CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Erectile dysfunction (ED) is an age-related health challenge with new cases of about 6-66 cases in every 1,000 men per year (Schouten *et al.,* 2004; Colson *et al.,* 2018). The condition is described as the failure to achieve or maintain a penile erection that is sufficient for satisfactory sexual performance (Cazzaniga *et al.,* 2020). It affects about 30% of men aged 40-70 years worldwide (Shabsigh *et al.,* 2004). Its prevalence could be as high as 46.9% among Italians, 42.5% among Germans and 41% among Chinese (Goldstein *et al.,* 2017). In South-western Nigeria, ED ranging from mild to severe was found to occur in 58.9% of men aged 30-80 years in Ogbomoso town (Oyelade *et al.,* 2016) and 55.1% of men aged 18-70 years attending a primary health care facility in Ibadan (Adebusoye *et al.,* 2012).

Physiologically, erectogenic functions of the penis depend on penile tissue relaxation whereas it contracts to be flaccid; this contraction or relaxation is determined by the phosphorylation state of myosin light chain (MLC) of its corpus cavernosa smooth muscle (Dean and Lue, 2005). The MLC is either phosphorylated for contraction or dephosphorylated for relaxation. Phosphorylation of the MLC is mediated by myosin light chain kinase (MLCK) while its dephosphorylation is mediated by myosin light chain phosphatase (MLCP), which are calcium (Ca^{2+})-dependent pro-contraction and Ca^{2+}-sensitization pro-relaxation enzymes, respectively (Dean and Lue, 2005; Hirano, 2007). Therefore, modulation of MLC responsiveness via inhibition of Ca^{2+}-dependent MLCK-mediated activity or Ca^{2+}-sensitization MLCP-mediated activity are required to achieve penile erection.

Key activators of Ca^{2+}-dependent pathway includes Nitric Oxide (NO) and prostaglandin, released into the corpus cavernosa smooth muscle cell by nitergenic fibres and blood vessels upon arousal (Andersson, 2003; Dean and Lue, 2005). Nitric oxide activates soluble Guanylate

Cyclase (sGC) which in turn activates cGMP (Ghalayini, 2004) while prostaglandin activates adenylate cyclase (AC) then cAMP (Moreland *et al.*, 2003) within the cavernous smooth muscle cell. Activation of cGMP or cAMP causes fall in free cytosolic calcium ion (Ca^{2+}), which inhibits the Ca^{2+}/calmodulin activation of MLCK to phosphorylate MLC, thus promoting relaxation to facilitate erection.

In the absence of sexual arousal, phosphodiesterase enzymes (PDEs), usually PDE-3, PDE-4 and PDE-5 are present within the corpus cavernosa smooth muscle cell to degrade cGMP and cAMP (Waldkirch *et al.*, 2005; Uckert *et al.*, 2006), hence, the MLC is continually phosphorylated to maintain penile flaccidity. Therefore, phosphodiesterase inhibitors such as sildenafil citrate is used in treatment of erectile dysfunction (Yuan *et al.*, 2013). Studies have however shown the failure of PDE inhibitors in some patients especially those with erectile dysfunction secondary to diabetes mellitus and hypertention (Francis *et al.*, 2006; Filippi *et al.*, 2009; McCullough *et al.*, 2010). Hence, other therapeutic targets like Rho-Kinase (ROCK) inhibitors that target Ca-sensitization pathway (Sopko *et al.*, 2014) are currently receiving attention as alternative to PDE inhibitors.

The ROCK is an enzyme that maintain cavernosa smooth muscle contraction for penile flaccidity by its ability to deactivate MLCP without alterations in cytosolic calcium ion level (Sopko *et al.*, 2014). Upon sexual arousal, the effect of ROCK is blocked by Protein kinase G (PKG) activated by cGMP. Blockade of ROCK leads to activation of MLCP to dissociate phosphate from the MLC, thereby reducing the responsiveness of MLC to Ca^{2+}/calmodulin modulated actions of MLCK (Dean and Lue, 2005). Therefore, ROCK inhibition also promotes smooth muscle relaxation for penile erection (Somlyo and Sumylo, 2000). Up regulation of ROCK observed in experimental diabetes, cavernous nerve injury and ageing may explain the erectile dysfunctions associated with these conditions; thus, modulation of ROCK activities is another target for the treatment of erectile dysfunction (Jin *et al.*, 2006; Gratzke *et al.*, 2010).

The efficiency of these drugs in modulating erectile activity is well proven but contraindications like cGMP accumulation and unpredicted hypotensive episodes in patients with history of myocardial infarction, stroke, arrhythmias or congestive heart failure are major limitations to their usage (Dong *et al.*, 2011; Vlachopoulos *et al.*, 2013; Sharma *et al.*, 2017). Thus, a window for new drug discovery to treat erectile dysfunction is widely open (Campbell *et al.*, 2018).

Ocimum gratissimum known as Efinrin, Ahuji/Nchanwu and Daidoya by Yoruba, Igbo and Hausa tribe of Nigeria respectively, is a medicinal plant with diverse range of pharmacological activities (Effraim *et al.*, 2010; Prabhu *et al.*, 2009). It has antibacterial (Nakamura *et al.*, 1999), antifungal (Terezinha *et al* 2006), antioxidant (Akinmoladun *et al.*, 2010; Shittu *et al.*, 2016), antimicrobial (Akinyemi *et al.*, 2005), anti-diarrhoeal (Offiah and Chikwendu, 1999), anti-inflammatory (Sahouo *et al.*, 2003), analgelsic (Aziba *et al.*,1999), anti-mutagenic (Obaseiki-Ebor *et al.*, 1993), immunostimulatory (Oladunmoye, 2006) and anti-diabetic (Mohammed *et al.*, 2007; Shittu *et al.*, 2018) activities. It was found efficacious in management of blood pressure via its relaxant effect on vascular smooth muscle *in vivo* and *ex-vivo* (Interaminense *et al.*, 2005; Interaminense *et al.*, 2007; Shaw *et al.*, 2017). Pande and Pathak (2009) reported that *Ocimum gratissimum* improved erectile function and percentage ejaculatory performance in male mice, however the mechanisms involved in this action was not elucidated. Given that smooth muscle relaxation is required for penile erection, it is imperative to know if the smooth muscle relaxant effect of *Ocimum gratissimum* may have bearing on penile erectogenic activity in Wistar rats

A gas chromatographic coupled mass spectrometry analysis of aqueous extract of *Ocimum gratissimum* leaf used for this study showed that it contains twelve compounds including thymol (47%), p-Cymene (6%), p-Menthatriene (6%) and Caryophyllene (4%). This distribution of its component is closely related to the report of Pandey *et al.* (2014). Thymol which is the most abundant is a terpenoid phenol that has been widely used in food industry as preservative and in dentistry as analgesic agent (Ozen *et al.*, 2011). It has calcium modulatory activity (Leal-Cardoso and Fonteles, 1999) and promotes relaxation of canine ventricular cardiomyocyte (Magyar *et al.*, 2002) and relaxation of isolated rat aorta (Peixoto-Neves *et al.*, 2010) by inhibiting calcium channel activities. It was also reported to reduce intracellular calcium ion by inhibiting (Ca^{2+})-ATPase activity in isoproterenol-induced myocardial necrosis in male Wistar rat (Nagoor-Meeran *et al.*, 2015). So far, reports on the effect of thymol on penile smooth muscle are lacking in literature.

