PHOSPHODIESTERASE 5 ACTIVITY AND ENDOTHELIAL MARKERS AFTER ADMINISTRATION OF *Cnestis ferruginea* AND *Fadogia agrestis* EXTRACTS TO PAROXETINE-TREATED RATS

BY

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CERTIFICATION

I certify that this work was carried out by Mrs. Muritala, Hamdalat Folake in the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria under my supervision.

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APPROVAL PAGE

This thesisi has been read and approved as having met the requirement of the Department of Biochemistry and Postgraduate School, University of Ilorin, Ilorin, Nigeria, for the award of the degree of Doctor of Philosophy (Ph. D.) in Biochemistry.

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DEDICATION

This thesis is dedicated to Almighty Allah (Subhanahu watahalla) the creator and sustainer of the universe, the Noble Prophet Muhammad (Salalahu alayhi wasalam) and the entire Muslim Ummah.

It is also dedicated to my wonderful family,

My sweetheart, my treasure;

My boys, my love;

My mother, my jewel of inestimable value;

My father, the best dad I can ever ask for;

My siblings, my bond, I love you all;

My in-laws, thanks for your understanding

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ABSTRACT

PDE-5 inhibitors such as sildenafil citrate (Viagra) commonly prescribed for the management of erectile dysfunction (ED) has been reported to have failure rate of 30-50% at the attempt of intercourse. This has thus necessitated the need to source for better alternatives in medicinal plants for managing ED and its associated risk factors like cardiovascular diseases (CVD).Therefore, this study investigated the PDE-5 inhibitory activities and modulatory properties of *Cnestis ferruginea* root, *Fadogia agrestis* stem and their combinations on endothelial markers of paroxetine-treated rats. The specific objectives of this study were to: (i) determine the secondary metabolites in the plants' parts; (ii) partially purify PDE-5 from rat penile and cardiac tissues (iii) carry out PDE-5 inhibitory assays of the extracts in the presence of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP); (iv) determine the effects of paroxetine on selected endothelial, erectile and cardiovascular functional biomarkers in rats; and (v) determine the effects of the extracts on the selected markers of erectile, cardiovascular and endothelial dysfunctions of male rats pre-treated with paroxetine.

Forty male Wistar rats (200.32 ± 6.33) were used for the *in vitro* PDE-5 inhibitory assays, after which Michealis-Menten constant ($K_{m)}$ and maximum velocity (V_{max}) were derived from the double reciprocal plots. For the *in vivo* study, 210 male Wistar rats (210.43 ± 4.04) g were grouped into two consisting of 40 rats in group A (control) and 170 rats in group B (received 10 mg/kg body weight of paroxetine) for 21 days. Eight animals from groups A and B were sacrificed on days 5,8,15 and 22 of paroxetine treatment. The remaining animals in group B were re-assigned into 5 groups of 16 animals each. 8 rats were sacrificed from each group on days 29 and 36 of experimental period. Biochemical assays were done on the tissues and plasma. Data were subjected to Analysis of Variance and Duncan multiple range Test. Statistical significance was set at $p<0.05$.

The results revealed that:

i *C. ferruginea* root extract contained six secondary metabolites while *F. agrestis* stem extract contained five secondary metabolites with alkaloids as major component of the plants (49.95 ± 0.25 x 10^{-2} and 59.15 ± 0.25 x 10^{-2} mg/g) respectively;

ii purification folds of isolated penile PDE-5 was 5.6 while that of cardiac PDE-5 was 7.5 when compared with the crude extracts of PDE-5;

iii the extracts at 0.025-0.125 µg/mL competitively inhibited PDE-5 activities in a manner similar to sildenafil;

iv paroxetine-treatment related PDE-5 inhibition was reversed by the extracts after post-treatment;

v paroxetine-treatment related reduction in nitric oxide concentration was significantly ($p>0.05$) elevated by the extracts and was sustained post-treatment;

vi endothelin-1, cGMP, and testosterone concentrations were significantly ($p>0.05$) decreased during treatment and post-treatment periods.

vii paroxetine related increase in creatine kinase, triacylglycerol, total cholesterol and low density lipoprotein cholesterol were significantly ($p>0.05$) decreased after administration of extracts.

The study concluded that the extracts exerted their aphrodisiac activities via inhibition of penile phosphodiesterase-5 in a similar mechanism as sildenafil. Moreover, the extracts can be used for managing ED and CVD.

CHAPTER ONE

INTRODUCTION

Erectile dysfunction (ED) is an imperative health concern that can extensively affect a man's psychosocial well-being (Nguyen *et al*., 2017). Traditionally, the term "impotence" has been used to connote a male's inability to attain and maintain an erection. Impotence, in most circumstances, is more precisely referred to as erectile dysfunction (ED). Erectile dysfunction is a type of male sexual dysfunction characterized by the inability to develop or maintain an erection of the penile organ during sexual intercourse. Although ED is a non-malignant disorder, it may affect physical, mental health and may have a major impact on the quality of life (QoL) of patients and their spouses (Feldman *et al*., 1994). An estimated 10-20 million men suffer from the condition. However, this number is expected to increase significantly, with an estimated figure of 322 million by 2025 (Aytac *et al.,* 1999). Epidemiological studies show a worldwide incidence of 20 - 30% and increased rate of male sexual dysfunction (MSD) as men ages (Lewis *et al*., 2004). Although there is a little or no information on the incidence of MSD in Nigeria, available studies have shown that 15%–50% of men experience varying degrees of sexual dysfunction (Akinade and Sulaiman, 2005; Idung *et al*., 2012).

Even though the prevalence of ED increases with age, it must be stressed that ageing itself is not a cause of ED as it is associated with metabolic syndrome such as cardiovascular disease, diabetes mellitus and obesity (Kassier and Veldman, 2014).

In human species, reproduction is made possible by the copulatory union between a male and a female; this is referred to as sexual intercourse (coitus) (Keele and Neil, 1974).

For sexual intercourse to occur, the sexual organs and factors relating to erection of the copulatory organ in the male must function normally. The repeated inability of the male to

perform this function effectively or a disorder that interferes with his full sexual response cycle is termed male sexual dysfunction (MSD) (Guay *et al*., 2003). The inability to achieve an erection sufficient for the purpose of satisfactory sexual intercourse, is known as erectile dysfunction (Ogah, 1999).

Male sexual dysfunction (MSD) is the persistent, unfulfilling sexual performance that occurs in one-quarter of all presented cases (Malviya *et al*., 2011). MSD which arises as a result of changes in the well-ordered sequence of sexual feed-back in men (APA, 1994) presents as disinterest in sexual activity, inability to keep and maintain penile erection, quick or absence of ejaculation during sexual intercourse, inability to control or go through the climax of sexual excitement (orgasm) and absence of satisfactory sexual fulfillment (Kayode and Yakubu, 2017).

Male sexual dysfunction (MSD) is common worldwide among men of all ages, ethnicities and cultural backgrounds. Generally, a prevalence of about 10% occurs across all ages. Because sexual dysfunction is an inevitable process of aging, the prevalence is over 50% in men between 50 and 70 years of age (Rendel *et al*., 1999).

Sexual dysfunction in men takes different forms, such as disorders of desire (persistently or recurrently deficient sexual craving and desire for sexual activity), disorders of orgasm (recurrent delay in, or absence of, orgasm after a normal sexual excitement phase), disorders of ejaculation (recurrent ejaculation with minimum sexual stimulation that occurs before, upon, or shortly after penetration and before a person wishes it or a situation where ejaculation does not occur at all), failure of detumescence (prolonged priapism lasting for more than 4 h) and erectile dysfunction (persistent failure to generate sufficient penile body pressure to achieve vaginal penetration and/or the inability to maintain this degree of penile rigidity until ejaculation).

Erection is basically a spinal reflex that can be initiated by recruitment of penile afferents, both autonomic and somatic, as well as supraspinal influences from visual, olfactory, and imaginary stimuli (Andersson, 2011).

Erectile dysfunction is defined as the persistent inability to attain and maintain an erection sufficient to permit satisfactory sexual performance. Erectile dysfunction is a serious clinical problem in adult men. The malfunction of penile erection could be as a result of impaired relaxation of the smooth muscle related to the elevated blood flow into the spaces of the corpus cavernosum of the penis (Hnatyszyn *et al*., 2003).

There is increasing evidence that ED can be an early manifestation of coronary artery and peripheral vascular disease; thus, ED should not be regarded only as a quality of life issue but also a potential sign of cardiovascular disease (Dong *et al.,* 2011; Guo *et al*., 2011).

Cardiovascular diseases (CVD) refer to all forms of diseases pertaining to blood vessels or organs that are heart related or go to/from the heart. Coronary is the name of the arteries that feed the muscle. When these arteries are blocked, such individual experiences coronary artery diseases (CAD) which is the most common form of CVD. Other forms of CVDs are angina- pain in the heart and myocardial infarction- heart attack.

Coronary artery disease (CAD) and erectile dysfunction (ED) are both highly prevalent conditions that frequently occur simultaneously (Tan and Pu, 2003; Montorsi *et al*., 2004; Gazzaruso *et al*., 2004). They share common risk factors, including diabetes mellitus (DM), hypertension, hyperlipidemia, obesity and tobacco abuse (Montorsi *et al*., 2004). As the number of cardiovascular risk factors increase, so does the incidence of both CAD and ED (Virag *et al*., 1985; Fung *et al*., 2004).

In all individuals with sexual and cardiac complains, their sexual symptoms occurred approximately 39 months prior to their cardiac symptoms (Montorsi *et al*., 2003). Epidemiological studies have reported the relationship between cardiovascular, metabolic risk factors and sexual dysfunction in men and women (Laumann *et al.,* 1999). ED can facilitate identification of diabetic men and women having cardiovascular problems without visible symptoms (Batty *et al*., 2010; Gazzaruso *et al*., 2011). ED drastically increases the risk of cardiovascular disease, coronary heart disease, stroke and the increase is probably independent of conventional cardiovascular risk factors (Dong *et al.,* 2011).

Cardiovascular diseases coupled with the increasing number of men seeking help for the treatment of impotence, has necessitated the need for more pharmacological research on naturally occurring treatment options (Cicero *et al*., 2001). To date, sildenafil citrate is a standard drug for the first line oral therapy for the management of erectile dysfunction and currently being utilized for the management or treatment of cardiovascular problems (Sable *et al.,* 2017).

Cyclic nucleotide phosphodiesterases (PDEs) are enzymes that hydrolyse phosphodiester bonds. They are widely distributed in various tissues in mammals where they perform their specific role of cell signaling through hydrolysis of second messengers –cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Phosphodiesterases comprise of 11 distinct gene families (PDE 1 - PDE 11), also referred to as the PDE Superfamily (Figure 1). Each family consists minimum of 1 and maximum of 4 genes resulting in more than 20 genes encoding over 50 various proteins in mammalian cells (Lugnier, 2006).

Reduction of cyclic nucleotide levels by adenylyl and guanylyl cyclases, the PDE family by hydrolyzing cyclic nucleotides (Figure 2), regulates cAMP and cGMP levels and facilitates their return to the basal state. The classification of PDEs (PDE1 to PDE11) (Table 1) was derived from

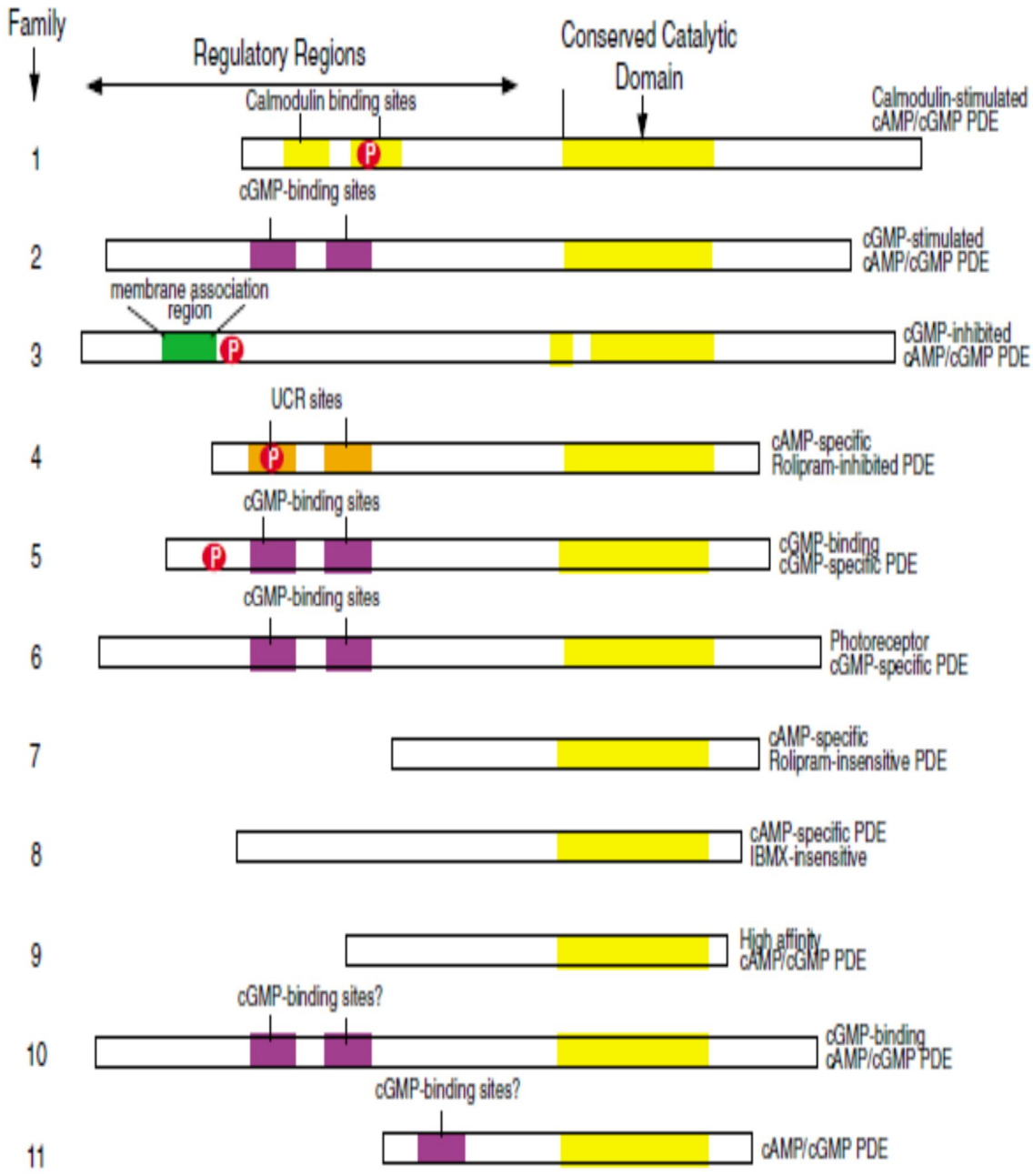


Figure 1: Phosphodiesterase families

Source: Corbin (2004)

the genes of their protein products, their biochemical features, regulation, and their sensitivity to modulators (Beavo, 1995). Their critical role in intracellular signaling has made them potential new therapeutic targets. Several leading pharmaceutical companies are searching for and developing new therapeutic agents on the basis of their ability to actively and selectively inhibit PDE isozymes, notably PDE4 in inflammation and PDE5 in human erectile and cardiovascular dysfunctions (Lugnier, 2006).

Alterations in intracellular signaling (the level of the second messengers) may be pointing at a clue to addressing specific disease conditions.

Several analyses comprising dozens of trials and thousands of patients, including patients with coronary artery disease who were also on antihypertensive medications, reported that PDE5 inhibitors did not significantly affect the incidence of adverse cardiovascular events (Kloner *et al.*, 2003; Mittleman *et al*., 2003; Kloner *et al.*, 2006). However, PDE 5 is distributed in many tissues, including platelets, veins, and arterial smooth muscle (pulmonary, coronary, and systemic arteries) (Wallis *et al*., 1999). Thus, PDE 5 inhibitors affect the cardiovascular system, mostly via vasodilation and often cause small decreases in blood pressure (BP). When PDE 5 inhibitors are co-administered with nitrates or α-blockers, pronounced systemic vasodilation and severe hypotension are possible (Simonsen, 2002). Many patients with ED are elderly and have the same risk factors as patients with coronary artery disease, so these diseases drug combinations are commonly considered or encountered in clinical practice (Seftel *et al*., 2004).

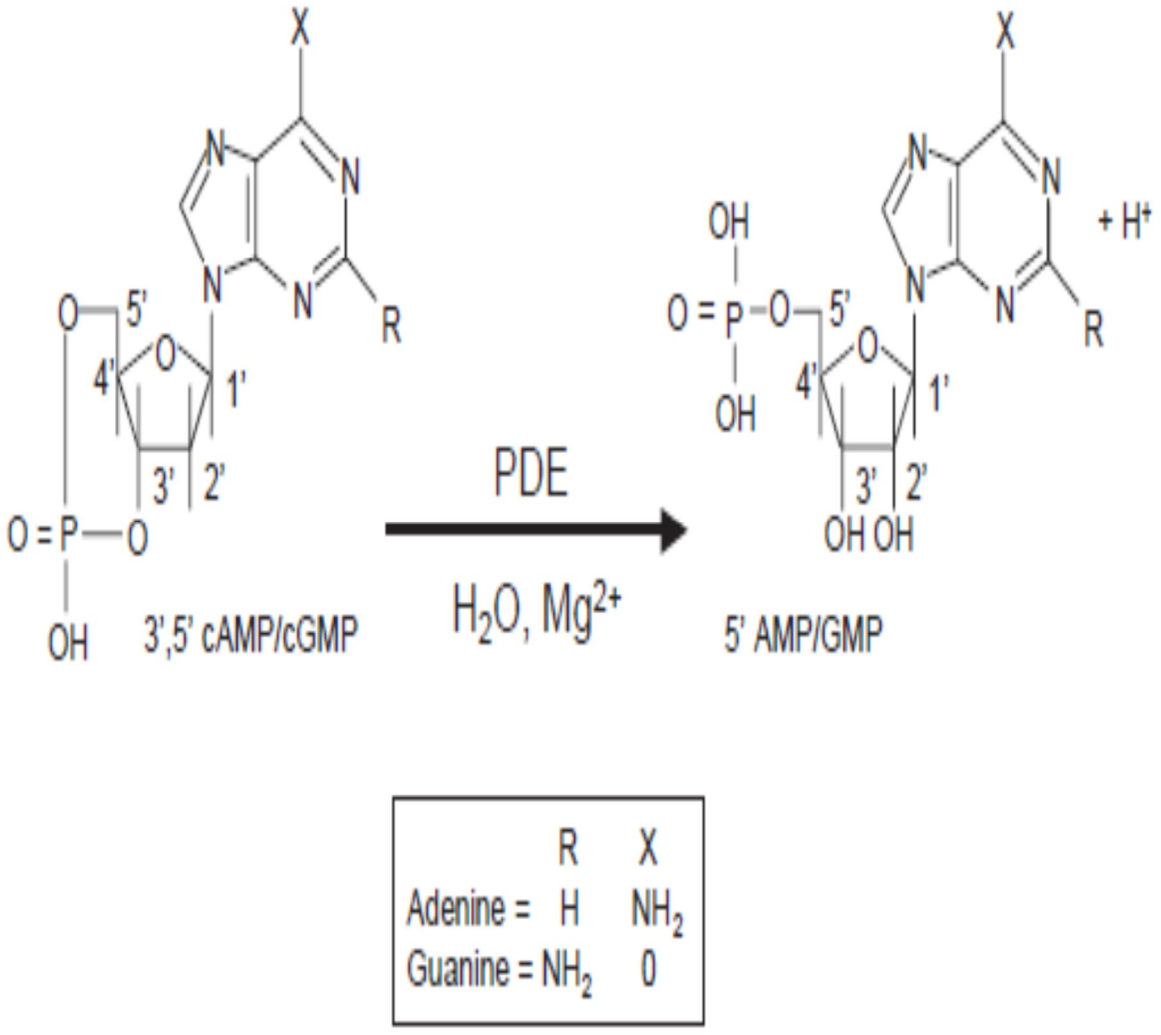


Figure 2: Cyclic nucleotide hydrolysis by Phosphodiesterase

Source: Lugnier (2006)

Table 1: Classification of Phosphodiesterases

PDE Family	Substrate specificity	Property	Specific inhibitors
PDE 1	cAMP, cGMP	Ca-calmodulin activated	Nimodipine
PDE 2	cAMP, cGMP	cGMP-activated	EHNA
PDE 3	cAMP, cGMP	cGMP-inhibited	Cilostamide, milrinone
PDE 4	cAMP	cGMP-insensitive	Rolipram, roflumilast
PDE 5	cGMP	PKA/PKG-phosphorylated	Zaprinast, Sildenafil, DMPPO
PDE 6	cGMP	Tranducin-activated	Zaprinast, Sildenafil, DMPPO
PDE 7	cAMP	Rolipram-insensitive	BRL 50481, ICI242
PDE 8	cAMP	Rolipram-insensitive IBMX-insensitive	Unknown
PDE 9	cGMP	IBMX-insensitive	Unknown
PDE 10	cAMP, cGMP	Unknown	Unknown
PDE 11	cAMP, cGMP	Unknown	Unknown

Key:

EHNA = erythro-9-(2- hydroxyl -3- nonyl) adenine

IBMX = isobutylmethylxanthine

DMPPO =

cAMP = cyclic adenosine monophosphate

cGMP = cyclic guanosine monophosphate

Source: Lugnier (2006)

Inhibition of intracellular enzyme, phosphodiesterase 5 (PDE 5), reduces cyclic guanylate monophosphate breakdown, promoting vascular relaxation in the corpora cavernosa and penile erection during sexual stimulation (Ghiadoni *et al*., 2008).

The relationship between erectile dysfunction (ED) and cardiovascular disease is well-established in the literature and both conditions share same risk factors. Therefore, it is difficult to distinguish the effect of underlying disease and adverse effects of the drugs and/or interactions between ED drugs and drugs used for cardiovascular disease. The known interactions of generally administered drugs for ED and cardiovascular disease are pharmacodynamically same. Thus, nitrates enhance the production of cyclic GMP and should not be used with phosphodiesterase type-5 inhibitors as it may lead to severe hypotension (Simonsen, 2002).

Endothelial dysfunction is an important symptom in the pathogenesis of atherosclerosis, contributing to plaque opening and succession. Reductions in circulating endothelial progenitor cells that participate in regeneration of the endothelium participate in endothelial pathophysiology. The severity of endothelial dysfunction has been shown to have prognostic value for cardiovascular events (Endemann and Schiffrin, 2004).

Repair of endothelial dysfunction may be accompanied with reduced cardiovascular risk. Circulating endothelial progenitor cells may represent a potential therapeutic approach for endothelial dysfunction (Endemann and Schiffrin, 2004).

Cyclic guanosine monophosphate (cGMP) is the intracellular trigger for penile erection. Cyclic GMP (Figure 3) activates cGMP-dependent protein kinase (PKG), which in turn phosphorylates several proteins. These protein kinase interactions result in reduced intracellular calcium levels and a consequent relaxation of arterial and trabecular smooth muscle, leading to arterial dilatation, venous constriction, and the rigidity of penile erection (Beavo, 1995). PDE 5 normally

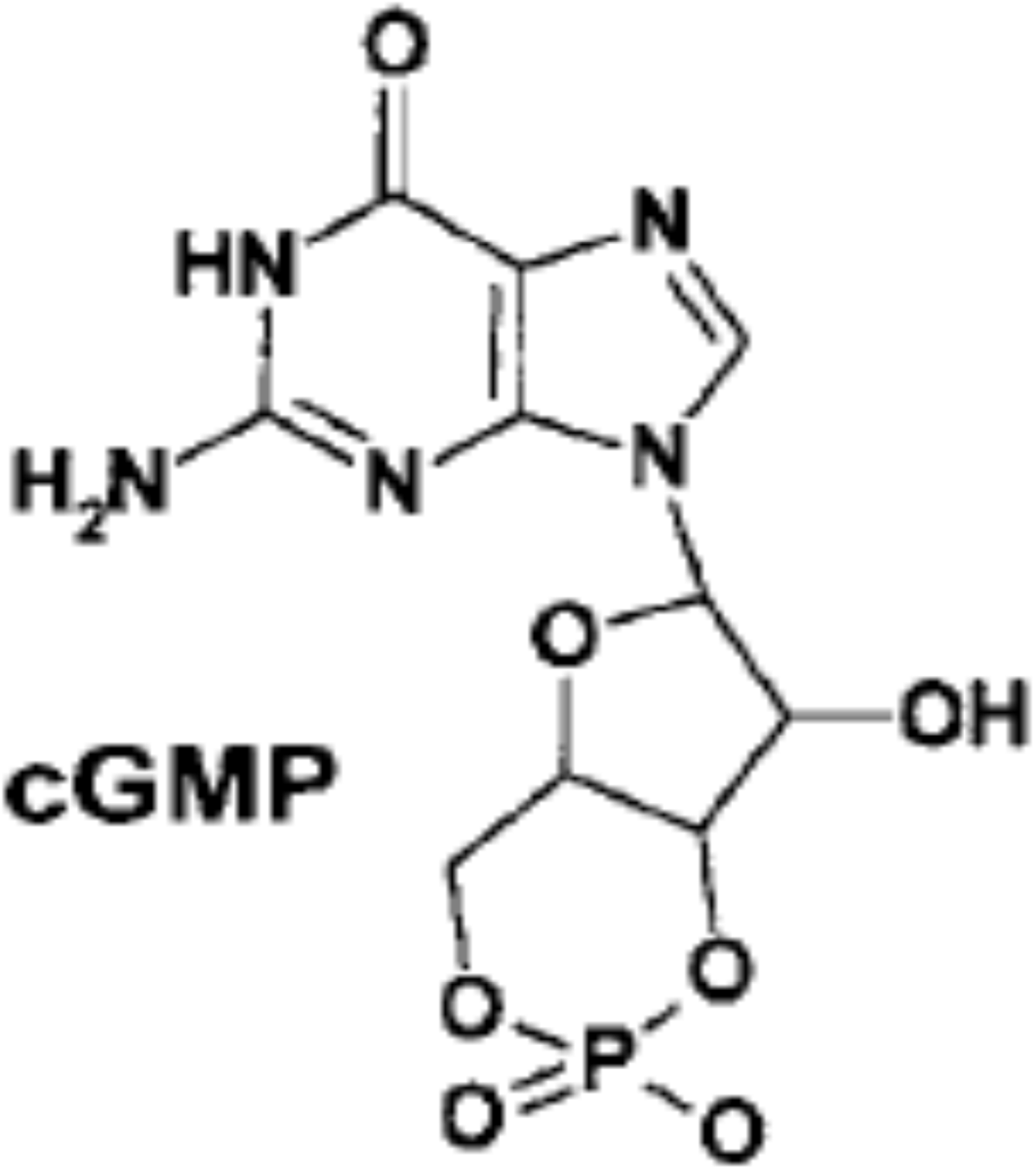


Figure 3: Structure of Cyclic Guanosine Monophosphate (cGMP)

Source: Kim *et al.* (2003)

inhibits penile erection by degrading cGMP. This degradation occurs at the catalytic site in the presence of bound zinc. Sildenafil citrate, a potent PDE 5 inhibitor lowers the activity of PDE5 by competing with cGMP and consequently raise the level of cGMP.

1.1 Literature Review

1.1.1 Male reproductive system

The human male reproductive system consists of organs located outside the body and around the pelvic region of a male that is responsible for the reproductive process. Various substances such as fluids, hormones and pheromones are important to the effective functioning of the reproductive system (Silva *et al*., 2012). The primary direct function of the male reproductive system is to provide the male (gamete) or (spermatozoa) for fertilization of the ovum. The male reproductive system (Figure 4) can be grouped into three categories. The first is the testes which function in sperm production and storage. Production takes place in the testes which are housed in the temperature regulating scrotum, immature sperm then travel to the epididymis for development and storage. The testes are carried in an external pouch called scrotum, where they normally remain slightly cooler than body temperature to facilitate sperm production. Having the testes outside the abdomen facilitates temperature regulation of the sperm, which requires specific temperatures to survive (Sah, 2002). The second category is the ejaculatory fluid producing glands which include the seminal vesicles, prostate and the vas deferens. The final categories are those used for copulation, and deposition of the spermatozoa (sperm) within the male, these include the penis, urethra, vas deferens, and Cowper's gland.

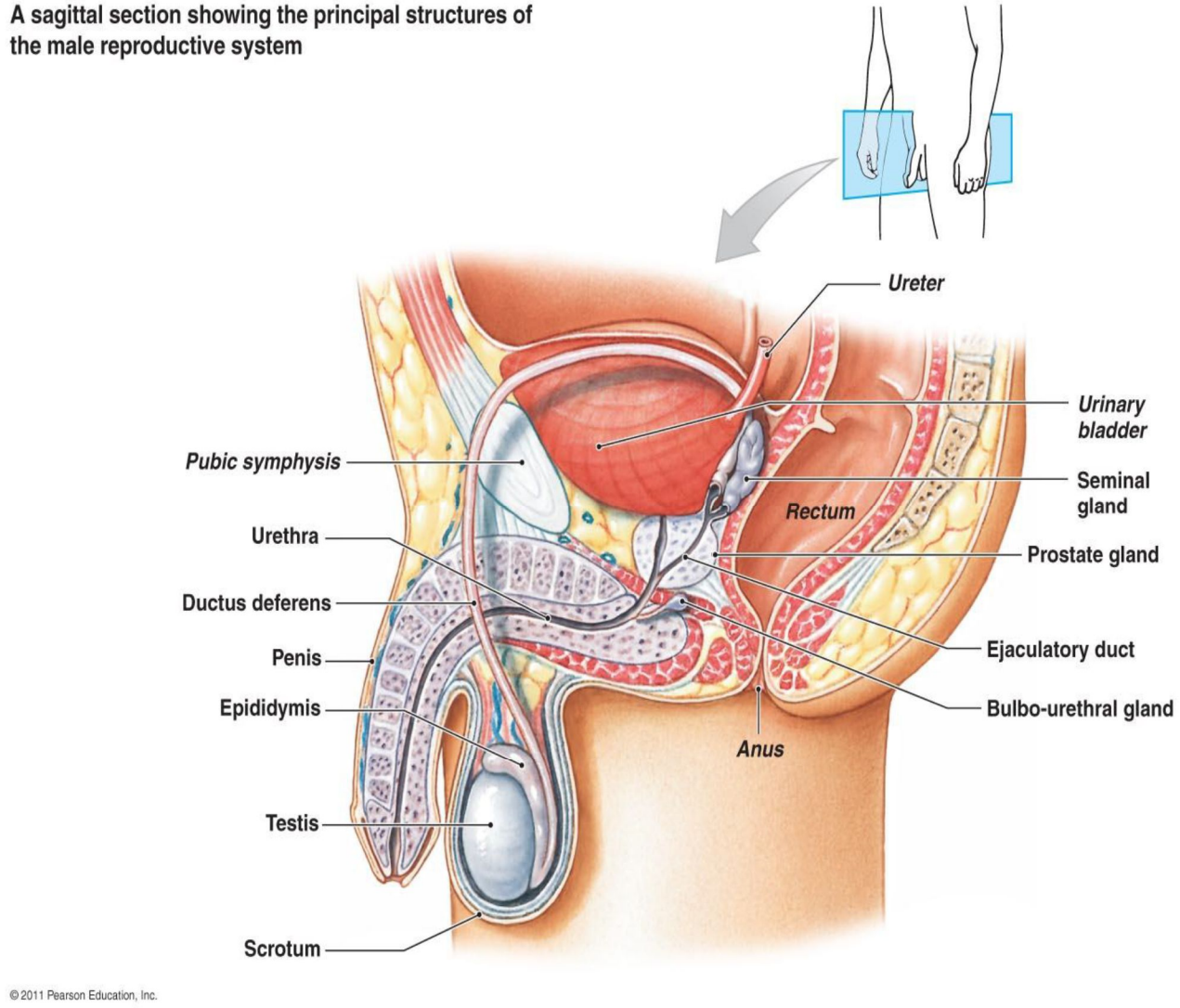


Figure 4: The male reproductive system

Source: Gutfeld *et al*. (2007)

1.1.1.1 Male sexual function

Male sexual function is a complex, multi-dimensional phenomenon that incorporates biological, psychological, interpersonal and behavioural dimensions. Sexual behaviour in male consists of three behaviours:

i. Mount: The animal assumes the copulatory position, but does not insert its copulatory organ into the vagina of the female
ii. Intromission: The copulatory organ enters the vagina during a mount
iii. Ejaculation: Forceful expulsion of semen.

Sexual stimulation of the human male results in a series of psychological, neuronal, vascular, and local genital changes.

1.1.1.1.1 Male sexual response cycle

Erection- Erection is the enlarged and rigid state of the sexually aroused penis sufficient enough for vaginal penetration. It results from multiple psychogenic and sensory stimuli arising from imaginative, visual, auditory, olfactory, gustatory, tactile, and genital reflexogenic sources (Saini *et al.,* 2011).

Ejaculation- Ejaculation is the act of ejecting semen. It is a reflex action that occurs as a result of sexual stimulation. It is made up of two sequential processes. The first process called emission is associated with deposition of seminal fluid into the posterior urethra while the second process is the true ejaculation, which is the expulsion of the seminal fluid from the posterior urethra through the penile meatus (Saini *et al.,* 2011).

Orgasm- This is the climax of sexual excitement. The entire period of emission and ejaculation is known as the male orgasm (Guyton and Hall, 2000).

Detumescence- This is the flaccidity of an erect penis after ejaculation (Wagner, 1981).

1.1.1.2 Male sexual dysfunction

Sex disorders of the male are classified into disorders of sexual function, sexual orientation, and sexual behaviour. In general, several factors must work in harmony to maintain normal sexual function. Male sexual dysfunction (MSD) results when these factors are altered. Such factors include neural activity, vascular events, intracavernosal nitric oxide system and androgens. Thus, malfunctioning of at least one of these could lead to sexual dysfunction of any kind. MSD therefore refers to a problem during any phase of the sexual response cycle that prevents the individual or couples from experiencing satisfaction in sexual activity. Sexual dysfunction in men refers to repeated inability to achieve normal sexual intercourse. It can also be viewed as disorders that interfere with a full sexual response cycle. These disorders make it difficult for a person to enjoy or to have sexual intercourse. Sexual dysfunction takes different forms in men. The primary male sexual dysfunctions include erectile dysfunction, inhibited or absent libido, premature ejaculation, and retarded ejaculation/anorgasmia (Laumann, 1994; Michael, 2003). It may also present as pain during intercourse.

1.1.1.3 Types of male sexual dysfunction

Male sexual dysfunction is present as disorders of desire, loss of libido, erectile dysfunction, ejaculation disorder, orgasmic disorder and failure of detumescence.

I. Disorders of desire: Disorders of desire can involve either a deficient or compulsive desire for sexual activity. Dysfunctions that can occur during the desire phase include:

(a) Hypoactive sexual desire (HSD): This is defined as persistently or recurrently deficient (or absent) sexual fantasy and desire for sexual activity leading to marked distress or interpersonal difficulty. It results in a complete or almost complete lack of desire to have any type of sexual relation (APA, 1994).

(b) Compulsive sexual behaviours (CSBs): These constitute a wide range of complex sexual behaviours that have strikingly repetitive, compelling, or driven qualities. They usually manifest as obsessive-compulsive sexuality (*e.g.* excessive masturbation and promiscuity), excessive sex-seeking in association with affective disorders (*e.g.* major depression or mood disorders), addictive sexuality (*e.g.* attachment to another person, object, or sensation for sexual gratification to the exclusion of everything else), and sexual impulsivity (failure to resist an impulse or temptation for sexual behaviour that is harmful to self or others such as exhibitionism, rape or child molestation) (Kaplan, 1996).

(II) Disorders of ejaculation: This disorder takes the form of mild premature to severe retarded or absent ejaculation. Premature ejaculation is the most common male sexual dysfunction (Metz *et al*., 1997) which could be persistent or recurrent ejaculation with minimum sexual stimulation that occurs before, upon, or shortly after penetration and before the person wishes it. It can also be painful ejaculation arising from side effects of tricyclic antidepressants (Aizenberg *et al*., 1991); inhibited or retarded ejaculation (absence of ejaculation) or retrograde ejaculation (forced ejaculation back into the bladder rather than through the urethra and out of the end of the penis at orgasm).

(III) Disorders of orgasm: This is a persistent or recurrent delay in or absence of orgasm after a normal sexual excitement phase during sexual activity (APA, 1994).

(IV) Failure of detumescence: This is a prolonged (usually >4 h duration) and extremely painful erection unaccompanied by sexual desire and is often preceeded by usual sexual stimuli.

(V) Erectile dysfunction (ED): This is a problem with sexual arousal and it is the difficulty in achieving or maintaining an erection sufficient for sexual activity or penetration

1.1.1.4 Etiologies of erectile dysfunction

Erectile dysfunction may occur as a result of either psychogenic causes (i.e. all related physiological and neurovascular pathways are intact but a psychological impairment is present) or organic causes (a hormonal, neurovascular, or anatomical risk factor causes erectile dysfunction). However, in most individuals suffering from ED, both factors are probably responsible for the failure to achieve an adequate erection (Muneer *et al*., 2014).

1.1.1.4.1 Psychogenic erectile dysfunction

Psychogenic erectile dysfunction may be ascribed to relationship stress, performance anxiety, or overt psychological disorders, such as depression or schizophrenia, which is further exacerbated by drugs prescribed to treat these conditions (Muneer *et al*., 2014).

1.1.1.4.2 Organic causes of erectile dysfunction

Blood flow within the corpus cavernosum needs tobe high in order to achieve adequate penile tumescence for successful penetrative intercourse. This requires proper coordination of neurovascular and biochemical events to allow relaxation of cavernosal smooth muscle and dilation of the cavernosal artery. A venous leak, which may be congenital or acquired, can also cause erectile dysfunction owing to a failure of blood remaining in the corpora (Muneer *et al*., 2014).

1.1.1.5 Management options for erectile dysfunction

Many cases of sexual dysfunction can be corrected by treating the underlying physical or psychological problems. Several treatment strategies available include:

1.1.1.5.1 PDE-5 inhibitors as first line oral therapy

Oral PDE inhibitors are a convenient, efficacious, and widely available treatment option for erectile dysfunction. They are contraindicated in patients taking nitrates, in patients in whom

vasodilatation or sexual activity is inadvisable, and in those with a history of non-arteritic optic neuropathy. PDE inhibitors should be used with caution in patients with renal or hepatic impairment, recent stroke, myocardial infarction, or unstable angina and in those taking α blockers for lower urinary tract symptoms. These drugs inhibit type 5 PDE within the cavernosal smooth muscle and prevent the breakdown of cyclic guanosine monophosphate (cGMP) to GMP. Nitric oxide mediated smooth muscle relaxation is therefore facilitated in both the corpus cavernosum and cavernosal arteries. Although several oral PDE-5 inhibitors are marketed globally, they all have the same mechanism of action but differ in their half life and potency. The most common side effects seen with sildenafil include headache, flushing, dyspepsia, and rhinitis. The adverse effects with tadalafil and vardenafil are similar to sildenafil, although tadalafil is associated with a higher incidence of back pain and myalgia (Brock *et al*., 2002).

1.1.1.5.2 Intracarvernosal and intraurethral prostaglandins as second line therapy

The synthetic prostaglandin E1 analogue alprostadil can be given as second line treatment. Alprostadil increases intracellular concentrations of cyclic AMP (cAMP), resulting in relaxation of smooth muscle. Currently, two methods of administration are available: direct intracavernosal injection of alprostadil (Caverject 2.5-20 μg) or intraurethral application of a small pellet (MUSE 250-1000 μg). In an open label flexible dosing study using intracavernosal alprostadil, 82% of men reported successful sexual intercourse (Linet and Ogrinc, 1996). The MUSE study group reported a 65.9% successful intercourse rate in a double blind placebo controlled trial of 1511 men (Padma-Nathan *et al*., 1997).

1.1.1.5.3 Vacuum erection devices as second line therapy

Vacuum devices can be used alone or combined with other treatments, regardless of the cause of erectile dysfunction.

Complications include pain, bruising, and penile numbness, with more serious adverse events such as skin necrosis occurring if the constriction ring is left on for too long. A questionnaire based study found patient and partner satisfaction rates of 82% and 84%, respectively, with long term use (Cookson and Nadig, 1993).

1.1.1.5.4 Penile prosthesis surgery as third line therapy

Third line treatment for erectile dysfunction involves surgery. Penile prosthesis surgery is suitable for patients with severe organic erectile dysfunction that are not responding to drug treatment. Two main types of prosthesis are available: a malleable prosthesis and an inflatable one. Complications related to the AMS inflatable prosthesis in a retrospective multicentre study include infection rates of 3.2% and device malfunction of 17.5%; 85.2% of patients were satisfied or highly satisfied with the prosthesis (Carson *et al*., 2000).

1.1.1.6 Prevalence of erectile dysfunction

Erectile dysfunction (ED) is a worldwide condition and its prevalence is anticipated to increase from 152 million in 1995 to 322 million by 2025 (Ismail, 2016). This projection indicates a growing need to re-evaluate ED therapeutic strategies and mandates robust steps to validate the innovative drugs and technologies that may revolutionise ED treatment.

1.1.2 Cardiovascular system and function

The cardiovascular system is a system that involves the transportation of blood, lymph, oxygen and other gases. The cardiovascular system consists of the heart, blood vessels, blood and lymph

(Guyton and Hall, 2000). The cardiovascular system is the transport system of the body by which food, oxygen, water and all other essential components are carried to the tissues and their waste products excreted. The heart pumps blood through the arteries to the arterioles and these passes the fluid to the smaller capillaries. The capillaries are thin-walled vessels interconnected between the arterioles and venules. Approximately, 7000 liters of blood is pumped every dayby the heart. In an average individual's lifetime, the heart contracts 2.5 billion times. The blood flow in humans is in an enclosed circuit which is divided into pulmonary and systemic circuit (Monahan-Earley *et al*., 2013).

The cardiovascular system can be divided into three main parts based on their major functions:

A) the blood : the fluid in which materials are carried to and from the tissues

B) the heart: the driving force that propels the blood

C) the blood vessels: the routes by which the blood travels to and through the tissues and back to the heart.

1.1.2.1 Cardiovascular dysfunction

Cardiovascular diseases (CVDs) are the results of cardiovascular dysfunction. CVDs are a group of disorders of the heart and blood vessels and they include:

- coronary heart disease – disease of the blood vessels supplying the heart muscle;
- cerebrovascular disease – disease of the blood vessels supplying the brain;
- peripheral arterial disease – disease of blood vessels supplying the arms and legs;
- rheumatic heart disease – damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria;
- congenital heart disease – malformations of heart structure existing at birth;

- deep vein thrombosis and pulmonary embolism – blood clots in the leg veins, which can dislodge and move to the heart and lungs.

Heart attacks and strokes are usually acute events and are mainly caused by a blockage that prevents blood from flowing to the heart or brain. The most common reason for this is a build-up of fatty deposits on the inner walls of the blood vessels that supply the heart or brain. Strokes can also be caused by bleeding from a blood vessel in the brain or from blood clots. The cause of heart attacks and strokes are usually the presence of a combination of risk factors, such as tobacco use, unhealthy diet and obesity, physical inactivity and harmful use of alcohol, hypertension, diabetes and hyperlipidaemia.

1.1.2.2 Etiologies of cardiovascular dysfunction

The most important behavioural risk factors of heart disease and stroke are unhealthy diet, physical inactivity, tobacco use and harmful use of alcohol. The effects of behavioural risk factors may show up in individuals as raised blood pressure, raised blood glucose, raised blood lipids, and overweight and obesity. These “intermediate risks factors” can be measured in primary care facilities and indicate an increased risk of developing a heart attack, stroke, heart failure and other complications (WHO, 2015).

There are also a number of underlying determinants of CVDs or "the causes of the causes". These are a reflection of the major forces driving social, economic and cultural change – globalization, urbanization and population ageing. Other determinants of CVDs include poverty, stress and hereditary factors (WHO, 2015).

1.1.2.3 Prevalence of cardiovascular dysfunction

Cardivascular diseases (CVDs) are the number 1 cause of death globally: more people die annually from CVDs than from any other cause. An estimated 17.7 million people died from

CVDs in 2015, representing 31% of all global deaths. Of these deaths, an estimated 7.4 million were due to coronary heart disease and 6.7 million were due to stroke. Over three quarters of CVD deaths take place in low- and middle-income countries. Out of the 17 million premature deaths (under the age of 70) due to noncommunicable diseases in 2015, 82% are in low- and middle-income countries, and 37% are caused by CVDs.

Most cardiovascular diseases can be prevented by addressing behavioural risk factors such as tobacco use, unhealthy diet and obesity, physical inactivity and harmful use of alcohol using population-wide strategies. People with cardiovascular disease or who are at high cardiovascular risk (due to the presence of one or more risk factors such as hypertension, diabetes, hyperlipidaemia or already established disease) need early detection and management using counselling and medications, as needed.

1.1.3 Types of cardiovascular diseases

1.1.3.1 Coronary heart disease

Disease of the blood vessels supplying the heart muscle. Major risk factors are high blood pressure, high blood cholesterol, tobacco use, unhealthy diet, physical inactivity, diabetes, advancing age, genetic disposition. Other risk factors include: Poverty, low educational status, poor mental health (depression), inflammation and blood clotting disorders.

1.1.3.2 Stroke

Strokes are caused by disruption of the blood supply to the brain. This may result from either blockage (ischaemic stroke) or rupture of a blood vessel (haemorrhagic stroke). The risk factors are high blood pressure, atrial fibrillation (a heart rhythm disorder), high blood cholesterol, tobacco use, unhealthy diet, physical inactivity, diabetes and ageing.

1.1.3.3 Congenital heart disease

Malformations of heart structures existing at birth may be caused by genetic factors or by adverse exposures during gestation. Examples are holes in the heart, abnormal valves, and abnormal heart chambers.Risk factors are maternal alcohol use, medicines (for example thalidomide, warfarin) used by the expectant mother, maternal infections such as rubella, poor maternal nutrition (low intake of folate), close blood relationship between parents.

1.1.4 Other cardiovascular diseases

Tumours of the heart; vascular tumours of the brain; disorders of heart muscle (cardiomyopathy); heart valve diseases; disorders of the lining of the heart.

1.1.4.1 Rheumatic heart disease

Damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria.

1.2 Endothelial cells

Endothelial cells are cells that are found lining the inside of every blood vessel in the body. They form a single thick layered cell known as the endothelium which is also found on the inner walls of the chambers of the heart and lymphatic vessels. They carry excess plasma around the body (). Endothelial cells occupy a crucial location between the blood and the tissues. This facilitates their involvement in numerous physiological processes. Blood vessels consist of a layer of smooth muscle surrounding an inner layer of endothelium. In addition to providing a selectively permeable barrier to blood, endothelial cells are vital to maintaining a physiological equilibrium relating to the processes of inflammation, platelet aggregation, thrombosis and vascular smooth muscle proliferation (Henderson, 1991). Endothelial cells also transform vascular tone and blood

flow, in doing so, have profound effects on the overall function of the cardiovascular system (Henderson, 1991).

The largest blood vessels are arteries and veins, which have a thick, tough wall of connective tissue and many layers of smooth muscle cells. The wall is lined by an exceedingly thin single sheet of endothelial cells, the endothelium, separated from the surrounding outer layers by a basal lamina. The amounts of connective tissue and smooth muscle in the vessel wall vary according to the vessel's diameter and function, but the endothelial lining is always present.

The endothelium is a very thin layer of cells lining vessels inwards. Apart from its function as a mechanical barrier, it plays crucial roles in immune and inflammatory responses, in haemostasis, and in vascular tone regulation. Also it has autocrine, paracrine, and endocrine functions (Haefliger *et al*., 1994; Haefliger *et al*., 2001).

1.2.1 Endothelial function

The endothelium plays important roles in modulating vascular tone by synthesizing and releasing a variety of endothelium-derived relaxing factors, including vasodilator prostaglandins, NO, and endothelium-dependent hyperpolarization factors, as well as endothelium-derived contracting factors. Endothelial dysfunction is mainly caused by reduced production or action of these relaxing mediators. Endothelial functions are essential to ensure proper maintenance of vascular homeostasis and that endothelial dysfunction is the hallmark of a wide range of cardiovascular diseases associated with pathological conditions toward vasoconstriction, thrombosis, and inflammatory state (Godo and Shimokawa, 2017).

Endothelial function has a more important role in penile erection than in other vascular systems. To sustain an erection, the cavernous and helicine arteries dilate up to 80%, whereas most

conduit arteries dilate only about 15% in response to flow-mediated dilation (FMD) (Kaiser *et al*., 2004).

1.2.2 Endothelial cell dysfunction

Endothelial cell dysfunction in modern cardiovascular medicine refers to abnormalities in the bioavailability of endothelial derived nitric oxide and resultant deleterious changes in vascular reactivity (Gimbrone and Garcia-Cardena, 2016).

The term 'endothelial cell dysfunction' may refer to disruption of any of the processes that require healthy endothelial cells. It is most commonly used to describe abnormal endothelial-dependent smooth muscle relaxation (i.e, vasodilation) due to impairment of the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway (Bivalacqua *et al*., 2003).

Endothelial dysfunction is an early manifestation of cardiovascular diseases (CVD) which occurs prior to the onset of the disease itself (Blick *et al*., 2016). Endothelial dysfunction can be identified as limited flow-mediated dilation (FMD) of the brachial artery. Flow-mediated dilation (FMD) is a technique used to measure endothelial function and it has predictive value for future cardiovascular diseases (Ras *et al*., 2013).

1.2.3 Endothelins

Endothelins are proteins consisting of 21 amino acid residues that are produced in various cells and tissues, playing a role in regulation of cell proliferation, hormone production and have been implicated in the development of vascular diseases.

Endothelins (ETs) have been demonstrated in penile erectile tissues and may have different roles in erectile function, including maintenance of corpus carvenosum smooth muscle tone (Andersson and Wagner, 1995; Andersson, 2001; Ritchie and Sullivan, 2011). In the endothelium of human corpus carvenosum (CC) tissue, intense ET-like immunoreactivity has

been observed; immunoreactivity has also been observed in the CC smooth muscle. Binding sites for ET-1 have been demonstrated by autoradiography in the vessels and in CC tissue. Both ET_A and ET_B receptors have been found in human CC smooth muscle membranes, and it cannot be excluded that both receptor subtypes are functional (Andersson, 2001).

Endothelin-1 (ET-1), a peptide of 21 amino acid residues, is a pleiotropic molecule best known for vasoconstriction (Kwanabe and Nauli, 2010). ET-1 is one of a family of three proteins encoded by distinct genes that includes ET-2 and ET-3 (Inuoue *et al*., 1989; Yanagisawa *et al*., 1988). ET-2 and ET-3 differ from ET-1 by 2 and 6 amino acids respectively (Figure 5) (Kwanabe and Nauli, 2010; Inuoue *et al*., 1989). The vascular endothelium is an abundant source of ET-1 (Yanagisawa *et al*., 1988; Yanagisawa, 1994).It may also be expressed by leucocytes, smooth muscle cells and cardiac myocytes (MacCumber *et al*., 1990; Luscher and Barton, 2000). ET-1 production is inhibited by nitric oxide (NO), prostacyclin and atrial natriuretic peptide (ANP) (Fujisaki *et al*., 1995).

1.3 Relationship between endothelial, cardiovascular and erectile dysfunctions

Erectile dysfunction shares several common risk factors with cardiovascular disease, for example, obesity, metabolic syndrome, smoking, lack of exercise, diabetes, and hypercholesterolaemia. A community based cross sectional study reported that erectile dysfunction was more prevalent in men with a body mass index (BMI) of 30 or more (Andersson *et al*., 2008). Another study found that men with a BMI above 25 are at a higher risk of erectile dysfunction (Esposito *et al*., 2004).

The common pathway linking cardiovascular disease and erectile dysfunction probably involves endothelial dysfunction and small vessel atherosclerosis, which impairs smooth muscle relaxation within the penis. This link with cardiac disease initially led to the publication of the

first Princeton consensus, (DeBusk *et al*., 2000) which proposed assessing men for exercise ability to ensure that they can meet the demands of sexual activity.

Coronary artery disease (CAD) and erectile dysfunction (ED) are both highly prevalent conditions that frequently occur concomitantly (Tan and Pu, 2003; Montorsi *et al*., 2004).

Additionally, they share mutual vascular risk factors, suggesting that they are both manifestations of systemic vascular disease. The role of endothelial dysfunction in CAD is well established. Normal erectile function is primarily a vascular event that relies heavily on endothelially derived, nitric oxide-induced vasodilation (Rodriguez *et al*., 2005).

As CAD and ED overlap in prevalence and risk factors, they are also thought to share pathological basis of etiology and progression (Kirby *et al*., 2005). The role of endothelial dysfunction is well established in coronary artery disease and its risk factors (Schachinger and Zeiher, 2001; Brunner *et al*., 2005).

Normal erectile function is primarily a vascular event that relies heavily on vasodilation, which occurs largely due to endothelially derived nitric oxide (NO) (Jeremy *et al*., 1997 Kloner and Zusman, 1999; Andersson and Steif, 2000;). Accordingly, endothelial dysfunction has been implicated as a common mechanism between CAD and ED (Solomon *et al*., 2003).

ED and vascular disease are thought to be coupled at the level of the endothelium. Endothelial dysfunction results in an incapability of the smooth muscle cells lining the arterioles to relax, thereby preventing blood flow (Solomon *et al.,* 2003).

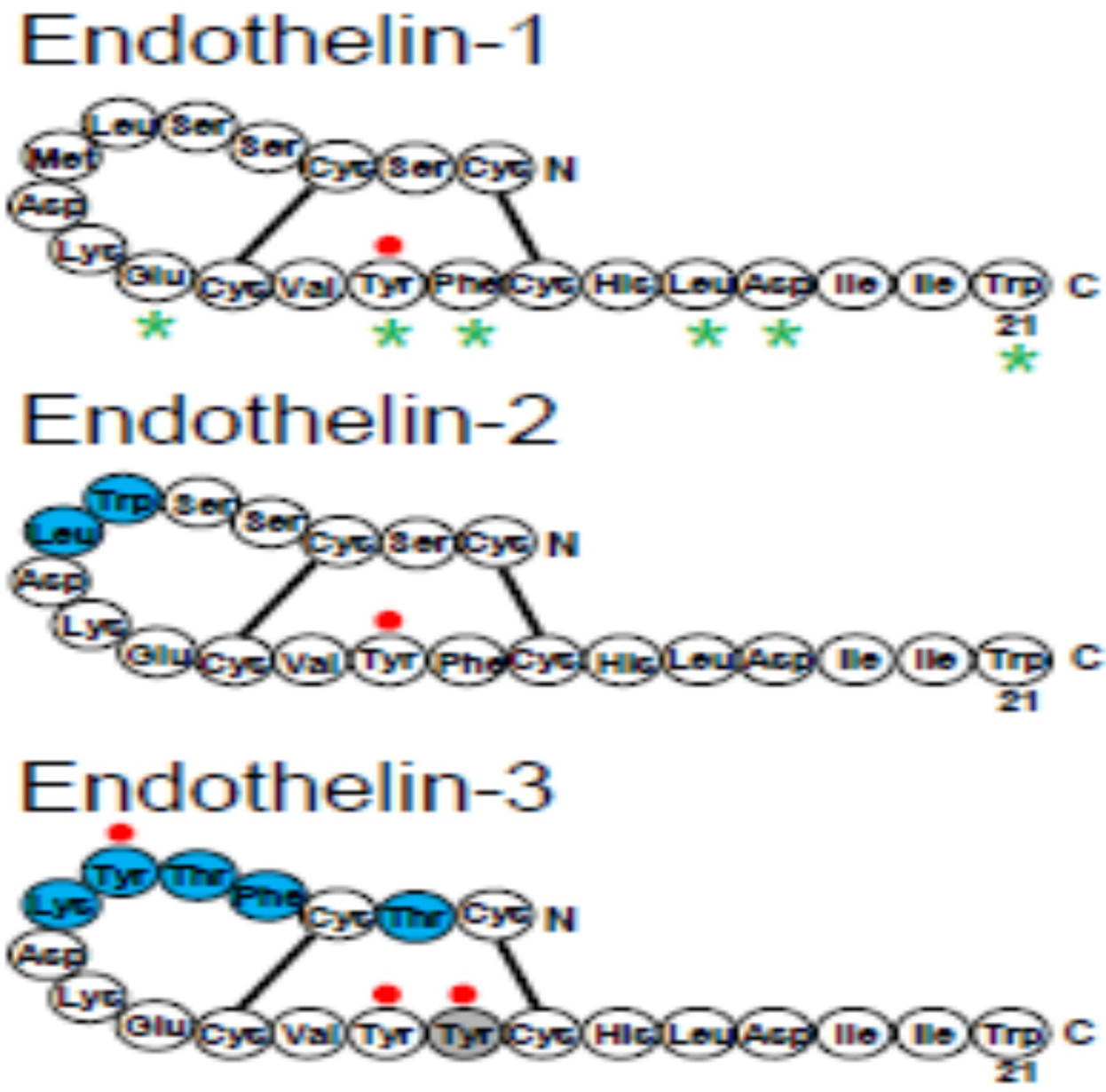


Figure 5: Structure of Endothelins

Source: Davenport *et al*. (2016)

Assessment of endothelial function is based on principle of conversion of amino acid L-arginine to NO by enzyme NO synthase (Figure 6). This process may be receptor-dependent (such as in response to acetylcholine) or via a non–receptor-dependent mechanism (such as shear stress induced by reactive hyperemia). NO diffuses to smooth muscle cells and mediates relaxation through cGMP mechanism.

Endothelial dysfunction has gained increasing notoriety as a key player in the pathogenesis of atherosclerosis (Ross, 1993). As atherosclerosis is the commonest cause of vasculogenic erectile dysfunction in older men, it is frequently considered another manifestation of vascular disease (Russel and Nehra, 2003; Montorsi *et al*., 2004). The mutual risk factors shared by ED and CAD each contribute to endothelial dysfunction. Just as the presence of these risk factors overlaps within patient populations, so do their effects on the endothelium.

1.4 Nitric oxide

Nitric oxide is a colourless poisonous gas formed by oxidation of nitrogen or ammonia present in the atmosphere. Nitric oxide can also be synthesized by mammals from arginine and oxygen and it acts as a vasodilator as well as a mediator of cell-to-cell communication between nerves. Nitric oxide (NO) is recognized as a mediator in a broad array of biologic systems. NO is the endothelium-derived relaxing factor, which causes vasodilation by relaxing vascular smooth muscle. NO also acts as a neurotransmitter, prevents platelet aggregation and plays an essential role in macrophage function.

Nitric oxide is an important mediator in the control of vascular smooth muscle tone. NO is synthesized by eNOS in endothelial cells, and diffuses to vascular smooth muscle, where it activates the cytosolic form of guanylyl cyclase to form cGMP (Figure 7).

