count and five-part differential count. This technique is based on the fact that the amount of light scattered at different specific angles is characteristic of the different subpopulations of WBCs. A helium neon laser is used as a light source and a series of mirrors, lenses, and slits guide and shape the beam along the light path. When cells pass through the beam of light, the light is scattered. Photo detectors measure the amount of light deflected at specific angles and the data are displayed on scattergrams.

On CELL-DYN analyzers, the MCV is essentially a measured parameter, derived from the average volume of the red blood cells, measured individually. This parameter is an important indicator of the average size of the RBCs in the sample, and thus how much room there is in each cell to transport oxygen. CELL-DYN analyzers also measure the Hgb and RBC. The MCH and MCHC are calculated, and represent the average weight of hemoglobin in each red cell (MCH) and the average concentration or percent of haemoglobin in the RBCs (MCHC). The haematocrit is calculated using the MCV and RBC. Measured parameters are determined by a direct analysis or count, and calculated parameters are determined by a mathematical manipulation of measured parameters or scientific constants (CELL-DYN analyzers manual).

2.7.3 Biochemical assays

Serum biochemistry was performed on the ATAC 8000 Random Access Chemistry System (Elan Diagnostics, Smithfied, RI, USA). Parameters that were determined include: liver function tests - aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (GGT), lactate dehydrogenase (LDH), total bilirubin (T-BIL), direct bilirubin (D-BIL), indirect bilirubin (I-BIL), total-protein (T-PROT), albumin globulin,; renal function tests – serum sodium (Na^+), serum potassium (K^+), blood urea nitrogen (BUN), serum creatinine, serum uric acid, a Anion gap. Also lipid profile which

include total cholesterol (T-CHO), triglycerides (TAG), high density lipoprotein (D-HDL), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and coronary risk were determined. The methods adopted by the automated instrument for the determination of the above parameters are as follows and all reagents are from JAS™ diagnostics, Inc. (JAS Diagnostics, Ine. Miami Florida, USA).

2.7.3.1 Albumin (BCG)

Observations of serum albumin level are useful as an aid in diagnosing disease states of the liver and kidneys. Moderate to large changes in the concentration of albumin have significant effects on the relative amounts of the bound and free concentrations of the ligands it carries. Hypoalbuminemia is very common in many illnesses and results in most instances from one or more of the following factors: 1) impaired synthesis, 2) increased catabolism, 3) reduced absorption of amino acids, 4) altered distribution which may sequester large amounts of albumin in an extravascular compartment, 5) protein loss by way of urine or feces.

Principle and Method

At a controlled pH, bromocresol green (BCG) forms a coloured complex with albumin. The intensity of colour at 630 nm is directly proportional to albumin content. The instantaneous initial absorbance is obtained as suggested by Webster, (1977). The method used by the JAS™ albumin reagent is based on that of Doumas *et al.,* (1971).

$$\boldsymbol{BCG + Albumin \xrightarrow{controlled\ pH} Green\ BCG/Albumin\ Complex}$$

2.7.3.2 Bilirubin

Red blood cells at the end of their circulating life are broken down in the recticuloendothelial system, mainly the spleen. The resulting haem, once the iron is removed, is then converted to bilirubin. Total bilirubin is the sum of the un-conjugated and conjugated fractions. Total bilirubin is elevated in conditions

causing obstruction of the bile duct, hepatitis, cirrhosis, in hemolytic disorders and several inherited enzyme deficiencies. Indirect bilirubin is elevated by pre-hepatic causes such as hemolytic disorders or liver diseases resulting in impaired entry, transport or conjugation within the liver.

Principle and Method

Most methods currently used for assaying bilirubin are based on the reaction between bilirubin and diazotized sulphanilic acid solutions. In aqueous solution only the direct (conjugated) bilirubin will react in this manner. The JAS™ direct bilirubin reagent uses an acid diazo method. Conjugated bilirubin reacts with diazotized sulphanilic acid to produce an acid azobilirubin, the absorbance of which is proportional to the concentration of direct bilirubin in the sample and can be measured at 550 nm.

The JAS™ total bilirubin method is based on a modification of the Pearlman and Lee, (1974) method in which a surfactant is used as a solubilizer. Sodium nitrite is added to sulphanilic acid to form diazotized sulphanilic acid. Bilirubin in the sample reacts with the diazotized sulphanilic acid to produce azobilirubin which absorbs strongly at 550 nm. The absorbance measured at 550 nm is directly proportional to the total Bilirubin concentration in the sample.

2.7.3.3Total Protein (Biuret)

Total protein is useful for monitoring changes in protein levels caused by various disease states. Hyperproteinemia is noted in dehydration, Addison's disease, or diabetic acidosis. Hemodilution is reflected as relative hypoproteinemia which occurs with water intoxication or salt retention syndromes.

Principle and Method

The present method is based on the modification of Gornall *et al.,* (1949). Protein in serum forms a blue coloured complex when reacted with cupric ions in an alkaline solution. The intensity of the violet colour is proportional to the amount of protein present when compared to a solution with known protein concentration.

$$\boldsymbol{Protein + Cu^{2+} \xrightarrow{Alkali} Colored\ Complex}$$

2.7.3.4 γ-glutamyltransferase (γ-GT)

γ-GT is an enzyme present in liver and bile duct which is the most sensitive indicator of hepatobiliary diseases. Due to a high negative predictive value for these diseases the measurement of γ-GT is widely used to rule out a hepatic or biliary origin. Together with other enzymes γ-GT is a valuable tool for the differential diagnosis in liver diseases.

Principle and Method

The JAS™ method is based on the kinetic photometric test, according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (Shaw *et al.,* 1983). γ-GT in the sample catalyzes the transfer of the glutamyl group from L-Gamma-glutamyl-3-carboxyl-4-nitroanalide to glycylglycine according to the reaction below. The amount of 5-amino-2-nitrobenzoate formed is proportional to γ-GT activity and may be measured kinetically at 405 nm by the increasing intensity of the yellow colour formed.

$$\boldsymbol{L-\gamma-glutamyl-3-carboxy-4-nitroanilide + Glycylglycine \xrightarrow{Gamma-GT} L-\gamma-glutamyl-glycylglycine + 5-amino-2-nitrobenzoate}$$

2.7.3.5 Aspartate aminotransferase (AST)

AST is widely distributed with high concentration in the heart, liver, skeletal muscle, kidney and erythrocytes. Damage or disease to any of these tissues such as myocardial infarction, hepatitis, liver necrosis, cirrhosis and muscular dystrophy may result in raised serum levels of AST.

Principle and Method

The present method is based on IFCC recommendations. AST catalyzes the transfer of the amino group from L-aspartate to 2-oxoglutarate to yield oxaloacetate and L-glutamate. The oxaloacetate undergoes reduction with simultaneous oxidation of NADH to NAD^+ in the malate dehydrogenase (MDH) catalyzes reaction. The resulting rate of decrease in absorbance at 340 nm is directly proportional to the AST activity. Lactate dehydrogenase (LDH) is added to prevent interference from endogenous pyruvate which is normally present in serum.

$$\boldsymbol{L-Aspartate + 2-Oxoglutarate \xrightarrow{AST} Oxaloacetate + L-Glutamate}$$

$$\boldsymbol{Oxaloacetate + NADH \xrightarrow{MDH} L-Malate + NAD^+}$$

2.7.3.6 Alanine aminotransferase (ALT)

ALT is widely distributed in tissues with highest concentrations found in the liver and kidney. Even so, ALT is considered more liver-specific than AST. Elevated levels of ALT are often only observed in liver diseases such as cirrhosis, hepatitis, or metastatic carcinoma. However, there can be elevated levels of ALT with infectious mononucleosis, muscular dystrophy, and dermatomyositis.

Principle and Method

The procedure described herein is based on the method of Bergmeyer and Hørder, (1980). Alanine aminotransferase (ALT) catalyzes the transfer of the amino group from L-alanine to 2-oxoglutarate to yield pyruvate and L-glutamate. Lactate

dehydrogenase (LDH) catalyzes the reduction of pyruvate and simultaneous oxidation of NADH to NAD^+. The resulting rate of decrease in absorbance at 340 nm is directly proportional to the ALT activity. Endogenous sample pyruvate is rapidly and completely reduced by LDH during the initial incubation period so that it does not interfere with the assay.

$$L-Alanine + 2-Oxoglutarate \xrightarrow{ALT} Pyruvate + L-Glutamate$$

$$Pyruvate + NADH \xrightarrow{LDH} L-Lactate + NAD^+$$

2.7.3.7 Alkaline phosphatase (Alk. Phos.)

Alkaline phosphatase is a hydrolytic enzyme found in serum in many distinct forms which originate mainly from bone and liver. Pathological increases are largely associated with hepatobiliary and bone diseases. Elevated activities are also observed in infectious hepatitis, bone disease, osteomalacia (rickets), bone metastases and hyperparathyroidism.

Principle and Method

Alkaline phosphatase in serum is determined by measuring the rate of hydrolysis of various phosphate esters under specified conditions. p-Nitrophenyl Phosphate is one such phosphate ester and was introduced as a substrate by Fujita, (1939). Bessey, Lon and Brock published an endpoint procedure (Bessey *et al.*, 1946) while Bowers and McComb, (1966) reported a kinetic procedure. The JAS™ method is based on the kinetic photometric test, according to the IFCC. Absorbance is read at 405 nm.

$$p-Nitrophenylphosphate + H_2O \xrightarrow{Alk.Phos.}$$

$$\boldsymbol{Phosphate + p - Nitrophenol}$$

2.7.3.8 Cholesterol

Principle and Method

The present method utilizes a phenol substitute (4-aminoantipyrine (4-AAP)) that performs like phenol but without being corrosive. The intensity of the red colour produced is directly proportional to the total cholesterol in the sample when read at 500 nm.

$$\boldsymbol{Cholesterol\ Esters \xrightarrow{Cholesterol\ Esterase} Cholesterol + Fatty\ Acids}$$

$$\boldsymbol{Cholesterol + O_2 \xrightarrow{Cholesterol\ oxidase} Cholest-4-en-3-one + H_2O}$$

$$\boldsymbol{2H_2O_2 + HBA + 4AAP \xrightarrow{Peroxidase} Quinoneimine\ (red\ dye) + 4H_2O}$$

2.7.3.9 Triglycerides

Principle and Method

The present method uses a modified Trinder (Barham and Trinder, 1972; Trinder, 1969) colour reaction to produce a fast, linear, endpoint reaction (Fossati and Prencipe, 1982; McGowan *et al.*, 1983). Triglycerides in the sample are hydrolyzed by lipase to glycerol and fatty acids. The glycerol is then phosphorylated by ATP to glycerol-3-phosphate (G3P) and ADP in a reaction catalyzed by glycerol kinase. G3P is then converted to dihydroxyacetone phosphate (DAP) and hydrogen peroxide by glycerophosphate oxidase (GPO). The hydrogen peroxide then reacts with 4-aminoantipyrine (4-AAP) and 3, 5-dichloro-2-hydroxybenzen (3,5-DHBS) in a reaction catalyzed by peroxidase to yield a red coloured quinoneimine dye. The intensity of the colour produced is directly proportional to the concentration of triglycerides in the sample.

$$\boldsymbol{Triglycerides + H_2O \xrightarrow{Lipase} Glycerol + Fatty\ Acids}$$

$$\boldsymbol{Glycerol + ATP \xrightarrow{Glycerol\ kinase} G3P + ADP}$$

$$\boldsymbol{G3P + O_2 \xrightarrow{Glycerolphosphate\ oxidase} DAP + H_2O_2}$$

$$\boldsymbol{H_2O_2 + 4AAP + 3,5 - DHBS \xrightarrow{Peroxidase} Quinoneimine\ (red\ dye) + 2H_2O}$$

2.7.3.10 HDL-Cholesterol

Principle and Method

The method employed herein is in a two reagent format. The first reagent contains anti human β-lipoprotein antibody which bind to lipoproteins (LDL, VLDL and chylomicrones) other than HDL. The second reagent contains enzymes which then selectively react with the cholesterol present in the HDL particles. Consequendy only HDL cholesterol is subject to cholesterol measurement. The primary reading is done at 600 nm and secondary at 700 nm.

2.7.3.11 Urea Nitrogen (BUN)

Determination of urea nitrogen in serum is widely used as a screening test for renal function. When used in conjunction with the determination of creatinine in serum, it is helpful in the differential diagnosis of the three types of azotemia; pre-renal, renal and post-renal.

Principle and Method

The present procedure is based on a modification of the method of Talke and Schubert, (1965). Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. The liberated ammonia reacts with α-ketoglutarate in the presence of NADH to yield glutamate. An equimolar quantity of NADH undergoes oxidation during the reaction catalysed by Glutamate dehydrogenase (GLDH) resulting in a decrease in absorbance (340 nm) that is directly proportional to the urea nitrogen concentration in the sample.

$$Urea + H_2O \xrightarrow{Urease} 2NH_3 + CO_2$$

$$NH_3 + \alpha - Ketoglutarate + NADH \xrightarrow{GLDH} L - Glutamate + NAD^+$$

2.7.3.12 Creatinine

Creatinine measurements are used in the assessement of renal dysfunction. Elevated creatinine levels are found in renal diseases and insufficiency with decreased glomerular filtration (uremia or azotemia if severe); urinary tract obstruction; reduced renal blood flow including congestive heart failure, shock and dehydration.

Principle and Method

This method is based on a modification of the kinetic procedure which is fast, simple and avoids interferences (Fabiny and Ertingshausen, 1971), incorporating a surfactant and other ingredients to minimize protein and carbohydrate interferences. Creatinine reacts with picric acid in alkaline conditions to form a colour complex (yellow-orange) which absorbs at 510 nm. The rate of formation of colour is proportional to the creatinine in the sample.

$$Creatinine + Sodium\ Picrate \xrightarrow{Alkali} Creatinine - picrate\ complex$$

2.7.3.13 Uric Acid

Uric Acid measurements are most commonly used in the diagnosis of gout. Increased levels (hyperuricaemia) may be observed in leukemia, polycythaemia, atherosclerosis, diabetes, hypothyroidism, and conditions associated with decreased renal function.

Principle and Method

The JAS™ procedure uses uricase, peroxidase and the chromogen TBHB to yield a colorimetric end product. Uric acid is oxidized by Uricase to allantoin and

hydrogen peroxide. TBHB + 4-aminoantipyrine + hydrogen peroxide, in the presence of peroxidase, produce a quinoneimine dye that is measured at 520 nm. The colour intensity at 520 nm is proportional to the concentration of Uric Acid in the sample.

$$\boldsymbol{Uric\ Acid + O_2 + H_2O \quad \overrightarrow{Uricase} \quad Allantoin + CO_2 + H_2O_2}$$

$$\boldsymbol{H_2O_2 + 4-Aminoantipyrine + TBHB \ \overrightarrow{Peroxidase} \ Quinoneimine + H_2O}$$

2.7.4 Histopathological examination

Portions of the tissue from liver, kidney, and testes were used for histopathological examinations. Tissues were fixed in 10% buffered formalin (pH 7.2) and dehydrated through a series of ethanol solutions, embedded in paraffin, and routinely processed for histological analysis. Sections of 2 μm thickness were cut and stained with haematoxylin-eosin for examination. The stained tissues were observed through an Olympus microscope (BX-51) and photographed by a chare-couple device (CCD) camera.

2.8 GENETIC TOXICOLOGY - MUTAGENISIS

2.8.1 The principle of Salmonella (Ames) Test for Mutagenicity

The *Salmonella typhimurium*/mammalian microsome assay (Ames test) is a generally accepted short-term test for detecting chemicals that induce mutations in the DNA of organisms. This is a reverse mutation assay that employs histidine-dependent *Salmonella* bacteria with mutations at various genes in their histidine operon that render them incapable of synthesizing the amino acid histidine. When these histidine-dependent cells are grown on minimal medium agar plates containing a trace of histidine, only those cells that revert (mutate) to histidine independence (his^+) are able to form colonies. The small amount of histidine allows all the plated bacteria (~ 1×10^8 cells) to undergo a few cell divisions; in many

cases, this growth is essential for mutagenesis to occur. The his^+ mutants are easily scored as colonies against the slight background growth of the his-dependent bacteria (bacterial lawn). The number of spontaneous mutant colonies per plate is relatively constant. However, when a mutagen is added to the plate, the number of revertant colonies is increased, usually in a dose-related manner.

2.8.2 Ames mutagenicity test

Ames mutagenicity test was performed according to the method of Maron and Ames, (1983). The Salmonella typhimurium TA_{97}, TA_{98}, TA_{100} and TA_{102} tester strains were used in the Ames reversion test. Assays were carried out on samples of fractionated liver homogenate, namely, whole homogenate, S9 fraction, nucleosomes, microsomes and the cytosolic fraction. All samples were assayed according to the standard plate incorporation test and challenged with S9-Mix (Maron and Ames, 1983). Dimethyl sulfoxide was used as a negative control whilst 5.0 µg/plate daunomycin (daunorubicin hydrochloride), 1.5 µg/plate of sodium azide were used as positive controls for TA_{102}, TA_{98} and TA_{100}, respectively and 3.0 µg/plate of sodium azide were used as positive controls for TA_{97}.

2.9 STATISTICAL ANALYSIS

The sexual behavioural data are presented as mean (± SEM). Data were analyzed using two-way repeated measures analysis of variance (ANOVA) with two between-subject factors *(time × drug treatment)* followed by Bonferroni's test. To further compare differences between groups, one-way ANOVA was performed with Tukey's test for selected pairs as *post hoc*.

The data obtained from CNS work were presented as mean (± SEM). To compare differences between groups, one way analysis of variance (ANOVA) followed by Newman-Keul's test as *post hoc*. Also, the behavioural data from light/dark box, open field and elevated plus maze were analyzed using two-way analysis of

variance (ANOVA) with groups as a between-subject factor and compartment as a within-subject factor followed by Benferroni's as *post hoc*.

To compute total distances travelled by the mice in the anxiety model, the software, Behaviour Collect was used to obtain raw XY data from the videos (http://cas.bellarmine.edu/tietjen/DownLoads/computer_programs_for_data_colle.htm). These data were then exported into Microsoft® Office Excel 2007 and further analyzed. Distance between two X-Y coordinate pairs was calculated from the formula:

$$\sqrt{[(X_1 - X_2)^2 + (Y_1 - Y_2)^2}$$

ED_{50} (dose responsible for 50% of the maximal effect) for each drug in the FST and TST was determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation

$$Y = \frac{a + (b - a)}{(1 + 10^{(Log\ ED_{50} - X)})}$$

Where, X is the logarithm of dose and Y is the response. Y starts at *a* (the bottom) and goes to *b* (the top) with a sigmoid shape.

The toxicological data were presented as mean ± SEM. The presence of significant differences among means of the groups was determined by one-way ANOVA using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.grphpad.com. Significant differences between pairs of groups were calculated using the Newman-Keuls Multiple Comparison Test. However, paired t-test was used for the analysis of total cytochrome P450 and the sleeping time.

In all statistical test, a value of $p<0.05$ was considered significant. All graphs were plotted using SigmaPlot for Windows, Version 10.0, Build 10.0.0.54, www.systat.com

Chapter 3

RESULTS

3.1 PHYTOCHEMICAL ANALYSIS

The phytochemical analysis of SJE showed it contains terpenoids, flavonoids and alkaloids compound. Alkaloid is the most dominant chemical constituents (Table 3.1).

Table 3.1 Chemical constituent of ethanolic extract of roots of *S. jollyanum*

TESTS	RESULTS
Tannins	
ferric chloride test	–
Lead acetate test	–
Phlobatannin's test	–
Flavonoids	
Lead acetate test	++
ferric chloride test	++
Sodium chloride test	++
Alkaloids	
Draggendoff's test	+++
mayer's test	+++
Wagner's test	+++
Saponins	
Benedict's test	–
Emulsion test	–
Frothing test	–
Steroids	
Lieberman's test	–
Terpenoids test	++
Glycosides general test	–

–: Not detected, +: Present in low concentration, ++: Present in moderate concentration, +++: Present in high concentration.

3.2 EFFECT OF SJE ON SEXUAL BEHAVIOUR TEST

3.2.1 Male sexual behaviours in mounting test

Figure 3.1 shows the acute effect of the extract on sexual behaviours in male mice. Compared with vehicle-treated males, SJE decreased significantly the latency to mount by 37.31 and 41.28 % at 100 mg kg^{-1} and by 58.2 and 16.23% at 300 mg kg^{-1} one hour and three hours respectively after treatment (Figure 1a). Two-way ANOVA revealed that time had no effect on the latency ($F_{1,40}$=0.57; P=0.46).

The frequency of each behavioural parameter after SJE treatment showed bell-shaped dose-effect curve. Only the 100 mg kg^{-1} SJE significantly increased the frequency of attempted mounting and genital sniffing in the 1st hour (P<0.05 for both parameters). In contrast, the 300 mg kg^{-1} treatment produced a higher frequency of mounts and penile licking than the lower dose.

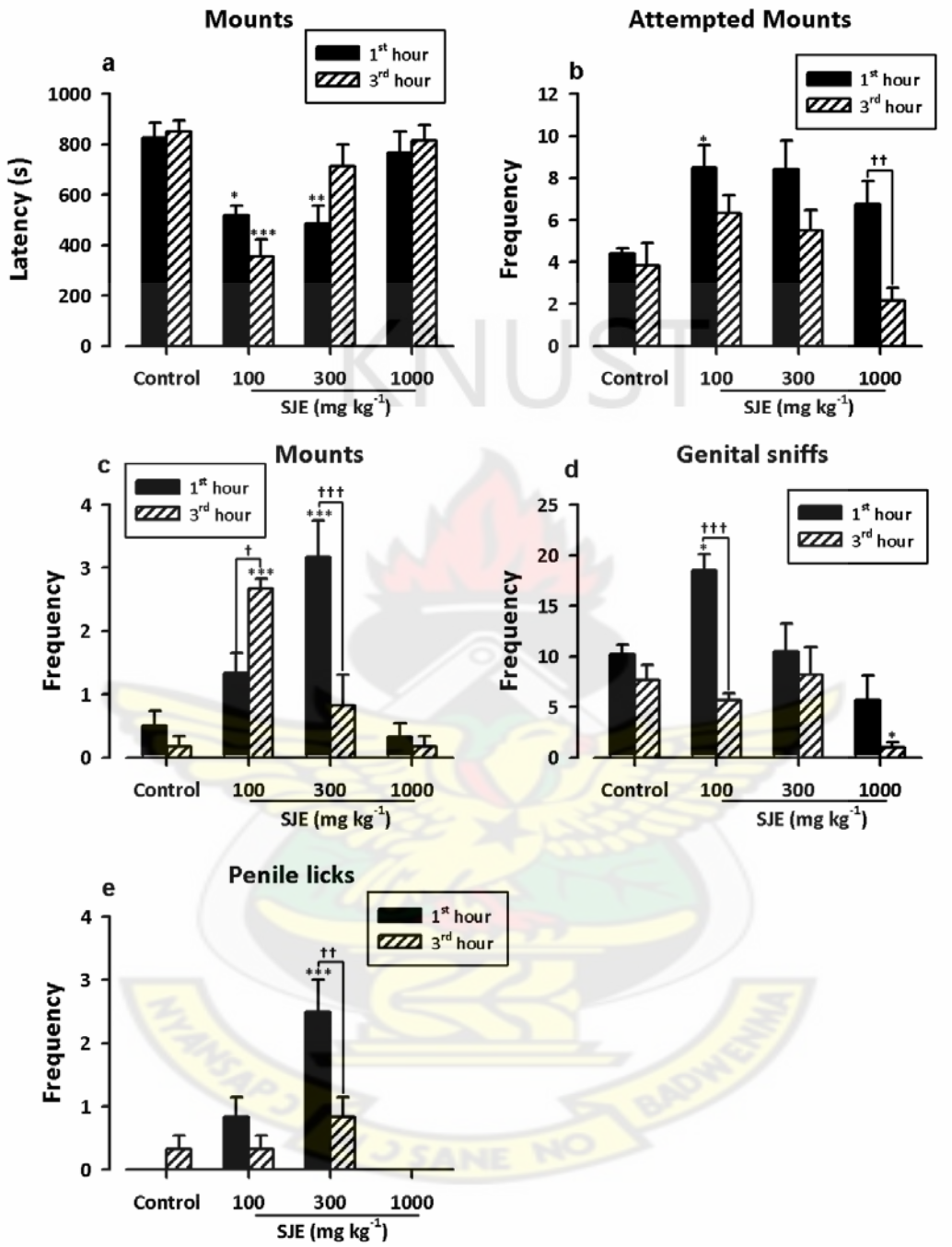

Figure 3.1 Effect of *SJE* on sexual behavioural parameters (mounting behaviour tests) in male mice. Results are presented as means ± SEM. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls *post hoc*); †*P* ≤ 0.05, ††*P* ≤ 0.01, †††*P* ≤ 0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*).

3.2.2 Male sexual behaviours in mating tests

In these experiments females were rendered receptive by pretreatment with an estrogen-progestogen combination. Figure 3.2 shows the acute effect of SJE on mounting, and intromission. Compared with the vehicle-treated group, SJE (100, 300, 1000 mg kg^{-1}; *p.o.*) dose-dependently decreased the latency of mounting (12.20%, 56.26% and 55.33% respectively), and intromission (26.00%, 33.99% and 38.55% respectively) in a dose-dependent manner. In addition, SJE significantly increased the incidence of mounting (144.43%, 266.67% and 300% respectively) and intromission (100%, 158.84% and 200.04% respectively) in a dose-dependently. Latency to ejaculate latency in both the first series (10.23%, 159.77% and 60.88%) and second series (5.38%, 110.33% and 52.80% respectively) of mating was also dose-dependently increased (Figure 3.3). Two-way ANOVA revealed a significant difference in the ejaculation latency for the 1st and 2nd series: the latencies were comparatively greater in the 2nd series ($F_{1,40}$=27.58; P<0.001). There was a significant decrease in the post-ejaculatory interval by 33.16%, 60.36% and 56.54% for the 100, 300 and 1000 mg kg^{-1} groups respectively, compared to vehicle-treated groups (Figure 3.3)

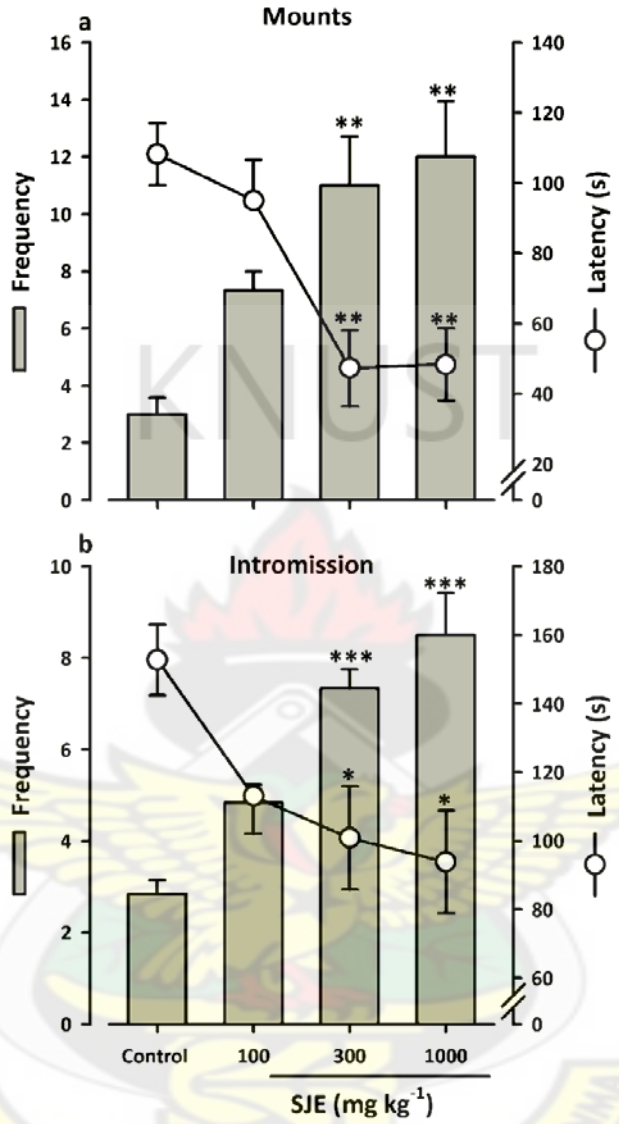

Figure 3.2 Effects of SJE on the latencies and frequencies to mount and intromit in the mating experiments. Results are presented as means ± SEM. ****P* **≤ 0.05, *****P* **≤ 0.01, ******P* **≤ 0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls *post hoc*).