1.2 Statement of the problem

Difficulties in penile erection is a worldwide problem that could start early in life and progress with age (Shabsigh *et al.*, 2004; Adebusoye *et al.*, 2012; Goldstein *et al.*, 2017). Hypertension and other cardiovascular diseases are among the leading risk factors of erectile dysfunction (Corona *et al.*, 2014). The efficacy of the available drugs for erectile dysfunction are not in doubt, however, contraindications like cGMP accumulation and unpredicted hypotensive episodes in patients with history of myocardial infarction, stroke, arrhythmias or congestive heart failure still mark their limitations (Dong *et al.*, 2011; Vlachopoulos *et al.*, 2013). Thus, the need for newer intervention with little or no complication is imperative.

1.3 Justification for the study

Ocimum gratissimum leaf is currently gaining fame as a natural therapeutic agent. Over 1,000 *in-vitro* and *in-vivo* studies with about 15 clinical trials have reported the beneficial effects of *Ocimum gratissimum* in health and diseases including its activity on mating behaviour (Pande and Pathak, 2009; Jamshidi and Cohen, 2017). There is however paucity of information on the mechanism involved in the erectogenic effect of *Ocimum gratissimum* and thymol with respect to penile smooth muscle relaxation.

1.4 Aim

This study was designed to investigate the erectogenic activities and mechanisms of action of aqueous extract of *Ocimum gratissimum* leaf and thymol on penile tissue in Wistar rats

1.5 Specific objectives

1. To identify the constituents of aqueous extract of *Ocimum gratissimum* leaf using Gas Chromatography Mass Spectrometry
2. To investigate the effects of aqueous extract of *Ocimum gratissimum* and thymol on mating behaviour, serum reproductive hormone profile, tissue histoarchitecture, nitric oxide, cyclic guanosine monophosphate (cGMP), calcium ion concentration and calcium ATPase activity in penile tissue of Wistar rats.

3. To investigate the effect of aqueous extract of *Ocimum gratissimum* leaf and thymol on the response of penile tissue to acetylcholine in the presence of penile erectogenic pathway modulators including nitric oxide, prostaglandin, cGMP, calcium channel, myosin light chain kinase and rhokinase inhibitors.

CHAPTER TWO

Literature Review

2.1 Structure of the Penis

The penis is a dangling organ comprising of erectile masses of tissues known as corporal bodies. The corpora bodies include a pair of corpora cavernosum lying dorsally and one corpus spongiosum lying ventrally. Each corpora body contains irregular endothelium-lined vascular spaces which are called cavernous spaces. The two corpus cavernosum and the corpus spongiosum is surrounded by a dense fascial sheath known as the tunica albuginea (Hsu *et al*., 2004). The structure and appearance of the penis and its supporting tissues during erection and when flaccid are shown in figure 2.1.

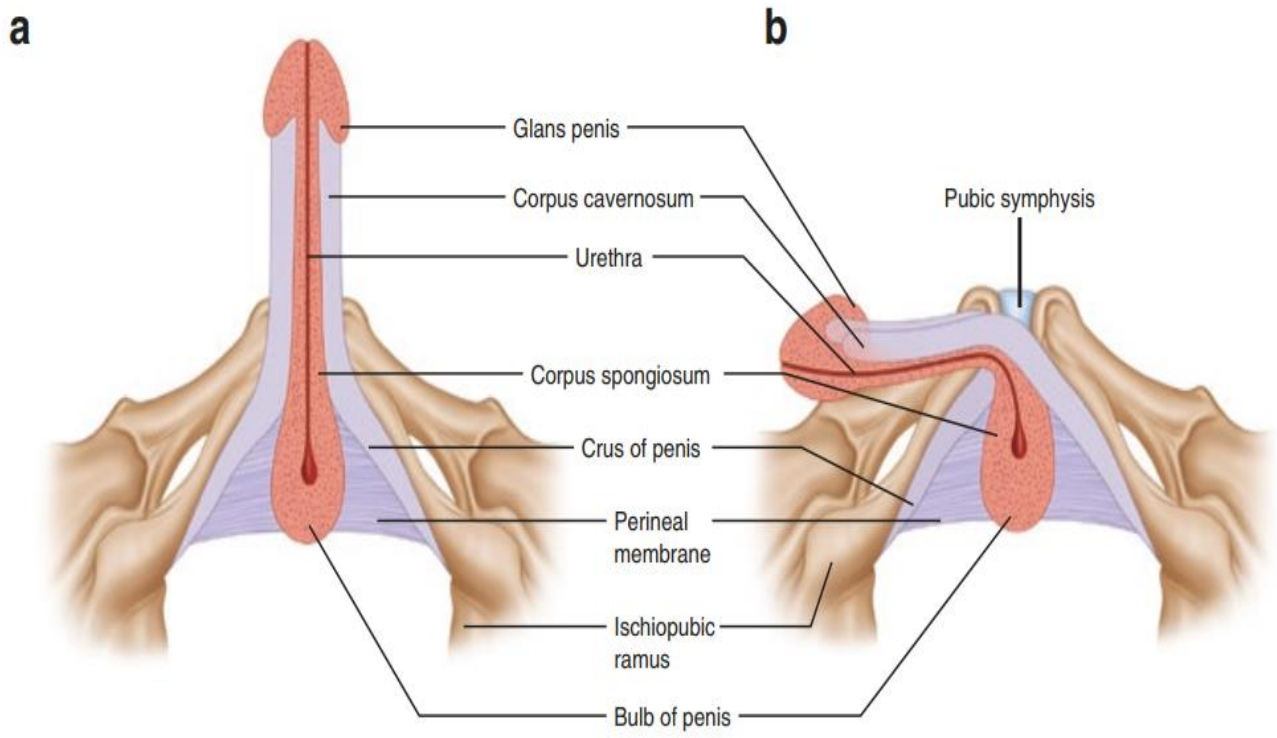
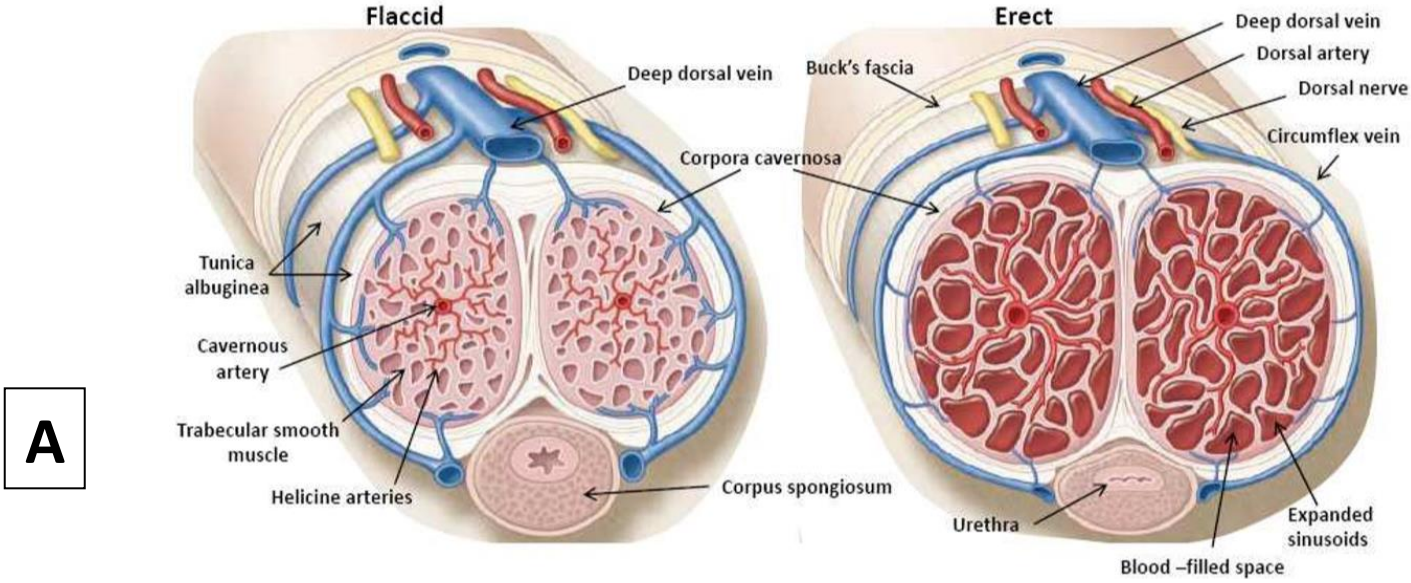