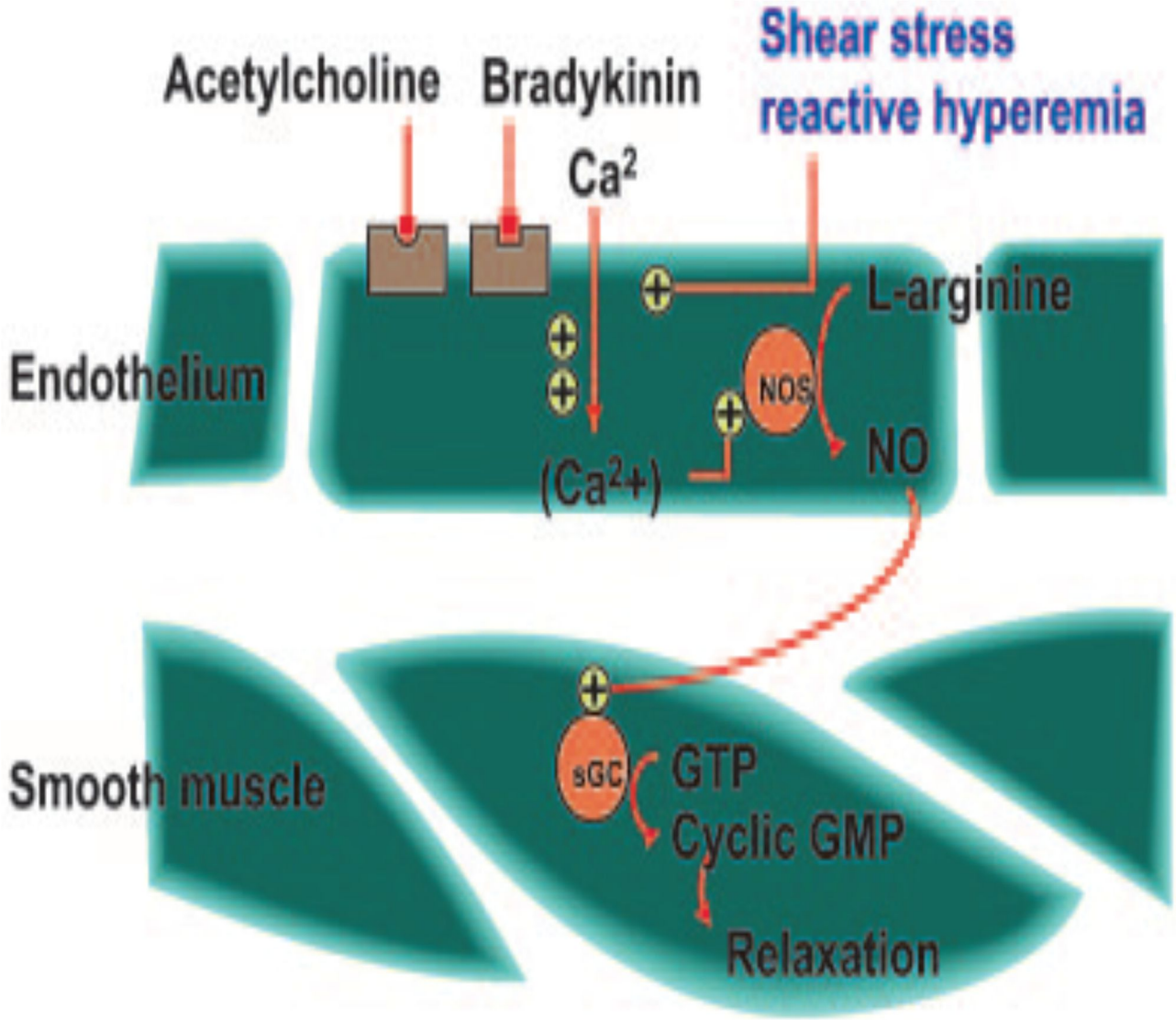


Figure 6: Mechanism of Assessing Endothelial Function

Source: Amir Lerman *et al*. (2005)

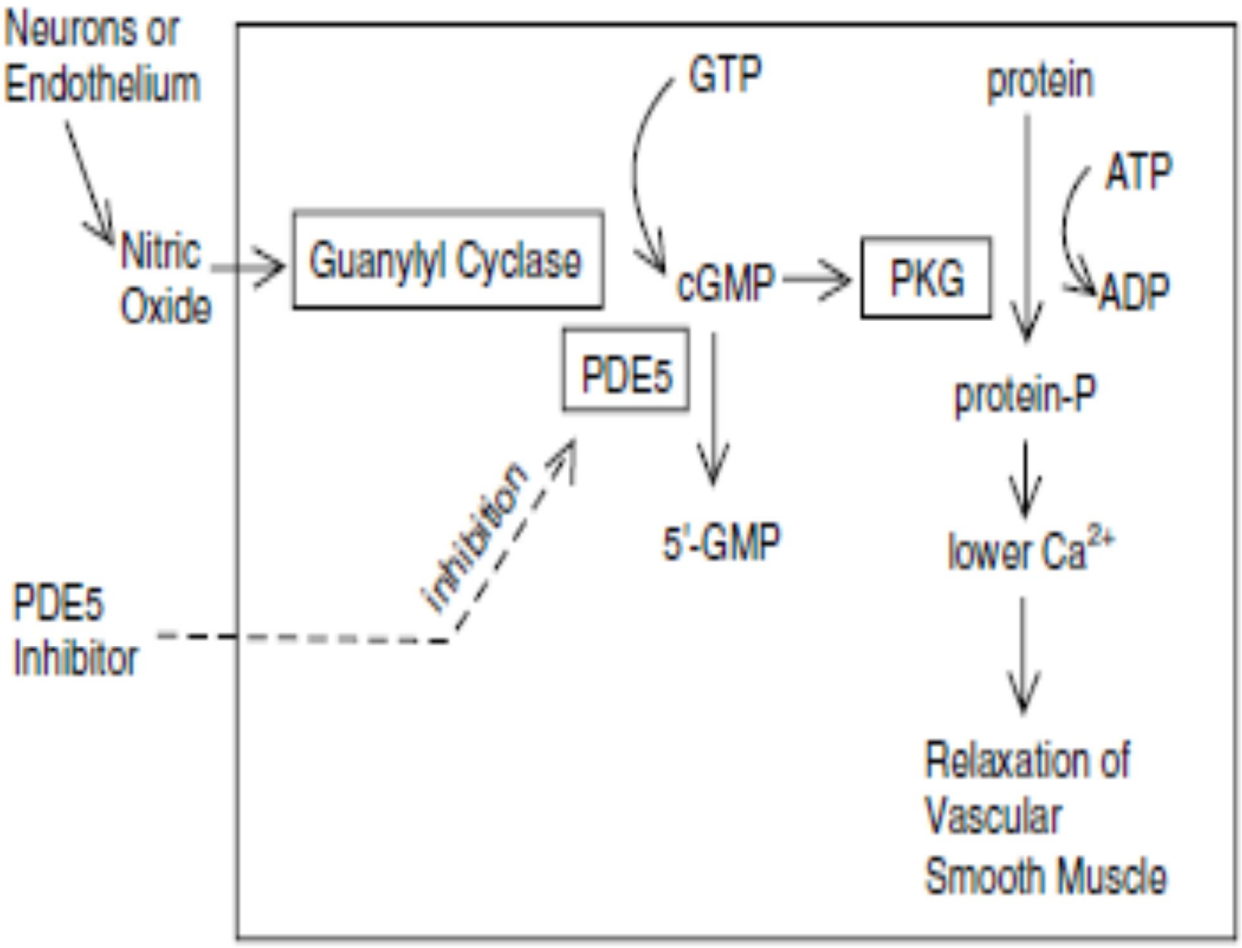


Figure 7: Nitric oxide–cGMP pathway for relaxation of smooth muscle

Source: Corbin (2004)

Nitric oxide (NO) is produced from oxygen and L-arginine under the control of nitric oxide synthase (NOS). Sexual arousal stimulates neural pathways that result in the release of NO from nerves and endothelial cells directly into the penis. NO penetrates into the cytoplasm of smooth muscle cells and binds to guanylyl cyclase. The interaction of NO with guanylyl cyclase causes a conformational change in this enzyme, which results in the catalytic production of 3'5'cyclic guanosine monophosphate from guanosine triphosphate (GTP) (Rajfer *et al*., 1992 and Trigo-Rocha *et al*., 1994). Accumulation of cyclic guanosine monophosphate activates protein kinase G which in turn phosphorylates several cascades of proteins leading to lowering of intracellular calcium levels and finally relaxation of vascular smooth muscle (erection of the penis) (Corbin, 2004).

The role of NO as the principal mediator of penile erection operating through a specific signal transduction mechanism cannot be overemphasized (Burnett, 1997; Cartledge *et al.,* 2001; Andersson, 2001). The molecule has an important messenger-signaling function, characterized by its release from nerves and endothelium in the cavernosal tissue and binding to soluble guanylyl cyclase in corporal smooth muscle cells, which results in the activation of the enzyme to produce the potent second messenger molecule, 3',5'-cyclic guanosine monophosphate (cGMP). This product then stimulates a cGMP-dependent protein kinase, protein kinase G (PKG), which critically induces the relaxant effects in the cavernosal tissue. The synthesis of NO depends upon the catalytic action of NOS and is derived from the precursor amino acid, L - arginine. The constitutive NOS isoforms: neuronal NOS (nNOS) and endothelial NOS (eNOS), account for its regulated formation, which is biochemically relevant for penile erection, relatively different from roles attributed to inducible NOS. These constitutive isoforms are regulated by

classically known interactions played by the NOS regulatory cofactors, calcium, calcium-binding protein calmodulin, oxygen, and reduced NADPH. They generate NO transiently and in low amounts, consistent with traditional tenets of cell– cell signaling, after a rise in intracellular calcium and calcium–calmodulin binding. Acute increases in 'shear stress,' the term used to indicate the mechanical forces of blood flow on the vascular lining, also supposedly drive rapid, but limited, amounts of NO release from eNOS by related biochemical mechanisms (Busse and Fleming, 1998). In the absence of stimulation of the NO pathway, PDE5 inhibition is ineffective. Since sexual arousal stimulates this pathway specifically in the penis, PDE5 inhibitors have a relatively small effect on smooth muscle in other tissues.

1.5 Antidepressants as chemical inducers of erectile dysfunction

Depression is conventionally seen as a state of chemical imbalance, and antidepressants are suggested to act through increasing monoaminergic neurotransmission (Andrade and Rao, 2010). Male reproductive function follows a defined pattern of events which can be influenced by both exogenic and endogenic substances. However, many of the male reproductive disorders that may afflict mammals are caused by ingested materials which could be from environmental or industrial sources (Hauser *et al.,* 2015). Majority of these substances may confer male reproductive toxic effects which may result into sexual dysfunction and infertility, hence may be referred to as chemical inducers of reproductive disorders. Examples of these chemical inducers include selective serotonin re-uptake inhibitors.

1.5.1 Selective Serotonin Re-uptake inhibitors (SSRIs)

Serotonin is a neurotransmitter that modulates neural activities and a wide range of neuropsychological processes (Berger *et al.,* 2009). Selective serotonin reuptake inhibitors (SSRIs) are a class of drugs that are typically used as antidepressants in the treatment of major

depressive disorder and anxiety disorders. SSRIs increase the level of serotonin by limiting its re-uptake into the presynaptic cell, increasing the amount of serotonin in the synaptic cleft available to bind to the postsynaptic receptor. SSRIs are useful therapeutic agents for the management of depression and other psychiatric disorders in many countries (Preskorn *et al.,* 2004). Treatment with these agents is associated with a high incidence of sexual dysfunction, reaching incidence rates above 50% in some studies (Zajecka *et al*., 1997). SSRI-induced sexual dysfunction affects men and women and includes orgasm delay, anorgasmia, delayed ejaculation and impotence (Labbate *et al*., 1998). A commonly known and available SSRI in Nigeria is paroxetine.

Paroxetine has often been reported to induce sexual dysfunction (Kennedy *et al*., 2000; Kayode and Yakubu, 2017), while citalopram has been shown to affect sexual function to a lesser degree (Mendels *et al*., 1999). Paroxetine has been reported to inhibit nitric oxide synthase (NOS) activity *in vitro* and *in vivo* (Finkel *et al*., 1996).

1.5.1.1 Paroxetine

Paroxetine or paroxetine hydrochloride (with trade name Seroxat), is an antidepressant medication of the selective serotonin reuptake inhibitors (SSRIs) type (Katzman, 2009). It is the hydrochloride salt of a phenylpiperidine compound identified chemically as (-)-trans-4R-(4'-fluorophenyl)-3S-[(3',4'-methylenedioxyphenoxy) methyl] piperidine hydrochloride hemihydrate and represented chemically as: $C_{19}H_20FNO_3$•HCl•1/2H_2O as shown in Figure 8. Its chemical structure is unrelated to other selective serotonin reuptake inhibitors (which are tricyclic or tetracyclic) and not related to other available antidepressant/ antipanic agents.

It is currently the treatment option for individuals suffering from depression. SSRIs, including paroxetine, increase the amount of serotonin in the synaptic clefts by inhibiting serotonin

reuptake pumps (Hyttel, 1994). Paroxetine is the most potent inhibitor of the reuptake of serotonin of all currently available antidepressants including the class of SSRIs and it potently and selectively inhibits neuronal serotonin reuptake through antagonism of the serotonin transporter. Inhibition of serotonin reuptake also enhances serotonergic neurotransmission by reducing turnover of the neurotransmitter via a negative feedback mechanism (Hyman and Nestler, 1996). However, it may take 5 to 12 days for paroxetine to clear out of the system (Alison, 2002).

1.6 Male reproductive hormones

Hormones are chemical substances synthesised in small amounts by an endocrine tissue and carried in the blood to another tissue where it acts as a messenger to regulate the function of the target tissue or organ (Nelson and Cox, 2005). Cells respond to hormone when they express a specific receptor for that hormone. The hormone binds to the receptor protein, resulting in the activation of a signal transduction mechanism that ultimately leads to cell type-specific responses (Nelson and Cox, 2005). They interfere with the synthesis, secretion, transport, binding, action, or elimination of other natural hormones in the body which can change the homeostasis, reproduction, development, and/or behaviour of an individual (Crisp *et al*., 1998).

1.6.1 Testosterone

Testosterone is a steroid hormone chemically referred to as 17β-hydroxyandrost-4-en-3-one-Δ-4-androstan-17β-ol-3-one, is a compound with molecular formula $C_{19}H_{28}O_2$. It is formed from the androgen group and is found in mammals, reptiles, birds (Cox and John-Alder, 2005) and other vertebrates. It is a hormone made primarily in the testicles.

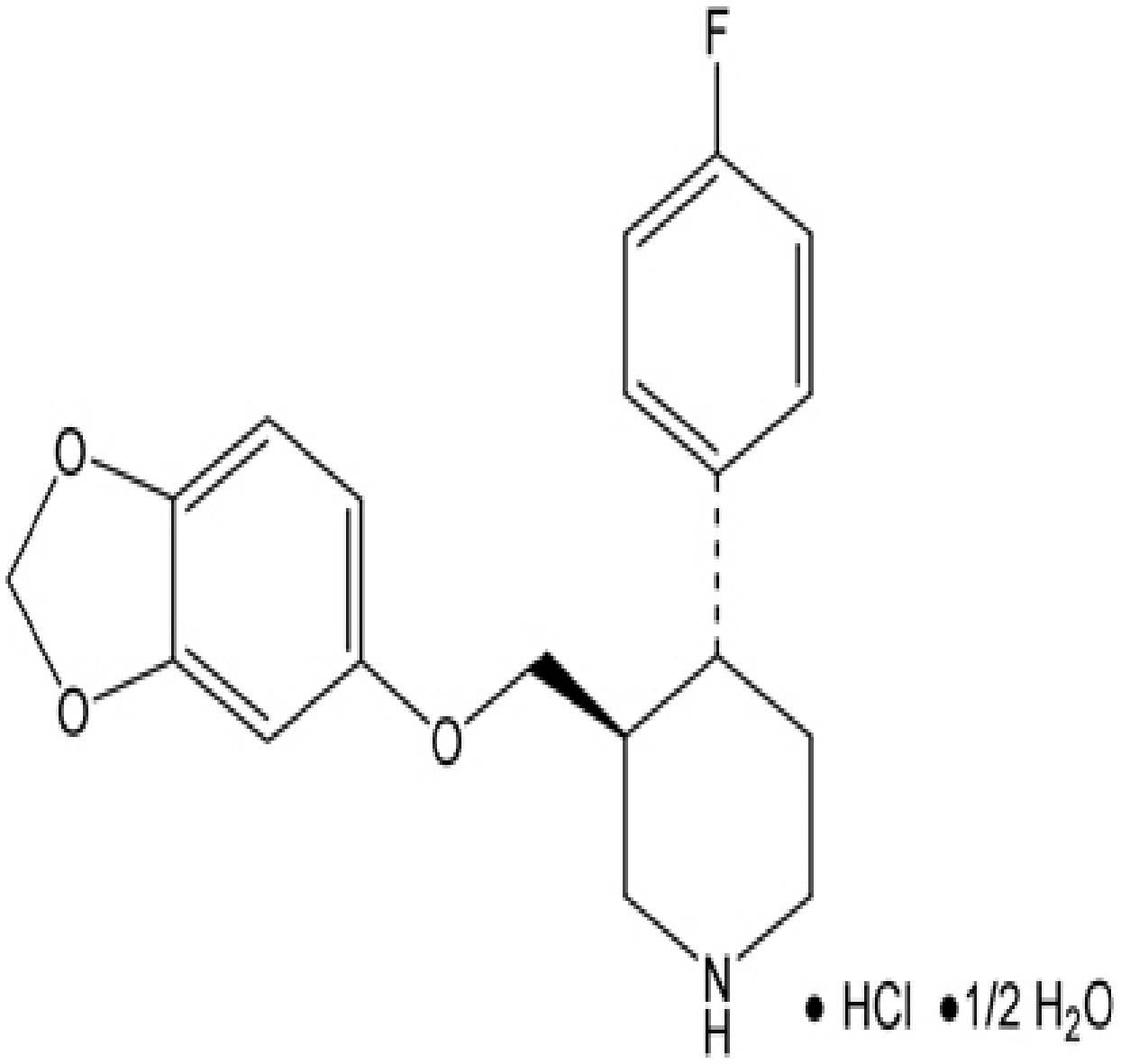


Figure 8: Chemical structure of paroxetine hydrochloride

Source: Alan and Charles (2009)

Testosterone is usually referred to as the “king of hormones” and it is produced mainly by the interstitial cells of the Leydig in the testes of males, in the ovarian theca in female and to a lesser extent in the adrenal cortex in both sexes (Hutson *et al*., 1997).

1.6.1.1 Distribution of testosterone

On the average, in adult human males, the plasma concentration of testosterone is about 7–8 times the concentration in the plasma of adult human females (Torjesen and Sandnes, 2004), but as the metabolic consumption of testosterone in males is greater, the daily production is about 20 times greater in men. Females are also sensitive to testosterone (Dabbs and Dabbs, 2000). Testosterone is found in all tissues of the body (with high concentration in gonads) as well as body fluids (Beth *et al*., 2005).

1.6.1.2 Mechanism of action of testosterone as a reproductive hormone

The effects of testosterone in humans and other vertebrates occur by way of two main mechanisms: by activation of the androgen receptor [directly or as Dihydrotestosterone (DHT)] and by conversion to estradiol and activation of certain estrogen receptors (Hiipakka and Liao, 1998; McPhaul and Young, 2001). Testosterone (T) is transported into the cytoplasm of target cells, where it can bind to the androgen receptor, or can be reduced to 5α-dihydrotestosterone (DHT) by the cytoplasmic enzyme 5-alpha reductase (Breiner *et al*., 1996). DHT binds to the same androgen receptor even more strongly than testosterone, so that its androgenic potency is about 5 times that of T (Breiner *et al*., 1996). The T-receptor or DHT-receptor complex undergoes a structural change that allows it to move into the cell nucleus and bind directly to specific nucleotide sequences of the chromosomal DNA. The areas of binding are called hormone response elements (HREs), and influence transcriptional activity of certain genes, producing the androgen effects. Androgen

receptors occur in many different vertebrate tissues, and both males and females respond similarly to similar levels.

1.6.1.3 Biochemical roles of testosterone

i Testosterone stimulates the development of the penis and testes (Bagatell and Bremner, 1996).

ii It maintains sex drive, increases libido (Anderson *et al*., 1992), enhances production of sperm cells, and causes baldness amongst others.

iii In men, testosterone promotes development of secondary sexual characteristics such as increased muscle, bone mass, and the growth of body hair (Anderson *et al*., 1992).

iv Testosterone is necessary for normal sperm development and activates genes in Sertoli cells, which promote differentiation of spermatogonia (Mehta *et al*., 2008).

v It regulates acute Hypothalamic–Pituitary–Adrenal axis (HPA) response under dominance challenge (Mehta *et al*., 2008).

vi Testosterone regulates cognitive and physical energy.

vii It maintains muscle trophism.

viii Testosterone regulates the population of thromboxane A_2 receptors on megakaryocytes and platelets and hence platelet aggregation in humans (Ajayi *et al*., 1995).

1.7 Second messengers

Hormones or neurotransmitters can be thought of as signals and their receptors as signal detectors. Each component serves as a link in the communication between extracellular events and chemical changes within the cell (Harvey and Ferrier, 2011). Many receptors signal their recognition of a bound ligand by initiating a series of reactions that ultimately result in a specific intracellular response (Harvey and Ferrier, 2011).

Second messengers are small molecules and ions that send signals received by cell surface receptors to effector proteins. They include a wide variety of chemical species and have diverse properties that allow them to signal within membranes (e.g., hydrophobic molecules such as lipids and lipid derivatives), within the cytosol (e.g., polar molecules such as nucleotides and ions), or between the two (e.g., gases and free radicals) (Newton *et al*, 2016). Biosignals received by receptors at the surface of the cell, or in some cases, within the cell are often sent to all parts of the cell via generation of small, rapidly diffusing molecules referred to as second messengers. These second messengers spread the initial signal (the "first message") that occurs when a ligand binds to a specific cellular receptor (Heldin *et al*., 2014) ligand binding alters the protein conformation of the receptor such that it stimulates nearby effector proteins that speed-up the production (or in the case of ions), release/influx of the second messenger (Newton *et al.,* 2016). The second messenger then diffuses rapidly to protein targets elsewhere within the cell, altering the activities as a response to the new information received by the receptor. Three classic second messenger pathways are illustrated in Figure 9:

(1) Activation of adenylyl cyclase by G-protein-coupled receptors (GPCRs) to generate the cyclic nucleotide second messenger, 3′-5′-cyclic adenosine monophosphate (cAMP);

(2) Stimulation of phosphoinositide 3-kinase (PI3K) by growth factor receptors to generate the lipid second messenger, phosphatidylinositol 3, 4, 5-trisphosphate (PIP3)

(3) Activation of phospholipase C by GPCRs to generate the two second messengers membrane-bound messenger diacylglycerol (DAG) and soluble messenger inositol 1,4,5-trisphosphate (IP3), which binds to receptors on subcellular organelles to release calcium into the cytosol.

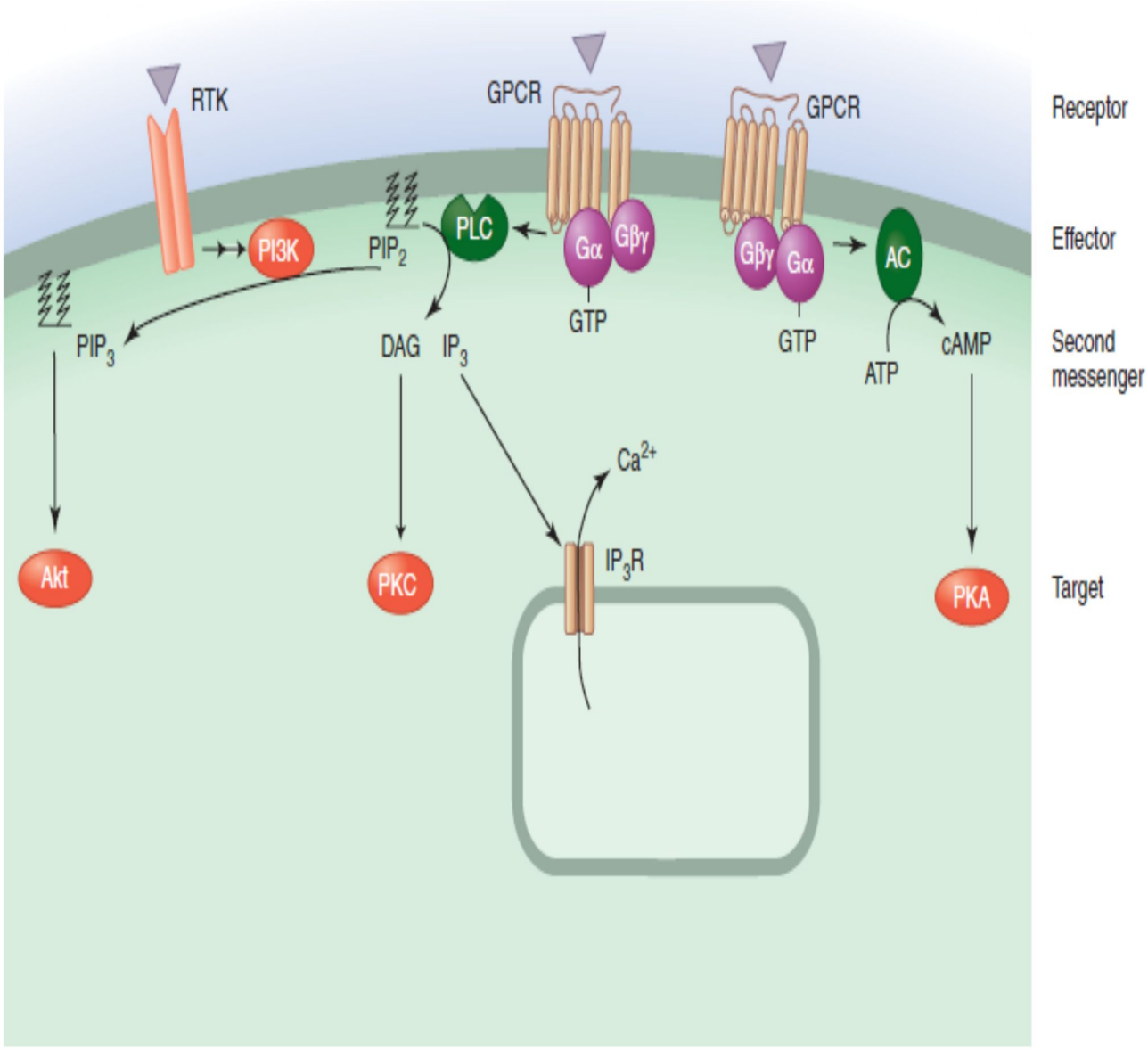


Figure 9: Three classic second messenger pathways and their targets

Source: Newton *et al*. (2016)

The activation of multiple effector pathways by a single plasma membrane receptor and the production of multiple second messengers by each effector can generate a high degree of amplification in signal transduction, and stimulate diverse, pleiotropic, responses depending on the cell type (Newton *et al*, 2016).

On the right, binding of agonists to a GPCR (the receptor) can activate adenylyl cyclase (the effector) to produce cAMP (the second messenger) which phosphorylates protein kinase A (PKA; the target). On the left, binding of growth factors to a receptor tyrosine kinase (RTK; the receptor) can activate PI3K (the effector) to generate PIP3 (the second messenger), which activates Akt (the target). In the center, binding of ligands to a GPCR (receptor) activates phospholipase C (PLC; the effector), to generate two second messengers, DAG and IP3, leading to phosphorylation and activation of protein kinase C (PKC; the target) and release calcium from intracellular stores, respectively.

Second messengers fall into four major classes:

(i) Cyclic nucleotides, such as cAMP, cGMP and other soluble molecules that signal within the cytosol;

(ii) Lipid messengers that signal within cell membranes;

(iii) Ions that signal within and between cellular compartments; and

(iv) Gases and free radicals that can signal throughout the cell and even to neighboring cells.

Second messengers from each of these classes bind to specific protein targets, altering their activity to relay downstream signals. In many cases, these targets are enzymes whose catalytic activity is modified by direct binding of the second messengers. The activation of multiple target

enzymes by a single second messenger molecule further amplifies the signal (Newton *et al..,* 2016).

1.7.1 Cyclic nucleotide signalling

Over the years, cyclic nucleotides i.e. cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) have been identified as essential signalling compounds within cells. Under normal physiological conditions, cyclic nucleotides regulate numerous biological processes such as cell growth and adhesion, energy homeostasis, neuronal signalling, and muscle relaxation. Also, alteration in cyclic nucleotide signalling has been observed in quite a number of pathophysiological situations which includes erectile dysfunction (Fajardo *et al.,* 2014). Cyclic nucleotides act as second messengers between an extracellular signal such as a hormone or neurotransmitter and their respective displayed intracellular responses. While the specific functions of a given signal varies according to the cell type, extracellular environment, stimulus activating the signal, localization of the signal, and the type of cyclic nucleotide formed, as depicted in Figure 10, an extracellular signal will normally activate a cyclase enzyme, which catalyzes the formation of the cyclic nucleotide (cNT) from its nucleotide triphosphate precursor (NTP). Once cyclic nucleotides are formed, they affect the activity of downstream effector molecules including kinases, ion channels, transcription factors, and scaffolding proteins. Both the amplitude and duration of a cyclic nucleotide signal also varies and is largely dependent on the expression and activity levels of cyclic nucleotide phosphodiesterase (PDE) enzymes, which catalyze the hydrolytic breakdown of cyclic nucleotides (Fajardo *et al.,* 2014).

Figure 10 demonstrates cyclic nucleotide (cNT) production through nucleotide precursor (NTP) and activation of selective cyclase and activation of specific receptor molecules and corresponding intracellular response or degradation of cNT through phosphodiesterase (PDE) activity.

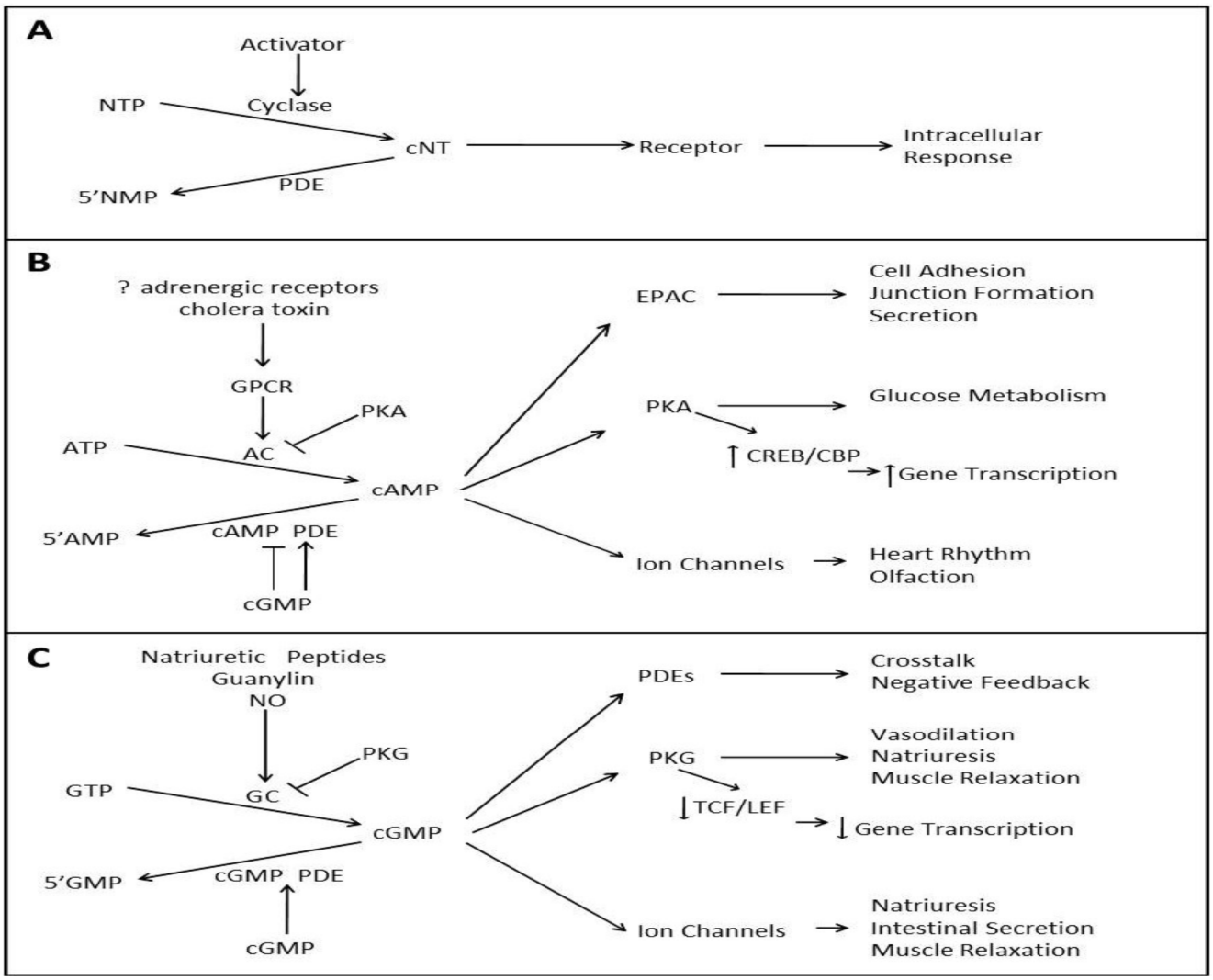


Figure 10: Cyclic nucleotide signalling

Source: Fajardo *et al.* (2014)

1.7.1.1 Cyclic Adenosine Monophosphate

Cyclic adenosine monophosphate (cAMP) was first described in the late 1950s and it is the most studied cyclic nucleotide. cAMP, is regulated by opposite activities of two enzymes, adenylyl cyclases (ACs) and phosphodiesterases (PDEs) (Gerits *et al.,* 2008; Sassone-Corsi, 2012). Both enzymes are regulated by numerous signalling pathways including, calcium signaling through calmodulin (CaM) and calcineurin, G-proteins, inositol lipids [e.g., protein kinase C (PKC)] and receptor tyrosine kinases (Sassone-Corsi, 2012). As reviewed previously (Rehmann *et al.,* 2007) cAMP is produced from its precursor, ATP, through the catalytic activity of the ACs. Differing primarily in tissue distribution and subcellular localization, nine membrane-bound and one soluble AC have been identified in mammals (Taussig and Gilman, 1995). The majority of ACs are indirectly activated by various stimuli including adrenergic agonists, which bind to G protein coupled receptors (GPCRs) on the cell membrane resulting in the activation of the GPCR and release of the Gα subunit that is subsequently responsible for binding and activating AC, thus stimulating the production of cAMP (Hanoune and Defer, 2001; Lee *et al.,* 2013). The balance of cAMP signaling is essential to multiple cellular processes, including immune function, growth, differentiation, gene expression and metabolism (Lee *et al.,* 2013). Buxton and Brunton (1983), demonstrated how different GPCR agonists could excite, in a sectionalized manner, corresponding increase in cAMP levels but yet result in receptor specific-mediated outcomes in cardiac muscle cells (Lee *et al.,* 2013; Buxton and Brunton, 1983). Concentrations of cAMP can be regulated by processes throughout the whole cell and within membrane regions.

1.7.1.2 Cyclic Guanine Monophosphate

In a manner similar to cAMP, cyclic guanosine monophosphate (cGMP) is formed through the activity of guanylyl cyclase (GC) enzymes from its precursor guanosine triphosphate (GTP). One

distinct difference between cAMP and cGMP signaling is that guanylyl cyclases are more evenly dispersed between the membrane and the cytosol of cells and are directly activated by their stimuli (Rehmann *et al.,* 2007). Seven particulate or membrane bound GCs (pGC) have been identified, each consisting of a single transmembrane region (Feil and Kemp-Harper, 2006). As with the membrane-bound AC isoforms, pGC isoforms differ largely in their tissue distribution but also in their sensitivity to ligands, which include natriuretic peptides, small paracrine peptide hormones such as, guanylin, enterotoxins, and certain cytokines. Conversely, the heme-containing soluble GC (sGC) enzyme is restricted to the cytoplasm and is exclusively activated by nitric oxide (NO) under physiological conditions (Rehmann *et al.,* 2007). Similar to cAMP, cGMP levels are mostly controlled by degradation via PDE enzymes. PDE1, 2, 3, 5, 6, 9, 10 and 11 families are capable of hydrolyzing cGMP with PDE5, 6, and 9 being selective for cGMP (Omori and Kotera). In most tissues, PDE 5 is the isoform predominantly responsible for cGMP hydrolysis and subsequent termination of a cGMP signal (Figure 11). The activity of the PDE 5 enzyme is tightly controlled by cGMP signalling. In the presence of a cGMP signal, cGMP will bind to the GAF A domain of the *N*-terminal region of the PDE 5 protein to promote its phosphorylation at a separate *N*-terminal site by the cGMP-dependent serine/threonine protein kinase (PKG), an event that produces a several-fold increase in the activity of the enzyme while simultaneously increasing the affinity of the catalytic site for cGMP (Beavo, 1995). This signalling pathway is involved in a number of important physiological processes, including smooth muscle relaxation and neurotransmission (Heldin *et al*. 2014).

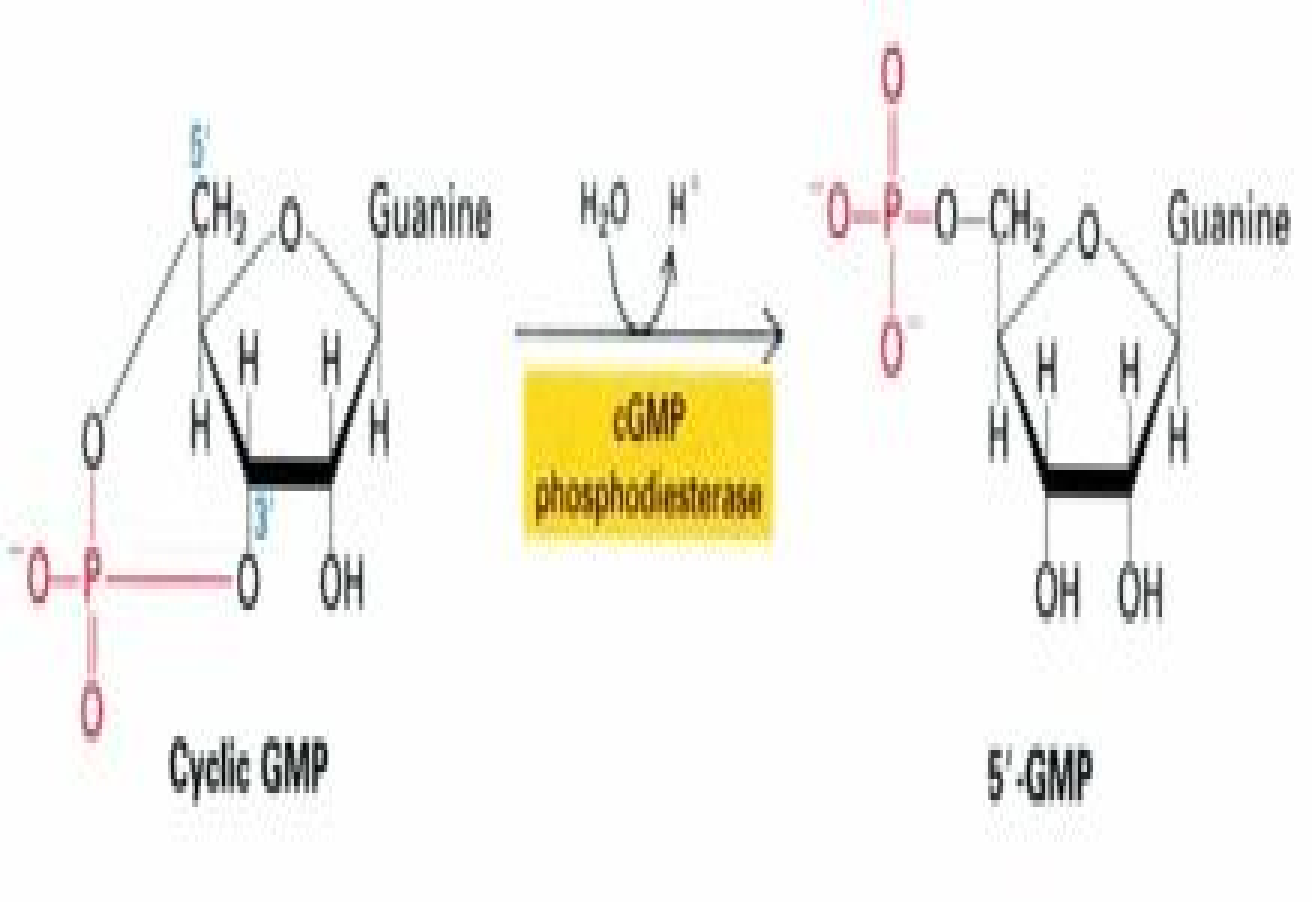


Figure 11: Reaction catalysed by Phosphodiesterase 5

Source: Kim *et al.* (2003)

Guanosine 3',5'-cyclic monophosphate (cGMP) is an important mediator of smooth muscle relaxation and has been shown to be a key molecular regulator of penile tumescence via the same mechanism earlier described.

A well-defined biochemical pathway leading to penile erection involves the binding of nerve- or endothelium-derived nitric oxide to the heme moiety of soluble guanylyl cyclase within the vascular smooth muscle of the resistance arteries and the trabeculae of the corpora cavernosa (Andersson and Wagner, 1995). This activation of guanylyl cyclase by nitric oxide catalyzes the formation of cGMP from GTP. Elevated levels of intracellular cGMP lead to the activation of cGMP-specific phosphodiesterases which hydrolyze cGMP to 5'-GMP. The degradation of cGMP by phosphodiesterases is the way by which nitric oxide signal transduction pathway is terminated within smooth muscle cells (Kim, 2003).

1.7.1.3 Cyclic nucleotide phosphodiesterases

The endpoint of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) initiated signals is mediated by phosphodiesterases (PDEs). These enzymes represent a vast gene family of 11 distinct subtypes and more than 100 isoforms that can break the phosphoester bond of either cyclic nucleotide to liberate AMP or GMP (Beavo and Brunton 2002). PDE activity can be regulated in a variety of ways. For example, calcium-dependent processes control the activity of the PDE1 and PDE2 isoforms, PKA phosphorylation attenuates the activity of PDE3 and PDE4 isoforms, and PKA or PKG phosphorylation participates in the control of PDE5. Less is known about the mechanisms of regulation for PDE6-11. More recently, there has been considerable interest in the development and clinical application of small molecule PDE inhibitors.

Selective PDE inhibitors that produce elevated levels of cAMP/cGMP have been used clinically to alleviate chronic obstructive pulmonary disease, asthma, and combat certain immune disorders, but their most celebrated therapeutic application has been in the treatment of male erectile dysfunction. Sildenafil citrate (Viagra) and its relatives act by inhibiting cGMP- specific PDE5 (Beavo and Brunton 2002) in the arterial wall smooth muscle of the penis, which elevates cGMP and increases blood flow.

1.8 Lipid profile

Lipids are fatty substances that are normally insoluble in an aqueous environment but are solubilised and transported in the plasma as water-soluble macromolecular complexes known as lipoproteins (Styrer, 1995). Lipids profile found in the serum that are of diagnositic importance include cholesterol, triglycerides, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol and phospholipids (Nelson and Cox, 2000).

1.8.1 Total cholesterol

Cholesterol is a waxy steroid biomolecule found in the cell membranes and transported in the blood plasma of all animals. It is an essential structural component of mammalian cell membranes, where it is required to establish proper membrane permeability and fluidity (Stryer, 1995). In this structural role, cholesterol reduces the permeability of the plasma membrane to protons (positive hydrogen ions) and sodium ions. In addition, cholesterol is an important component for the manufacture of bile acids, steroid hormones, and fat-soluble vitamins including vitamins A, D, E, and K (Emma-Leah, 2009).

Within the cell membrane, cholesterol also functions in intracellular transport, cell signaling and nerve conduction. Cholesterol is essential for the structure and function of invaginated caveolae and clathrin-coated pits, including caveola-dependent and clathrin-dependent endocytosis.

Recently, cholesterol has also been implicated in cell signaling processes, assisting in the formation of lipid rafts in the plasma membrane (Pawlina *et al.,* 2006).

1.8.2 Triglyceride

A triglyceride (triacylglycerol, TAG, or triacylglyceride) is an ester derived from glycerol and three fatty acids. It is the main constituent of vegetable oil and animal fats (Nelson and Cox, 2000).

Triglycerides, as major components of very-low-density lipoprotein (VLDL) and chylomicrons, play an important role in metabolism as energy sources and transporters of dietary fat. They contain twice as much energy (9 kcal/g or 38 kJ/g) as carbohydrates and proteins. In the intestine, triglycerides are split into monoacylglycerol and free fatty acids in a process called lipolysis, with the secretion of lipases and bile, which are subsequently moved to absorptive enterocytes, cells lining the intestines. The triglycerides are rebuilt in the enterocytes from their fragments and packaged together with cholesterol and proteins to form chylomicrons. These are excreted from the cells and collected by the lymph system and transported to the large vessels near the heart before being mixed into the blood. Various tissues can capture the chylomicrons, releasing the triglycerides to be used as a source of energy. Fat and liver cells can synthesize and store triglycerides. When the body requires fatty acids as an energy source, glucagon signals the breakdown of the triglycerides by hormone-sensitive lipase to release free fatty acids. As the brain cannot utilize fatty acids as energy source (unless converted to a ketone), the glycerol component of triglycerides can be converted into glucose, via glycolysis by conversion into dihydroxyacetone phosphate and consequently into glyceraldehyde 3-phosphate, for brain fuel when it is broken down. Fat cells may also be broken down for that reason, if the need of the brain ever outweigh that of the body (Parks, 2002).

Triglycerides cannot pass through cell membranes freely. Special enzymes on the walls of blood vessels called lipoprotein lipases must break down triglycerides into free fatty acids and glycerol. Fatty acids can then be taken up by cells via the fatty acid transporter (FAT) (Balch and Phyllis, 2006).

1.8.3 High-density lipoprotein cholesterol

This is composed of 13% triglycerides, 46% phospholipids, 29% cholesterol esters, 6% cholesterol and 6% free fatty acids (Nelson and Cox, 2000). It is the smallest lipoprotein with a density of 1.063 – 1.210 mmol/L. It is known as 'good cholesterol' becauses it mediates the removal of cellular cholesterol by carrying cholesterol away from the body cells and tissues to the liver for excretion (Murray *et al*., 2000). The increase in the concentration of HDL-C correlates inversely with coronary heart disease (Philip, 1995).

1.8.4 Low-density lipoprotein cholesterol

This is also called beta lipoprotein and has a density of 1.006 – 1.063 g/mL. They are primary carriers of cholesterol and are known as the 'bad cholesterol' because excess LDL-C in the blood with other substances can cause atherosclerosis which may lead to stroke (Stryer, 1995). LDL-C is composed of 3% triglycerides, 28% phospholipids, 48% cholesterol esters, 10% cholesterol and 1% free fatty acids (Nelson and Cox, 2000). LDL is formed from VLDL and it transport cholesterol to cells (Philip, 1995). The normal level of LDL-C is less than 3.88 mmol/L (Ghatak and Asthana, 1995).

1.9 Medicinal plants

Medicinal plants have been used and is still being used to manage a lot of diseases/ailments. Approximately 80% of the populations of the world still rely on local medicines and traditional treatments mainly from plant extracts (Akerele, 1993; Sagga *et al*., 2007). It is worthy to note

that herbal medicine is now very popular in the developing countries (Obici *et al*., 2008). Nigerian traditional medicines are administered to treat a myriad of health problems including mental disorders, insomnia, broken bones and infertility as well as other reproductive health challenges (Enwereji, 1999).

1.9.1 Aphrodisiacs

An aphrodisiac is defined as any food or drug that arouses the sexual instinct, induces veneral desire and increases pleasure and performance. This word is derived from *ëAphroditaeí* the Greek Goddess of love and these substances are derived from plants, animals or minerals and since time immemorial they have been the passion of man (Yakubu *et al.,* 2005). A lot of natural substances have historically been known as aphrodisiacs in Africa and Europe, like yohimbine and the mandrake plant, as well as ground rhinoceros horn in the Chinese culture and "Spanish fly" which is actually toxic (Ang *et al.,* 1997; Evans, 1969). Even in today's culture, there are certain foods that are used as aphrodisiacs, including strawberries and raw oysters. Chocolate, coffee, and honey are also believed to have aphrodisiac potential. Although these natural items are claimed as aphrodisiacs, there is no or little scientific confirmation supporting those assertions.

Enhanced sexual behaviour may provide increased relationship satisfaction and self-esteem in humans (Montorsi *et al.,* 1995). The hunt for an effective aphrodisiac has been a constant pursuit throughout history. The role of various dopaminergic, adrenergic, and serotonergic agents has been intensively examined in both human and animal studies. Some of these drugs have been considered for their potential role for the treatment of sexual dysfunction, while some others have contributed to the basic neurophysiological processes in sexual arousal (Rosen and Ashton, 1993).

Aphrodisiacs can be classified by their mode of action into three types: Those that increase libido, potency, or sexual pleasure. Various substances of animal and plant origin have been used in folk medicines of different cultures to energize, vitalize and improve sexual function, and physical performance in men, out of these very few have been identified pharmacologically. For increasing libido, ambrein, a major constituent of *Ambra grisea*, is used in Arab countries. It contains a tricyclic triterpene alcohol which increases the concentration of several anterior pituitary hormones and serum testosterone. Bufo toad skin and glands contain bufotenine (and other bufadienolides), a hallucinogenic congener of serotonin. It is the active ingredient in West Indian "love stone" and the Chinese medication *chan su*. In traditional Chinese medicine, *Panax ginseng* is used as a sex stimulant. It works as an antioxidant by enhancing nitric oxide (NO) synthesis in the endothelium of corpora cavernosa (CC); ginsenosides also cause transmural nerve stimulation-activated relaxation associated with increased tissue cyclic guanosine monophosphate. For increasing sexual pleasure, cantharidin ("Spanish fly") from blister beetles, which have been used for millennia as a sexual stimulant (Sandroni, 2001).

1.9.1.1 Mechanism of action of Aphrodisiacs

Sexual desire is controlled and regulated by the central nervous system which integrates tactile, olfactory and mental stimuli (Patel *et al*., 2011). On sexual stimulation (visual or otherwise) the famines of the axons of parasympathetic nerves release endothelial nitric oxide (NO). Nitric oxide diffuses into smooth muscle cells that line the arteries of the corpus cavernosum (spongy erectile tissue) and activates the enzyme soluble guanylyl cyclase (sGC). The later converts the nucleotide guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). The cGMP in turn causes the smooth muscle cells around the penis to relax, leading to dilation and elevated influx of blood into the penile tissue. This blood is essentially trapped in the penis and

results in an erection. The erection ceases after a while because cGMP is hydrolyzed by the enzyme phosphodiesterase 5 (PDE 5) to 5'GMP which is inactive. Plants with aphrodisiac potentials inhibit the hydrolyzing action of PDE 5 which will lead to the accumulation of cGMP, undisturbed and prolong the erection through increased blood flow (Chew *et al*., 2000).

1.9.2 Plants studied

1.9.2.1 *Cnestis ferruginea*

Cnestis ferruginea Vahl ex DC belong to the family Connaraceae. It is usually referred to as *gboyin gboyin* or *Omu aja* (Yoruba), *Fura amarya* (Hausa), *Amu nkita* (Igbo), *Ukpo-ibieka* (Edo) and *Usiere ebua* (Efik). It is a perennial shrub found mainly in the savannah region of tropical West Africa. The plant is about 3.0-3.6 m high with densely, rusty brown, pubescent branches, indecidous leaves with more or less alternate or sometimes opposite, ovate to narrowly oblong leaflets and reddish-orange fruits. The ovoid follicles which are 1-5 in fruit are often united at the base and contain one seed each (Garon *et al*., 2007). *C. ferruginea* leaf has been acclaimed in herbal medicine and some literatures to have diverse therapeutic uses such as the management of conjunctivitis, bronchitis, tuberculosis, migraines, sinusitis, and oral infection (fruits); snakebite, dysentery, syphilis, gonorrhea, cough, dysmenorrhea, enema, ovarian troubles and aphrodisiac (roots) Plate 1; abortion, constipation, fever and pain (leaves) (Gill, 1992; Okafor and Ham, 1999). Studies have shown that aqueous root extract of *Cnestis ferruginea* contained alkaloids, flavonoids, saponins, anthraquinones and tannins (Yakubu *et al*., 2011).

Plate 1: ***Cnestis ferruginea*** **root**

The fruits have been reported to have anti-microbial effects especially against gram-positive bacteria (Lewis and Elvin-Lewis, 2003), while the aqueous root extract has been reported to possess anti-stress and laxative activities (Ishola and Ashorobi, 2007; Yakubu *et al*., 2011). Also, the methanolic root extract has been reported to possess analgesic and anti-inflammatory activity (Ishola *et al.*, 2011).

The aqueous root extract of *C. ferruginea* have been reported to have elicited aphrodisiac properties by reversing male sexual dysfunction (MSD) in rats induced with paroxetine especially at 52 mg/kg body weight (Yakubu and Nurudeen, 2012). The toxicological implications of the crude alkaloidal fraction from *C. ferruginea* root on the liver function indices of male rats as well as the cytotoxic activity of the leaves have been reported (Garon *et al*., 2007; Atere and Ajao, 2009). The hypoglycemic activity and acute toxicity of the methanolic extract of *C. ferruginea* have also been substantiated with scientific evidence (Adisa *et al*., 2010).

1.9.2.2 *Fadogia agrestis*

Fadogia agrestis Schweinf belongs to the family, Rubiaceae. It is also known as Black aphrodisiac (English), Baakin gagai (Hausa) is an erect shrub of 1–3 ft high. The leaves and stem are yellowish and tomentellous. The aqueous extract of the stem contain saponins, alkaloids, anthraquinones and flavonoids (Yakubu *et al.,* 2005). The stem of the plant (Plate 2) are largely used in folklore medicine as aphrodisiac (Irvine, 1961) and the use of this plant as sexual invigorators has been scientifically proven by Yakubu *et al*. (2005).

Fadogia agrestis possess significant aphrodisiac properties. Yakubu *et al*. (2008) evaluated the aphrodisiac potential of the aqueous extract of *F. agrestis* in male rats. Their sexual behaviour parameters and serum testosterone concentration were evaluated. There was also a significant increase in serum testosterone concentrations in all the groups in a dose –dependent manner.

Plate 2: ***Fadogia agrestis*** **plant**

The aqueous extract of *F. agrestis* stem increased the blood testosterone concentrations and this may be the mechanism responsible for its aphrodisiac effects and various masculine behaviours. It may be used to modify impaired sexual functions in animals, especially those arising from hypotestosteronemia.

1.10 Secondary metabolites

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism (Fraenkel, 1959; Bidlack, 2000). Unlike primary metabolites, absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of the organism's survivability, fecundity, or aesthetics, or perhaps in no significant change at all (Bidlack, 2000). Secondary metabolites are often restricted to plant species and can be classified on the basis of their chemical structure, composition, solubility in various solvents, or the pathway by which they are synthesised (Bidlack, 2000). A simple classification according to their biosynthetic pathways includes three main groups: the terpenes (mevalonic acid), phenols (from simple sugars), and alkaloids (Harborne, 1999). Secondary metabolites often play an important role in plant defense system against herbivores (Stamp, 2003) and other interspecies defenses (Samuni-Blank *et al*., 2012). Secondary metabolites are used by human beings as medicines, flavouring agents and recreational drugs (Nafiseh and Mohammad, 2013).

In this study, the secondary metabolites investigated are: alkaloids, saponins, phenolics, phlobatannins, cardiac glycosides, chalcones, steroids, flavonoids and terpenoids.

1.10.1 Saponins

Saponins are amphipathic glycosides grouped logically by the soap-like bubbles they produce when shaken in aqueous solutions, and structurally by their composition of one or more hydrophilic

glycoside moieties combined with a lipophilic triterpene derivative (Hostettmann and Marston, 2000). Saponins are indeed found in many plants and derive their name from the soapwort plant (Genus *Saponaria*, Family *Caryophyllaceae*). Saponins have been found to have aphrodisiac properties due to its ability to serve as precursor of sexual hormones (Abo-Doma *et al*., 1991). They inhibit or kill cancer cell without killing normal cells by binding to cholesterol using the hydroxyl group and thus interfere with abnormal cell growth and division.

1.10.2 Alkaloids

Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms but this group also includes some related compounds with neutral and even weakly acidic properties (Manske, 2003). The alkaloids are found mainly in plants (10-25% in higher plants), but also to a lesser extent in microorganisms and animals (Aniszewski, 2007). They are synthesised predominantly from amino acids (Aniszewski, 2007). Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke a bitter taste (Rhoades, 1979). Alkaloids exhibit a wide variety of pharmacological activities and clinical uses such as analgesics and narcotics, anti-asthmatics, expectorants, anti-hypertensive, antihelmintics and antiparasitics (Doughari, 2012). Some animals use specific alkaloids in their metabolism, especially in the production of neurotransmitters like serotonin, dopamine and histamine. Also, Yohimbine, a form of alkaloid, serves as a stimulant of the central nervous system and an aphrodisiac (Hesse, 2002). Alkaloids have been reported to be responsible for increase in spermatogenesis, weight of testes, seminal vesicle, prostate and facilitate erection in rats (Saksena and Dixit, 1987).

1.10.3 Phenolics

Phenols are chemical components that occur ubiquitously as natural coloured pigments responsible for the colour of fruits of plants. Phenolic compounds have been reported to be universally distributed in plants (Dai and Mumper, 2010). They are classified into (i) phenolic acids and (ii) flavonoid polyphenolics (flavonones, flavones, xanthones and catechins) and (iii) non-flavonoid polyphenolies. Caffeic acid is regarded as the most common of the phenolic compound distributed in the plant flora followed by chlorogenic acid known to cause allergic dermatitis among humans (Kar, 2007). Phenolics in plants are mostly synthesised from phenylalanine via the action of phenylalanine ammonia lyase. They are very important to plants and have multiple functions. Dietary phenolics possess antioxidant properties and function as reducing agents, free radical scavengers and quenchers of singlet oxygen formation.

1.10.4 Cardiac glycosides

The terms ‘cardiac glycosides’ or ‘digitalis’ are steroid or steroidal glycoside compounds that exert a prominent effect on heart muscle and heart rhythm (specifically on myocardial contraction). Cardiac glycosides are composed of two structural features: the sugar (glycoside) and the non-sugar (aglycone-steroid) moieties. Two classes have been observed in nature; the cardenolides and dienolides. They show considerable structural diversity, but all members of this family share a common structural motif. The core structure consists of a steroidal framework, which is considered the pharmacophoric moiety responsible for the activity of these compounds. More than a hundred cardiac glycosides have been identified as secondary metabolites in plants, with most belonging to the angiosperms (Melero *et al*., 2000).

1.10.5 Tannins

Tannins are naturally occurring plant polyphenols which are water soluble polymeric phenolics that bind and precipitate protein (Haslam, 1989). They are widely distributed in the plant kingdom and exist in mixtures with many other classes of plants phenolic compounds (Reed, 1995). They are responsible for the astringent taste of wine and unripe fruits and for enhancing colours seen in flowers and in autumn leaves. They also act as a defense mechanism in plants against pathogens, herbivores and hostile environmental condition. Effects of tannins may largely depend on tannin type, composition, and source and on other nutritional and physiological factors (Chung-MacCoubrey *et al*., 1997). Tannins have diverse effects on biological systems because they are potential metal ion chelators, protein precipitating agents, and biological antioxidants (Ann *et al*., 1998). Tannins have shown potential anti-viral (Lu *et al*., 2004), antibacterial (Akiyama *et al*., 2001) and antiparasitic effects (Kolodziej and Kiderlen, 2005).

1.10.6 Phlobatannins

Phlobatannins are chemically referred to as tetrahydropyranochromenes. They are condensed tannins (complex of tannin and phlobaphene). The treatment of phlobatannins with acids or heat forms tannin and phlobaphene (Cronjr *et al*., 1995). Phlobaphene has a Greek root meaning bark or dye and natural phlobaphenes are the common bark, pericarp, cob glume and seed coat (testa) pigments. Phlobaphenes are reddish, alcohol soluble and water insoluble phenolic substances. They can be extracted from plants, or products that result from treatment of tannin extracts with mineral acids.