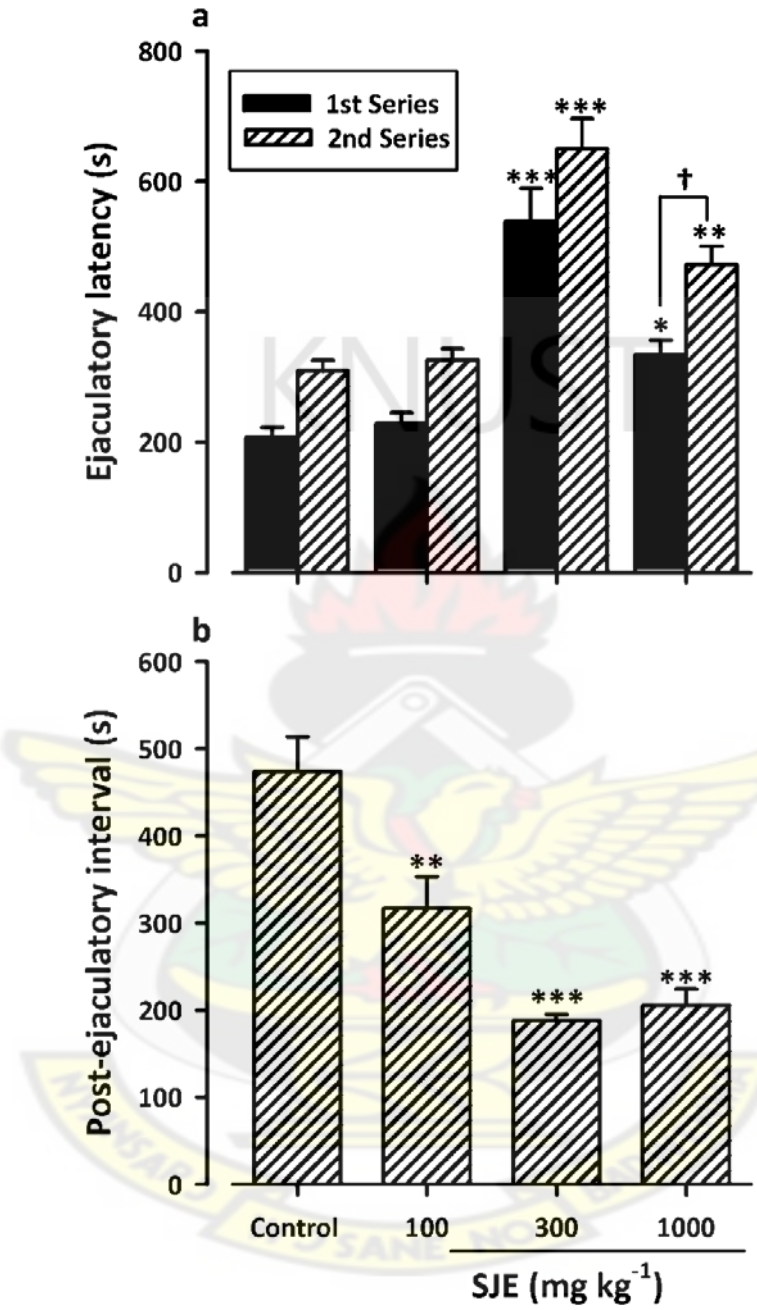

Figure 3.3 Effects of SJE on ejaculatory latency and post-ejaculatory latency in male mice. Bars represent means ± SEM. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (one-way ANOVA followed by Newman-Keuls *post hoc*); †$P \leq 0.05$ (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*).

3.3 EFFECTS OF SJE ON HORMONAL LEVELS

SJE treatment had no effect on prolactin levels over the treatment period (Figure 3.4). However, FSH levels were significantly increased by the 2nd week of treatment in a dose-dependent manner ($P < 0.0001$). Though luteinizing hormones were decreased in all the test groups, the level of testosterone was greatly increased by the 3rd week of treatment ($P < 0.0001$).

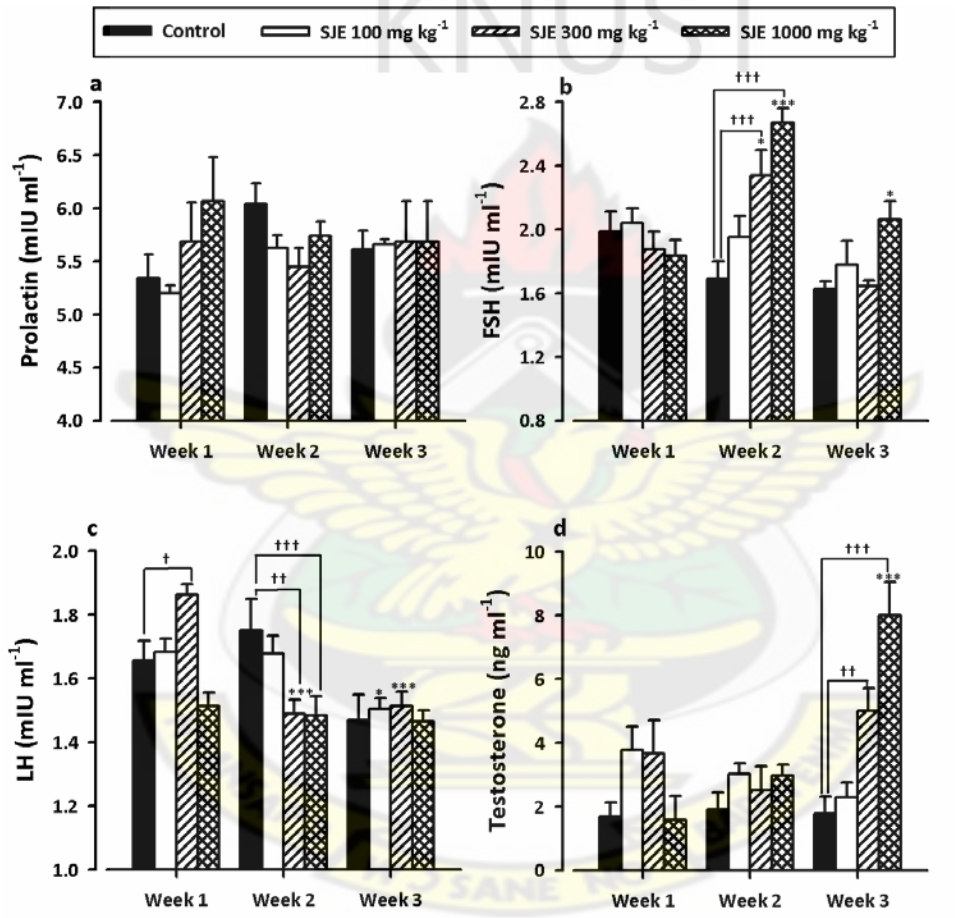

Figure 3.4 Effect of SJE on reproductive hormones in male Sprague-Dawley rats. Results are presented as means ± SEM. *$P \leq 0.05$, ***$P \leq 0.001$ compared to respective controls (one-way ANOVA followed by Newman-Keuls *post hoc*); †$P \leq 0.05$ ††$P \leq 0.01$ †††$P \leq 0.001$ (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*).

3.4 *IN VITRO* EXPERIMENT ON ISOLATED TISSUE

3.4.1 Effect of SJE on isolated rat anococcygeus

SJE, phentolamine (PHT) and sodium nitroprusside (SNP) dose-dependently relaxed isolated anococcygeus muscle pre-contracted with K^+ (Figure 3.5). The relaxation were expressed as percentage of total relaxation (loss in tone) induced by the addition of 1.0 mM papaverine HCl to the bath at the end of the experiment. EC_{50} and E_{max} values for SJE are 6.31 ± 3.07 mg ml^{-1} and 79.84 ± 3.67 % respectively, PHT are 0.97 ± 2.75 µg ml^{-1} and 87.38 ± 2.66 % respectively, and SNP are 0.98 ± 2.82 µg ml^{-1} and 88.79 ± 1.47 % respectively.

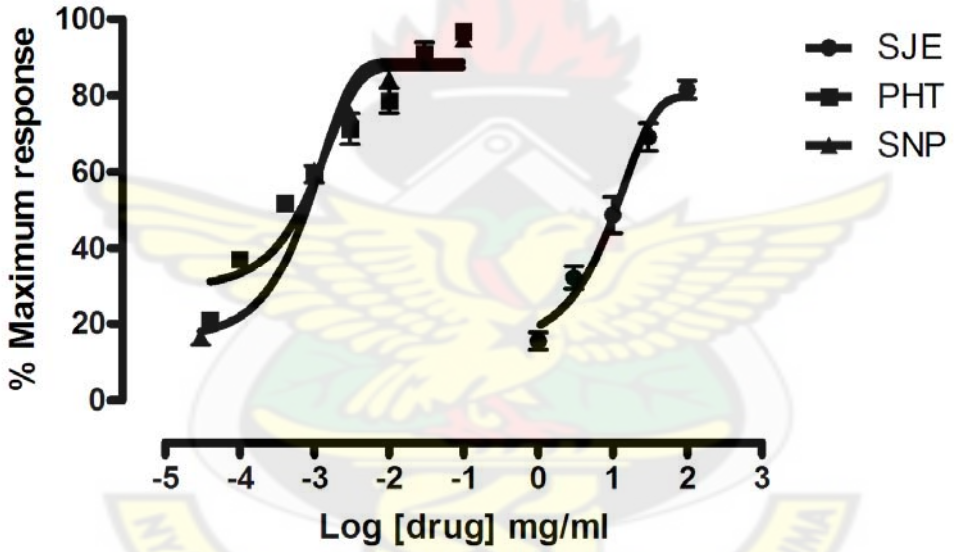

Figure 3.5 Effect of SJE, phentolamine and sodium nitroprusside on (potassium pre-contracted) isolated rat anococcygeus muscle. Data are presented as mean ± SEM. Responses are expressed as percentages of maximum relaxation induced by papaverine (1.0 mM).

Pre-treatment of the tissue with atropine (0.1 µg ml^{-1}) caused rightward parallel shift of the carbachol-induced contractions while increasing concentrations of SJE (0.1, 1.0 mg ml^{-1}) caused rightward shift with depression of maximal response (Figure 3.6). EC_{50} and E_{max} values are shown in Table 3.2.

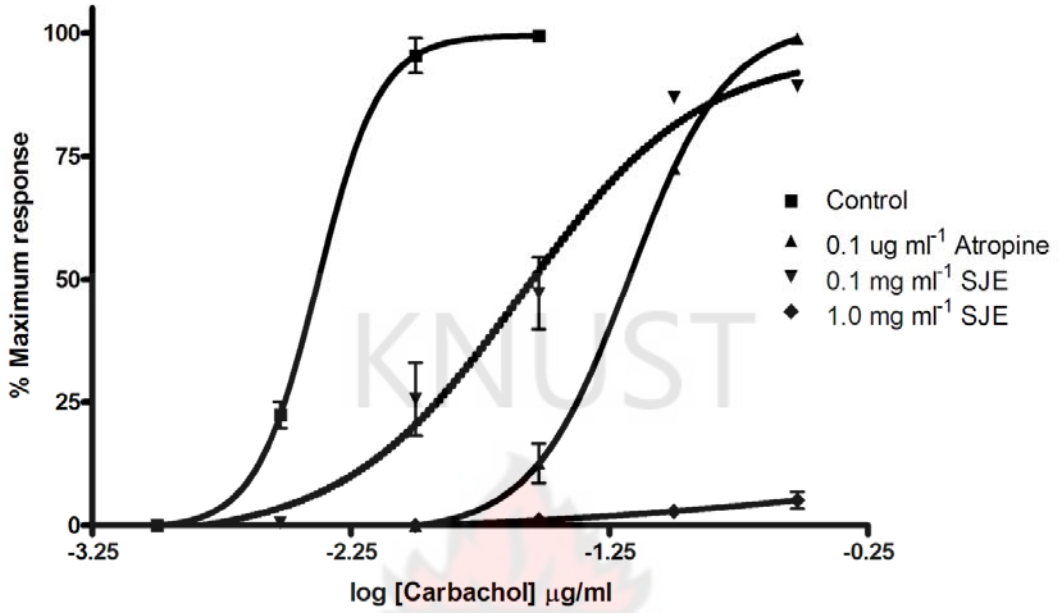

Figure 3.6 Effect of atropine and SJE on concentration-response curves of carbachol on isolated rat anococcygeus muscle. Responses are expressed as percentages of maximum control response.

Table 3.2 E_{max} and EC_{50} values for effect of SJE and atropine on carbachol induced contractions

	E_{max} (%)	EC_{50} (mg ml^{-1})
Control	99.51±3.07	0.0041±0.03
0.1 µg/ml Atropine	102.00±2.75	0.0672±0.02
0.1 mg/ml SJE	96.13±8.03	0.0251±0.09
1.0 mg/ml SJE	16.35±2.80	0.8031±16.99

Pre-treatment of the tissue with prazosin (0.1 and 1.0 µg ml^{-1}) caused a rightward shift of phenylephrine-induced contractions (Figures 3.7) while increasing doses of SJE (0.1, 1.0 mg ml^{-1}) caused a rightward shift and depression of maximal response

of the repeated curves (Figures 3.8). EC_{50} and E_{max} values are shown in Table 3.3 and 3.4.

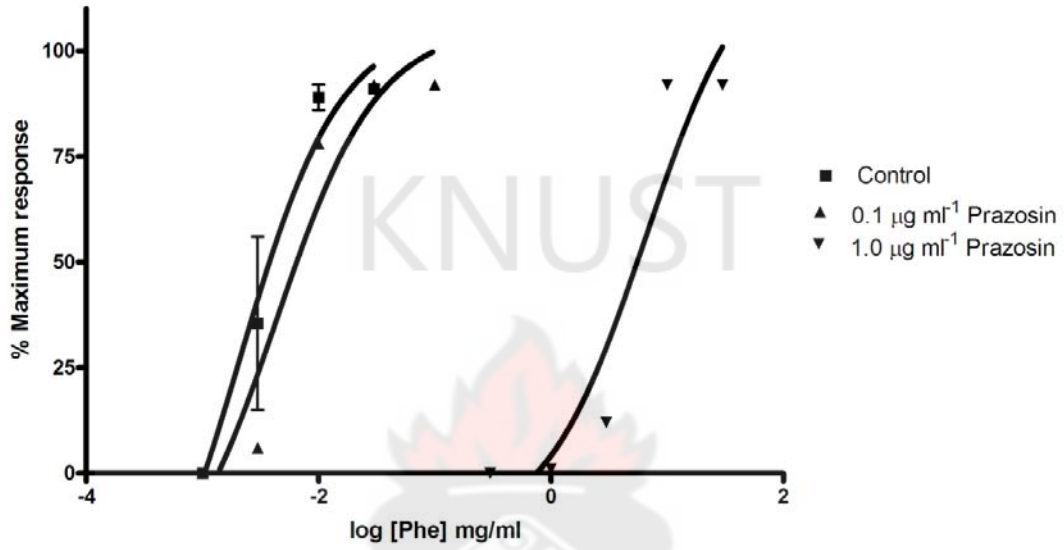

Figure 3.7 Effect of prazosin on concentration-response curves of phenylephrine on isolated rat anococcygeus muscle. Responses are expressed as percentages of maximum control response.

Table 3.3 EC_{50} and E_{max} values effect of prazosin on phenylephrine induced contractions.

	E_{max} (%)	EC_{50} (μg ml^{-1})
Control	106.90±14.82	0.0021±0.37
0.1 μg ml^{-1}Prazosin	105.70±18.63	0.0043±0.43
1.0 μg ml^{-1}Prazosin	126.00±46.16	6.5170±0.51

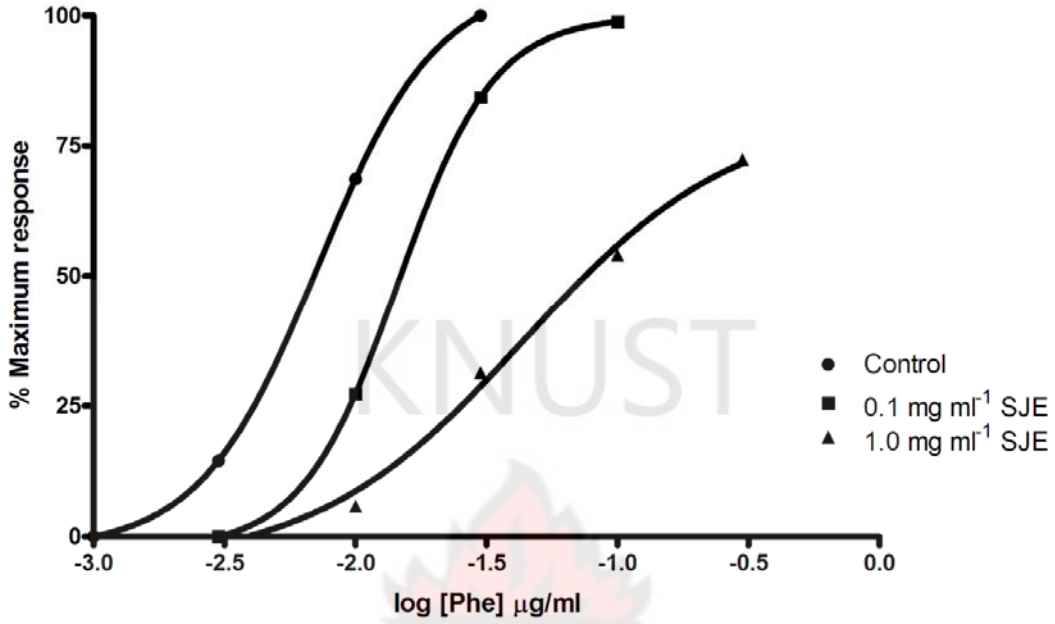

Figure 3.8 Effect of SJE on concentration-response curves of phenylephrine on isolated rat anococcygeus muscle. Responses are expressed as percentages of maximum control response.

Table 3.4 EC_{50} and E_{max} values for effect of SJE on phenylephrine induced contractions.

	E_{max} (%)	EC_{50} (mg ml^{-1})
Control	106.90±14.82	0.0020±0.37
0.1 mg ml^{-1} SJE	99.88±2.69	0.0145±0.02
1.0 mg ml^{-1}SJE	81.31±7.24	0.0443±0.07

Pre-treatment of the isolated anococcygeus muscle with 0.1, and 1.0 mg ml^{-1} of SJE caused a dose-dependent inhibition of maximal response obtained with calcium (Figure 3.9). EC_{50} and E_{max} values are shown in Table 3.5.

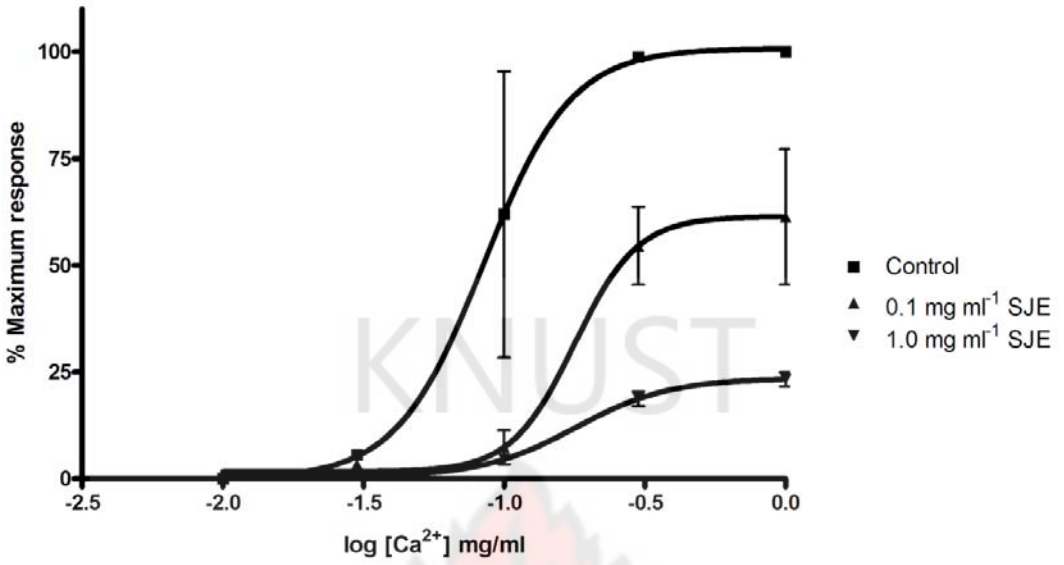

Figure 3.9 Effect of SJE on concentration-response curves of calcium on isolated rat anococcygeus muscle. Responses are expressed as percentages of maximum control response.

Table 3.5 EC_{50} and E_{max} values for concentration-response curves for calcium in the presence of SJE on isolated anococcygeus muscle preparation.

	E_{max} (%)	EC_{50} (mg ml^{-1})
Control	100.80±28.11	0.0847±0.27
0.1 mg ml^{-1}SJE	61.44±9.62	0.1778±0.18
1.0 mg ml^{-1}SJE	23.57±1.54	0.1796±0.06

3.4.2 Effect of SJE on rabbit corpus cavernosum

In another set of experiments, SJE, phentolamine (PHT) and sodium nitroprusside (SNP) dose-dependently relax phenylephrine-precontracted corpus cavernosum smooth muscle (CCSM) (Figure 3.10). The relaxation responses were expressed as percentage of total relaxation (loss in tone) induced by the addition of 1.0 mM papaverine HCl to the chambers at the end of the experiment. EC_{50} and E_{max} values

for SJE are 10.31 ± 3.07 mg ml^{-1} and 92.90 ± 1.90 % respectively, PHT are 1.75 ± 0.11 μg ml^{-1} and 76.23 ± 4.58 % respectively, and SNP are 1.10 ± 0.02 μg ml^{-1} and 87.51 ± 8.06 % respectively.

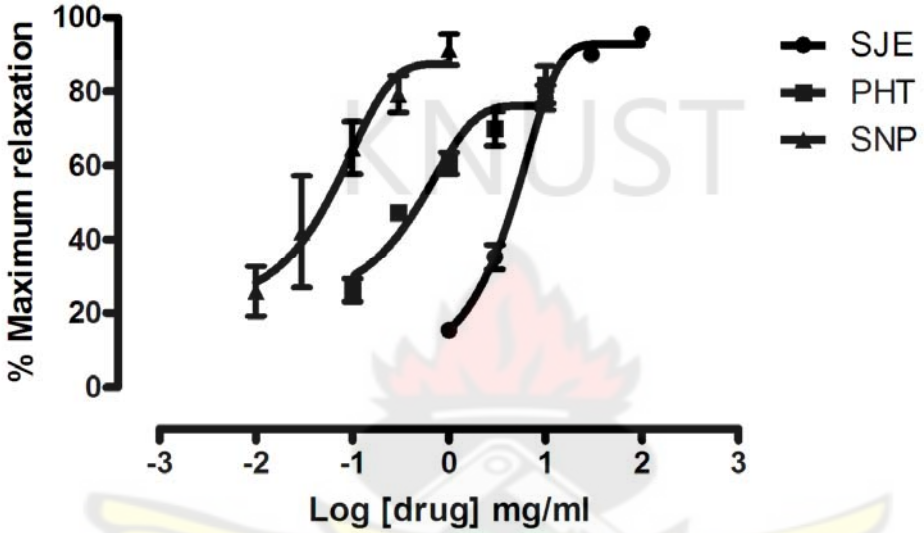

Figure 3.10 Effect of SJE, phentolamine and sodium nitroprusside on phenylephrine pre-contracted isolated rabbit corpus cavernosum smooth muscle. Responses are expressed as percentages of maximum relaxation induced by papaverine.

3.5 CENTRAL NERVOUS SYSTEM EFFECT OF SJE

3.5.1 Spontaneous locomotor activity

Spontaneous locomotor activity of the caffeine and SJE treated mice were significantly decreased as compared to the control animal in a dose dependent manner ($F_{3,27}$ = 27.96; P<0.0001 and $F_{3,32}$ = 14.26; P<0.0001 respectively). Though spontaneous locomotor activity of mice treated with diazepam was lower than the control group, there was gradual increase in activity as the dose increased ($F_{3,32}$ = 13.05; P<0.0001) (Figure 3.11).

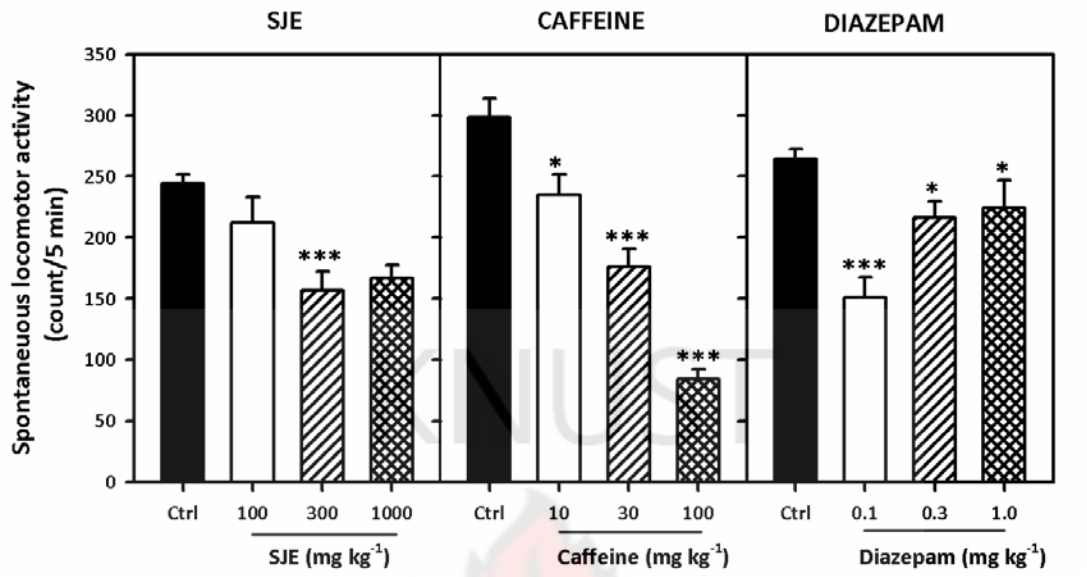

Figure 3.11 Effects of acute SJE (100, 300 and 1000 mg kg^{-1}), caffeine (10, 30 and 100.0 mg kg^{-1}) and diazepam (0.1, 0.3 and 1.0 mg kg^{-1}), all 30 min pretest on spontaneous locomotor activity in the activity cage. Data are presented as group means (±SEM). Significantly different from control (*Ctrl): *P<0.05, ***P<0.001 by Newman-Keuls test.

3.5.2 Elevated plus-maze test

In the elevated plus-maze test, acute administration of caffeine (10-30-100 mg kg^{-1} *i.p.*) significantly decreased [$F_{3,32}$ = 5.849; P = 0.0026] the percentage time spent by the mice in the open arms in a dose related manner and the percentage entry into the open arms [$F_{3,32}$ = 4.280, P = 0.0120] with respect to total arms entry. These are indication of an anxiogenic effect in the EPM test (Figure 3.12). In addition, caffeine induced a significant reduction in the number of head dips, rearing, nose poke and stretch attends posture made during the 5 min periods (Figure 3.13). Besides that, total distance travelled was significantly reduced [$F_{3,30}$ = 24.39, P<0.0001] (Figure 3.14) and the time spent in the closed arm was also significantly increased (Figure 3.12). These (distance travelled and duration in closed arm) are indicators of decreased locomotor activity. The results of the other parameters measured are listed in Table 3.6.

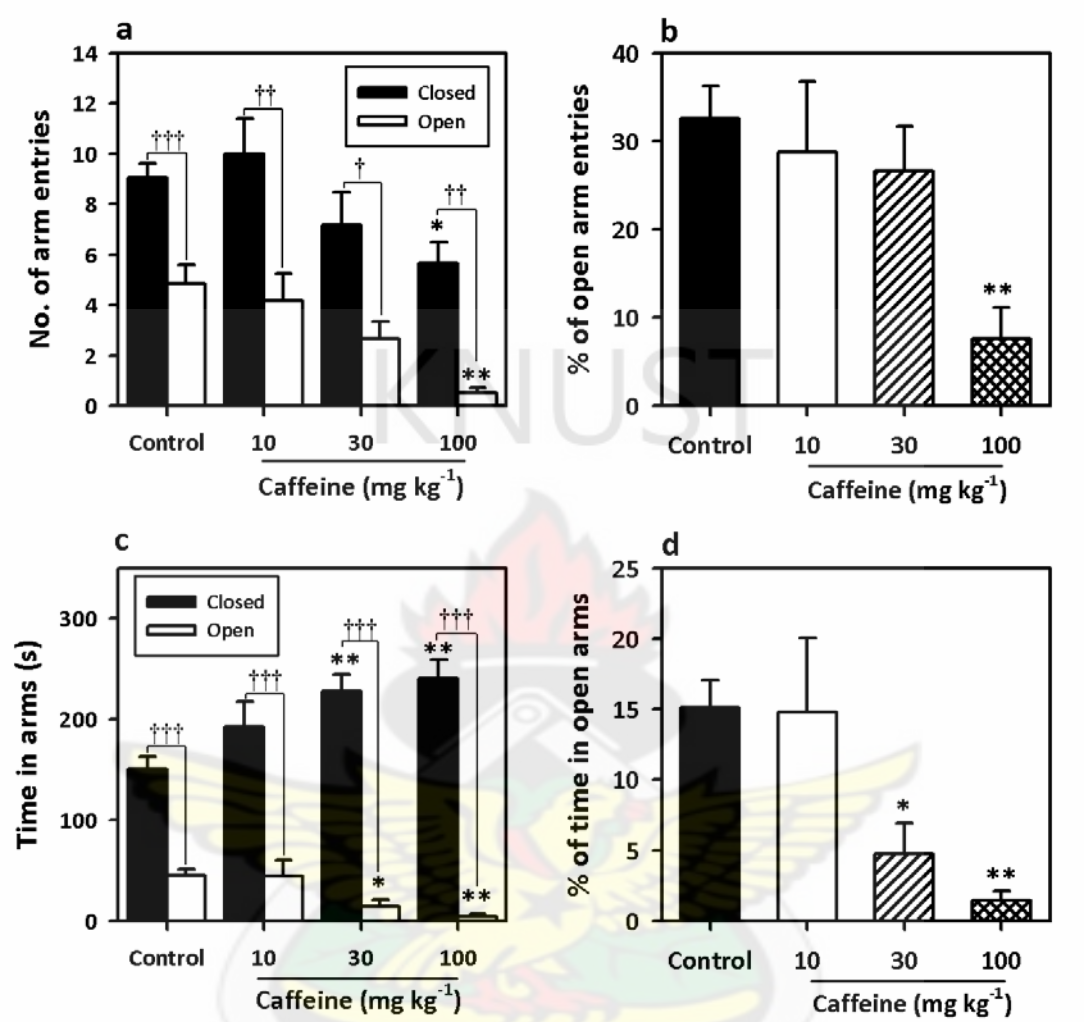

Figure 3.12 Effects of acute caffeine (10, 30 and 100 mg kg^{-1}) 30 min pretest on number of arm entries (a), % entries open arm (b), time in arms (c) and % time open arm (d) in the EPM. Data are presented as group means (±SEM). Significantly different from control (*Ctrl*): **P*<0.05, ***P*<0.01 by Newman-Keuls test and significant difference when open arm and closed arm where compared: †*P*<0.05, ††*P*<0.01, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*).