Figure 2.1: Diagram showing the penis and the supporting tissues at (a) erect and (b) flaccid states (Sharma and Kumar, 2017).

2.2 Blood and Nerve supply to the penis

The penis is supplied by pudendal artery, which branches to dorsal artery, cavernosal artery, bulbar artery and urethral artery. The cavernosal arteries nourishes the corpus cavernosum, which is the primary penile erectile body. The venous drainage occurs through the superficial dorsal vein into the pudendal branches of the saphenous vein (Quartey, 1997). The blood supply to the penis is shown in figure 2.2(A).

The penile tissue is supplied by both sympathetic and parasympathetic nerve fibres. The sympathetic penile innervation is from thoracolumbar spinal segment T10-L2 via inferior mesenteric and superior hypogastric plexuses to the pelvic plexus through the hypogastric nerve. Parasympathetic penile innervation arises from sacral spinal segment S2-S4 to the pelvic plexus via the pelvic nerve. The pudendal nerve innervates the external sphincter, bulbo-spongiosus and ischiocavernosus muscles and also provides sensory fibres to the dorsal nerve of the penis. The sacral parasympathetic neurons are responsible for the erectile function and are influenced by a cortical-sacral efferent pathway while sympathetic innervation of the penis mediates the detumescence after the orgasmic relief, and in the absence of sexual arousal it maintains the penis in the flaccid state (Ibrahim *et al.*, 2016). The innervation of the penis is shown in figure 2.2(B).



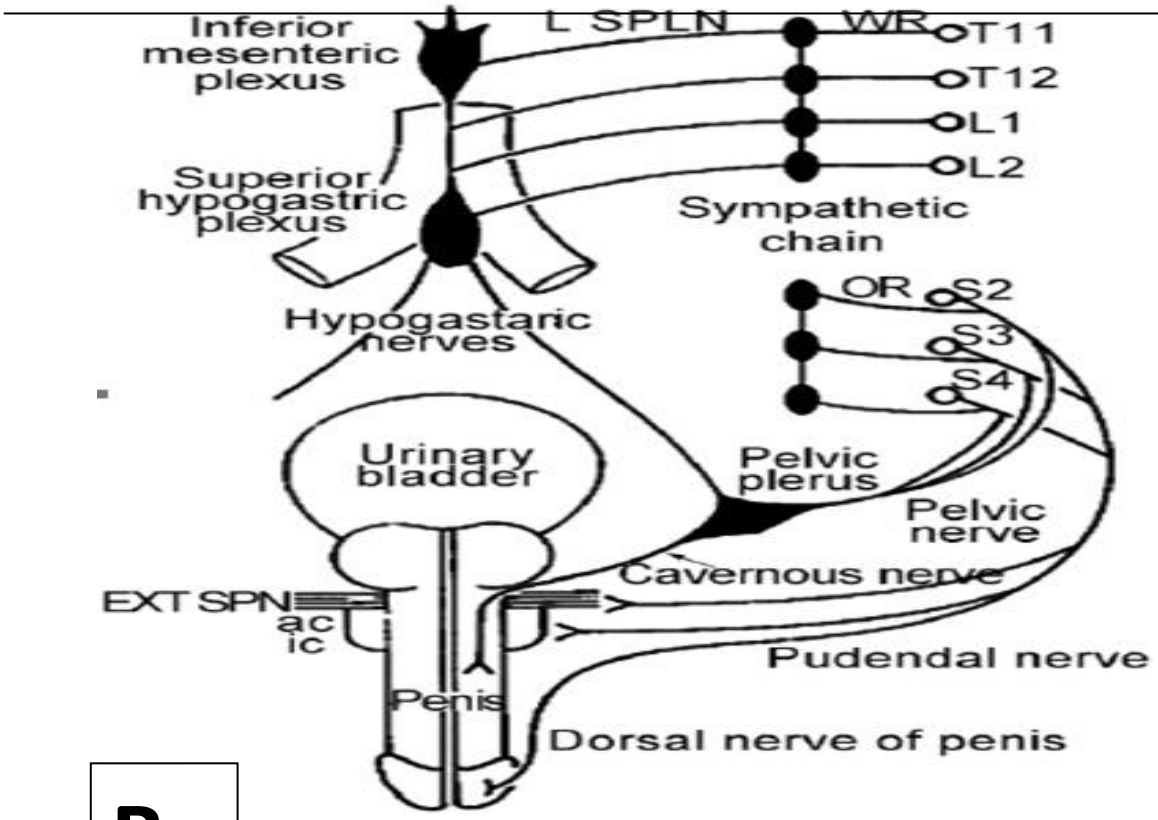


Figure 2.2: Diagram showing the (A) Blood supply (Sharma and Kumar, 2017) and (B) Nerve supply to the penis (Ibrahim *et al.*, 2016)

2.3 Penile Erection

Penile erection is a neurovascular event that depends on neural integrity, a functional vascular system, and healthy cavernosa tissue. The process involves the regulation of penile hemodynamics (Kandeel, *et al.*, 2001) relying on signal transfer from the nervous systems (Gratzke *et al.*, 2010) and on the interplay between local physiological mediators including neurotransmitters, vasoactive agents and endocrine factors (Steers *et al*., 2000).