1.11 Phosphodiesterase Family

Phosphodiesterases (PDEs, EC 3.1.4.17) are metallohydrolases that regulate the intercellular levels of two important second messengers, cyclic adenosine 3′,5′ monophosphate (cAMP) and cyclic guanosine 3′,5′monophosphate (cGMP), by controlling their degradation (Temkitthawon *et al*., 2008; Karami-Tehrani *et al*., 2012; Das *et al*., 2015).

PDEs participate in the regulation of signal transduction by regulating cyclic nucleotides so that the response to cell stimuli is both specific and activates the appropriate third messengers (Daniela *et al*., 2008).

In all, 11 main types of phosphodiesterases (some types with multiple isoforms) have been identified (Conti, 2000; Soderling and Beavo, 2000). Each type of phosphodiesterase has differing substrate specificities based upon their ability to discriminate between cGMP and cAMP (Table 2).

Although several different types of phosphodiesterases have been identified within penile cavernosal tissue, human trabecular smooth muscle cells express phosphodiesterase type 5 (PDE 5) as the major cGMP hydrolyzing enzyme (Moreland *et al*., 1998; Stacey *et al*., 1998).

1.11.1 Phosphodiesterase 5

Phosphodiesterase 5(PDE 5) is a homodimer of 99.6 kDa subunits, binds two zinc atoms per monomer which are necessary for its catalysis (Francis *et al*., 1994). PDE 5 is a major regulator of the intercellular concentration of cGMP (Kumazoe *et al*., 2013; Das *et al*., 2015). PDE 5 has two allosteric sites for cGMP binding. Occupying these sitesby cGMP is necessary for phosphorylation of PDE 5 which has been shown to occur via protein kinase G activation (Turko *et al*., 1998, Rybalkin *et al*., 2002).

Table 2: Subtrate specificities and tissue distribution of PDE families

PDE Family	Substrate	Tissue localization
1	cGMPand cAMP	Brain, heart, kdney, liver, skeletal muscle, vascular and visceral smooth muscle
2	cAMP and cGMP	Adrenal cortex, brain, kidney, liver, heart, visceral smooth muscle, skeletal muscle
3	cAMP and cGMP	Heart, platelets, Vascular and visceral smooth muscle, liver, kidney, adipose tissue
4	cAMP	Kidney, lung, mast cells, brain, heart, skeletal muscle, Vascular and visceral smooth muscle, thyroid, testes, neural tissue
5	cGMP	Corpus carvenosum, platelets, heart, vascular airway and visceral smooth muscle
6	cGMP	Retina
7	cAMP	Skeletal muscle, heart, lymphocyte, caudate, nucleus, pancreas
8	cAMP	Testes, ovaries, small intestine, colon
9	cGMP	Spleen, small intestine, brain
10	cAMP and cGMP	Striatum, testes, thyroid
11	cAMP and cGMP	Corpus carvenosum, pituitary, liver, kidney, prostate, skeletal muscle, thymus, testes

Sorce: Corbin (2004)

Cyclic guanosine 3′,5′ monophosphate plays a key role in physiologic functions, including platelet aggregation, neurotransmission, vascular smooth muscle modulation, cell proliferation, differentiation, and apoptosis (Saravani *et al*., 2012).

The cyclic nucleotide phosphodiesterases (PDEs) play the predominant role in the degradation of second messengers i.e. cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). The PDEs function in conjunction with adenylate cyclase (AC) and guanylate cyclase (GC) to regulate the amplitude and duration of intracellular signaling mechanisms (mediated via cAMP and cGMP, respectively). Detailed sequence analyses suggest that there are at least 11 different families of mammalian PDEs. Most of the PDEs families include more than one gene product (Bender and Beavo, 2006).

1.11.1.1 Distribution of Phosphodesterase 5

Phosphodiesterase 5 (PDE 5) was first isolated from rat lungs but now identified in many species. The three isoforms, PDE 5A1, PDE 5A2, and PDE 5A3, differ only in their N-terminal sequence. PDE 5A1 and PDE 5A2 are ubiquitous, but PDE 5A3 is specific to smooth muscles (Table 3) (Lin, 2004). PDE 5 is found in high concentration in smooth muscle cells of the corpora cavernosa (Wallis *et al*., 1999).

1.11.1.2 Structure of Phosphodesterase 5

Phosphodiesterase 5 is a homodimer of 99.6 kDa subunits some have one or more GAF domains, which bind cGMP in PDE 5 and thus represent allosteric (noncatalytic) sites. In addition to its cGMP-selective catalytic site, PDE 5 contains two potential allosteric cGMP-binding sites and at least one phosphorylation site for PKG on each subunit (Thomas *et al.,* 1990; Liu *et al.,* 2002) (Figure 12). cGMP can bind to allosteric binding sites of PDE 5, and cGMP occupation of one or both of these sites stimulates the catalytic site for cGMP.

Table 3: Tissue distribution of PDE 5 isoforms

	PDE 5 isoforms		
Tissue	A1	A2	A3
Brain	+	+	-
Lung	+	+	-
Heart	+	+	+
Liver	+	+	-
Kidney	+	+	-
Bladder	+	+	+
Prostate	+	+	+
Urethra	+	+	+
Penis	+	+	+
Uterus	+	+	+
Skeletal muscle	+	+	-

Key: + = present - = absent

Source: Lin (2004)

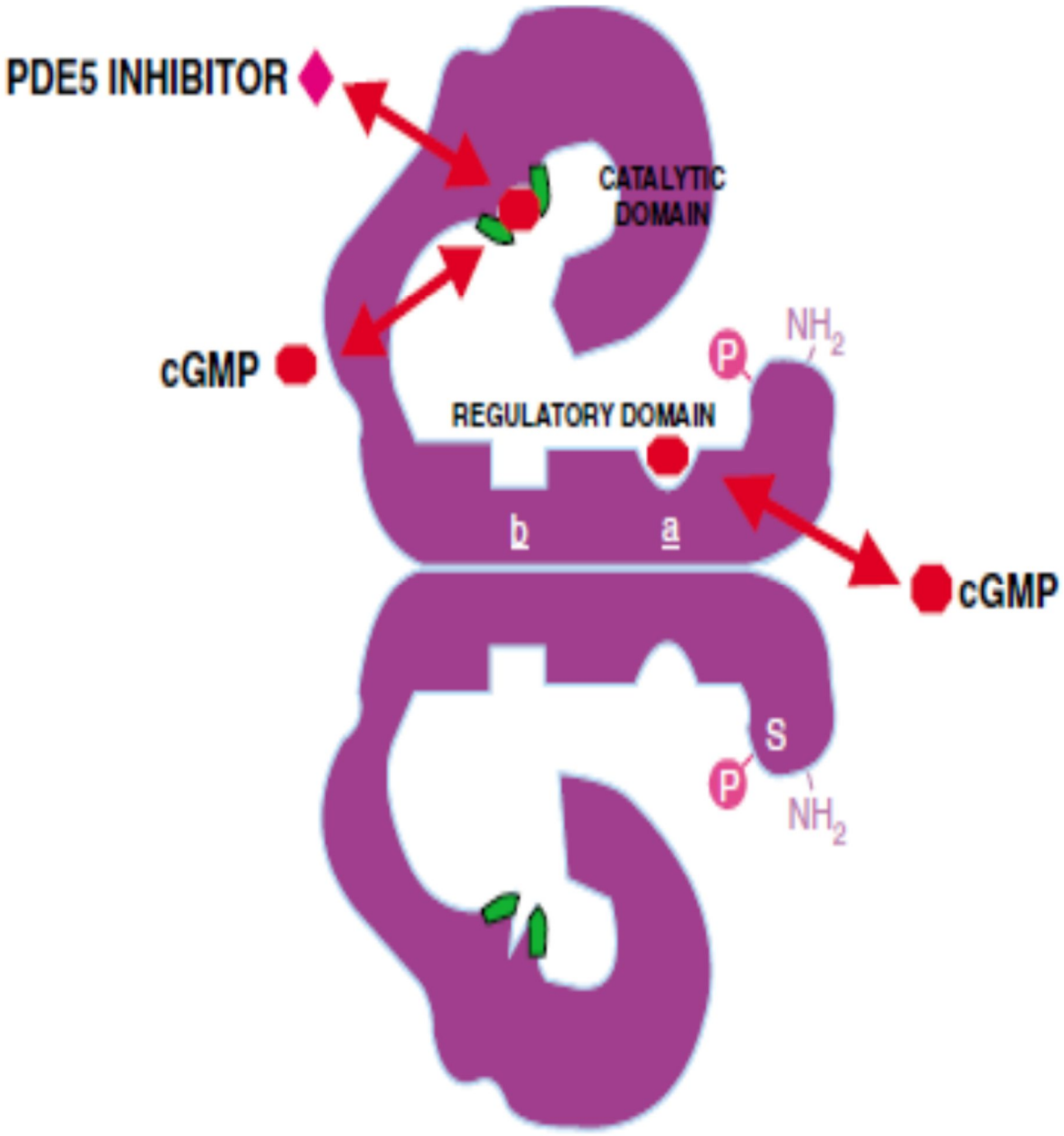


Figure 12: Structure of Phosphodiesterase 5

Source: Corbin (2004)

Occupation of the allosteric binding site by cGMP alters the conformation of PDE 5, which exposes a single serine residue as the phosphorylation site. Phosphorylation of PDE 5 by protein kinase G (PKG) augments the enzymatic activity as well as the affinity of PDE 5 allosteric sites for cGMP (Gopal *et al*., 2001; Corbin *et al.,* 2000). The level of enzymatic activity has been shown to increase with increase in phosphorylation, and the increase in activity is on average about 1.6-folds.

1.11.1.3 Biological functions of Phosphodesterase 5

PDE 5 is a key enzyme involved in the regulation of cGMP-specific signaling pathways in normal physiological processes such as smooth muscle contraction and relaxation. For this reason, inhibition of the enzyme can alter those pathophysiological conditions associated with a lowering cGMP level in tissues. For example, selective PDE 5 inhibitors such as sildenafil and tadalafil have been successfully used to treat the condition of human erectile dysfunction (Zhu and Strada, 2007).

The involvement of this enzyme has been proposed to influence antiproliferation and proapoptotic mechanism in multiple carcinomas. It has been reported that increases in PDE 5 activities in many carcinomas and the ability of PDE 5 inhibitors such as exisulind and its analogs related to anticancer activities. Inhibition of PDE 5 that result in sustained increase in cGMP concentraions are required to modify the process of apoptosis and mitotic arrest in those cancer cells with enhanced PDE 5 expressions (Zhu and Strada, 2007).

PDE5 are also involved in contributing to the pathological changes in the pulmonary system resulting in hyperproliferative remodeling of both smooth muscle and endothelium in models of pulmonary hypertension. For this reason, the use of PDE 5 inhibitors in the treatment of human pulmonary hypertension has met with some success (Zhu and Strada, 2007).

1.12 Organs/Tissues studied in relation to erectile and cardiovascular dysfunctions

1.12.1 Penis

A penis is the primary sexual organ that male animals use to inseminate sexually receptive mates during copulation (Janet and Alex, 2010). Such organs occur in both vertebrate and invertebrate animals.

1.12.1.1 Anatomy of the penis

Rat sensory genital cortex contains a large sexually monomorphic representation of the penis in males (Lenschow and Brecht, 2018). The rat penis is an external sex organ located above the scrotum. It is made of spongy tissue and blood vessels. The main parts of the penis are the root (radix) (consisting of the bulb of penis in the middle and the crus of penis, one on either side of the bulb); the body (corpus); and the epithelium (consisting of the shaft skin, the foreskin, and the preputial mucosa on the inside of the foreskin and covering the glans penis). The rat penis is made up of three columns of tissue: two corpora cavernosa that lie next to each other on the dorsal side and one corpus spongiosum which lies between them on the ventral side (Brooks, 2007) (Figure 13). The enlarged and bulbous-shaped end of the corpus spongiosum forms the glans penis, which supports the foreskin, or prepuce, a loose fold of skin that in adults can retract to expose the glans. The area on the underside of the penis, where the foreskin is attached, is called the frenum, or frenulum. The rounded base of the glans is called the corona. The perineal raphe is the noticeable line along the underside of the penis. The urethra, which is the last part of the urinary tract, traverses the corpus spongiosum, and its opening, known as the meatus, lies on the tip of the glans penis (Figure 13). It is a passage for both urine and for the ejaculation of semen (Sinclair *et al.,* 2016).

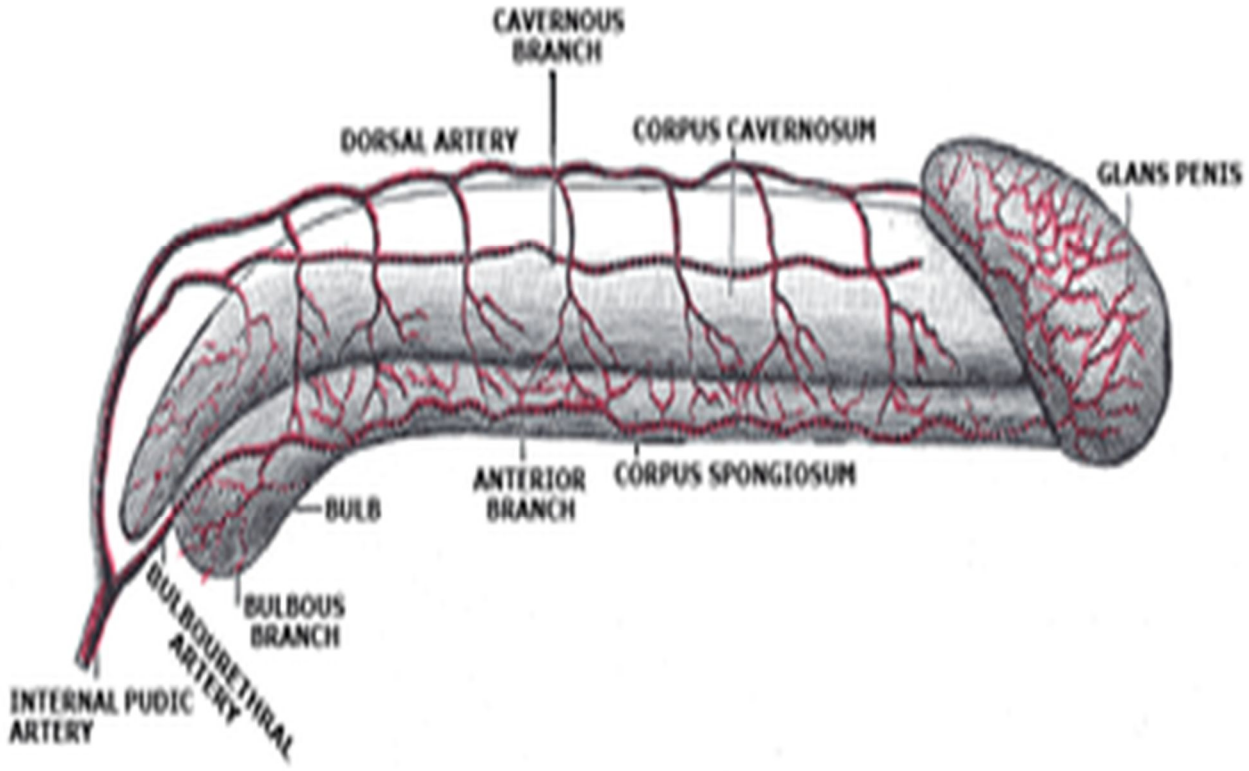


Figure 13: Anatomy of the penis

Source: Brooks (2007)

1.12.1.2 Functions of the penis

The physiological functions of the penis include, exclusively, urination and ejaculation.

1.12.2 The heart

The heart is a hollow, muscular organ about the size of a fist. It is responsible for pumping blood through the blood vessels by repeated, rhythmic contractions. The heart is composed of cardiac muscle, an involuntary muscle tissue that is found only within this organ.

The cardiac muscle is self-exciting, meaning it has its own conduction system. This is in contrast with skeletal muscle, which requires either conscious or reflex nervous stimuli. The heart's rhythmic contractions occur spontaneously, although the frequency or heart rate can be changed by nervous or hormonal influence such as exercise or the perception of danger.

1.12.2.1 Anatomy of the heart

Animals have a four-chambered heart (Figure 14) consisting of the right atrium, left atrium, right ventricle, and left ventricle. The atria are the two upper chambers. The right atrium receives and holds deoxygenated blood from the superior vena cava, inferior vena cava, anterior cardiac veins and smallest cardiac veins and the coronary sinus, it then sends down to the right ventricle (through the tricuspid valve) which in turn sends it to the pulmonary artery for pulmonary circulation. The left atrium receives the oxygenated blood from the left and right pulmonary veins, which it pumps to the left ventricle (through the mitral valve) for pumping out through the aorta for systemic circulation (Whitaker, 2014).

The right atrium and right ventricle are often referred to as the right heart and similarly the left atrium and left ventricle are often referred to as the left heart. The atria do not have valves at their inlets and as a result, a venous pulsation is normal and can be detected in the jugular vein as

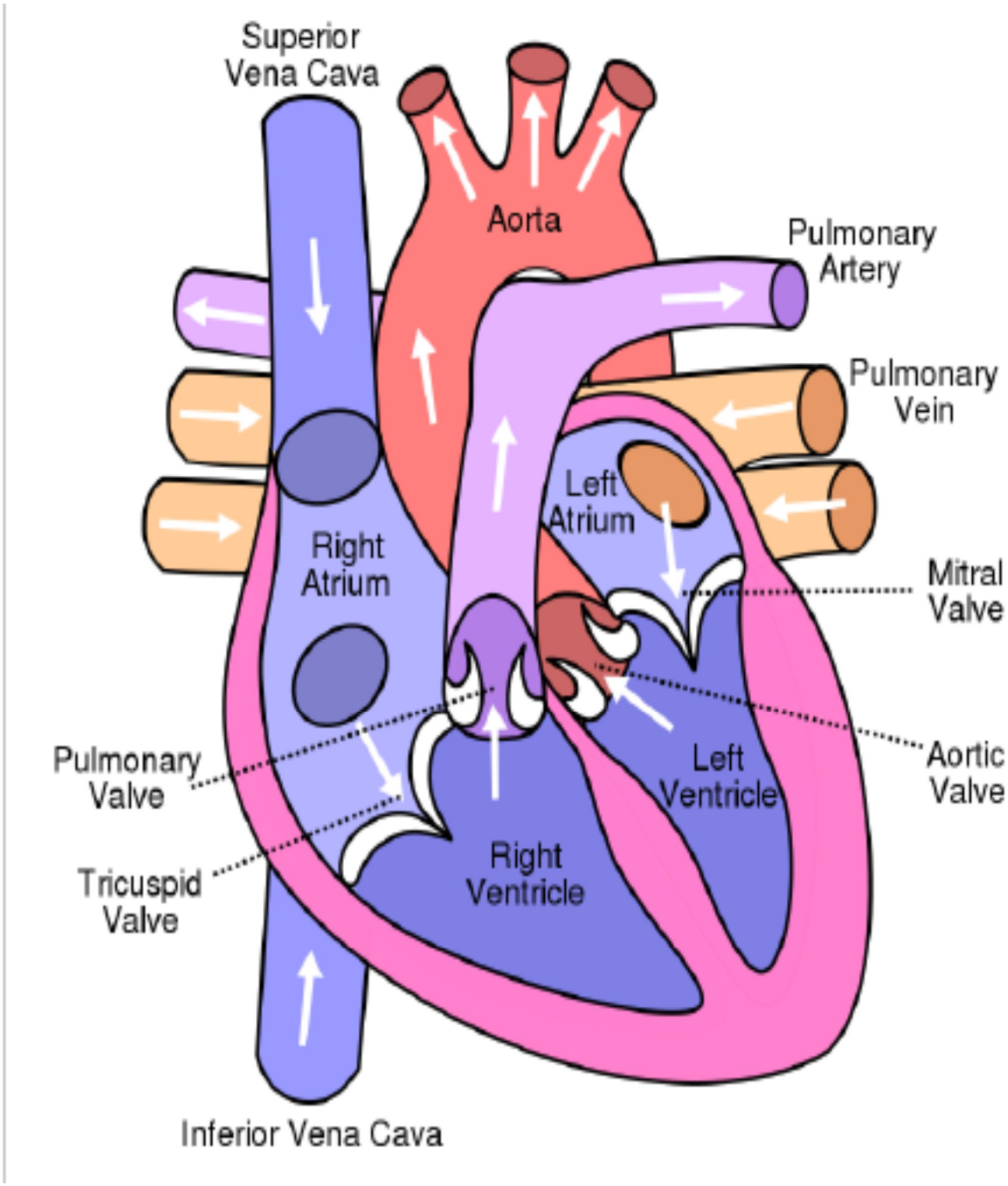


Figure 14: Anatomy of the Heart

Source: Gutfeld (2007)

the jugular venous pressure (Mahadevan, 2012). Internally, there are the rough pectinate muscles and crista terminalis of His, which act as a boundary inside the atrium and the smooth walled part of the right atrium, the *sinus venarum* derived from the sinus venosus. The sinus venarum is the adult remnant of the sinus venous and it surrounds the openings of the venae cavae and the coronary sinus (Mahadevan, 2012). Attached to the right atrium is the right atrial appendage – a pouch-like extension of the pectinate muscles. The interatrial septum separates the right atrium from the left atrium and this is marked by a depression in the right atrium –the fossa ovalis. The atria are depolarised by calcium. High in the upper part of the left atrium is a muscular ear-shaped pouch – the left atrial appendage. This appears to "function as a decompression chamber during left ventricular systole and during other periods when left atrial pressure is high" (Whitaker, 2014).

1.12.2.2 Functions of the heart

The main function of the heart is to pump blood through the body, setting in place a system for the distribution of different substances, mainly oxygen (O_2), carbon dioxide (CO_2) and hormones. The heart sends desoxygenated blood to the lungs which it then receives after the oxygenation process and sends to the rest of the body. When it receives it again without oxygen the whole cycle starts over. This is a key physiological activity because all of our cells need oxygen to function properly and lack of it causes severe trauma to the tissue/organ affected.

1.12.3 Blood

Blood is a vital substance made up of red (erythrocytes) and white blood cells (leukocytes) suspended in a liquid called blood plasma. The blood contain many proteins which are involved in blood clotting. Blood circulates around the body in arteries and veins; and acts as a transport system for many substances including oxygen, amino acids (proteins), lipids (fats), sugar,

glucose (carbohydrate), hormones and waste products (ammonia and carbon dioxide) (Tietz, 1986).

Almost all tissues depend on a blood supply, and the blood supply depends on endothelial cells, which form the linings of the blood vessels.

1.13 Biomarkers of erectile dysfunction

1.13.1 Arginase

Arginase (EC 3.5.3.1) is a metalloenzyme (containing manganese) that catalyzes the hydrolysis of L-arginine to produce L-ornithine and urea. It is proposed that arginase competes for L-arginine and reduces NOS activity in genital tissues, thus modulating sexual function (Kim *et al*., 2004). The reaction catalyzed by arginase is depicted by the following chemical equation:

$$\text{Arginine} + H_2O \rightarrow \text{Ornithine} + \text{Urea}.$$

It is ubiquitous to all domains of life. Arginase catalyzes the fifth and final step in the urea cycle, a series of biochemical reactions in mammals during which the body disposes of harmful ammonia. Specifically, arginase converts L-arginine into L-ornithine and urea (Wu and Morris, 1998). Mammalian arginase is active as a trimer, but some bacterial arginases are hexameric (Dowling *et al.,* 2008). The enzyme requires a two-molecule metal cluster of manganese in order to maintain proper function. These Mn^{2+} ions coordinate with water, orienting and stabilizing the molecule and allowing water to act as a nucleophile and attack L-arginine, hydrolyzing it into ornithine and urea (Di Costanzo *et al*., 2007).

In most mammals, two isozymes of the enzyme exist; the first, Arginase I, functions in the urea cycle, and is located primarily in the cytoplasm of the liver. The second isoenzyme, Arginase II, has been implicated in the regulation of the arginine/ornithine concentrations in the cell. It is located in mitochondria of several tissues in the body, with most abundance in the kidney and

prostate. It may be found at lower levels in macrophages, lactating mammary glands, and brain (Morris, 2002). The second isozyme may be found in the absence of other urea cycle enzymes (Di Costanzo *et al*., 2007).

Arginase is co-expressed with nitric oxide (NO) synthase in smooth muscle tissue, such as the muscle in the genitals of both men and women (Kim *et al.,* 2004). The contraction and relaxation of these muscles has been attributed to NO synthase, which causes rapid relaxation of smooth muscle tissue and facilitates engorgement of tissue necessary for normal sexual response. However, since NO synthase and arginase compete for the same substrate (L-arginine), over-expression of arginase can affect NO synthase activity and NO-dependent smooth muscle relaxation by depleting the substrate pool of L-arginine that would otherwise be available to NO synthase. In contrast, inhibiting arginase with 2(S)-Amino-6-boronohexanoic acid (ABH) or other boronic acid inhibitors will maintain normal cellular levels of arginine, thus allowing for normal muscle relaxation and sexual response (Cama *et al*., 2003).

Arginase is a controlling factor in both male erectile function and female sexual arousal, and is therefore a potential target for treatment of sexual dysfunction in both sexes. Arginase is an indirect regulator of penile blood flow (Kim *et al.,* 2004).

1.13.2 Nitric oxide

Nitric oxide (NO) may also mediate smooth muscle relaxation independent of cGMP. In aortic smooth muscle cells NO has been shown to directly activate Ca^{2+}- dependent potassium (K^{+}) channels (Bolotina *et al.,* 1994). In human corpus cavernosum smooth muscle cells, NO was shown to directly activate sodium-potassium ATPase to cause hyperpolarization. An important factor underlying the pathology of erectile dysfunction may be attenuation in NO/cGMP signaling (Cartledge *et al.,* 2001). NO production in penile tissue is reduced in aging (Garban *et*

al., 1995) and diabetes (Bivalacqua *et al.,* 2001), conditions having a high degree of association with erectile dysfunction. Several reports suggested that decreased NO production due to endothelial dysfunction or nerve injury may represent a central mechanism of erectile dysfunction. Reduced production of NO by the endothelium may be the result of decreased NOS protein expression and activity, increased NO scavenging, increased endogenous inhibitors of NOS, or decreased levels of substrate (L-arginine and oxygen) and cofactors.

1.14 Biomarkers of cardiac dysfunction

1.14.1 Creatinine kinase

Creatine kinase (CK) is also referred to as creatine phosphokinase (CPK) or phospho-creatine kinase. It is an enzyme expressed by various tissues and cell types. CK catalyses the conversion of creatine and utilizes adenosine triphosphate (ATP) to create phosphocreatine (PCr) and adenosine diphosphate (ADP) (Figure 15). This CK enzyme reaction is reversible and thus ATP can be generated from PCr and ADP.

In tissues and cells that consume ATP rapidly, especially skeletal muscle, but also brain, photoreceptor cells of the retina, hair cells of the inner ear, spermatozoa and smooth muscle, PCr serves as an energy reservoir for the rapid buffering and regeneration of ATP *in situ*, as well as for intracellular energy transport by the PCr shuttle or circuit (Wallimann *et al*., 1992). Thus creatine kinase is an important enzyme in such tissues (Wallimann and Hemmer, 1994).

Clinically, creatine kinase is assayed in plasma as a marker of damage of CK-rich tissue such as in myocardial infarction (heart attack), rhabdomyolysis (severe muscle breakdown), muscular dystrophy, autoimmune myositides, and acute kidney injury (Maghadam-Kia *et al*., 2016).

1.14.2 Atherogenic index

Atherogenic index is the molar ratio of either plasma total cholesterol, triglyceride or LDLC to

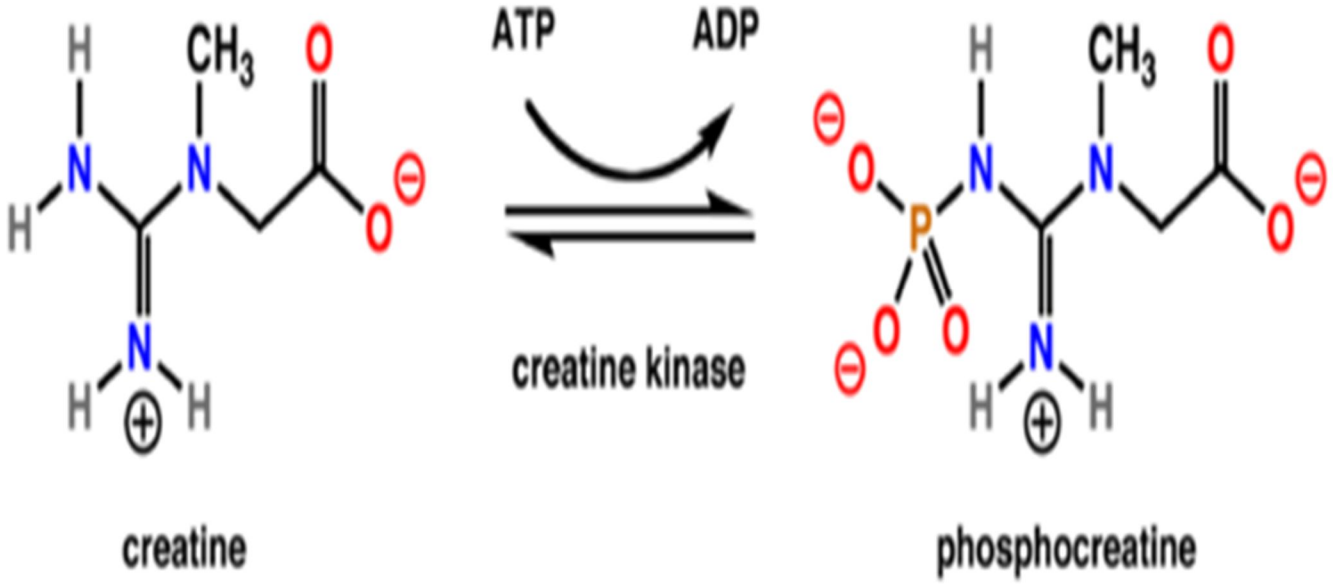


Figure 15: Reaction catalysed by creatine kinase

Source: Boghog (2009)

HDLC which has good predictive values for coronary heart disease risk (Gaziano *et al*., 1997) i.e. it is usually the logarithm ratio of a negative index of atherosclerosis (TG, LDL or TC) to that of the positive index of atherosclerosis (HDLC). The lower the atherogenic value, the lower the risk of atherosclerosis hence, the lower the risk of cardiovascular disease (Udoh, 1998).

1.14.3 Cardiac index

Cardiac index (CI) is a haemodynamic parameter that relates the cardiac output (CO) from left ventricle in one minute to body surface area (BSA) thus relating heart performance to the size of the individual.the higher the value of CI, the higher the risk of developing cardiovascular diseases (Ansell *et al.,* 2005; Ajiboye *et al.,* 2014).

1.15 Statement of the problem

Selective serotonin re-uptake inhibitors (SSRIs) such as paroxetine are used for the treatment of depression and other psychotic conditions are associated with several incidence of sexual dysfunction (Aguilo *et al.,* 2003). Erectile dysfunction (ED) is one of the most common sexual dysfunction in men (Young *et al.,* 2013). Studies have shown that ED shares risk factors with vascular diseases such as coronary artery (CAD) and cardiovascular heart (CHD) diseases and often the triad co-exists (Aguilo *et al.,* 2009; Montesori *et al.,* 2009). The pathophysiological linkage between ED and vascular diseases is endothelial dysfunction (Kaya *et al*., 2006). Endothelial dysfunction is the initial step of atherosclerotic process involving many vascular districts including penile and coronary circulation (Angelis *et al*., 2013). The key point for the prevention of ED is the early identification of endothelial dysfunction and the treatment of the co-existing risk factors. Modulators of endothelial derived relaxation factor which involves nitric oxide (NO) pathway leading to impairment of endothelium dependent vasodilation (early phase) and structural vascular abnormalities (late phase) that are observed in erectile dysfunctional state

are required for effective treatment of ED patients. There is a need to develop ED therapeutics that promotes endothelial functions from wide range of known PDE-5 inhibitors and/or screen new candidates with PDE-5 inhibitory activities.

1.16 Justification for the study

Phosphodiesterase 5 has been implicated as a marker for erectile function (Boswell-Smith *et al.,* 2006). Sildenafil citrate (Viagra), one of the potent drugs used in the treatment of erectile dysfunction, act by inhibiting the activity of PDE 5.

PDE 5 inhibitors such as sildenafil and tadalafil are commonly prescribed as first line oral therapy for ED patients. However, significant proportions are non-responsive to PDE 5 inhibitors with about 30-50 % failure rate at the attempt of intercourse leading to high discontinuation rate (Souverein *et al.,* 2002; Fusco *et al.,* 2010; Lusker *et al.,* 2010). Also, undesirable adverse side effects have been associated with several synthetic ED drugs (Siddig *et al*., 2012), increasing the needs for better alternatives. Many botanical extracts used by traditional medicine practitioners (TMPs) in Nigeria as decoctions for the treatment of various reproductive and sexual abnormalities have been investigated, established and shown to enhance sexual functions. These includes but not limited to *Fadogia agrestis* (Yakubu *et al.,* 2007), *Cnestis ferruginea* (Yakubu and Nurudeen, 2012). However, the mechanism of action of aqueous extract *Cnestis ferruginea* root and *Fadogia agrestis* stem as aphrodisiacs have not been established. This study was therefore proposed to identify the probable mechanism of action of the aqueous extracts of these plants by which they bring about relief to patients with erectile dysfunction and/or cardiovascular diseases. Screening of plants that have both potential to improve sexual and circulatory functions for PDE 5 inhibitory activities is the first step in the development of new better drug candidates

not only for treatment of ED or its co-existing risk factors but also for eradication of the underlying endothelial dysfunctional linkage.

1.17 Overall Objective

The overall objective of this study was to investigate the interactive effects of aqueous extracts of *Cnestis ferruginea* root and *Fadogia agrestis* stem as well as their combination on biomarkers of erectile, cardiovascular and endothelial dysfuctions in paroxetine- treated male rats.

1.17.1 Specific objectives of the study

The specific objectives were to:

(i) determine the quantity of secondary metabolites in the plant extracts;

(ii) isolate and partially purify PDE 5 from rat penile and cardiac tissues;

(iii) determine the PDE 5 inhibitory activities of aqueous extract of *Cnestis ferruginea* (CF) root, *Fadogia agrestis* stem (FA) and their combinations(CF+FA) in the presence of cGMP and cAMP;

(iv) determine the Kinetic parameters (K_m and V_{max}) of PDE 5 in the presence of the CF, FA and CF+FA;

(v) assess the effect of paroxetine on:

  (a) selected endothelial (NO, Arginase, cGMP, endothelin-1) functional biomarkers in male rats;

  (b) selected cardiovascular (CK, TC, LDLc) functional biomarkers in male rats;

  (c) selected erectile (testosterone, PDE 5) functional biomarkers in male rats;

(vi) study the effect of CF, FA and CF+FA on the selected markers of erectile, cardiovascular and endothelial dysfunctions induced by paroxetine in male rats.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant materials and authentication

Fresh *Cnestis ferruginea* roots and *Fadogia agrestis* stem were obtained from herb sellers at *Oja tuntun* and *Kulende* markets, Ilorin, Kwara State respectively. Identification of *Cnestis ferruginea* roots was done at the herbarium unit of the Department of Plant Biology, Faculty of Life Sciences, University of Ilorin where a voucher no- IUV No 007 was assigned while *Fadogia agrestis* stem was identified at the Department of Horticulture and Landscape Technology, Federal School of Forestry, Jos, Nigeria where a voucher number 2:108 was assigned.

2.1.2 Reagents, chemicals and assay kits

Trizma base (Lot no.: BCBJ8442V), Ammonium sulphate (Lot no.: BCBJ0661V), cyclic guanosine monophosphate (cGMP) (Lot no.: BCBJ1515V), cyclic adenosine monophosphate (cAMP) (Lot no.: SLBJ8361V) and *Crotalus adamanteus* venom (SLBR1148V) were products of Sigma Aldrich Chemical Company, St. Louis, United States of America; Imidazole (Lot no.: B0212) was a product of Santa Cruz Biotechnology, Dallas, Texas; Sildenafil citrate (Lot no.: A471719) was a product of Pfizer, New York; Paroxetine hydrochloride (Lot no.: 615M) was a product of Glaxo Smith Klein (GSK), Brasov, Romania; Tween-80 was a product of BDH Laboratory Chemicals, Poole,England; L-arginine was a product of Burgoyne Burbidges & Co., Mumbai, India; Assay kit for testosterone was a product of Monobind Inc., Lake Forest, USA; Assay kits for cGMP (Lot no.: P104126) and Endothelin-1 (P109997) were products of Bio-techne Ltd, Abingdon, United Kingdom; Assay kits for creatine kinase, total cholesterol, triglyceride, low density lipoprotein cholesterol (LDLC), high density lipoprotein cholesterol

(HDLC) were products of Randox laboratories Co-Artrim, United Kingdom. All other reagents used were of analytical grade from the Laboratory in Department of Biochemistry.

2.1.3 Experimental animals

Two hundred and fifty adult male Wistar rats (weighing between 210.43 ± 10.04 g) were obtained from the Animal Holding Unit of the Department of Biochemistry, University of Ilorin, Ilorin.

2.2 Methods

2.2.1 Extract preparation

The root of *Cnestis ferruginea* and *Fadogia agrestis* stem were separately rinsed with distilled water to remove soil debris, cut into pieces and oven-dried at 40^{o}C until a uniform weight was obtained. The dried plant parts were pulverized with an electric blender in preparation for extraction.

The extract preparation was carried out as described by Yakubu and Nurudeen (2012). Each of the powdered plant of *Cnestis ferruginea* root and *Fadogia agrestis* stem (100 g) was measured and extracted in 400 ml of distilled water in separate containers, and in combination (50:50) for 48 hours after which they were filtered with Whatman No.1 filter paper (GE Healthcare Limited, United Kingdom). The resulting filtrate was concentrated using a lyophilizer (Zirbus technologies, GMBH, Germany). The resulting samples were stored in labeled glass containers accordingly as CF for *Cnestis ferruginea*, FA for *Fadogia agrestis* and their combinations CF+FA and refrigerated at 4^{o}C to prevent fermentation. The lyophilized CF gave a yield of 115.32 g of extract corresponding to a percentage yield of 19.22%; FA gave a yield of 18.43 g of extract corresponding to a percentage yield of 18.43% and CF+FA produced a yield of 66.72 g corresponding to 11.12%.

2.2.2 Screening of secondary metabolites

Chemical tests (using standard procedures) were carried out for the determination of the qualitative and quantitive analyses of the secondary metabolites in the plants.

2.2.2.1 Qualitative analysis

A known quantity (1.0 g) of the powdered *C. ferruginea* roots and *F. agrestis* stem were extracted in 100.0 cm^3 of distilled water (1% w/v) and used for phytochemical screening. A portion of the extract were subjected to standard chemical tests as described for alkaloid (Harborne, 2008); steroids, anthraquinones, cardenolides and dienolides, phlobatannins (Trease and Evans, 1996); saponins (Wall *et al*., 1954), phenolics and flavonoids (Awe and Sodipo, 2001), cardiac glycoside (Sofowora, 2006); tannins and terpenes (Odebiyi and Sofowora, 1990).

2.2.2.1.1 Alkaloids

Exactly 1.0 cm^3 each of the extracts were stirred with 5.0 cm^3 of 1% (v/v) aqueous HCl on a steam bath and filtered while hot. Distilled water was added to the residue and 1.0 cm^3 of the filtrate was treated with two drops of Mayer's reagent (Potassium mercuric iodide- solution), Wagner's reagent (solution of iodine in Potassium iodide) and Dragendorff's reagent (solution of Potassium bismuth iodide). The formation of a cream colour with Mayer's reagent and reddish-brown precipitate with Wagner's and Dragendorff's reagents give a positive result for alkaloids.

2.2.2.1.2 Tannins

Exactly 1.0 cm^3 of freshly prepared 10% (w/v) ethanolic KOH was added to 1.0 cm^3 each of the extract. A white precipitate indicated the presence of tannins.

2.2.2.1.3 Phlobatannins

Boiling 3.0 cm^3 each of the extracts with 1% aqueous hydrochloric acid which resulted in the formation of red precipitate indicated the presence of phlobatannins.

2.2.2.1.4 Saponins

(Frothing Test) – A known volume (5.0 cm^3) each of the extracts was boiled in 20 cm^3 of distilled water in a water bath and filtered. The filtrate (10.0 cm^3) was mixed with 5.0 cm^3 of distilled water and shaken vigorously for a stable persistent froth which confirms the presence of saponins.

2.2.2.1.5 Phenolics

Two drops of 5% (w/v) of $FeCl_3$ was added to 1.0 cm^3 each of the plant extracts. Presence of a greenish precipitate indicated the presence of phenolics.

2.2.2.1.6 Cardiac glycosides

A known volume (1.0 cm^3) of the extract was added to 2.0 cm^3 of chloroform. Thereafter, 2.0 cm^3 H_2SO_4 was carefully added. A reddish brown colour at the interface indicated the presence of aglycone portion of cardiac glycosides.

2.2.2.1.7 Chalcones

A known volume (2.0 cm^3) of NH_4OH was added to 5.0 cm^3 each of the extracts. Absence of red colour confirmed absence of chalcones.

2.2.2.1.8 Steroids

Five drops of concentrated H_2SO_4 was added to 1.0 cm^3 each of the extracts. Absence of red colouration indicated the absence of steroids.

2.2.2.1.9 Terpenes

A known volume (1.0 cm^3) each of the extracts was added to 5 drops of acetic acid anhydride followed by a drop of concentrated H_2SO_4. The mixture was steamed for 1 hour and neutralised with NaOH followed by the addition of chloroform. A bluish-green colour confirmed the presence of terpenes.

2.2.2.1.10 Anthraquinones

Exactly 3.0 cm^3 each of the extract was shaken with 10.0 cm^3 of benzene, filtered and 5.0 cm^3 of 10% v/v NH_4OH was added to the filtrate. The absence of a pink colour in the ammonical (lower) phase indicated the absence of anthraquinones.

2.2.2.1.11 Flavonoids

Exactly 3.0 cm^3 each of the filtrates were mixed with 4.0 cm^3 of 1% potassium hydroxide in a test tube. Absence of a dark yellow colour indicated that flavonoids were absent.

2.2.2.1.12 Cardenolides

A portion (5.0 cm^3) each of the extracts was added to 2.0 cm^3 of glacial acetic acid containing one drop of 5% w/v $FeCl_3$ solution. This was then followed by the addition of 1.0 cm^3 of concentrated H_2SO_4. Absence of a brown ring at the interface indicated the absence of a deoxy sugar characteristic of cardenolides.

2.2.2.2 Quantitative analysis of secondary metabolites

2.2.2.2.1 Determination of alkaloid concentration

The quantitative determination of alkaloids was done by distillation and titrimetric methods as described by Henry (1996). Finely ground sample (2.0 g) was placed in a 100.0 cm^3 beaker and 20.0 cm^3 of 80% (v/v) ethanol added. The mixture was transferred to a 250.0 cm^3 flask and more ethanol was added to make up to 100.0 cm^3 after which 1.0 g of magnesium oxide was then added. The mixture was digested in a boiling water bath for 1.5 hours under refluxing with occasional shaking. The mixture was filtered while hot through a Buchner funnel. The residue was redigested for another 30 minutes with 50.0 cm^3 ethanol and filtered. The ethanol was evaporated from the filtrate. Three drops of 10% (v/v) HCl was added to the filtrate. The solution was transferred into a 250.0 cm^3 volumetric flask; 5.0 cm^3 of 5% (w/v) zinc acetate solution and 5.0 cm^3 of 24% (w/v)

potassium ferrocyanide solution were added and thoroughly mixed. The flask was left undisturbed for 10 minutes; filtered and 10.0 cm^3 of the filtrate was transferred into a separating funnel. The alkaloids were extracted vigorously by shaking with 50.0 cm^3 of chloroform. The residue was dissolved in 10.0 cm^3 of hot distilled water, transferred into a Kjeldahl flask followed by the addition of 0.20 g sucrose, 10.0 cm^3 concentrated H_2SO_4, and 0.02 g of selenium for digestion. The clear solution was used to determine Nitrogen content using Kjeldahl distillation apparatus. The distillate was back titrated with 0.01N HCl and the titre value obtained was used to calculate the % Nitrogen using the expression:

$$\% N = \frac{\text{Titre value x Atomic mass of Nitrogen x Normality of HCl x Dilution Factor x 100}}{\text{Weight of Sample (mg)}}$$

% Alkaloid = % Nitrogen x 3.26

where 3.26 is a constant.

2.2.2.2.2 Determination of tannin concentration

A known quantity (0.20 g) of the powdered sample was placed in a 50.0 cm^3 beaker. 20.0 cm^3 of 50% (v/v) methanol was added and covered with paraffin and placed in a water bath at 80^0C for 1 hour. The mixture was thoroughly shaken and filtered using a double layered Whatman No 41 filter paper into a 100.0 cm^3 volumetric flask. Then, 20.0 cm^3 of distilled water, 2.5 cm^3 of Folin-Denis reagent and 10.0 cm^3 of 17% (w/v) Na_2CO_3 were added and mixed properly. The mixture was made up to the marked level with distilled water, mixed properly and left undisturbed for 20 minutes after which a bluish–green colour developed. Standard tannin of concentration range 0-7 ppm was treated similarly like the sample as earlier described. The absorbances of sample as well as that of standard

concentration of tannin were read after colour development on a Spectronic 21D Spectrophotometer at a wavelength of 760 nm. The average gradient obtained from standard curve for tannins (Figure 61) was used to calculate the percentage of tannins using the expression:

$$\text{Total tannin content (\%)} = \frac{\text{Absorbance of sample x Average gradient} \times \text{Dilution factor}}{\text{Weight of sample x 10,000}}$$

2.2.2.2.3 Determination of phlobatannin concentration

A known quantity (0.50 g) of the powdered sample was placed in a 50.0 cm^3 beaker after which 20.0 cm^3 of 50% (v/v) methanol was added and covered with paraffin. The mixture was boiled in a water bath at 80^0C for 1 hour with constant shaking to ensure uniformity. It was later filtered with Whatman No 1 filter paper into a 50.0 cm^3 volumetric flask using aqueous methanol to rinse. The filtrate was then made up to the marked level with distilled water. The filtrate (1.0 cm^3) was pipetted into a 50.0 cm^3 volumetric flask, 20.0 cm^3 distilled water, 2.5 cm^3 Folin-Denis' reagent and 10.0 cm^3 of 17% Sodium carbonate were then added. This mixture was homogenised thoroughly for 20 minutes. 0-7 ppm of phlobatannin standard concentration were prepared from 100 ppm phlobatannin stock solution and treated like sample as earlier described. The absorbances of standard solutions as well as sample were read on a Spectronic 21D spectrophotometer at wavelength of 550 nm. The average gradient obtained from standard curve for phlobatannins (Figure 62) was used to calculate the percentage of phlobatannins using the expression:

$$\text{Total phlobatannin content (\%)} = \frac{\text{Absorbance of sample x Average gradient} \times \text{Dilution factor}}{\text{Weight of sample x 10,000}}$$

2.2.2.2.4 Determination of saponin concentration

The spectrophotometric method of Brunner (1984) was used for the quantification of saponins. Finely ground sample (1.0 g) was placed in a 250.0 cm^3 beaker and isobutyl alcohol (100.0 cm^3) added. The suspension was shaken for 5 hour to ensure uniform mixing. The mixture was filtered through a Whatman No. 1 filter paper into 20.0 cm^3 of 40% w/v saturated solution of $MgCO_3$. The resulting mixture was again filtered. The filtrate (1.0 cm^3) was pipetted into 50.0 cm^3 volumetric flask and 2.0 cm^3 of 5% w/v $FeCl_3$ solution was added. The volume was adjusted to the marked level with distilled water. This was left undisturbed for 30 minutes for blood red colour to develop. Saponin standard solution (0-5 ppm) was prepared from saponin stock solution. The standard solutions were treated similarly with 2.0 cm^3 of 5% w/v $FeCl_3$ solution as earlier described. The absorbance of the samples as well as standard saponin solutions was read using a Spectronic 21D Spectrophotometer at 380 nm. The average gradient obtained from standard curve for saponins (Figure 59) was used to calculate the percentage of saponins using the expression:

$$\text{Total saponin content (\%)} = \frac{\text{Absorbance of sample x Average gradient} \times \text{Dilution factor}}{\text{Weight of sample x 10,000}}$$

2.2.2.2.5 Determination of phenolic concentration

A known quantity (0.20 g) of the powdered sample was placed in a 50.0 cm^3 beaker then 20.0 cm^3 of acetone added. This was homogenised properly for 1hour to prevent lumping. The mixture was filtered through a Whatman No.1 filter paper into a 100.0 cm^3 volumetric flask using acetone to rinse and made up to the marked level with distilled water. Known volume (1.0 cm^3) of sample mixture was pipetted into a 50.0 cm^3 volumetric flask. Subsequently, 20.0 cm^3 water, 3.0 cm^3 of phosphomolybdic acid and 5.0 cm^3 of 23% (w/v) Na_2CO_3 were added, thoroughly mixed, made up

to the marked level with distilled water and left undisturbed for 10 minutes to develop bluish-green colour. Standard phenol of concentration range 0-7 ppm was prepared from 100 ppm stock phenol solution. The absorbance of the sample as well as that of standard concentrations of phenol were read on a Spectronic 21D Spectrophotometer at a wavelength of 510 nm. The average gradient obtained from standard curve for phenolics (Figure 60) was used to calculate the percentage of phenolics using the expression:

$$\text{Total phenol content (\%)} = \frac{\text{Absorbance of sample x Average gradient} \times \text{Dilution factor}}{\text{Weight of sample x 10,000}}$$

2.2.2.2.6 Determination of cardiac glycoside concentration

A known volume (10.0 cm^3) of extract was pipetted into 250.0 cm^3 conical flask. Chloroform (50.0 cm^3) was added and shaken on a Vortex Mixer for 1hour. The mixture was filtered into 100.0 cm^3 conical flask and 10.0 cm^3 pyridine, 2.0 cm^3 of 2% sodium nitroprusside were added and then shaken thoroughly for 10 minutes.A known volume (3.0 cm^3) of 20% NaOH was later added to develop a brownish yellow colour. Glycoside standard of concentrations with range 0-5 ppm were prepared from 100 ppm stock glycoside standard. The series of standards 0-5 mg/ml were treated similarly like sample as earlier described. The absorbance of sample as well as standard were read on a Spectronic 21D Digital Spectrophotometer at a wavelength of 510 nm. Percentage glycoside was calculated using the expression:

$$\text{Total glycoside content (\%)} = \frac{\text{Absorbance of sample x Average gradient} \times \text{Dilution factor}}{\text{Weight of sample x 10,000}}$$

2.3 Isolation of partially purified phosphodiesterase 5

The method of Butcher and Sutherland (1962) was used for the isolation and partial purification of phosphodiesterase 5. Forty male rats were used in this experimental study. The animals were housed in well ventilated cages and allowed to acclimatize for 2 weeks. They were fed with normal rat pellets (Grand Cereals Limited, a subsidiary of UAC Nigeria Plc, Jos) and water *ad libitum*. After acclimatization, the rats were sacrificed by cervical dislocation, penile and cardiac tissues were quickly excised and cleansed with blotting paper to remove blood stains and weighed, then collected and preserved in ice-cold 0.25 M sucrose-tris buffer (pH 7.4). The partial purification process was carried out in three steps:

Step 1: The penis and heart were cleared of fat and connective tissue and then washed in 0.25M sucrose and 10 mM Tris buffer. The tissues were cut into pieces with the use of a sterile scissors and homogenized using a Teflon homogenizer on ice. The homogenates were centrifuged at 2000 g for 45 minutes in the cold centrifuge. The resulting supernatants were decanted through glass wool prewashed in 0.25 M sucrose-tris buffer.

Step 2: The supernatants were kept in an ice water bath and adjusted to 0.5 saturation with 100 % ammonium sulfate. Neutrality was maintained by adding 1 N KOH as required and then left undisturbed for 30 min, the precipitates were collected by centrifugation at 8000 g for 40 minutes. The supernatants were discarded and the precipitate taken up in 15 % of the extract volume containing 1 x 10^{-3} M $MgS0_4$ and 1 x 10^{-3}M Imidazole pH 7.5, in cold glass-distilled water. Neutralized saturated ammonium sulfate was added to a final concentration (0.45% saturation).

Step 3: After 30 minutes of stirring, the precipitates were again collected by centrifugation at 8000 g for 40 minutes. The precipitates were added up to 5 % of the homogenate volume in 1 x

10^{-3}M $MgS0_4$ and 1 x 10^{-3}M Imidazole. The pellet was discarded and the supernatant subjected to dialysis. After dialysis, the supernatant was frozen, torred andcentrifuged at 8000 g for 40 minutes. The supernatant contains the isolated PDE 5 (Figure 16).

Ammonium sulphate precipitation

Principle

It relies on the principle that most proteins are less soluble in solutions of high salt concentrations because the addition of salt ions shields proteins with multi-ion charges.

Procedure

The supernatants were subjected to 50% ammonium sulphate precipitation by mixing a 300 ml of 100% saturated ammonium sulphate solution with 150 ml of the supernatant. The precipitate obtained was left overnight at 4°C and then centrifuged at 8000 xg for 40 minutes. The pellet obtained was collected by gently slanting the container and decanting the supernatant. The pellet was then re-dissolve with small amount of ice cold 0.05M Tris buffer, pH 7.4

Calculation:

In order to obtain 80% ammonium sulphate precipitation, the following expression was used:

$Cf \times Vf = Cn \times Vn$

Where:

Cf = Final Concentration

Vf = Final Volume

Vn = Volume of $(NH_4)_2SO_4$ added

Cn = Concentration of the $(NH_4)_2SO_4$

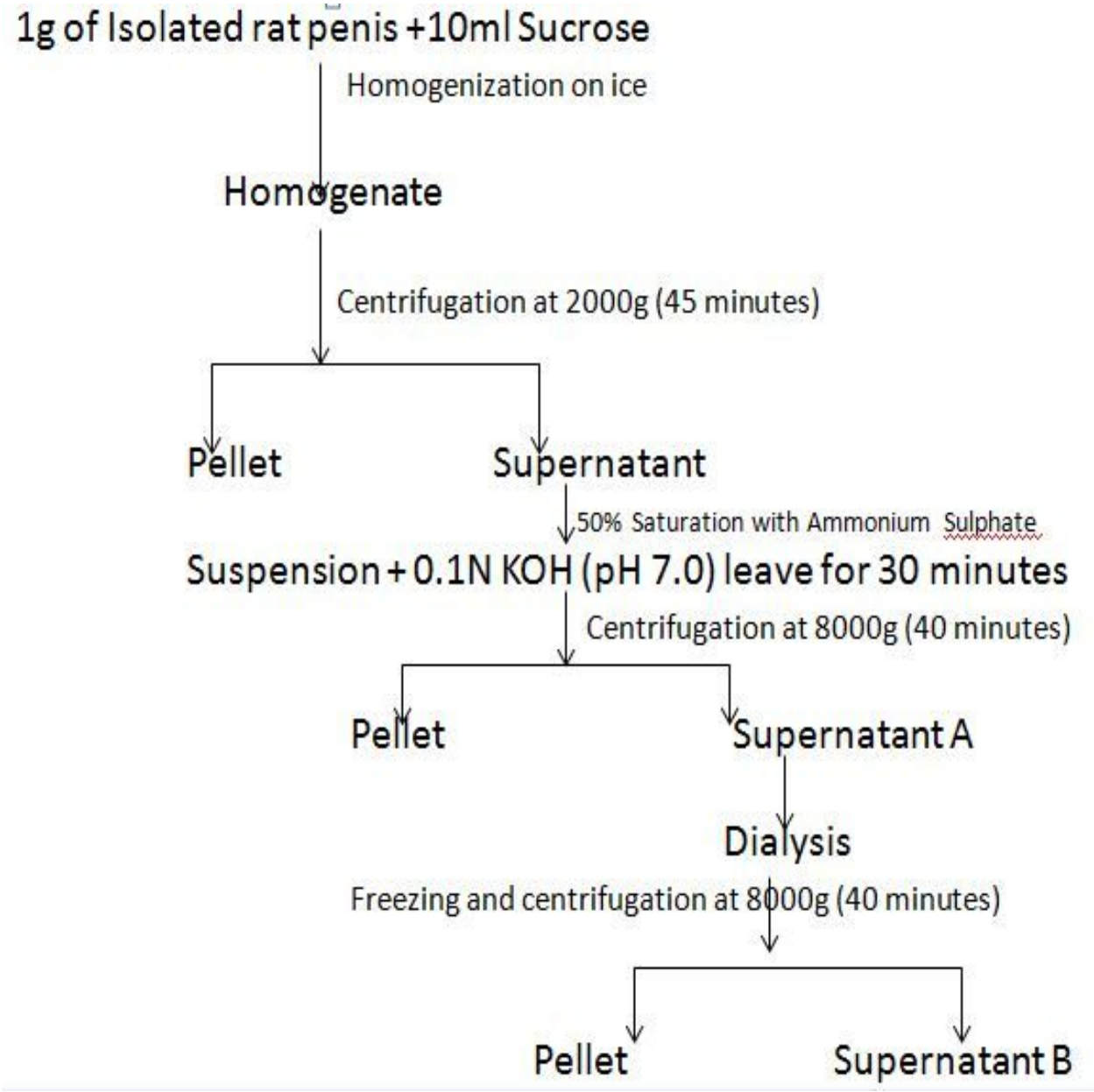


Figure 16: Isolation and purification of rat penile phosphodiesterase 5

Adapted from Butcher and Sutherland (1962)

Dialysis

Principle

The principle of dialysis is based on selective and passive diffusion of proteins through a semi-permeable membrane (dialysis bag).

Pretreatment of the dialysis bag

Dialysis bag was cut in the dimension 6 cm x 12 cm and pretreated before the dialysis. This was done by immersing the bag in $MgSO_4$ and imidazole buffer for 24 hours to remove glycerol and to open the bag from both ends according to method described by Ibraheem *et al*. (2016). The tube was washed again with distilled water, in order to remove metal traces, was further placed in a solution of 2 % sodium bicarbonate and 1 mM EDTA for 3 hours. The opened bag was rinsed with distilled water.

Procedure

The dialysis bag (6 cm x 12 cm) was tied at one of the open ends with thread. The solution was gently poured into the tube ensuring that the tube was half-filled. The half-filled tube was then suspended in 1000 ml beaker filled with ice-cold 0.05M Tris buffer, pH 7.4. The suspension of the dialysis tube was achieved by attaching the ends of the tube to a glass rod and carefully placing across the beaker. The solution was then subjected to dialysis against the buffer for 24 hours with continuous stirring using magnetic stirrer and intermittent replenishment of the buffer at 3 hours interval.

2.3.1 Determination of phosphodiesterase 5 activity

The method of Butcher and Sutherland (1962) was used for the determination of phosphodiesterase 5 activity.

Principle

The principle involves measuring the release of inorganic phosphate with use of an excess of 5' nucleotidase.