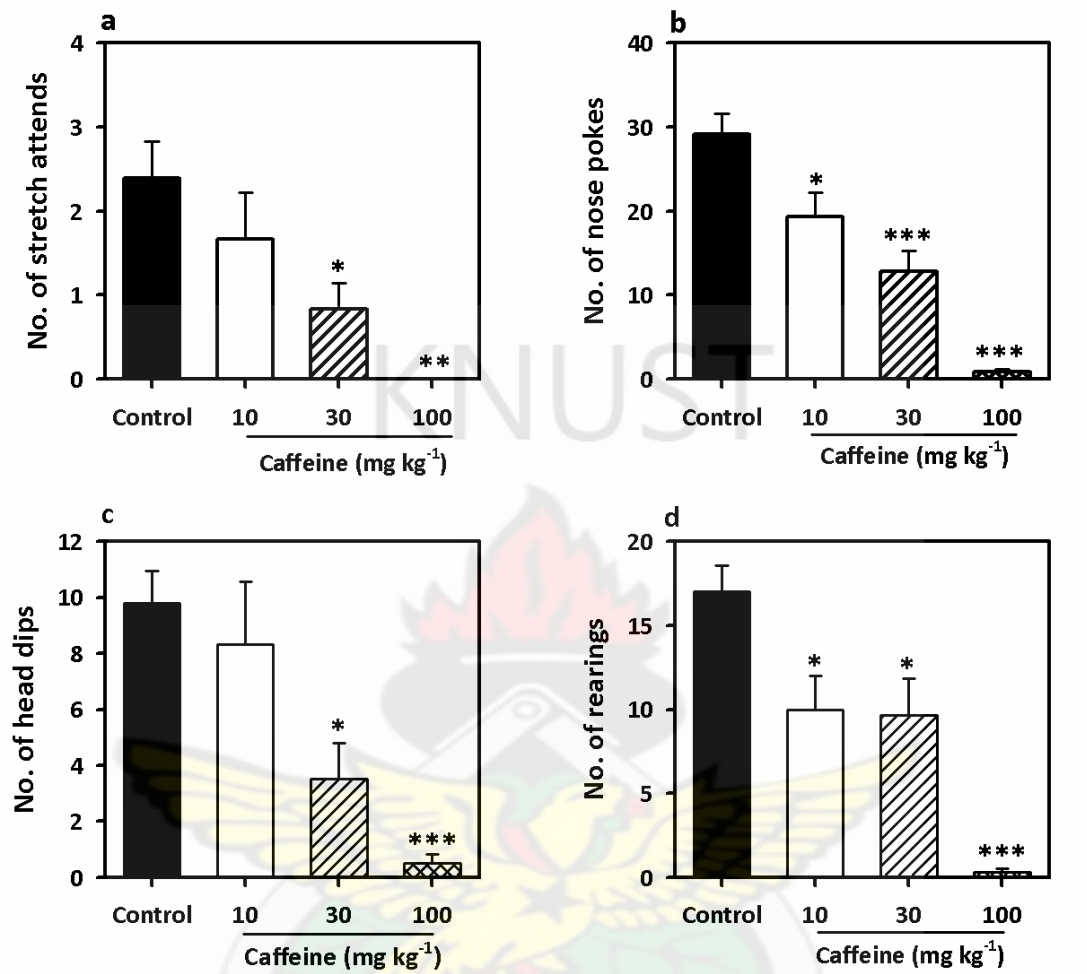

Figure 3.13 Effects of acute caffeine (10, 30 and 100 mg kg^{-1}) 30 min pretest on number of stretch attend (a), number of nose poke (a), number of head dips (c) and number of rearing (d) in the EPM. Data are presented as group means (±SEM). Significantly different from control (*Ctrl*): **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test.

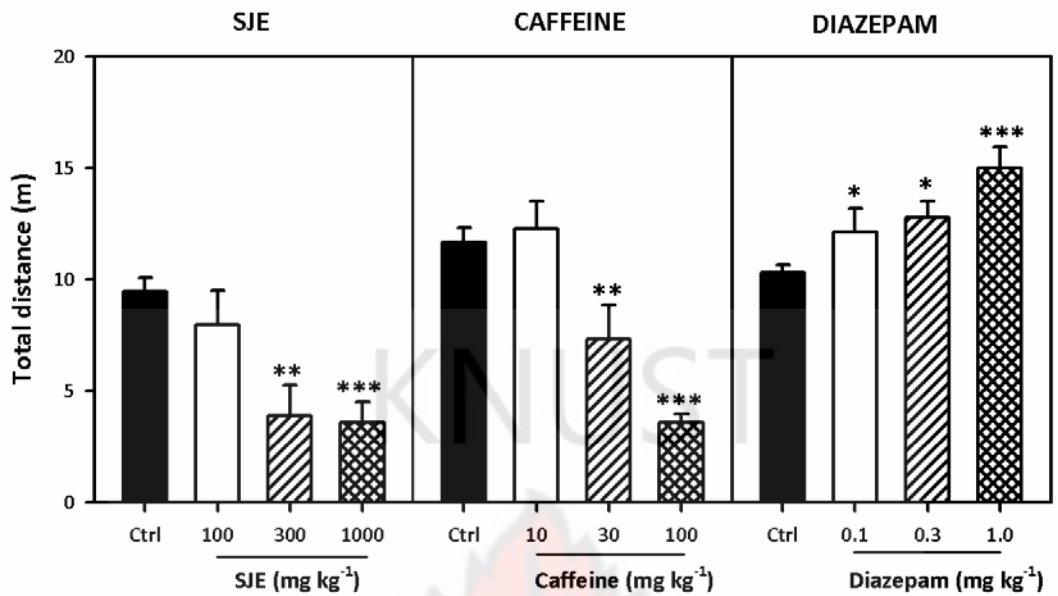

Figure 3.14 Effects of acute SJE (100, 300 and 1000 mg kg^{-1}), caffeine (10, 30 and 100.0 mg kg^{-1}) and diazepam (0.1, 0.3 and 1.0 mg kg^{-1}), all 30 min pretest on locomotion in the EPM. Data are presented as group means (±SEM). Significantly different from control (*Ctrl*): **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test.

Table 3.6 Effect of acute caffeine on other parameters of elevated plus maze.

	CONTROL	CAFFEINE (mg kg^{-1})		
PARAMETERS	**NS**	**10**	**30**	**100**
Latency for movement (s)	4.33±1.14	4.33±1.67	11.67±5.02*	4.00±1.86
No. center entry	8.56±0.77	9.33±2.20	6.00±1.37	3.17±0.79**
Duration in center (s)	94.06±9.75	72.67±23.25	65.00±23.48	61.83±24.04
Duration of head-dipping (s)	13.44±1.78	14.33±4.73	7.83±3.24	1.00±0.68***
Freezing	1.72±0.34	3.17±0.54*	2.67±0.67	14.83±1.49***
Duration of freezing (s)	10.50±4.28	15.17±2.48	43.17±22.11*	227.80±12.28***
Duration of stretch (s)	3.16±0.64	3.00±1.46	2.00±1.61	0.17±0.17*
Grooming	0.72±0.14	0.83±0.17	1.50±0.76	0.50±0.50
Duration of grooming (s)	11.83±3.23	16.33±9.04	11.67±7.85	1.33±1.33
Fecal boli	1.17±0.25	1.00±0.45	1.00±0.52	0.17±0.17*

Data are presented as group means (±SEM). NS = normal saline. Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 by Newman-Keuls test.

SJE (100-300-1000 mg kg^{-1} *p.o.*) also significantly decreased [$F_{3,38}$ = 5.831, P = 0.0022] the percentage time spent by the mice in the open arms in a dose-related manner and the percentage entry into the open arms [$F_{3,32}$ = 5.638, P = 0.0027] with respect to total arms entry. These indicate an anxiogenic effect in the EPM test (Figure 3.15). The number of head dips, rearing, nose poke and stretch attends posture were also significantly reduced (Figure 3.16). SJE also significantly reduced [$F_{3,31}$ = 10.30, P<0.0001] the total distance covered (Figure 3.14) and the duration in the closed arm (Figure 3.15) was increased in confirmation of the decreased in locomotor activity of SJE in EPM. The results of the other parameters measured are listed in Table 3.7.

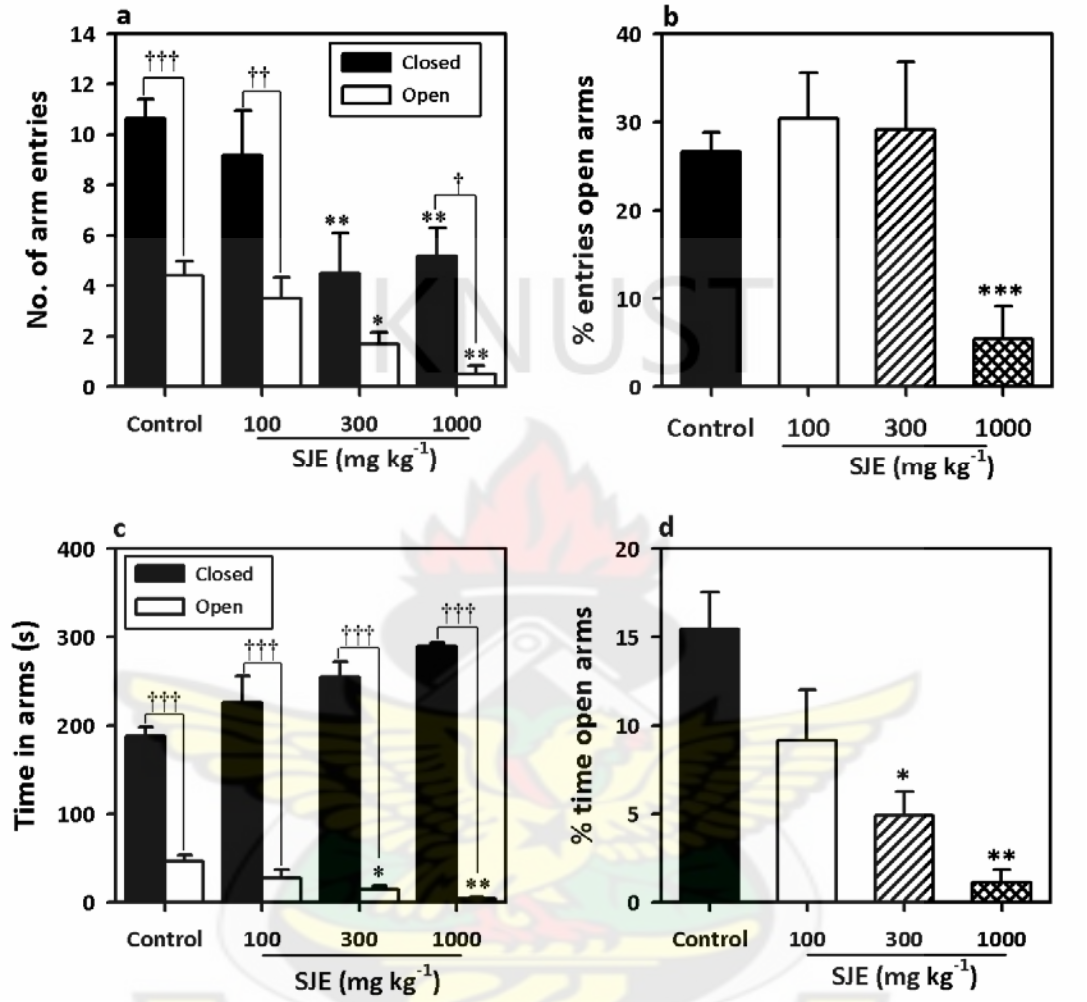

Figure 3.15 Effects of acute SJE (100, 300 and 1000 mg kg^{-1}) 30 min pretest on number of arm entries (a), % entries open arm (b), time in arms (c) and % time open arm (d) in the EPM. Data are presented as group means (±SEM).*Ctrl* control. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test and significant difference when open arm and closed arm where compared: †*P*<0.05, ††*P*<0.01, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*).

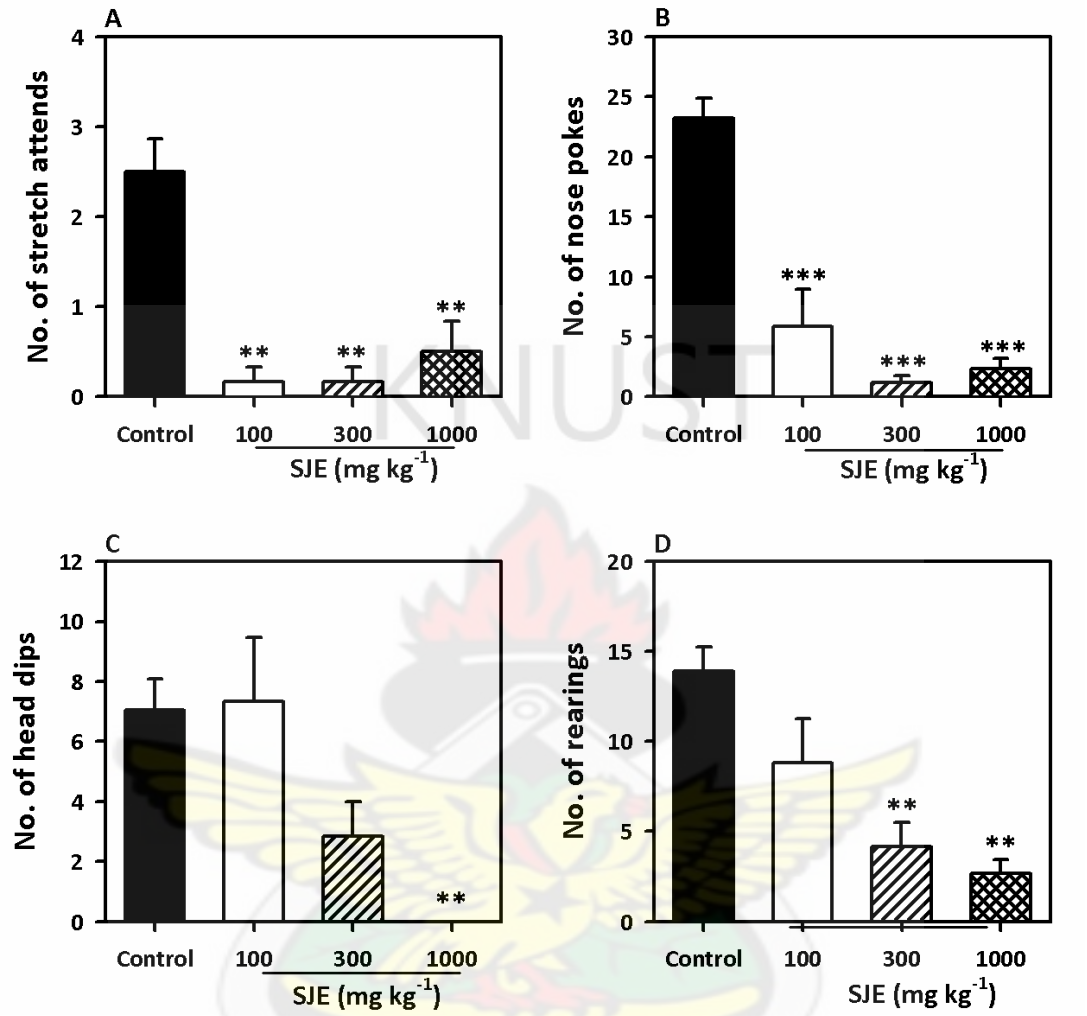

Figure 3.16 Effects of acute SJE (100, 300 and 1000 mg kg^{-1}) 30 min pretest on number of stretch attend (A), number of nose poke (B), number of head dips (C) and number of rearing (D) in the EPM. Data are presented as group means (±SEM). Significantly different from control: **P<0.01, ***P<0.001 by Newman-Keuls test

Table 3.7 Effect of acute SJE on other parameters of elevated plus maze.

	CONTROL	SJE (mg kg^{-1})		
PARAMETERS	**NS**	**100**	**300**	**1000**
Latency for movement(s)	2.28±0.40	3.83±1.22	2.33±0.42	2.00±1.00
No. center entry	9.22±1.01	4.17±1.38*	2.50±0.92**	1.00±0.63***
Duration in center (s)	65.44±7.15	22.67±8.07**	19.50±12.47**	3.67±2.65***
Duration of head-dipping (s)	11.17±1.99	20.50±8.35	15.17±7.56	0.17±0.17*
Freezing	2.42±0.43	6.17±1.25**	4.50±1.61	5.17±1.01*
Duration of freezing (s)	17.54±4.20	80.67±34.91**	141.50±52.02***	185.00±28.74***
Duration of stretch (s)	5.21±1.18	0.17±0.17*	0.17±0.17*	0.33±0.21
Grooming	1.54±0.38	1.17±0.31	1.50±0.72	1.00±0.68
Duration of grooming (s)	32.75±9.20	14.83±5.90	32.50±16.14	22.33±17.50
Fecal boli	0.44±0.15	0.17±0.17	0.33±0.21	0.17±0.17

Data are presented as group means (±SEM). NS = normal saline. Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 by Newman-Keuls test.

On the contrary, diazepam (0.1-0.3-1.0 mg kg^{-1} i.p.) increased both the percentage of time spent in open arms and the percentage entries into open arms (Table 3.8). The number of rearing ($F_{3,32}$ = 3.68; P = 0.0220), grooming ($F_{3,20}$ = 10.54; P = 0.0002) and stretch attends ($F_{3,32}$ = 8.55; P = 0.0003) were significantly reduced with significant increased in the number of head dips ($F_{3,32}$ = 11.13; P < 0.0001) (Table 3.8). The total distance traveled and duration in the closed arm gives a reflection of the locomotor activity. The total distance traveled was significantly increased [$F_{3,31}$ = 3.907, P = 0.0178] (Figure 3.14) and the duration in closed arm was also significantly reduced ($F_{3,20}$ = 9.46; P = 0.0004) (Table 3.8). The results of the other parameters measured are listed in Table 3.9.

Table 3.8 Effects of acute diazepam (0.1, 0.3 and 1.0, mg/kg) 30 min pretest on the parameters of elevated plus maze test.

Parameters	Control	Diazepam (mg kg^{-1})		
		0.1	0.3	1.0
Arm entries				
Closed arm	19.17±1.97	27.83±2.76*	33.33±2.43**	33.50±3.53**
Open arm	2.83±0.60	5.67±1.31	7.83±1.05	13.67±2.36***
% entries in open arm	23.90±3.15	32.46±4.39	47.90±3.72**	56.65±6.99***
Time spent in arm entries				
Closed arm	173.30±15.58	159.70±23.47	81.83±16.35**	70.67±10.43**
Open arm	36.83±9.57	72.60±15.97	97.00±17.15*	145.70±10.31***
% time in open arm	9.50±1.84	20.39±5.78	32.33±5.72**	48.56±3.44***
Other parameters				
Head dips	8.00±0.83	9.83±1.42	15.00±1.32***	15.83±1.47***
Rearing	16.56±2.06	10.83±2.94	8.67±1.20	7.17±1.38*
Grooming	1.67±0.33	2.33±0.42	0.17±0.17**	0.67±0.21*
Stretch attends	4.50±0.62	1.33±0.72**	1.83±0.70*	0.00±0.00***

Data are presented as group means (±SEM). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 by Newman-Keuls test.

Table 3.9 Effect of acute Diazepam on other parameters of elevated plus maze.

	CONTROL	DIAZEPAM (mg kg^{-1})		
PARAMETERS	**NS**	**0.1**	**0.3**	**1.0**
Latency for movement(s)	7.39±1.22	1.83±0.48*	1.33±0.56*	1.83±0.31*
No. center entry	6.78±0.54	12.67±2.09***	14.17±1.68***	20.50±2.01***
Duration in center (s)	77.67±8.79	73.67±13.60	117.80±12.29*	104.80±18.70
Duration of head-dipping (s)	10.89±1.53	24.83±8.32*	53.33±11.00***	52.17±9.92***
Freezing	3.89±0.58	4.83±1.20	4.83±1.08	4.33±0.95
Duration of freezing (s)	19.78±4.83	27.67±12.37	33.33±10.82	33.00±11.27
Duration of stretch (s)	9.78±1.86	2.00±1.18*	4.50±2.20	0.17±0.17**
Grooming	1.39±0.38	2.00±0.73	0.17±0.17	0.67±0.21
Duration of grooming (s)	23.00±8.23	28.17±13.74	5.00±5.00	8.33±4.37
Fecal boli	0.89±0.25	0.33±0.33	2.50±0.43**	0.33±0.21

Data are presented as group means (±SEM). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 by Newman-Keuls test.

3.5.3 Open field test

In the open field test, acute administration of caffeine (10-30-100 mg kg^{-1} i.p.) significantly decreased [$F(3,31) = 5.334$, $P = 0.0044$] the percentage center visited as well as the percentage time spent in the center [$F(3,31)=3.670$, $P=0.0226$] in a dose-related manner. The percentage entries and percentage time spent at the corner visited significantly increased ($F_{3,32} = 4.54$; $P = 0.0093$ and $F_{3,31} = 15.03$; $P < 0.0001$ respectively) whereas percentage entries and percentage time spent at the periphery were significantly decreased ($F_{3,32} = 4.85$; $P = 0.0068$ and $F_{3,31} = 3.79$; $P < 0.0201$ respectively). All these demonstrated the anxiogenic property of caffeine in open field (Figure 3.17). In addition, caffeine induced a significant reduction [$F_{3,32}=41.42$, $P<0.0001$] in locomotor activity in a dose related manner, reflected in the total distance traveled during the test (Figure 3.18). The results of the other parameters measured are listed in Table 3.10.

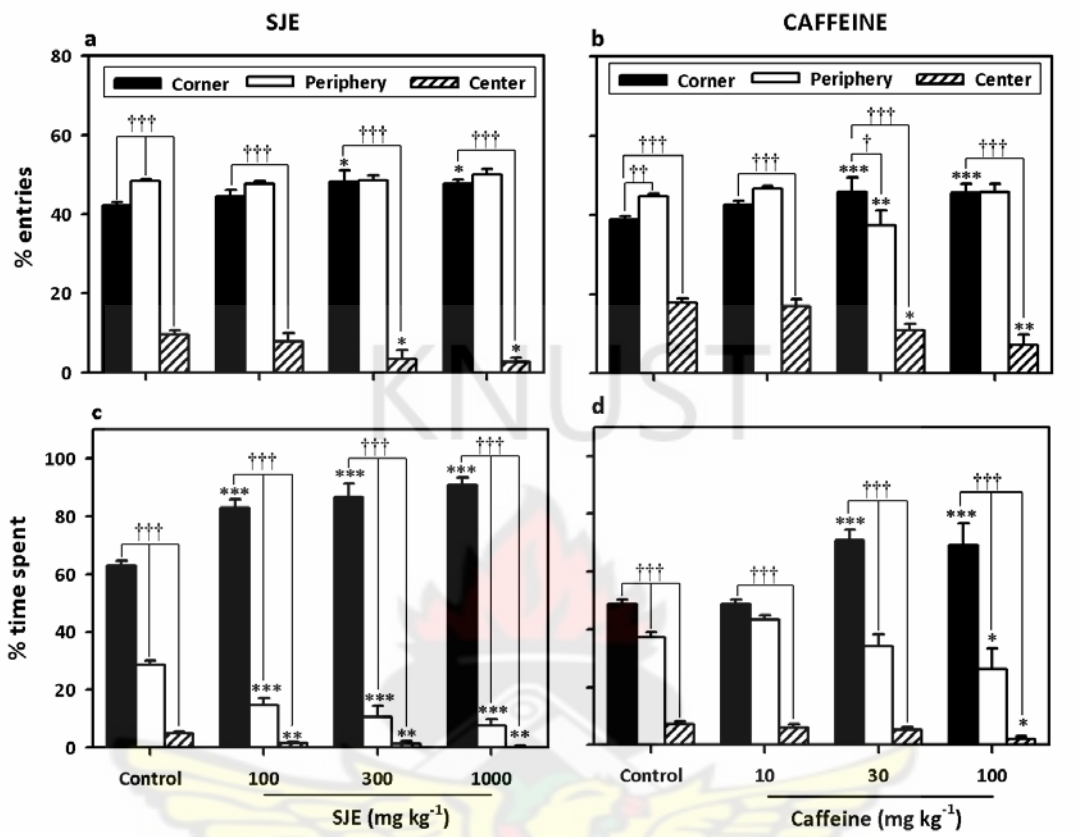

Figure 3.17 Effects of acute SJE (100, 300 and 1000 mg kg^{-1}) and caffeine (10, 30 and 100 mg kg^{-1}) 30 min pretest on the % entries for SJE (a), caffeine (b) and % time spent for SJE (c), caffeine (d) in the open field test. Data are presented as group means (±SEM). Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test and significant difference when the zonal entries where compared to each other: †*P*<0.05, ††*P*<0.01, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*).

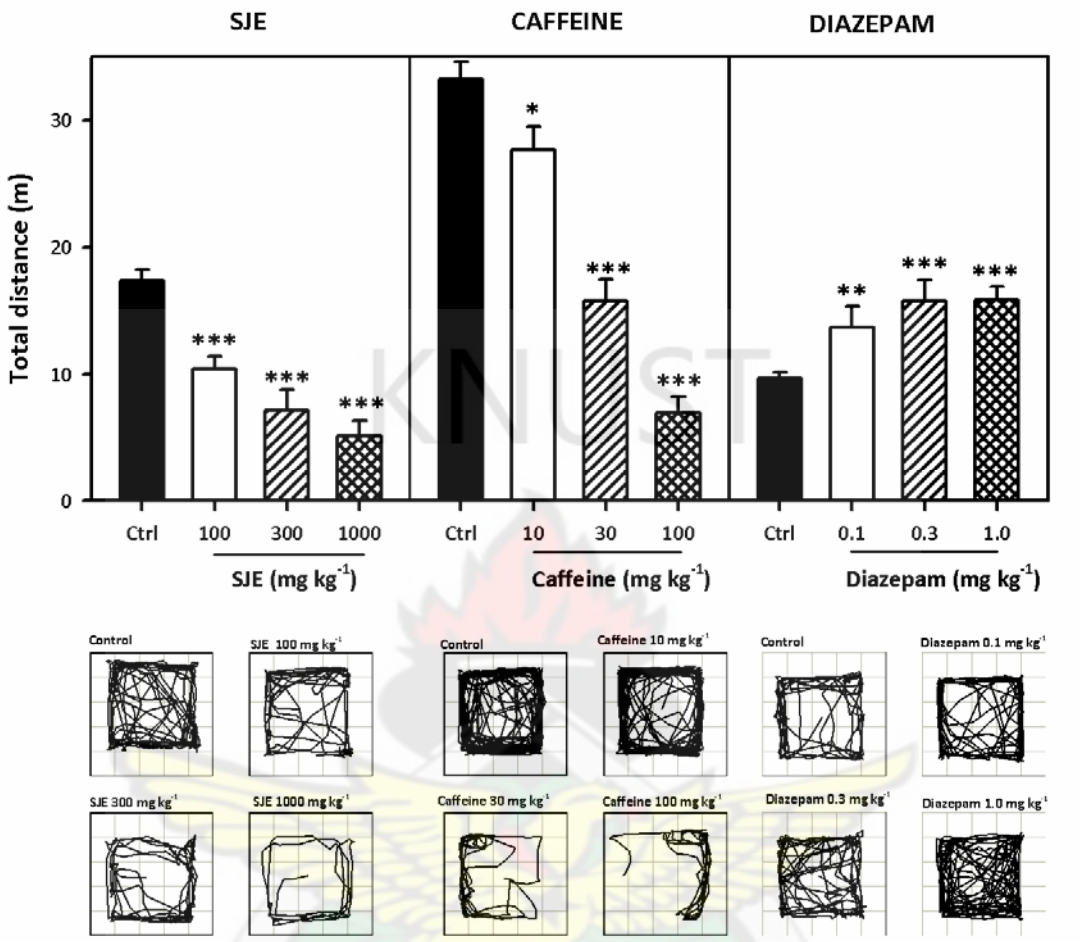

Figure 3.18 Effects of acute SJE (100, 300 and 1000 mg kg^{-1}), caffeine (10, 30 and 100.0 mg kg^{-1}) and diazepam (0.1, 0.3 and 1.0 mg kg^{-1}), all 30 min pretest on total distance travelled in the open field test. Data are presented as group means (±SEM). Significantly different from control (*Ctrl*): **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test. Below the corresponding graph are the tracking paths of mice in separate open field test (n = 6 in each tracking path).

Table 3.10 Effect of acute caffeine on other parameters of open field test

	CONTROL	CAFFEINE (mg kg^{-1})		
PARAMETERS	**NS**	**10**	**30**	**100**
Latency for movement (s)	13.11±3.82	0.83±0.65	1.83±0.95	1.40±0.51
No. of nosepoke	11.22±1.86	6.83±1.56	2.00±0.58**	1.20±0.58*
No. of supported rearing	35.00±3.46	41.33±8.55	19.67±1.28*	0.60±0.24***
No. of unsupported rearing	1.72±0.55	2.17±1.45	0.50±0.34	0.20±0.20
No. of grooming	3.00±0.52	2.33±0.88	3.17±0.79	3.60±0.75
Duration for grooming (s)	16.78±4.89	9.67±3.37	8.67±2.33	10.20±3.14
No. of stop	0.11±0.08	0.17±0.17	3.50±1.09**	6.80±2.48***
Duration for stop (s)	1.39±1.09	2.00±2.00	48.00±18.99*	85.80±42.94**
No. of rest	0.72±0.39	2.33±1.45	8.83±0.91***	15.80±4.20***
Duration for rest (s)	2.06±1.18	3.00±2.30	32.17±2.82**	68.60±20.58***
No. of right curve	32.28±2.01	35.33±3.90	16.67±2.45***	12.40±1.86***
No. of left curve	31.89±2.31	41.83±6.13*	21.17±2.85*	11.20±2.20***
No. Stretch attend posture	10.72±1.19	9.50±2.19	10.00±2.73	8.80±2.87
Duration of stretch (s)	53.33±4.43	25.83±6.15*	29.33±7.39*	36.00±13.72
Fecal Boli	2.37±0.56	0.50±0.34	0.83±0.54	0.17±0.17

Data are presented as group means (±SEM). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 by Newman-Keuls test.