Tactile, visual, olfactory or imaginative stimuli leading to penile erection are integrated in septal portion of the hippocampus, the anterior cingulate gyrus, the anterior thalamic nuclei and the mammillothalamic tract and the mammillary bodies of the brain (Giuliano *et al*., 1995; Azadzoi *et al*., 2013). The sensory impulse leading to reflexogenic penile erection is generated by the touch, vibration, or temperature receptors under the penile skin and glans where it travels through the pudendal nerve to the dorsal roots of the second, third and fourth sacral nerves (Yang and Bradley., 1998). The efferent motor impulses are generated in the ventral roots of the spinal sacral segments two, three and four (S_2-S_4) and carried through the pelvic parasympathetic nerves to the penis where it facilitates the relaxation of the corporal bodies and arterioles hence, more blood flow to the penile tissue (Wespes *et al*., 1990). The intracorporeal pressure increases as the penile sinusoids engorge due to increased blood flow and the veins between the tunica albuginea and corporal bodies are compressed and blocked because of increased intracorporeal pressure. This does not allow venous blood to return, thereby maintaining rigidity of organ (Dean and Lue 2005).

Therefore, irrespective of the stimuli, penile erection is dependent on the relaxation of the penile arterioles and the corpus cavernosum (Giuliano et al., 1995).

2.4 Molecular Mechanism of Penile Smooth Muscle Relaxation

The molecular mechanism underlying penile erection is a cumulative activity of two interconnected pathways, calcium-dependent pathway and Rho-A kinase pathway, both of which are characterised by different stages of signal transduction and each stage can serve as point of intervention in translational studies (Mas, 2010).

2.4.1 Calcium-Dependent Penile Smooth Muscle Relaxation Pathway

Calcium/calmodulin-dependent pathway of penile smooth muscle relaxation involves the modulation of intracellular calcium ion level of the penile smooth, through the activation of cyclic Guanosine MonoPhosphate (cGMP); which may be dependent or independent on carvernosal endothelium (Dean and Lue, 2005). While the cavernosal endothelial independent activation of calcium/calmodulin-dependent relaxation pathway is initiated by direct release of nitric oxide from non-adrenergic non-cholinergic nerves, the endothelial dependent activation of calcium/calmodulin-dependent relaxation pathway involves the synthesis of nitric oxide and prostacyclins which are endothelium relaxing factors (Morelli *et al*., 2004).

Therefore, inputs from (1) cavernosal endothelium, (2) cyclic nucleotides (3) cytosolic calcium ion and (4) myosin light chain make up the calcium-dependent penile smooth muscle relaxation pathway.

2.4.1.1 Role of endothelium in Calcium-Dependent Penile Smooth Muscle Relaxation Pathway

Acetylcholine binds to muscarinic receptors on endothelial cells, which increase inositol triphosphate (IP_3), which in turn increases Ca^{2+} level in the endothelium. The rise in endothelia Ca^{2+} stimulates the release of endothelial nitric oxide synthase, an enzyme that facilitate nitric oxide synthesis from l-citruline (Leite *et al.* 2007). Also, the binding of acetylcholine to muscarinic receptor of the endothelium leads to activation of phospholipase A_2, which liberates arachidonic acid from membrane-bound phospholipids in endothelial cells. The liberated arachidonic acid is converted to cyclooxygenase (COX) which leads to production of smooth muscle relaxing prostaglandin E_2 and I_2 (Moreland *et al*., 2003).

The released nitric oxide and prostaglandin diffuses into adjacent smooth muscle cells of corpora cavernosum where they respectively activate cyclic guanosine monophosphate and

cyclic adenosine monophosphate (Dean and Lue 2005). The synthesis and release of endothelium relaxing factors is shown in figure 2.3.

2.4.1.2 Role of cyclic nucleotides in the Calcium-Dependent Penile Smooth Muscle Relaxation Pathway

Cyclic nucleotides, cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) are the intracellular mediator of nitric oxide and prostaglandin relaxant activities (Trigo-Rocha *et al*., 1993). While nitric oxide activates soluble guanylyl cyclase (sGC) to generate cyclic Guanosine MonoPhosphate (cGMP), Prostaglandin activates adenyl cyclase (AC) to generate cyclic adenosine monophosphate (cAMP). These second messengers, cGMP and cAMP respectively activate protein kinase G and protein kinase A, the enzymes that facilitate reduction of cytosolic calcium ion level in corpus cavernosum (Saenz de Tejada *et al.* 2000). The role of cyclic nucleotide in penile smooth muscle relaxation is summarize in figure 2.5.

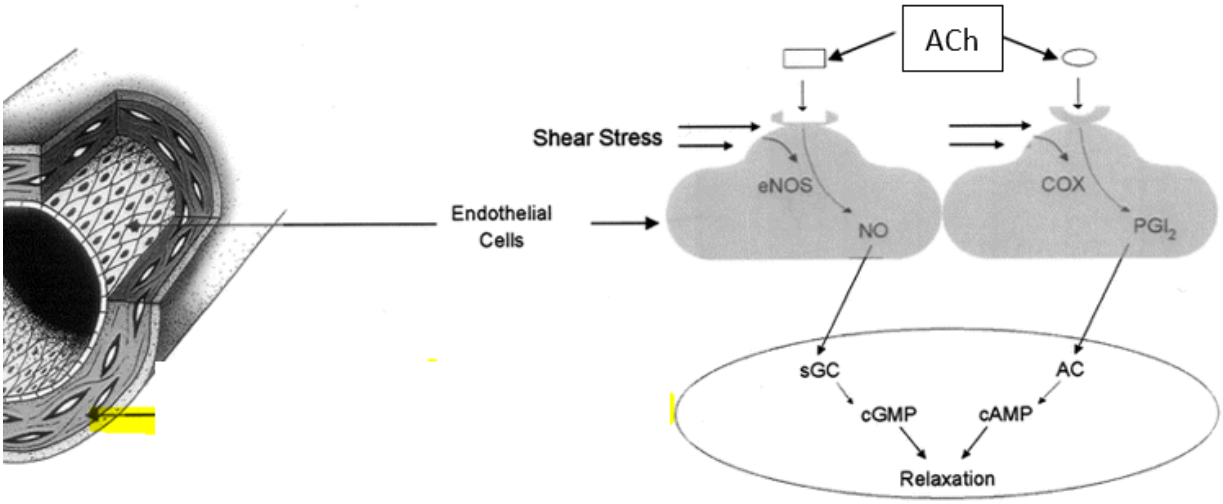


Figure 2.3: synthesis and release of endothelium relaxing factors (Bryan *et al*., 2005).

nitric oxide (NO); prostacyclin (PGI2); AC (adenylyl cyclase); (cAMP) cyclic adenosine monophosphate; cGMP (cyclic guanosine monophosphate); COX (cyclooxygenase); eNOS (endothelial nitric oxide synthase); sGC (soluble guanylyl cyclase).