Procedure

Briefly, the dialysed fraction of the extract containing the enzyme was suspended in a reaction mixture containing 0.36 µmole of cGMP, 1.8 µmoles of $MgS0_4$, and 36 µmoles of Tris buffer, pH 7.5, with a suitable dilution of the phosphodiesterase sample being tested in a total volume of 0.9 ml. This mixture was incubated at 37 ºC for 30 minutes. After the first 20 minutes of the incubation, 0.1 ml of a *Crotalus adamanteus venom* solution was added containing 0.1 mg of venom in 1 x 10^{-2} M Tris, pH 7.5. The entire reaction was terminated by the addition of 0.1 ml of ice-cold 55% trichloroacetic acid. After addition of trichloroacetic acid, the solution was centrifuged at 2000 g for 10 minutes, the precipitate was removed, and 500 µl of the supernatant fluids were analyzed for inorganic phosphate as described by Fiske and Subbarow (1925), modified by Butcher and Sutherland (1962) with a slight modification as described below:

0.5ml of the supernatant solution was taken and 0.5 ml of distilled water was added in a cuvette. The colour was developed by the addition of 1 ml of reagent C (9% ascorbic acid and ammonium molybdate solution in ratio 4:1 which was prepared just before use) and read at 820 nm on a spectrophotometer. The amount of inorganic phosphate released per unit time was extrapolated from the caliberation curve (Figure 58).

2.3.2 Protein determination

Protein concentration was determined according to the procedure described by Lowry *et al*. (1951) using bovine serum albumin (BSA) as standard.

Principle

The colour reagent used is a phosphor-18-molybdictungstic complex (a mixture of several molecular forms such as $3H_2O.P_2O_5.9MoO_3$ and $3H_2O.P_2O_5$ ¯$.10Wo_3.8MoO_3$) which can be reduced by phenol groups giving a blue colour at alkaline pH.

Procedure

The reaction of protein in solution with Folin's reagent occurs in two steps which contributes to the final blue colour obtained in protein determination:

(a) Reaction with copper in an alkaline medium as depicted below:

$$Cu + \text{protein} \longrightarrow Cu^{2+}\text{---protein}$$

(b) Reduction of phosphomolybdictungstic reagent by the copper- treated protein

To determine the total protein content of samples, the standard protein curve must be plotted at various concentrations of BSA following this procedure:

The assay was done in triplicates and the corresponding average absorbances were plotted against the concentration of BSA used. Six set of test tubes were used for the experiment, the first was used as blank which contained every reagent except the BSA while other tubes contained BSA at different concentrations.

Step 1: the first test tube (blank) had 1000 µl of distilled water while the other five test tubes had 900 µl, 700 µl, 500 µl, 300 µl and 100 µl respectively.

Step 2: BSA was added to all the test tubes except the blank with the first tube 100 µl, 300 µl, 500 µl, 700 µl, and 900 µl respectively.

Step 3: 3 ml of reagent D (a mixture of reagents A, B and C in the ratio 100:1:1 respectively) was added to all the test tubes and left undisturbed for 10 minutes at room temperature.

Step 4: 300 µl of reagent E (Folin ciocalteau) was added to all the test tubes, the mixture shaken vigorously and left undisturbed for the next 30 minutes.

Step 5: Absorbance was read at 750 nm wavelength.

Step 6: the result obtained was used to plot a standard calibration curve for protein (Figure 63).

Determination of total protein content in experimental samples

For the experimental samples, 100 µl of sample and 900 µl of distilled water were used and every other step was as described earlier. The absorbance readings were extrapolated from the protein calibration curve (Figure 63).

2.3.3 Inorganic phosphate determination

Inorganic phosphate was determined using the method of Fiske and Subarrow (1925) as modified by Butcher and Sutherland (1962).

Principle

The principle is based on the fact that inorganic phosphate reacts with ammonium molybdate in an acidic medium to form phosphomolybdate (phosphomolybdic acid). The addition of a reducing agent causes the reduction of molybdenum resulting in a blue colour which can be measured spectrophotometrically.

Procedure

This involves the use of 1mM disodium hydrogen orthophosphate as a standard, reagents A and B mixed in ratio 1:4 which gave rise to reagent C. reagent A is Ammonium molybdate solution and reagent B is 9% L-ascorbic acid.

2.3.4 Kinetic parameters

2.3.4.1 Phosphodiesterase 5 inhibitory assay

Exacly 20 µl of partially purified phosphodiesterase 5 was pre-incubated in 36 µM Tris buffer (pH 7.5) with 0-10 µM sildenafil citrate (SC), 0-20 µg/ml aqueous extracts of *Cnestis ferruginea* (CF) root, *Fadogia agrestis* (FA) stem and combination of CF+FA, at 37 ºC for 10 minutes. Reaction was initiated by the addition of 200 µM cyclic guanosine monophosphate (cGMP) to the reaction mixture and incubated at 37 ºC for 10 minutes. The reaction was stopped by the addition of 10 µl of ice cold trichloroacetic acid. The solution was centrifuged and 500 µl of the supernatant was used for the assaywith 2 ml of reagent C. The amount of inorganic phosphate liberated was determined by extrapolation from standard inorganic phosphate calibration curve (Figure 64).

2.3.4.2 Mode of phosphodiesterase 5 inhibition

This was done following the method described by Elmar (1984). The mode of inhibition of the extracts was determined using 0.00033-0.0033 µg/ml of each extract and their combination. Briefly, 10-100 µL of the extracts were pre-incubated with 20 µl of partially purified enzyme solutions for 10 minutes at 37 ºC in a set of test tubes. Reaction was initiated by the addition of various concentrations of 1.38 – 41.36 µM cyclic guanosine monophosphate (cGMP) and 1.29 – 38.71 µM cyclic adenosine monophosphate (cAMP) respectively. The amount of inorganic phosphate released were determined spectrophotometrically using the equation obtained from the calibration curve (Figure 60) and converted to reaction velocity (amount of inorganic phosphate released (Pi) divided by incubation time of 20 minutes) and enzyme activity. A double reciprocal plot (1/V versus 1/[S]) where V is reaction velocity and [S] is substrate concentration was

plotted. The mode of inhibition of the crude extract on PDE 5 activity was determined by analysing the double reciprocal (Lineweaver-Burk) plot using Michaelis-Menten kinetics.

One unit of enzyme activity was defined as the amount of enzyme that liberates inorganic phosphate equivalent to 1 µM of substrate (cGMP/cAMP) per minute at 20 ºC (Butcher and Sutherland 1962; Patil and Shastri, 1981).

Calculation

The activity (P) of phosphodiesterase 5 was calculated according to the following formular:

$$P = \frac{V\Delta C}{V_3} \quad \text{(units/ml)}$$

Where P is enzyme activity

V is total volume of reaction

ΔC is reaction velocity (Pi/ incubation time of 20 minutes)

V_3 is Volume of enzyme source

While specific enzyme activity (P') was defined as enzyme activity divided by total protein in the sample and was determinined using the formular:

$$P' = \frac{V\Delta C}{V' \times V_3} \quad \text{(units/ml/mg protein)}$$

Where P is enzyme activity

V is total volume of reaction

ΔC is reaction velocity (Pi/incubation time of 20 minutes)

V_3 is Volume of enzyme source

V' is protein content

2.4 *In vivo* experimental studies

2.4.1 Induction of erectile and endothelial dysfunction

One hundred and eighty male rats were induced with erectile dysfunction by oral administration of 10 mg/kg of paroxetine suspension which was prepared daily in Tween-80 (BDH Chemicals, Ltd.; Poole, England), suspended in 9 g/L saline solution) for twenty-one days using an oropharyngeal cannula (Chan *et al*., 2010; Malviya *et al.,* 2011; Yakubu and Nurudeen, 2012). Thirty none - ED rats received only normal saline solution to serve as control. Erectile/ cardiac dysfunctions were confirmed with nitric oxide depletion and elevated levels of phosphodiesterase 5 in the tissues.

2.4.1.1 Animal grouping and extract administration

The animals were randomly selected into six groups of ten animals each as follows:

Control: Normal Control (non-induced rats that received 0.75 ml of physiological saline solution)

PXT: Paroxetine Control (PXT-induced rats that received 0.75ml of physiological saline solution)

PXT SC: PXT-induced rats treated with Standard Drug (Sildenafil citrate)

PXT CF: PXT-induced rats treated with 100 mg/kg b. wt. *Cnestis ferruginea* (CF) aqueous root extract

PXT FA: PXT-induced rats treated with 100 mg/kg b. wt. *Fadogia agrestis* (FA) aqueous stem extract

PXT CF+FA: PXT-induced rats treated with combinations of *Cnestis ferruginea* and *Fadogia agrestis* in ratio 1:1.

2.4.2 Preparation of plasma

The animals (six rats each from the paroxetine and control groups respectively) were sacrificed after the 4th day, 7th day 14th day and 21st day of paroxetine treatment. Under diethyl ether anaesthesia, the neck areas were quickly cleared of fur and skin to expose the jugular veins. The jugular veins were slightly displaced from the neck region (to prevent contamination of the blood with interstitial fluid) and then cut with a sharp sterile blade. The rats were made to bleed into lithium-heparinized tubes which were left at room temperature for 10 minutes. After that, the tubes were centrifuged at 3500 rpm for 10 minutes using Uniscope Laboratory Centrifuge (model SM800B, Surgifriend Medicals, England).

The plasma was thereafter collected using Pasteur pipettes into clean, dry, sample bottles and then stored frozen overnight before being used for the testosterone, lipid profile and creatinine kinase assays.

2.4.3 Tissue homogenates

The animals were dissected, the penis and heart excised and immersed in ice-cold 0.25 M sucrose-tris buffer solution (to maintain the integrity of the tissues). Homogenates were prepared for the heart and penis. This was done by cleaning the penis and heart in Tris Buffer Solution (TBS) and then blotted. The tissues were thereafter homogenized in 0.1 M TBS (pH 7.4). All experiments were carried out at 4 ºC. The homogenates were stored in the freezer (- 4 ºC) (each in a labeled specimen bottle) and used for analysis within 2 weeks.

2.5 Erectile function assays

2.5.1 Endothelial function parameters

2.5.1.1 Determination of concentration of cyclic guanosine monophosphate

This was done according to the manufacturer's guide (R and D Systems) as described by Motterlini *et al.* (2002).

Principle

This assay is based on the competitive binding technique in which cGMP present in the sample competes with a fixed amount of horseradish peroxidase (HRP)-labelled cGMP for sites on a rabbit polyclonal antiboby. During the incubation, the polyclonal antibody becomes bound to the goat anti-rabbit antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The colour development is stopped and absorbance is read at 450 nm. The intensity of the colour is inversely proportional to the concentration of cGMP in the sample.

Procedure

One hundred and fifty micro litre (150 µl) of appropriate diluents were added to the non-specific binding (NSB) wells and 100 µl was added to the zero standard (B_0) wells. 100 µl of sample and standard were added to the respective wells. 50 µl of cGMP conjugate was added to the wells (wells were now red in colour). 50 µl of the primary antibody solution was added to the wells (except the NSB wells). The colour in the wells now changed to violet except the NSB wells. It was covered with adhesive strip that was provided and incubated for 3 hours at room temperature on a microplate shaker. After incubation period, each well was aspirated and washed. This process was repeated three times to make four washes. After the last wash, the remaining buffer was decanted, inverted and blotted against clean paper towels. 200 µl of substrate (combination

of hydrogen peroxide and tetramethylbenzidine) solution was added to each of the wells and incubated at room temperature for 30 minutes in a dark cupboard (protecting it from light). 50 µl of stop solution was added to each well (there was a colour change from blue to yellow). The optical density was determined within 30 minutes using a microplate reader at 450 nm.

Calculation

The average of the duplicated readings for each standard, control and samples were calculated from which the NSB absorbance was subtracted. The concentration of cGMP was extrapolated from the standard curve generated (Figure 66).

2.5.1.2 Nitric oxide

The nitric oxide level in tissues were determined using the Griess reagent following the method of Green *et al*. (1982).

Principle:

The principle is based on the fact that nitrate is reduced by Copper-coated Cadmium, and the nitrite produced is determined by diazotization of Griess reagent under acidic condition. Griess reaction measures nitrite which reflects nitric oxide production rate.

Procedure:

Tissue homogenate was deproteinised by treating 500 µl of appropriately diluted homogenate with 100 µl of 5% $ZnSO_4$ and 100 µl of 0.3 M NaOH. The reaction mixture was then centrifuged at 4000 g for 20 minutes. Thereafter, 100 µl of the supernatant was treated with 1000 µl of Griess reagent and the absorbance was read at 540 nm. Griess reagent was used as the blank. The nitrite concentration was determined using calibration curve for sodium nitrite (Figure 68).

2.5.1.3 Determination of concentration of Endothelin-1

This was done according to the manufacturer's guide (R and D Systems) as described by Yanagisawa (1994).

Principle

This assay employs the qauantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for endothelin-1 was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any endothelin-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for endothelin-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of endothelin-1 bound in the initial step. The colour development is stopped and the intensity of the colour is read using a microtitre plate reader.

Procedure

Exactly 150 µl of assay diluents was added to each of the wells of the microplate. 75 µl of standard, control, or sample were added per well. These were covered with an adhesive strip and incubated for 1 hour at room temperature on a horizontal orbital microplate shaker. After incubation, the wells were aspirated and washed, repeating the process three times for a total of four washes. After the last wash, the remaining wash buffer was removed by decanting. The plate was inverted and blotted against clean paper towels. A known volume (200 µl) of Endothelin-1 conjugate was added to each wells, covered with a new adhesive strip and incubated for 3 hours at room temperature on a shaker. The washing and aspiration were repeated after which 200 µl of substrate (combination of hydrogen peroxide and tetramethylbenzidine) solution was added to each of the wells and incubated for 30 minutes at

room temperature in the drawer (to protect it from light). 50 µl of stop solution (2N sulfuric acid) was added to each well, at this point the colour in the wells changed from blue to yellow. The absorbance was determined within 30 minutes using a microtitre plate reader at 450 nm.

Calculation

The average of the duplicated readings for each standard, control and samples were calculated from which the average zero standard absorbance was subtracted. The concentration of endothelin-1was extrapolated from the calibration curve (Figure 67).

2.6 Determination of cardiovascular function parameters

2.6.1 Determination of plasma lipid profile

2.6.1.1 Total cholesterol

The total cholesterol concentration was determined by colorimetric method as described by Fredrickson *et al.* (1967).

Principle

Cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator, quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase as represented by the following chemical equations:.

$$\text{Cholesterol-ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids} \quad \text{(i)}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestene-3-one} + H_2O_2 \quad \text{(ii)}$$

$$2H_2O + \text{4-aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O \quad \text{(iii)}$$

Procedure

Three test tubes were set and labelled blank, sample and standard. Then, 1.0 ml of the working reagent was pipetted into the three test tubes after which 0.01ml of distilled water, appropriately diluted plasma and standard solution was respectively added into the blank, sample and standard

test tubes. The resulting solution in each test tube was properly mixed and incubated at 37 ºC for 5 minutes. The absorbance of the sample was read against the reagent blank at 500 nm.

Calculation

Total cholesterol = $\frac{\text{Absorbance of Sample x Concentration of standard}}{\text{Absorbance of Standard}}$

Concentration of the standard was 208 mg/dl.

2.6.1.2 Triglyceride

Triglyceride concentrations in the plasma were determined as described by Searcy (1969).

Principle:

Triglycerides incubated with lipase liberate glycerol and fatty acids. Glycerol formed is then converted to glycerol phosphate by glycerol kinase (GK) and ATP. Glycerol phosphate is oxidized to dihydroxyacetone phosphate by glycerol phosphate oxidase (GPO) and the hydrogen peroxidase (H_2O_2) formed is detected by a chromogenic oxygen acceptor, 4-aminophenazone and 4-chlorophenol in the presence of peroxidase (POD). The red quinine formed is proportional to the amount of triglycerides present in the sample as represented by the following chemical equations:

Triglycerides + H_2O $\xrightarrow{\text{lipases}}$ Glycerol + Fatty acids (i)

Glycerol + ATP $\xrightarrow{\text{GK}}$ Glycerol- 3 – phosphate + ADP (ii)

Glycerol-3-phosphate + O_2 $\xrightarrow{\text{GPO}}$ Dihydroxyacetone phosphate + H_2O_2 (iii)

$2H_2O_2$+ 4-aminophenazone + 4-chlorophenol $\xrightarrow{\text{POD}}$ Quinoneimine + HCl + $4H_2O$ iv)

Procedure

Exactly 1.0 ml of the reconstituted reagents was pipette into three test tubes labelled blank, standard and sample. Then 0.01ml each of the standard solution and appropriately diluted

plasma sample was pipetted into test tubes labelled standard and sample respectively. The contents of the tubes were mixed and incubated at 25 ºC for 10 minutes. The absorbance of the standard and sample was determined at 500 nm against the reagent blank.

Calculation:

$$\text{Triglyceride in sample} = \frac{\text{Absorbance of Sample} \times \text{Concentration of Standard}}{\text{Absorbance of Standard}}$$

Concentration of the standard was 192 mg/dl

2.6.1.3 High-density lipoprotein cholesterol

HDL-Cholesterol was determined by a colorimeteric method described by Burstein and Mortin (1969).

Principle

Low density lipoproteins (LDL), very low density lipoprotein (VLDL) and chylomicron fractions were precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation the cholesterol in the high density lipoprotein (HDL) fraction, which remains in the supernatant is determined.

Procedure

Specifically, 1.0 ml of phosphotungstic acid in the presence of magnesium ions (precipitating agent) was added to 0.5ml of plasma in a test tube, mixed and left for 10 minutes at room temperature. The mixture was then centrifuged at 4000 rpm for 10 minutes to obtain the supernatant. Now, 0.1 ml each of distilled water, supernatant and standard solutions were added to test tubes labelled blank, sample and standard, respectively. 1.0 ml of the cholesterol reagent was added into the three tubes. The contents were thoroughly mixed and incubated for 5 minutes

at 37 ºC. The absorbance of the sample and standard was read at 500 nm against the reagent blank.

Calculation:

HDL-Cholesterol conc. (mg/dl) = Absorbance of Sample x Conc. of Standard

Absorbance of Standard

Concentration of standard was 0.443 mg/dl.

2.6.1.4 Low-density lipoprotein Cholesterol

The serum low-density lipoprotein cholesterol was assayed using the polyvinyl sulphate (PVS) reaction as described by Demacker *et al*. (1984).

Principle

LDLC was determined as the difference between total cholesterol and cholesterol content of the supernatant after precipitation of the LDL fraction by polyvinyl sulphate in the presence of polyethylene-glycol monomethyl ether.

Procedure

The procedure involved two assays which were the precipitation reaction assay and the cholesterol assay. The precipitation reaction assay involved the addition of 0.1ml of the precipitation solution to 0.2ml of the serum sample. The mixture was thoroughly shaken and left for 15 minutes at room temperature. It was centrifuged at 2000 g for 15 minutes. The cholesterol concentration was then determined in the supernatant. For the cholesterol assay, the cholesterol in the supernatant was determined according to the CHOD-PAP reaction as described by Fredrickson *et al.* (1967).

Calculation:

LDL-C (mg/dl) = Total cholesterol (mg/dl) – 1.5 x Supernatant cholesterol (mg/dl)

2.6.1.5 Atherogenic index

The atherogenic index was computed using the method described by Ng *et al* (1997). The computation was done using the expression LDLC/ HDLC.

2.6.2 Determination of cardiovascular heart disease risk parameters

2.6.2.1 Plasma creatine kinase activities

The method described by Witt and Tren-Delenburg (1982) was used for the quantitative determination of creatine kinase in the plasma.

Kinetic determination of creatine kinase (CK) activity as described by Witt and Tren-Delenburg (1982) was employed based on the following reactions:

$$\text{Creatine Phosphate} + \text{ADP} \xrightarrow{\text{Creatine kinase}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{Hexokinase}} \text{ADP} + \text{Glucose} - 6\text{- phosphate}$$

$$\text{G-6-P} + \text{NADP}^{+} \xrightarrow{\text{Glucose-6-phosphate dehydrogenase}} \text{D-Gluconate -6-phosphate} + \text{NADPH} + \text{H}^{+}$$

Procedure

Creatine phosphate (0.5 ml of 250 mM) solution was mixed with 0.2 ml of buffered substrate containing D-glucose (25 mM), N-acetyl-L-cysteine (25 mM), magnesium acetate (12.5 mM), NADP (2.4 mM), EDTA (2.0 mM) and Hexokinase (> 6800 U/L) prepared in immidazole buffer (125 mmol/L). Enzyme source (0.01 ml of the serum sample of rats) was added to the reaction mixture. The resulting solution was incubated for 2 minutes at 37 ^{o}C. Absorbance was read at 340 nm per minute for three min. Enzyme activity was calculated using the following expression:

Calculation:

Creatine kinase (U/l) = 4127 x (ΔAbsorbance/minute x dilution factor)

4127 is the standard factor used in preparation of the enzyme kit.

2.7 Testosterone

The serum testosterone concentration was quantitatively determined using the direct human testosterone enzyme immunoassay (EIA) kit described by Tietz (1995).

Principle:

The testosterone Enzyme Immuno Assay (EIA) is based on the competitive binding between testosterone in the test sample and testosterone-Horseradish peroxide (HRP) conjugate for a constant amount of rabbit anti-testosterone.

Procedure:

A desired number of coated wells in the holders were secured. 10.0 µl each of standards, samples and controls were dispensed into appropriate wells. A known volume (50 µl) of testosterone-HRP conjugate reagent and 50 µl of rabbit anti-testosterone were dispensed into all wells. This was then mixed for 30 seconds after which it was incubated at 37^0C for 1 hour. The microwells were rinsed and flicked 5 times with distilled water. 350 µl of 3, 3, 5, 5-tetramethylbenzidine (TMB) reagent (a surfactant) was dispensed into each well, gently mixed for 5 seconds and incubated at room temperature for 15 minutes. The reaction was stopped by adding 50 µl of 1N HCl to each well and then gently mixed for 20 seconds. The resultant yellow colour solution formed from blue colour was read at 405 nm with a microtitre plate reader within 15 minutes.

Calculation of testosterone concentration:

The serum testosterone concentration of the sample was extrapolated from the calibration curve for testosterone (Figure 65).

2.8 Statistical analysis

All data were presented as the mean of six determinations ± standard error of mean (S.E.M.). Statistical evaluation was done by one-way analysis of variance (ANOVA), followed by Duncan's posthoc test for multiple comparisons using Graphpad prism 5. Values were considered statistically significant at $p<0.05$.

Table 4: Dilution factors for the various assays

Parameter/Organs	Penis	Heart	Plasma
NO	10	30	5
PDE 5	10	30	5
Arginase	10	30	5
cGMP	-	-	5
Endothelin-1	-	-	5
Testosterone	-	-	5
Cholesterol	-	-	5
Protein	10	30	5
CK	-	-	5

NO – Nitric oxide; PDE 5- Phosphodiesterase 5; cGMP- cyclic guanosine monophosphate; CK-Creatine kinase

CHAPTER THREE

RESULTS

3.1 Secondary metabolites

Table 5 shows the secondary metabolites quantified in aqueous extracts of *C. ferruginea* root and *F. agrestis* stem. Alkaloids predominates the extracts of both plants; *F. agretis* has significantly ($p < 0.05$) higher amount of alkaloids than *C. ferruginea.* Saponins and phenolics were also detected in appreciable amount; values in both plants were not significantly ($p \geq 0.05$) different from each other. *C. ferruginea* root aqueous extract also has substantial amount of cardiac glycosides, whereas this class of metabolites was not detected in *F. agrestis* extract. Trace amount of tannins and phlobatannins were also detected in both plants.

3.2 PDE 5 from rat penile, and cardiac tissues

The isolation and partial purification of phosphodiesterase 5 from rat penile and cardiac tissues were carried out in this study. The results obtained in Tables 6 and 7 showed that a high level of the activities found in the homogenate was located in the precipitate after centrifugation. There was an increase in the specific enzyme activities as purification progressed with a concomitant increase in the purification yields. The ammonium sulphate precipitation of the penile PDE 5 gave a purification yield of 1.5 folds with dialysed fraction of approximately 5.6 folds (Table 6). The result of Ammonium sulphate precipitation of the cardiac PDE 5 gave a purification fold of 5 and dialysed fraction was approximately 7.5 folds (Table 7) respectively.

Table 5: Secondary Metabolite Constituents of Aqueous Extracts of *Cnestis ferruginea* Root and *Fadogia agrestis* stem

Secondary metabolites	*Cnestis ferruginea* Root (mg/g) $x10^{-2}$	*Fadogia agrestis* stem (mg/g) $x10^{-2}$
Alkaloids	49.95 ± 0.25	59.15 ± 0.25
Tannins	0.21 ± 0.01	0.20 ±0.02
Phlobatannins	0.16 ± 0.01	0.12 ± 0.01
Saponins	12.40 ± 0.10	11.00 ± 0.20
Phenolics	6.50 ± 0.10	6.05 ± 0.20
Cardiac glycosides	3.60 ± 0.10	Not detected
Chalcones	Not detected	Not detected
Steroids	Not detected	Not detected
Flavonoids	Not detected	Not detected
Terpenoids	Not detected	Not detected
Anthraquinones	Not detected	Not detected
Cardenolides	Not detected	Not detected

No. of replicates=3

Table 6: Purification and yield of penile phosphodiesterase 5

Purification Step	Total Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/ml/mg protein)	Purification fold (%)
Crude Extract	1.69	5.60	0.30	0.00
Ammonium Sulphate	0.26	0.52	0.51	1.70
Dialysed Fraction	0.36	0.22	1.64	5.47

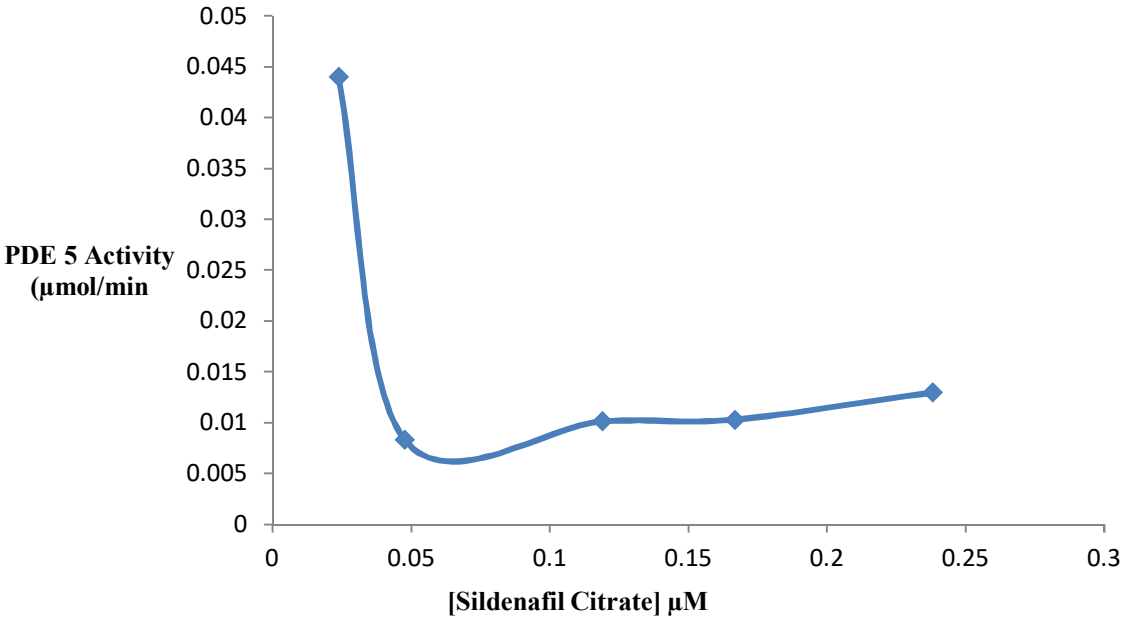
Table 7: Purification and yield of cardiac phosphodiesterase 5

Purification Step	Total Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/ml/mg protein)	Purification fold (%)
Crude Extract	1.52	6.80	0.22	0.00
Ammonium Sulphate	1.79	1.62	1.10	5.00
Dialysed Fraction	0.46	0.28	1.64	7.46

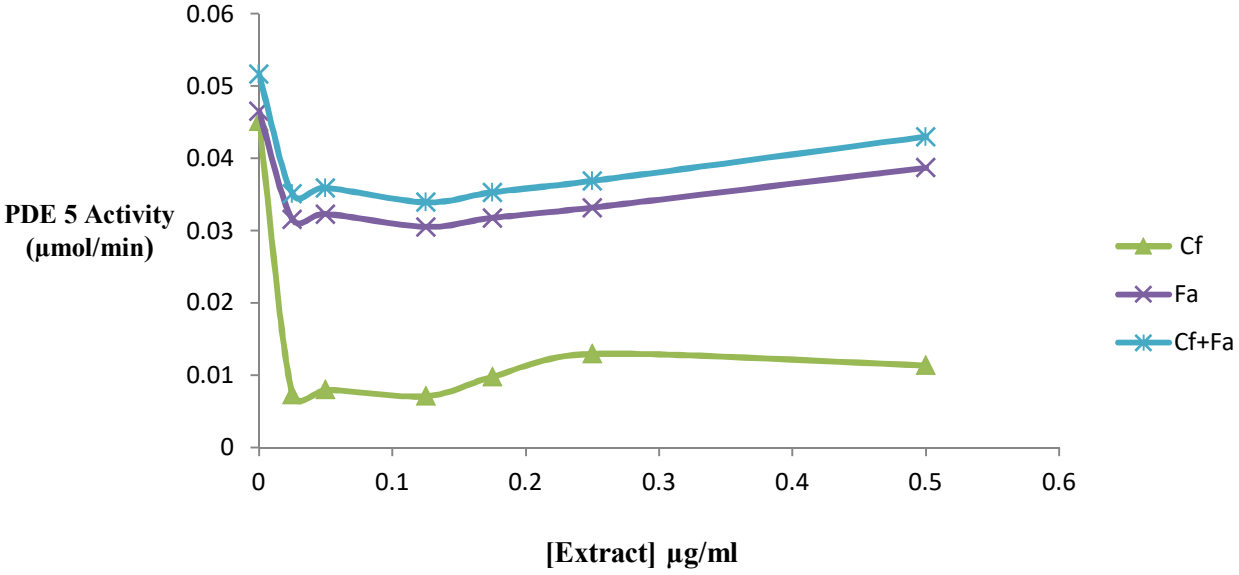
3.3 Phosphodiesterase 5 inhibitory activities of aqueous extract of *Cnestis ferruginea* (CF) root and *Fadogia agrestis* stem (FA) as well as their combinations (CF+FA) in the presence of cGMP

The rates of hydrolysis of cGMP in the dialysed fraction of penile PDE 5 in the presence of aqueous extracts of *C. ferruginea, F. agrestis* and their combination were compared in this study. The results obtained in Figures 17(b) showed that the effects of the extracts on hydrolysis of cGMP by penile phosphodiesterase 5 were similar to the effects of the standard phosphodiesterase 5 inhibitor (sildenafil citrate) as seen in Figure 17 (a) which inhibited penile phosphodiesterase 5 between 0.05 µM – 0.1 µM. There was a decrease in the enzyme activity in the presence of the extracts with *C. ferruginea* showing the highest inhibition between 0.025 µg/ml – 0.125 µg/ml and higher concentrations of the extract lead to increase in penile phosphodiesterase 5 activity. The penile PDE 5 inhibition was in the order: CF>FA>CF+FA.

The rates of hydrolysis of cGMP in the dialysed fraction of cardiac PDE 5 in the presence of aqueous extracts of *C. ferruginea, F. agrestis* and their combination were compared in this study. The results obtained in Figures 18(b) showed that the effects of the extracts on hydrolysis of cGMP by cardiac phosphodiesterase 5 were similar to the effects of the standard phosphodiesterase 5 inhibitor (sildenafil citrate) as seen in Figure 18 (a) which inhibited cardiac phosphodiesterase 5 between 0.05 µM – 0.1 µM. There was a decrease in the enzyme activity in the presence of the extracts with *C. ferruginea* showing the highest inhibition between 0.025 µg/ml – 0.125 µg/ml and higher concentrations of the extract lead to increase in cardiac phosphodiesterase 5 activity. The cardiac PDE 5 inhibition was in the order: CF> CF+FA> FA.



(a)



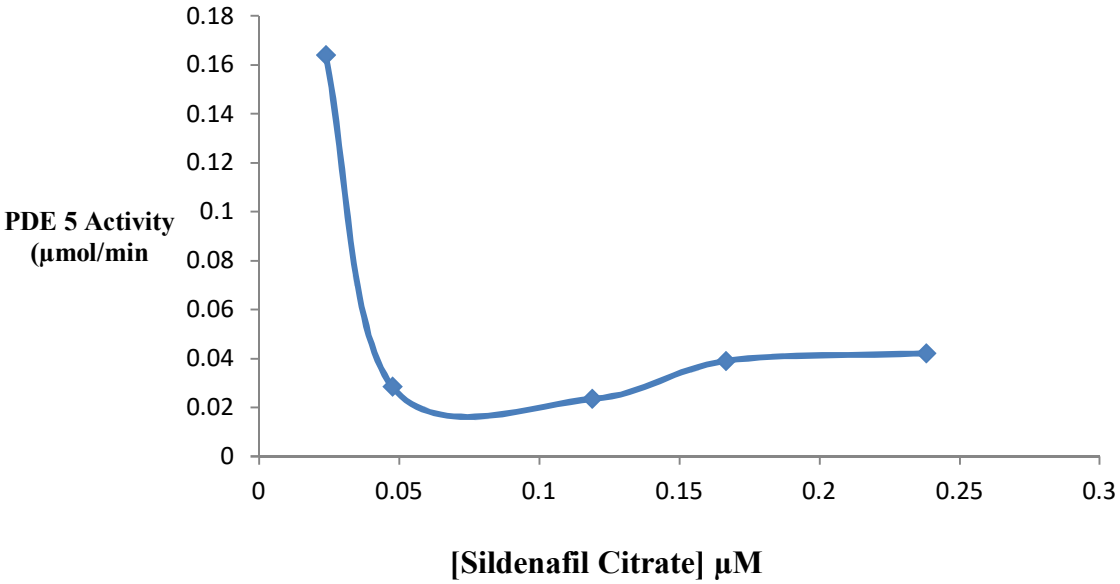
(b)

Figure 17: Effect of (a) sildenafil citrate and (b) aqueous extracts of *Cnestis ferruginea*, *Fadogia agrestis* and combination of *C. ferruginea* and *F. agrestis* on penile PDE 5-catalysed hydrolysis of cGMP

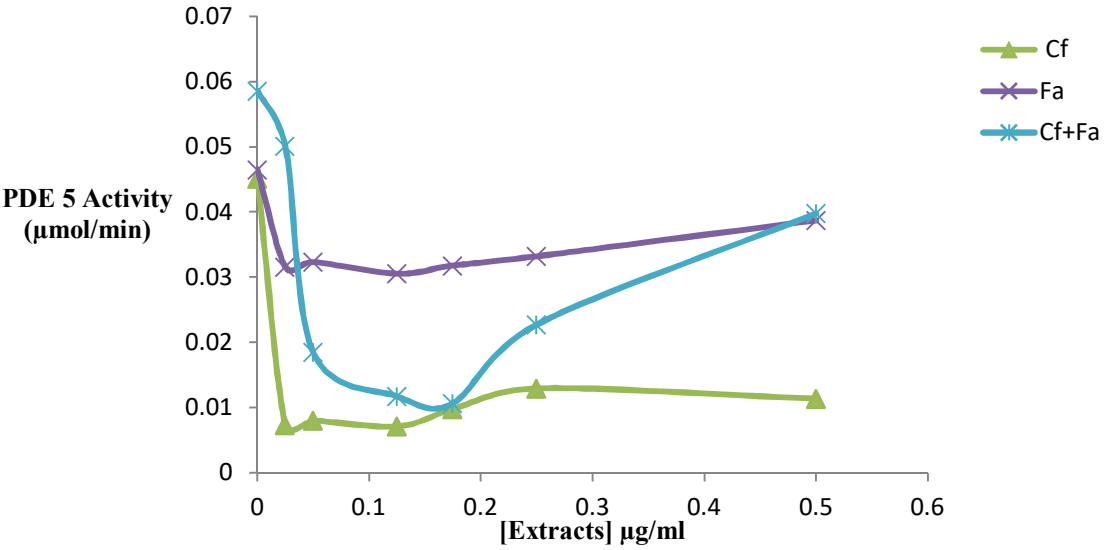
Cf= *Cnestis ferruginea*

Fa= *Fadogia agrestis*

Cf+Fa= Combination of *C. ferruginea* and *F. agrestis*



(a)



(b)

Figure 18: Effect of (a) sildenafil citrate and (b) aqueous extracts of *Cnestis ferruginea*, *Fadogia agrestis* and combination of *C. ferruginea* and *F. agrestis* on cardiac PDE 5-catalysed hydrolysis of cGMP

Cf= *Cnestis ferruginea*
Fa= *Fadogia agrestis*
Cf+Fa= Combination of *C. ferruginea* and *F. agrestis*

3.4 Effects of various extracts on substrate kinetics of dialysed fraction of penile PDE 5

Figure 19 shows the effects of aqueous extracts of *C. ferruginea, F. agrestis* and their combination on substrate kinetics of penile PDE 5 catalysed hydrolysis of cGMP. There was a steady increase in the enzyme activity as the substrate increased, the curve in the absence of standard drug and extracts obeys the Michaelis-Menten kinetics giving hyperbolic curve whereas, the curves in the presence of the sildenafil and the extracts produced sigmoidal shape. There was a steady increase in PDE 5 activity until 13.786 µM of cGMP, where increase in substrate concentration produced a concomitant sharp increase in PDE 5 activity, at 27.572 µM, increase in substrate concentration had no effect on the enzyme activity. The effects of the individual extracts and their combination on the kinetic parameters (V_{max} and K_m) as obtained in Lineweaver-Burk plots are presented on Figures 20, 21, 22, 23 and values of the kinetic parameters are shown in Table 8. Looking critically at Figures 20, 21, 22, and 23, the double reciprocal plots revealed the meeting points of the line graphs at the intercept of x and y axis.

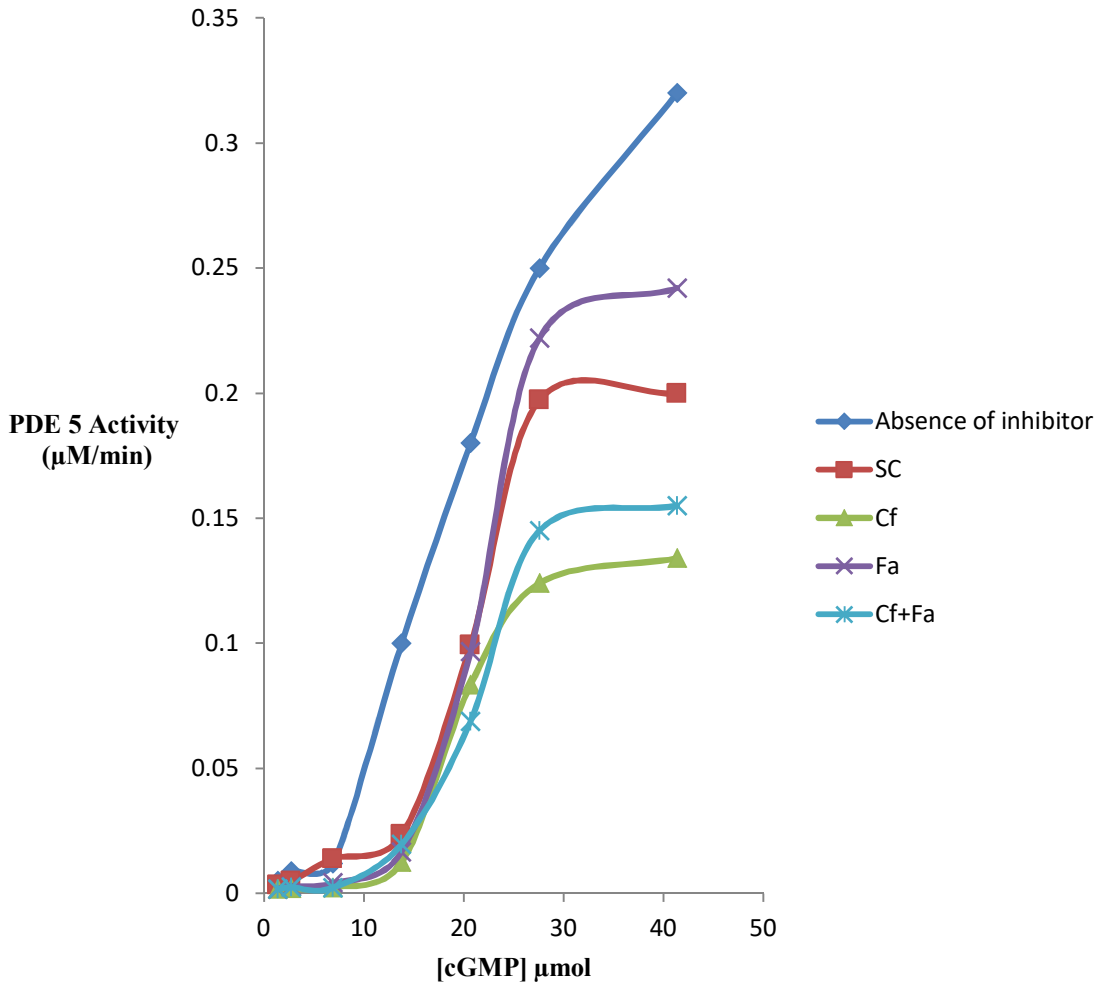


Figure 19: Effects of some inhibitors on substrate kinetics of penile PDE 5 catalysed hydrolysis of cGMP

SC= Sildenafil Citrate

Cf= *Cnestis ferruginea*

Fa= *Fadogia agrestis*

Cf+Fa= Combination of *C. ferruginea* and *F. agrestis*

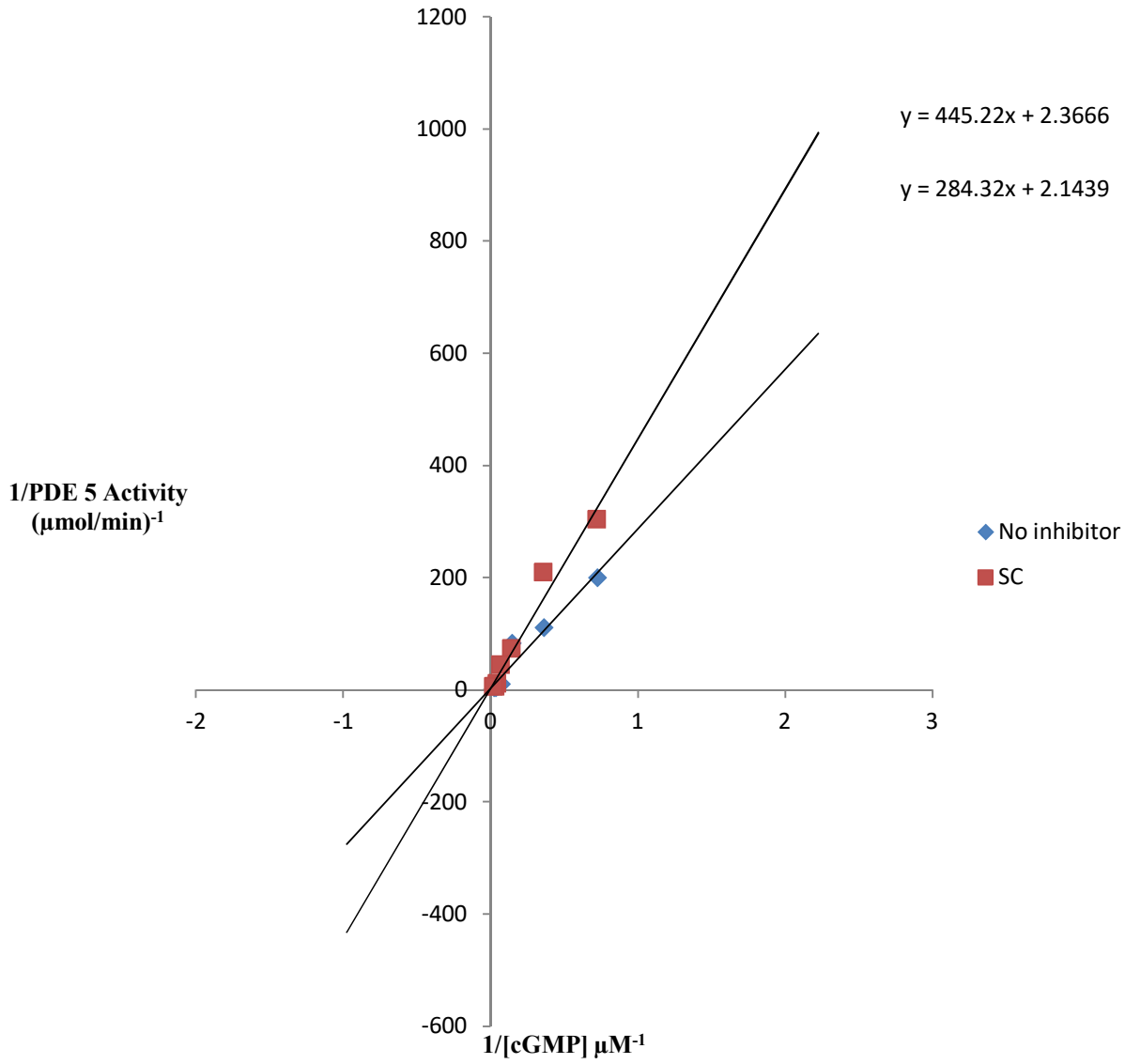


Figure 20: Lineweaver-Burk plot of the effects of sildenafil citrate on substrate kinetics of penile PDE 5-catalysed hydrolysis of cGMP

SC= Sildenafil Citrate

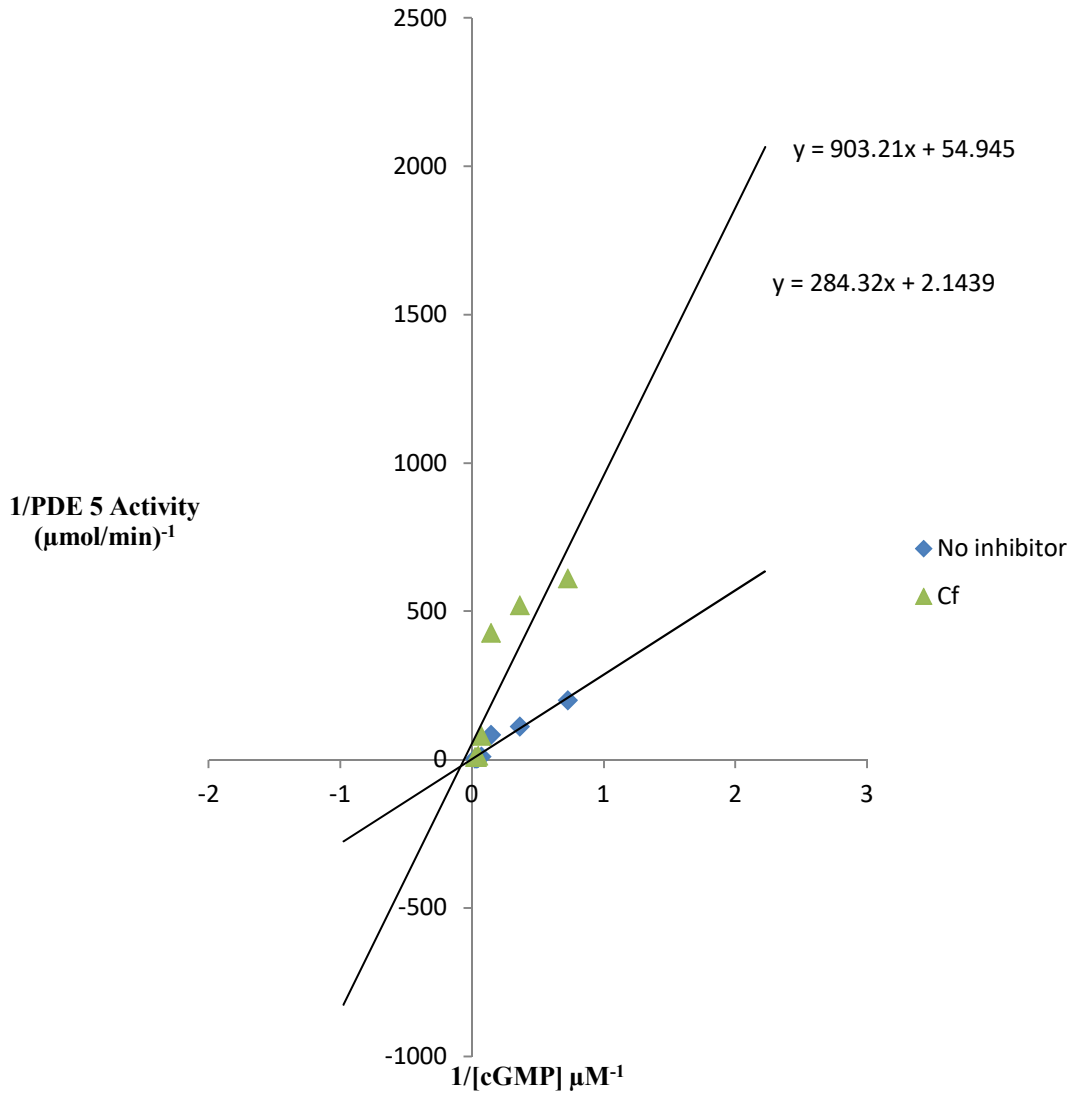


Figure 21: Lineweaver-Burk Plot of the effects of aqueous extract of *cnestis ferruginea* root on substrate kinetics of penile PDE 5-catalysed hydrolysis of cgmp

Cf= *Cnestis ferruginea*

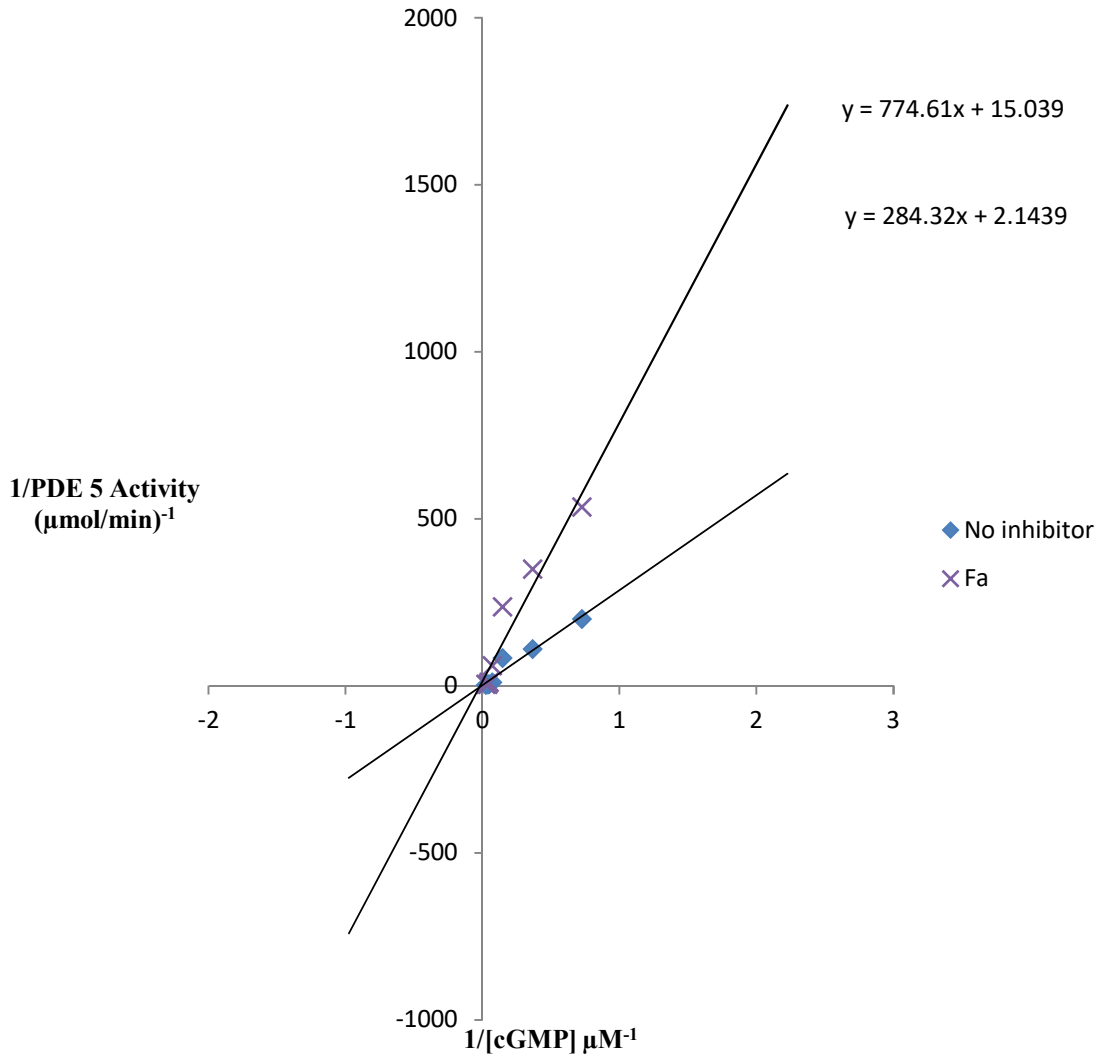


Figure 22: Lineweaver-Burk plot of the effects of aqueous extract of *Fadogia agrestis* stem on substrate kinetics of penile PDE 5-catalysed hydrolysis of cGMP

Fa= *Fadogia agrestis*

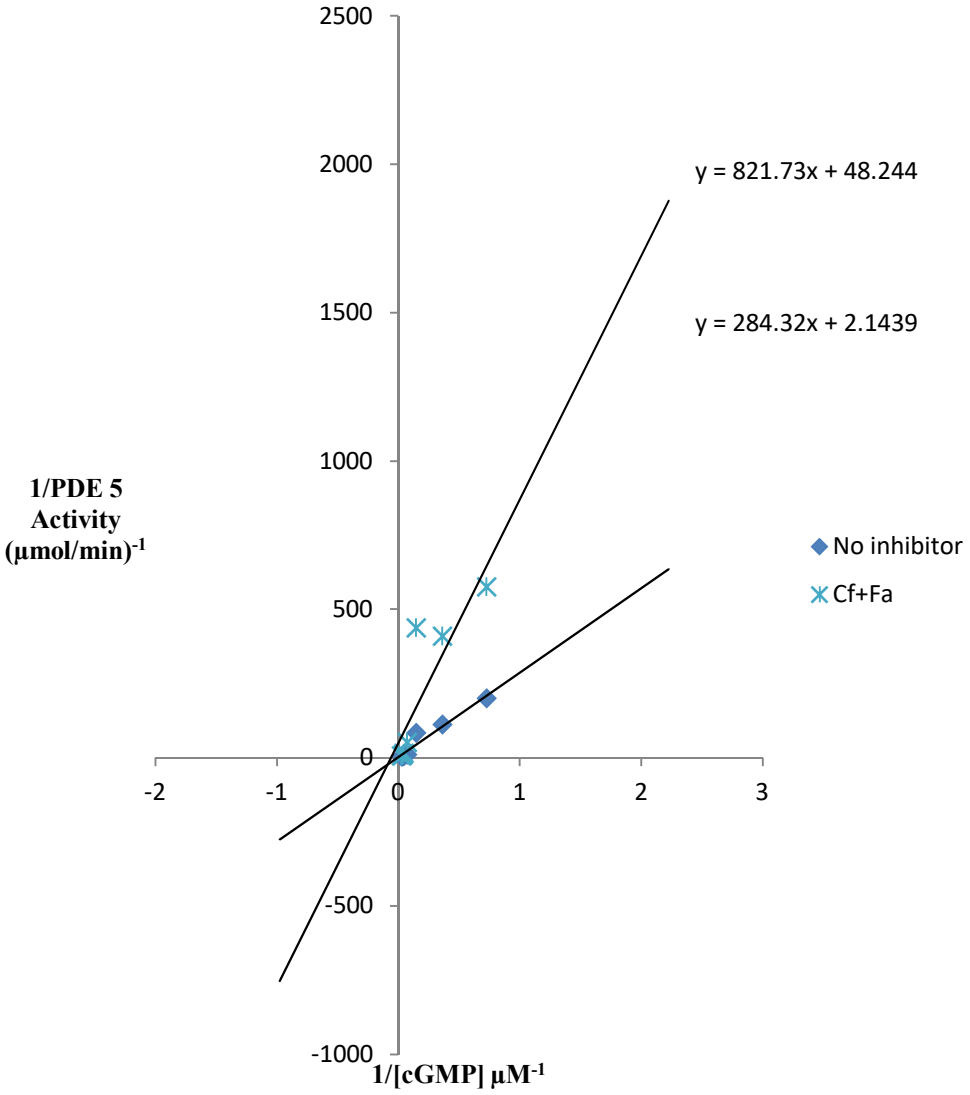


Figure 23: Lineweaver-Burk plot of the effects of combination of aqueous extracts of *C. ferruginea* root and *F. agrestis* stem on substrate kinetics of penile PDE 5- catalysed hydrolysis of cGMP

Cf+Fa= Combination of *C. ferruginea* and *F. agrestis*

Table 8: Effects of some inhibitory agents on the kinetic parameters of penile PDE 5 catalysed hydrolysis of cGMP

	V_{max} (µmol/min)	K_m (µM)
Absence of inhibitory agent	0.47	132.49
Sildenafil Citrate	0.42	188.33
C. ferruginea root extract (Cf)	0.02	16.26
F. agrestis stem extract (Fa)	0.07	51.12
Combination (Cf+Fa)	0.02	17.26

3.5 Effects of various extracts on substrate kinetics of cardiac PDE 5

The effects of aqueous extracts of *C. ferruginea, F. agrestis* and their combinations on substrate kinetics of cardiac phosphodiesterase 5 catalysed hydrolysis of cGMP can be seen in Figure 24. There was a steady increase in the enzyme activity as the substrate increased, enzyme activity in the absence of inhibitor obeyed the Michaelis-Menten kinetics giving hyperbolic curve whereas, the curves in the presence of the inhibitors produced sigmoidal shape. There was a gradual and steady increase until 13.786 µM of cGMP, where increase in substrate concentration produced a sharp increase in enzyme activity, at 27.572 µM, increase in substrate concentration had no effect on the enzyme activity. The effects of the individual extracts and their combination on the kinetic parameters (V_{max} and K_m) as obtained in Lineweaver-Burk plots are presented on Figures 25, 26, 27, 28 and values of the kinetic parameters are shown in Table 9. Looking critically at Figures 20, 21, 22, and 23, the double reciprocal plots revealed the meeting points of the line graphs at the intercept of x and y axis which are indications of competitive inhibition. the combination of the extracts (Cf + Fa) inhibited PDE 5 activities better than the individual extracts.