SJE (100-300-1000 mg kg^{-1} *p.o.*) also significantly decreased the % entries ($F_{3,31}$ = 7.132, P = 0.0008) and percentage time spent at the center ($F_{3,31}$=7.357, P=0.0007) in a dose-related manner. The percentage entries and percentage time spent at the corner visited significantly increased ($F_{3,32}$ = 4.01; P = 0.0043 and $F_{3,31}$ = 4.11; P < 0.0300 respectively) whereas percentage time spent at the periphery were significantly decreased ($F_{3,32}$=16.49, P = 0.0031). These trends demonstrate the anxiogenic property of SJE in open field (Figure 3.17). Apart from that, SJE also induced a significant reduction ($F_{3,32}$=27.29, P<0.0001) in locomotor activity in a dose-related manner, reflected in the total distance traveled during the test (Figure 3.18). The results of the other parameters measured are listed in Table 3.11.

Table 3.11 Effect of acute SJE on other parameters of open field test

	CONTROL	SJE (mg kg^{-1})		
PARAMETERS	**NS**	**100**	**300**	**1000**
Latency for movement (s)	9.00±1.13	1.17±0.17***	2.50±1.31**	2.00±1.00**
No. of nosepoke	3.56±0.73	0.17±0.17*	0.67±0.67*	0.17±0.17**
No. of supported rearing	34.67±1.92	14.83±3.58***	9.8±3.70***	4.67±1.96***
No. of unsupported rearing	4.61±1.15	3.17±1.70	3.17±2.20	0.67±0.33
No. of grooming	2.83±0.33	5.50±0.67**	2.83±0.70	2.17±0.98
Duration for grooming (s)	20.22±4.73	64.83±19.23**	36.50±11.84	16.00±9.65
No. of stop	1.78±0.55	4.50±1.34*	2.33±0.67	0.83±0.31
Duration for stop (s)	4.50±1.93	12.17±3.94	6.83±2.33	2.00±0.77
No. of rest	0.44±0.20	1.67±0.76	2.83±1.30*	4.50±1.18***
Duration for rest (s)	1.94±0.82	13.42±6.86	49.50±25.54**	71.25±27.07***
No. of right curve	22.67±1.63	20.83±1.78	14.83±4.69	14.17±3.54
No. of left curve	28.71±1.71	13.67±1.98***	12.67±3.73***	9.00±3.10***
No. Stretch attend posture	2.39±0.34	0.17±0.17**	0.50±0.34**	0.17±0.17***
Duration of stretch (s)	5.72±1.42	0.33±0.33***	1.00±0.63***	0.33±0.33***
Fecal Boli	0.50±0.20	0.17±0.17	0.33±0.21	0.83±0.65

Data are presented as group means (±SEM). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 by Newman-Keuls test.

However, percentage entries into the corner and periphery showed significant reduction ($F_{3,32}$ = 5.32; P = 0.0033 and $F_{3,31}$ = 2.32; P = 0.0311 respectively) whereas percentage entries into center showed statistically significant increased when the mice were treated with diazepam in open field test ($F_{3,31}$=6.53, P = 0.0041) (Table 3.12). Also, the percentage time spent in the corner was significantly reduced ($F_{3,31}$=15.55, P<0.0001) with significant increased in the percentage time spent at the center ($F_{3,32}$=2.02, P = 0.0301) (Table 3.12). The total distance as a measure of locomotor activity which usually increased in anxiolytic drugs was significantly increased ($F_{3,31}$=10.59, P<0.0001) in the open field of diazepam (Figure 3.18). The results of the other parameters measured are listed in Table 3.13

Table 3.12 Effects of acute diazepam (0.1, 0.3 and 1.0, mg/kg) 30 min pretest on the % entries and % time spent in the open field test.

Parameters	Control	DIAZEPAM (mg kg^{-1})		
		0.1	0.3	1.0
% zonal entries				
Corner	47.37±0.96	40.26±0.28***	36.73±0.91***	37.25±0.16***
Periphery	43.51±0.64	48.62±1.57**	44.42±0.59	38.23±0.98**
Center	9.12±1.58	11.12±1.83	18.86±1.50***	24.52±0.90***
% time in zones				
Corner	63.11±2.84	52.89±3.28*	48.22±3.47**	44.61±2.89**
Periphery	31.39±4.45	38.50±5.35	42.33±5.91	43.83±5.14
Center	4.20±1.15	9.87±2.00	10.93±2.37	12.93±2.19*

Data are presented as group means (±SEM). Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test.

Table 3.13 Effect of acute diazepam on other parameters of open field test

	CONTROL	DIAZEPAM (mg kg^{-1})		
PARAMETERS	**NS**	**0.1**	**0.3**	**1**
Latency for movement (s)	5.05±1.09	0.50±0.22*	0.83±0.17*	0.17±0.17*
No. of nosepoke	17.67±1.56	9.33±2.11*	15.67±4.77	5.50±0.50**
No. of supported rearing	34.56±2.56	25.83±7.68	23.50±6.89	22.50±6.51
No. of unsupported rearing	4.15±1.36	3.67±3.67	2.00±1.48	0.50±0.34
No. of grooming	2.89±0.37	5.33±0.99	4.00±1.03	9.17±6.38
Duration for grooming (s)	14.72±3.69	36.83±13.59	47.67±27.48	32.50±11.05
No. of stop	0.70±0.39	1.67±1.12	0.67±0.33	1.50±0.56
Duration for stop (s)	2.35±1.06	10.00±6.99	12.33±9.82	40.00±24.34*
No. of rest	5.80±1.37	9.33±2.19	4.33±1.56	7.33±1.63
Duration for rest (s)	11.05±2.56	22.17±5.12	14.83±6.64	15.00±5.03
No. of right curve	27.65±2.49	25.50±4.82	23.83±3.36	18.17±2.98
No. of left curve	22.15±2.08	20.67±3.22	21.33±6.13	20.00±2.81
No. Stretch attend posture	12.61±1.09	12.33±3.11	9.67±2.65	5.00±1.65*
Duration of stretch (s)	36.11±3.08	30.67±12.98	22.83±6.32	12.50±4.99*
Fecal Boli	2.72±0.54	0.17±0.17	1.50±0.96	1.83±0.87

Data are presented as group means (±SEM). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 by Newman-Keuls test.

3.5.4 Hole board test

Though acute administration of caffeine (10-30-100 mg kg^{-1} *i.p.*) did not have any significant effect on the number of head dips as well as the locomotor activity as measured by total distance travelled (Figure 3.19), the duration of head dipping was significantly decreased ($F_{3,18}$ = 9.88; P = 0.0005) in a dose related manner (Figure 3.20). SJE (100-300-1000 mg kg^{-1} *p.o.*) significantly decreased the locomotor activity (total distances travelled) ($F_{3,21}$ = 1.82; P = 0.0405) (Figure 3.19) as well as the number and duration of head dipping ($F_{3,21}$ = 4.22; P = 0.0283 and $F_{3,21}$ = 5.37; P = 0.0222 respectively). These further confirm the anxiogenic property of SJE (Figure 3.20). In contrast, diazepam caused an increase in the duration of head dipping (Figure 3.20) as well as the locomotor activity (Figure 3.19).

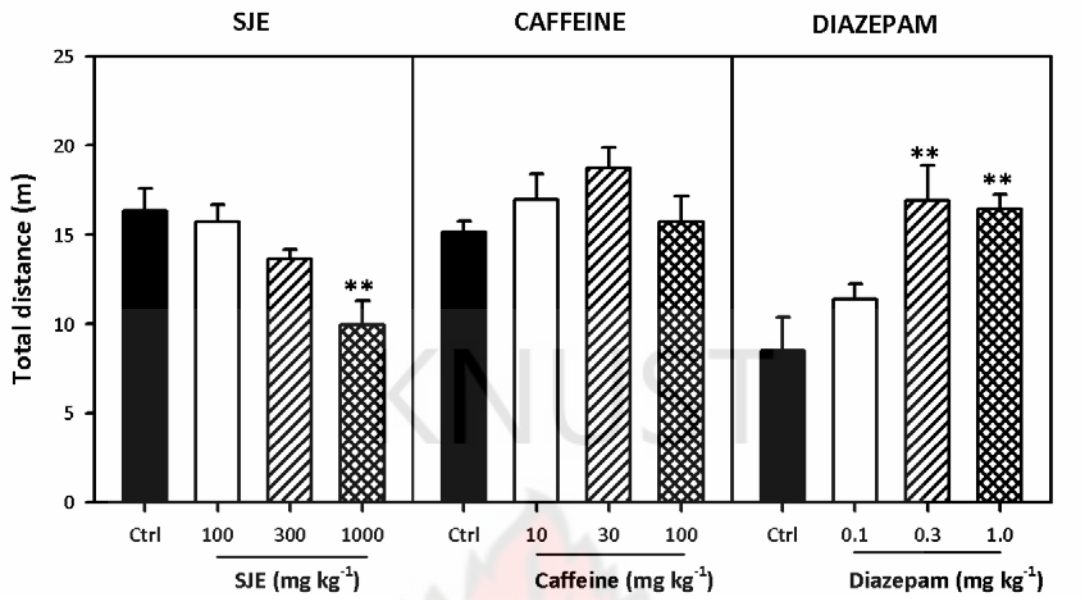

Figure 3.19 Effects of acute SJE (100, 300 and 1000 mg kg^{-1}), caffeine (10, 30 and 100.0 mg kg^{-1}) and diazepam (0.1, 0.3 and 1.0 mg kg^{-1}), all 30 min pretest on total distance travelled in the hole board test. Data are presented as group means (±SEM). Significantly different from control (*Ctrl*): **P<0.01 by Newman-Keuls test.

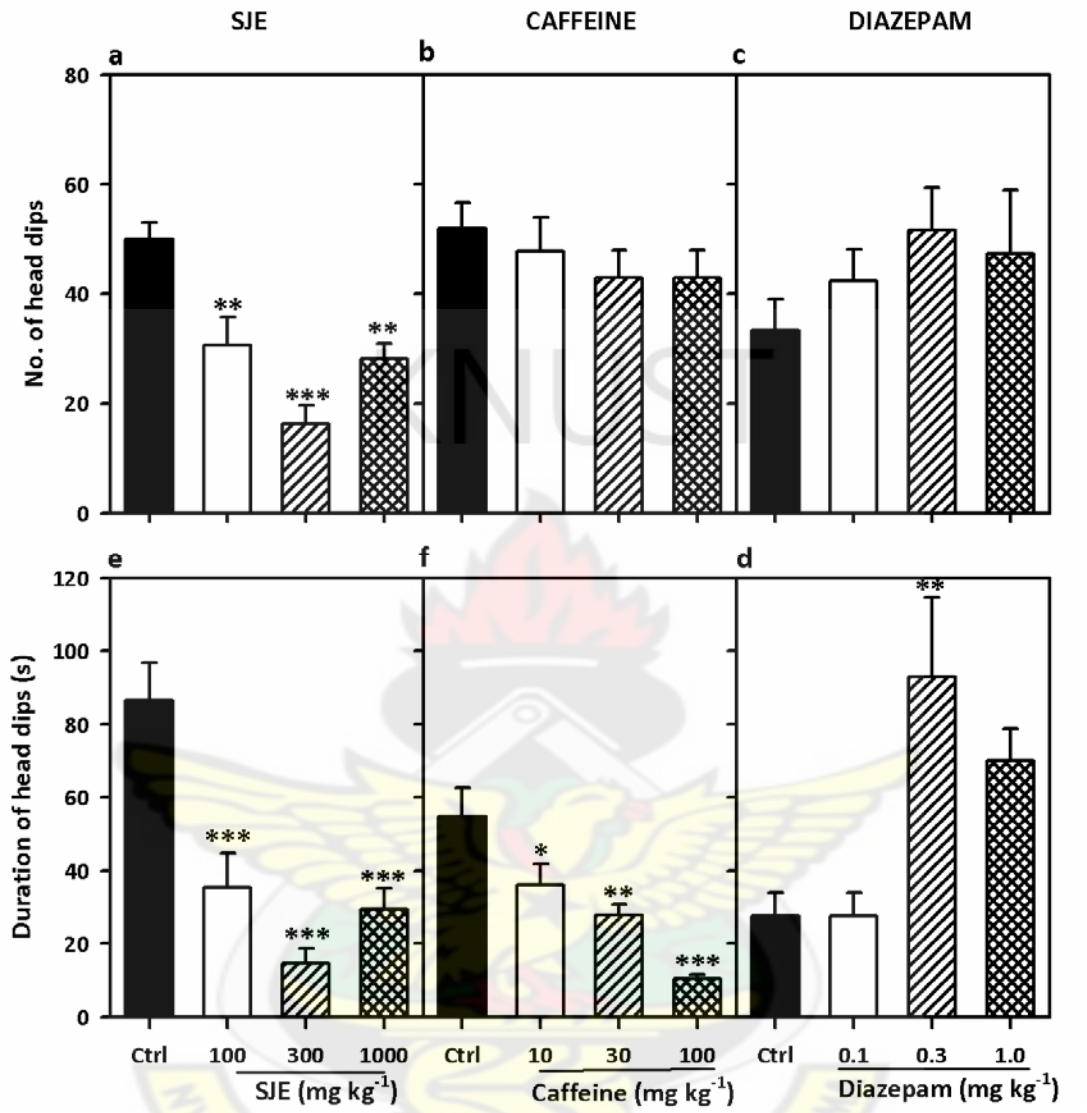

Figure 3.20 Effects of acute SJE (100, 300 and 1000 mg kg^{-1}), caffeine (10, 30 and 100.0 mg kg^{-1}) and diazepam (0.1, 0.3 and 1.0 mg kg^{-1}), all 30 min pretest on the number of head dips and duration of head dipping in the hole board test. Data are presented as group means (±SEM). Significantly different from control (*Ctrl)*: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test.

3.5.5 Effects on anxiety in the light/dark test

In the light/dark test, acute administration of caffeine (10-100 mg kg^{-1} *i.p.*) induced anxiety-like effects (Figure 3.21). Caffeine significantly decreased ($F_{3,20}$= 4.839; *P*=0.0108) the time spent by mice in the lit box and increased significantly ($F_{3,20}$= 4.839; *P*=0.0108) the time spent in the dark box. The frequency of entry into the light and dark box as well as the frequency of transition between compartments also increased significantly ($F_{3,20}$= 6.310; *P*=0.0035, $F_{3,20}$= 4.373; *P*=0.0160 and $F_{3,20}$= 4.307; *P*=0.0169 respectively). Two-way ANOVA revealed that there was significant difference ($F_{1,30}$=250.99; *P*<0.0001) between the duration of entries into the light and dark box but concentration does not affect the result ($F_{3,30}$=0.00; *P*=1.0000).

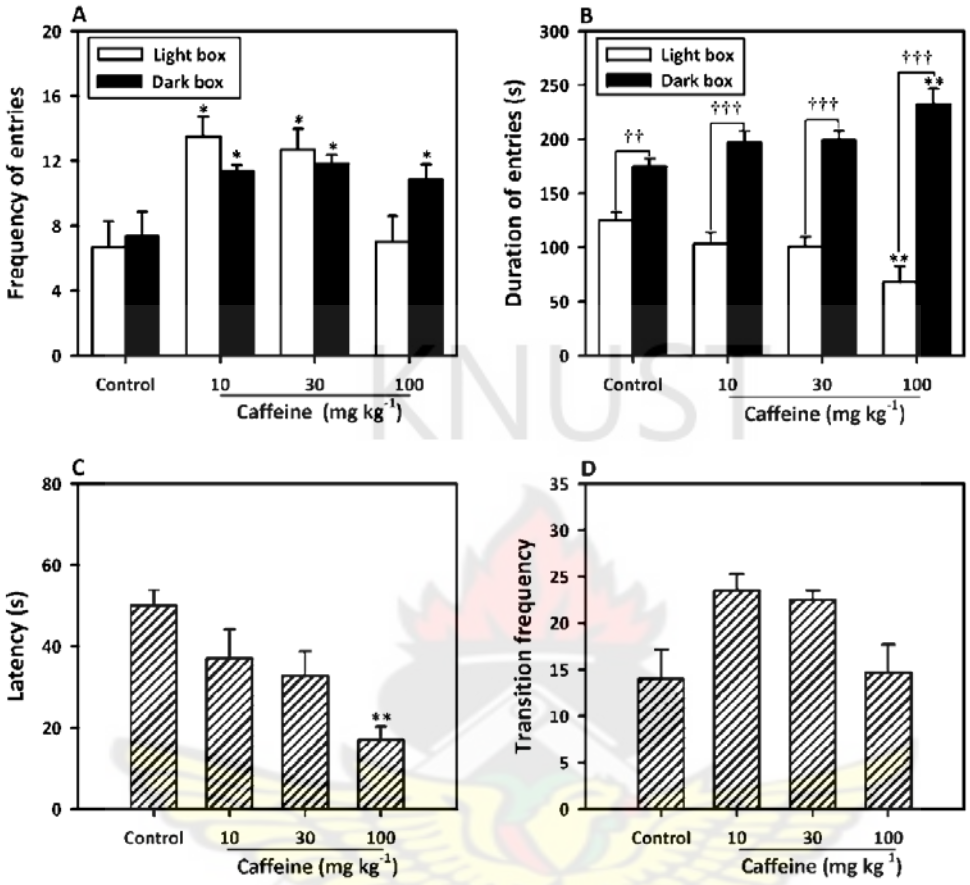

Figure 3.21 Effects of acute administration of caffeine in the light/dark test. Data are presented as group means (±SEM). Significantly different from control (*Ctrl)*: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test and significant difference when open arm and closed arm where compared: †*P*<0.05, ††*P*<0.01, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*).

SJE (100-1000 mg kg^{-1} *p.o.*) also induced anxiety-like effects like caffeine by significantly decreasing the time spent in the lit box ($F_{3,20}$=4.461; *P*=0.0149) and increased the time spent in the dark box ($F_{3,20}$= 4.461; *P*=0.0149). There were no significant changes in the frequency of entries into the light and dark box as well as in the frequency of transition between compartments. Two-way ANOVA revealed that there was a significant difference ($F_{1,30}$=298.07; *P*<0.0001) in the duration of entries into the light and dark box but concentration did not have any significant effect ($F_{3,30}$=0.00; *P*=1.0000) (Figure 3.22).

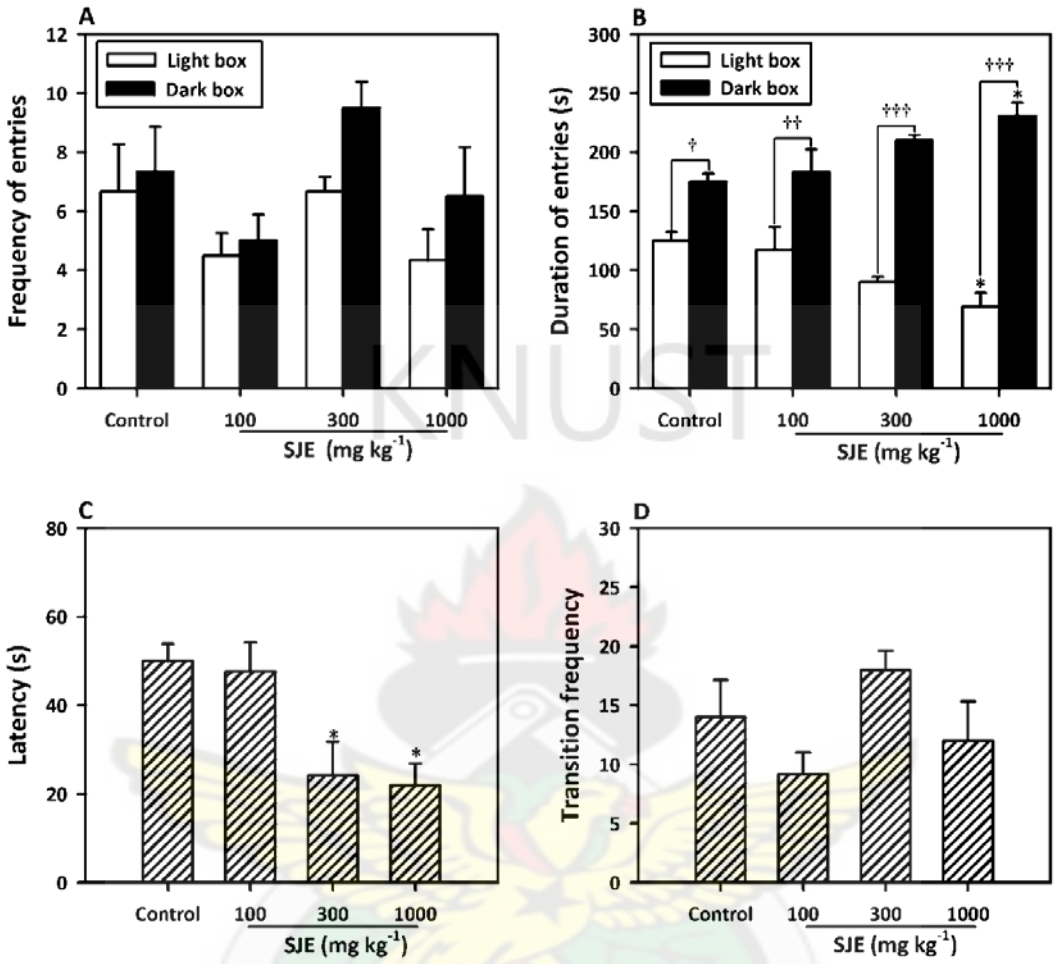

Figure 3.22 Effects of acute administration of SJE in the light/dark test. Data are presented as group means (±SEM). Significantly different from control (*Ctrl*): **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test and significant difference when open arm and closed arm were compared: †*P*<0.05, ††*P*<0.01, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*).

In contrast, diazepam (0.1-1.0 mg kg^{-1} *i.p.*) induced anxiolytic-related measures in the light/dark test (Figure 3.23). Diazepam significantly increased the duration of time spent in the lit box ($F_{3,20}$=26.73; P<0.0001) and decreased the time spent in the dark box ($F_{3,20}$= 26.73; P<0.0001). Frequency of entries into the light/dark and transition between compartments did not change significantly. Two-way ANOVA revealed that there was a significant difference ($F_{1,30}$=65.13; P<0.0001) in the duration of entries into the light and dark box but concentration did not have any significant effect ($F_{3,30}$=0.00; P=1.0000).

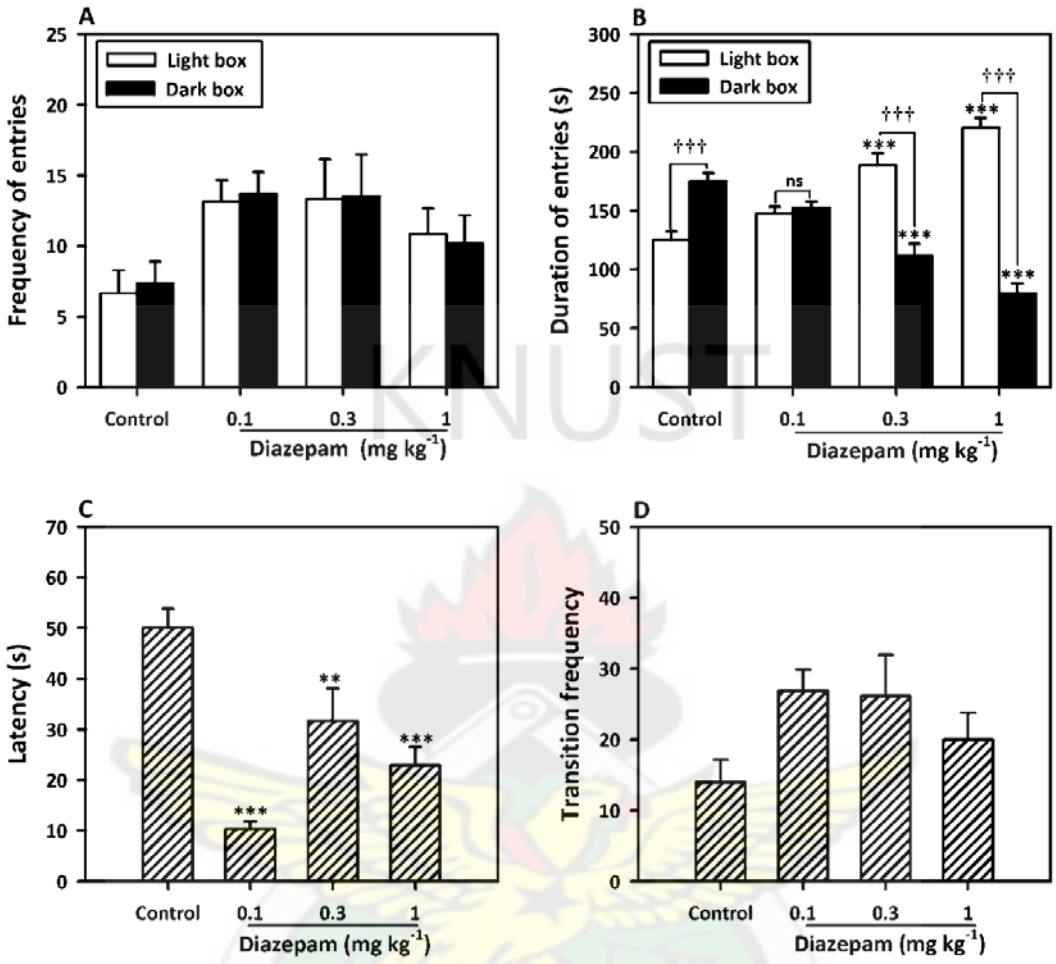

Figure 3.23 Effects of acute administration of diazepam in the light/dark test. Data are presented as group means (±SEM). Significantly different from control (*Ctrl*): **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test and significant difference when open arm and closed arm were compared: †*P*<0.05, ††*P*<0.01, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*).

3.6 ANTIDEPRESSANT PROPERTY OF SJE

3.6.1 Effects on motor coordination in rota-rod

The results indicate that there is no significant difference in the time spent on the rotating drum when the SJE treated mice ($F_{3,19}$= 2.793; P=0.0684), imipramine treated mice ($F_{3,19}$= 0.9428; P=0.4396) or fluoxetine treated mice was compared to the control group ($F_{3,20}$= 0.9711; P=0.4256) (Figure 3.24).

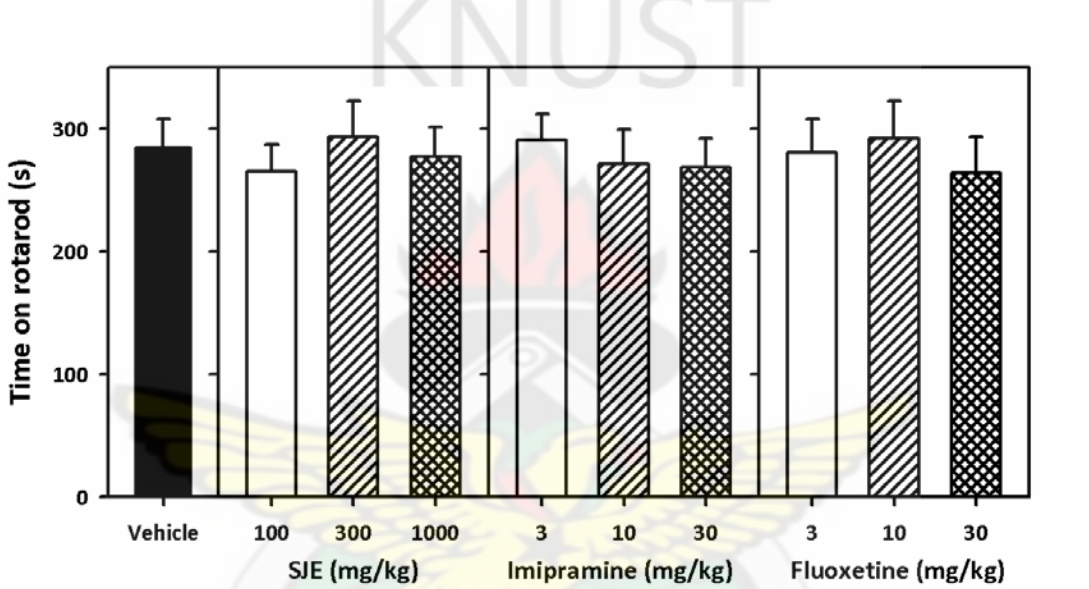

Figure 3.24 Behavioural effect of acute SJE (100, 300 and 1000 mg kg^{-1}), Imipramine (3, 10 and 30.0 mg kg^{-1}) and Fluoxetine (3, 10 and 30 mg kg^{-1}), on motor coordination on the rotarod. Data are presented as group means (±SEM).