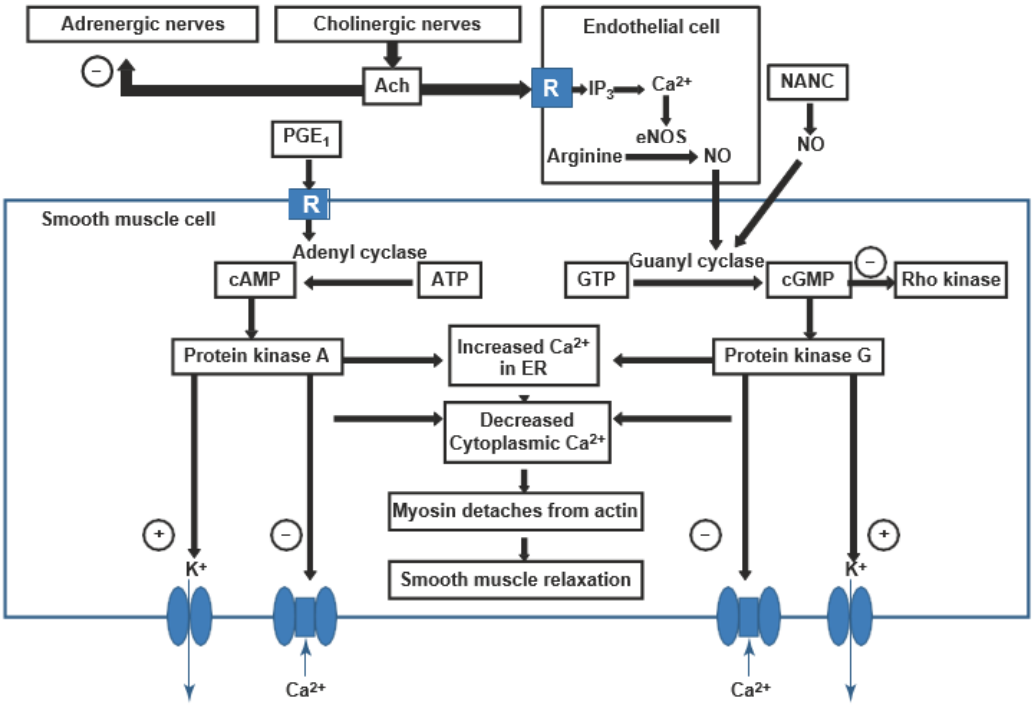


Figure 2.4: Schematic diagram showing the role of cyclic nucleotide in penile smooth muscle relaxation. (Sharma and Kumar, 2017).

Ach - acetylcholine), *PGE1* - prostaglandin E_1, *IP3* - inositol triphosphate, *Ca^{2+}* - calcium, *K^+* - potassium, *eNOS* - endothelial nitric oxide synthase, *NANC* - noradrenergic noncholinergic, *NO* - nitric oxide, *R* receptor, *ATP* - adenosine triphosphate, *cAMP* - cyclic adenosine monophosphate, *GTP* - guanosine triphosphate, *cGMP* - cyclic guanosine monophosphate, *ER* – endoplasmic reticulum.

2.4.1.3 Modulation of cytosolic calcium ion level in Calcium-Dependent Penile Smooth Muscle Relaxation Pathway

Reduction of intracellular calcium ion level is the major activity that differentiate calcium/calmodulin-dependent penile smooth muscle relaxation pathway from Rho kinase pathway (Berridge, 2008). While relaxation occurs through calcium calmodulin pathway when the intracellular calcium ion level decreases, relaxation may still occur without changes in the intracellular calcium ion level through rho-a kinase modulation pathway (Wang *et al*., 2002; Sumlyo and Sumlyo, 2003). Modulation of intracellular calcium ion level for penile erection in the calcium-dependent pathway could be achieved by: (1) reducing Ca^{2+} influx from cavernosal extracellular to intracellular space (2) reducing Ca^{2+} release from the sarcoplasmic reticulum (SR), the intracellular calcium store or (3) removal of intracellular Ca^{2+} by ATPase driven ion pumps (House *et al*., 2008).

Passage of calcium from extracellular to intracellular cavernosal space occurs through voltage gated calcium channels expressed in the corpus cavernosum (Zeng *et al*., 2005). The rise in nitric oxide has been documented to bring about closure of voltage gated calcium channel thereby facilitating smooth muscle relaxation Yuill *et al*., (2009). Beyond closing of voltage gated calcium channel, Yuill *et al*. (2009) also showed that nitric oxide suppresses the activities of ryanodine receptors, the receptors that initiate calcium release from intracellular store to the cytosol (Essin and Gollasch, 2009). Furthermore, Ca^{2+}-ATPases activity is also activated by NO and cGMP to promote the transfer of calcium ion from the cytosol to the sarcoplasmic reticulum thereby maintaining a low intracellular calcium level for smooth muscle relaxation (Cohen *et al*., 1999; Pritchard *et al*., 2010). The cumulative effect of this intracellular calcium ion level modulation is to regulate the myosin light chain phosphorylation within the penile smooth muscle (Sumlyo and Sumlyo, 2000).

2.4.1.4 Role of myosin light chain Modulation in Calcium-Dependent Penile Smooth Muscle Relaxation Pathway

The phosphorylation state of myosin light chains (MLC) is the prerequisite for penile smooth muscle contraction or relaxation. The penile smooth muscle with phosphorylated myosin light chain contracts, the smooth muscle relaxes upon dephosphorylation of its myosin light chain (Sumlyo and Sumlyo, 2000). The enzyme responsible for the initiation of MLC phosphorylation is myosin light-chain kinase (MLCK), the activity of which depends largely on free intracellular calcium ion (Ca^{2+}). In the absence of sexual arousal or penile erectile stimulus, Ca^{2+} binds to calmodulin (CaM) to form a Ca^{2+}-CaM complex. This complex activates myosin light chain kinase (MLCK), which in turn phosphorylates myosin light chain (p-MLC) followed by the onset of the smooth muscle contraction (Takahashi *et al*., 2003). Therefore, reduction of free intracellular calcium level prevents the activation of myosin light chain kinase and phosphorylation of myosin light chain thereby initiates smooth muscle relaxation.

Besides this Ca^{2+}-dependent contractile mechanism, there is also evidence for a Ca^{2+}-independent mechanism operating to maintain smooth muscle contraction and relaxation (Chitaley *et al.,* 2001). This was evidenced from the observation of DeFao and Morgan (1985), Karaki (1990) and Hannan *et al*., (2013) that in agonist-induced smooth muscle contraction, the contraction response still persists even after the rise in calcium level has becomes normal. This was corroborated by the findings of Andersson (2003) and Jin *et al.* (2006) that the phosphorylation of myosin light chain through calcium-dependent pathway only initiates smooth muscle contraction, which is transient, the sustenance of the contraction is achieved via Rho kinase pathway. Therefore, modulation of rho kinase pathway is an integral process in penile smooth muscle relaxation

2.4.2 Rho-kinase Pathway in Penile Smooth Muscle Relaxation

Rho-kinase is an enzyme that act to ensure the sensitivity of myosin light chain to calcium-induced contraction even at low cytosolic calcium level, hence, the pathway is otherwise known as calcium-sensitization pathway (Uehata *et al*., 1997). As earlier noted, the contraction resulting from myosin light chain phosphorylation in calcium dependent pathway is transient, this is because another enzyme known as Myosin Light Chain Phosphatase (MLCP) is

intrinsically expressed in smooth muscle (Sumlyo and Sumlyo 2000). The function of MLCP is to prevent contractions by dephosphorylating the MLC immediately after they are phosphorylated by MLCK (Takahashi *et al*., 2003), hence, contractions induced by MLCK are sustained via the inhibitory activity of rho-kinase on MLCP (Kimura *et al*., 1996).