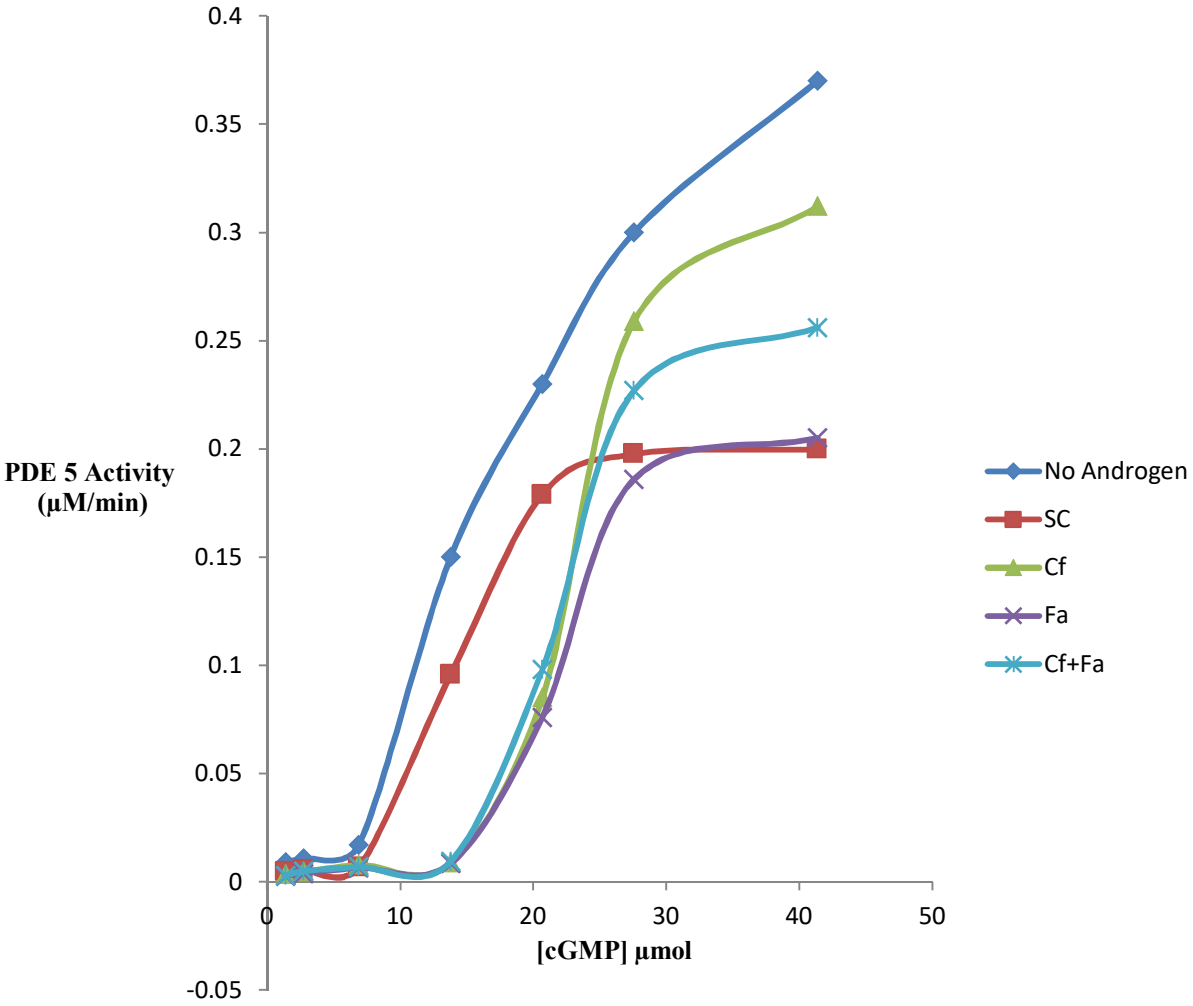


Figure 24: Effects of some inhibitory agents on substrate kinetics of cardiac PDE 5 catalysed hydrolysis of cGMP

SC= Sildenafil Citrate

Cf= *Cnestis ferruginea*

Fa= *Fadogia agrestis*

Cf+Fa= Combination of *C. ferruginea* and *F. agrestis*

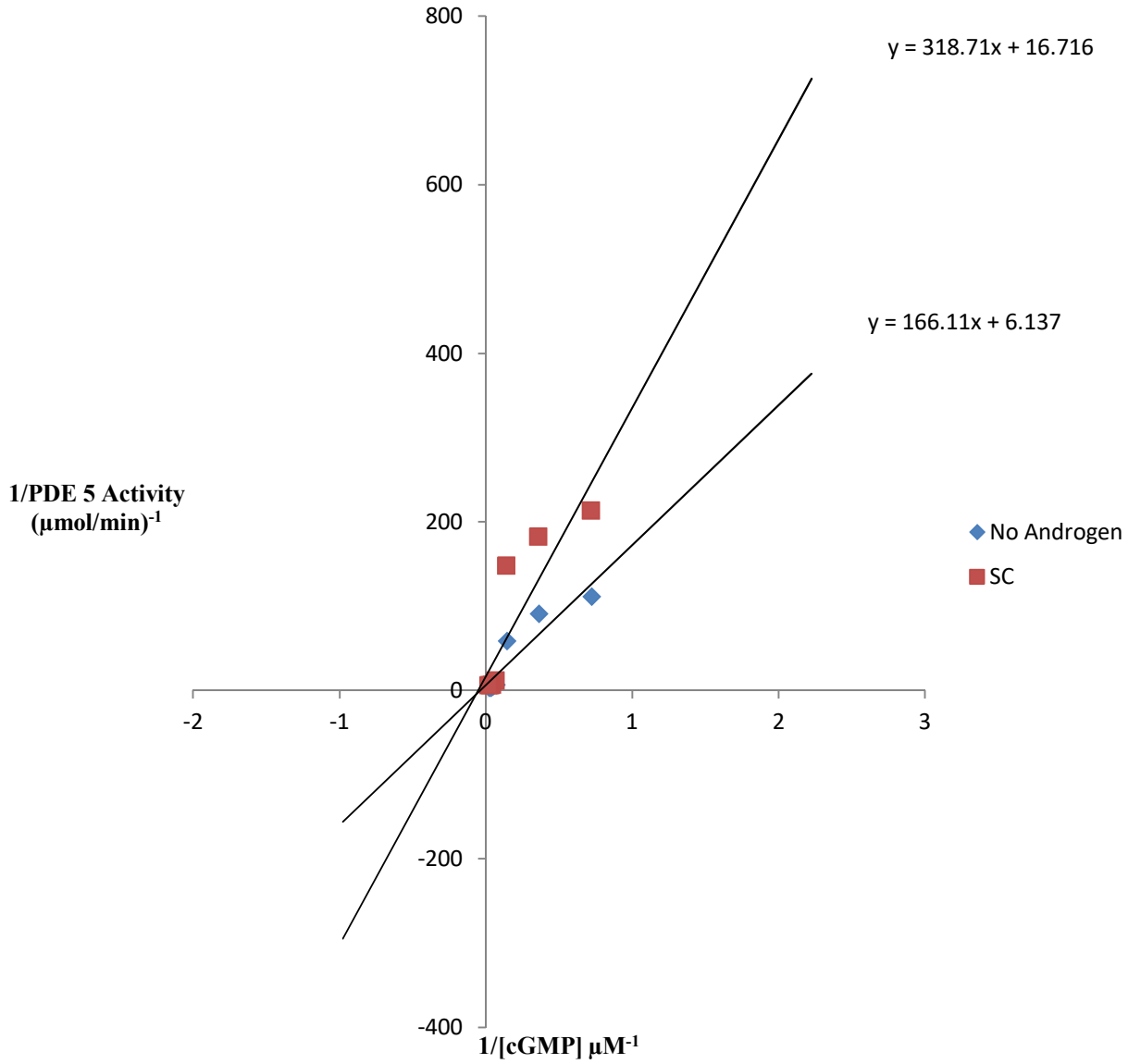


Figure 25: Lineweaver-Burk plot of the effects of sildenafil citrate on substrate kinetics of cardiac PDE 5-catalysed hydrolysis of cGMP

SC= Sildenafil Citrate

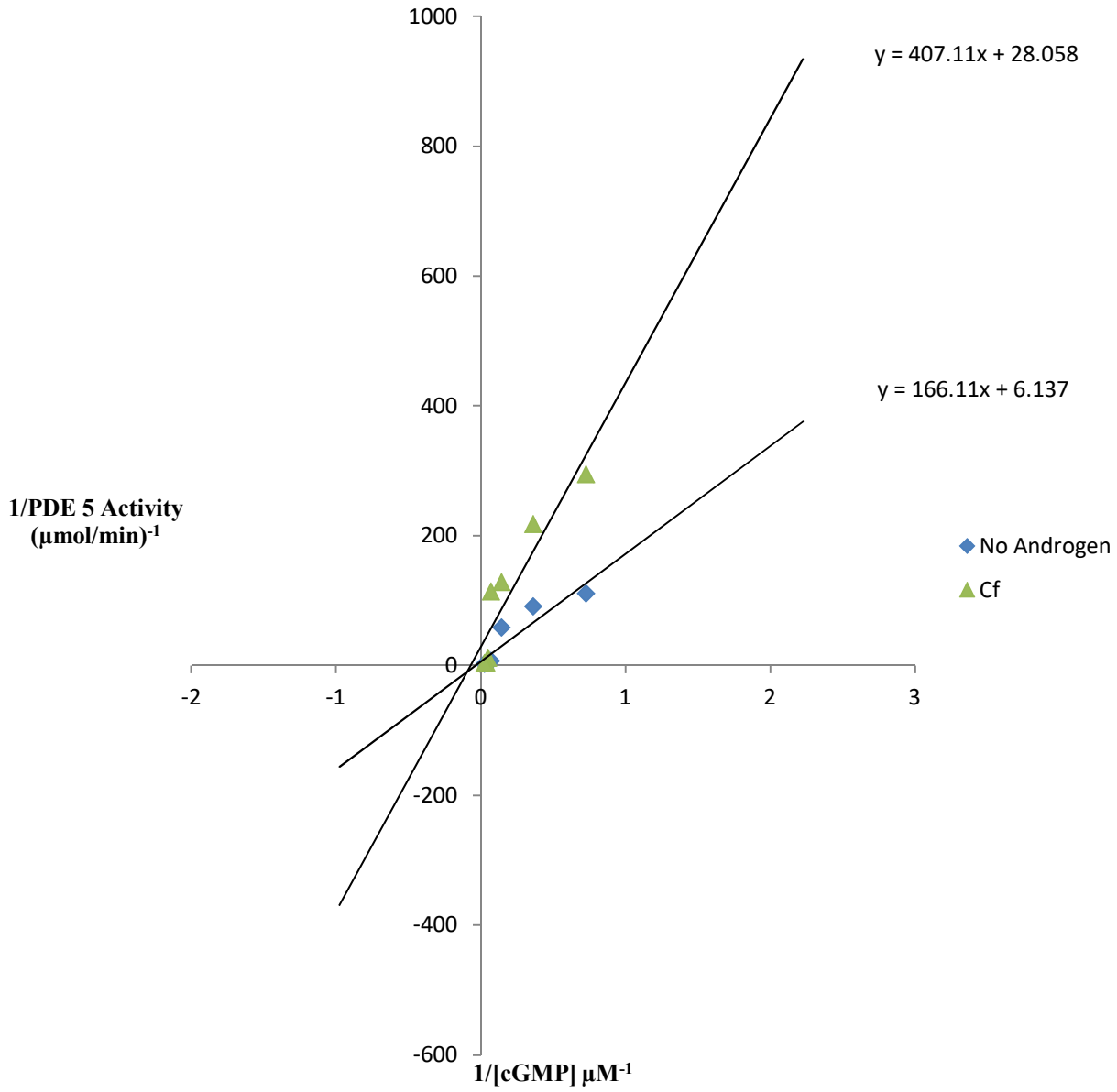


Figure 26: Lineweaver-Burk plot of the effects of aqueous extract of *Cnestis ferruginea* root on substrate kinetics of cardiac PDE 5-catalysed hydrolysis of cGMP

Cf= *Cnestis ferruginea*

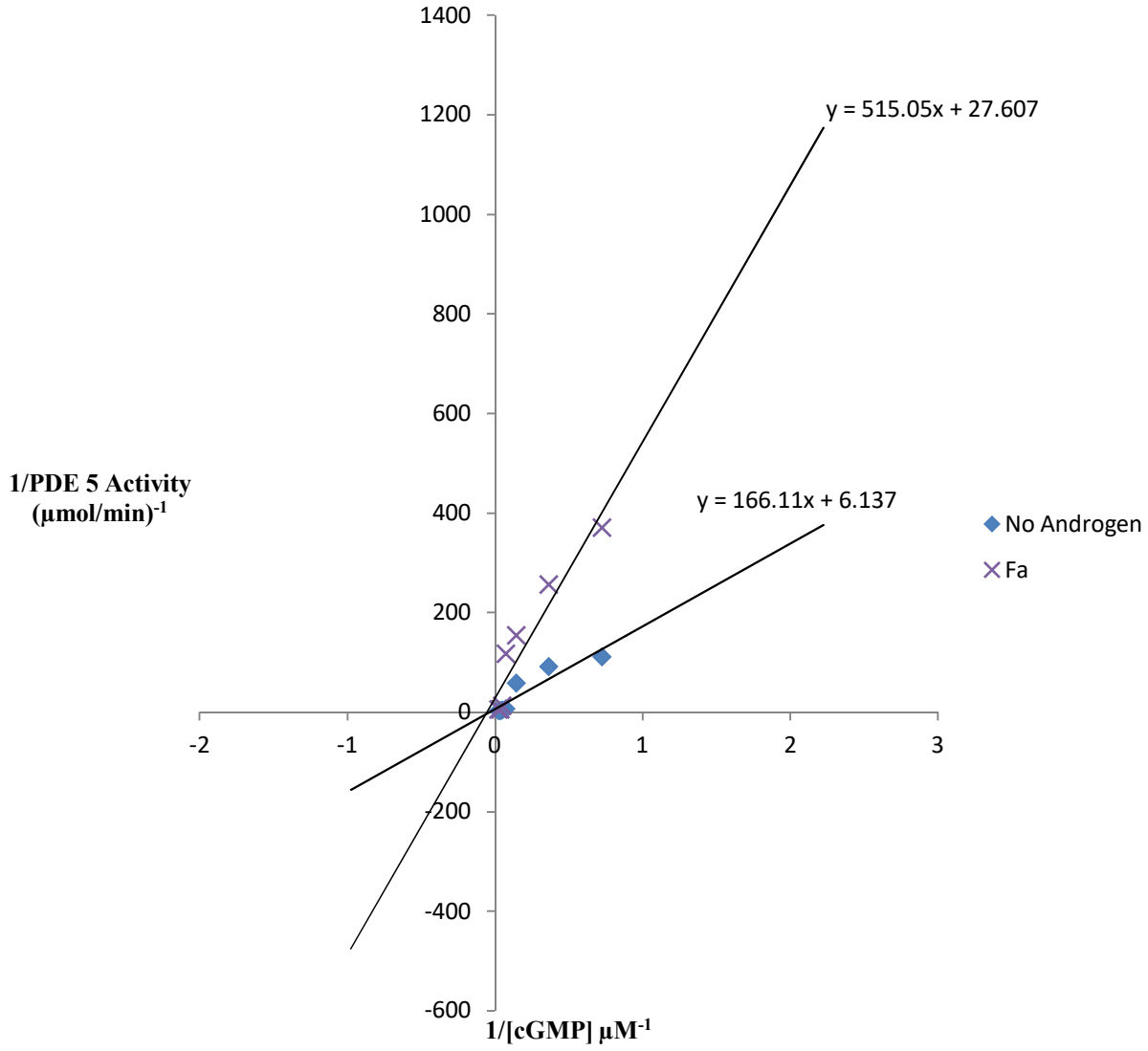


Figure 27: Lineweaver-Burk plot of the effects of aqueous extract of *Fadogia agrestis* stem on substrate kinetics of cardiac PDE 5-catalysed hydrolysis of cGMP

Fa= *Fadogia agrestis*

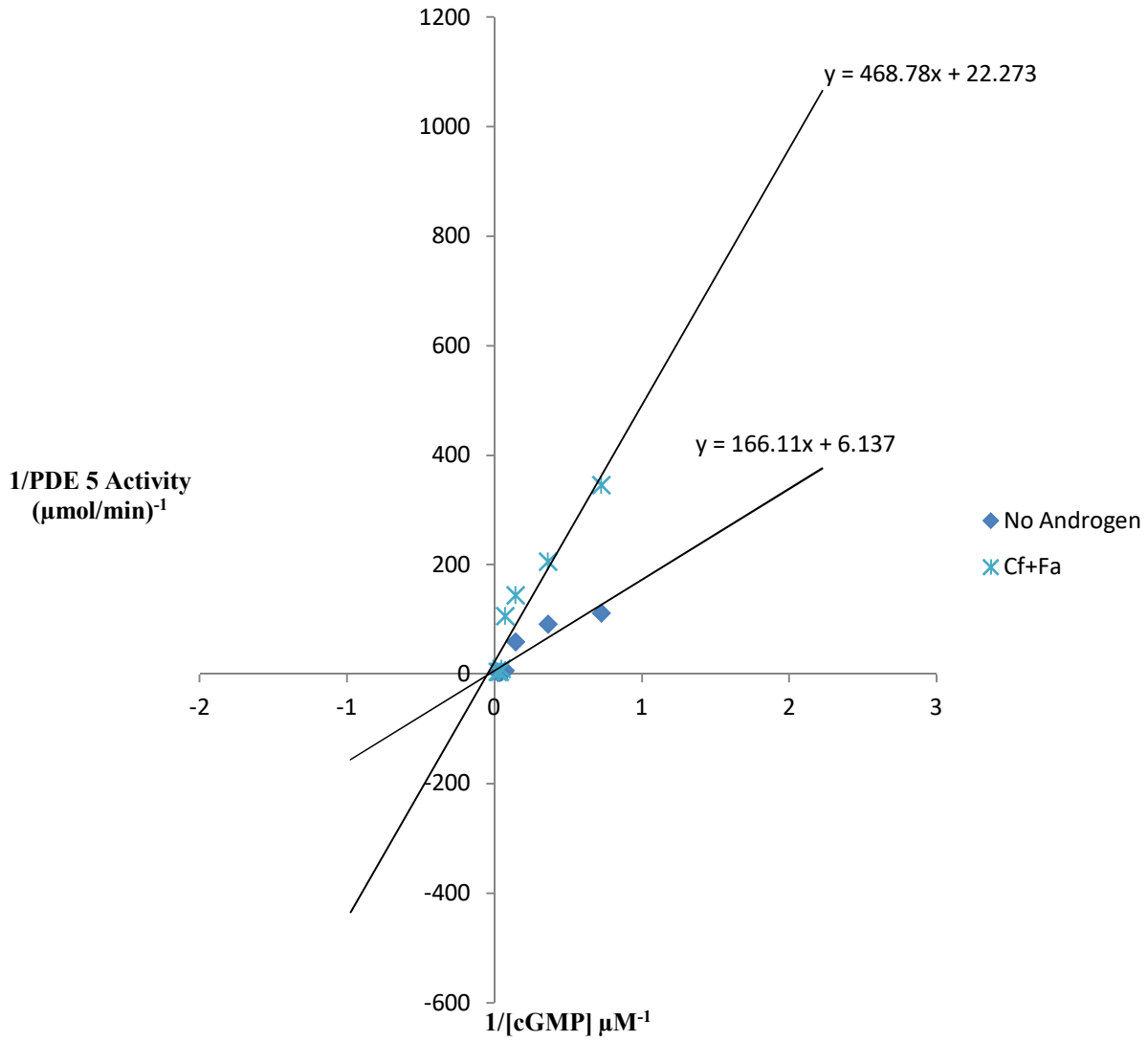


Figure 28: Lineweaver-Burk plot of the effects of combination of aqueous extracts of *C. ferruginea* root and *F. agrestis* stem on substrate kinetics of cardiac pde 5-catalysed hydrolysis of cGMP

Cf+Fa= Combination of *C. ferruginea* and *F. agrestis*

Table 9: Effect of Some PDE 5 inhibitors on the kinetic parameters of cardiac PDE 5-catalysed hydrolysis of cGMP

	V_{max} (µmol/min)	K_m (µM)
Absence of inhibitors	0.16	27.06
Sildenafil Citrate	0.06	19.12
C. ferruginea extract (Cf)	0.04	14.66
F. agrestis stem extract (Fa)	0.04	18.54
Combination (Cf+Fa)	0.05	21.10

3.6 Effects of various extracts on substrate kinetics of dialysed fraction of penile PDE 5

The effects of aqueous extracts of *C. ferruginea, F. agrestis* and extracts combination on substrate kinetics of penile PDE 5 catalysed hydrolysis of cAMP is shown in Figure 29. There was a sharp increase in the enzyme activity as the substrate increases, the curve in the absence of androgen obeyed the Michaelis-Menten kinetics giving hyperbolic curve while in the presence of the inhibitors (standard drug and extracts) enzyme activities also increased as the substrate increased but the enzyme activities was reduced when compared with the curve without inhibitors. There was a sharp increase in penile PDE 5 activities in the presence of 0.645-1.29 µM of cAMP after which increase in substrate concentration produced steady increase in enzyme activity. The effects of the individual extracts and their combination on the kinetic parameters (V_{max} and K_m) as obtained from Lineweaver-Burk plots are presented in Figures 30, 31, 32, 33 respectively and kinetic parameters are shown in Table 10.

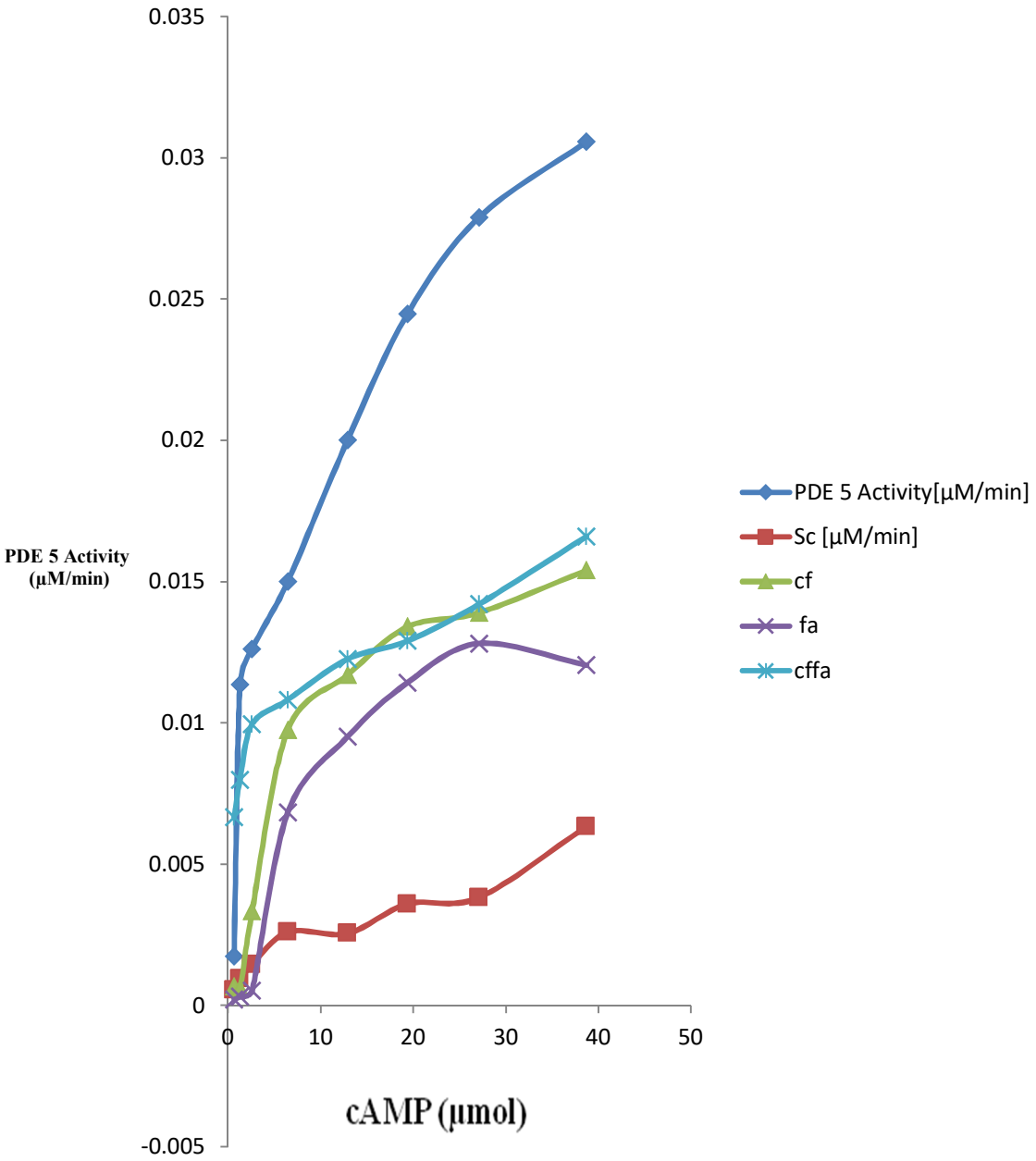


Figure 29: Effects of some inhibitory agents on substrate kinetics of penile PDE 5-catalysed hydrolysis of cAMP

SC= Sildenafil Citrate

Cf= *Cnestis ferruginea*

Fa= *Fadogia agrestis*

Cf+Fa= Combination of *C. ferruginea* and *F. agrestis*

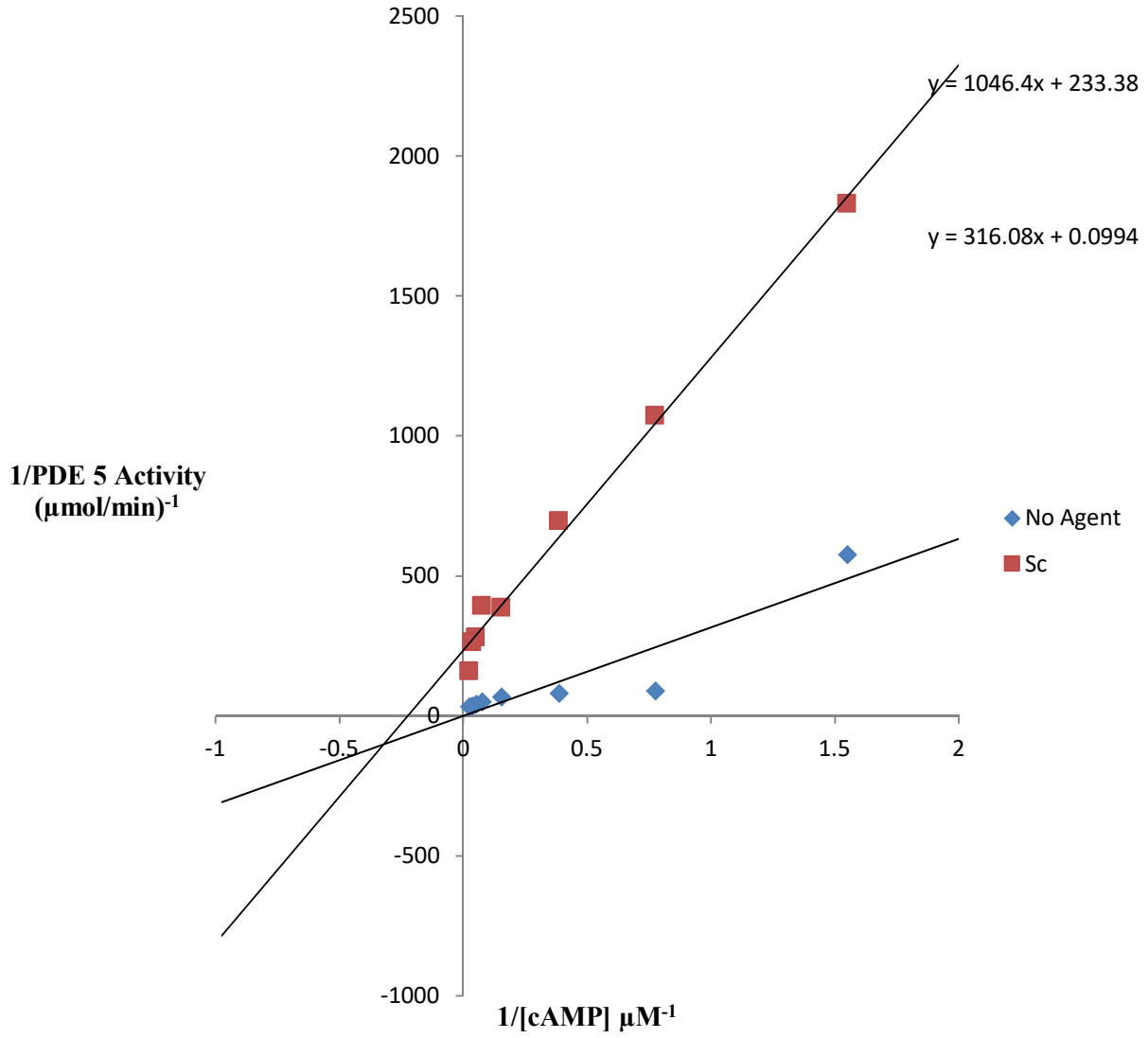


Figure 30: Lineweaver-Burk plot of the effects of sildenafil citrate on substrate kinetics of penile PDE 5-catalysed hydrolysis of cAMP

SC= Sildenafil Citrate

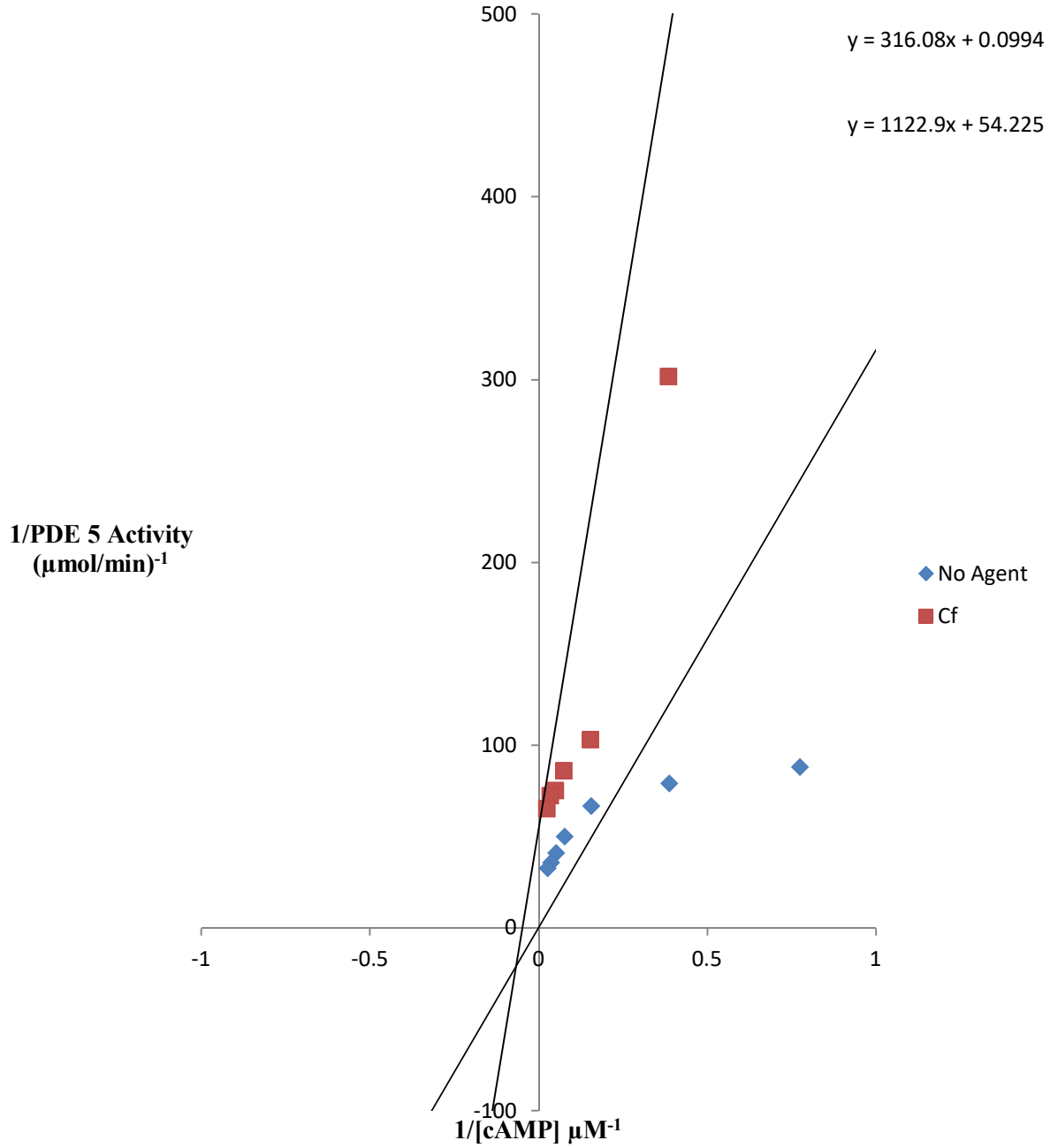


Figure 31: Lineweaver-Burk plot of the effects of aqueous extract of *Cnestis ferruginea* root on substrate kinetics of penile PDE 5-catalysed hydrolysis of cAMP

Cf= *Cnestis ferruginea*

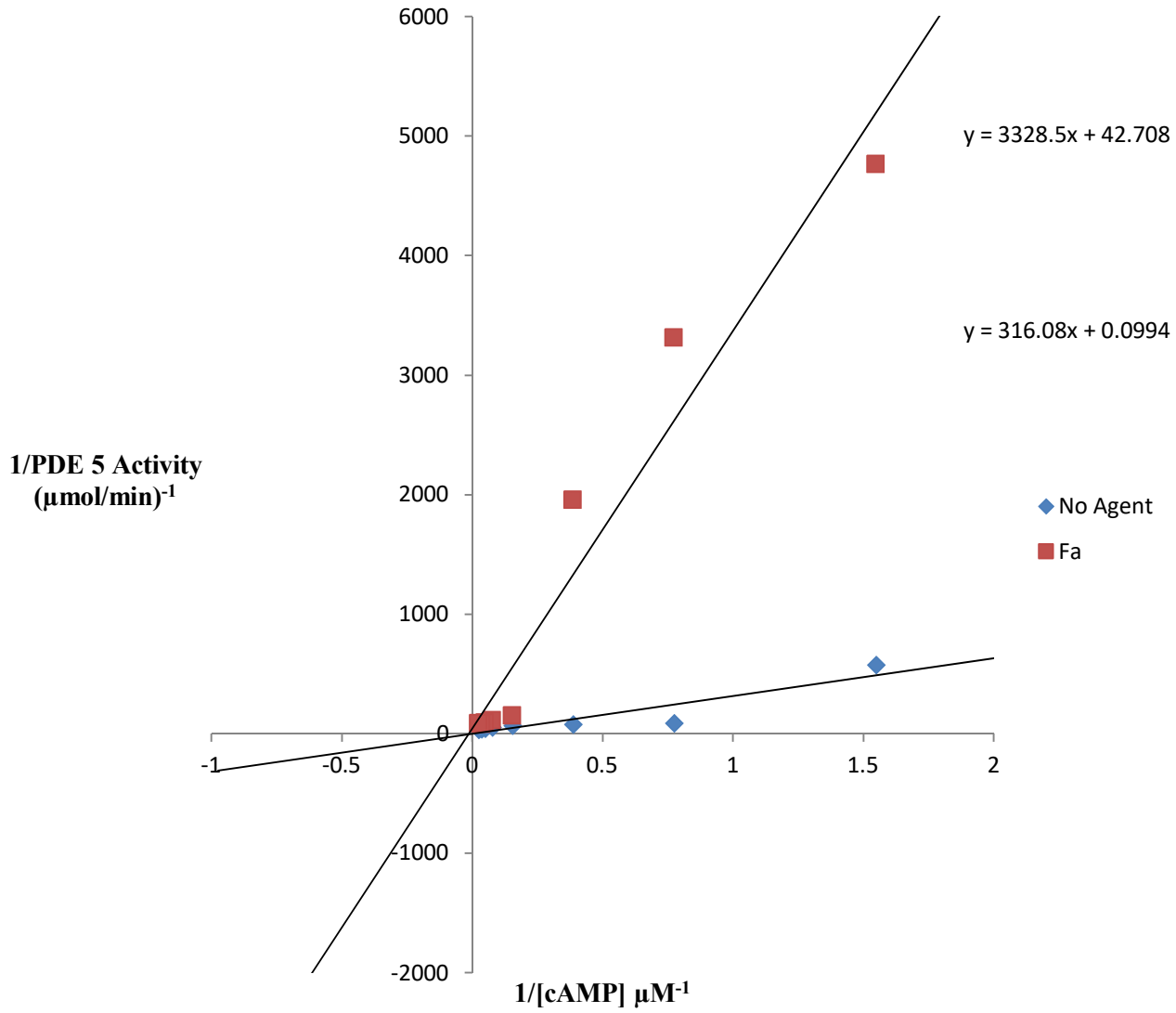


Figure 32: Lineweaver-Burk plot of the effects of aqueous extract of *Fadogia agrestis* stem on substrate kinetics of penile PDE 5-catalysed hydrolysis of cAMP

Fa= *Fadogia agrestis*

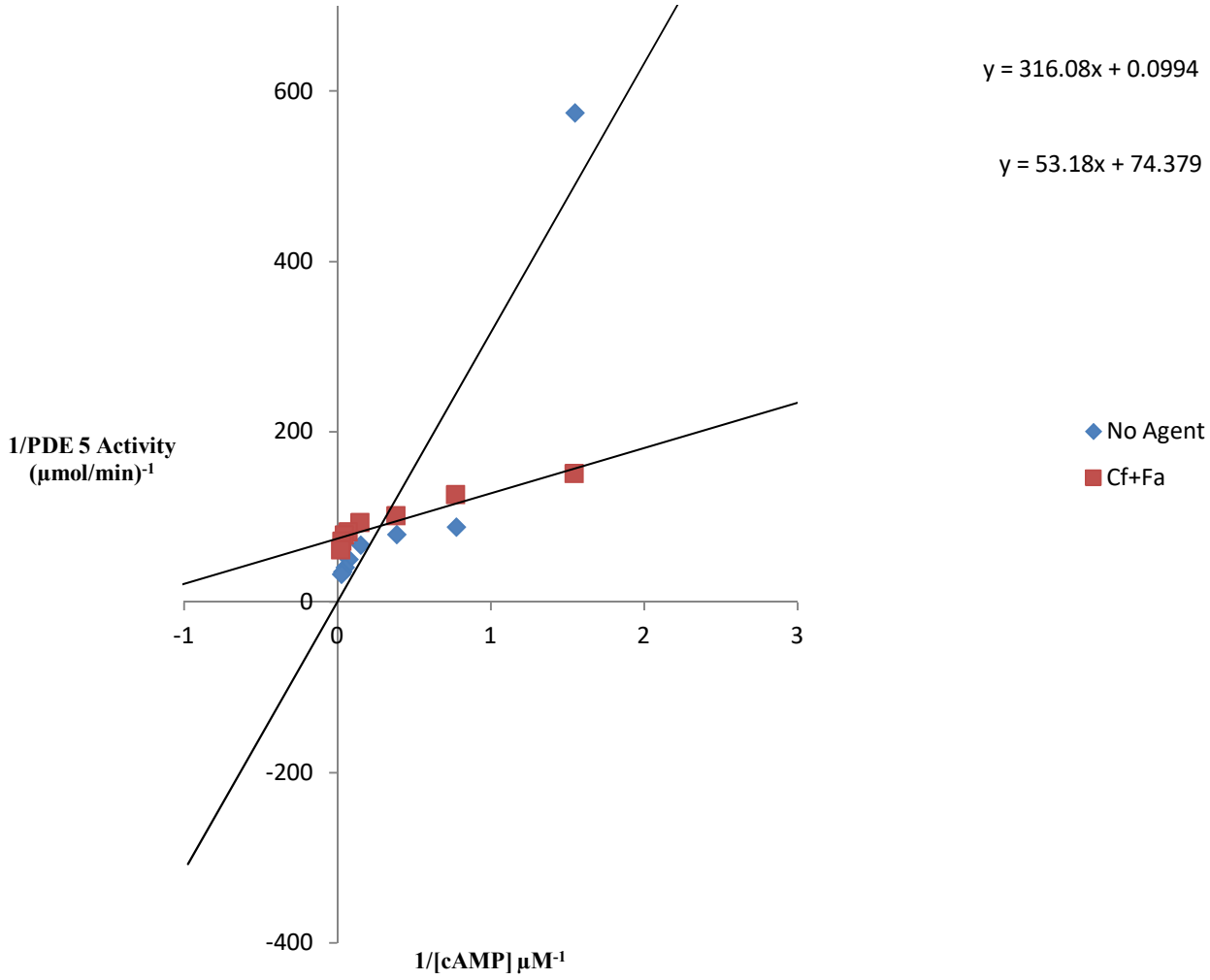


Figure 33: Lineweaver-Burk plot of the effects of combination of aqueous extracts of *C. ferruginea* root and *F. agrestis* stem on substrate kinetics of penile PDE 5-catalysed hydrolysis of cAMP

Cf+Fa= Combination of *C. ferruginea* and *F. agrestis*

Table 10: Effect of Some inhibitory agents on kinetic parameters of penile PDE 5-catalysed hydrolysis of cAMP

	V_{max} (µmol/min)	K_m (µM)
Absence of inhibitory agent	10.06	3179.70
Sildenafil Citrate	0.0043	4.50
C. ferruginea root extract (Cf)	0.018	20.21
F. agrestis stem extract (Fa)	0.023	76.56
Combination of extracts (Cf+Fa)	0.013	0.69

3.7 Effects of various extracts on substrate kinetics of cardiac PDE 5

Figure 34 shows the effects of aqueous extracts of *C. ferruginea, F. agrestis* and their combination on substrate kinetics of cardiac PDE 5 catalysed hydrolysis of cAMP. There was a sharp increase in the enzyme activity as the substrate increases, the curve in the absence of agents obeyed the Michaelis-Menten kinetics giving hyperbolic curve while in the presence of the agents, enzyme activities also increased as the substrate increased but the enzyme activities declined when compared with the curve without agents. There was a sharp increase in penile PDE 5 activities from 0.645-1.29 μM of cAMP after which increase in substrate concentration produced steady increase in enzyme activity. The effects of the individual extracts and their combination on the kinetic parameters (V_{max} and K_m) as obtained in Lineweaver-Burk plots are presented on Figures 35, 36, 37, 38 and actual values V_{max} and K_m are shown in Table 11. The agents inhibited cardiac PDE 5 in the presence of cAMP.

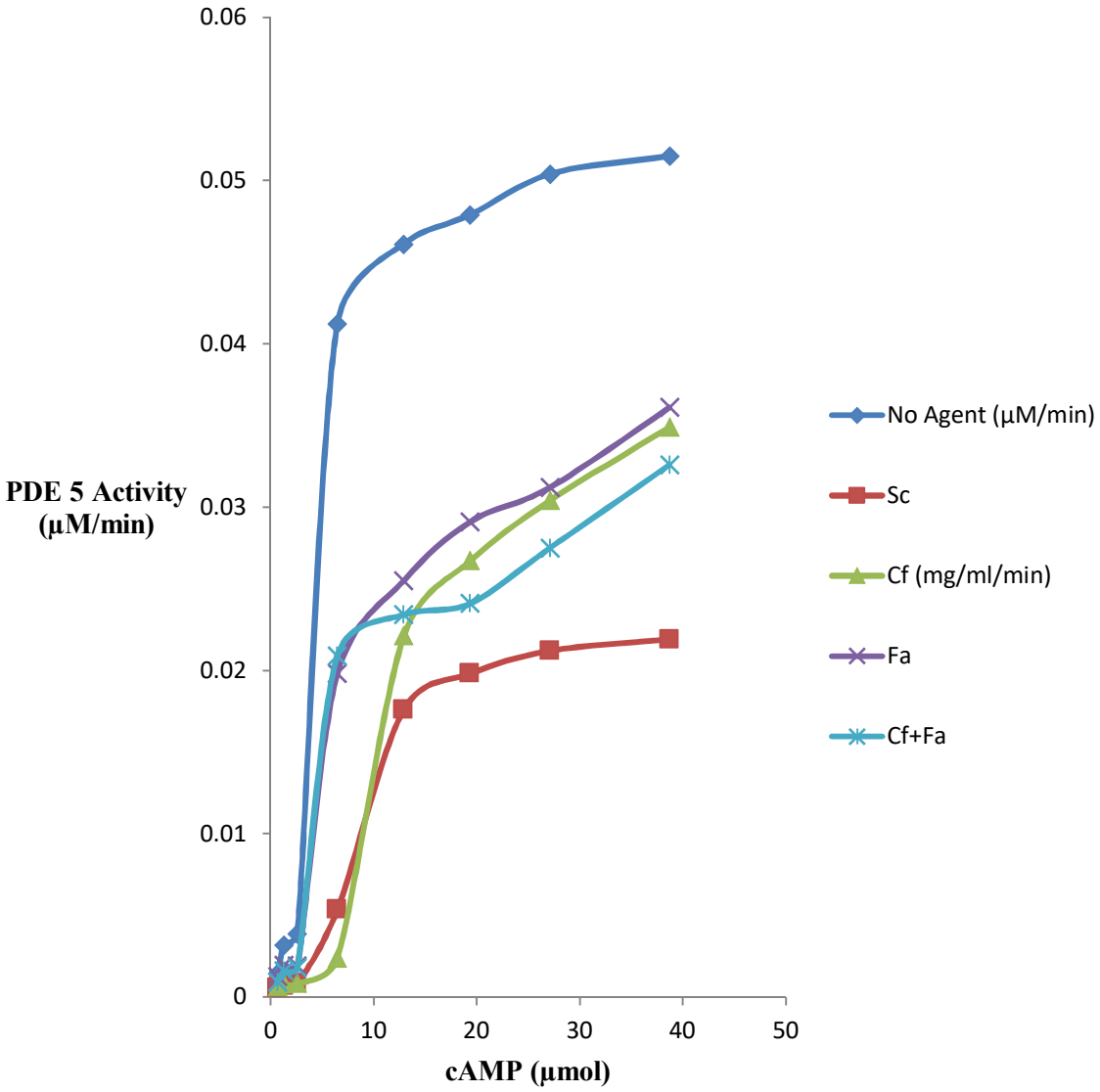


Figure 34: Effects of some androgenic agents on substrate kinetics of cardiac PDE 5-catalysed hydrolysis of cAMP

SC= Sildenafil Citrate

Cf= *Cnestis ferruginea*

Fa= *Fadogia agrestis*

Cf+Fa= Combination of *C. ferruginea* and *F. agrestis*

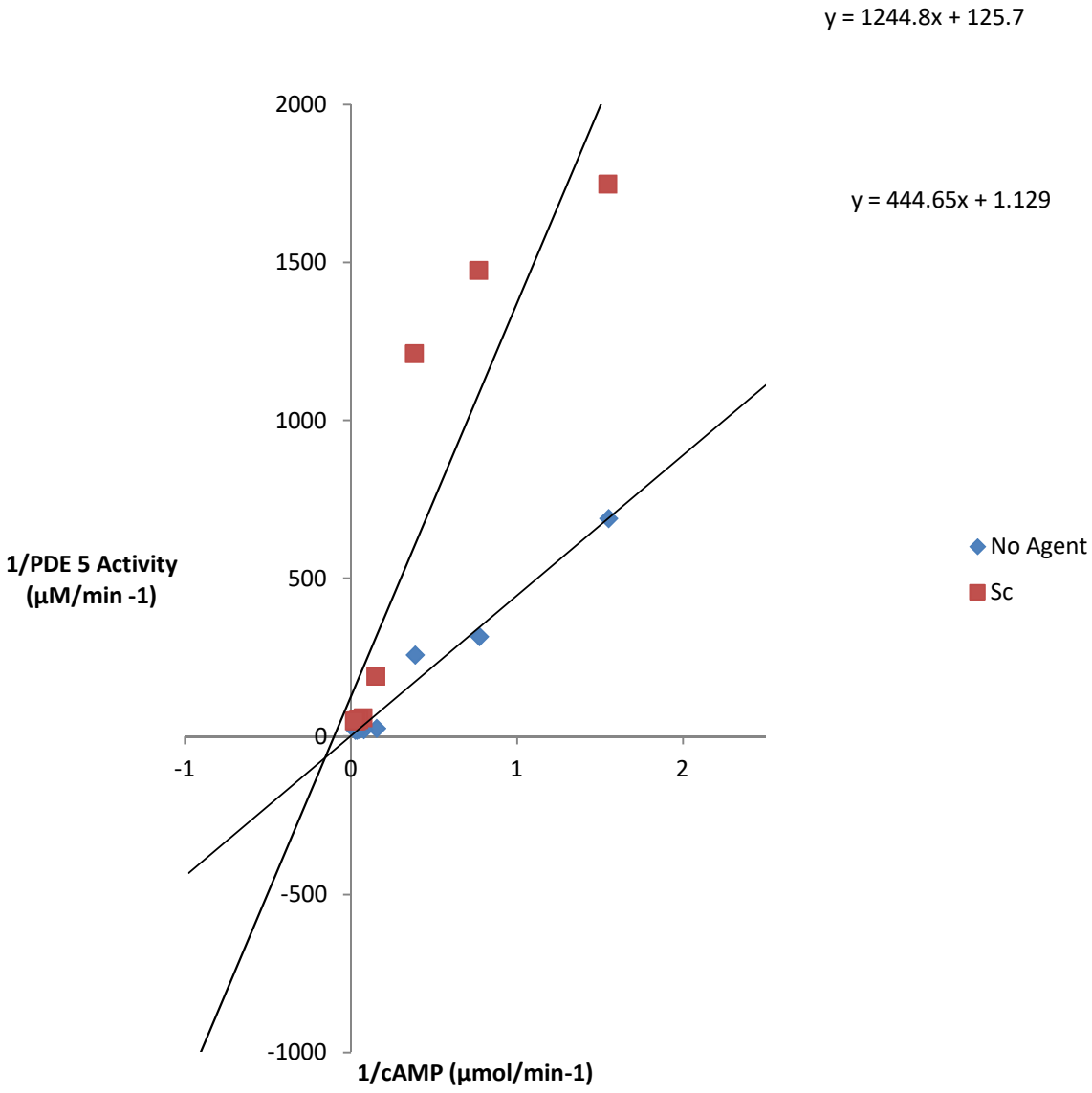


Figure 35: Lineweaver-Burk plot of the effects of sildenafil citrate on substrate kinetics of cardiac PDE 5-catalysed hydrolysis of cAMP

Sc= Sildenafil citrate

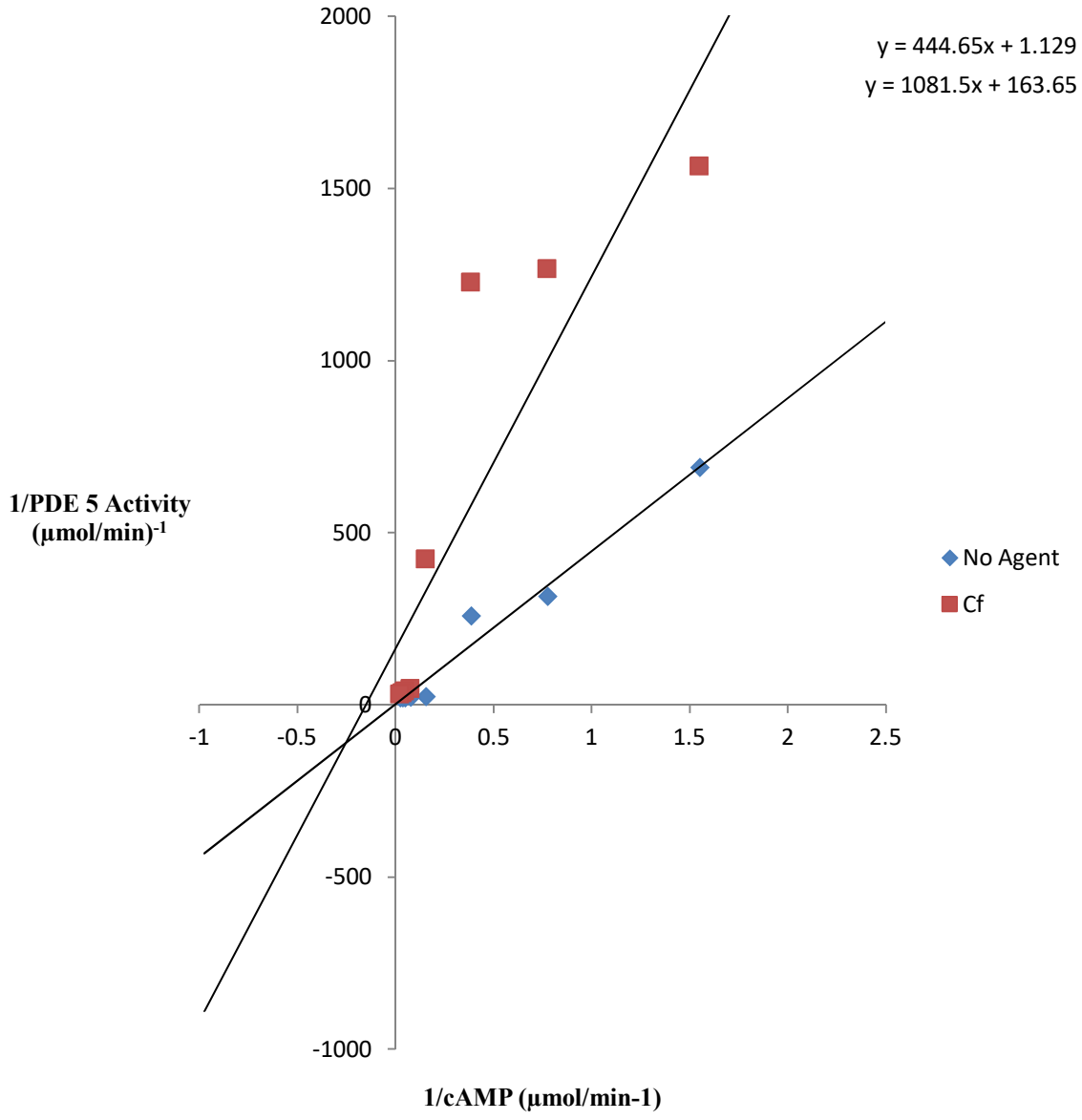


Figure 36: Lineweaver-Burk plot of the effects of aqueous extracts of *Cnestis ferruginea* on substrate kinetics of cardiac PDE 5-catalysed hydrolysis of cAMP

Cf= *Cnestis ferruginea*

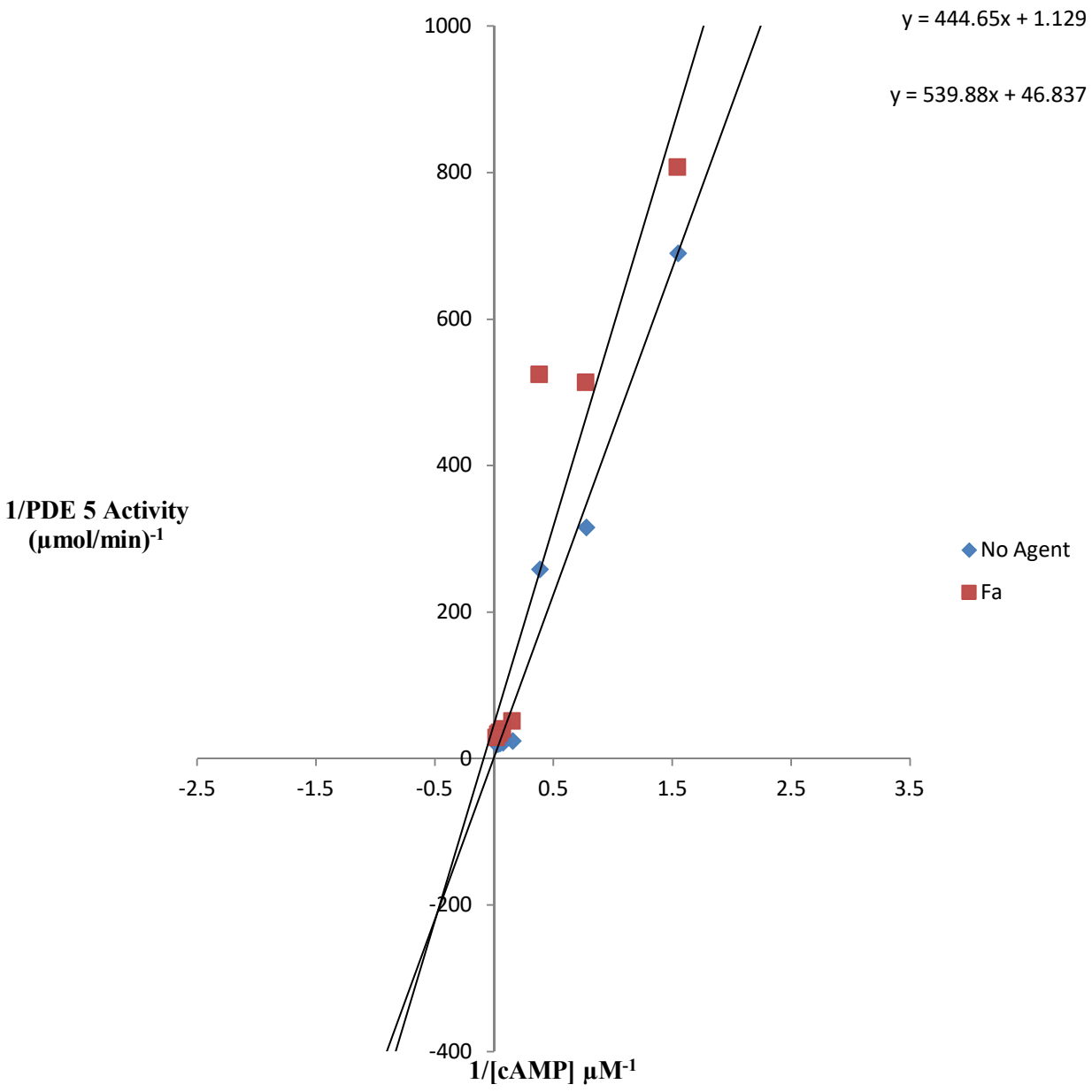


Figure 37: Lineweaver-Burk plot of the effects of aqueous extract of *Fadogia agrestis* stem on substrate kinetics of cardiac PDE 5-catalysed hydrolysis of cAMP

Fa= *Fadogia agrestis*

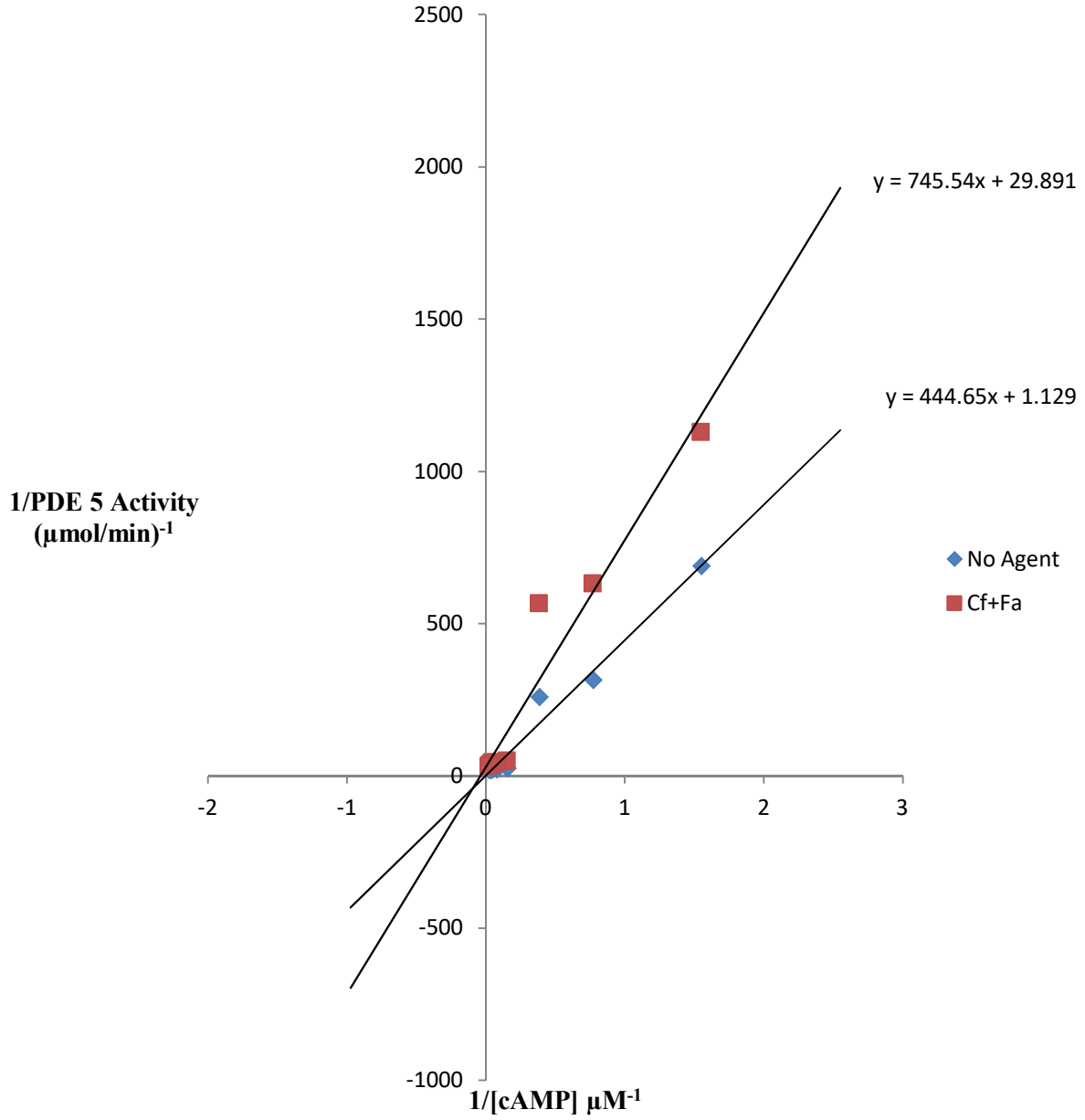


Figure 38: Lineweaver-Burk Plot of the Effects of Combination of Aqueous Extracts of *C. ferruginea* root and *F. agrestis* stem on Substrate Kinetics of Cardiac PDE 5-Catalysed Hydrolysis of cAMP

Cf+Fa= Combination of *C. ferruginea* and *F. agrestis*

Table 11: Effect of Some Agents on the Kinetic Parameters of Cardiac PDE 5-Catalysed Hydrolysis of cAMP

	V_{max} (µmol/min)	K_m (µM)
Absence of inhibitory agent	0.89	395.74
Sildenafil Citrate	0.008	9.96
C. ferruginea root extract (Cf)	0.006	6.49
F. agrestis stem extract (Fa)	0.021	11.34
Combination of extracts (Cf+Fa)	0.033	24.60

The penile PDE 5 activity in paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days is as shown in Figure 39. PDE 5 activities significantly ($p < 0.05$) increased in penile of rats treated with paroxetine (10 mg/Kg b. wt.) when compared to those administered normal saline (control). The enzyme activity in paroxetine-treated rats increased as the period of administration increased; activity of the enzyme after 21 days of administration was significantly ($p < 0.05$) higher than on days 4, 7 or 14. The increased activity of PDE 5 decreased significantly ($p < 0.05$) one week (day 28) after paroxetine was discontinued but was not significantly increased ($p < 0.05$) during the post-treatment week (day 35) after the administration of paroxetine was discontinued and the values determined were significantly ($p < 0.05$) different from those of day 21.