3.6.2 Effect on immobility periods in FST and TST

SJE (100, 300, and 1000 mg kg^{-1} *p.o.*) administered for 30 min before the test period significantly decreased the immobility periods of mice in a dose-dependent manner in both the FST ($F_{3,20}$ = 19.16; P < 0.0001) and the TST ($F_{3,15}$ = 21.45; P < 0.0001), indicating significant antidepressant activity. Fluoxetine (3, 10 and 30 mg kg^{-1} *p.o.*) significantly reduced ($F_{3,20}$ = 31.51; P < 0.0001 and, $F_{3,15}$ = 13.73; P = 0.0001 for FST and TST respectively) immobility periods in a dose-related manner

compared with the control. Imipramine (3, 10 and 30 mg kg^{-1} *p.o.*) also significantly reduced ($F_{3,20}$ = 26.02; P < 0.0001 and, $F_{3,15}$ = 18.92; P < 0.0001 for FST and TST respectively) immobility periods in a dose-related manner compared with the control (Figure 3.25). The reference drugs possess greater efficacy than SJE as indicated by the EC_{50} at the doses tested (Table 3.14).

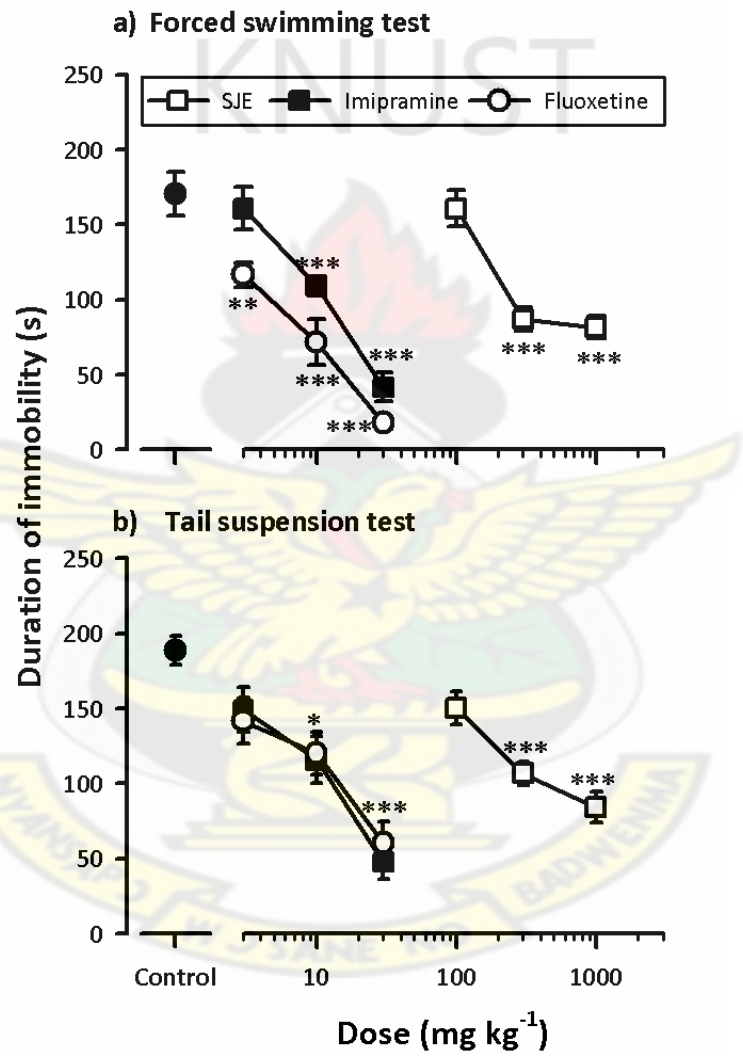

Figure 3.25 Effects of acute treatment on immobility periods in FST and TST. Data are presented as group means (±SEM). Significantly different from vehicle: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test.

Table 3.14 EC_{50} for SJE, Imipramine and Fluoxetine in the FST and TST

Drugs	EC_{50} (mg kg^{-1})	
	FST	TST
S. jollyanum	540.76±174.14	246.60±128.63
Imipramine	15.52±3.28	12.97±10.51
Fluoxetine	6.29±1.18	12.13±11.05

3.6.3 The effects of catecholamine depletion on behavioural responses in the TST

3.6.3.1Pretreatment with MeDOPA

The effects of α-methyldopa (MeDOPA) pretreatment on the behavioural effects of antidepressants in the TST are shown in Figures 3.26-3.28. Mice were pretreated with vehicle or MeDOPA (400 mg kg^{-1}, *p.o.*) 3.5 h before the TST and received either fluoxetine (3, 10 or 30 mg kg^{-1}), imipramine (3, 10 or 30 mg kg^{-1}), or SJE (100, 300 or 1000 mg kg^{-1}) 30 min before the TST. Pretreatment with MeDOPA did not completely block the effects of SJE. The results display a U-shape dose-response curve with only the 300 mg kg^{-1} being able to significantly reverse the effects of MeDOPA ($P < 0.05$) (Figure 3.26). Fluoxetine was able to reverse the percentage immobility in a dose-related manner (Figure 3.27). However, imipramine was not able to attenuate or reverse the immobility induced by MeDOPA (Figure 3.28). Pretreatment with MeDOPA significantly inhibited catecholamine synthesis as revealed by the inability of imipramine to reduce or reverses the immobility induced by MeDOPA without producing significant effect on serotonin content as revealed by the ability of fluoxetine to reverse the immobility induced by MeDOPA.

3.6.3.2 Pretreatment with reserpine

The effects of reserpine pretreatment on the behavioural effects of antidepressants in the TST are shown in Figure 3.26-3.28. Mice were pretreated with vehicle or

reserpine (1 mg kg^{-1}, s.c) 24 h before the TST and received either fluoxetine (3, 10 or 30 mg kg^{-1}), imipramine (3, 10 or 30 mg kg^{-1}), or SJE (100, 300 or 1000 mg kg^{-1}) 30 min before the TST. The extract (Figure 3.26) and fluoxetine (Figure 3.27) were able to reverse or attenuate the reserpine induced immobility in a dose-dependent manner whereas imipramine (Figure 3.28) was not able to reverse this effect of reserpine-induced immobility. However, the reversal effect of the fluoxetine is not as pronounced as it is when the monoamines were depleted with MeDOPA

3.6.3.3 Pretreatment with reserpine + MeDOPA

Because the behavioural effects of antidepressants could involve catecholamines located in different cellular pools or compartments, the responses to antidepressants were evaluated after reserpine and MeDOPA were given together. It is known that, vesicular stores of catecholamines were depleted by pretreating mice with reserpine (1 mg kg^{-1}, *s.c.*) 24 h before the TST, whereas newly formed stores of catecholamines were depleted by pretreatment with MeDOPA (200 mg kg^{-1}, *i.p.*) 3.5 h before the TST (O'Leary *et al.*, 2007). Separate groups of mice were tested with the specific serotonin re-uptake inhibitor (SSRIs) fluoxetine (3, 10 or 30 mg kg^{-1}), with the norepinephrine re-uptake inhibitor (NRIs) imipramine (3, 10 or 30 mg kg^{-1}), and the extract (100, 300 or 1000 mg kg^{-1}) as shown in Figures 3.26-3.28.

Although the behavioural effects of all the test antidepressant drugs were blocked in the reserpine + MeDOPA group, fluoxetine still had a significant reduction in the percentage change in immobility at the dose of 30 mg kg^{-1} after reserpine + MeDOPA pretreatment.

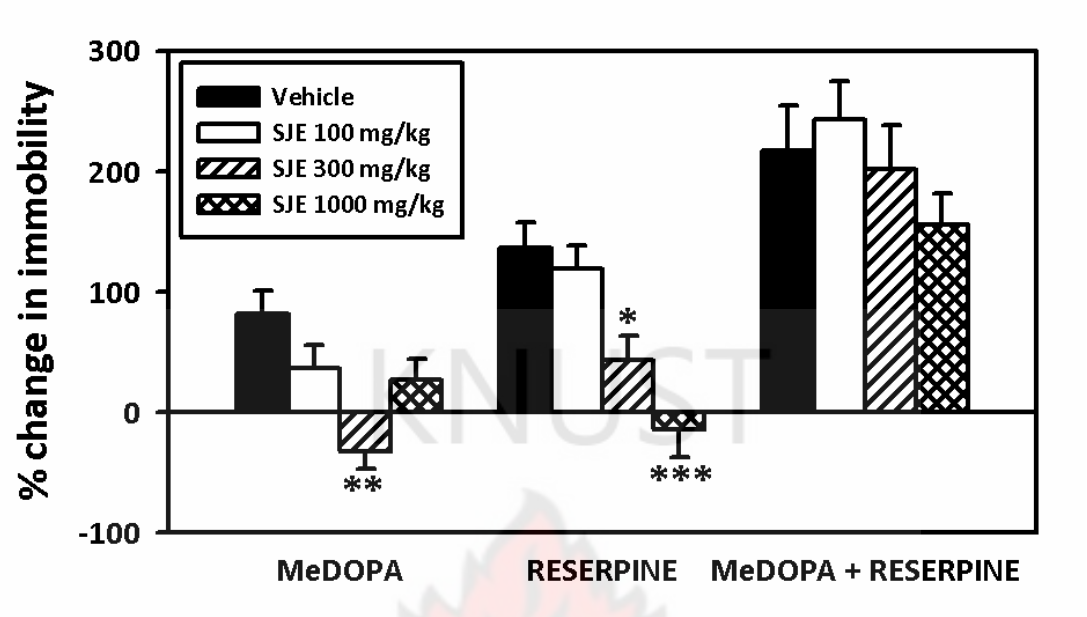

Figure 3.26 Behavioural effect of acute SJE (100, 300 and 1000 mg kg^{-1}) in the TST. Data are presented as group means (±SEM). Significantly different from vehicle: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test.

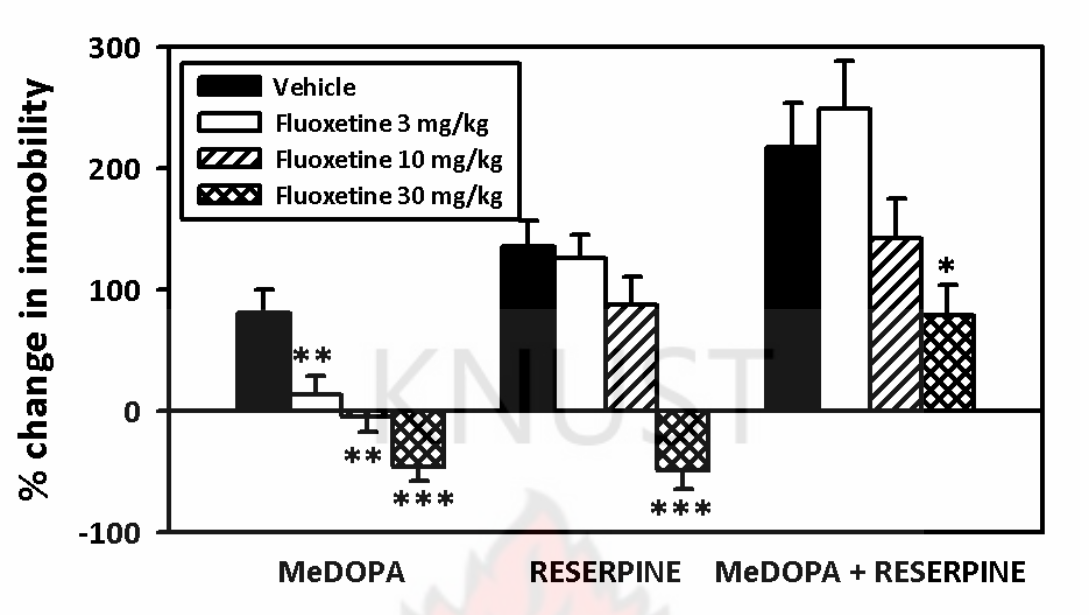

Figure 3.27 Behavioural effect of acute fluoxetine (3, 10 and 30 mg kg^{-1}) in the TST. Data are presented as group means (±SEM). Significantly different from vehicle: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test.

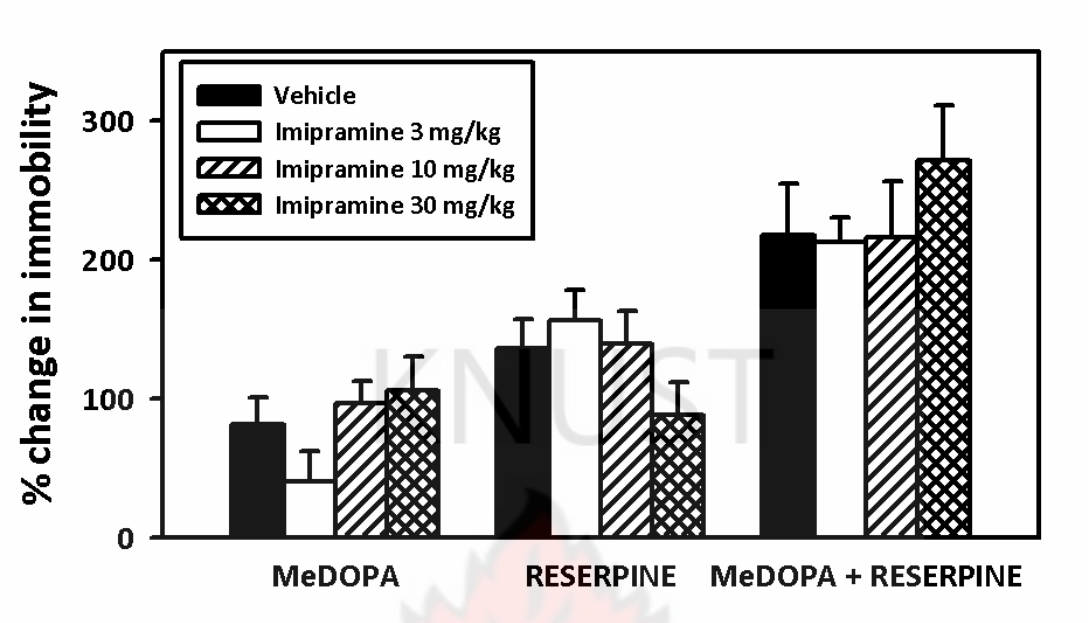

Figure 3.28 Behavioural effect of acute fluoxetine (3, 10 and 30 mg kg^{-1}) in the TST. Data are presented as group means (±SEM). Significantly different from vehicle: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test.

3.7 EFFECT OF SJE ON DRUG METABOLIZING ENZYMES

3.7.1 Total Cytochrome P450

The enzyme inducer phenobarbitone significantly increased the levels of the total cytochrome P450 (P = 0.0026) whilst that of the enzyme inhibitor ketoconazole could not reduce the levels of cytochrome P450 compared to the control group in this study (P = 0.7355). SJE like phenobarbitone significantly induced the drug metabolizing enzyme (cytochrome P450) as compared to the control groups (P = 0.0001) as shown in Figure 3.29.

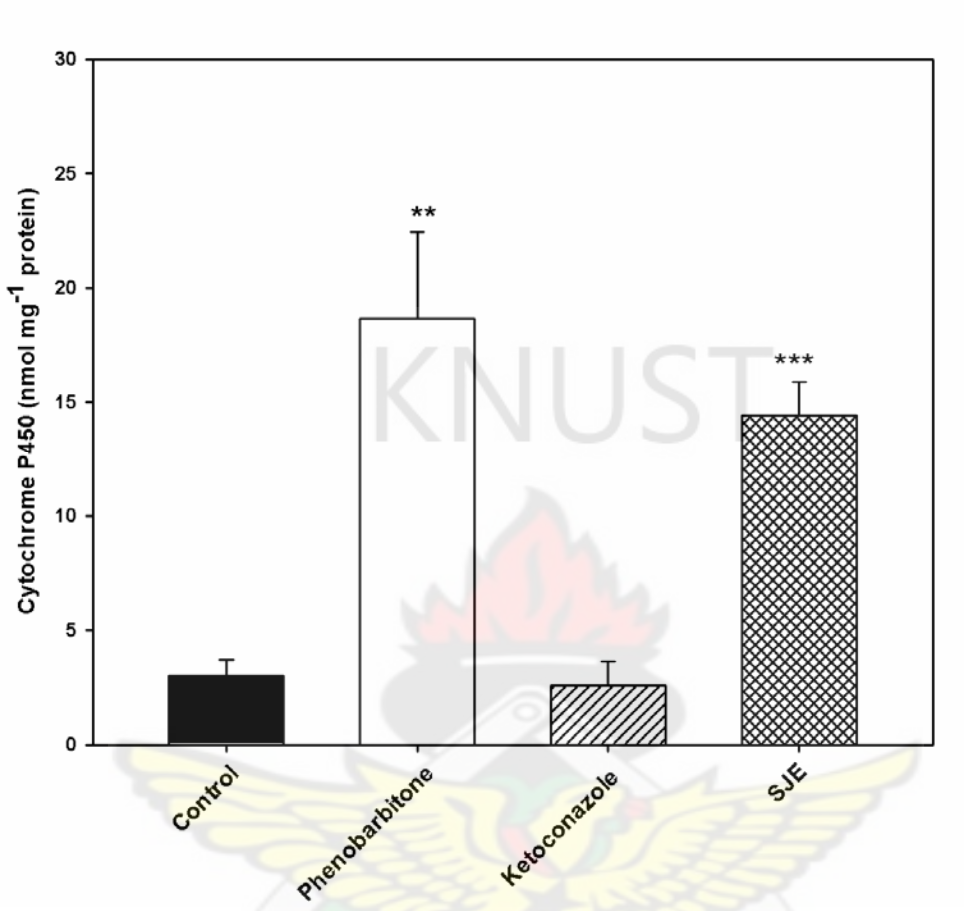

Figure 3.29 Effect of SJE (300 mg kg^{-1} p.o), phenobarbitone (80 mg kg^{-1} p.o) and ketoconazole (100 mg kg^{-1} p.o) on the levels of total cytochrome P450 of rats after 8 days treatment period. Values are mean ± SEM and significantly different from control: $^{**}P < 0.01$ and $^{***}P < 0.001$.

3.7.2 Pentobarbitone induced sleeping time

Phenobarbitone caused a profound decrease in pentobarbitone-induced sleeping time ($p < 0.0001$) as compared to the control group. SJE also produced a statistically significant decrease in the duration of pentobarbitone-induced sleeping time ($P = 0.0085$) as compared to the control group. However, ketoconazole did not have any significant effect on the pentobarbitone induced sleeping time ($P = 0.2739$) when compared to the control group. None of the test drugs had any significant effect on the onset of pentobarbitone induced sleeping time (Figure 3.30).

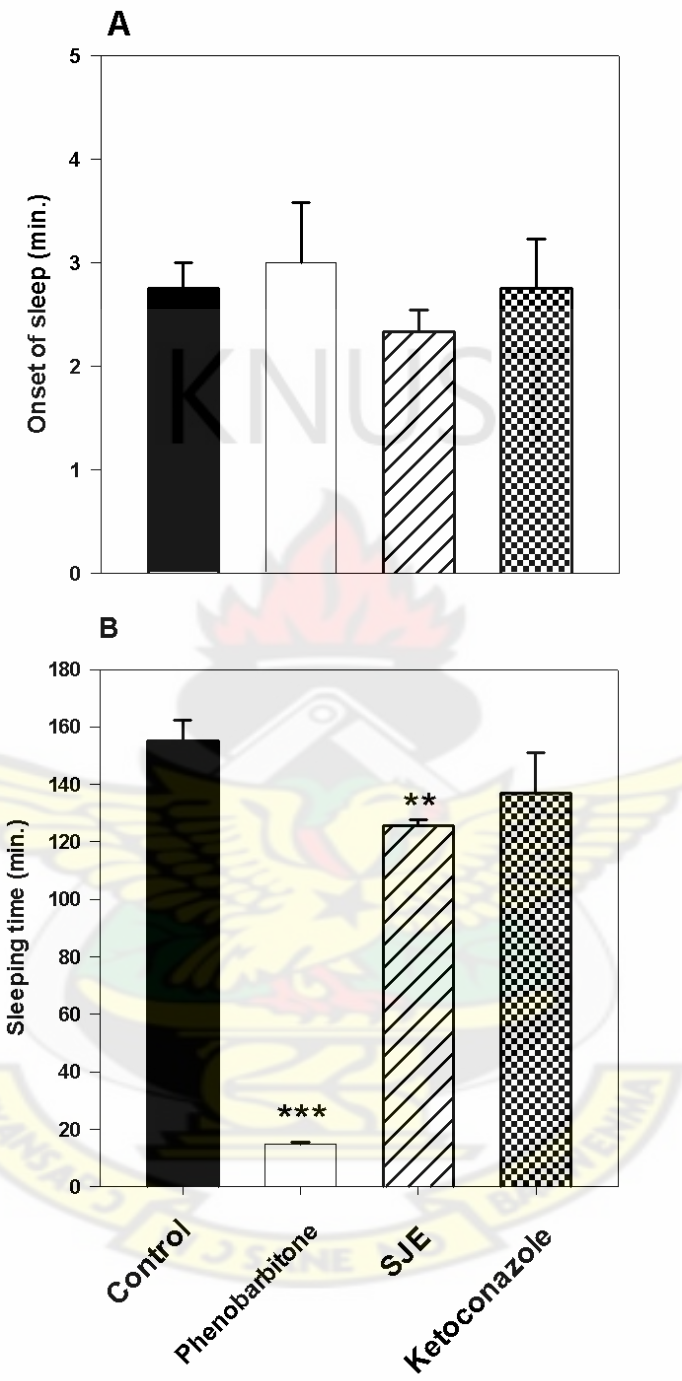

Figure 3.30 Effect of SJE (300 mg kg^{-1} p.o.), phenobarbitone (80 mg kg^{-1} p.o.) and ketoconazole (100 mg kg^{-1} p.o.) on pentobarbitone induced sleeping time after 8 days treatment period in rats. Values are mean ± SEM and significantly different from control:$^{**}P < 0.01$ and $^{***}P < 0.001$.

3.8 TOXICOLOGICAL PROFILE OF SJE

3.8.1 Clinical signs and mortality

All animals survived until the end of the study period. Furthermore, no remarkable clinical signs of toxicity were observed either immediately or during the post-treatment period even at the highest dose of 1000 mg kg^{-1} body weight in any of the animals during the study period. There were also no changes noted during the duration of the study in behaviour, activity, posture, gait, or external appearance that were considered to be test drug related.

3.8.2 Animals body weight and organ weight

Overall, mean body weights were comparable for SJE treated groups and control (Figure 3.31) with no significant difference ($P > 0.05$ in all comparison). Rats in all experimental groups gained weight over the course of this study.

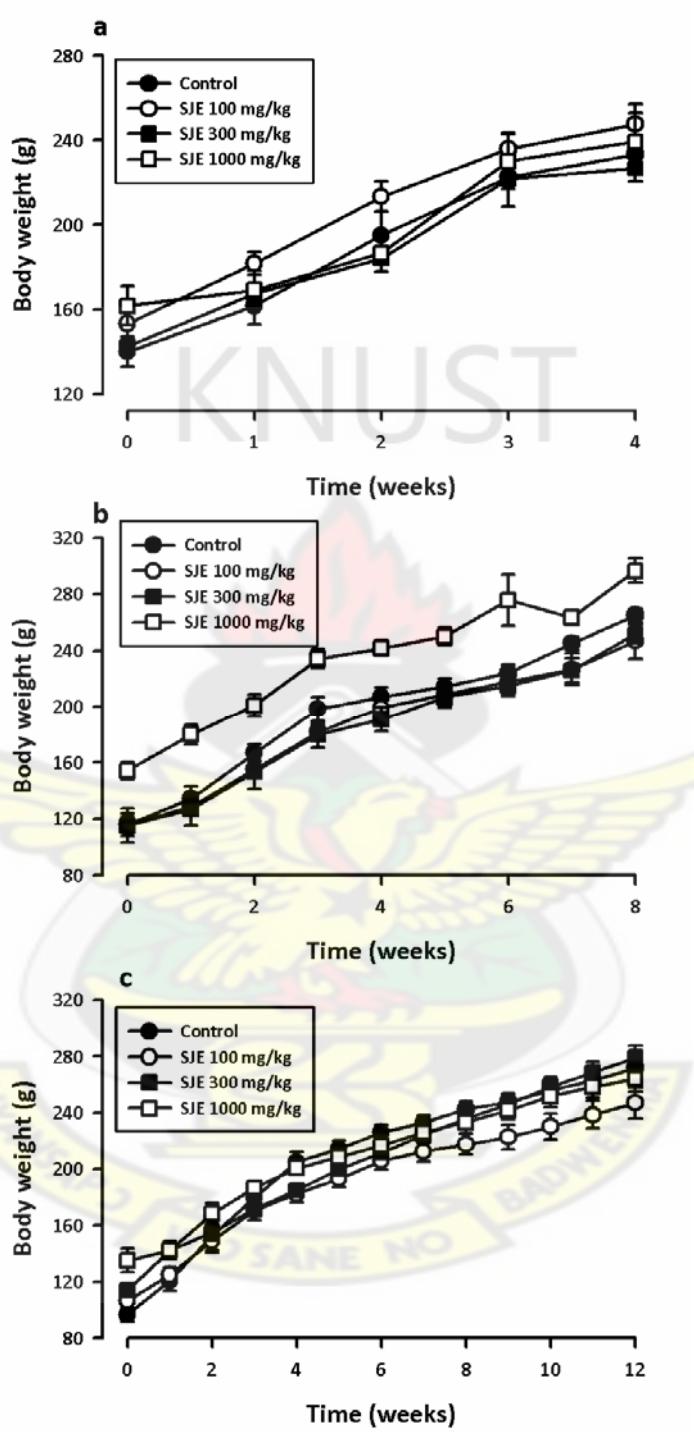

Figure 3.31 Mean body weights (mean ± SEM) of animals treated for (a) one month, (b) two months and (c) three months.

There were generally no statistically significant differences noted in relative organ weights between treated and control animals except for the liver [F(3,19)=6.391, P=0.0035]. Liver weight relative to body weights at the third month increased in a dose-dependent manner in all groups dosed with the test drug (Table 3.15). However, the magnitudes of the alterations were small and were not considered treatment-related.

Table 3.15 Relative organ weights (mean ± SEM) of animals treated for one, two and three months of SJE

PARAMETERS (g)	CONTROL	SJE (mg kg^{-1}) 100	300	1000
One month				
Lungs weight	0.44±0.08	0.55±0.03	0.61±0.04	0.61±0.05
Heart weight	0.31±0.01	0.33±0.01	0.34±0.02	0.33±0.02
Liver weight	2.58±0.09	2.40±0.05	2.58±0.06	2.77±0.05
Kidney weight	0.55±0.03	0.55±0.03	0.53±0.01	0.56±0.01
Spleen weight	0.32±0.03	0.30±0.03	0.33±0.03	0.34±0.03
Testes weight	1.03±0.05	0.85±0.15	0.91±0.09	1.04±0.06
Two months				
Lungs weight	0.62±0.04	0.66±0.07	0.67±0.04	0.75±0.06
Heart weight	0.31±0.01	0.31±0.02	0.32±0.01	0.26±0.02
Liver weight	3.27±0.19	3.29±0.23	3.04±0.17	3.55±0.25
Kidney weight	0.55±0.02	0.56±0.03	0.54±0.02	0.59±0.03
Spleen weight	0.39±0.03	0.37±0.01	0.38±0.02	0.49±0.06
Testes weight	0.97±0.05	1.06±0.08	1.06±0.06	0.83±0.03
Three months				
Lungs weight	0.63±0.07	0.65±0.05	0.76±0.10	0.63±0.08
Heart weight	0.30±0.01	0.33±0.02	0.34±0.01	0.32±0.02
Liver weight	2.38±0.06	2.70±0.06*	2.68± 0.08*	2.81±0.07**
Kidney weight	0.54±0.02	0.53±0.02	0.58±0.03	0.57±0.01
Spleen weight	0.32±0.02	0.36±0.01	0.39±0.03	0.41±0.01
Testes weight	1.08±0.04	0.97±0.06	0.98±0.02	1.05±0.04

Statistically significant difference $^{*}p < 0.05$, $^{**}p < 0.001$

3.8.3 Hematology

Results for one, two and three months are contained in tables 3.16, 3.17 and 3.18, respectively. There were generally no significant difference noted between the treated and the control groups for most of the parameters measured. However, there were some statistically significant differences for the first month i.e. WBC (P=0.0089), HGB (P=0.0001), HCT (P<0.0001), MCH (P<0.0001) MCHC (P<0.0001) and PLT (P=0.0064); for the second month i.e. WBC (P=0.0399), HGB (P=0.0025) and HCT (P=0.0011) and for the third month HGB (P=0.0060) HCT (P=0.0014) and PLT (P=0.0026). Because significant statistical differences of HGB and HCT occurred consistently over the period, they are regarded as relevant and study related.