Rho-kinase is a cytoplasmic serine/threonine-specific kinase that serves as an effector of RhoA protein (Pfitzer and Arner, 1998). The molecular processes responsible for RhoA activation is via activation of G-protein Couple Receptor (GPCR) by contraction agonists like norepinephrine and angiotensin (Amano *et al*., 2000), as well, RhoA may also be activated by membrane depolarization (Wang *et al*., 2002). Upon activation, RhoA activates Rho Kinase, which in turn phosphorylates MLCP to make it inactive. The inactivation of MLCP there allows the contraction induced by MLCK to be sustained (Kimura *et al*., 1996)

The modulation of rho-kinase activity in order to achieve penile erection has been well studied. Sauzeau *et al*. (2000), Sawada *et al*. (2001), Chitaley and Webb (2002) among other researchers reported that the activity of the released nitric oxide and the activated cyclic nucleotide is not limited to lowering of intracellular calcium ion level only, they also act to inhibit the activation of RhoA protein and Rho kinase thereby spearing the pro-relaxant activity of MLCP. The schematic representation of the smooth muscle relaxation via Rho kinase pathway is shown in figure 2.5.

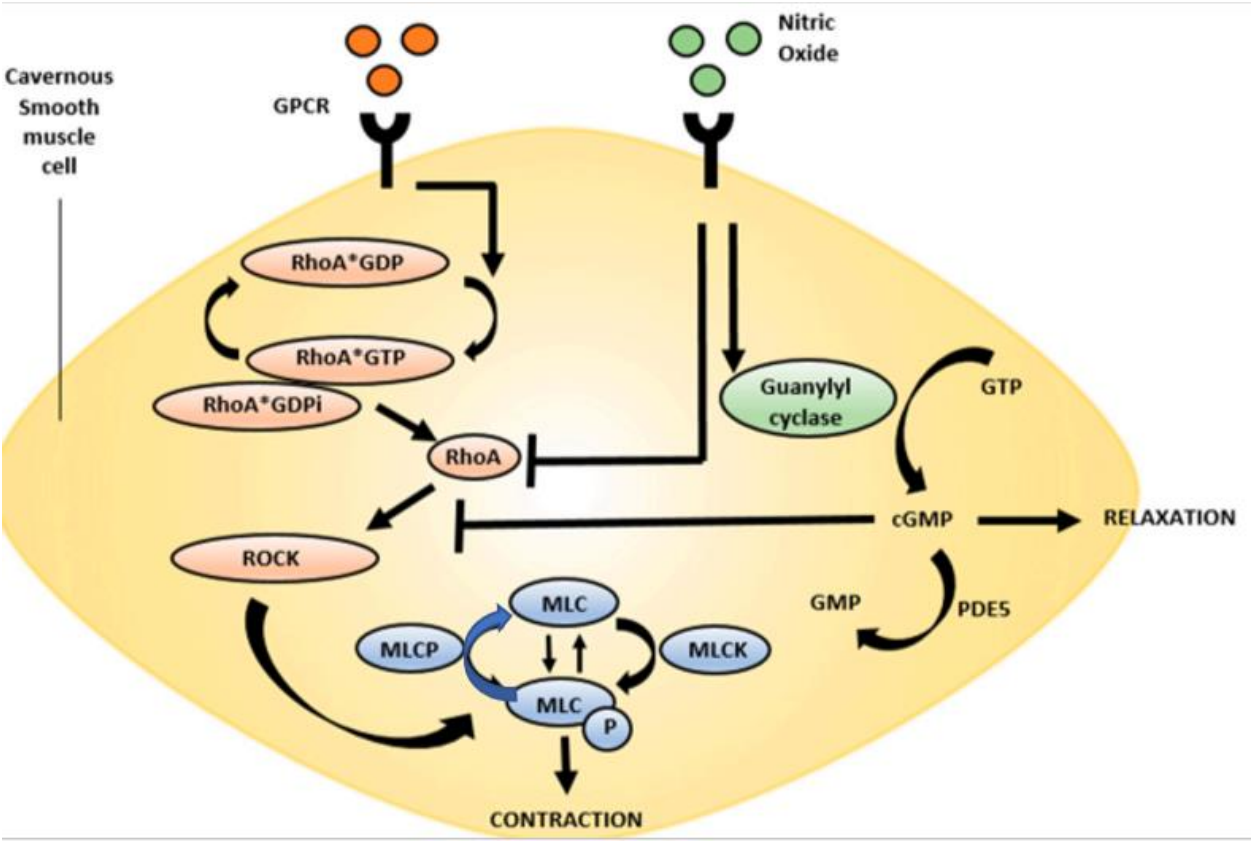


Figure 2.5: Schematic representation of smooth muscle relaxation via Rho kinase pathway (Campbell and Burnett, 2017)

2.5 Detumescence

Detumescence is the return of penis from erect to flaccid state, a process, which depends on the balance between contraction and relaxation factors of the penile tissue. It has been postulated that the process of detumescence involves the cessation and reversal of all the activities leading to penile tissue relaxation, hence the commencement of tissue contraction, expulsion of the trapped blood and reopening of venous channel (Andersson and Wagner, 1995). The penile smooth muscle contraction is controlled by sympathetic stimulus initiated during coitus by penile tactile receptors for ejaculation or as a result of the myogenic response of the penile tissue. Myogenic response is defined as the event leading to constriction of arterioles and corporeal body when the intracorporeal pressure due to the entrapped blood gets to the threshold at which the stretch receptor is activated (Davis and Hill, 1999).

The stimuli from the tactile receptor or stretch receptor travels to the T11–L2 spinal segments via sympathetic innervation exits the spinal cord and reaches the penis leading to corporeal vasoconstriction and corporeal smooth muscle contraction to cause penile flacidity. The resulting penile flaccidity is maintained in the absence of sexual arousal, otherwise, the sequence of penile erection is initiated again.

2.6. Penile erectile dysfunction

Erectile dysfunction (ED) is described as the failure to achieve or maintain a penile erection that is sufficient for satisfactory sexual performance (Salonia *et al.*, 2012). The condition is an age-related health issue that affect men from age 40-70 years (Shabsigh *et al.*, 2004). Its prevalence could be as high as 46.9% among Italians, 42.5% among Germans and 41% among the Chinese (Goldstein *et al.*, 2017). In South-western Nigeria, ED ranging from mild to severe was found to occur in 58.9% of men aged 30-80 years in *Ogbomoso* town (Oyelade *et al.*, 2016) and 55.1% of men aged 18-70 years attending a primary health care facility in *Ibadan* (Adebusoye *et al.*, 2012). Generally, the prevalence is continuously increasing with the projection of about 322 million men being affected worldwide by year 2025, translating to about 111% increase from the value in 1995 (Goldstein *et al.*, 2020).