Activity of cardiac PDE 5 in paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days is shown on Figure 40. PDE 5 activities significantly ($p < 0.05$) increased in cardiac tissues of rats treated with paroxetine when compared with the control. The enzyme activity in paroxetine-treated rats at 21 days of administration was significantly ($p < 0.05$) higher than on day 4 (first plot on the curve). The elevated activity of cardiac PDE 5 persisted for two weeks after the administration of paroxetine was discontinued and the values determined on day 28 or 35 were not significantly ($p < 0.05$) different from those of day 21.

Figure 41 shows the activity of penile arginase in paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days. Arginase activities significantly ($p < 0.05$) increased in penile tissues of rats treated with paroxetine when compared with the control. The enzyme activity in paroxetine-treated rats was not significantly ($p < 0.05$) different on the various days monitored; although activity of the enzyme on day 7 was significantly ($p < 0.05$) higher than on day 4, penile arginase activities on days 14 and 21 was not significantly ($p < 0.05$) different from those of day 4. The increased activity of penile arginase persisted after the discontinuation of paroxetine.

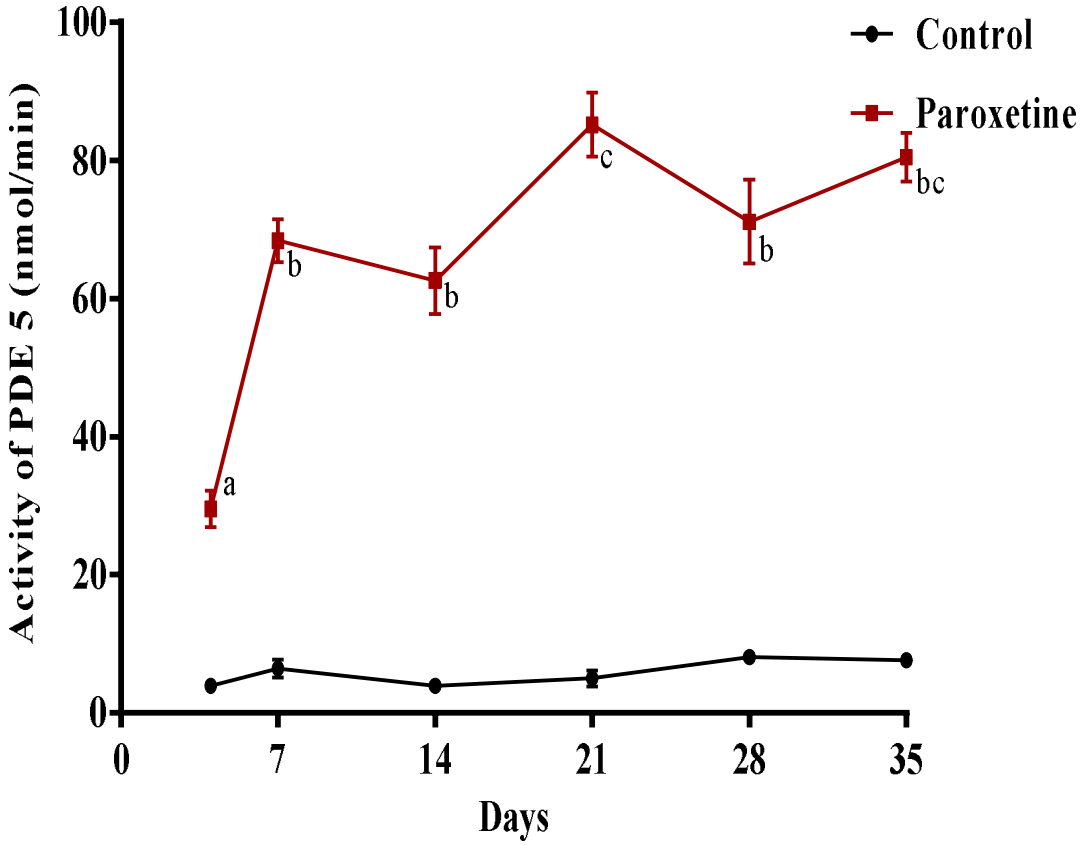


Figure 39: **Time course of penile phosphodiesterase 5 activities in paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days**

Values are mean of six determinations ± SEM; plotted values with different superscript are significantly ($p < 0.05$) different from each other.

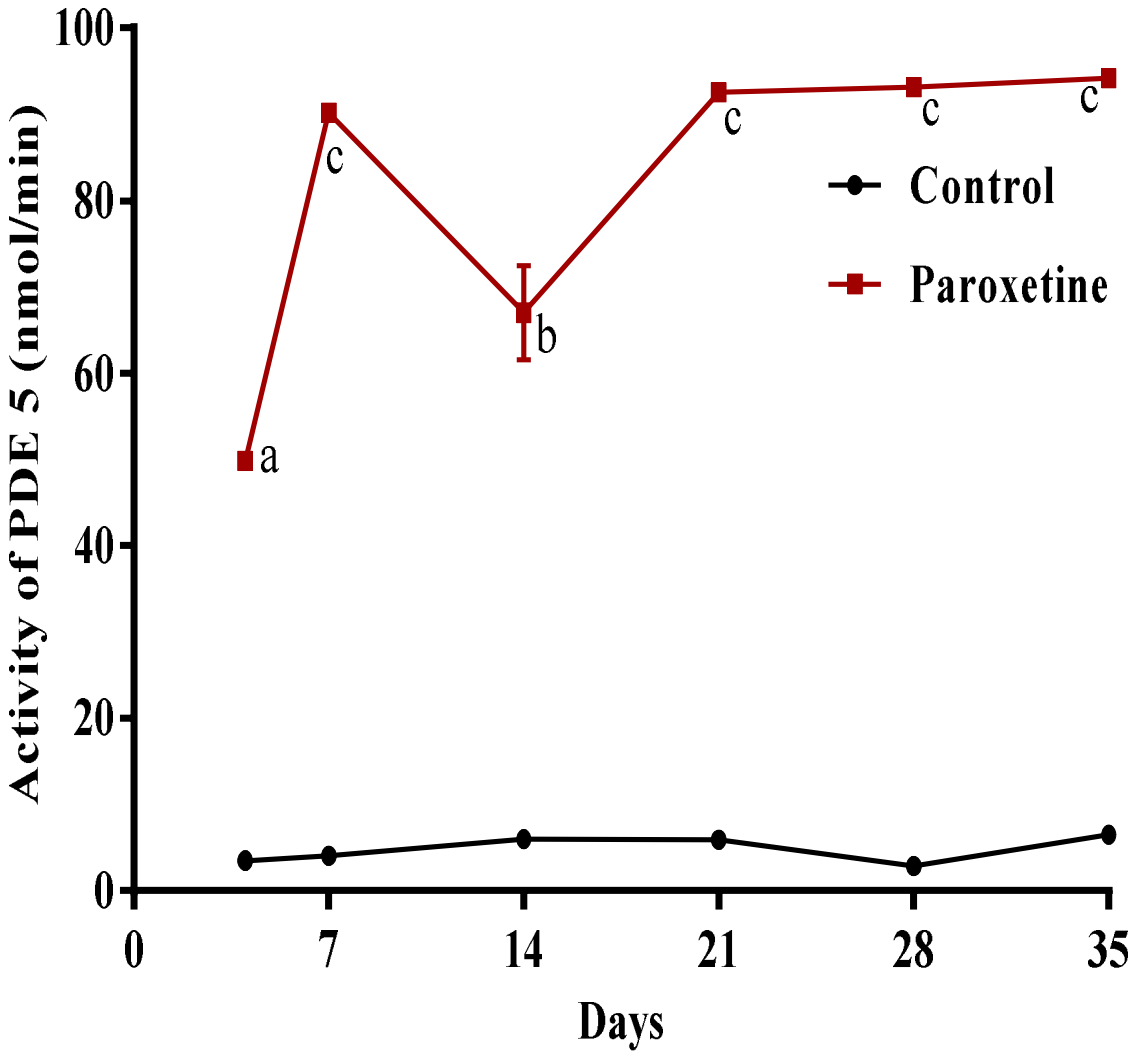


Figure 40: Time course of cardiac phosphodiesterase 5 activities in paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days

Values are mean of six determinations ± SEM; plotted values with different superscript are significantly ($p < 0.05$) different from each other.

Results for the activity of penile arginase seven days after withdrawal of paroxetine treatment (day 28) was significantly ($p < 0.05$) different from other days of treatment (days 4, 7, 14, 21 and 35) while that of 14 days after paroxetine discontinuation (day 35) were significantly ($p < 0.05$) lower than the penile arginase activity observed on days 7 and 28 but not significantly ($p \geq 0.05$) different from enzyme activity of days 4, 14, and 21 (Figure 41). Generally, there was a significant ($p < 0.05$) difference between the paroxetine-treated group and the control.

Arginase activity of heart of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days is shown in Figure 42. Arginase activities significantly ($p < 0.05$) increased in heart of rats treated with paroxetine when compared to the control. In contrast to arginase activities in the penile (Figure 41), the enzyme activity in heart of paroxetine-treated rats significantly ($p < 0.05$) increased as days increased. The elevated activity of cardiac arginase persisted for weeks after the administration of paroxetine was stopped, although post-treatment plotted values determined after 14 days were significantly ($p < 0.05$) lower than plotted values noted during treatment period on day 21.

Concentration of nitric oxide in the penile and heart of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days are shown on Figures 43 and 44 respectively. Concentration of nitric oxide significantly ($p < 0.05$) reduced in both tissues following administration of paroxetine to rats for more than 7 days, when compared to control. Concentration of nitric oxide estimated in the penile decreased as days of treatment increased; nitric oxide level observed on day 21 was significantly ($p < 0.05$) lower than those determined on day 4, 7 and 14 (Figure 43). Similarly, cardiac nitric oxide concentration following administration of paroxetine to rats was also time dependent; concentration on days 14 and 21 was significantly ($p < 0.05$) lower than that of days 4 and 7 (Figure 44). The reduced concentration was partly restored in both tissues after the administration of paroxetine was discontinued for a week (day 28) and penile nitric oxide concentration on day 35 was observed to be significantly ($p < 0.05$) different from every other day except day 28.

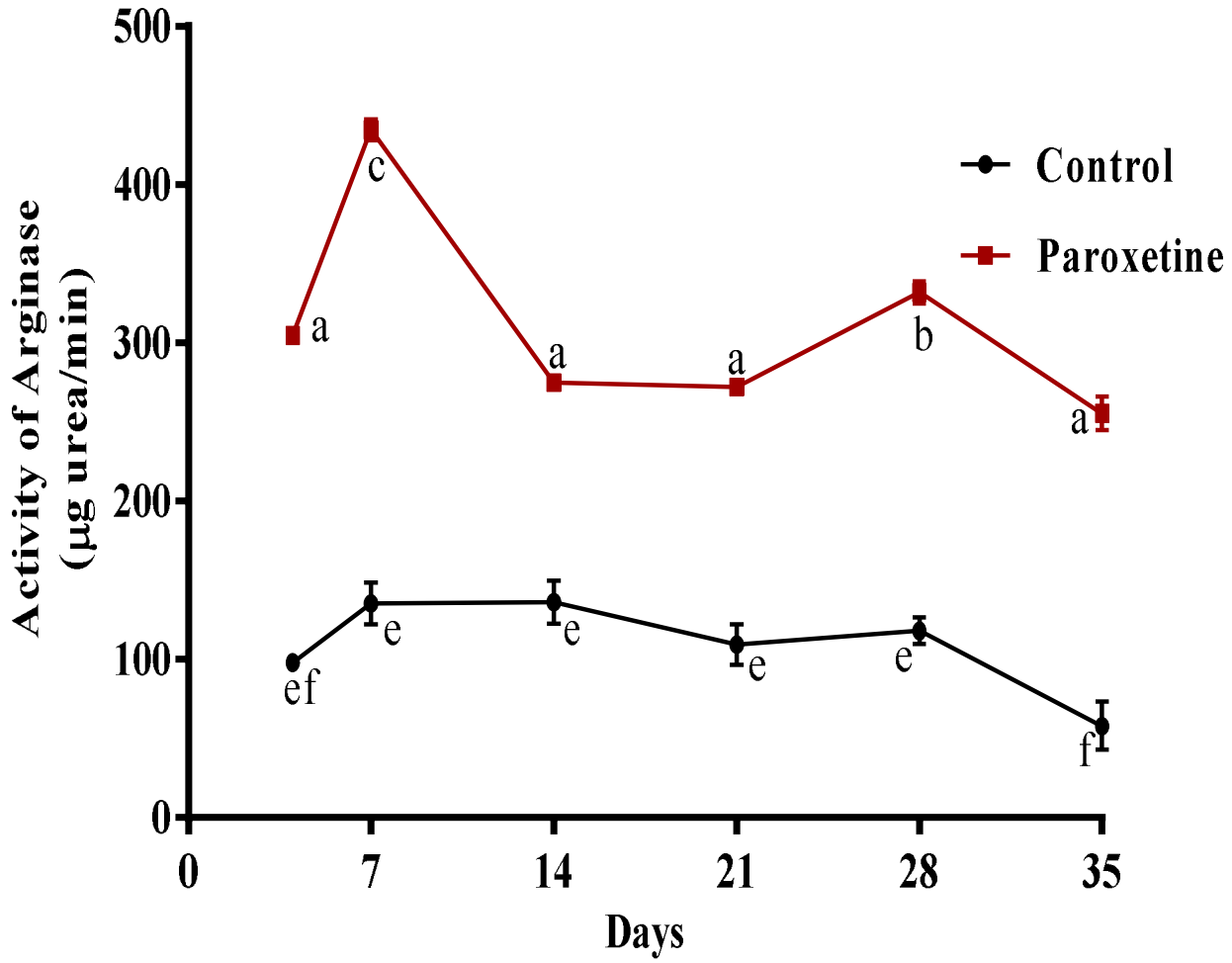


Figure 41: **Time course of penile arginase activities in paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days**

Values are mean of six determinations ± SEM; plotted values with different superscript are significantly ($p < 0.05$) different from each other.

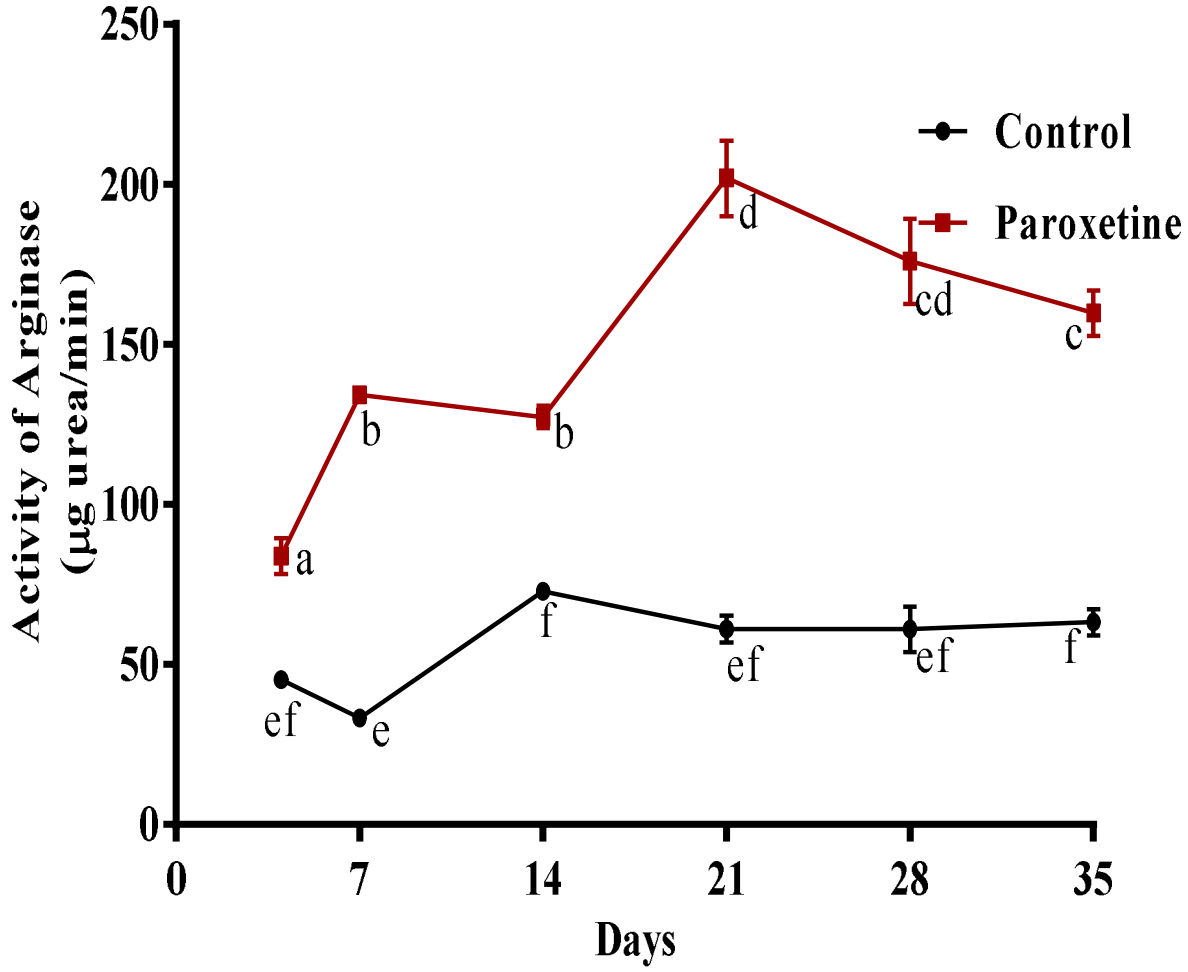


Figure 42: **Time course of cardiac arginase activities in paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days.**

Values are mean of six determinations ± SEM; plotted values on the graph with different superscript are significantly ($p < 0.05$) different from each other.

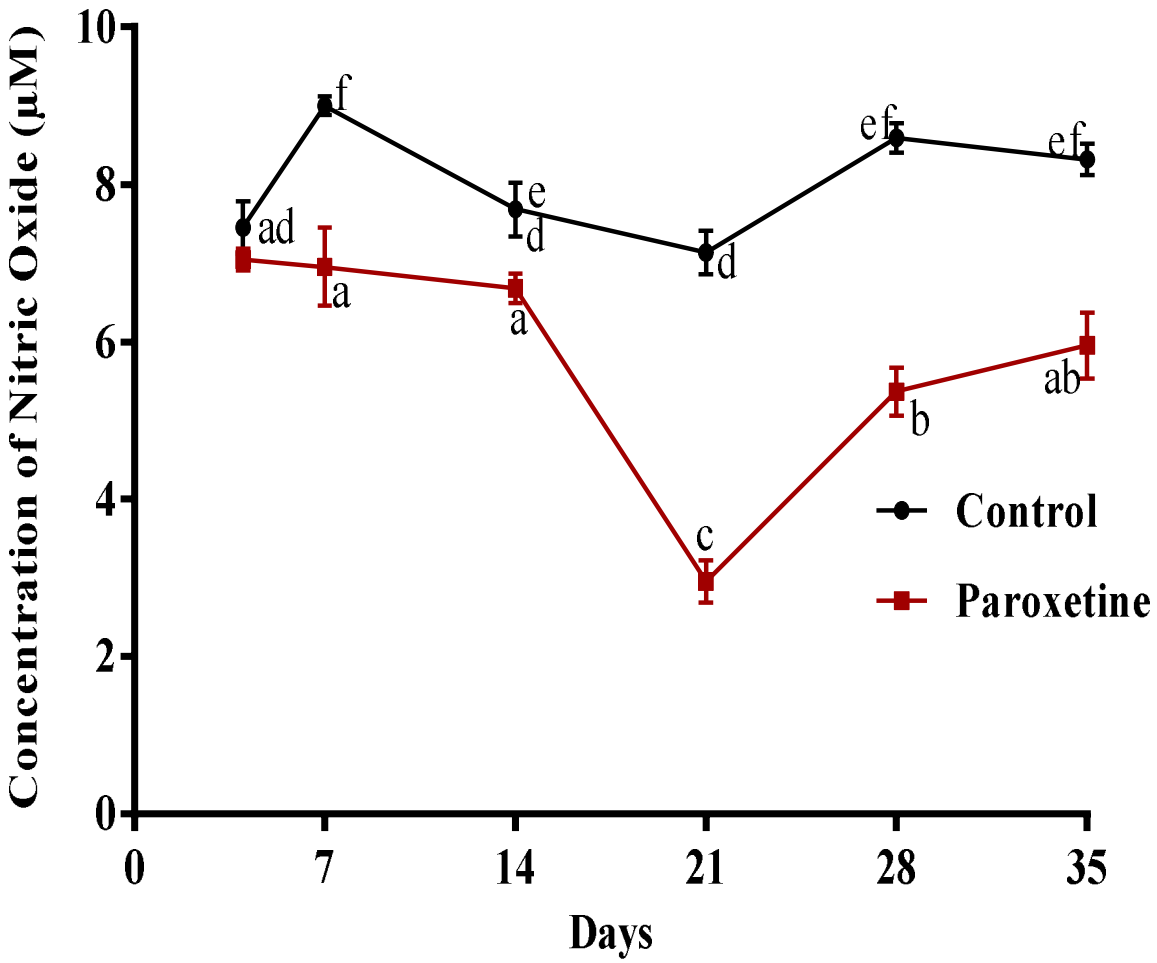


Figure 43: **Time course of concentration of penile nitric oxide of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days**

Values are mean of six determinations ± SEM; plotted values with different superscript are significantly ($p < 0.05$) different from each other.

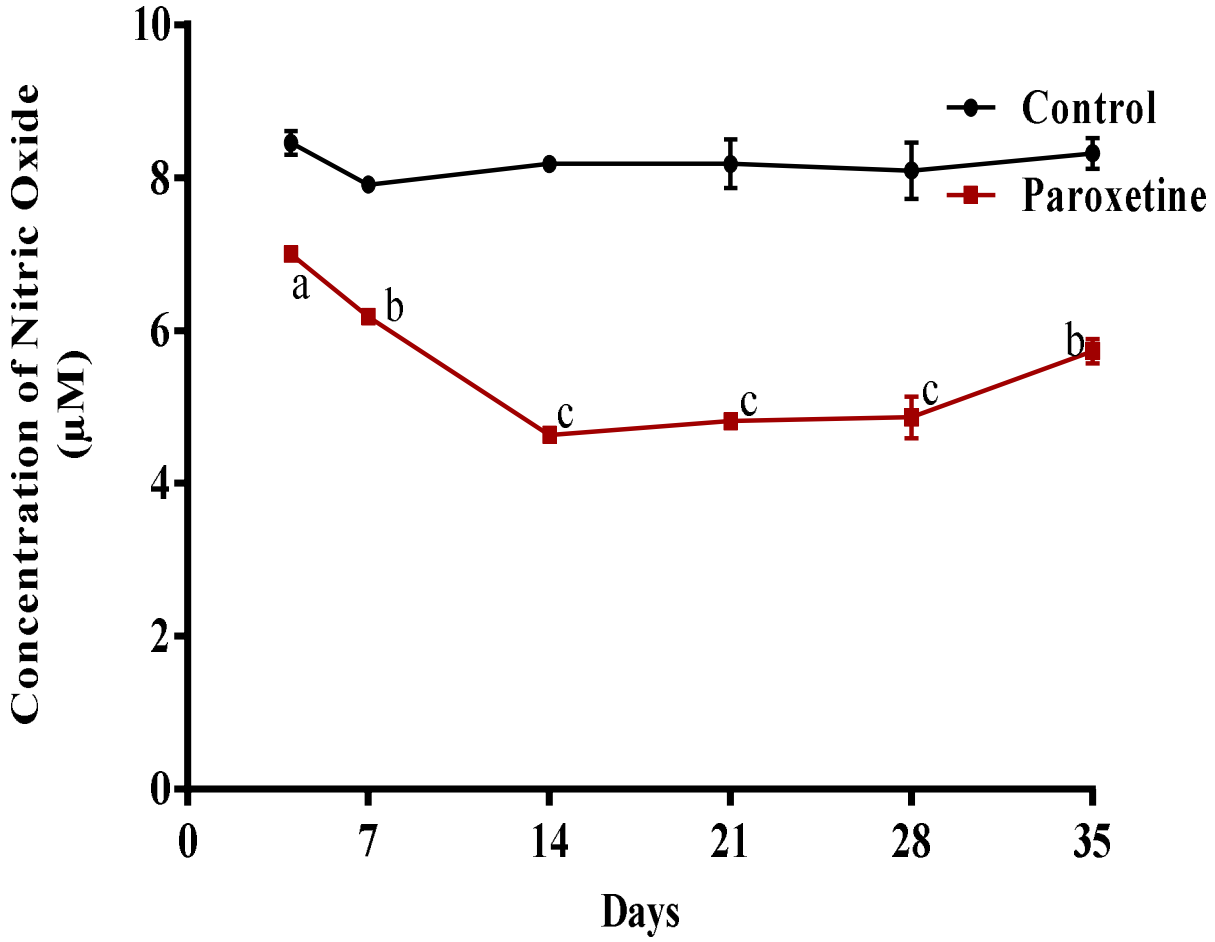


Figure 44: **Time course of concentration of cardiac nitric oxide of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days**

Values are mean of six determinations ± SEM; point values with different superscript are significantly ($p < 0.05$) different from each other.

Figure 45 shows concentration of plasma cGMP of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days. Concentration of cGMP significantly ($p < 0.05$) reduced following administration of paroxetine to rats when compared to the control. cGMP concentration in plasma was not significantly ($p \geq 0.05$) different as days of treatment with paroxetine increased. The reduced concentration of plasma cGMP persisted even after paroxetine had been discontinued for two weeks (day 21 - 35).

Concentration of endothelin-1 in the plasma of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days is shown on Figure 46. In contrast to concentration of plasma cGMP, concentration of plasma endothelin-1 significantly ($p < 0.05$) increased in rats treated with paroxetine when compared to the control. However, as days of treatment with paroxetine increased, plasma endothelin-1 significantly ($p < 0.05$) decreased. Conversely, endothelin-1 concentration was significantly ($p < 0.05$) increased for two weeks after the administration of paroxetine was discontinued (days 28 and 35).

Figure 47 shows concentration of plasma testosterone of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days. Concentration of testosterone significantly ($p < 0.05$) reduced following administration of paroxetine to rats, when compared to control. Plasma concentration of testosterone decreased as the days increased, plotted values determined after 21 days of administration of paroxetine to rats was significantly ($p < 0.05$) lower than on day 4 (first plot on the curve). The reduction in concentration of plasma testosterone persisted for two weeks (days 21 - 35) after the administration of paroxetine was discontinued.

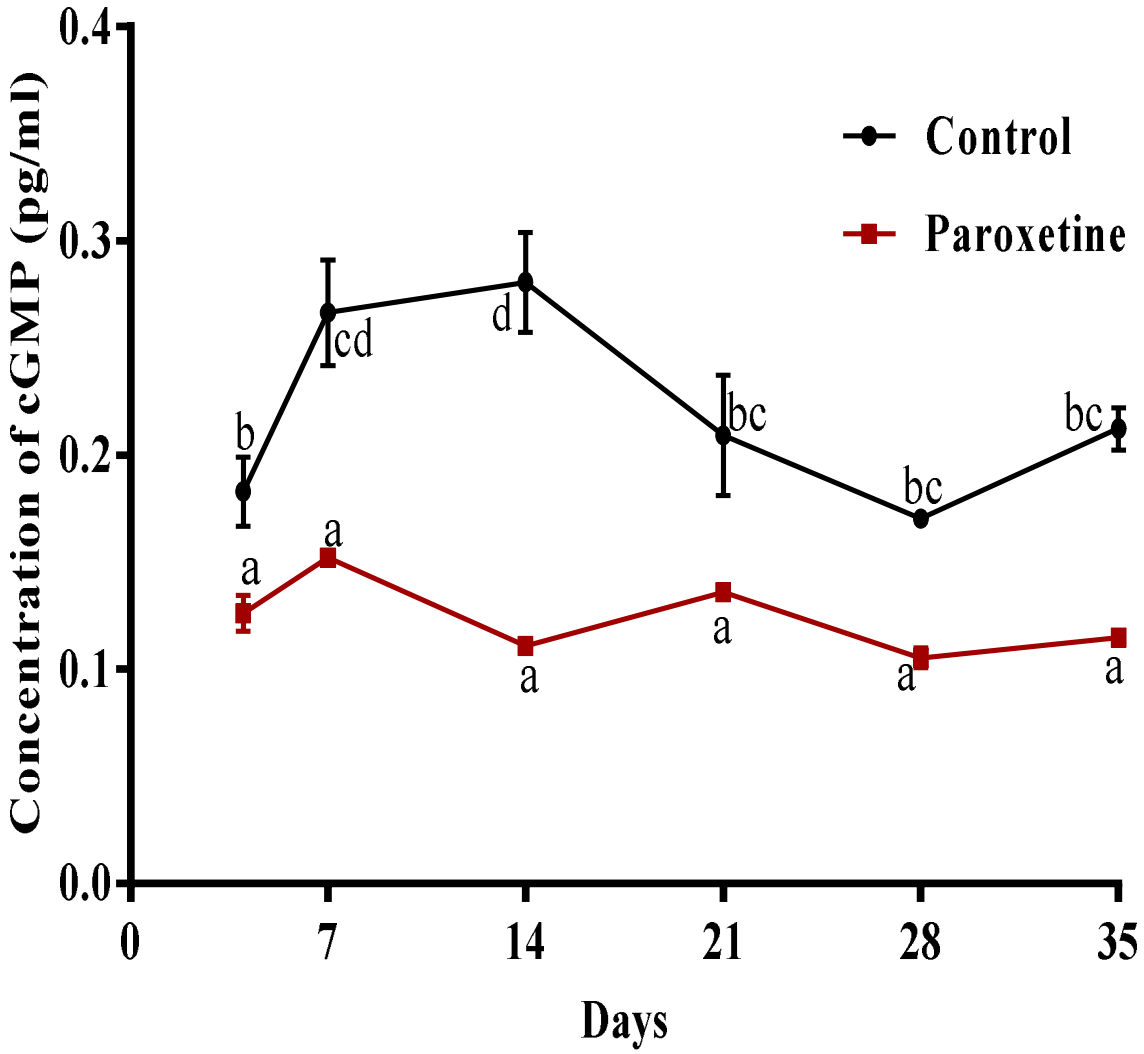


Figure 45: Time course of concentration of cyclic guanosine monophosphate (cGMP) in plasma of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days

Values are mean of six determinations ± SEM; plotted values with different superscript are significantly ($p < 0.05$) different from each other.

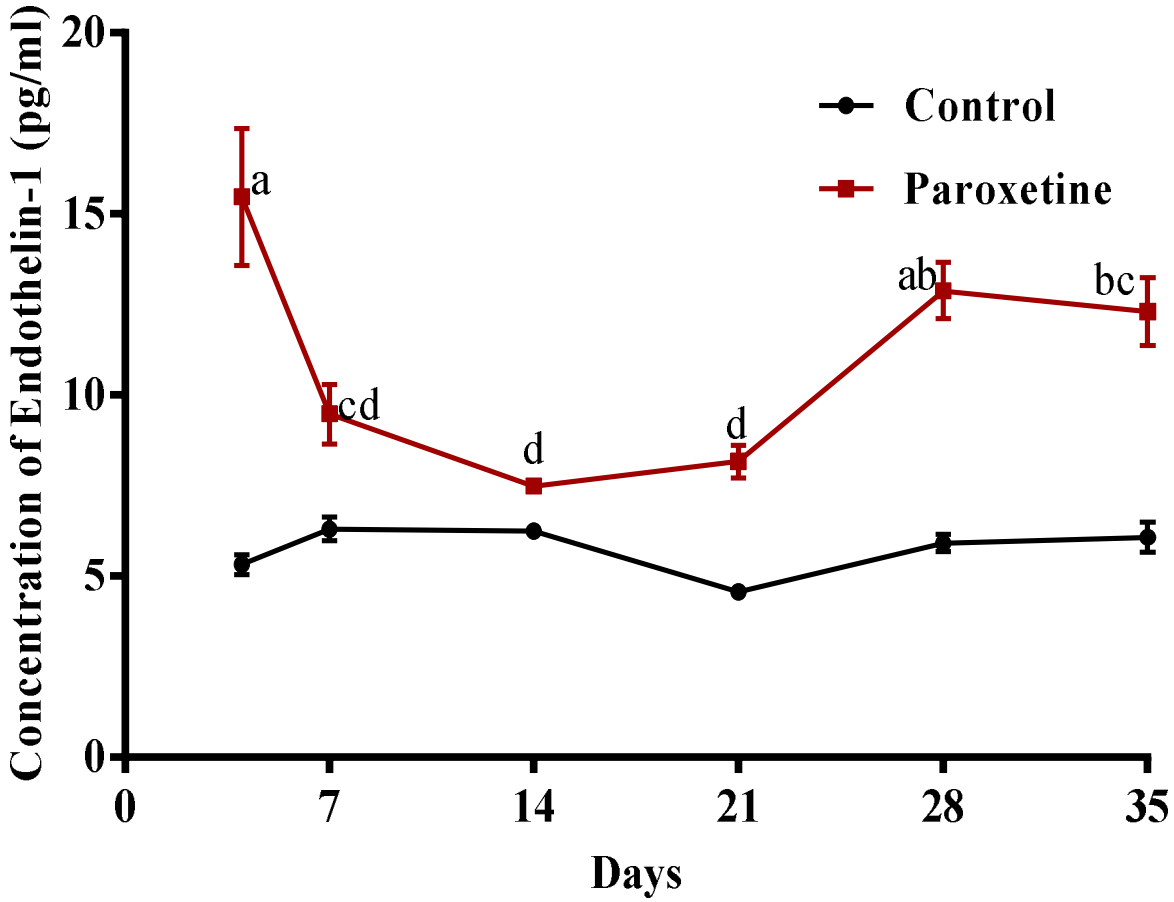


Figure 46: **Time course of concentration of endothelin-1 in the plasma of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days**

Values are mean of six determinations ± SEM; plotted values with different superscript are significantly ($p < 0.05$) different from each other.

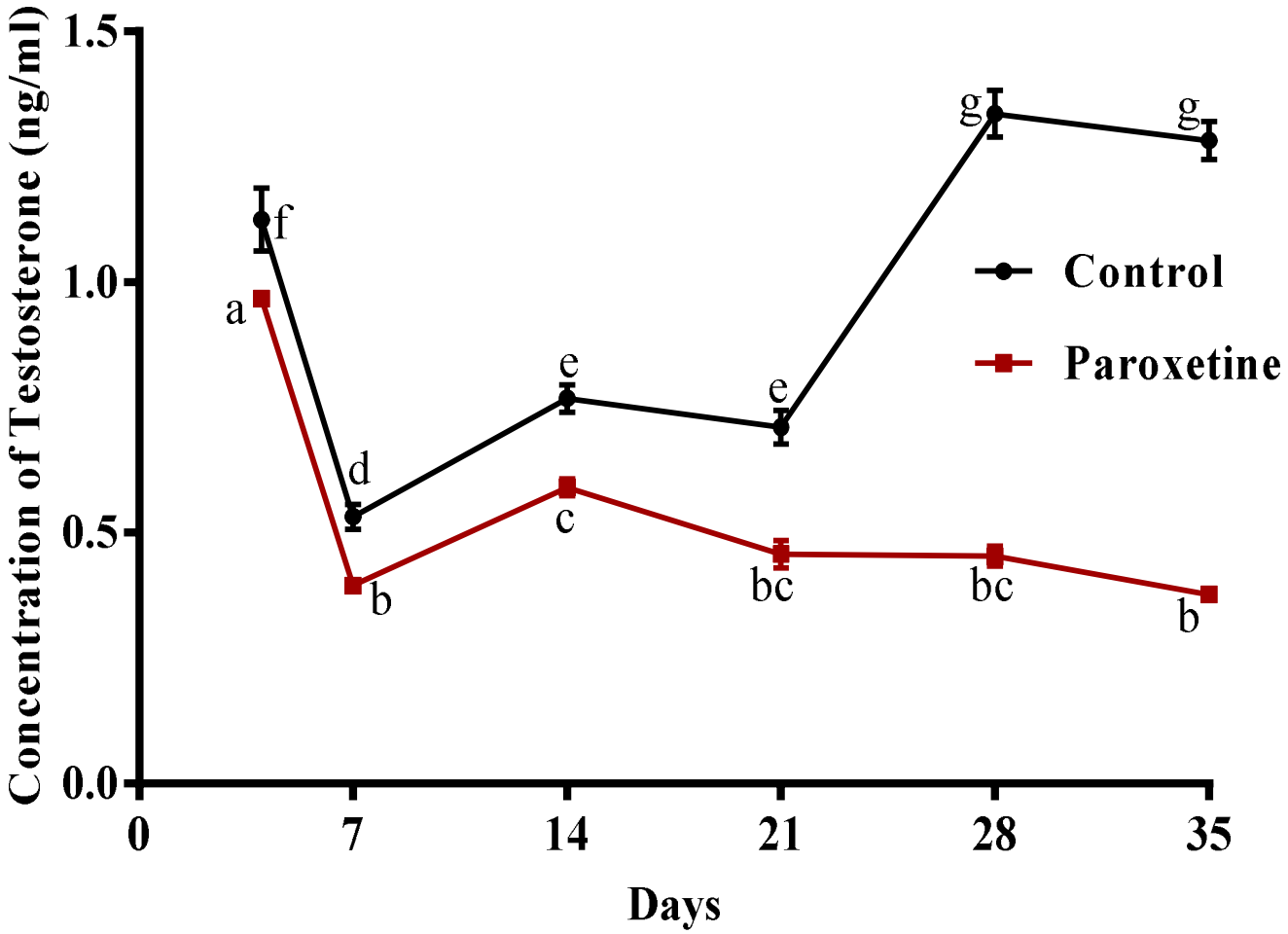


Figure 47: **Time course of concentration of testosterone in the plasma of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days**

Values are mean of six determinations ± SEM; plotted values with different superscript are significantly ($p < 0.05$) different from each other.

Activity of creatine kinase in the plasma of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days is shown in Figure 48. Plasma creatine kinase activity significantly ($p < 0.05$) increased in rats treated with paroxetine when compared to the control. Plasma creatine kinase activity was significantly ($p < 0.05$) different over paroxetine treatment period of 21 days. The increased activity of creatine kinase persisted for two weeks after the administration of paroxetine was discontinued, although post-treatment values (on days 28 and 35) were significantly ($p < 0.05$) lower than paroxetine-treatment values on day 14 (Figure 46; Red Line).

Concentrations of cholesterol and triglycerides in plasma of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days is shown in Table 12. Concentration of cholesterol and triglycerides in rats administered normal saline (control) were not significantly ($p \geq 0.05$) different from each other over the experimental period of 35 days. Administration of paroxetine to rats for ≥ 7 days resulted in significant ($p < 0.05$) increased in concentration of plasma cholesterol and triglycerides, when compared to the control. Concentration of the lipids observed in the plasma increased as days of treatment increased; levels determined on day 21 were significantly ($p < 0.05$) higher than those determined on days 4 and 7 (Table 12). The elevated concentration of cholesterol and triglycerides persisted after the administration of paroxetine was discontinued for two weeks (days 28 and 35), although values obtained for cholesterol were significantly ($p < 0.05$) lower than those of treatment values on day 21 but higher than values on day 4, 7 and 14 (Table 12).

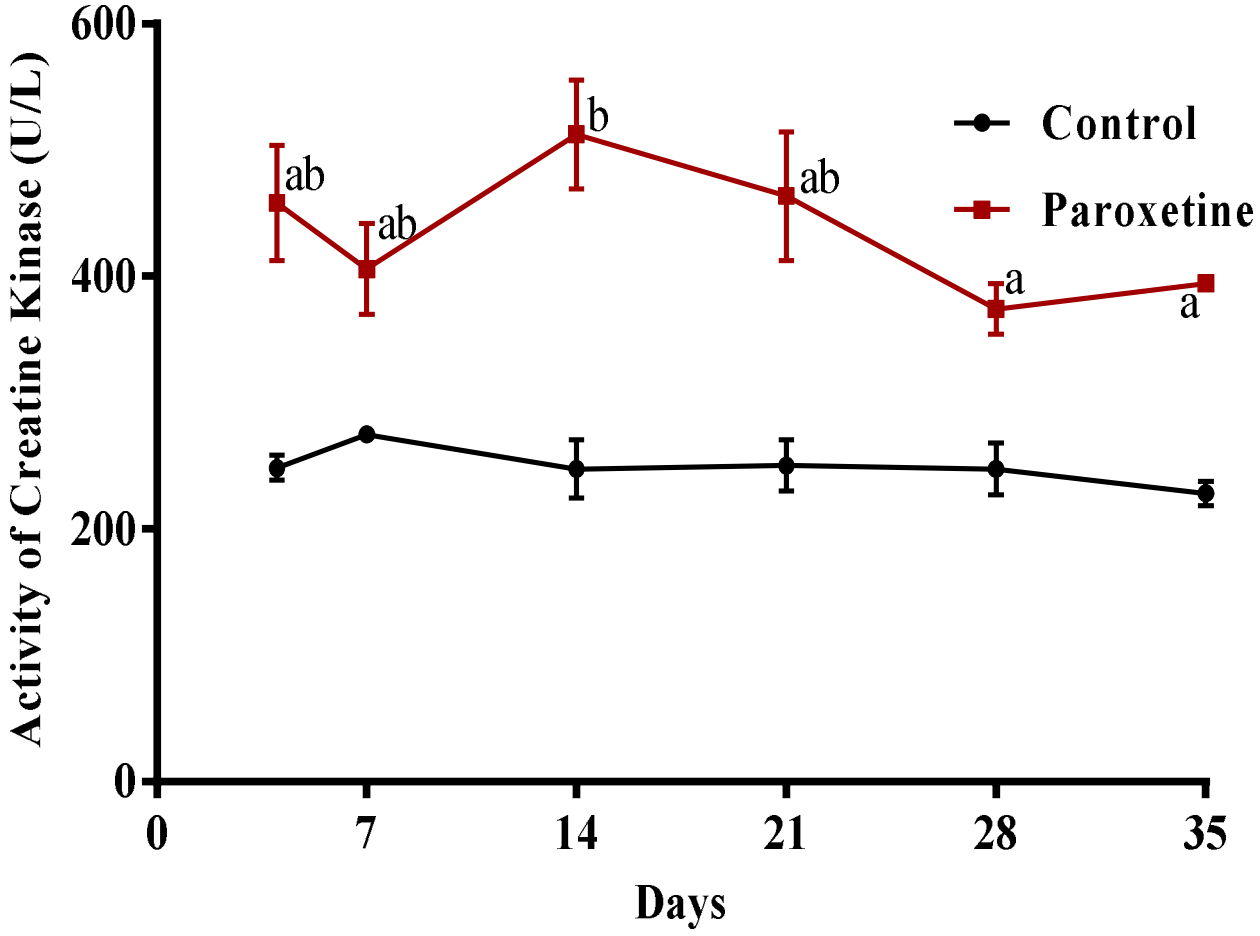


Figure 48: Time course of creatine kinase activities in plasma of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days

Values are mean of six determinations ± SEM; plotted values with different superscript are significantly ($p < 0.05$) different from each other.

Table 12: Time course of concentrations of cholesterol and triglycerides in the plasma of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days

	Cholesterol (mg/dl)		Triglycerides (mg/dl)	
Days	Control	Paroxetine	Control	Paroxetine
4	23.80 ± 0.74^{a}	36.67 ± 1.74^{a}	24.37 ± 0.23^{a}	30.74 ± 1.20^{ab}
7	24.80 ± 0.26^{a}	48.39 ± 3.30^{b}	22.14 ± 1.34^{a}	36.94 ± 3.26^{bc}
14	23.60 ± 1.82^{a}	59.11 ± 5.90^{b}	26.43 ± 1.61^{a}	51.65 ± 1.30^{de}
21	25.25 ± 1.39^{a}	181.55 ± 9.72^{d}	23.58 ± 0.04^{a}	58.61 ± 5.49^{e}
28*	22.94 ± 1.53^{a}	83.13 ± 7.15^{c}	18.78 ± 0.29^{a}	42.78 ± 3.50^{cd}
35**	23.46 ± 0.35^{a}	85.06 ± 0.17^{c}	25.52 ± 0.05^{a}	49.68 ± 2.63^{de}

Values are mean of six determinations ± SEM; values along a column with different superscript are significantly ($p < 0.05$) different from each other; *= 7 and ** =14 days after paroxetine was discontinued.

Table 9 shows the concentration of lipoprotein-cholesterol concentrations in plasma of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days. Although calculated values were between 7 - 10 mg/dl, fluctuations were recorded in the level of HDL-cholesterol over the experimental period in rats administered normal saline (control). Similarly, administration of paroxetine over a period of 21 days did not significantly ($p > 0.05$) alter the concentration of HDL-cholesterol. However, concentration of LDL-cholesterol was significantly ($p < 0.05$) higher in rats treated with paroxetine, when compared with the control. The LDL-cholesterol concentration progressively and significantly ($p < 0.05$) increased as days of treatment with paroxetine increased. Withdrawal of the paroxetine treatment for two weeks partly reduced the increased concentration of LDL-cholesterol, values obtained were significantly ($p < 0.05$) lower than those of paroxetine - treatment values on day 21 but higher than values obtained on day 4, 7 and 14 (Table 13).

Time course of cardiac and artherogenic indices of paroxentine-treated rats are shown in Table 14. Similar trend in relation to LDL-cholesterol were observed. There was progressive and significant ($p < 0.05$) increase in the level of paroxetine-treated rats over a period of 21 days and discontinuation of drugs partly reduced the predisposition of the rats to arherogenic and cardiac risks (Table 14).

Table 15 shows correlation analyses between erectile, endothelial and cardiovascular functional markers evaluated in paroxetine-treated rats. Activity of penile PDE 5 in paroxetine-treated rats indirectly and strongly correlated (-0.9333) to the concentration of plasma testosterone and moderately correlated (-0.5909) to plasma endothelin-1 as well as to the amount of nitric oxide in the penile (-0.6562), whereas the enzyme activities in the cardiac and penile tissues are directly and strongly correlated (0.9211).

Table 13: Time course of lipoprotein-cholesterol profiles in the plasma of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days

	HDL-cholesterol (mg/dl)		LDL-cholesterol (mg/dl)	
Days	Control	Paroxetine	Control	Paroxetine
4	9.58 ± 0.16^{b}	8.51 ± 0.23^{ab}	4.75 ± 0.74^{a}	19.53 ± 1.74^{bc}
7	8.59 ± 0.42^{b}	9.42 ± 0.41^{ab}	7.21 ± 0.26^{a}	29.90 ± 3.30^{cd}
14	7.51 ± 0.27^{a}	7.69 ± 0.15^{a}	8.41 ± 1.82^{a}	43.83 ± 5.90^{d}
21	8.45 ± 0.54^{ab}	8.10 ± 0.69^{a}	8.03 ± 1.34^{a}	165.84 ± 9.71^{f}
28*	7.44 ± 0.37^{a}	8.07 ± 0.46^{a}	8.22 ± 1.53^{a}	66.84 ± 7.15^{e}
35**	8.69 ± 0.41^{ab}	10.10 ± 0.68^{b}	15.32 ± 0.34^{b}	65.26 ± 0.17^{e}

Values are mean of six determinations ± SEM; values along a column with different superscript are significantly ($p < 0.05$) different from each other; * =7 and ** =14 days after paroxetine was discontinued.

Table 14: Time course of cardiac and artherogenic indices of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days

	Cardiac index		Artherogenic index	
Days	Control	Paroxetine	Control	Paroxetine
4	2.49 ± 0.04^{a}	4.31 ± 0.08^{a}	0.48 ± 0.06^{a}	2.29 ± 0.11^{a}
7	2.91 ± 0.12^{ab}	5.13 ± 0.25^{a}	0.91 ± 0.07^{ab}	3.13 ± 0.26^{a}
14	3.13 ± 0.13^{b}	7.68 ± 0.57^{b}	1.10 ± 0.17^{b}	5.65 ± 0.60^{b}
21	2.99 ± 0.03^{b}	22.61 ± 0.78^{c}	0.98 ± 0.04^{b}	20.60 ± 0.70^{c}
28*	3.08 ± 0.09^{b}	9.85 ± 0.34^{b}	1.09 ± 0.12^{b}	7.88 ± 0.09^{b}
35**	3.76 ± 0.14^{c}	8.54 ± 0.56^{b}	1.75 ± 0.09^{c}	6.53 ± 0.49^{b}

Values are mean of six determinations ± SEM; values along a column with different superscript are significantly different from each other; * =7 and ** =14 days after paroxetine was discontinued.

Table 15: Correlation between erectile, endothelial and cardiac functions of paroxentine-treated rats

	Penile PDE 5	Penile Arginase	Penile NO	cGMP	Testos-terone	Endo thelin-1	Cardiac PDE 5	Cardiac Arginase	Cardiac NO	Creatine Kinase	Choles-terol	Trigly-cerides	HDL-c	LDL-c	CI	AI
Penile PDE5																
Penile Arginase	-0.1222															
Penile NO	**-0.6562**	0.3841														
cGMP	0.0202	**0.6208**	-0.0275													
Testos-terone	**-0.9333**	-0.1642	0.3816	-0.0877												
Endo thelin-1	**-0.5909**	-0.0122	**0.3416**	**-0.2509**	**0.5268**											
Cardiac PDE 5	**0.9211**	0.1504	-0.5370	0.1036	-0.9501	-0.3392										
Cardiac Arginase	0.9120	**-0.1977**	-0.8675	-0.0648	-0.7668	-0.4347	**0.8590**									
Cardiac NO	-0.6849	0.3455	**0.5723**	0.4304	0.5509	**0.6726**	-0.4747	**-0.720**6								
Creatine Kinase	-0.2963	-0.3911	0.0055	0.0118	0.4757	-0.4626	-0.6268	-0.3087	-0.1738							
Choles-terol	0.7001	-0.4106	-0.9874	0.0870	-0.4214	-0.4302	**0.5453**	**0.8591**	-0.5698	0.0602						
Trigly-cerides	0.7867	-0.5881	-0.7351	-0.2034	-0.5521	-0.7032	0.4961	**0.7620**	-0.8235	0.2837	**0.7972**					
HDL-c	0.1948	0.1975	0.2664	0.3090	-0.3532	0.2737	0.3603	-0.0535	**0.5119**	**-0.5809**	-0.2005	-0.2108				
LDL-c	0.6896	-0.4135	-0.9887	0.0781	-0.4079	-0.4384	**0.5300**	**0.8546**	-0.5827	0.0800	**0.9995**	**0.7994**	-0.2310			
CI	0.6447	-0.4062	-0.9844	0.0833	-0.3554	-0.4651	0.4716	**0.8246**	-0.5973	0.1498	**0.9922**	**0.7917**	-0.3139	**0.9955**		
AI	0.6452	-0.4051	-0.9848	0.0826	-0.3564	-0.4640	0.4731	**0.8258**	-0.5979	0.1474	**0.9922**	**0.7911**	-0.3141	**0.9955**	1.**0000**	

Values were derived from 36 (6 periods x 6 rats) determinations; - sign = indirect (negative) correlation; 0.7500 – 1.000 = strong correlation; 0.5000 - 0.7499 = moderate correlation; 0.1000 – 0.4999 = weak correlation; PDE 5 = Phosphodiesterase 5; NO = Nitric oxide; CI = Cardiac index; AI = Artherogenic index

Similarly, the concentration of nitric oxide in the cardiac and penile tissues is directly and moderately correlated (0.5723). Concentration of plasma endothelin-1 is also moderately linked to concentration of plasma testosterone (0.5268) and cardiac nitric oxide (0.6726), but weakly correlated to penile nitric oxide (0.3416) and indirectly to concentration of plasma cGMP (-0.2509).

In contrast to PDE-5 activity, the coefficient between arginase activities in the cardiac and penile tissues is indirectly and weakly correlated (-0.1977). However, cardiac arginase activity is directly and strongly linked (0.8590) to cardiac PDE 5 and moderately correlated (0.6208) to cGMP but indirectly to cardiac nitric oxide (-0.7206).

Strong correlation (0.7911 – 1.000) exist among cardiac arginase and functional indices (cholesterol, triglycerides, LDL-cholesterol and computed artherogenic and cardiac indices), whereas concentration of HDL-cholesterol is moderately and directly linked (0.5199) to concentration of cardiac nitric oxide but indirectly correlated (-0.5809) to activity of cardiac arginase.

Activities of penile and cardiac PDE 5 in paroxetine-treated rats following oral administration extracts of *C. ferruginea* root, *F. agretis* stem and their combination are shown in Figures 49 and 50 respectively. Treatment of rats with paroxetine for 21 days significantly ($p < 0.05$) resulted in higher activities of PDE 5 in penile of rats, when compared to rats administered normal saline to serve as control. Administration of the extracts (*C. ferruginea, F. agrestis, C. ferruginea* and *F. agrestis*) significantly ($p < 0.05$) reduced PDE 5 enzyme activity in the penile of rats after 7 days which compared favourably well with the enzyme activity in the penile of rats administered with sildenafil citrate (PXT-SC) reference drug (Figure 49). However, it was observed that the PDE 5 activity in the penile of rats administered the combination of the extract once a day for 7 days significantly ($p < 0.05$) reduced PDE 5 activities in the penile when compared with all other groups except the control.

Phosphodiesterase 5 activity of the PXT-CFFA group was not significantly ($p \geq 0.05$) different from those of control (Figure 49).

Treatment of rats with paroxetine for 21 days significantly ($p < 0.05$) resulted in high activities of PDE 5 in heart of rats when compared to rats administered normal saline to serve as control. Administration of the extracts (*C. ferruginea, F. agrestis, C. ferruginea* and *F. agrestis*) significantly ($p < 0.05$) reduced PDE 5 enzyme activity in the heart of rats after 7 days which compared favourably well with the enzyme activity in the heart of rats administered with sildenafil citrate (PXT-SC) reference drug (Figure 50). However, it was observed that the PDE 5 activity in the heart of rats administered the sildenafil citrate had a significantly ($p < 0.05$) reduced PDE 5 activity when compared to the groups administered the extracts (PXT-CF, PXT-FA and PXT-CFFA).

After administration of the extracts for a week, some of the rats were left for the next 7 days and later sacrificed after the 7th day (post-treatment). Penile PDE 5 activities during treatment or post-treatment period were not significantly ($p > 0.05$) different from each other when compared with the control, paroxetine-induced and extract treated groups. PDE 5 activity was significantly ($p < 0.05$) increased in the heart of rats, although values were still significantly ($p < 0.05$) lower than those determined in the untreated group (Figure 50). There was significant ($p < 0.05$) difference in the FA post-treatment group when compared with other extracts and the reference drug.

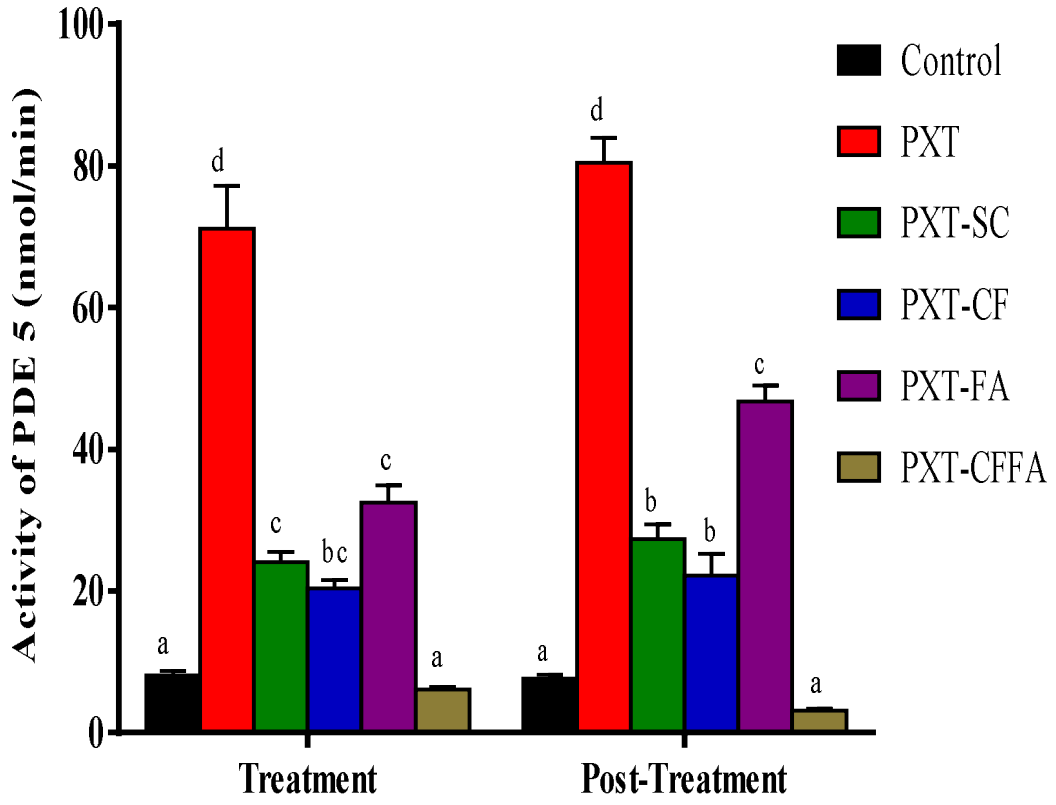


Figure 49: **Activity of penile phosphodiesterase 5 in paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days**

Values are mean of six determinations ± SEM; values within a group of bars with different superscripts are significantly ($p < 0.05$) different from each other.