Table 3.16 Haematology parameters of animals treated for 30 days (mean ± SEM)

		SJE (mg kg^{-1})		
PARAMETERS	CONTROL	100	300	1000
WBC (k/μL)	6.57±0.67	2.92±0.49**	5.00±0.53	5.30±0.91
LYM (%)	90.93±0.50	91.22±0.81	91.52±0.83	92.83±0.35
MID (%)	7.18±0.45	6.05±0.63	6.60±0.57	5.47±0.35
GRAN (%)	1.88±0.12	2.73±0.38	1.88±0.29	1.70±0.13
RBC (M/μL)	4.39±0.12	3.82±0.23	4.35±0.17	4.11±0.18
HGB (g/dl)	8.48±0.38	7.27±0.48	10.82±0.47*	9.97±0.51
HCT (%)	24.25±0.95	20.78±1.28	31.77±1.59**	28.67±1.57
MCV (fL)	55.27±1.15	54.37±0.73	56.47±0.27	54.45±1.15
MCH (pg)	19.32±0.48	19.07±0.84	24.85±0.16**	24.27±0.56**
MCHC (g/dl)	34.95±0.24	35.05±1.40	44.03±0.31**	44.53±0.25**
RDW (%)	16.62±0.56	15.57±0.40	16.05±0.22	15.95±0.64
PLT (k/μL)	295.70±30.62	159.50±26.55**	232.80±19.77	210.70±16.80

Statistically significant difference $^{*}p < 0.05$, $^{**}p < 0.001$

Table 3.17 Haematology parameters of animals treated for 60 days (mean ± SEM)

		SJE (mg/kg)		
PARAMETERS	CONTROL	100	300	1000
WBC (k/μL)	9.85±0.54	6.77±0.27*	8.13±1.07	9.20±0.82
LYM (%)	93.57±0.64	92.85±0.82	94.45±0.48	90.02±1.70
MID (%)	5.23±0.52	5.45±0.66	4.18±0.38	7.55±1.24
GRAN (%)	2.41±0.50	1.70±0.16	1.37±0.18	2.42±0.50
RBC (M/μL)	5.00±0.27	4.90±0.18	5.08±0.2319	5.51±0.24
HGB (g/dl)	8.82±0.29	9.62±0.46	10.25±0.31	11.12±0.41**
HCT (%)	25.33±0.73	27.97±1.29	29.62±1.01	32.47±1.12**
MCV (fL)	52.75±0.99	53.77±0.53	52.53±0.63	54.18±0.52
MCH (pg)	18.78±0.42	19.62±0.27	18.82±0.26	19.88±0.22
MCHC (g/dl)	35.32±0.37	36.45±0.18	35.88±0.37	36.60±0.13
RDW (%)	14.83±0.48	14.62±0.15	13.98±0.39	15.58±1.25
PLT (k/μL)	217.50±6.99	187.50±10.51	180.20±5.05	241.70±12.95

Statistically significant difference $^{*}p < 0.05$, $^{**}p < 0.001$

Table 3.18 Haematology parameters of animals treated for 90 days (mean ± SEM)

		SJE (mg/kg)		
PARAMETERS	CONTROL	100	300	1000
WBC (k/μL)	5.98±0.87	6.67±0.87	9.38±0.73	10.32±1.90
LYM (%)	86.98±1.20	86.05±1.14	85.08±1.38	86.80±0.57
MID (%)	9.60±0.62	10.22±0.72	10.63±0.76	9.82±0.59
GRAN (%)	3.42±0.60	3.73±0.52	4.28±0.83	3.38±0.22
RBC (M/μL)	4.87±0.17	5.51±0.31	5.46±0.17	5.48±0.13
HGB (g/dl)	9.90±0.33	11.20±0.47	11.52±0.28*	11.78±0.24**
HCT (%)	27.1±0.96	31.45±1.63	32.97±0.94 **	34.40±0.53**
MCV (fL)	51.63±0.78	51.23±0.91	53.80±0.74	54.14±1.02
MCH (pg)	20.35±0.35	20.07±0.34	21.12±0.30	20.80±0.56
MCHC (g/dl)	39.42±0.13	39.22±0.32	39.30±0.20	38.38±0.52
RDW (%)	14.63±0.31	15.27±0.31	14.82±0.28	14.60±0.23
PLT (k/μL)	145.80±13.08	222.00±26.77	278.20±24.96**	232.00±15.48

Statistically significant difference $^{*}p < 0.05$, $^{**}p < 0.001$

3.8.4 Clinical chemistry

There were few significant observations noted (e.g., Na (P=0.0001), BUN (P=0.0139), Alkaline Phosphatase (P=0.0078) and coronary risk (P=0.0009) for the first month; Na^+ (P=0.0028), BUN (P=0.0091), Anion gap (P=0.0113), Protein-total (P=0.0210), Globulin (P=0.0049), A/G (P=0.0025), D-HDL (P<0.0001) and coronary risk (P<0.0001) for the second month and K^+ (P=0.0367) and coronary risk (P=0.0012) for the third month) as shown in tables 3.19-3.24. Even though coronary risk was significant at a particular dose for each of the months, it was not judged test drug-related because it was not consistent, not related to dose and it is a calculated value and as such will depend on the degree of deviation of cholesterol and HDL from the control value. The cholesterol and HDL did not show any significant difference from the control value. Therefore, none of these changes was judged to be of toxicological significance.

Table 3.19 Serum liver and renal function test (mean ± SEM) following 30 days of exposure to SJE

PARAMETERS		SJE (mg kg^{-1})		
	CONTROL	100	300	1000
Sodium (mmol/L)	151.00±0.97	143.80±1.67*	142.70±1.45**	152.70±1.69
Potassium (mmol/L)	7.12±0.45	7.22±0.37	7.05±0.25	8.55±0.57
BUN (SI) (mmol/L)	7.57±0.22	7.32±0.40	9.27±0.48*	7.90±0.48
Creatinine (SI) (µmol/L)	47.13±1.86	47.17±6.31	50.08±2.95	47.13±3.71
Anion Gap (mmol/L)	30.12±1.04	28.72±2.46	28.38±0.78	32.38±1.04
GOT (AST) (U/L)	304.50±60.02	405.70±74.76	293.00±31.14	347.50±49.93
GPT (ALT) (U/L)	112.30±17.41	115.80±16.46	111.30±6.71	114.50±13.32
Alkaline Phos (ALP) (U/L)	512.70±37.60	327.20±39.29**	421.50±29.56	370.30±31.81
GGT (U/L)	2.50±0.34	1.50±0.22	2.00±0.52	1.67±0.33
Bilirubin-Total (SI) (µmol/L)	5.10±0.62	6.83±0.78	4.82±0.52	6.55±0.83
Bilirubin-Direct (SI) (µmol/L	3.40±0.62	3.97±0.84	2.83±0.57	3.68±0.68
Bilirubin-Indirect (SI) (µmol/L)	1.70±0.00	2.87±0.85	1.98±0.28	2.88±0.85
Protein-Total (SI) (g/L)	79.50±2.05	78.83±2.39	81.67±1.48	82.67±4.51
Albumin (SI) (g/L)	39.17±2.07	36.83±1.20	41.00±0.52	40.67±1.45
Globulin (g/dl)	4.03±0.20	4.20±0.13	4.07±0.16	4.20±0.36
A/G (Ratio)	0.98±0.08	0.88±0.03	1.02±0.05	1.02±0.10
Bun/Crt (Ratio)	40.05±1.35	41.47±4.80	46.55±2.75	42.88±3.96

Statistical significant difference *$p < 0.05$, **$p < 0.001$

Table 3.20 Lipid profile, uric acid and calcium (mean ± SEM) following 30 days of exposure to SJE

PARAMETERS		SJE (mg kg^{-1})		
	CONTROL	100	300	1000
Cholesterol-Total (SI) (mmol/L)	1.92±0.15	2.08±0.16	1.95±0.10	2.03±0.14
Triglyceride (SI) (mmol/L)	0.63±0.07	0.55±0.08	0.60±0.05	0.55±0.04
D-HDL (SI) (mmol/L)	1.03±0.06	0.86±0.05	1.09±0.06	0.94±0.07
VLDL (mg/dl)	11.17±1.25	9.50±1.41	10.50±0.92	9.83±0.83
LDL (mg/dl)	33.50±3.97	46.50±7.00	34.33±2.61	41.67±3.95
Coronary Risk (Ratio)	2.53±0.12	3.37±0.24**	2.48±0.09	2.97±0.07
Uric acid (SI) (μmol/L)	118.80±30.73	119.00±21.73	89.25±13.30	119.00±0.00
Calcium (SI) (mmol/l)	6.10±0.09	2.65±0.08	4.57±0.40	7.03±0.11

Statistical significant difference ** $p < 0.001$

Table 3.21 Serum liver and renal function test (mean ± SEM) following 60 days of exposure to SJE

PARAMETERS		SJE (mg kg^{-1})		
	CONTROL	100	300	1000
Sodium (mmol/L)	158.70±1.67	150.50±1.43**	150.50±1.63**	153.70±1.23
Potassium (mmol/L)	7.27±0.20	6.52±0.24	6.43±0.31	7.30±0.34
BUN (SI) (mmol/L)	8.28±0.25	7.15±0.34	6.55±0.27**	7.40±0.40
Creatinine (SI) (µmol/L)	22.10±1.97	25.05±5.32	20.62±2.95	17.68±2.29
Anion Gap (mmol/L)	41.15±1.88	33.68±1.03*	35.27±1.01	34.13±2.10*
GOT (AST) (U/L)	226.20±29.01	190.00±10.12	177.50±17.14	191.30±8.87
GPT (ALT) (U/L)	158.00±17.94	134.20±4.25	127.20±13.50	139.00±5.97
Alkaline Phos (ALP) (U/L)	492.70±31.46	430.70±50.34	349.50±32.07	469.80±29.62
GGT (U/L)	2.67±0.92	2.00±0.29	1.67±0.33	2.17±0.65
LDH (U/L)	2012.00±41.53	1660.00±140.30	1750.00±153.70	1604.00±82.54
Bilirubin-Total (SI) (µmol/L)	4.25±0.58	4.53±0.36	4.53±0.36	4.53±0.36
Bilirubin-Direct (SI) (µmol/L	1.82±0.36	2.82±0.35	2.27±0.36	2.55±0.38
Bilirubin-Indirect (SI) (µmol/L)	2.27±0.57	1.70±0.00	2.27±0.36	1.98±0.28
Protein-Total (SI) (g/L)	79.33±2.06	74.33±1.73	71.00±1.10*	73.67±1.87
Albumin (SI) (g/L)	37.17±0.79	36.50±0.99	37.67±0.76	37.50±0.96
Globulin (g/dl)	4.10±0.21	3.78±0.10	3.33±0.08**	3.62±0.10
A/G (Ratio)	0.92±0.06	0.95±0.02	1.13±0.03**	1.05±0.02
Bun/Crt (Ratio)	96.88±8.99	92.35±22.68	97.72±19.52	118.80±21.36

Statistical significant difference *$p < 0.05$, **$p < 0.001$

Table 3.22 Lipid profile and uric acid (mean ± SEM) following 60 days of exposure to SJE

PARAMETERS		SJE (mg kg^{-1})		
	CONTROL	100	300	1000
Cholesterol-Total (SI) (mmol/L)	2.08±0.19	2.15±0.11	5.25±3.15	1.55±0.11
Triglyceride (SI) (mmol/L)	1.47±0.09	1.82±0.20	1.72±0.28	1.28±0.11
D-HDL (SI) (mmol/L)	0.63±0.07	0.90±0.07*	0.67±0.04	0.34±0.05*
VLDL (mg/dl)	26.17±1.72	31.50±3.59	29.67±4.94	22.50±1.84
LDL (mg/dl)	37.00±4.26	29.00±2.48	32.50±7.25	28.67±3.51
Coronary Risk (Ratio)	4.63±0.25	3.38±0.26	4.42±0.32	6.80±0.53**
Uric acid (SI) (μmol/L)	89.25±13.30	59.50±0.00	79.33±12.54	59.50±0.00

Statistical significant difference *$p < 0.05$, **$p < 0.001$

Table 3.23 Serum liver and renal function test (mean ± SEM) following 90 days of exposure to SJE

PARAMETERS		SJE (mg kg^{-1})		
	CONTROL	100	300	1000
Sodium (mmol/L)	151.80±2.23	150.50±1.61	147.70±1.50	149.60±5.18
Potassium (mmol/L)	6.20±0.28	6.38±0.25	6.67±0.24	7.36±0.28*
BUN (SI) (mmol/L)	7.73±0.32	7.70±0.46	7.90±0.63	6.90±1.34
Creatinine (SI) (µmol/L)	22.10±4.98	14.73±1.88	23.58±7.80	30.06±12.70
Anion Gap (mmol/L)	37.20±2.16	37.72±2.22	36.67±1.52	38.56±1.59
GOT (AST) (U/L)	206.00±8.91	213.00±16.48	200.50±15.68	224.20±26.79
GPT (ALT) (U/L)	129.50±11.56	141.50±9.29	114.00±6.56	130.20±11.20
Alkaline Phos (ALP) (U/L)	452.3±19.50	486.50±68.13	381.30±52.82	371.60±53.43
GGT (U/L)	1.33±0.21	1.67±0.21	2.33±0.21	3.20±1.07
Bilirubin-Total (SI) (µmol/L)	4.53±0.57	4.53±0.36	4.82±0.52	5.44±0.64
Bilirubin-Direct (SI) (µmol/L	2.27±0.36	2.27±0.36	2.27±0.56	3.40±0.54
Bilirubin-Indirect (SI) (µmol/L)	2.27±0.36	2.27±0.36	2.55±0.38	2.04±0.34
Protein-Total (SI) (g/L)	75.67±2.73	79.83±1.87	74.83±2.73	76.00±2.95
Albumin (SI) (g/L)	42.00±1.24	45.67±2.26	41.50±1.26	44.60±2.18
Globulin (g/dl)	3.37±0.23	3.42±0.19	3.33±0.20	3.14±0.27
A/G (Ratio)	1.27±0.11	1.38±0.12	1.27±0.07	1.46±0.15
Bun/Crt (Ratio)	111.70±25.53	138.90±15.05	117.50±29.65	112.70±46.82

Statistical significant difference *$p < 0.05$

Table 3.24 Lipid profile, uric acid and calcium (mean ± SEM) following 90 days of exposure to SJE

PARAMETERS		SJE (mg kg^{-1})		
	CONTROL	100	300	1000
Cholesterol-Total (SI) (mmol/L)	2.03±0.11	1.95±0.15	5.10±3.18	1.72±0.19
Triglyceride (SI) (mmol/L)	1.03±0.19	1.33±0.30	1.30±0.37	0.98±0.20
D-HDL (SI) (mmol/L)	0.73±0.09	0.80±0.11	0.50±0.06	0.39±0.07
VLDL (mg/dl)	18.33±3.51	22.83±5.21	22.67±6.40	16.80±3.38
LDL (mg/dl)	39.00±4.60	32.83±3.96	37.33±7.07	38.60±7.58
Coronary Risk (Ratio)	4.02±0.34	3.67±0.42	5.50±0.46	6.42±0.58**
Uric acid (SI) (μmol/L)	79.33±12.54	89.25±13.30	89.25±13.30	119.00±0.00
Calcium (SI) (mmol/l)	1.93±0.04	1.88±0.05	1.87±0.04	2.04±0.10

Statistical significant difference **$p < 0.001$

3.8.5 Histopathology

No test drug-related changes were observed in this study. For all the rats in the treated and control groups, the morphological structure of the livers, portal tract, central vein, hepatocytes and hepatic lobule were normal and no necrosis, parenchymal damage or denaturation were found. No infiltration of inflammatory cells was observed in the portal area and no hyperplasia was found in connective tissues (Plate 3.2-3.3). The morphological structure of renal glomerulus was also found normal for each rat in the study. No renal glomerulus, tubules, intestitium, arteriolies or parenchymal renal damage. There were also no thickened of the epithelia of renal capsule (Plate 3.4-3.6). There were no pathologic changes observed for testis. Spermatogenesis and seminiferous tubules were normal (Plate 3.7-3.9).

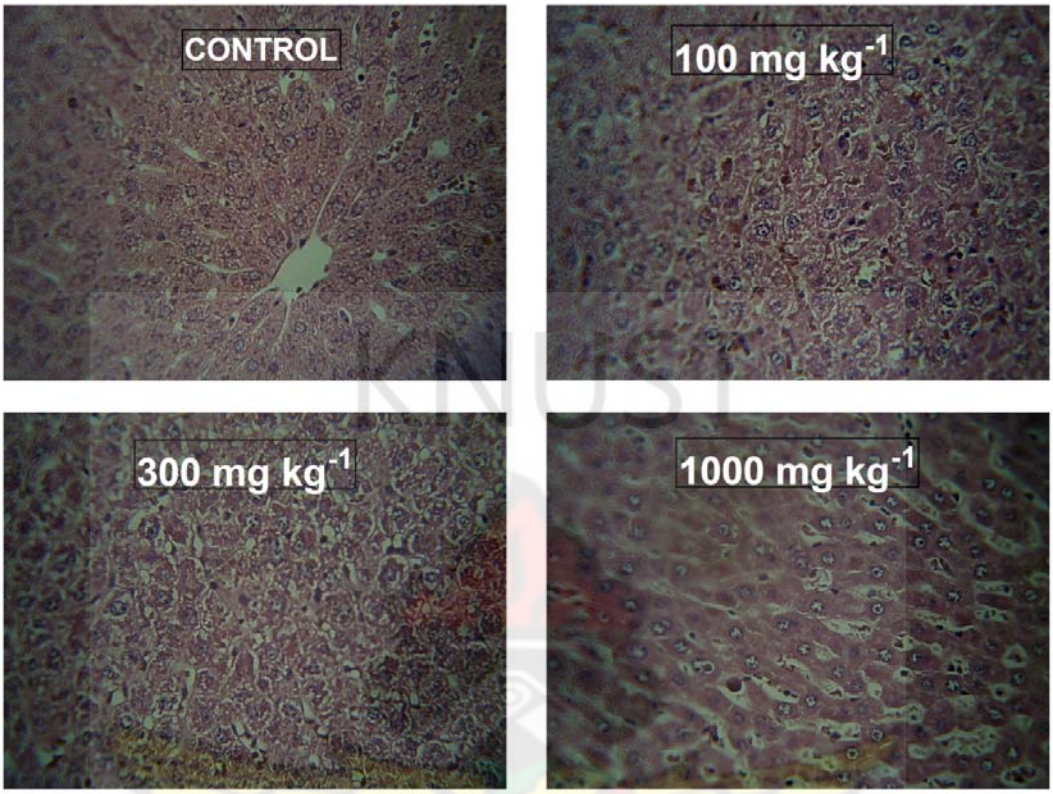

Plate 3.1 Photomicrographs of a transverse section of the liver of control and treated groups for 30 days repeated dose (H & E, x 400).

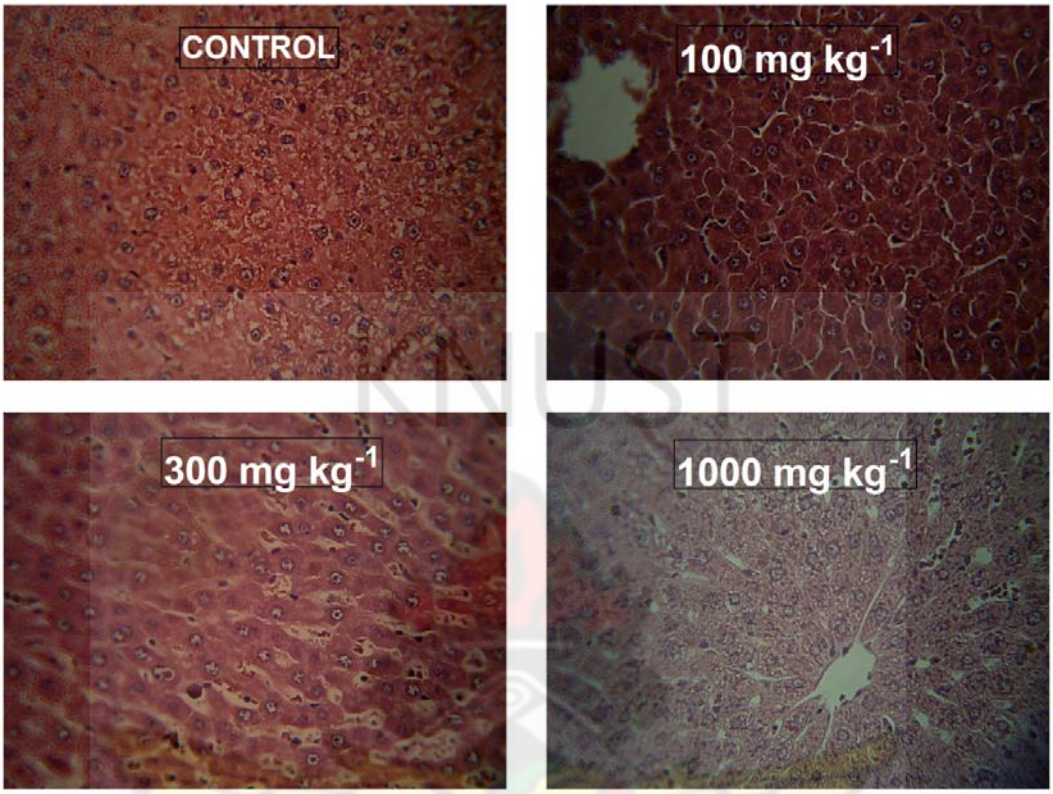

Plate 3.2 Photomicrographs of a transverse section of the liver of control and treated groups for 60 days repeated dose (H & E, x 400).

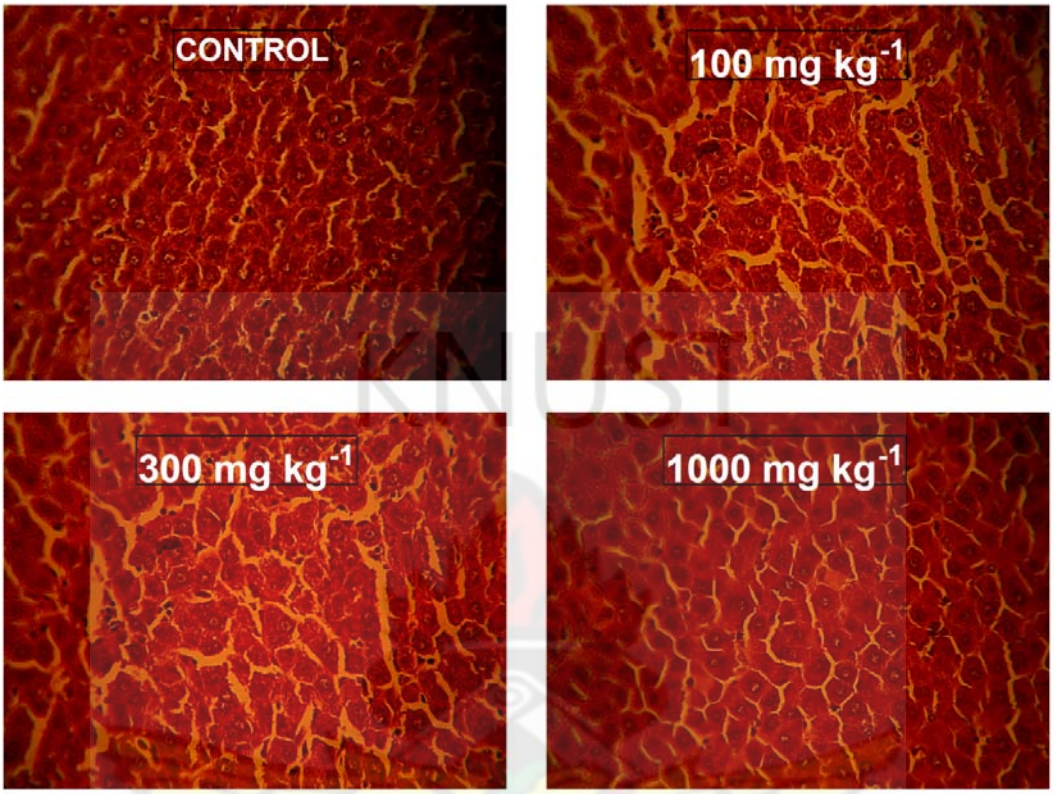

Plate 3.3 Photomicrographs of a transverse section of the liver of control and treated groups for 90 days repeated dose (H & E, x 400).

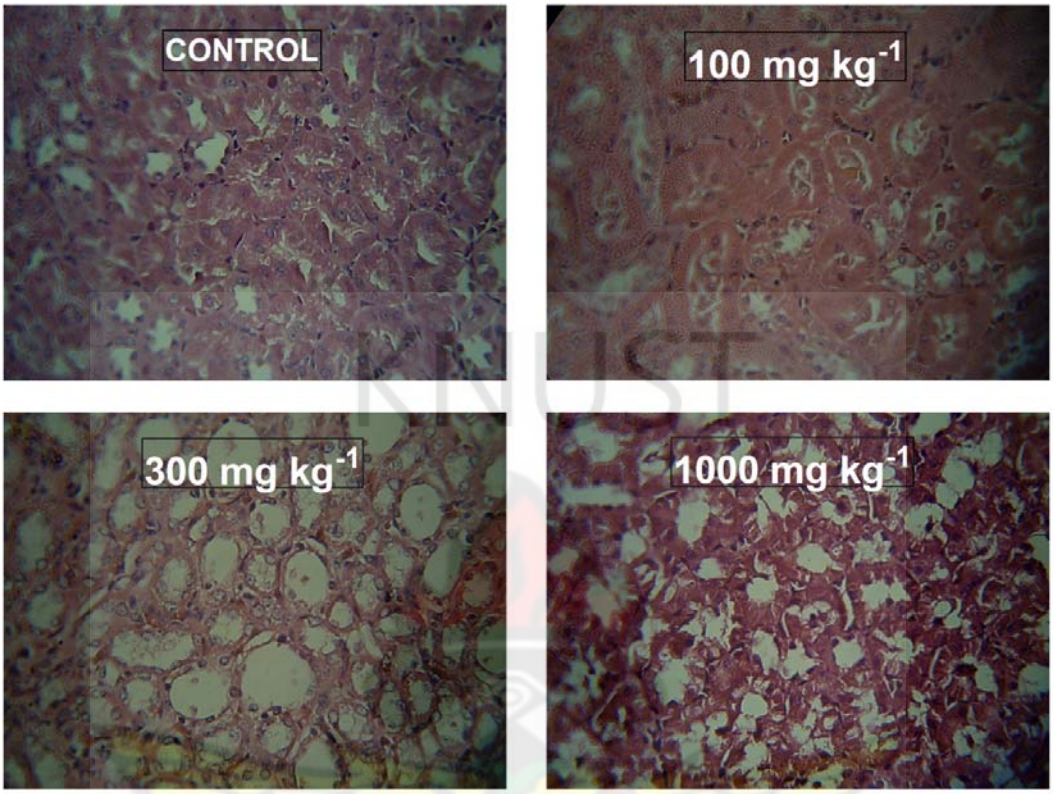

Plate 3.4 Photomicrographs of a transverse section of the kidney of control and treated groups for 30 days repeated dose (H & E, x 400).

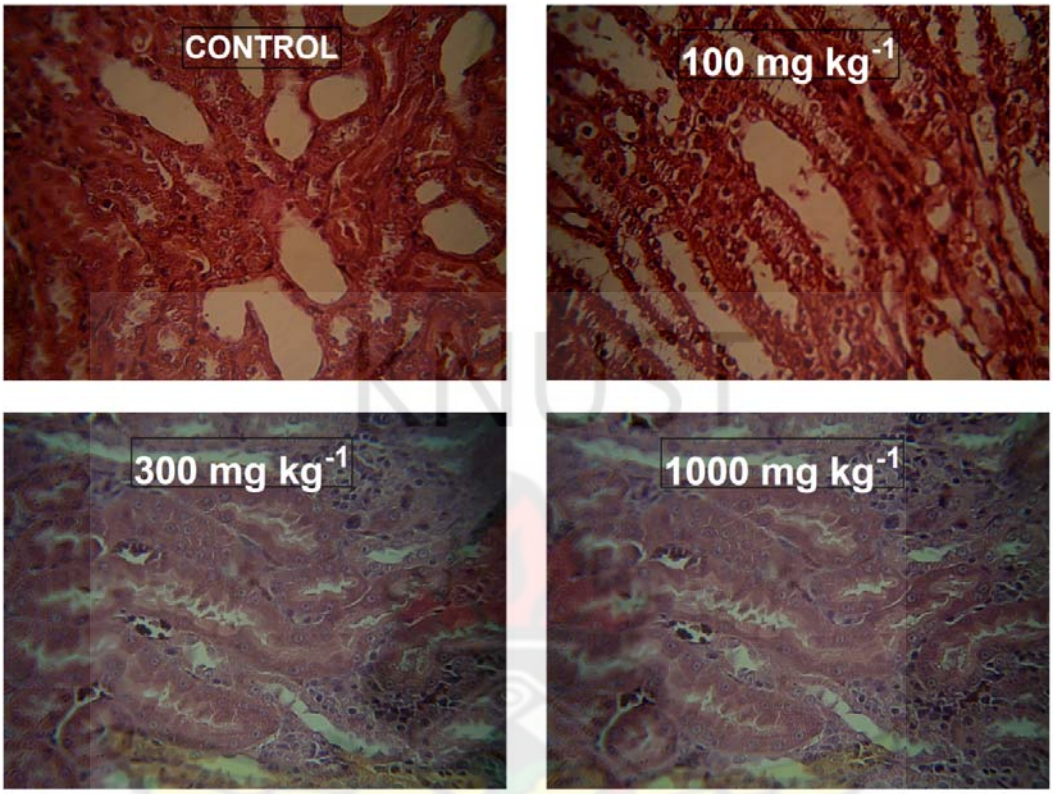

Plate 3.5 Photomicrographs of a transverse section of the kidney of control and treated groups for 60 days repeated dose (H & E, x 400).