2.7 Predisposing factors for penile disorder

The risk factors for the disorder in the penile tissue function include but not limited to sexual trauma and psychological issues (Corona *et al.*, 2014), diabetes mellitus (Kalter-Leibovici *et al.*, 2005; DeLay *et al.*, 2016), cardiovascular diseases (Viigimaa, 2014; Uddin *et al.*, 2018), androgen dysregulation (Sidori *et al.*, 2014), feeding lifestyle and sedentary activities (Dong *et al.*, 2011; Allen, 2019; Duca *et al.*, 2019).

2.8 Available interventions for erectile dysfunction and their limitations

Interventions for erectile dysfunction can be broadly categorized into mechanical, surgical and pharmacological approach (Hatzimouratidis and Hatzichristou, 2005).

2.8.1 Mechanical Intervention (Vacuum erectile device)

Mechanical approach to solving the problem of penile erection involves the use of vacuum pump device (Levine and Dimitriou, 2001). The components of this device include: constriction rings, a cylinder and vacuum pump. The rings are placed at the open end of cylinder. The cylinder is placed over the penis with the ring holding it firm to the penis while pump is operated to create a vacuum until penile engorgement occurs (Sharma *et al.*, 2017). The success rate of vacuum erectile device on penile erection was reported to be over 75% and up to 90%, however, discontinuation rate was also up to 30% due to bruising at the base of penis, decreased orgasm, and temporary change to penile sensation (Liu *et al.*, 2017; Lee and Sharifi, 2018). An example of a vacuum erectile device is shown in figure 2.6.

2.8.2 Surgical Approach (Penile Prosthesis Implantation)

This is an intervention where an inflatable device surgically implanted in the penis (Segal *et al*., 2013). Patient satisfaction rate ranges from 90% to 100% and varies by brands of prosthetic device (Levine *et al.*, 2016; Dick *et al.*, 2019). However, complications associated with inflatable penile prosthesis implantation include auto-inflation, distal cylinder erosion, pump migration, and reservoir displacement (Scherzer *et al.*, 2019). An example of a vacuum erectile device is shown in figure 2.7.

2.8.3 Pharmacological approach

Pharmacological approach to the treatment of erectile dysfunction involves the use of drugs which may be given via oral, intra-cavernosal injection or intraurethral routes administration (Johannes *et al*., 2000).

2.8.3.1 Oral Phosphodiesterase 5 inhibitors

Phosphodiesterase 5 inhibitors (PDE5-Is) is a first line pharmacological approach to the treatment of erectile dysfunction with satisfactory report from up to 60% of patients with erectile dysfunction (Hatzimouratidis *et al.*, 2016). Table 2.1 shows some brands of phosphodiesterase 5 inhibitor, prescribed doses, onset of actions and half-life.

The expression of the PDE5 gene is not limited to penile corpora cavernosa but also found in other organs including skeletal muscle, lung, stomach, thyroid, and adrenal gland (Morelli *et al.*, 2004; Yuan *et al.*, 2013). Therefore, the use of PDE 5 inhibitors is associated with some possible adverse effects like myalgia, headache, heartburn, facial flushing, nasal congestion, and vision-related conditions (Giuliano *et al.*, 2010). Also, unresponsiveness to Phosphodiesterase 5 inhibitors in patients with denervation after radical prostatectomy (Montorsi and McCullough, 2005) has limited the use of this drug in erectile dysfunction.

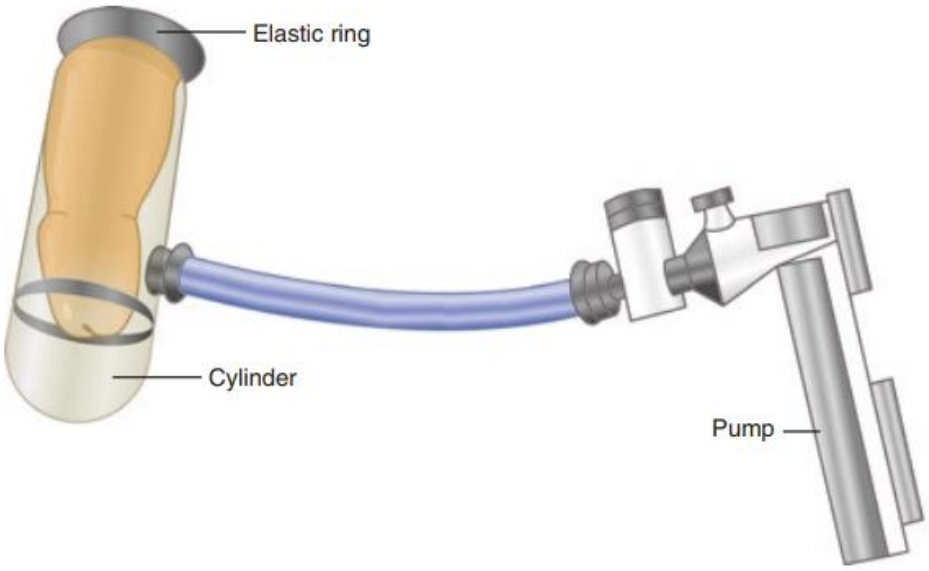


Figure 2.6: Diagram showing vacuum pump device used in treatment of erectile dysfunction (Lee and Sharifi, 2018)

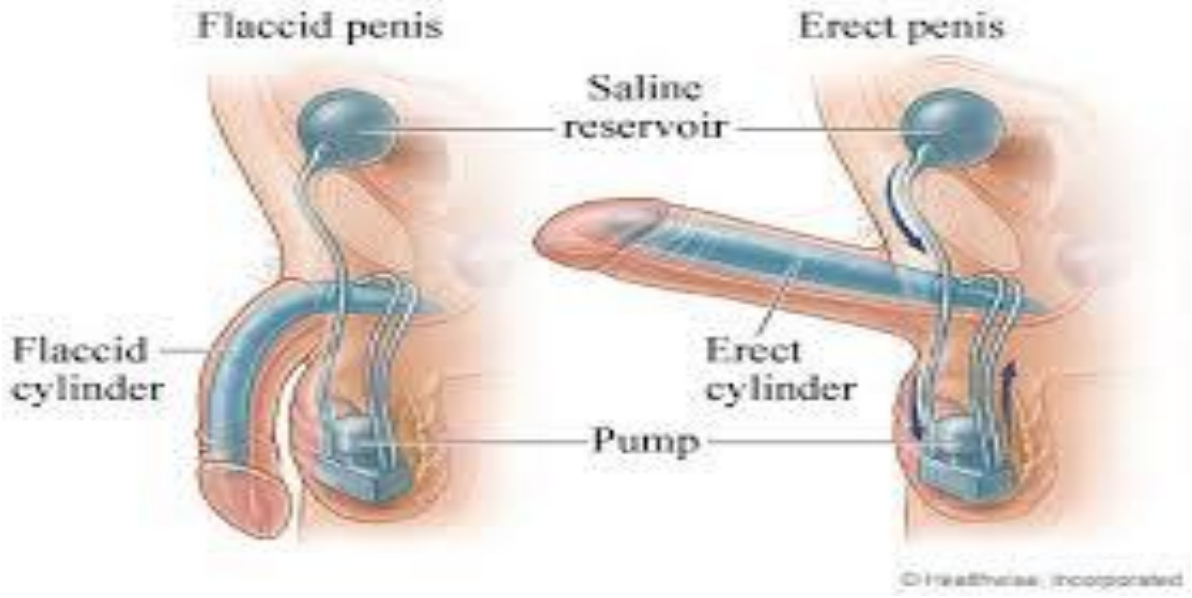


Figure 2.7: Diagram showing penile prosthetic device used in treatment of erectile dysfunction