PXT = Paroxetine-induced treated with distilled water (negative control); PXT-SC= Treated with sildenafil citrate; PXT-CF= Treated with aqueous *Cnestis ferruginea* root extract; PXT-FA= Treated with aqueous *Fadogia agrestis s*tem extract; PXT-CFFA= Treated with the combination of aqueous *Cnestis ferruginea* root and *Fadogia agrestis* stem extracts

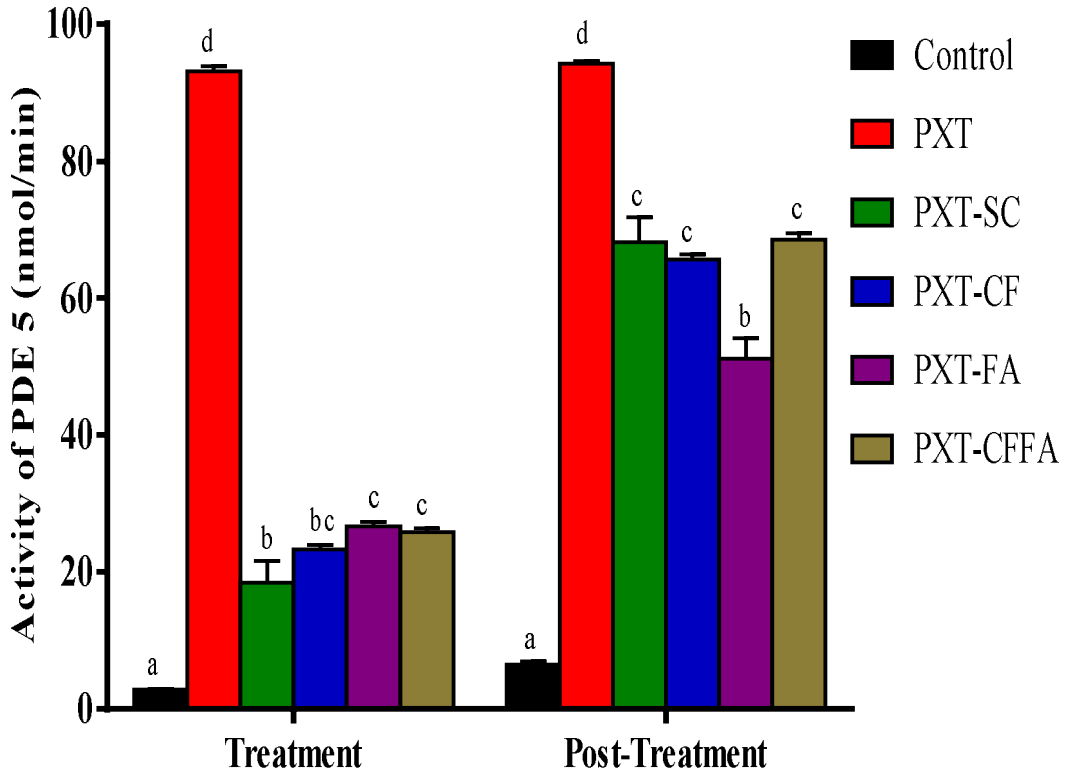


Figure 50: **Activity of cardiac phosphodiesterase 5 in paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination once a day for 7 days and post-treated for another 7 days**

Values are mean of six determinations ± SEM; values within a group of bars with different superscripts are significantly ($p < 0.05$) different from each other.

PXT = Paroxetine-induced treated with distilled water (negative control); PXT-SC= Treated with sildenafil citrate; PXT-CF= Treated with aqueous *Cnestis ferruginea* root extract; PXT-FA= Treated with aqueous *Fadogia agrestis s*tem extract; PXT-CFFA= Treated with the combination of aqueous *Cnestis ferruginea* root and *Fadogia agrestis* stem extracts

Figures 51 and 52 respectively show the effect of extracts (*C. ferruginea, F. agrestis, C. ferruginea* and *F. agrestis*) on activities of penile and cardiac arginase of paroxetine-induced rats respectively. Treatment of rats with paroxetine for 21 days significantly ($p < 0.05$) increased the activities of arginase in penile of rats, when compared to the control. Administration of extracts (*C. ferruginea, F. agrestis, C. ferruginea* and *F. agrestis*) significantly ($p < 0.05$) reduced activities of arginase in the penile tissues of paroxetine-treated rats, whereas administration of sildenafil citrate only significantly ($p < 0.05$) reduced penile arginase and was not significantly ($p \geq 0.05$) different from the control as well as the group treated with *F. agrestis*. There was no significant ($p \geq 0.05$) difference between the penile arginase activities of the rats treated with CF and CFFA but penile arginase activities in these two groups were significantly ($p < 0.05$) different from all other groups (Figure 51). Treatment of rats with paroxetine for 21 days significantly ($p < 0.05$) increased arginase activities in heart when compared to the control. However, the administration of the reference drug significantly ($p < 0.05$) increased arginase activities in the heart of rats in that group. There was a significant reduction in the cardiac arginase activities of rats administered *C. ferruginea* and combination of the two extracts (CFFA) which was not significantly ($p \geq 0.05$) different from those of control (Figure 52). Discontinuation of the extracts and reference drug for a week increased the activities of penile and cardiac arginase in rats administered the combined extracts, although values were still significantly ($p < 0.05$) lower than those determined in the untreated group. Cardiac arginase activity during treatment or post-treatment period were not significantly ($p > 0.05$) different from each other in rats administered *C. ferruginea* extract alone (Figure 52).

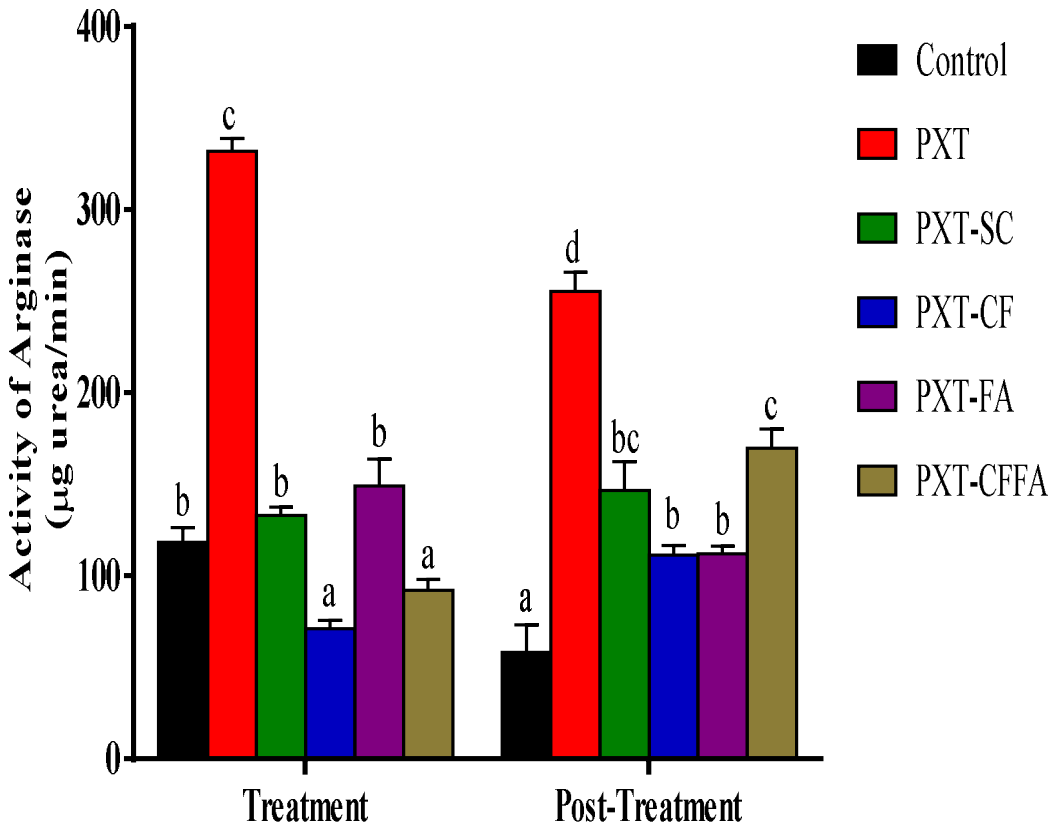


Figure 51: **Activity of penile arginase in paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days**

Values are mean of six determinations ± SEM; values within a group of bars with different superscript are significantly ($p < 0.05$) different from each other.

PXT = Paroxetine-induced treated with distilled water (negative control); PXT-SC= Treated with sildenafil citrate; PXT-CF= Treated with aqueous *Cnestis ferruginea* root extract; PXT-FA= Treated with aqueous *Fadogia agrestis s*tem extract; PXT-CFFA= Treated with the combination of aqueous *Cnestis ferruginea* root and *Fadogia agrestis* stem extracts.

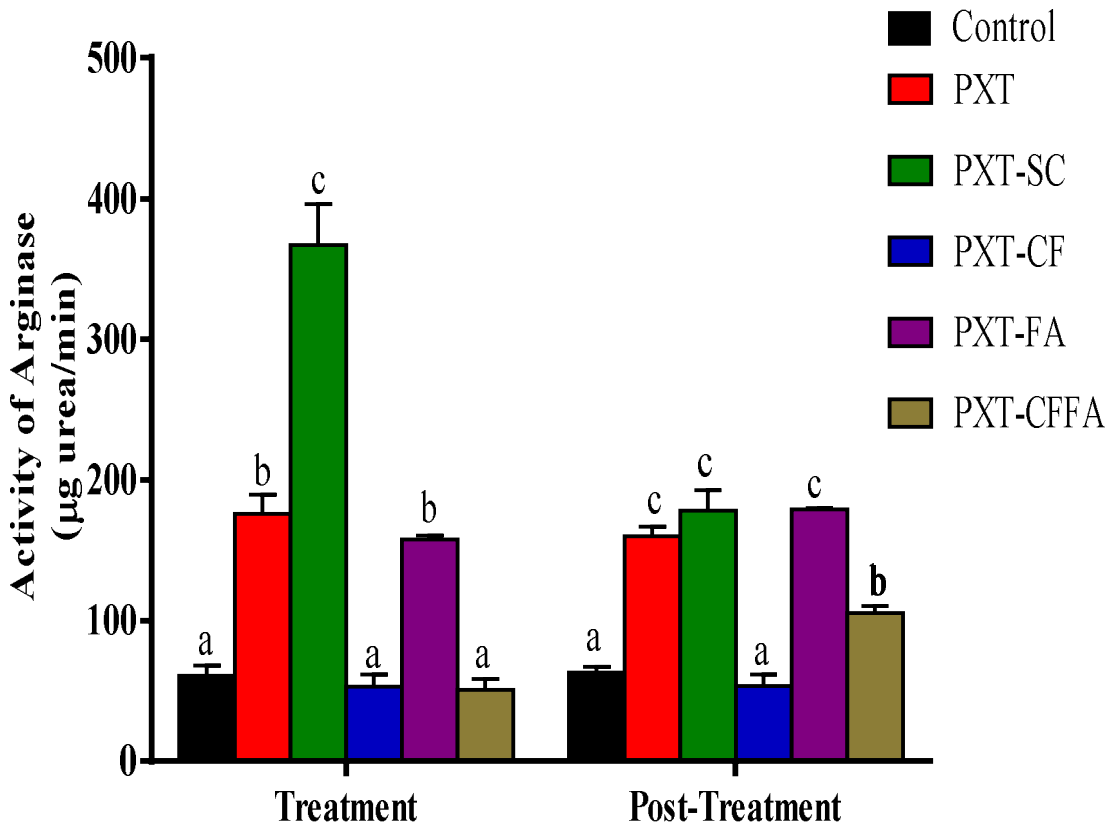


Figure 52: **Activity of cardiac arginase in paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days**

Values are mean of six determinations ± SEM; values within a group of bars with different superscript are significantly ($p < 0.05$) different from each other.

PXT = Paroxetine-induced treated with distilled water (negative control); PXT-SC= Treated with sildenafil citrate; PXT-CF= Treated with aqueous *Cnestis ferruginea* root extract; PXT-FA= Treated with aqueous *Fadogia agrestis s*tem extract; PXT-CFFA= Treated with the combination of aqueous *Cnestis ferruginea* root and *Fadogia agrestis* stem extracts.

Concentration of nitric oxide in the penile and heart of paroxetine-treated rats following oral administration of extracts of *C. ferruginea* root, *F. agretis* stem and their combination are shown on Figures 53 and 54 respectively. Treatment of rats with paroxetine for 21 days significantly ($p < 0.05$) reduced concentration of nitric oxide in the penile and cardiac of rats, when compared to the control. Administration of the extracts (*C. ferruginea* root, *F. agretis* stem and their combination) significantly ($p < 0.05$) increased concentration of nitric oxide in both penile and heart tissues of paroxetine-induced rats, whereas administration of sildenafil citrate only significantly ($p < 0.05$) increased level of nitric oxide in the heart but did not significantly ($p \geq 0.05$) affect the nitric oxide concentration in the penile, when compared to the untreated group. Nitric oxide concentration was normal in the penile of rats administered *C. ferruginea* extract (Figure 53), whereas in the cardiac tissue, concentration of nitric oxide was significantly ($p < 0.05$) higher in rats administered combined extract, when values were compared to the control (Figure 54). Discontinuation of the extracts (*C. ferruginea* root, *F. agretis* stem and their combination) for a week increased the level of nitric oxide in the penile of sildenafil citrate-treated rats, whereas significant ($p < 0.05$) reduction in post-treatment concentration of penile nitric oxide was observed in rats administered the extracts, when compared to values obtained during treatment period or that of the untreated group.

Figure 55 shows concentration of plasma cGMP of paroxetine-treated rats orally administered the extracts. Concentration of cGMP significantly ($p < 0.05$) reduced following treatment of rats with paroxetine for 21 days, when compared to the control. Administration of *C. ferruginea* extract to paroxetine-treated rats over a period of 7 days did not significantly ($p \geq 0.05$) alter concentration of plasma cGMP, whereas those administered *F. agretis* extract or its combination with *C. ferruginea* extract significantly ($p < 0.05$) increased plasma cGMP when compared to the untreated group or the sildenafil citrate-treated group or the non-induced control group.

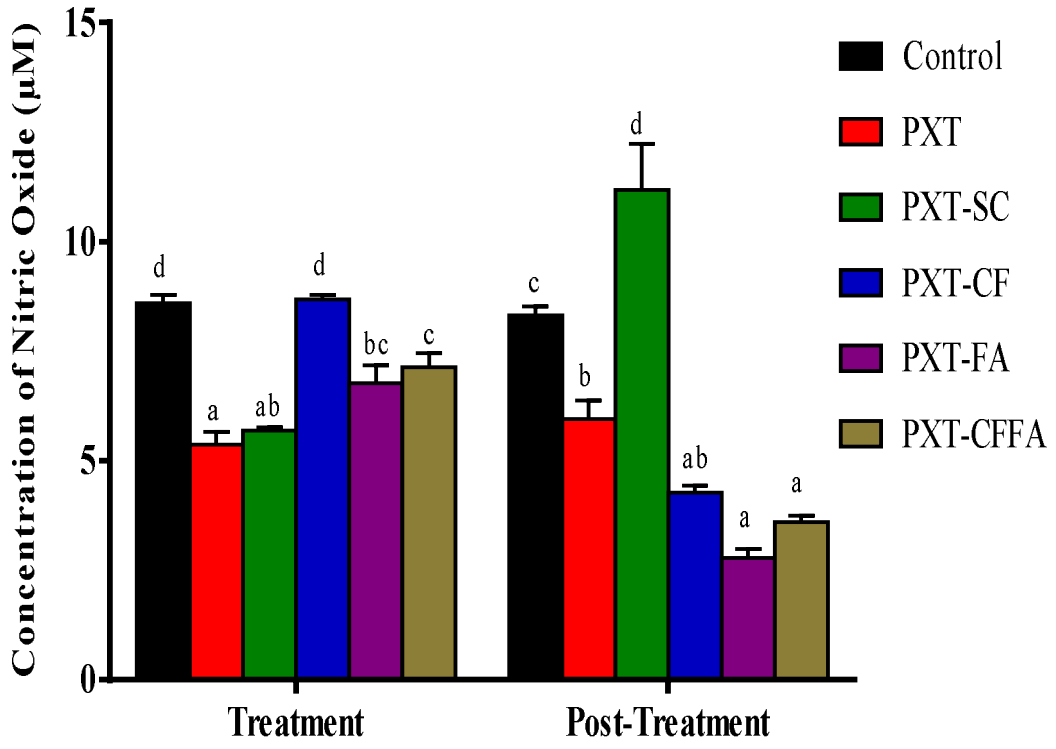


Figure 53: **Concentration of nitric oxide in the penile of paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days**

Values are mean of six determinations ± SEM; values within a group of bars with different superscript are significantly ($p < 0.05$) different from each other.

PXT = Paroxetine-induced treated with distilled water (negative control); PXT-SC= Treated with sildenafil citrate; PXT-CF= Treated with aqueous *Cnestis ferruginea* root extract; PXT-FA= Treated with aqueous *Fadogia agrestis s*tem extract; PXT-CFFA= Treated with the combination of aqueous *Cnestis ferruginea* root and *Fadogia agrestis* stem extracts.

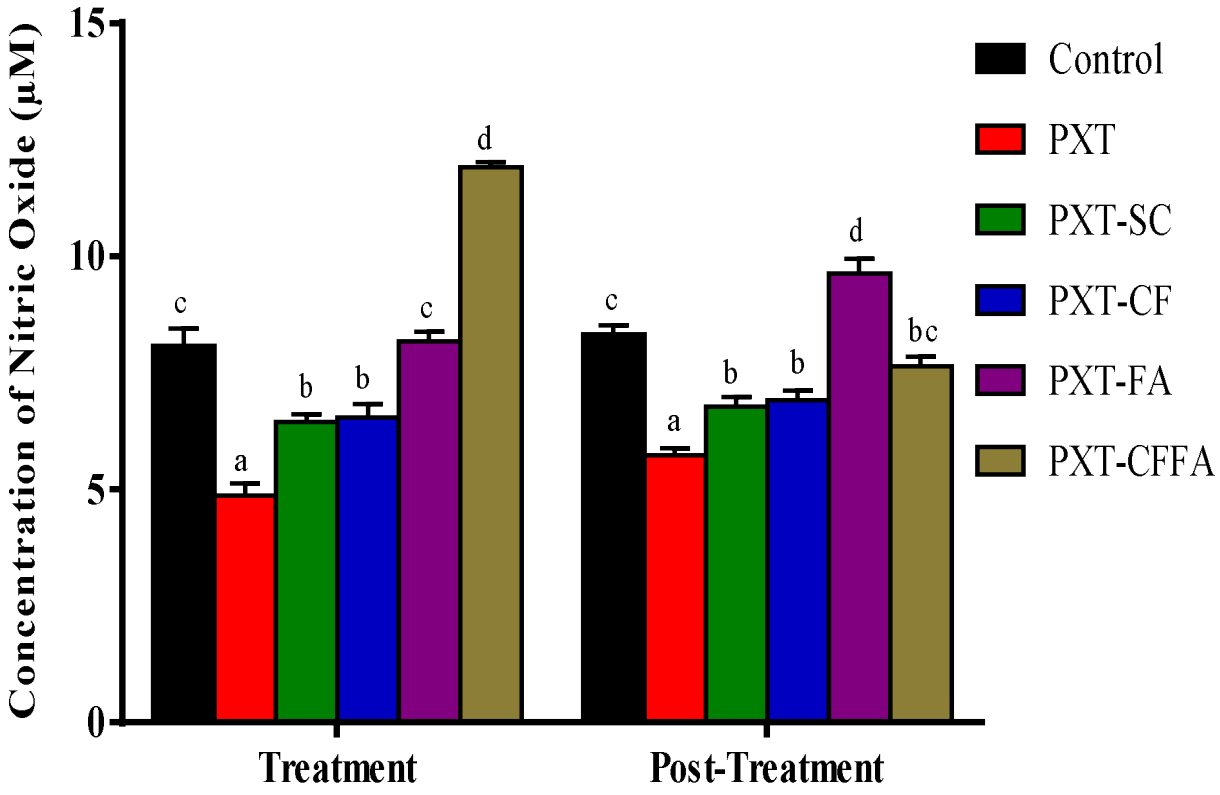


Figure 54: **Concentration of nitric oxide in the heart of paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days**

Values are mean of six determinations ± SEM; values within a group of bars with different superscript are significantly ($p < 0.05$) different from each other.

PXT = Paroxetine-induced treated with distilled water (negative control); PXT-SC= Treated with sildenafil citrate; PXT-CF= Treated with aqueous *Cnestis ferruginea* root extract; PXT-FA= Treated with aqueous *Fadogia agrestis s*tem extract; PXT-CFFA= Treated with the combination of aqueous *Cnestis ferruginea* root and *Fadogia agrestis* stem extracts.

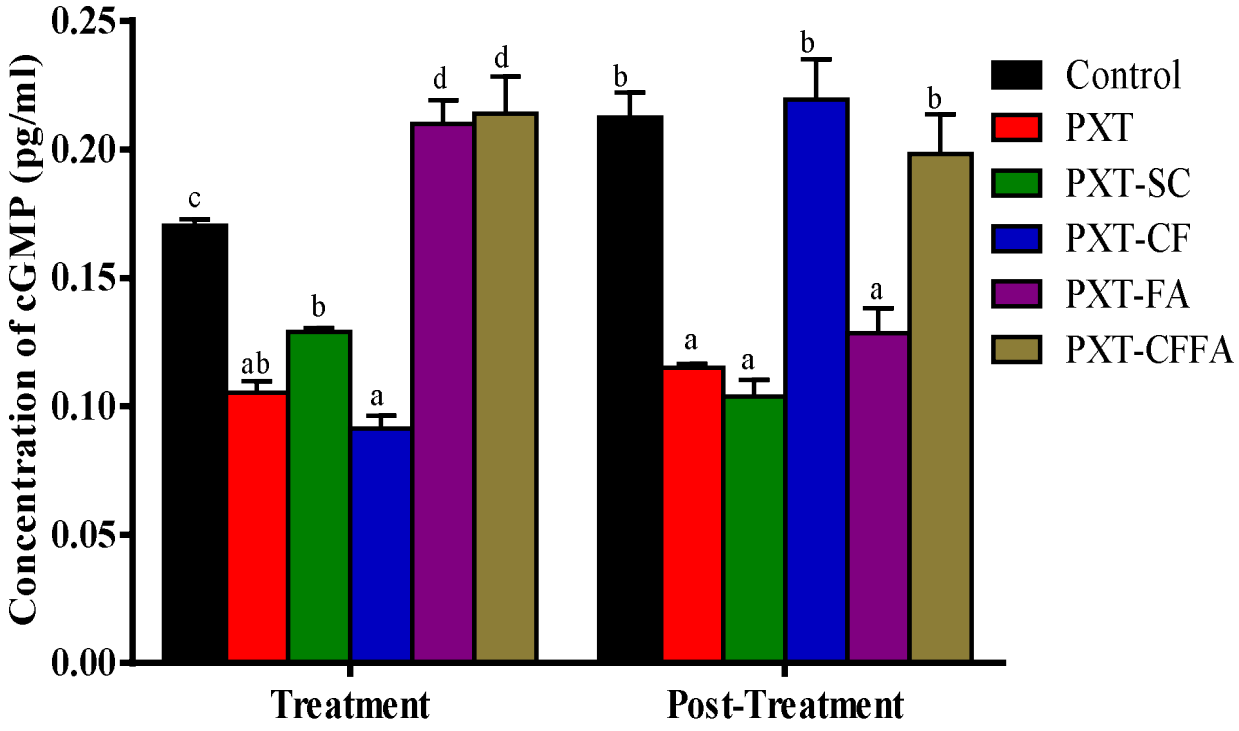


Figure 55: **Concentration of cyclic guanosine monophosphate (cGMP) in plasma of paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days**

Values are mean of six determinations ± SEM; values within a group of bars with different superscript are significantly ($p < 0.05$) different from each other.

PXT = Paroxetine-induced treated with distilled water (negative control); PXT-SC= Treated with sildenafil citrate; PXT-CF= Treated with aqueous *Cnestis ferruginea* root extract; PXT-FA= Treated with aqueous *Fadogia agrestis s*tem extract; PXT-CFFA= Treated with the combination of aqueous *Cnestis ferruginea* root and *Fadogia agrestis* stem extracts.

Withdrawal of the extracts for a week increased the level of cGMP in the plasma of *C. ferruginea*-treated rats, whereas significant ($p < 0.05$) reduction in level of plasma cGMP during the post-treatment period was observed in rats administered *F. agretis* extract, when compared to values obtained during the treatment period or that of the untreated group (Figure 55).

Concentration of plasma endothelin-1 in paroxetine-treated rats orally administered extracts (CF, FA, and CFFA) is shown in Figure 56. Administration of paroxetine to rats significantly ($p < 0.05$) increased concentration of plasma endothelin-1 in rats. Concentration of plasma endothelin-1 was significantly ($p < 0.05$) reduced following administration of the extracts (CF, FA, and CFFA) to paroxetine-treated rats when compared to the untreated group but not significantly ($p \geq 0.05$) different from the control. Discontinuation of the administration of the extracts and reference drug for a week increased the post-treatment level of plasma endothelin-1 of paroxetine-treated rats administered the combined extracts of *F. agretis and C. ferruginea* (PXT-CFFA; post-treatment), although values were still significantly ($p < 0.05$) lower than those determined in the untreated group.

Figure 57 shows concentration of plasma testosterone of paroxetine-treated rats orally administered extracts (CF, FA, and CFFA). Concentration of testosterone significantly ($p < 0.05$) reduced following treatment of rats with paroxetine for 21 days, when compared to the control. Administration of extracts to paroxetine-treated rats over a period of 7 days significantly ($p < 0.05$) increased plasma testosterone concentrations when compared to the untreated group and values were not significantly ($p \geq 0.05$) different from the control group. However, there was a significant ($p < 0.05$) difference between the groups treated with all the extracts and that treated with the reference drug; there was no significant ($p \geq 0.05$) defference in the testosterone concentration of animals in the untreated group and that treated with the reference drug.

Withdrwal of extracts for a week increased the level of testosterone in the plasma of rats treated with the combination of *C. ferruginea* and *F. agretis* extracts, however post-treatment values determined in the combined group was not significantly ($p \geq 0.05$) different from that of the control group (Figure 57).

Activity of plasma creatine kinase in paroxetine-treated rats orally administered aqueous extracts of *C. ferruginea*, *F. agretis* and their combination is shown in Figure 58. Administration of paroxetine to rats significantly ($p < 0.05$) increased creatine kinase activity in the plasma of rats. Activity of plasma creatine kinase was significantly ($p < 0.05$) reduced following administration of the extracts (*C. ferruginea* root, *F. agretis* stem and their combination) to paroxetine-treated rats when compared to creatine kinase activity of the untreated group, values were not significantly ($p \geq 0.05$) different from that of the control, except for those treated with *C. ferruginea* extract that recorded creatine kinase activity significantly ($p < 0.05$) lower than that of control. Discontinuation of the extracts (*C. ferruginea* root, *F. agretis* stem and their combination) for a week (Figure 58) did not alter the post-treatment values of creatine-kinase activity of paroxetine-treated rats administered the extracts and values were not significantly ($p \geq 0.05$) different from those determined in the untreated group.

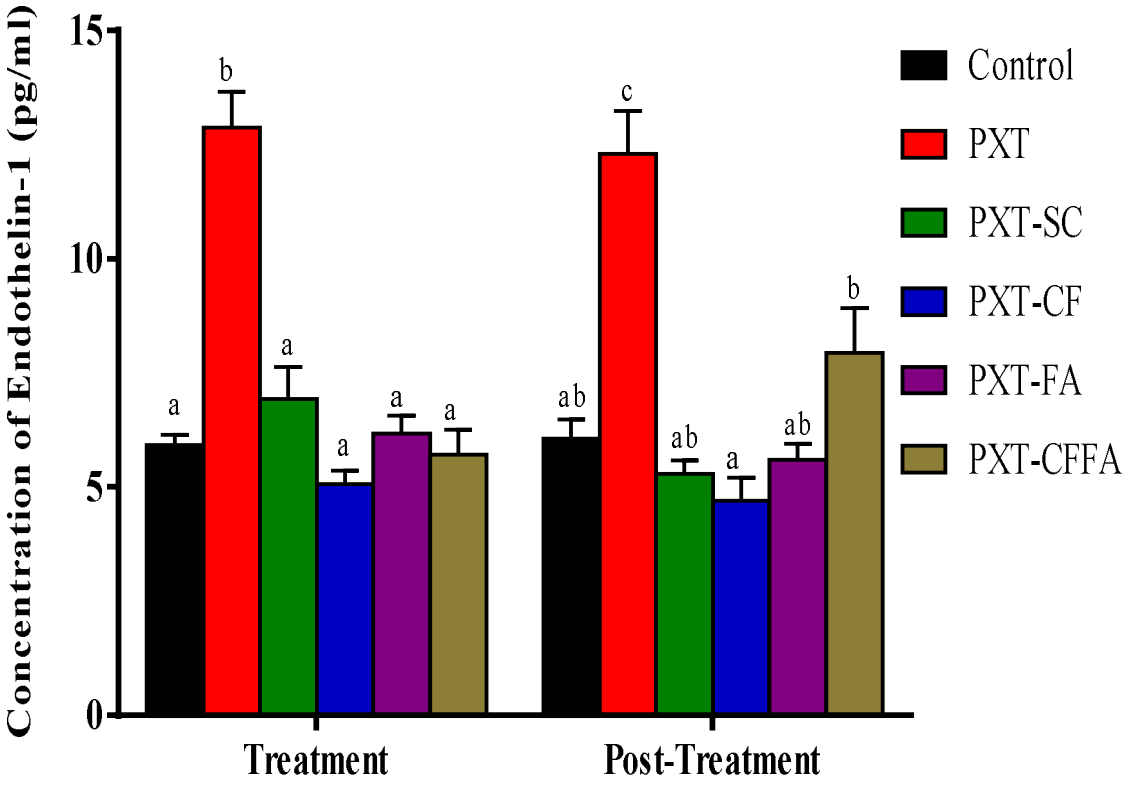


Figure 56: **Concentration of endothelin-1 in plasma of paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days**

Values are mean of six determinations ± SEM; values within a group of bars with different superscript are significantly ($p < 0.05$) different from each other.

PXT = Paroxetine-induced treated with distilled water (negative control); PXT-SC= Treated with sildenafil citrate; PXT-CF= Treated with aqueous *Cnestis ferruginea* root extract; PXT-FA= Treated with aqueous *Fadogia agrestis s*tem extract; PXT-CFFA= Treated with the combination of aqueous *Cnestis ferruginea* root and *Fadogia agrestis* stem extracts.

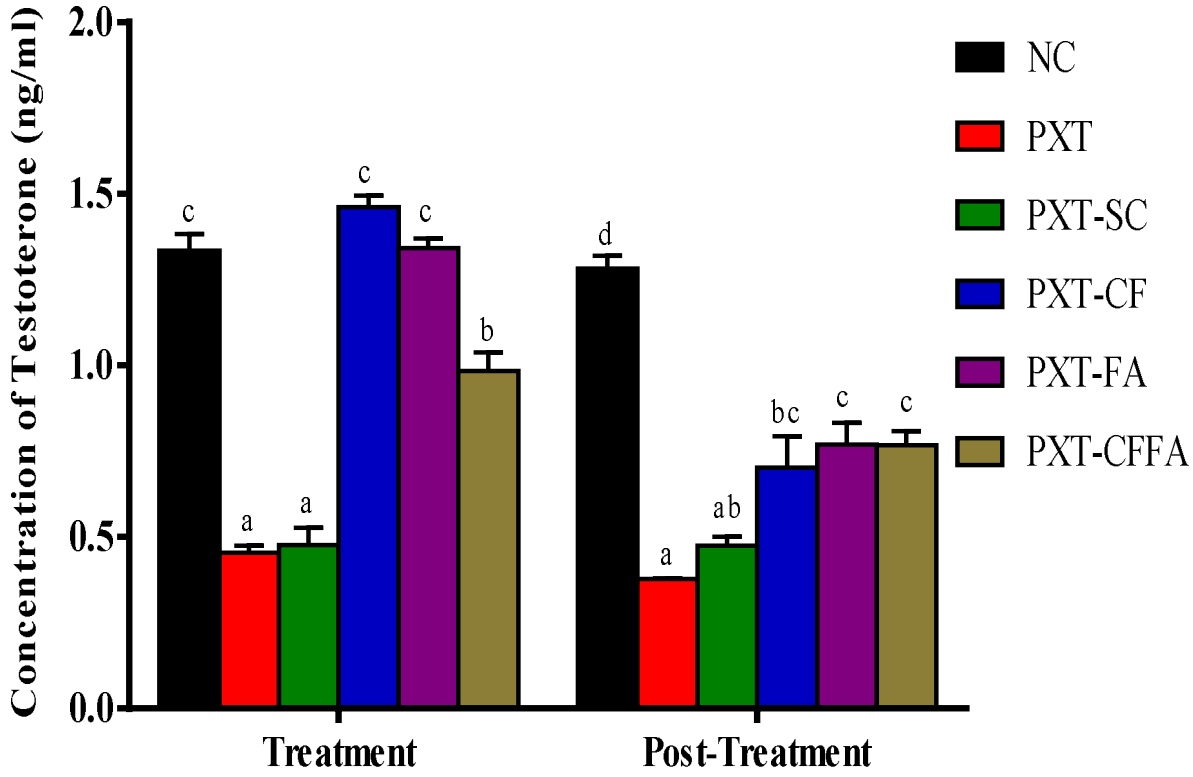


Figure 57: **Concentration of testosterone in plasma of paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days**

Values are mean of six determinations ± SEM; values within a group of bars with different superscript are significantly ($p < 0.05$) different from each other.

PXT = Paroxetine-induced treated with distilled water (negative control); PXT-SC= Treated with sildenafil citrate; PXT-CF= Treated with aqueous *Cnestis ferruginea* root extract; PXT-FA= Treated with aqueous *Fadogia agrestis s*tem extract; PXT-CFFA= Treated with the combination of aqueous *Cnestis ferruginea* root and *Fadogia agrestis* stem extracts.

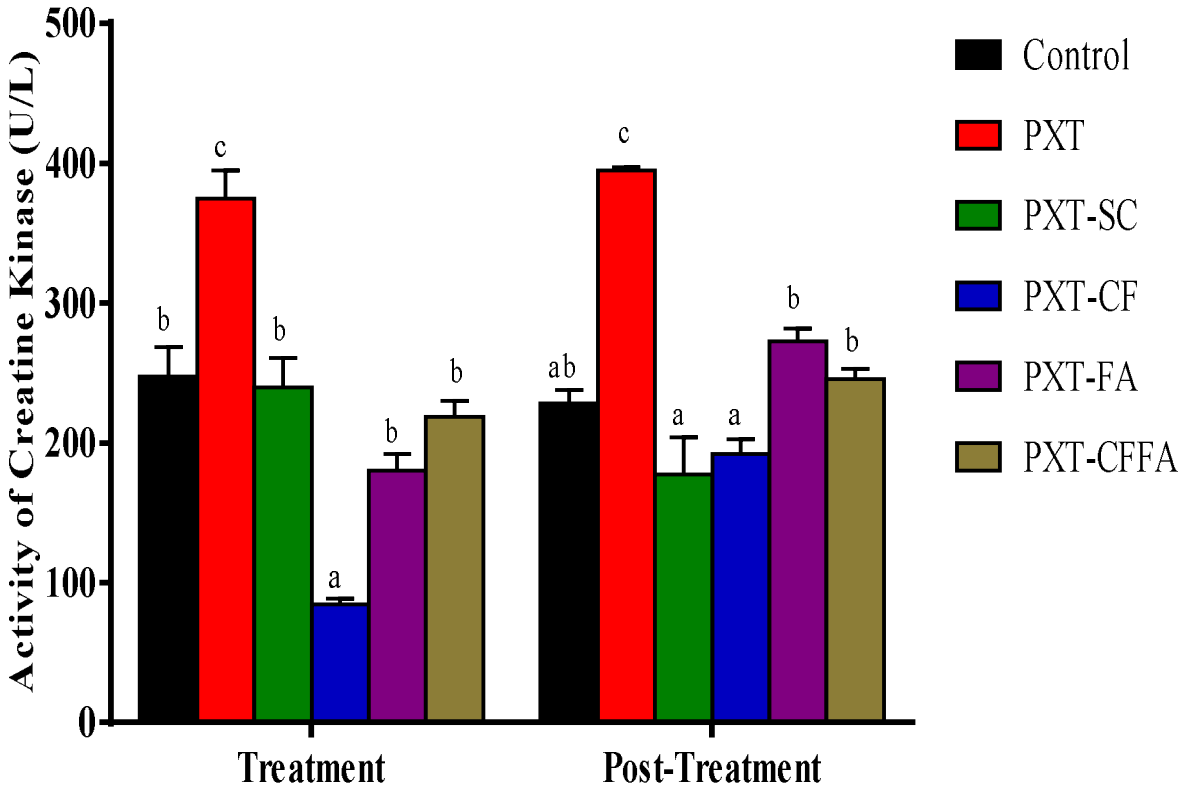


Figure 58: **Activity of creatine kinase in plasma of paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days**

Values are mean of six determinations ± SEM; values within a group of bars with different superscript are significantly ($p < 0.05$) different from each other.

PXT = Paroxetine-induced treated with distilled water (negative control); PXT-SC= Treated with sildenafil citrate; PXT-CF= Treated with aqueous *Cnestis ferruginea* root extract; PXT-FA= Treated with aqueous *Fadogia agrestis s*tem extract; PXT-CFFA= Treated with the combination of aqueous *Cnestis ferruginea* root and *Fadogia agrestis* stem extracts.

Concentration of cholesterol in plasma of rats administered normal saline (control) were not significantly ($p \geq 0.05$) different from each other over the experimental period (Table 16). Continuous administration of paroxetine to rats resulted in significant ($p < 0.05$) increase in concentration of plasma cholesterol, when compared to the control. Concentration of the cholesterol in the plasma of rats treated with the extracts and reference drug were significantly ($p < 0.05$) different from the untreated group and control (Table 16). The extracts significantly ($p < 0.05$) reduced concentration of cholesterol in all the treatment groups though not as low as the control. However, there was significant ($p < 0.05$) increase in plasma cholesterol concentration in all the groups after post treatment period of 7 days.

Concentration of triglycerides in plasma of rats administered normal saline (control) were not significantly ($p \geq 0.05$) different from each other over the experimental period of 35 days. Administration of paroxetine to rats resulted in significant ($p < 0.05$) increase in concentration of plasma triglycerides when compared to the control. Concentration of the cholesterol in the plasma of rats treated with the extracts and reference drug were significantly ($p < 0.05$) reduced when compared with the untreated group (Table 16). The extracts significantly ($p < 0.05$) reduced concentration of triglycerides in all the treatment groups which was not significantly different from the control except for the group treated with *C. ferruginea* which had a concentration significantly ($p < 0.05$) different from the control but not significantly ($p \geq 0.05$) different from the untreated group. However, there was significant ($p < 0.05$) increase in plasma triglyceride concentration in all the groups after post treatment period of 7 days. There was no significant ($p \geq 0.05$) difference between the group treated with CF and he untreated group after the post-treatment period. However, a significant ($p < 0.05$) reduction in triglyceride concentration was observed in the group treated with the combination of the extracts (CFFA) which persisted when the extract administration was withdrawn (Table 16).

Table 16: Concentrations of cholesterol and triglycerides in the plasma of paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days

	Cholesterol (mg/dl)		Triglycerides (mg/dl)	
Groups	Treatment	Post-Treatment	Treatment	Post-Treatment
Control	22.94 ± 1.53^{a}	32.46 ± 0.35^{a}	18.78 ± 0.29^{ab}	18.78 ± 0.29^{b}
PXT	83.13 ± 7.15^{d}	85.06 ± 0.17^{e}	42.78 ± 3.50^{c}	49.68 ± 2.63^{d}
PXT-SC	31.77 ± 1.14^{ab}	74.38 ± 1.19^{d}	25.94 ± 0.39^{b}	23.92 ± 2.92^{b}
PXT-CF	37.00 ± 1.01^{b}	51.10 ± 0.69^{b}	35.03 ± 2.36^{c}	46.82 ± 0.78^{d}
PXT-FA	49.24 ± 2.52^{c}	58.31 ± 0.35^{c}	19.20 ± 0.19^{ab}	35.14 ± 1.50^{c}
PXT-CFFA	39.98 ± 0.87^{bc}	76.79 ± 0.44^{d}	15.72 ± 1.25^{a}	13.36 ± 0.30^{a}

Values are mean of six determinations ± SEM; values along a column with different superscript are significantly ($p < 0.05$) different from each other.

Table 17 shows the concentration of lipoprotein-cholesterol profile in plasma of paroxetine-treated rats over a period of 21 days and post-treatment period of 14 days. There was no significant ($p \geq 0.05$) difference between the HDL- cholesterol of all the treatment groups except that significant ($p < 0.05$) increase was observed in the group treated with *F. agrestis* extract throughout the treatment period. Interestingly, there was significant ($p < 0.05$) increase in the HDL-C of rats during the post-treatment period. There was significant ($p < 0.05$) increase in the HDL-C of rats in all the groups when compared to the treatment period and also when compared with the control. However, concentration of LDL-cholesterol was significantly ($p < 0.05$) higher in rats treated with paroxetine, when compared with the control. The LDL-cholesterol level progressively and significantly ($p < 0.05$) increased as days of treatment with paroxetine increased. Significant ($p < 0.05$) increase was observed when the administration was discontinued for 7 days (post-treatment; Table 17). There was significant ($p < 0.05$) differences between LDL-C of rats in all the post-treatment groups when compared with control.

Cardiac and atherogenic indices of paroxetine-treated rats are shown on Table 18. There was a significant ($p < 0.05$) increase in the cardiac index of rats in the group of rats treated with the reference drug when compared with all other groups during the treatment period. There was no significant difference between the cardiac indices during treatment and post-treatment periods in all the groups.

There was no significant ($p \geq 0.05$) difference in the artherogenic indices of rats administered the reference drug and control during the treatment period but was significantly ($p < 0.05$) increased upon administration of all the extracts. There is a significant ($p < 0.05$) increase in the atherogenic indices of rats in the untreated (PXT) group when compared with all other groups during the treatment period. Similar trend was observed during the post-treatment periods (Table 18).

Table 17: HDL-cholesterol and LDL-cholesterol concentrations in the plasma of paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days

	HDL-cholesterol (mg/dl)		LDL-cholesterol (mg/dl)	
Groups	Treatment	Post-Treatment	Treatment	Post-Treatment
Control	7.44 ± 0.37^{a}	8.69 ± 0.41^{a}	8.22 ± 1.53^{a}	15.32 ± 0.34^{a}
PXT	8.07 ± 0.46^{a}	10.10 ± 0.68^{ab}	66.84 ± 7.15^{d}	65.26 ± 0.17^{f}
PXT-SC	8.92 ± 0.32^{a}	11.27 ± 0.54^{b}	14.00 ± 1.14^{ab}	52.16 ± 1.29^{e}
PXT-CF	8.51 ± 0.34^{a}	15.03 ± 0.54^{c}	20.39 ± 0.89^{bc}	20.73 ± 0.69^{b}
PXT-FA	10.51 ± 0.55^{b}	13.85 ± 0.41^{c}	27.86 ± 2.52^{c}	30.38 ± 0.35^{c}
PXT-CFFA	8.92 ± 0.56^{ab}	15.73 ± 0.14^{c}	22.53 ± 0.87^{bc}	45.25 ± 0.44^{d}

Values are mean of six determinations ± SEM; values along a column with different superscript are significantly ($p < 0.05$) different from each other.

Table 18: Cardiac and atherogenic indices of paroxetine-treated rats orally administered *Cnestis ferruginea* root extract, *Fadogia agrestis* stem extract and their combination, once a day for 7 days and post-treated for another 7 days

	Cardiac index		Artherogenic index	
Groups	Treatment	Post-Treatment	Treatment	Post-Treatment
Control	3.08 ± 0.09^{a}	3.76 ± 0.14^{c}	1.09 ± 0.12^{a}	1.75 ± 0.09^{a}
PXT	9.85 ± 0.34^{b}	8.54 ± 0.56^{c}	7.88 ± 0.09^{c}	6.53 ± 0.49^{b}
PXT-SC	3.56 ± 0.09^{a}	6.63 ± 0.20^{c}	1.58 ± 0.09^{a}	4.62 ± 0.16^{c}
PXT-CF	4.37 ± 0.13^{b}	3.41 ± 0.08^{a}	2.43 ± 0.11^{b}	1.38 ± 0.04^{a}
PXT-FA	4.69 ± 0.08^{b}	4.22 ± 0.10^{ab}	2.73 ± 0.09^{b}	2.22 ± 0.07^{ab}
PXT-CFFA	4.52 ± 0.19^{b}	4.88 ± 0.07^{b}	2.55 ± 0.13^{b}	2.88 ± 0.06^{b}

Values are mean of six determinations ± SEM; values along a column with different superscript are significantly ($p < 0.05$) different from each other.

CHAPTER FOUR

DISCUSSION

4.1 Secondary metabolites of aqueous extracts of *Cnestis ferruginea* root and *Fadogia agrestis* stem

The medicinal value of plants lies in their bioactive secondary metabolites which show various physiological effects on animals. The positive influence of plants on male reproductive health and their performance enhancement properties have been attributed to the presence of alkaloids, flavonoids, tannins and saponins (Lesile, 2003).

Alkaloids are natural nitrogen-containing secondary metabolites mostly derived from amino acids. Alkaloids possess great bioactive potentials particularly as inhibitors of phosphodiesterases (Silver, 2006) which serves as the basis for the design and development of new and more selective drugs with reduced side effects. Seftel (2002) also reported that alkaloids were found to be effective in the drugs prescribed to patients with erectile dysfunction of various etiologies and levels of severity. Alkaloids act as dopamine agonists due to their structural similarity. They improve central pro-erectile mechanisms by binding to receptors in the para-ventricular nucleus of the hypothalamus which sends signals to the erection-generating centre of the spinal cord (Seftel, 2002).

Saponins are vast group of non-nitrogenous compounds. They are soluble in water and form colloidal solutions that foam upon shaking (Sparg *et al*., 2004; Schenkel *et al*., 2007). Some saponins have been reported to inhibit PDE 5 like those present in *Allium tuberosum* and *Tribulus terrestris* but the mechanism by which they do this is not yet clear (Schenkel *et al*., 2007).

These secondary metabolites also work peripherally by enhancing the activity of nitric oxide synthase (an enzyme that synthesises nitric oxide from L-argine), releasing the nitric oxide, dilating the blood vessels and relaxing the Corpus carvenosum smooth muscles of the

corpulatory organ, thus leading to enhanced penile erection (Shukla and Khanuja, 2004; Singh and Gupta, 2011; Yakubu *et al*., 2011). In this study, the detection of these bioactive principles in appreciable quantity may account for the acclaimed folkloric aphrodisiac effects of the plant extracts.

4.2 Purification of phosphodiesterase 5 from rats penile and heart tissues

The more the purification step, the more the enzyme activity (Avery *et al.,* 2017). This was also in agreement with what was found in this study, the purification yield of phosphodiesterase 5 (PDE 5) was more in the heart than than the penis. This enzyme required magnesium and was stimulated by imidazole in an unknown manner and was inhibited by sildenafil and the extracts (*C. ferruginea* root, *F. agretis* stem and their combination) in the presence of cyclic nucleotides.

4.3 Effect of various extracts (*C. ferruginea* root, *F. agretis* stem and their combination) on the hydrolysis of cGMP

Despite extensive literature reports concerning the effects of aqueous extracts of *C. ferruginea* root, and *F. agretis* stem in the treatment of erectile dysfunction, their mechanism of action have not been documented in literature. Inhibition of phosphodiesterase 5 activity plays a major role in the regulation of blood flow and blood flow is important in relaxation of smooth muscles (Zhang *et al*., 2015). Monitoring the behaviour of the enzymes within their simulated environment, however, is essential for understanding their physiological functioning and the possible modulation of their activity by cellular signaling pathways (Bellamy and Garthwaite, 2001). The penis and the heart used for this study have the advantage of being richly endowed with phosphodiesterase 5, such that cGMP hydrolysis was monitored over several minutes. Sildenafil is a very potent PDE 5 inhibitor, active in low micromolar concentrations. The extracts (*C. ferruginea* root, *F. agretis* stem and their combination) inhibited PDE 5 competitively indicating a reversible type of inhibition. This

might be due to the presence of saponin and alkaloids in the various extracts. Saponnins have been adduced to the PDE inhibitory activities of some plant extracts such as *Lilium regale* and *Lilium henryi* have PDE inhibitory effects on cAMP phosphodiesterases (Mimaki *et al.,* 1993). A number of plant alkaloids have been reported to have inhibitory properties on PDEs (Ohmoto *et al.,* 1988). Chen *et al.*(2008) isolated neferin (bis-benzyl isoquinoline alkaloid) from *Nelubo nucifera* and reported that neferin enhanced accumulation of cyclic nucleotide in cavernosum tissue of rabbits probably via inhibition of PDE activity.

The extracts inhibited PDE 5 in the presence of cAMP non competitively implies that the substrate may be inappropriate for the analyses but may be used for such experiments. The inhibition can also be as a result of the presence of saponins in the plant extracts. Kuroda *et al.* (1995) reported that ethanol extract of *Allium chinense* has inhibitory effects on CAMP PDE probably because of its saponin content.

4.4 Effects of paroxetine and the exracts on biochemical parameters of male rats

Paroxetine, an antidepressant, is a selective serotonin reuptake inhibitor (SSRI) that reduces the mesolimbic dopaminergic activity due to inhibitory serotonergic midbrain raphe nuclei projections or inactivation of 5-HT1A receptor-mediated norepinephrine neurotransmission (Prabhakar and Richard, 2010). It also delays orgasm/sex drive and inhibits synthesis of nitric oxide in male rats (Prabhakar and Richard, 2010).

Aphrodisiacs that displayed excellent *in vitro* PDE 5 inhibitory activity may fail to replicate such biological activity in *in vivo* models. PDE 5 is a cGMP specific enzyme that is most prominently found in the corpus cavernosum of the penis and also in appreciable amount in the heart tissue (Bobin *et al.,* 2016). Elevation in the activity of PDE 5 has been reported in cases of erectile dysfunction (Yang *et al.,* 2013).The use of anti-psychotics such as paroxetine has also been linked to erectile dysfunction (Garcia *et al.,* 2017). Paroxetine is an antidepressant of the selective serotonin reuptake inhibitors (SSRIs) that is used majorly to

treat depressive disorders. One of the adverse effects of SSRIs administration to psychotic patients is erectile dysfunction and symptoms of sexual dysfunction have been reported to persist after discontinuation of SSRIs (Simonsen *et al.* 2017). In this study, sub-chronic administration of paroxetine for 21 days caused progressive increase in the activity of PDE 5 by ~ 16 folds, establishing erectile dysfunction in rats. Persistence in elevated levels of PDE 5 was observed following discontinuation of paroxetine for two weeks, indicating post-paroxetine induced sexual dysfunction in rats.

Similarly, PDE 5 activity in the cardiac tissue of rats treated with paroxetine was also elevated. Correlation analysis showed a strong relationship between cardiac and penile PDE-5 activities (0.9211). Recent studies have highlighted relationship between erectile dysfunction and cardiovascular diseases (Gandaglia *et al.,* 2014). The role of nitric oxide and endothelial factors in modulating cardiac and erectile functions have also been elucidated (Montorsi *et al.,* 2009). Levels of nitric oxide in both (penile and cardiac) tissues of paroxetine-induced rats were reduced, signifying attenuated activities of nitric oxide synthase. Paroxetine is a novel inhibitor of nitric oxide synthase (Finkel *et al.,* 1996). Nitric oxide synthase catalyses conversion of L-arginine to nitric oxide; leading to vasodilation of the smooth muscles and penile erection.

Soluble guanylyl cyclase is activated in the presence of nitric oxide and the enzyme converts guanine triphosphate (GTP) to cyclic guanine momophosphate (cGMP). In paroxetine-induced erectile dysfunction state, reduction in concentration of plasma cGMP was observed in rats following admistration of paroxetine for 4 days and the reduced concentration persisted during the 21 days of treatment as well as following discontinuation of paraoxetine for 2 weeks. Concentration of cGMP in corpus cavernosum smooth muscle cells is dependent on the balance between its production and utilization by soluble guanylyl cyclase and PDE 5 respectively (Lau *et al.,* 2006). Decreased production of enthodelium-derived nitric oxide has

been implicated in the pathogenesis of not only erectile dysfunction but also cardiovascular diseases (Lara *et al.,* 2003).

Treatment of rats with paroxetine may also influence the activities of other enzymes involved in erectile, endothelial and vascular functions such as arginase. Thakur and Nivsarkar (2017) reported that rats exposed to paroxetine for 5 days showed alterations in the expression of arginase activity; similarly in the study, 4 days exposure of rats to paroxetine resulted in increased activity of arginase to ~ 1.5 and 2.3 folds in penile and cardiac tissues respectively. Arginase is a manganese-containing enzyme that converts arginine to urea and ornithine. In penile tissue, arginase competes with nitric oxide synthase for L-arginine (Ruth *et al.,* 2014; Lacchini *et al.,* 2015); thus when there is reduction in nitric oxide synthase activity as evident by low nitric oxide activity in the penile tissue, L-arginine was readily utilized by arginase and this phenomenon may be responsible for higher activity of arginase in the tissues. Arginase reciprocally regulates nitric oxide synthesis as well as endothelial and cardiac functions (Berkowitz *et al.,* 2003; Durante *et al.,* 2007).

In correlation with activity of arginase especially in the cardiac tissue, level of plasma endothelin-1 was increased in paroxetine-induced erectile dysfunction rats. In contrast to vasodilating nitric oxide, endothelins are vasoconstricting peptides that are produced primarily in the endothelium with a crucial role in vascular homeostatis and are implicated in a number of vascular diseases (Agapitov and Haynes, 2002). Enthothelin-1 encoded by *EDN1* gene is a pleiotropic molecule and one of three isoforms found in humans. Endothelin-1 is a putative modulator of erectile dysfunction; Christ *et al.* (1995) suggested that the physiological relevance of endothelin-1 to corporal physiology may be related to its ability to augment the contractile responses of other vasomodulators present in the human corpora, in particular, perhaps modulating the contractile responses to sympatheic activity. Similarly, Kaiser *et al.* (2004) concluded that endothelin-1 is the best independent predictor of erectile

dysfunction suggesting that biochemical measures of endothelial damage are detectable independent of other risk factors. Inhibition of this peptide may prevent pulmonary vasoconstriction and decreases pulmonary vascular resistance (Angus *et al.,* 2017).

In this study, prolonged administration of paroxetine resulted not only in elevation of plasma endothelin-1 but also concomitant reduction in concentration of plasma testosterone. A component increased risk conferred by erectile dysfunction could be testosterone deficiency (Johnson, 2010). Testosterone is an anoblic streroid and the primary sex hormone that plays a crucial role in the development of the male reproductive tissues. Yakubu and Jimoh (2015) showed that paroxetine administration to rats significantly reduced concentration of testosterone, concluding that sexual function was impaired in paroxetine-treated rats. Similar findings have also been reported by other researchers (Ajiboye *et al.,* 2016; El-Sheikh *et al.,* 2017). Low testosterone may increase levels of total and LDL cholesterol and thus, predisposed rats to cardiovascular risks (Carson and Rosano *et al.,* 2012).

In this study, administration of paroxetine increased concentration of creatine kinase, indicating myocardial infarction may be associated with erectile dysfunction. Evidence from study involving large number of patients showed clinical relationship between the triad of erectile dysfunction, endothelial dysfunction and cardiovascular diseases (Montorsi *et al.,* 2009). Endothelial dysfunction is the initial step of the atherosclerotic process involving many vascular districts, including penile and coronary circulation. The onset of sexual dysfunction is sometimes considered as a marker of subclinical systemic vascular disease, thus there is need for treatment of risk factors and conditions associated with erectile dysfunction.

PDE 5 inhibitors such as sildenafil citrate are prescribed as first line oral therapeutics for the management of erectile dysfunction. However, adverse effects of this synthetic drug which includes blurred vision, persistent headache, heartburn, nasal congestion, delayed flaccidity,

diarrhoea and dyspepsia (Moreira *et al.,* 2000; Lichtblau *et al.*, 2015). More so, patients suffering from pulmonary arterial hypertension, incidences of vascular, gastrointestinal and neurologic side effects have been reported and in some patients, adverse effects may remain intolerable leading to discontinuation of treatment (Lichtblau *et al.*, 2015; Siehr *et al.,* 2015). Locally, aphrodisiacs such as *C. ferruginea and F. agrestis* are used for the treatment of erectile dysfunction. Yakubu *et al.* (2005) reported the aphrodisiac potentials of *F. agestis* stem extract and attributed the sexual stimulatory functions of the extract in rats to its ability to increase testosterone concentration. More so, in sexual dysfunction rat model induced by prolonged administration of paroxetine to male rats; the aqueous extract of *C. ferruginea* root restored sexual competence, thus providing scientific evidence to support use of the plant by traditional medicine practitioners for the management of sexual disorders in men (Yakubu and Nurudeen, 2012).

Similarly, in this study, extracts from *C. ferruginea* and *F. agrestis* improved markers of erectile function in paroxetine-treated rats. In correlation to *in vitro* study, the extracts inhibited the activity of penile PDE 5 in paroxetine-treated rats. PDE 5 plays a key role in the regulation of a cascade of events leading to erection. Inhibition of PDE 5 will prevent the breakdown of cGMP to 5'GMP hindering the relaxation of smooth muscle (Corbin, 2004). In paroxetine-treated rats, reduced concentration of cGMP was observed; this may be as a result of decreased in nitric oxide production (Lara *et al*., 2003). During erotic stimulus, nitric oxide is released from both the neurons and endothelial cells of the penile, leading to activation of NO-dependent soluble guanylyl cyclase and increasing conversion of GTP to cGMP (Francis *et al.,* 2008). Previous study by Nurudeen *et al.* (2015) showed that administration of extract from *Lecaniodiscus cupanioides* increased concentration of cGMP in paroxetine-treated rats. Similarly, in this study, administration of *C. ferruginea* increased concentration of cGMP, however, when administered in combination with *F. agrestis*, twice

the amount of cGMP was quantified in the paroxetine-treated rats indicating a synergistic effect of the combined extracts. Results from this study showed that administration of *C. ferruginea* and *F. agrestis* extracts as a mono- or combined therapy for erectile dysfunction have the ability to modulate NO-cGMP pathway. Penile erection is a neurovascular process that involves dilation of the corpus cavernosum smooth muscle, in which NO and cGMP plays an important role (Gocmez *et al.,* 2011). In addition to elevation of cGMP level, the extracts also modulated other markers of erectile dysfunction, inhibiting the activity of penile PDE 5 and increasing concentration of penile nitric oxide as well as plasma concentration of testosterone.