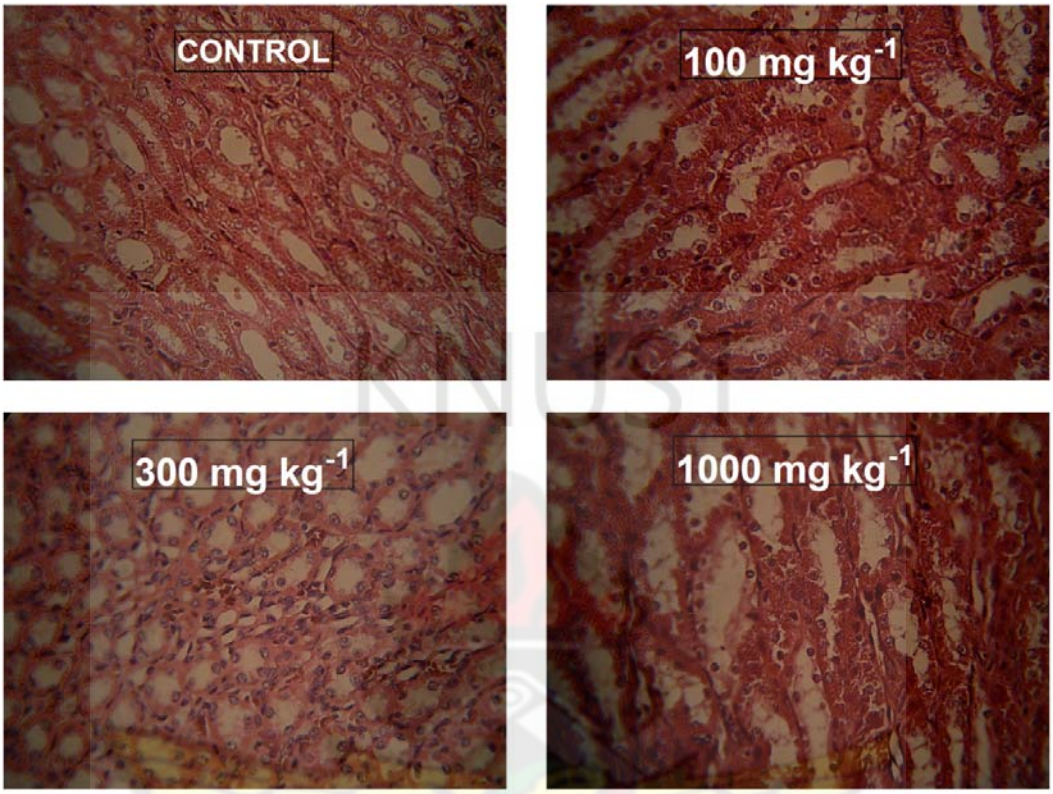

Plate 3.6 Photomicrographs of a transverse section of the kidney of control and treated groups for 90 days repeated dose (H & E, x 400).

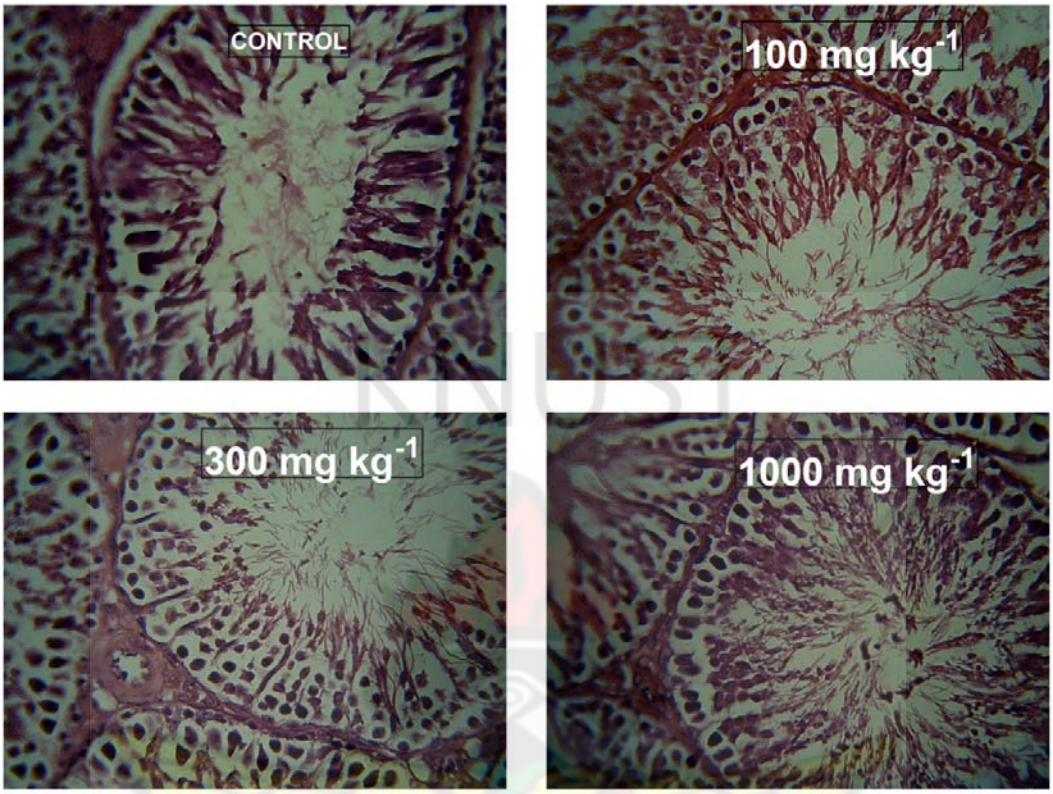

Plate 3.7 Photomicrographs of a transverse section of the testes of control and treated groups for 30 days repeated dose (H & E, x 400).

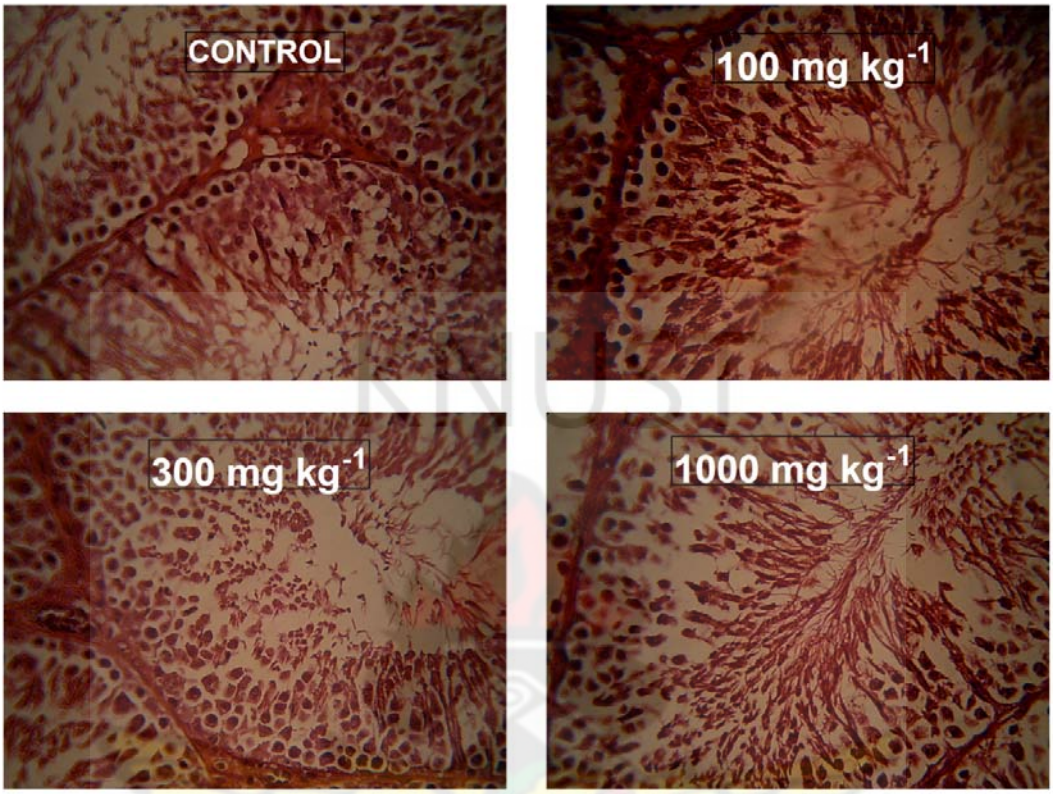

Plate 3.8 Photomicrographs of a transverse section of the testes of control and treated groups for 60 days repeated dose (H & E, x 400).

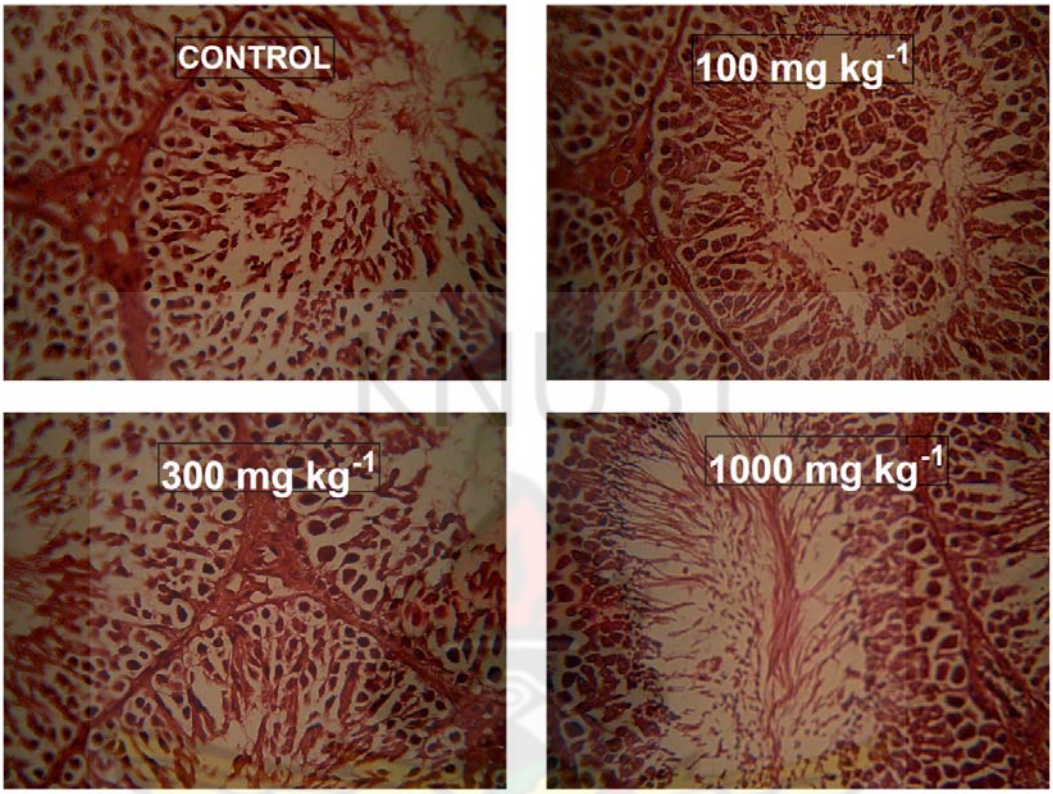

Plate 3.9 Photomicrographs of a transverse section of the testes of control and treated groups for 90 days repeated dose (H & E, x 400).

3.8.6 Ames test

The reverse mutation test showed that no significant increase in the number of revertant colonies occurred in the four *S. typhimurium* strains TA_{97}, TA_{98}, TA_{100} and TA_{102} at any tested concentrations of SJE (Figure 3.32). However, the colonies for the positive controls were five to eleven times more than those for the negative control samples. Similar results were obtained from the duplicate test. No obvious dose–response relationship for SJE was found.

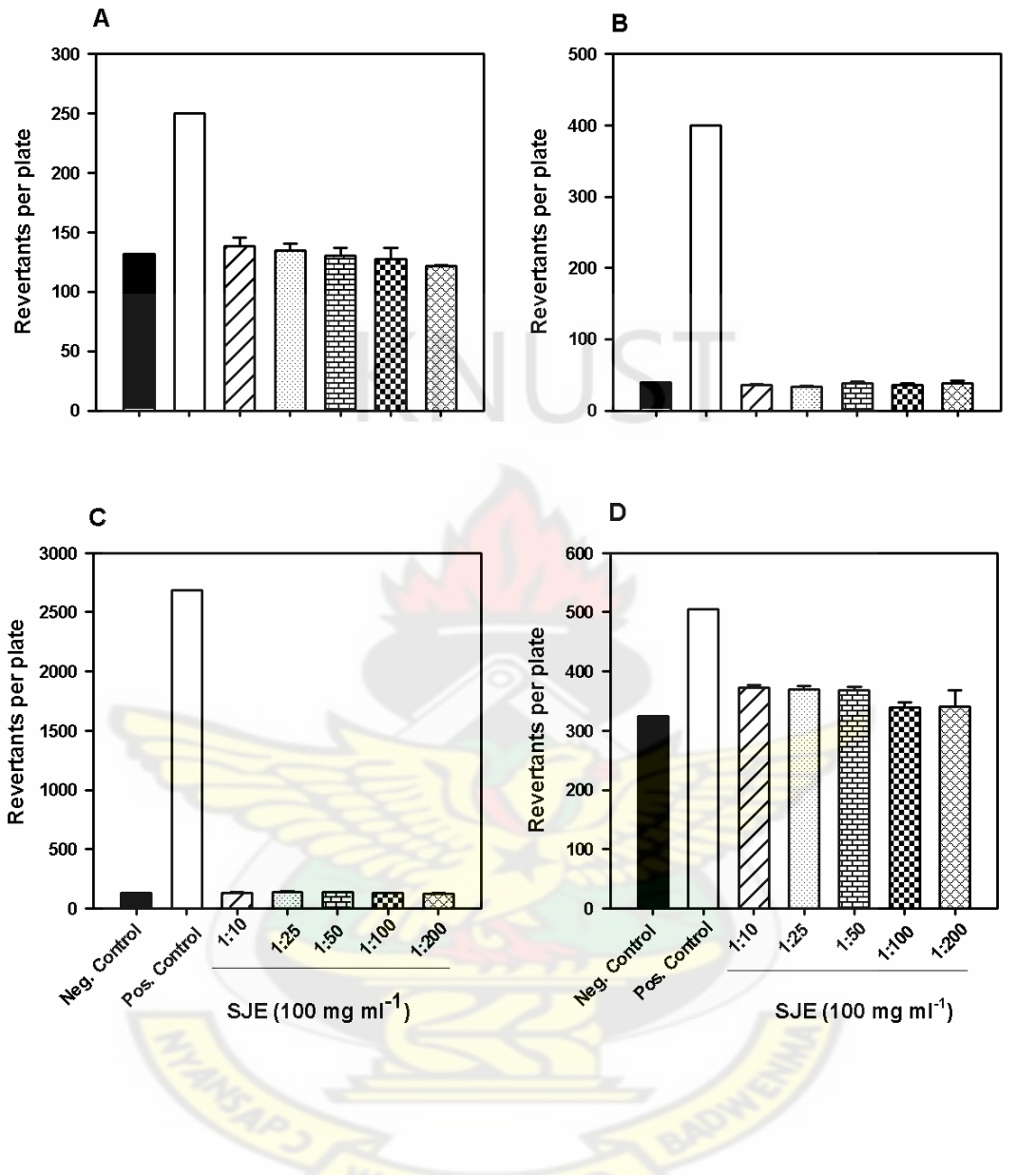

Figure 3.32 Mutagenicity of SJE tested in the presence of rat S9 fractions using the Ames test with S. typhimurium (A) TA97, (B) TA98, (C) TA100 or (D) TA102. The positive control in A is 3.0 µg, B and C is 1.5 µg of Sodium azide per plate and D is 5.0 µg of daunomycin per plate.

Chapter 4

DISCUSSION

4.1 EFFECT OF SJE ON SEXUAL BEHAVIOUR TEST

Roots of *S. jollyanum* are chewed in Ghana as a CNS stimulant and aphrodisiac (Irvine, 1961; Abbiw, 1990). This study aimed at investigating the sexual behavioural effects of an extract of the plant in mice as a means of validating the traditional use. Mice were used for the behavioural experiments since they share many features at the anatomical, cellular, biochemical, and molecular level with humans as well as sharing with humans brain functions, such as anxiety, hunger, circadian rhythm, aggression, memory, sexual behaviour and other emotional responses (Greenwood-Van Meerveld *et al.*, 2005). SJE modified the mice mating behaviour as well as mounting behaviour (orientation activity), the two main determinants for measuring male sexual behaviour (Malmnas, 1973).

4.1.1 Male sexual behaviour in mounting test

In these series of experiments, the males were paired with non-estrus females. Non oestrus females are usually non-receptive and thus number of mounts and attempted mounts are direct effects of the extract on libido (Doherty *et al.*, 1986; Ferrari *et al.*, 1985; Taha *et al.*, 1995). Increases in anogenital sniffing and penile licking are indicative of arousal (Doherty *et al.*, 1986; Ferrari *et al.*, 1985; Taha *et al.*, 1995). Reasons for the U-shaped dose-response curves are not very clear. However, roots of SJE are chewed in Ghana as a CNS stimulant, a property that have been confirmed in murine behavioural model (Woode *et al.*, 2006). Psychostimulants would normally show anxiogenic property in murine model of anxiety (Lapin, 1993; Lister, 1987; Pellow and File, 1985; Varty *et al.*, 2002). Anxiety has been shown to decrease sexual behaviour in rodents (Bale *et al.*, 2001; Barrot *et al.*, 2005; Brien *et al.*, 2002). Though stimulants may be useful in managing erectile dysfunction, they are only effective when the dysfunction is psychogenic. That is when there is hypoactive sexual desire in which case anxiety may lead to excitement inhibition

(Kandeel *et al.*, 2001). Apomorphine, a dopamine agonist and CNS stimulant, stimulates copulatory behaviour in mice and rats and also exhibits a hormetic dose-response curve (Sugiura *et al.*, 1997). Also the extract contains several components so at higher doses the inhibitory components tends to predominate over the stimulatory components. In some instances, the observed effects had worn out by the 3rd hour whilst in some others the effect was more pronounced in the 3rd hour. This may be due to interplay of onset and duration of action of this extract.

4.1.2 Male sexual behaviour in mating tests

In these experiments, the females were rendered receptive by pretreatment with an oestrogen-progestogen combination. The decrease in ML and IL on one hand and the increase in MF and IF on the other hand are suggestive of an improved sexual performance (Ferrari *et al.*, 1985; Ratnasooriya and Dharmasiri, 2000; Taha *et al.*, 1995) and indicate the extract in the present study as a sexual stimulant. Premature ejaculation is one of the important causes of sexual dysfunction, so the assessment of ejaculatory latency in first series (EL1) and in second series (EL2) were studied (Gauthaman *et al.*, 2002). The study revealed that the SJE administered to the male mice, showed a significant delayed initiation of the second series of copulation. Post-ejaculation interval (PEI) is considered as an index of potency and libido, and also a parameter of the rate of recovery from exhaustion after first series of mating (Gauthaman *et al.*, 2002). The significant decrease in this parameter (Figure 3.3) indicates that, the extract decreased PEI either by enhancing the potency and libido or by producing lesser exhaustion in the first series of mating or both.

Phytochemical screening shows that SJE contains alkaloids, terpenoids and flavonoids. Philardeau and Debray, (1965) confirmed the presence of an alkaloid that corresponded to berberine iodide and berberine has been shown to exert direct effects on penile tissue that could contribute to the copulatory performance-enhancing actions of SJE (Chiou *et al.*, 1998). Also, Moody *et al.,* (2006) isolated two furanoditerpene constituents of SJE (Isocolumbin and columbin) and other plants

of the same family Menispermaceae with similar constituents are used for their reputed aphrodisiac properties worldwide. *Tinospora cordifolia* belong to the family Menispermaceae like SJE and are used to enhance libido and sexual function (Singh *et al.*, 2002; Tharakan and Manyam, 2005).

Apart from the desire that is essential for initiation of sex, penile tumescence and rigidity as well as the accessory muscles that help in providing additional penile rigidity and ejaculation are dependent on testosterone for normal sexual activity (Gauthaman *et al.*, 2002).

4.1.3 Effect of SJE on hormonal levels

The levels of reproductive hormones were measured in an attempt to elucidate the possible mechanism of the extract. The increase in testosterone (Figure 3.4d) confirms the findings of Raji *et al.*, (2006) who also reported that, a methanolic extract of SJE caused a dose-dependent significant reduction in count, motility and viability of spermatozoa in albino rats.

Plasma levels of androgens and luteinizing hormone (LH) offer information on the physiological situation that the treatment mediates. The notion that testosterone production by Leydig cells is primarily under the control of the pituitary gonadotrophin luteinizing hormone (LH) has been documented for several years (Catt *et al.*, 1980; Desjardins, 1981; Huhtaniemi *et al.*, 1982). Besides that, LH secretion is in pulses and the frequency and amplitude of these pulses is crucial for proper activation of gonadal function (Bannister *et al.*, 1986; Bartke *et al.*, 1973; Steiner *et al.*, 1982; White *et al.*, 2007). In the rat, earlier studies have indicated that there is no direct relationship between the trains of LH pulses and the initiation of testosterone secretory episodes, with often an active LH secretory period being dissociated from the testicular response (Ellis and Desjardins, 1982; Pierroz *et al.*, 1999; Sodersten *et al.*, 1983). The lack of correlation between LH and testosterone production is in conformity with earlier studies, which has shown that the production of testosterone in rat differs from that seen in humans, as more direct

testosterone responses were observed in male subjects after trains of LH peaks (Spratt *et al.*, 1988). Nevertheless, in humans, testosterone secretion lags behind LH secretion by 40 min (Spratt *et al.*, 1988).

Male sexual behaviour and erection are dependent on testosterone that may act both centrally and peripherally (Mills and Sethia, 1996). Testosterone supplementation has been shown to improve sexual function and libido (Aversa and Fabbri, 2001) as well as intensifying orgasm and ejaculations (Morales *et al.*, 1996). Though androgens are known to influence male masculine behaviour, it has been observed that in many animals this behaviour requires weeks, and in some cases longer, to extinguish after castration (Crews, 1983; Meisel and Sachs, 1994), suggesting that some aspects of masculine sexual behaviour can be maintained in a steroid-independent manner. It is well established that dopamine released from the medial preoptic area (MPOA) is essential for activation of adult male sexual behaviour in rats and mice (Cowan, 1992; Hull *et al.*, 1997; Hull *et al.*, 1999; Kudwa *et al.*, 2005). Furthermore, it is postulated that testosterone may increase DA release by upregulating nitric oxide synthase, which produces nitric oxide, which in turn increases DA release (Hull *et al.*, 1999). As stated earlier, the roots of the plant are chewed as a CNS stimulant in Ghana and its anxiogenic properties have been shown in a preliminary report in mice in elevated plus maze and open field behavioural models (Woode *et al.*, 2006). This finding supports the possible involvement of central neuronal circuits in the actions of SJE.

4.2 IN VITRO EXPERIMENT ON ISOLATED TISSUE

4.2.1 Effects of SJE on isolated rat anococcygeus

The degree of contraction of cells of smooth muscle determines the lumen in blood vessels and airways as well as the propulsive function of the gastrointestinal and genitourinary tract. Abnormalities in the contraction are related to a variety of clinical conditions including male erectile dysfunction. For instance, the degree of contraction of corpus cavernosal smooth muscle determines the functional states of

penile flaccidity, tumescence, erection and detumescence (Andersson and Wagner, 1995). Contraction of cavernosal smooth muscle depends on a balance between contractant and relaxant factors which are controlled by both central and peripheral mechanisms, and involve many transmitters and transmitter systems (Andersson, 2001a; Moreland *et al.*, 2001). Substances that act to control contractility of smooth muscle are useful in the treatment of disorders due to contraction abnormalities in such muscles.

Smooth muscle can relax through: (a) increase of permeability of the membrane to potassium; (b) mobilization of ions of calcium; (c) increase of the cyclical nucleotides (cGMP) and cAMP); (d) direct action on the contractile proteins; and (e) reduction of sensibility to calcium (Barreto, 2002; Cox, 1990). SJE caused a dose-dependent relaxation of isolated rat anococcygeus smooth muscles. Its inhibitory effect at α-adrenergic sites (indicated by antagonism of PE-induced contraction) lends some credence to its use as an aphrodisiac. This is because, blockade of α-adrenergic pathway has been shown to be a mechanism by which some aphrodisiacs resolve problems of erectile dysfunction (Andersson, 2001b). However, non-surmountable antagonism was observed with SJE at the other receptor sites studied. Since SJE blocked non-specifically, the contraction of anococcygeus produced by all the agonists tested, it was speculated to inhibit a common pathway in the signal transduction mechanism.

Further more, SJE relaxed in a concentration-dependent manner, maintained contractions of the anococcygeus evoked by a solution containing 80 mM K^+ (Figure 3.5). Potential sensitive calcium channels are activated by depolarization of the plasma membrane when the extracellular K^+ concentration is increased. Several evidences revealed that, the relaxant effect of a calcium channel blocker becomes more pronounced when smooth muscle is depolarized (Beavo, 1995; Bender and Beavo, 2006). Thus SJE could probably relax the maintained contraction induced by potassium through the Ca^{2+}-dependent voltage channel modulation. Coupled with

the fact that, inhibitory effects of SJE were non-specific (at both α-adrenergic and cholinergic receptor sites); the possibility that the inhibitory action of the extract might be through some calcium-regulated mechanism was therefore explored.

To confirm this hypothesis, another experimental procedure analyzed the relationship between the influx of Ca^{2+} and the contraction it produced. The protocol consisted of pre-incubating the muscle with a solution free of Ca^{2+}, to stimulate it with a high-potassium solution free of Ca^{2+}, and to increase gradually the solution of Ca^{2+} in the bath. In this study, the rightward displacement of the concentration-response curve and the depression of the maximum response to Ca^{2+} by SJE suggest that the interaction between SJE and Ca^{2+} may not be competitive. Signifying that one of the actions probably involved in the relaxation induced by SJE is a Ca^{2+}-dependent voltage channel blockade.

4.2.2 Effects of SJE on rabbit corpus cavernosum

SJE showed a potent relaxing effect on the phenylephrine induced contraction in isolated rabbit corpus cavernosum tissue (Figure 3.10). Phenylephrine-stimulated contraction of corporal smooth muscle is mediated by the activation of α-adrenergic receptors. Activation of α-receptors stimulate contraction through the translocation of extracellular calcium through receptor operated channels and through the release of intracellular calcium from the sarcoplasmic reticulum (SR) (Levin *et al.*, 1997; Saito *et al.*, 1996). Corporal smooth muscle relaxation plays a critical role in erection. Smooth muscle relaxation is mediated by nitric oxide (NO) which, during sexual stimulation, is synthesized in the nerve terminals of parasympathetic nonadrenergic, non-cholinergic (NANC) neurons in the penis and also by the endothelial cells lining blood vessels and the lacunar spaces of the corpora cavernosum (Burnett, 2006; Burnett, 1995; Trigo-Rocha *et al.*, 1994). Nitric oxide is considered the principal stimulator of cavernosal smooth muscle relaxation (Escrig *et al.*, 2002) however, the inhibition of vasoconstrictors (that is, norepinephrine and endothelin-1) cannot be ignored as a potential regulator of

penile erection (Chitaley *et al.*, 2001). Since SJE causes relaxation of agonist induced contractions of CSM (Figure 3.10), this direct effect exerted on the penile tissue by SJE could also contribute to its copulatory performance-enhancing actions.

The overall nonspecificity of SJE's blockade of the explored receptor types is not surprising considering that plant extracts contain several chemical agents with a multiplicity of pharmacological activity (Aherne *et al.*, 2007). The alkaloid berberine has been isolated from this plant (Philardeau and Debray, 1965) and is known to have a variety of unique pharmacological effects: antimicrobial, diuretic, smooth muscle relaxant and cardiac depressant activities (Bruneton, 1995). A very detailed study on the relaxation of the corpus cavernosum showed that berberine possesses a relaxant effect on the rabbit corpus cavernosum tissue which is attributed to both endothelial-dependent and –independent properties (Chiou *et al.*, 1998). It was pointed out that, while papaverine appears to be more effective than berberine in inducing penile erection, the duration of tumescence caused by berberine is considerably longer.

Besides, it is possible that SJE acts peripherally by ultimately antagonising Ca^{2+} because it contains flavonoids which have been shown to have Ca^{2+} mobilisation effects (Duan and Tang, 1996; Kempuraj *et al.*, 2005). The calcium inhibitory property of SJE (Figure 3.9) suggests that the mechanism by which it exerts its smooth muscle relaxant effects are mediated partly by inhibition of calcium mobilization. This may perhaps justify the use of SJE as a spasmolytic agent in traditional medicine (Moody *et al.*, 2006).

4.3 NEUROPHARMACOLOGICAL EXPERIMENTS

Sexual desire may be affected directly by increasing serum testosterone levels or by having a testosterone-like effect, or indirectly by affecting behavioural depression, stress (Kumar *et al.*, 2001), anxiety (Rowland *et al.*, 1987) and sedation (Ratnasooriya and Jayakody, 2000). Improvement of depression by selective

serotonin reuptake inhibitors in depressed patients has been associated with improvement in sexual function (Ekselitus and von Knorring, 2001; Michelson *et al.*, 2001).

4.3.1 Effects of SJE on anxiety

In this work, the effect of SJE was studied in several behaviour animal models to evaluate possible central activity. There are numerous animal models of anxiety-like behaviour. The relevance of these models to human anxiety disorders is derived first and foremost from pharmacological validity. Benzodiazepine, an anxiolytic drug, reduce the level of anxiety-like behaviour in virtually every animal model of anxiety (Borsini *et al.*, 2002) whereas yohimbine and caffeine are well known as an anxiogenic agents in rodents in the social interaction and elevated plus-maze (Baldwin *et al.*, 1989; Bhattacharya *et al.*, 1997; Lister, 1987; Pellow *et al.*, 1985a).

The elevated plus-maze, open field, hole board and light/dark box tests allow behaviour in a conflict situation to be examined. The validity of these measures as a model of anxiety has been confirmed pharmacologically, ethologically and physiologically. The elevated plus-maze, a bidirectional test is based on the natural aversion of rodents for open spaces and height (Lister, 1987). The open field allows examination of behaviour related to the aversion of rodents to an open brightly lit area (Asano, 1986; Whimbey and Denenberg, 1967) and the hole board test has been claimed to be suitable to assess directed exploratory behaviour, since it allows the discrimination between exploratory activity and nonspecific motor activity in rodents (Lister, 1987; Wei *et al.*, 2007). The light/dark test is based on the conflict between the inherent tendency of mice to explore a novel environment against their natural avoidance of a brightly lighted open field (Blumstein and Crawley, 1983). All these models were used to examine differences in anxiety-like state of mice treated with SJE.

Overall, the current data demonstrate that there are marked differences in the behaviour of diazepam treated animals as compared to caffeine and SJE treated animals in all the behavioural tests model used. Also, these differences show consistency between the tests. Briefly, an anxiolytic drug, diazepam (Borsini *et al.*, 2002), displays a much lower degree of anxiety-like behaviour and much higher locomotor activity. On the other hand, the caffeine, a well known anxiogenic agent in rodents (Baldwin *et al.*, 1989; Bhattacharya *et al.*, 1997; Lister, 1987; Pellow *et al.*, 1985a) and SJE treated group display a much higher degree of anxiety-like behaviuor and lower locomotor activity. These data are now considered in more detail.

The EPM consists of an elevated, plus-shaped platform, two arms of which are enclosed by high walls. The other two arms are unenclosed or surrounded by a small lip. Consistent with the idea that the open arms are known to provoke an anxiety-like behavioural state, mice exposed to the maze will make fewer entries into and spend less time in the open arms than the closed arms (Lister, 1987; Pellow *et al.*, 1985b). In agreement with this study, benzodiazepines are known to increase the number of open-arm entries and time spent in the open arms, whereas caffeine and other anxiogenic agents decrease these parameters (Lister, 1987; Pellow *et al.*, 1985b; Pellow and File, 1986). SJE like caffeine induced anxiogenic-like behaviour in this experiment. Ethological analysis has demonstrated that animals perform more defensive and anxiety-related behaviours in the open arms than in the closed arms (Cruz *et al.*, 1994; Pellow *et al.*, 1985b). In this study, the diazepam-treated mice are displayed a much lower level of anxiety-like behaviour and had a much greater capacity for 'risk-taking' behaviour as compared to caffeine and SJE treated mice with higher level of anxiety and lower capacity for 'risk-taking' behaviour.

Data in the literature demonstrate that decrease in spontaneous motor activity gives an indication of the level of excitability of the CNS (de Sousa *et al.*, 2005). Increase in locomotor activity as well as the increase in time spent on the open arms, can be interpreted has less emotional reactivity and perhaps increased impulsivity (diazepam treated mice) and decrease in these parameters means more emotional reactivity and perhaps decreased impulsivity (Escorihuela *et al.*, 1999; Liebsch *et al.*, 1998). Animals with increased anxiety had higher plasma corticosterone concentrations and produced more fecal boli than those with lower anxiety (Pellow *et al.*, 1985). However, fecal boli did not give consistent results from this study (Table 3.6 and 3.7).

With regards to ethological variables measured, there was a significant dose-dependent increase in the number of head dips performed by diazepam-treated mice. Caffeine and SJE mice showed dose-dependent decrease in the number of head dips. Increase in the number of head dips is an indication of a low-anxiety state and decrease indicates high-anxiety state (Cruz *et al.*, 1994; Griebel *et al.*, 1997). Stretch attend posture (SAP), a behaviour sometimes seen to decrease in animals given anxiolytic drugs (Cruz *et al.*, 1994; Griebel *et al.*, 1997), was consistent with our results of diazepam. However, the observed lower level of SAP in the caffeine and SJE treated mice is inconsistent (Figure 3.13 and 3.16).

Rearing activity in rodents is described as a complex pattern of stereotyped behaviour (de Sousa *et al.*, 2005). Rearing was lower in all the treated groups (diazepam, caffeine and SJE), as observed in the EPM. This is surprising as it is a behaviour that has been claimed to be reflective of increased motility (Cruz *et al.*, 1994; Griebel *et al.*, 1997) and is, therefore, inconsistent with the observed high locomotor activity of the diazepam-treated mice in the EPM.

Lower anxiety-like behaviour of diazepam-treated mice was similarly observed in the open field, where the mice spent more time at the center (Table 3.12). This may

indicate increased risk taking by the diazepam-treated mice, as the caffeine and SJE (anxiogenic-like behaviour) tended to remain in the corner, the 'safer' environment nearest to the high surrounding wall of the open-field arena. These results confirm the suitability of the method used in the present study and agree with previous literature data (Lister, 1987; Wolfman *et al.*, 1994; Yasumatsu *et al.*, 1994).

The light/dark box test, is a model in which benzodiazepines produced a facilitation of exploratory behaviour between an illuminated open field and a dark enclosure (Crawley and Goodwin, 1980). Mice placed in the white area would generally move around the periphery until they found an opening, at floor level, to enable access to the black compartment. The essential feature was the measurement of the time spent in each compartment (Bourin and Hascoet, 2003; Crawley and Goodwin, 1980). Bourin and Hascoet, (2003) found that increased exploratory behaviour was associated with an increased number and time spent in the illuminated area. In this procedure, decrease in the first time entry from the white chamber to the black chamber (latency) reflects the aversive properties of the brightly lit area, an indication for anxiogenic effect. Unexpectedly, all the drugs influenced this parameter in the same way. However, this effect might be non-specific. Nevertheless, the behaviour observed using the light/dark test in the present study confirmed the anxiolytic activity of diazepam (increased time spent in lit area) as reported previously (Corda and Biggio, 1986) and anxiogenic-like effects of caffeine and SJE (decreased time spent in the lit area).

The hole-board test is a measure of exploratory behaviour in rodents (File and Wardill, 1975a). In addition, head dips as well as duration of head dipping of mice observed in the hole board experiments have been accepted as a parameter for the evaluation of anxiety conditions in animals. In this model, non-sedative doses of benzodiazepines and other anxiolytic drugs have been reported to increase head dips as well as its duration in mice while their antagonists decrease it (Crawley, 1985). In the present experiments, compared to controls, diazepam-treated animals

manifested significant dose dependent increase in the number of head dips and its duration indicating an anxiolytic-like effect (Figure 3.20). Biochemical and electrophysiological studies show that benzodiazepine anxiolytics facilitate GABAergic transmission in the central nervous system via positive allosteric modulation of the $GABA_A$ receptor complex in a dose-dependent manner. Low dose of benzodiazepines enhance and higher doses antagonize GABAergic transmission (Rao *et al.*, 1999; Sieghart, 1995; Tallman *et al.*, 1980). Thus the diazepam-induced increase of mouse head dips in hole board test may be a result of facilitation of GABAergic transmission.

Caffeine (anxiogenic drug) and SJE however, decreased the number and duration of head dipping (Figure 3.20). Increase in the number of head dips and duration of head dipping is an indication of a low-anxiety state and decrease indicates high-anxiety state (Cruz *et al.*, 1994; Griebel *et al.*, 1997). Blockade of both A_1 and A_{2A} receptors is the most probable mechanism for the anxiogenic activity of caffeine, since caffeine is a non-selective antagonist of adenosine receptors, and adenosine can activate only A_1 and A_{2A} receptors at physiological concentrations (Fredholm *et al.*, 1999). Activation of A_1 receptors decreases the release of several neurotransmitters such as glutamate (Fredholm and Dunwiddie, 1988), serotonin (5-HT) (Feuerstein *et al.*, 1985), and noradrenaline (Jonzon and Fredholm, 1984), which may be involved in anxiety-related brain circuits. The selective adenosine A_1 receptor agonist, N^6-cyclopentyladenosine, has anxiolytic activity in mice (Jain *et al.*, 1995). Moreover, mice lacking A_1 receptors were found to have increased anxiety-related behaviour (Johansson *et al.*, 2001). Adenosine A_{2A} receptors, in contrast to A_1 receptors, tend to increase neurotransmitter release, such as 5-HT (Okada *et al.*, 2001), glutamate (Popoli *et al.*, 1995), and γ-aminobutyric acid (Mayfield *et al.*, 1993). However, A_{2A} receptor knockouts mice like A_1 receptor knockout, had higher anxiety scores (Ledent *et al.*, 1997). Therefore, both adenosine receptor subtypes might be involved in the anxiogenic effects of caffeine.

Like caffeine, SJE in a preliminary report induced anxiety-like behaviour in laboratory animals (Woode *et al.*, 2006). It should be remembered that, in Ghana, the root of *S. jollyanum* is chewed as a central nervous system (CNS) stimulant and aphrodisiac (Abbiw, 1990; Irvine, 1961) and we have recently established its aphrodisiac effects (Owiredu *et al.*, 2007) where SJE caused an increase in testosterone level (Owiredu *et al.*, 2007; Raji *et al.*, 2006). It is postulated that testosterone may increase dopamine (DA) release by upregulating nitric oxide synthase, which produces nitric oxide, which in turn increases DA release (Hull *et al.*, 1999). Administration of pyschostimulant amphetamine and apomorphine to rodents, which released both dopamine and norepinephrine, causes a cessation of normal rodent behaviour (exploration and grooming) and possesses anxiogenic-like effect (Cancela *et al.*, 2001; Silva *et al.*, 2002) unrelated to external stimuli. These effects are prevented by dopamine antagonist, and by destruction of dopamine-containing cell bodies in the midbrain, but not by drug that inhibit the noradrenergic system (Cancela *et al.*, 2001; Silva *et al.*, 2002). The anxiogenic effect of SJE could be due to an interaction with monoamine oxidase neurotransmission.

4.3.2 Antidepressant property of SJE

The brain area most associated with sexual behaviour is the limbic system. Research with various animal and human models indicates a relationship between brain monoamines and sexual behaviour (Ramachandran *et al.*, 2004). Both dopamine and 5HT are implicated in depression. The relationship of dopamine to human sexual behaviour is supported by reports of per sexuality behaviour induced by L-dopa in parkinsonian patients. Stimulants and antidepressants are known to effect libido, erection, ejaculation and orgasm.

In the present study, SJE (100 - 1000 mg kg^{-1}) was evaluated for possible antidepressant effects. SJE produced significant antidepressant-like effect in mice in both FST and TST (Figure 3.25). The effect of SJE in the FST and TST was similar to the effect produced by the oral administration of fluoxetine and imipramine,

used as control (Figure 3.25). However, it had lower efficacy than fluoxetine and imipramine at the doses used. Both models of depression are widely used to screen new antidepressant drugs (Porsolt, 1979; Steru *et al.*, 1985). These tests are quite sensitive and relatively specific to all major classes of antidepressant drugs including tricyclics, serotonin-specific reuptake inhibitors, MAO inhibitors, and atypicals (Porsolt, 1979; Steru *et al.*, 1985). In FST, mice are forced to swim in a restricted space from which they cannot escape, and are induced to a characteristic behaviour of immobility. This behaviour reflects a state of despair which can be reduced by several agents which are therapeutically effective in human depression. The TST also induces a state of immobility in animals like that in FST. This immobility, referred to as behavioural despair in animals, is claimed to reproduce a condition similar to human depression (Porsolt, 1979; Steru *et al.*, 1985; Willner, 2005). Drugs with antidepressant activity reduce the time during which the animals remain immobile (Porsolt, 1979; Steru *et al.*, 1985; Willner, 2005).

SJE did not cause alteration in motor coordination on the rotarod test (Figure 3.24), suggesting that the decreased locomotor activity observed earlier in EPM, OF and HB may not be exerted through peripheral neuromuscular blockage. The antidepressant-like effect of SJE also seems not to be associated with any motor coordination effects, since it had no effect on motor coordination of mice as assessed by rotarod test. This indicates that motor coordination activity was not involved in the antidepressant-like action of the SJE in the FST and TST and confirms the assumption that the antidepressant-like effect is specific.

Though, the precise mechanism by which SJE produced antidepressant-like activity is not yet known. The monoamine theory, proposed in 1965, suggests that depression results from functionally deficient monoaminergic (norepinephrine (NE) and/or serotonin (5-HT)) transmission in the CNS. Prior depletion of these neurotransmitters would be expected to block their behavioural effects. Hence, studying the effects of monoamine depletions on antidepressant activity is an

important strategy for directly assessing the relative contribution of specific neurotransmitters to the behavioural effects of different types of antidepressant.

The aim of the present study was therefore to systemically assess the relative contribution of monoamine neurotransmitters, either 5-HT or NE and dopamine (DA), to the behavioural effects of SJE in TST. This study examined the role of NE and DA in the acute behavioural effects of SJE in the TST by using drugs that interfere with neurotransmitter synthesis or release.

The results from the present study indicate that depletion of NE and DA with MeDOPA increased baseline immobility and attenuated the effects of imipramine (non-selective inhibitor of NE and DA reuptake) but not fluoxetine (selective inhibitor of 5HT) and the extract SJE in the TST. MeDOPA when administered is decarboxylated and hydrolysed to α-methylnorepinerphrine which is a false transmitter with a stronger effect on presynaptic α_2 receptors so the autoinhibitory feedback mechanism operates more strongly than normal thus reducing transmitter release below normal levels (Rang *et al.*, 2003). MeDOPA preferentially inhibits the formation of newly synthesized pools of DA and NE (O'Leary *et al.*, 2007; Weissman *et al.*, 1966), so as expected, the effects of imipramine was blocked completely by the depletion of NE and DA by the MeDOPA but the antidepressant effects of fluoxetine and SJE were still significant. This can possibly be explained by the fact that NE and DA may be more important for regulating the effect of imipramine than that of fluoxetine and SJE. The findings of the present study with MeDOPA agree with the results of catecholamine depletion studies by Delgado who showed that MeDOPA caused relapse in depressed patients that were successfully treated with NRIs but not SSRIs (Delgado *et al.,* 1993; Miller *et al.,* 1996).

Pretreatment with reserpine, which is known to disrupt vesicular storage and release of monoamine neurotransmitters (Corrodi and Hanson, 1966), increased baseline immobility and attenuated the effects of imipramine in the TST but did

not affect that of SJE and fluoxetine. Reserpine has also been shown to cause a substantial depletion of 5-HT (O'Leary *et al.,* 2007) but then pretreatment with reserpine did not completely block the response to fluoxetine. This can be explained from the fact that, disruption of vesicular storage and release of 5-HT by reserpine may be less effective than inhibition of transmitter synthesis at preventing 5HT-mediated behaviours (O'Leary *et al.,* 2007). In addition, pretreatment with reserpine did not completely block the response to SJE also which further strengthens the role of NE, DA and 5-HT in the effects of SJE.

When MeDOPA and reserpine were given together to inhibit synthesis and deplete vesicular pools of NE, DA and to some extent 5HT (O'Leary *et al.*, 2007), this combination was realized to be capable of completely blocking the effects of the imipramine because reserpine + MeDOPA completely prevented the behavioural effects of imipramine whereas imipramine was still active in reducing the immobility periods though not significant after reserpine was given alone. It is also unlikely that depletion of 5-HT contributed to the blockade of the behavioural effects of imipramine, as previous study by O'Leary *et al.*, (2007) showed that similar reductions in 5-HT tissue content by 5HT synthesis inhibitors such as parachlorophenylalanine did not prevent the behavioural effects of other NRIs in the TST. Taken together, the present studies suggest that depletion of both vesicular and cytoplasmic pools of NE and DA is required to completely prevent the behavioural effects of imipramine.

The effects of fluoxetine and SJE were also blocked by the reserpine + MeDOPA combination to some extent (Figure 3.26 and 3.27). An important observation of these studies was that pretreatment with reserpine alone did not block the effects of the SSRI and the extract neither did the pretreatment with MeDOPA alone. Fluoxetine, a selective serotonin reuptake inhibitor has also been shown to increase dopamine and norepinephrine extracellular levels in the hypothalamus,

the cortex and the prefrontal cortex of the brain (Gobert *et al.*, 1997; Perry and Fuller, 1997; Pozzi *et al.*, 1994; Pozzi *et al.*, 1999). The increase in NE and DA as well as 5-HT by fluoxetine in the prefrontal cortex is of particular interest because modulation of NE and DA are also involved in the therapy of depression (Delgado *et al.*, 1993; Feighner, 1999; Schildkraut, 1965). From this, it can be said that, with the combination of the reserpine and the MeDOPA depleting the levels of NE and DA robustly, fluoxetine could not exhibit its maximal effect only by increasing the levels of 5HT through transmitter synthesis in the presence of the reserpine and the MeDOPA since NE and DA are also important in its actions as an antidepressant (Bymaster *et al.*, 2002; Pozzi *et al.*, 1999). It can also be said from the results that even though SJE and fluoxetine are acting similarly, SJE has a stronger adrenergic component than that of fluoxetine comparatively, since the combination of the reserpine and MeDOPA had a greater effect on the SJE than the fluoxetine (Figure 3.26 and 3.27).

From the phytochemical analysis of SJE from this study, SJE contains flavonoids and terpenoids among others. What is more, most of these plant constituents have also been shown to exhibit antidepressant activities. Recently, several studies have suggested the antidepressant effect of flavonoid fraction obtained from the crude extract of *Hypericum perforatum* (St. John's Wort) using the positive results of FST (Butterweck *et al.*, 2000; Butterweck *et al.*, 2001; Noldner and Schotz, 2002). Also, extracts from *Ginko biloba*, which contains flavonoids and terpenoids have been reported to show antidepressant activities in both the FST and TST (Sakakibara *et al.*, 2006).

Meanwhile, effects of flavonoids on the central nervous system are complex and involve different mechanisms including actions on the synaptic receptor or ionic channels (Saponara *et al.*, 2002). Some studies have indicated that Hypericum

species and their flavonoid constituents act via a blockade of monoamines uptake into rat brain synaptic vesicles (Roz *et al.,* 2002), as well as into rat synaptosomes (Viana *et al.,* 2006), and it has been suggested that, this effect might explain its antidepressant properties. In addition, flavonoid do not only possess antidepressant property in the FST (Butterweck *et al.,* 2000; Butterweck *et al.,* 2001; Noldner and Schotz, 2002) but also, the greatest MAO inhibition is found in the fraction with the greatest amounts of flavonoids (Bladt and Wagner, 1994).

It is worth noting that, in Ghana, the root of *S. jollyanum* is chewed as a central nervous system (CNS) stimulant and aphrodisiac (Abbiw, 1990; Irvine, 1961). In a preliminary report, its anxiogenic-like effects in laboratory animals (Woode *et al.,* 2006) and its aphrodisiac effects (Owiredu *et al.,* 2007) have been established. SJE is known to cause an increase in testosterone levels (Owiredu *et al.,* 2007; Raji *et al.,* 2006), and it has been postulated that testosterone may increase MAO release by upregulating nitric oxide synthase, which produces nitric oxide, which in turn increases MAO release (Hull *et al.,* 1999). Subsequently, this can be used to explain the basis of the antidepressant effect of SJE by increasing MAO levels.

4.4 TOXICOLOGICAL PROFILE OF SJE

S. jollyanum has been used since time immemorial in traditional medicine in Ghana to treat several diseases including male erectile dysfunction (Abbiw, 1990; Owiredu *et al*., 2007). However, a survey of available literature has indicated that the toxicity of its extract has not been thoroughly evaluated. The assessment of toxicity is indispensable, if the extract has to be used for human applications, for reasons of safe dose management. Therefore, the present study was carried out to evaluate the toxicity and genotoxic effects of the ethanolic root extract of *S. jollyanum* in laboratory animals.

4.4.1 General toxicity

When SJE was administered to Fischer 344 rats at 100 - 1000 mg kg^{-1} (*p.o.*) body weight per day for 90 days, there were no significant adverse toxicological effects attributable to the treatment. Clinical signs were unremarkable, and there were no ocular findings in any animal. Monitoring of body weight gain and food consumption in drug studies can be a sensitive indicator of overall animal health (Borzelleca, 1996). The absence of any significant differences in these measured parameters across the studied groups therefore provides support for the safety of the extract (Figure 3.31).

Analysis of clinical pathologies, revealed only random statistically significant effects. There were also occasional effects noted on relative organ weights (Table 3.15). There were no findings at the end of the study in macroscopic examinations that indicated that any of these effects were related to treatment with the test material.

The no-observable- adverse-effect level (NOAEL) for SJE was found to be more than 1000 mg kg^{-1} b.w./day when administered orally for 90 consecutive days. This finding is contrary to the work of Raji *et al.*, (2006) who reported an LD_{50} of 136.5 mg kg^{-1} using methanolic extract of the root. Probably, the disparity may be related to the medium of extraction; since the methanolic extract and ethanolic extract of the root might contain different components. This observation may justify why the roots are chewed raw or cut into pieces and infused in gin for 3 days to be taken as "bitters" in the traditional methods of drug preparation.

4.4.2 Biochemical tests

Liver function was determined in order to detect possible hepatic dysfunction, tissue damage or changes in biliary excretion evoked by prolonged exposure to SJE. Well known markers of liver function such as levels of serum albumin, lipoproteins, cholesterol and triglycerides were unchanged in animals chronically exposed to SJE, clearly demonstrating that liver function was preserved in these

animals. Frequently, when hepatic injury occurs, serum aminotransferase (AST and ALT) activity increases. Chronic treatment of the male animals did not change these parameters indicating that the SJE treatment did not induce liver tissue damage. Similarly, since there were no significant changes in direct, indirect and total bilirubin fractions after treatment with the SJE it indicates that the extract does not alter hepatic metabolism or biliary excretion. Alterations observed in relative liver weight were not correlated with clinical and biochemical changes and could not be characterized as a hepatotoxic effect of the SJE.

Similarly, renal function tests were performed to assess the possible nephrotoxicity of chronic treatment with SJE. Besides a slight change in BUN, sodium and potassium levels in blood samples from animals exposed to SJE, none of the other parameters evaluated were changed, indicating that renal function was unaffected by SJE treatment. These differences were not considered test drug-related since they were of small magnitude and within ±2SD of the mean for the population of control groups and not dose related (observed at the low dose but not at the high dose), and/or occurred only after a particular month and not other months of the study.

4.4.3 Haematological tests

Some alterations in hematological parameters were found in male rats subjected to the 30, 60 and 90-day treatment period with SJE (Table 3.16-3.18). Besides the significant increase in HGB, HCT, MCH and MCHC in a dose dependent manner in blood samples from animals exposed to SJE, other parameters evaluated showed differences that were not considered test drug related since they were of a small magnitude and within ±2SD of the mean for the population of control groups and not dose related (observed at the low dose but not at the high dose), and/or occurred only after a particular month and not other months of the study. These imply that the extract probably has a haemopoietic effect. An increase in the HGB and HCT concentrations indicates that the extract enhances the oxygen-transport

capacity of the blood (Massey, 1992), where as the increase in the MCH and MCHC may be further evidence of the haemopoietic effect of the extract. This is probably the basis for the relief experienced by sickle cell patients who are treated with the extract in the West African sub-region (Abbiw, 1990; Iwu, 1993).

4.4.4 Histopathological tests

Cells, which are unable to adapt to stimuli exhibit a variety of morphological changes, first seen ultrastructurally and later as visible light microscopic abnormality. Cells that have failed to adapt to metabolic stress, cease to produce structural proteins and begin to have difficulty in supplying energy to preserve electrolyte gradients to sustain other membrane functions. Light microscopic examination of cells typically shows fluid accumulation in cells, which make them pale-staining or vacuolated. This is described as cloudy swelling or hydropic degeneration. Cells failing to metabolize fatty acid, accumulate lipid within cytoplasmic vacuoles giving rise to the term fatty change. If the injury is irreversible, there is a progressive failure of key structural and metabolic components leading to death of cells; this is termed necrosis (Sokol, 2002). Therefore the criteria for assessing histopathological changes include necrosis, cloudy swelling, fatty infiltration of cells, inflammatory infiltrations among other parameters. There were no findings at the end of the study in either macroscopic or histopathologic examinations that indicated that any of these effects were related to treatment with the test material. No test drug-related changes were observed in this study. All changes were considered normal background lesions in this strain and age of rat (Plate 3.1-3.9).

4.4.5 Cytochrome P450 and sleeping time

The liver is the major organ responsible for drug metabolism in most species. The duration and intensity of action of many drugs is critically influenced by the rate of their metabolism or biotransformation or that of others. Several indices have been proposed to study enzyme induction or inhibition. These include, increased drug clearance, decreased drug plasma half life, increased plasma γ-glutamyl

transferase and bilirubin levels. Although none of these methods can equivocally substantiate induction or inhibition, they provide reasonable indication of the state of liver function (Parker and Orton, 1980).

Cytochrome P450s (CYP) comprise a superfamily of enzymes that catalyze the oxidation of a wide variety of xenobiotic chemicals, including drugs and carcinogens (Gonzalez, 1988; Gonzalez, 1990; Guengerich, 1992a). Multiple drug therapy is a common therapeutic practice, particularly in patients with several diseases or conditions, and many drug–drug interactions involving metabolic induction are being reported. The possibility of interactions between SJE and other drugs exists as it is an enzyme inducer. This warrants a careful use of SJE as a remedy for male impotence and/or erectile dysfunction because toxicity and therapeutic failure of drugs have long been recognized as possible consequences of the interactions among drugs.

Pentobarbitone is a hypnotic drug whose duration of action can be measured in rats by the sleeping time i.e. the time from when the animal loses its righting reflex to when it regains its righting reflex. Since pentobarbitone is metabolized by the hepatic microsomal cytochrome P450 enzyme action, its duration of action can be altered by inducing agents or by inhibitors.

The effect of inducers, phenobarbitone and SJE as well as inhibitor ketoconazole on the liver drug metabolizing enzymes (cytochrome P450) correlated well with the *in vivo* duration of action of pentobarbitone on the sleeping time in this study. Because pentobarbitone is metabolized by the hepatic microsomal cytochrome P450 enzyme system, its duration of action was significantly reduced by phenobarbitone and SJE. However, ketoconazole had no effect on the pentobarbitone action probably because it was not able to inhibit the drug metabolizing enzyme significantly at the dose used (Figure 3.29).

4.4.6 Mutagenicity tests

The *Salmonella* assay is particularly well designed to detect chemically induced mutations (Ames *et al.*, 1975). The primary impetus for its use is the high predictivity of a positive mutagenic response in *Salmonella* for rodent carcinogenicity (McCann *et al.*, 1975; Sugimura *et al.*, 2004; Tennant *et al.*, 1987). The *Salmonella* test is also required or recommended by regulatory agencies prior to registration or acceptance of many chemicals, drugs, and biocides (Auletta *et al.*, 1993; Dearfield *et al.*, 1991; FDA, 1993; HPBGC, 1993; Kirkland, 1993; Kramers *et al.*, 1991a; Kramers *et al.*, 1991b; Sofuni, 1993).

The supposed '2-fold' rule (Mortelmans and Zeiger, 2000) was used to evaluate the mutagenicity test. Thus, drugs were concluded to be mutagenic if the following factors were satisfied: (1) the greatest number of revertants was 2-fold or more relative to the negative control, (2) a dose-dependent increase in the number of revertants was observed and (3) the dose-finding and main assays produced reproducible results. If no increase in the number of revertants was observed, the drugs were concluded to have a negative result. If the 'mutagenic' and 'negative' criteria were not met, then the drugs were judged to have exhibited an equivocal response. Mutagenic activity (equal to the number of induced revertants/mg/ plate) was calculated as a measure of mutagenicity. The Ames microbial mutagenicity test also found no evidence of point mutations *in vitro*.

The results of this study demonstrate that, the extract has no apparent mutagenic effect on the four mutant *Salmonella* strains in the presence or absence of a metabolism activation system. This indicates that the SJE had no mutagenic effect *in vitro* within the dosage range applied.

SJE is rich in alkaloids, particularly in the leaves and roots (Odebiyi and Sofowora, 1978; Philardeau and Debray, 1965; Smolenski *et al.*, 1975). Saponins were not present (Odebiyi and Sofowora, 1978) as confirmed by this study. Philardeau and Debray, (1965) confirmed the presence of an alkaloid that corresponded to

berberine iodide and in 1967, columbine was isolated as the bitter principle of the seed (Gilbert et al., 1967). Moody *et al.,* (2006) also confirmed the presence of columbine and isocolumbine in the stem back.

Columbine has been found to have no toxic effect in cats and dogs up to 200 mg kg^{-1} s.c and internally administered (Husemann, 1871). The alkaloid berberine has a variety of unique pharmacological effects: antimicrobial, diuretic, smooth muscle relaxant and cardiac depressant activities (Bruneton, 1995). More recently, berberine has been found to inhibit cell death (apoptosis) of thymocytes which are critical immune effector cells. It is well known that the effectiveness of cancer chemotherapy is limited by toxicity to immune effector cells. Hence this alkaloid has become of interest for new therapeutic modalities for cancer..

Chapter 5

CONCLUSION

5.1 GENERAL CONCLUSION

The results of this novel study have provided evidence to support the use of SJE as an aphrodisiac in traditional medicine and that the effect may be due in part to the central stimulatory effect for the acute action whilst the long term effects are due to testosterone. The calcium inhibitory property of SJE suggests that the mechanism by which it exerts its smooth muscle relaxant effects are mediated partly by inhibition of calcium mobilization. This may perhaps justify the use of SJE as a spasmolytic agent in traditional medicine. It is also possible that SJE acts peripherally by ultimately antagonizing Ca^{2+}. This direct effect exerted on the penile tissue by *S. jollyanum* could also contribute to its copulatory performance-enhancing actions.

The present study also demonstrates that SJE can exert anxiogenic-like activity similar to caffeine in the EPM, open field, light/dark box and hole board test. Ethanolic extract of *S. jollyanum* roots produced antidepressant-like effect in mice as assessed by both FST and the TST, and this effect seems most likely to be mediated through an interaction with adrenergic, dopaminergic, and serotonergic system. Thus, SJE may have potential therapeutic value for the management of depressive disorders.

The toxicological finding of this study indicates that, the ethanolic root extract of *S. jollyanum* is safe for consumption. However, SJE induces drug metabolizing enzyme which warrant its careful use as a remedy for male impotence and many other illnesses.

5.2 RECOMMENDATIONS

Since *S. jollyanum* induced cytochrome P450 enzyme, it is important to investigate further the mechanism whereby *S. jollyanum* changes the penetration, elimination, and or metabolizing processes of drugs.

As part of the search to establish the pharmacological profile of *S. jollyanum*, effort should be made to isolate and identify the active compound (s) having the aphrodisiac, antidepressant, and smooth muscle relaxant properties.

Furthermore, after establishing the relaxant effect of *S. jollyanum* on anococcygeus and corpus cavernosum, other tissues as well as the exact mechanism of action of *S. jollyanum* on smooth muscles must be investigated.

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