Testosterone is an established marker of male androgenicity (Yakubu *and* Jimoh*,* 2015). Decrease in the concentration of testosterone of paroxetine-treated rats may be due toxic effects of the selective serotonin reuptake inhibitor on the gonads or o the pituitary glands (Inass *et al.,* 2005). Reversal in the level of testosterone following treatment of the rats with the extracts suggest that the plants have aphrodisiac potentials and contain phytochemicals with androgenic properties which might have stimulated the synthetic capacity of the pituitary glands to produce testosterone. Phytochemical analysis of *F. agrestis* and *C. ferruginea* showed that the extracts were predominated by alkaloids, with appreciable amount of saponins and phenolics. This agrees with the study of Yakubu *et al.* (2005), which implicated the saponin component of *F. agrestis* in enhancing androgenic properties of rats. Similarly, the sexual stimulatory activity of *C. ferruginea* has also been attributed to saponins and alkaloids (Yakubu and Nurudeen, 2015). Sexual behaviours can be boosted by elevated levels of testosterone following administration of aphrodisiacs probably via increase in the action 5α-reductase and conversion of testosterone to its more active derivative dihydrotestoterone and thus triggering libido enhancing effects (Howet and Shale, 2001; Yakubu and Akanji, 2011).

Data from this study showed that in paroxetine-treated rats, testosterone deficiency could be an increased risk conferred by erectile dysfunction as evidenced in literature (Jackson *et al.,* 2010). Low level of testosterone in men coupled with erectile dysfunction increased the risk of cardiovascular diseases (Ho *et al.,* 2016). Low level of testosterone leads to elevated levels of total and low density lipoprotein cholesterol (Carson and Rosano, 2012). Similar findings were observed in this study; prolonged administration of paroxetine to rats resulted in hypotestosteronaemia and dyslipidemia.

Conclusion

From the results obtained in this study, it can be deduced that:

1. Alkaloids and saponins were the major secondary metabolites found in aqueous *C. ferruginea* root and *F. agrestis* stem extracts.
2. Sildenafil citrate inhibited PDE 5 activity between 0.0238-0.119 µM while the aqueous root extracts of *C.ferruginea* root and stem of *F.agrestis* stem inhibited PDE 5 activity between 0.01-0.05 µg/ml. However, concentrations of the extracts higher than 0.05 µg/ml activated Phosphodiesterase 5 activity.
3. Sildenafil citrate, *C. ferruginea* and *F.agrestis* exerted their inhibitory effects on PDE 5 activity via decreased tunover rate of cGMP. However, the presence of these androgenic agents enhanced the binding affinity of PDE 5 for cGMP suggesting that these agents exerted their inhibitory effects upon the formation of [ES] complex.
4. The extracts inhibited PDE 5 activities in the tissues after seven days extract administration.
5. The extracts increased nitric oxide concentration, which may facilitate the pathway leading to blood flow and relaxation of smooth muscles.
6. Paroxetine reduced testosterone concentration of animals used in this study, the individual extract and their combination signicantly increased testosterone concentration after the treatment period.
7. The extracts reduced the risk of cadiovascular disease, coronary artery disease and atherosclerosis after the treatment period.

Overall, this study suggests that aqueous extracts of *C. ferruginea* root and of *F . agrestis* stem and their combination exerted their androgenic properties via inhibition of PDE 5 in a competitive manner comparable to that of sildenafil citrate.

Recommendation for further studies

Findings from this study revealed that aqueous extract of *C. ferruginea* root and stem of *F. agrestis* possesses aphrodisiac and anti-atherosclerotic properties. In view of this, the following are recommended for further investigations:

i. Identify, isolate and characterise the active principles in the plant that are related to male erectile and cardiovascular health

ii. Investigate the erectile and cardiovascular effects of the isolated principles in male rats using similar parameters considered in this study

iii. Propose potential mechanisms of action using purified phosphodiesterase 5

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APPENDIX I

PREPARATION OF SOLUTIONS

Preparation of Paroxetine

Paroxetine was dissolved in tween 80 dispersed in saline solution.

10 mg/kg body weight of rats

1000g rat will receive 10 mg paroxetine

Assuming a rat weighs 200g

5 rats will weigh 1000g

So 10 mg of paroxetine to be given to 1kg of rats (5 rats)

Individual rat will receive a solution of 0.75 ml

Dissolve 10 mg paroxetine in (0.75 x 5) ml = 375 ml

Each tablet contains 20 mg (10 rats) = 7.5 ml, and this will be enough for 10 rats

200 g will receive 0.75 ml

A rat of 180 g will therefore receive 0.75/200 x 180g

0.00375 (factor) x 180 = 0.675 ml

Preparation of Sildenafil citrate

4 mg/kg body weight of rats

5 rats will take 20mg = 3.75 ml

20 rats (3 days) 80 mg = 15 ml

2 doses (50 mg each) = 100 mg was dissolved in 18.75 ml

Ammonium sulphate precipitation

That is:

To determine what amount of 100% saturated ammonium sulphate solution would be needed to add to 50 ml supernatanant to make 50% ammonium sulphate saturation, we have;

$(50 + x)$ x (50%) = (x) x (100%)

Where x is the volume of 100% saturated ammonium sulphate solution needed to be added to the supernatant.

$(50 + x)$ x $(50) = (x)$ x (100)

$2500 + 50x = 100x$

$2500 = 50x$

$x = 50$.

Therefore, to make 50 ml supernatant 50% ammonium sulphate saturated 50 ml of 100% ammonium sulphate saturation was added to the supernatant.

PREPARATION OF SOLUTIONS

1. 0.33M Sucrose solution

$$\text{Molarity} = \frac{\text{No of moles}}{\text{Volume}}$$

$$\text{No of moles} = \frac{\text{mass}}{\text{Molar mass}}$$

Mass =No of moles × molar mass

=0.33M ×342g/mol

=112.86g in 1000ml of distilled water

1×10^{-3} M $MgSO_4$

1×10^{-3} M Imidazole 0.068g

pH = 7.5

Molar mass of $MgSO_4$ = 121g/mol

Molar mass of Imidazole = 68.077g/mol

0.001 is present in 1000mls of distilled water

0.001 × 121 in 1000mls of distilled water

Therefore 0.121g of $MgSO_4$ will be weighed and dissolved in 1000mls

For Imidazole: 0.001 × 0.068g

= 0.000068g of Imidazole will be dissolved in 1000mls

0.121g of $MgSO_4$ and 0.000068g of Imidazole were weighed and dissolved in 1000ml of distilled water and pH was adjusted to 7.5. The solution was refrigerated afterwards.

2. 100µmoles $MgSO_4$

Molar mass = 121g/mol

$$\text{Molarity} = \frac{\text{No of moles}}{\text{Volume}}$$

$$\text{No of moles} = \frac{\text{Mass}}{\text{Molar mass}}$$

$$\frac{100}{100000} = \frac{\text{mass}}{121}$$

$$100 \times 1000 = 1000000 \times \frac{\text{mass}}{121}$$

$$\frac{100000 \times 121}{1000000} = \text{mass}$$

Mass = 12.1g in 1000mls of distilled H_2O

3. 500µmoles of Imidazole

Molar mass = 68.077g/mol

$$\frac{500}{1000000} = \frac{\text{Mass}}{68.077}$$

$$\frac{500}{1000}$$

$$\frac{500}{000000} \times \frac{500}{1000} = \frac{\text{Mass}}{68.077}$$

$$\frac{25}{100000} = \frac{\text{Mass}}{68.077}$$

$$\text{Mass} = \frac{68.077 \times 25}{100{,}000}$$

= 0.01702g in 500mls of distilled H_2O

4. **100% saturation Ammonium sulphate solution**

Into 200mls of distilled water in a beaker, ammonium sulphate was added until it could no longer dissolve in the medium i.e 100% saturation was attained. The solution was stored in a bottle and kept on the shelf until when needed.

5. 55% Trichloroacetic acid

55g of TCA was weighed and dissolved in 100mls of distilled water. The solution was stored in a bottle and refrigerated.

6. 1.0N KOH

Molar mass = 56g/mol

Molarity = Normality × No of replaceable H^+

1.0N KOH = 1.0M KOH

1M in 1000mls

xM in 1000mls

$\frac{100 \times 1M}{1000}$ = 0.1 in 100mls

5.6g in 100mls

Weigh 5.6g and dissolve in 100mls of distilled H_2O

PREPARPTION OF SOLUTIONS FOR PDE V ASSAY

Stock concentration

1.8µM – $MgSO_4$

36µM – Tris buffer

cGMP – 0.36µM

Trichloroacetic acid – 55%

1. $C_1V_1 = C_2V_2$

$C_1 \times 0.1 = 1.8 \times 1.1$

$C_1 = \frac{1.8 \times 1.1}{0.1}$ = 19.8µM would be prepared

Having prepared 100 µM MgSO4 before

$C_1V_1 = C_2V_2$

$100 \times 25 = 19.8 \times V_2$

$V_2 = \frac{100 \times 25}{19.8} = 126.26ml$

Therefore, 19.8µmoles stock = 25ml of 100µmoles + 121.26µM of H_2O

2. $C_1 \times 0.2 = 36 \times 1.1$

$C_1 = \frac{36 \times 1.1}{0.2} = 198$ µMoles

Having prepared 500µM of Tris buffer before

$C_1V_1 = C_2V_2$

$500 \times 25 = 198 \times V_2$

$V_2 = \frac{500 \times 25}{198} = 63.131$

Take 25ml of 500µM + 38.131mls of H_2O

3. $C_1 \times 0.5 = \frac{0.36 \times 1.1}{0.5} = 0.792$ µmoles of cGMP

To prepare 100mls of 0.792 µmoles of cGMP

$\text{Molarity} = \frac{\text{No of moles}}{\text{Volume}}$

No of moles = Mass

Molar mass

Molar mass of cGMP = 345.21g/mol

$\frac{0.792}{1000000} = \frac{\text{Mass}}{345.21} \div \frac{100}{1000}$

$\frac{0.792}{1000000} \times \frac{100}{1000} = \frac{\text{Mass}}{345.21}$

$7.92 \times 10^{-8} = \frac{\text{Mass}}{345.21}$

Mass = 2.734×10^{-5}

In order to avoid wastage of the substrate

Mass to prepare = 0.005g

Molar mass of cGMP =345.21

Volume to be prepared = 250mls

No of moles = $\frac{\text{Mass}}{\text{Molar mass}} = \frac{0.005}{345.21} = 1.448 \times 10^{-5}$

Molarity = $\frac{\text{No of moles}}{\text{Volume}}$

$= 1.448 \times 10^{-5} \div \frac{250}{1000}$

$= \frac{1.448 \times 10^{-5} \times 1000}{250}$

$= 5.79 \times 10^{-5}$ M

$C_1V_1 = C_2V_2$

$5.79 \times 10^{-5} \times 5\text{mls} = 0.792 \times V_2$

$V_2 = \frac{5.79 \times 10^{-5} \times 5}{0.792 \times 10^{-4}} = 3.655$

$5.79 \times 10^{-5} \times 20 = 0.792 \times V_2$

$V_2 = \frac{5.79 \times 10^{-5} \times 20}{0.792}$

$V_2 =$

PROTOCOL FOR PHOSPHODIESTERASE V ASSAY

	Test 1	Test 2	Test 3
Tris buffer (ml)	0.2	0.2	0.2
Enzyme(ml)	0.1	0.1	0.1
$MgSO_4$(ml)	0.1	0.1	0.1

Incubate at 37ºC for 20minutes

cGMP (ml)	0.5	0.5	0.5

Incubate at 37ºC for 20minutes

Crotalus atrox (µl)	0.1	0.1	0.1

Incubate at 37ºC for 10minutes

Trichloroacetic acid (µl)	0.1	0.1	0.1

Spin for 4000g for 10minutes

		Test 1	Test 2	Test 3
Supernatant(ml)	Blank	0.5	0.5	0.5
Distilled H_2O(ml)	1.0	0.5	0.5	0.5
Reagent C (ml)	2.0	2.0	2.0	0.5

Read absorbance at 820nm

TO ESTABLISH THE SUBSTRATE CONCENTRATION FOR PDE V ASSAY

	1	2	3	4	5	6	7
Tris buffer (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled H_2O(ml)	3.2	3.1	2.8	2.3	1.8	1.3	0.3
Enzyme(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$MgSO_4$(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Incubate at 37ºC for 20minutes cGMP (ml)	0.1	0.2	0.5	1.00	1.50	2.00	3.00
Incubate at 37ºC for 10minutes Crotalus atrox (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Incubate at 37ºC for 10minutes Trichloroacetic acid (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Allow to stand for 10minutes

	Blank	1	2	3	4	5	6	7
Supernatant(ml)	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled H_2O(ml)	1.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Reagent C (ml)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Read Absorbance at 820nm

Test	1	2	3	4	5	6	7
	0.004	0.021	0.009	0.044	0.067	0.177	0.215
	0.001	0.008	0.009	0.045	0.056	0.204	0.257
	0.001	0.009	0.046	0.041	0.066	0.175	0.161
Average	0.002	0.0085	0.021	0.043	0.063	0.176	0.236

	P_i (mg/ml)	ΔC/ min	Total Activity	Specific Activity
1	0.001	5×10^{-5}	9.0×10^{-4}	4.091×10^{-3}
2	0.006	3×10^{-4}	5.4×10^{-3}	0.0245
3	0.05	2.5×10^{-3}	0.045	0.205
4	0.11	5.5×10^{-3}	0.099	0.450
5	0.16	8.0×10^{-3}	0.144	0.655
6	0.47	0.024	0.432	1.964
7	0.64	0.032	0.576	2.618

EFFECT OF SILDENAFIL CITRATE ON PDE V ACTIVITY

	0	1	2	3	4	5	6	7	8	9
Tris buffer (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled H_2O(ml)	1.3	1.29	1.28	1.25	1.23	1.20	1.10	0.8	0.3	-
Enzyme(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$MgSO_4$(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Incubate at 37ºC for 20minutes cGMP (ml)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
SC	0.00	0.01	0.02	0.05	0.07	0.1	0.2	0.5	1.0	1.3
Incubate at 37ºC for 20minutes										
Crotalus atrox (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Incubate at 37ºC for 10minutes										
Trichloroacetic acid (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Allow to stand for 10minutes

	Blank	0	1	2	3	4	5	6	7	8	9
Supernatant(ml)	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled H_2O(ml)	1.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Reagent C (ml)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Absorbance was read at 820nm

Test	0	1	2	3	4	5	6	7	8	9
1	0.128	0.059	0.047	0.042	0.039	0.279	0.456	0.697	0.783	0.806
2	0.129	0.058	0.048	0.040	0.040	0.275	0.454	0.698	0.780	0.800
Average	0.1285		0.0475	0.041	0.0395	0.277	0.455	0.6975	0.7815	0.803

EFFECT OF SILDENAFIL, *Fadogia agresis,* and *Cnestis ferruginea* ON PDE V ACTIVITY

	0	1	2	3	4	5	6	7	8	9
Tris buffer (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled H_2O(ml)	1.3	1.29	1.28	1.25	1.23	1.20	1.10	0.8	0.3	-
Enzyme(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$MgSO_4$(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sildenafil citrate (Sc)	0.00	0.01	0.02	0.05	0.07	0.1	0.2	0.5	1.0	1.3
Fadogia agresis(Fa)	0.00	0.01	0.02	0.05	0.07	0.1	0.2	0.5	1.0	1.3
Cnestis ferruginea (Cf)	0.00	0.01	0.02	0.05	0.07	0.1	0.2	0.5	1.0	1.3
Incubate at 37ºC for 20minutes cGMP (ml)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Incubate at 37ºC for 20minutes										
Crotalus atrox (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Incubate at 37ºC for 10minutes										
Trichloroacetic acid (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Allow to stand for 10minutes

	Blank	0	1	2	3	4	5	6	7	8	9
Supernatant(ml)	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled H_2O(ml)	1.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Reagent C (ml)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Read Absorbance at 820nm

Sc Test Sample	0	1	2	3	4	5	6	7	8	9
1	0.123	0.024	0.028	0.026	0.027	0.036	0.038	0.047	0.056	0.806
2	0.125	0.023	0.029	0.030	0.031	0.037	0.039	0.050	0.059	0.800
Average	0.124	0.0235	0.0285	0.028	0.029	0.0365	0.0385	0.0485	0.0575	0.803

STANDARD PROTEIN DETERMINATION

Using LOWRY'S METHOD

REAGENTS FOR PROTEIN DETERMINATION

Reagent A

2.0g of Na_2CO_3

0.4g of NaOH- Dissolve 100mls of distilled water

Reagent B

2.0g of sodium- potassium tartrate in 100mls of distilled water

Reagent C

1.0g 0f $CuSO_4.5H_2O$ in 100mls of distilled water

Reagent D = Reagent A: reagent B : Reagent C

100 1 1

Reagent E

1N Folin-ciocalteau reagent is diluted with 5mls of distilled water

PREPARATION OF STANDARD PROTEIN BOVINE SERUM ALBUMIN (BSA)

0.5g of Bovine serum albumin (BSA) is dissolved in 500mls of distilled water. It was stirred using a magnetic stirrer in order to obtain a homogenous solution. 16 test tubes were used, one of the test tubes was used to prepare the blank which contained all other reagents in other test tubes except BSA (the sample protein)

Step1 – Distilled water was put into the test tubes in their respective volumes that is

Blank	Test tube1	Test tube2	Test tube 3	Test tube4	Test tube5
1000µl	900 µl	700 µl	500 µl	300 µl	100 µl

Test tube 1-5 in triplicates

Step 2 – BSA was added to all other test tubes except the blank to make up 1000µl respectively

Step 3 – 3ml of reagent D was then added to all the test tubes and allowed to stand at room temperature for10minutes

Step 4 – 0.3ml of reagent E (Folin C) was then added to all the test tubes, the mixture was shaken vigorously and allowed to stand for another 30minutees.

Step 5- Absorbance was read at 750nm wavelength

Step 6- The result obtained was used to plot a standard protein curve

PROTOCOL FOR STANDARD PROTEIN DETERMINATION

	Blank	1	2	3	4	5
Distilled water (µl)	1000	900	700	500	300	100
BSA	-	100	300	500	700	900
RGT D	3.00	3.00	3.00	3.00	3.00	3.00
RGT E	300	300	300	300	300	300
Total reaction volume	4.30	4.30	4.30	4.30	4.30	4.30

Absorbance was read at 750nm

STOCK CONCENTRATIONS OF EXTRACT FOR *IN VITRO* ANALYSIS

Fadogia agresis (Fa) = 0.1 mg/ml

Cnestis ferruginea (Cf) = 0.1 mg/ml

Cf + Fa = 0.05mg/ml

Sildenafil citrate (Sc) =1µM

cAMP = 0.054mM

cGMP = 0.0579mM

Reaction volume for Fa, Cf, Cf + Fa = 3.0ml

Reaction volume for cAMP, cGMP = 4.2mls

Volume	Sc (µM)	Cf (µg/ml)	Fa (µg/ml)	Cf + Fa (µg/ml)
0.00	0.00	0.00	0.00	0.00
0.01	0.00238	0.00033	0.00033	0.000167
0.02	0.00476	0.00067	0.00067	0.00033
0.03	0.00714	0.001	0.001	0.0005
0.05	0.0119	0.00167	0.00167	0.00083
0.06	0.0143	0.002	0.002	0.001
0.07	0.0167	0.00233	0.00233	0.00117
0.1	0.0238	0.00333	0.0033	0.00167

Volumes	cGMP (µM)	cAMP (µM)
0.1	1.379	1.290
0.2	2.757	2.581
0.5	6.893	6.452
1.00	13.786	12.905
1.50	20.679	19.357
2.00	27.572	27.100
3.00	41.358	38.714

ARGINASE ASSAY

Assay reaction mixture contained in 1ml: 50 µmol $NaHCO_3$ buffer pH 9.5, 20 µmol arginine and 0.79ml of 10%rat heart homogenate. The reaction mixture was incubated at 37ºCfor 1hour. The reaction was stopped with 1ml of 0.5mol/L $HClO_4$ and centrifuged to obtain clear supernatant and the urea formed was determined using urea kits.

Items (Reagents used)

- 50 µmol $NaHCO_3$ (pH 9.5)
- 20 µmol arginine
- 0.5 µmol $MnCl_2$
- 0.2ml crude extracts (10-1000µg)
- 0.79ml of 10% rat heart homogenate
- 0.5mol/L $HClO_4$

1a. $NaHCO_3$ buffer (pH 9.5)

$NaHCO_3 = 23 + 1+12 + 48 = 84gmol$

$NaHCO_3$ mass = 84×0.043

=3.612g in 1L of solution

=1.806g in 500ml of solution

$pH = PKa + \underline{\log [A^-]}$

$[HA]$

$9.5 = 10.3 + \log \underline{[CO_3^{2-}]}$

$[HCO_3]^-$

$\underline{[CO_3^{2-}]} = \underline{1}$

$[HCO_3]^- \quad 6.31$

$Na_2CO_3 = 1 \div 7.31 \times 0.05mol = 0.0068molin\ 1L$

$NaHCO_3 = 6.31 \div 7.31 \times 0.05ml = 0.043mol\ in\ 1L$

1b. Na_2CO_3 = [(23×2) + 1+12 + 48] g/mol = 106gmol

Mass = Molar mass × mole

= 106 × 0.0068g

= 0.7208g in 1L of solution

= 0.3604g in 500ml of solution

Dissolve 0.36g of Na_2CO_3 and 1.81g of $NaHCO_3$ in about 350ml of distilled H_2O confirm pH to be 9.5

2. 20mmol of Arginine in 1L of solution

Molar mass = 174.2g/mol

Mass = 174.2 × 0.02

= 3.48g in 1Lof solution

= 1.74g in 500ml of solution

3.0.5mmol of MnCl2.4H2O (125.844 g/mol) + 72 = 197.844

Mass = 125.844 × 0.0005

=0.0629g in 1L 0.098922 in 1L

=0.03145g in 500ml solution

0.0495g in 500ml solution

APPENDIX II

CALIBRATION CURVES

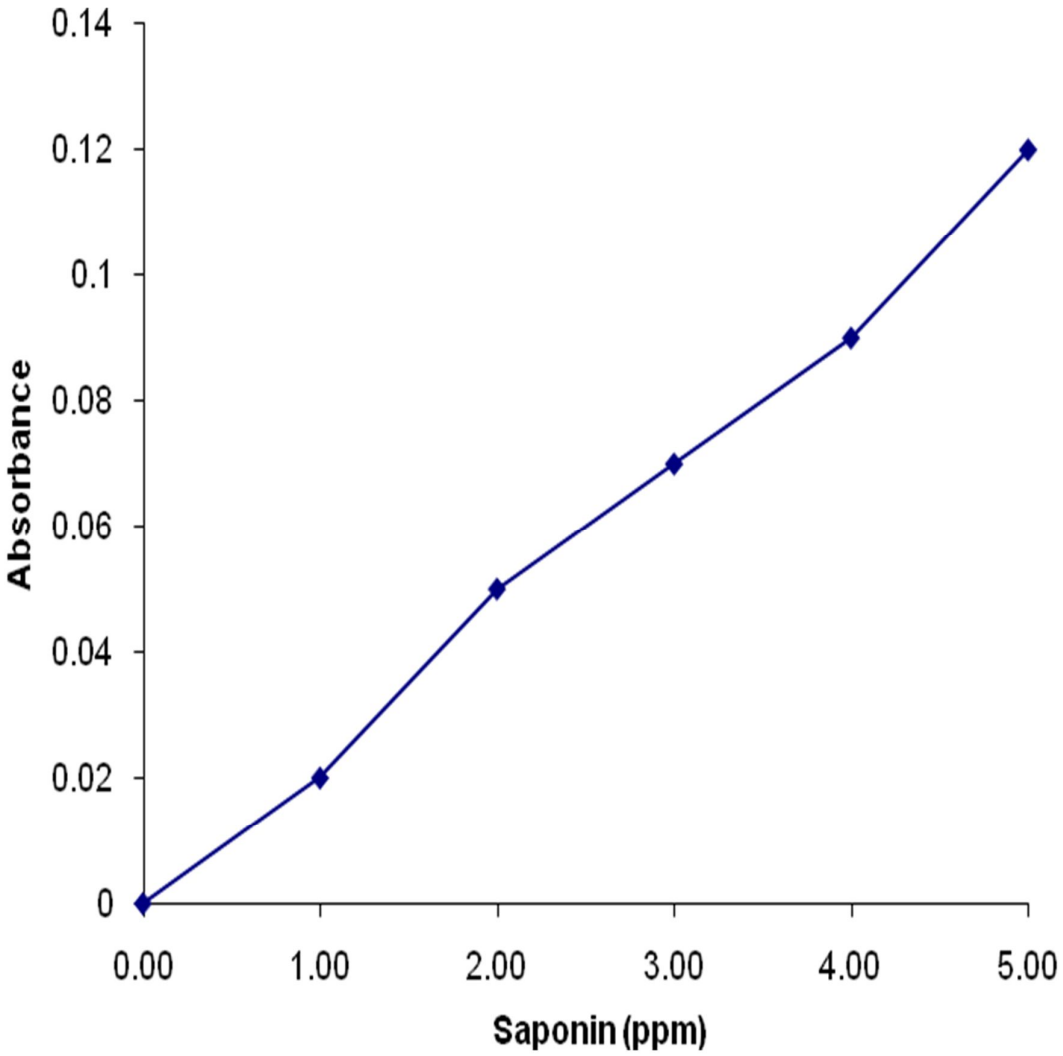

Figure 59: Calibration curve for saponins

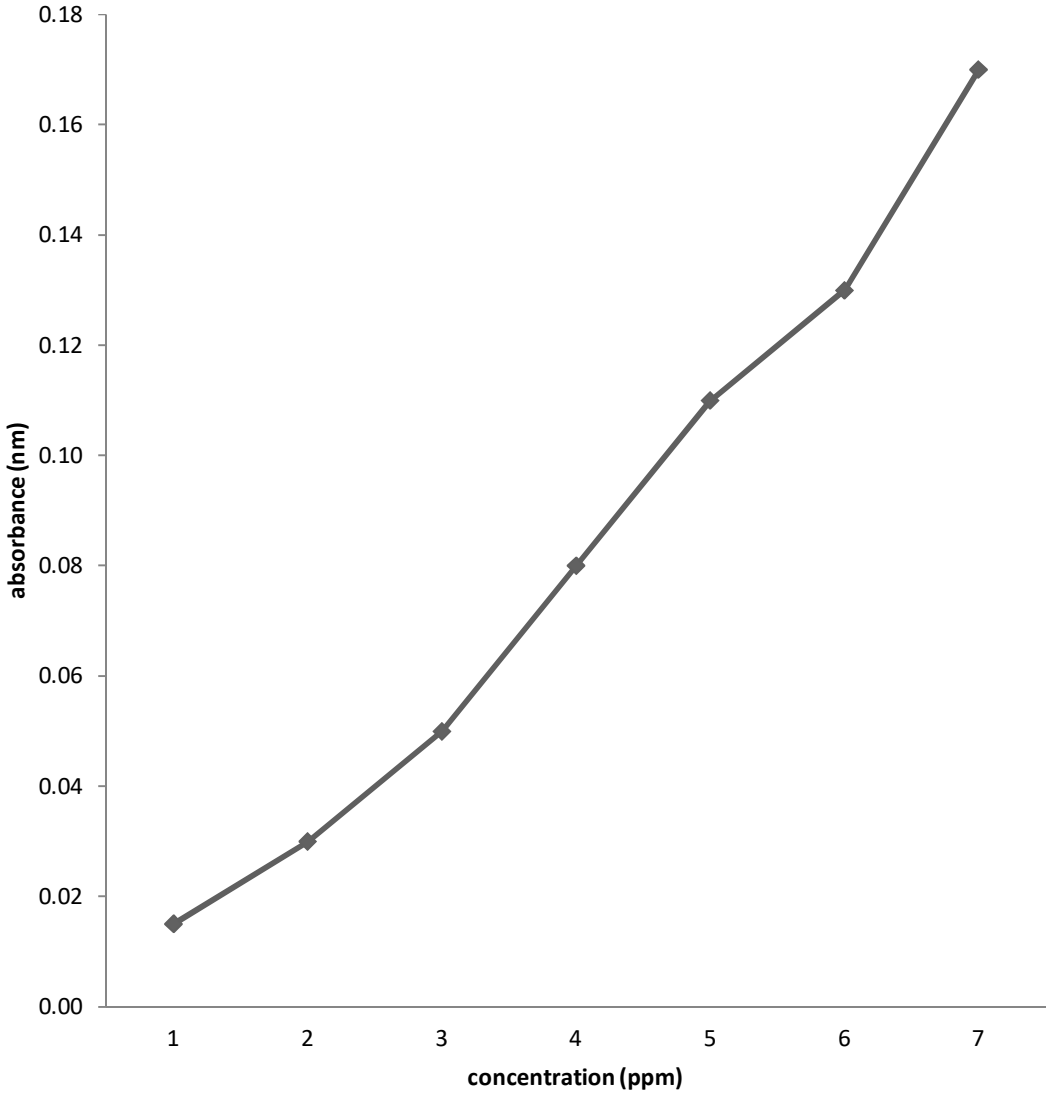

Figure 60: Calibration curve for phenolics

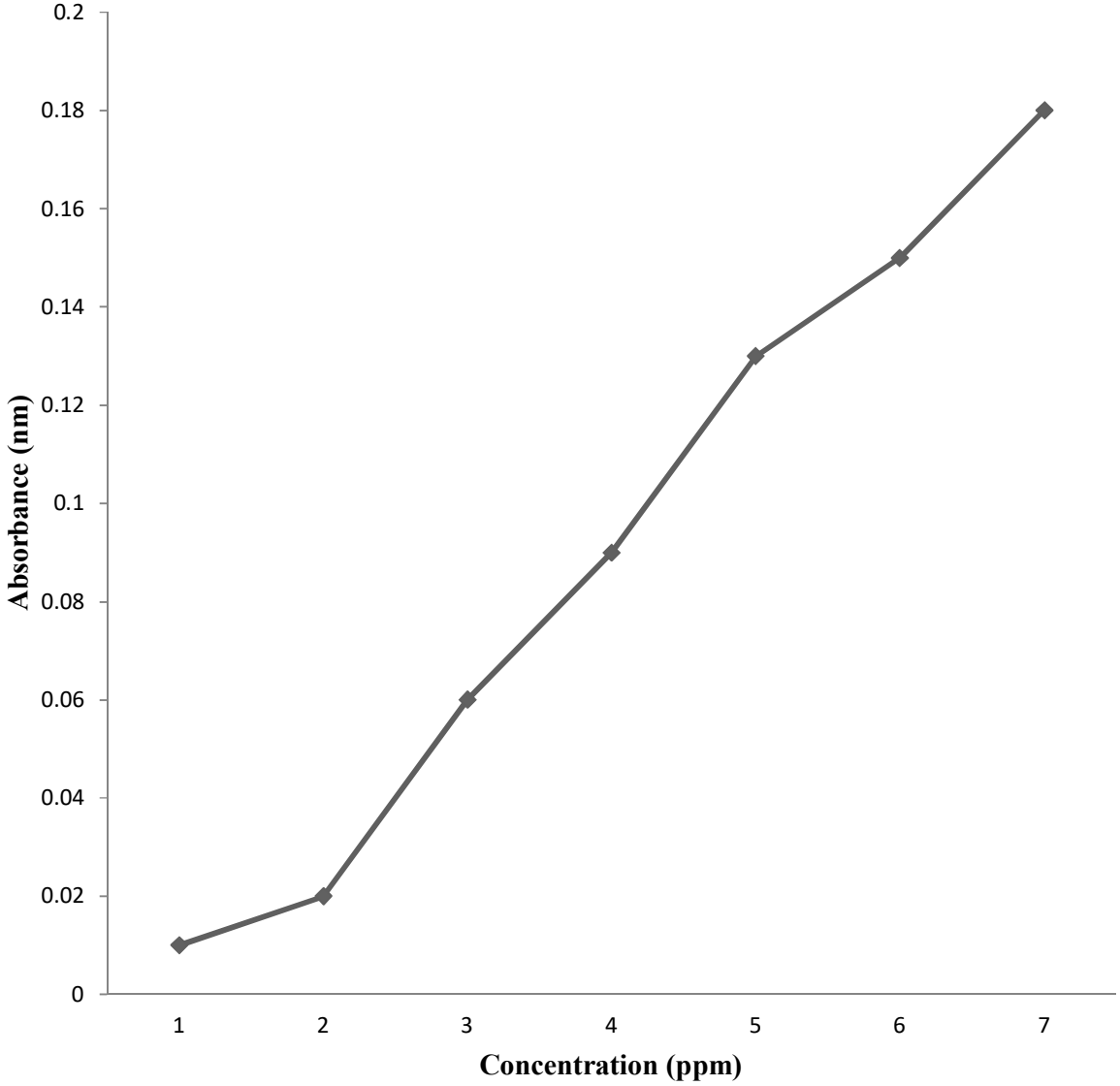

Figure 61: Calibration curve for tannins

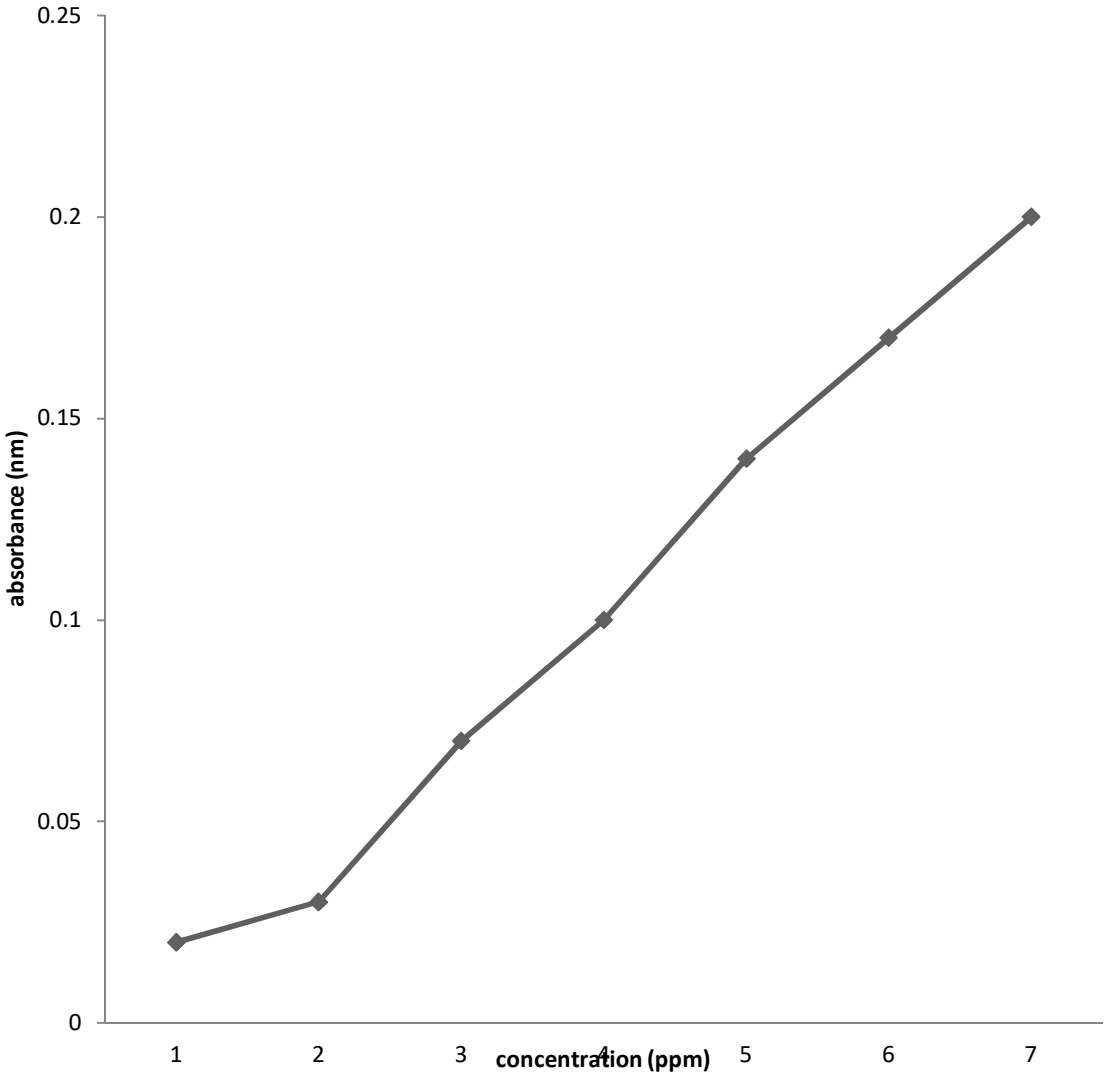

Figure 62: Calibration curve for phlobatannins

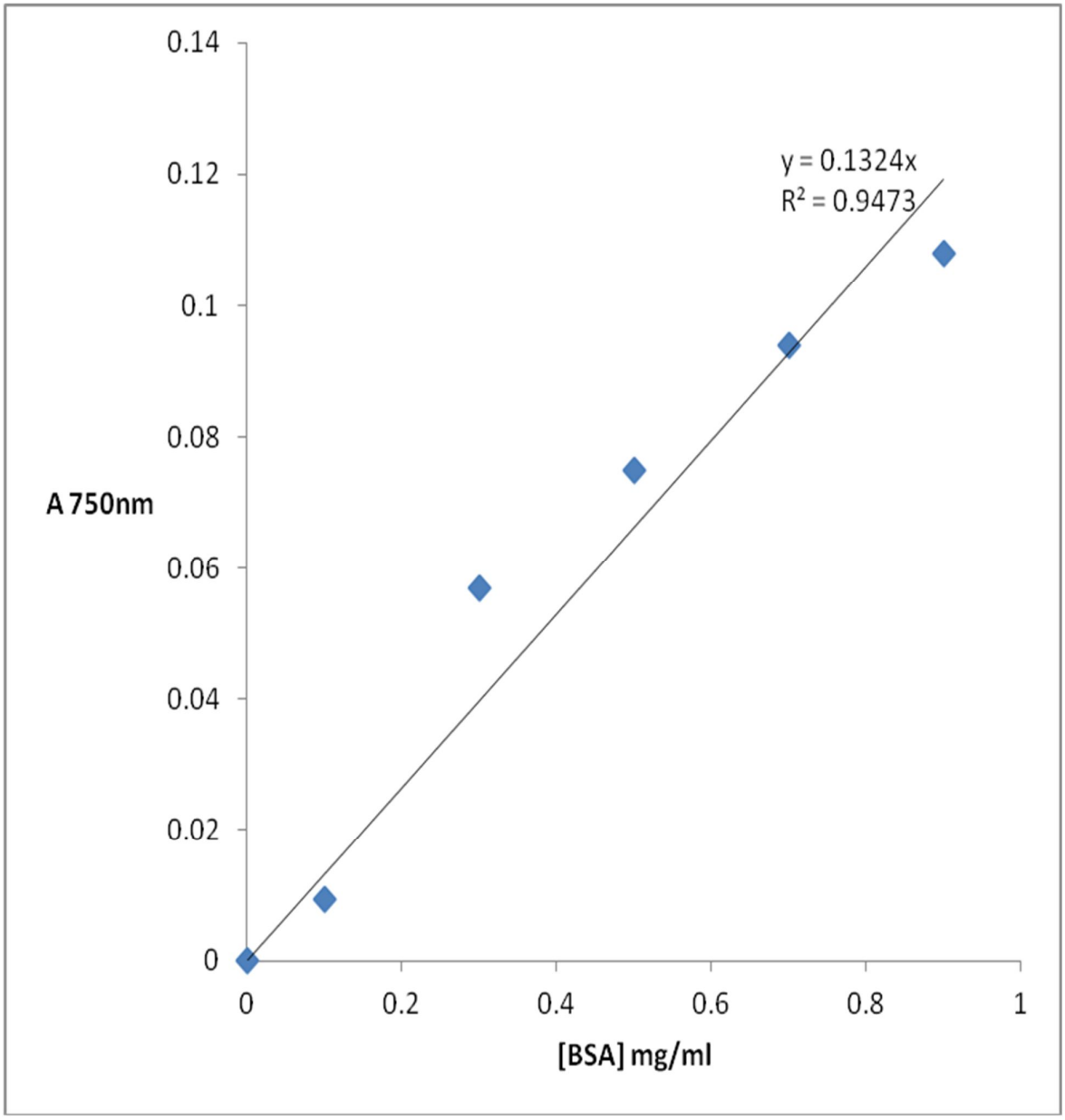

Figure 63: Calibration curve for protein

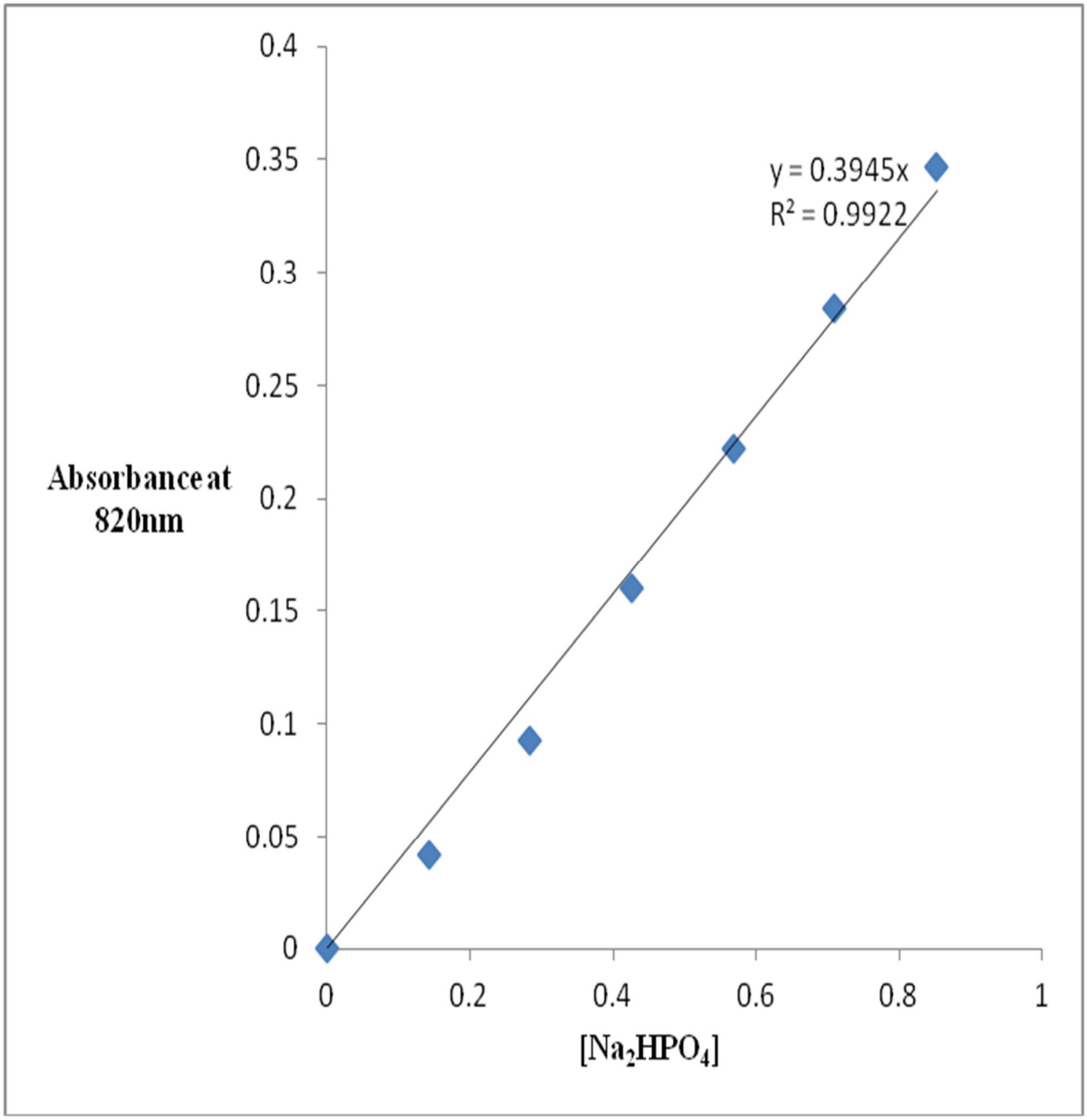

Figure 64: **Calibration curve for inorganic phosphate**

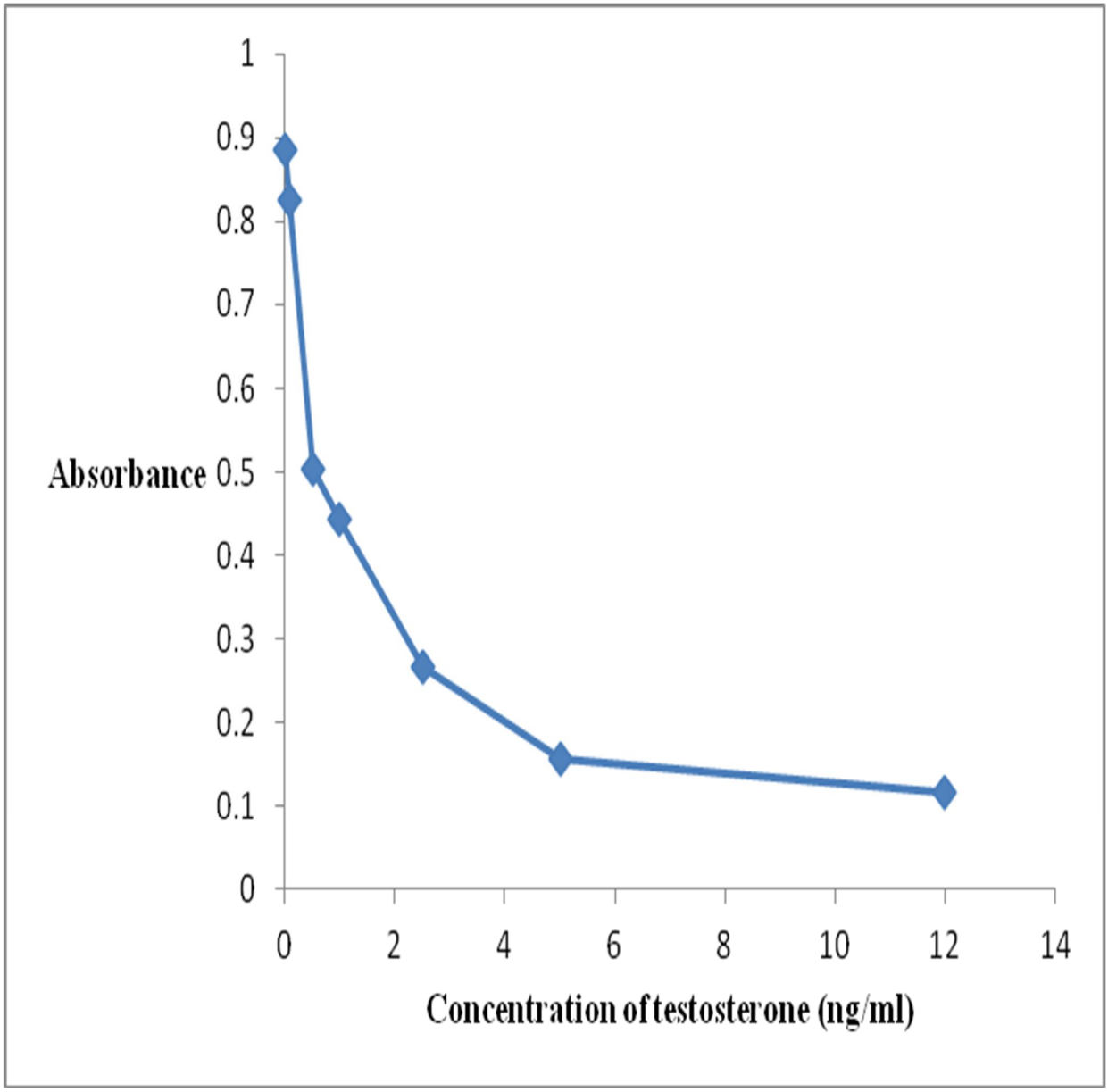

Figure 65: **Calibration curve for testosterone concentration**

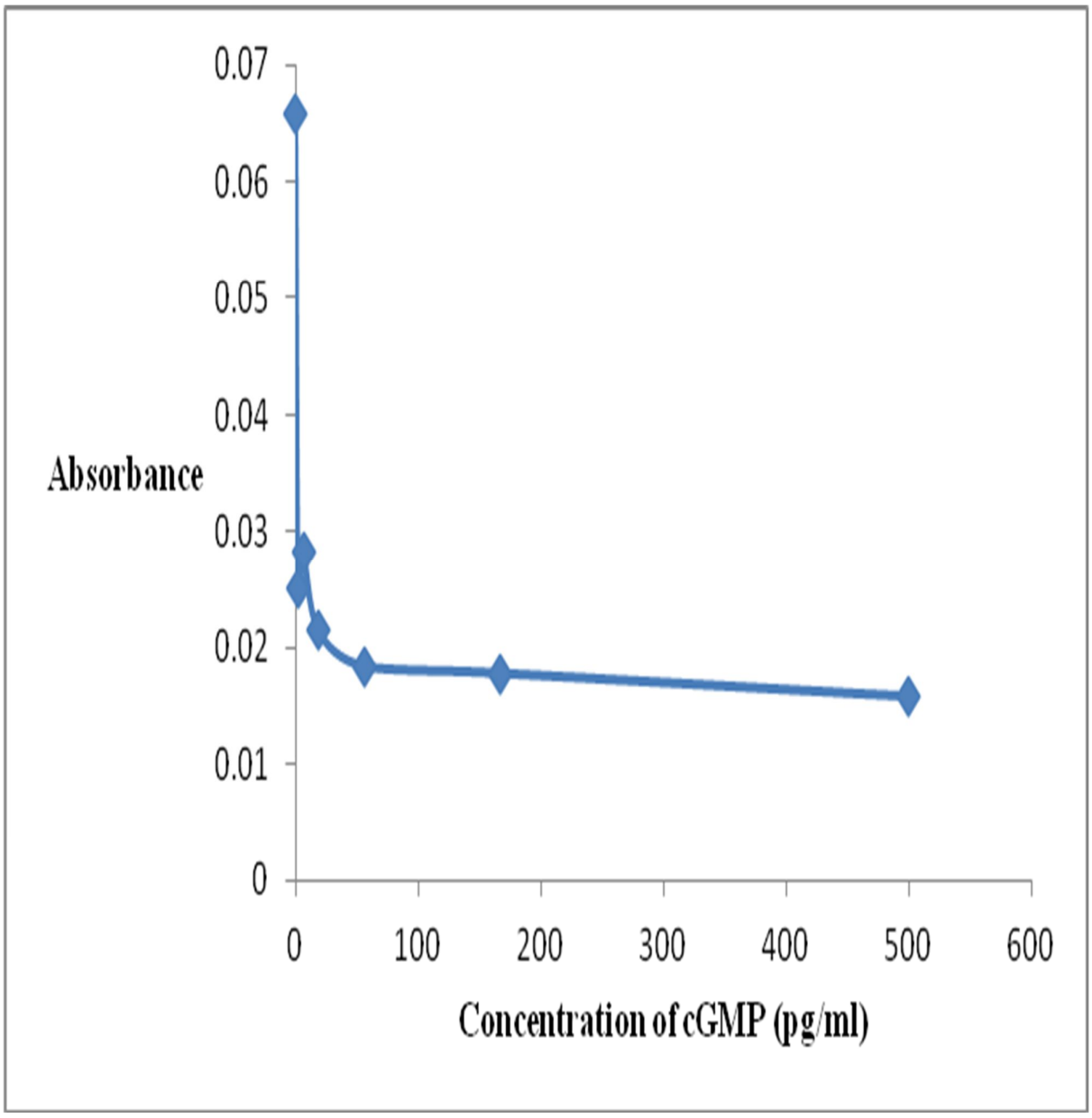

Figure 66: **Calibration curve for the concentration of cyclic guanosine monophosphate**

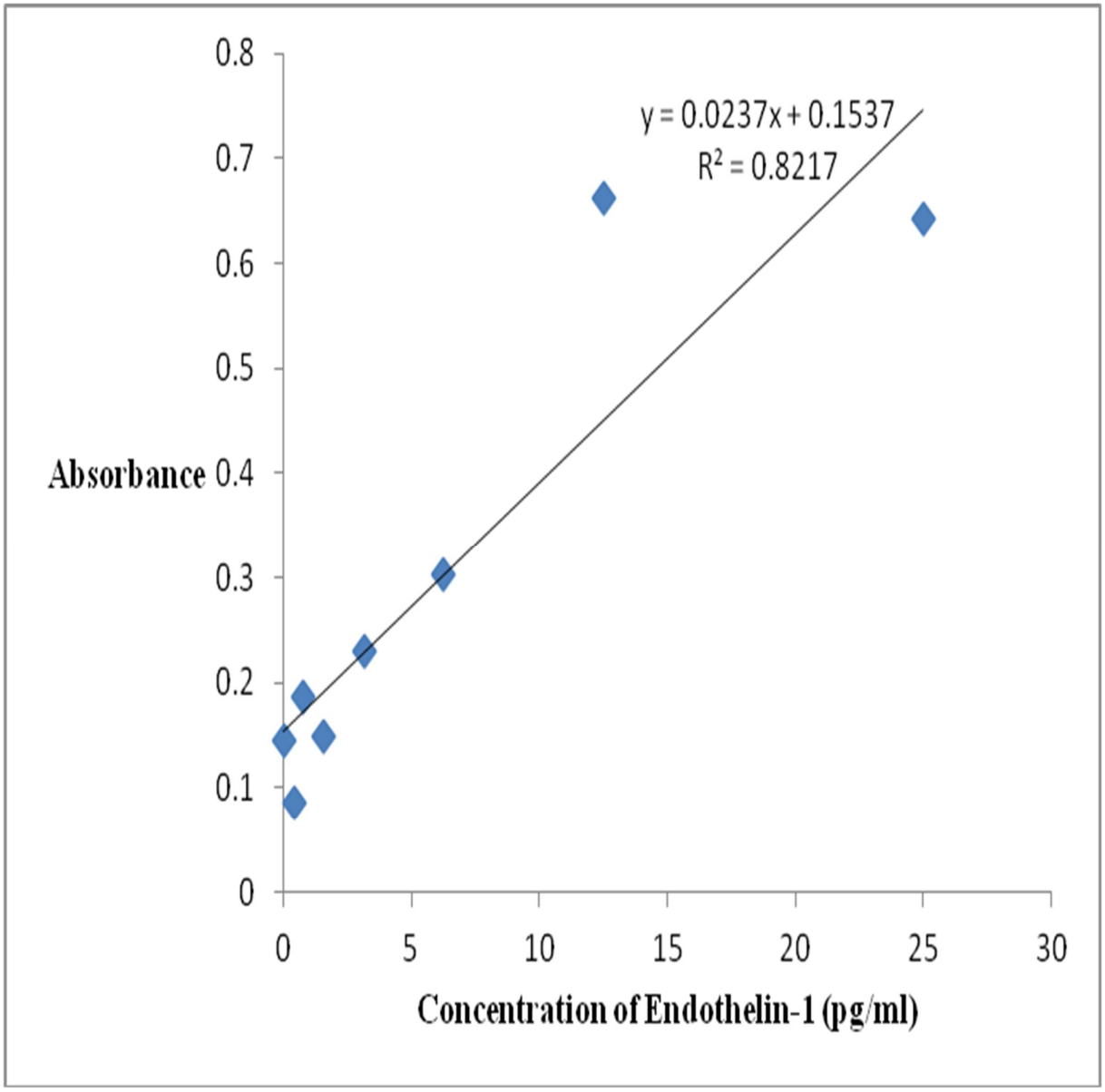

Figure 67: **Calibration curve for the concentration of Endothelin-1**

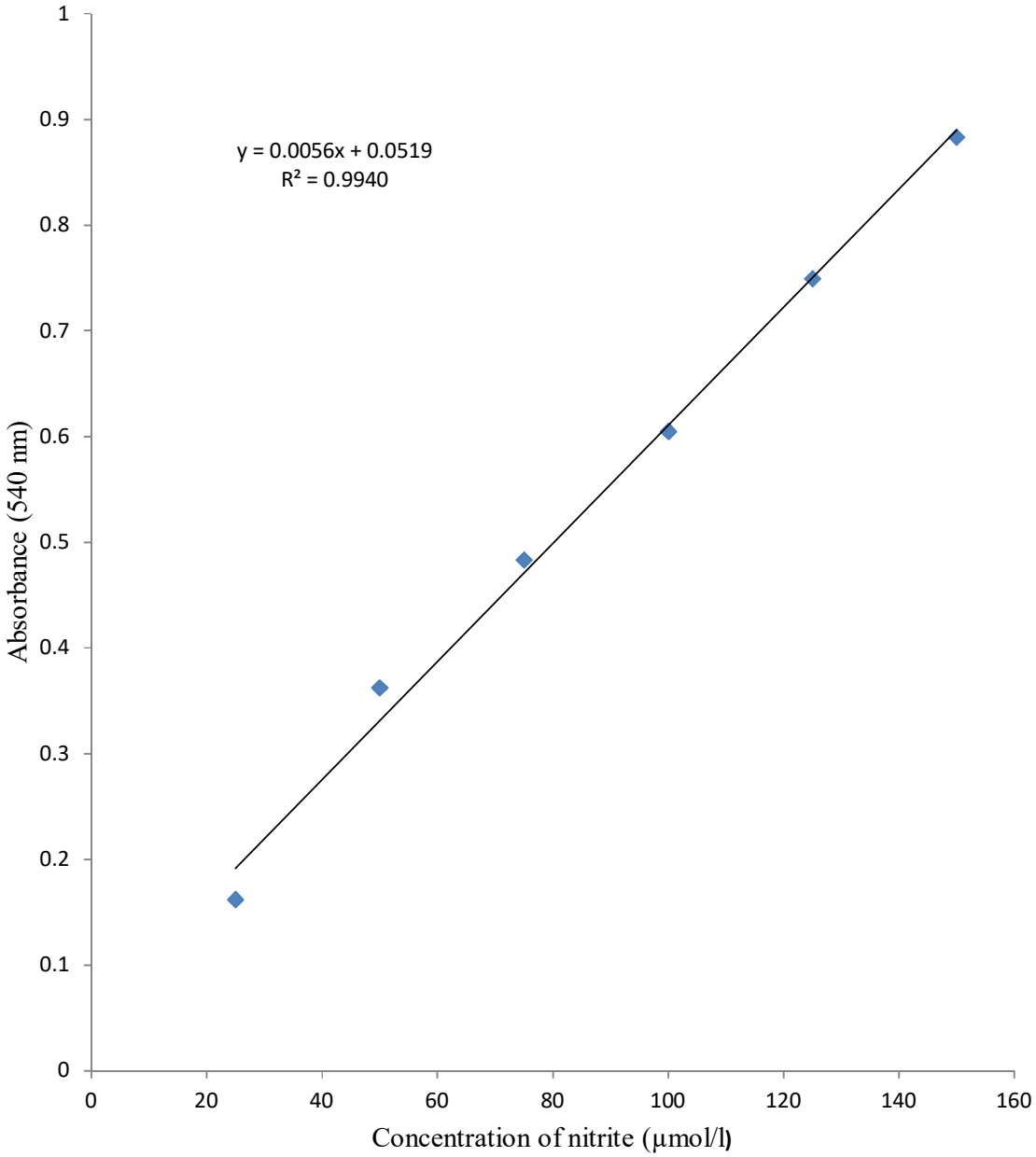

Figure 68: Calibration curve for the concentration of nitrite

Table 19: Calibration of nitrite standard curve

Absorbance	Concentration of nitrite (µmole/l)
0.162	25
0.362	50
0.483	75
0.605	100
0.749	125
0.883	150