Table 2.1: Brands of Phosphodiesterase 5 inhibitor

PDE 5 inhibitors	Onset of Action (minutes)	Half-life (hours)	Dosage (mg)
Sildenafil (Viagra®, Dynafil®)	As early as 11 minutes, with a median time of 36 minutes	3-5	25-100
Vardenafil (Levitra®)	As early as 16 minutes	4-5	5-20
Tadalafil (Cialis®)	16-30 minutes (The effects can last up to 36 hours post dosage)	17.5	2.5-20

2.8.4 Intracavernosal injection

Intracavernosal injection mode of treatment involves the injection of vasoactive substances directly into the corpus cavernosum at the lateral base of the penis via a small needle. These vasoactive medications include Prostaglandin E1, papaverine, and phentolamine (Belew *et al.*, 2015). Much as the initial satisfaction rates for intracavernosal injection mode of treatment could be as high as 94% of patients including patients with damaged nerve, dropout rates could also go up to 80% within the first year of treatment commencement (Nelson *et al.*, 2013). Causes of dropouts included high cost, problem of injection, lack of partner, and desire for a permanent solution (Mulhall *et al.*, 1999).

2.8.5 Intraurethral prostaglandin E1 suppository

Unlike intracavernosal injection mode of treatment, intraurethral prostaglandin E1 suppository uses a small intraurethral delivery catheter to introduce prostaglandin E1 within the urethra for absorption through the corpus cavernosum shortly before sexual intercourse (Mulhall *et al.*, 1999; Costa and Potempa., 2012). The efficacy of intraurethral prostaglandin E1 suppository is similar to oral phosphodiesterase 5 inhibitor but the drop-out rate is higher in intraurethral prostaglandin E1 suppository than in oral phosphodiesterase 5 inhibitor (McCullough *et al.*, 2010).

The common side effect and cause of withdrawal was urethral pain, dizziness, sweating, and hypotension (Shabsigh *et al.*, 2000).

2.9 Medicinal plants and male erectile function

Although the efficacy of the existing treatments including PDE5-Is, vacuum erection devices, and penile prosthesis implants are not in doubt, the percentage of patients that are unresponsive to the treatment or unsatisfied with the limitations remains high (Corona *et al.*, 2016). Accordingly, diversity of medicinal plant species and bioactive compounds are preferred as therapeutic options because they are natural, abundant, low-cost and with fewer or no side effects (Masuku *et al.*, 2020). Several plants including *Ocimum gratissimum* shown in table 2.2 have been reported to possess aphrodisiac properties (Enema *et al.*, 2018). However, the complexity of penile erection pathway and the pathophysiology of erectile dysfunction

necessitates the studies on the exact targets of the plants (Campbell *et al.*, 2018). This opens the research windows for other therapeutic option more natural, readily available and safe to better meet the needs of patients to overcome the huddle of erectile dysfunction.

Table 2.2: Some medicinal plants with aphrodisiac properties

Plant	Parts used	Effect	References
Mondia whitei (Periplocaceae)	Roots	Changes in levels of neurotransmitters, modulating the action neurotransmitters, or by increasing androgen levels	Watcho *et al*., (2006)
Tribulus terrestris (Zygophyllaceae)	Whole plant	Increased the androgenic status both centrally and peripherally	Adimoelja *et al*., (1997); Gauthaman *et al*., (2002)
*Myristica fragrans (*Myristicaceae)	Dried kernel	membranes and genetic content Nervous stimulating property	Tajuddin *et al*., (2003)
Lepidium meyenii (Cruciferae)	Root	Contains arginine sterols, such as campesterol, stigmasterol and sitosterol that enhance fertility	Cicero *et al*., (2001); Gonzales *et al*., (2003)
Eurycoma longifolia (Simaroubaceae)	Roots	Proandrogenic effect	Ang *et al*., (2001a, b, c)
Panax ginseng (Araliaceae)	Root	Promotes smooth muscle relaxation	Ang *et al*., (2001a, b, c)
Montanoa tomentosa (Asteraceae)	Leaves and flowers	By pro-sexual effects. Mechanisms also shows a steroid-like effect	Carro-Juárez *et al*., (2004; 2006)
Casimiroa edulis (Rutaceae)	Seeds	Neuro sexual mode of action	Ali and Rakkah, (2008)
Turnera diffusa (Turneraceae)	leaves	As an adaptogen	Estrada-Reyes *et al*., 2009
Ocimum gratissimum (Lamiaceae)	leaves	Promotes mounting frequency and latency in male mice through an unknown mechanism	Pande and Pathak, (2009); Kankara *et al*., (2015)

2.10 *Ocimum gratissimum*

Ocimum gratissimum, scent leaf (Effraim *et al*, 2010) is a widely distributed plant which belongs to the *labiatae* family commonly found in the savannah and coastal regions of the world including India, Polynesia, Hawaii, Mexico, Panama, West Indies, Brazil, and Bolivia and Nigeria (Prabhu *et al*, 2009).

The plant is commonly used in folk medicine to treat different diseases such as upper respiratory tract infections, diarrhoea, headache, ophthalmic, skin diseases, pneumonia, cough fever, conjunctivitis etc. (Onajobi, 1986).

Figure 2.8: Picture showing *Ocimum gratissimum* leaf (source: http://www.onlyfoods.net/ocimum-gratissimum.html) accessed on february 8, 2022

2.11 **Pharmacological Studies on the medicinal properties of *Ocimum gratissimum* leaf**

The usefulness of *Ocimum gratissimum* in complementary and alternative medicine have well been researched scientifically. Among the reported medicinal properties of the plant include antidiarrheal, analgesic, anti-hypertensive, anti-diabetic, antioxidant and aphrodisiac effects.

2.11.1 Anti-diarrheal effects of *Ocimum gratissimum leaf*

Anti-diarrhoeal property of the aqueous extract of *Ocimum gratissimum* was reported by Offiah and Chikwendu (1999). The extract decreased the number of wet faeces and inhibited the propulsive movement of the intestinal contents. This effect was shown to be partly elicited via inhibition of muscarinic receptors.

The anti-diarrhoeal property of the aqueous extract of *Ocimum gratissimum* was investigated in Wistar rats. The aqueous leaf extract of this plant, at various doses tested (25, 50 and 100 mg/kg body weight) displayed remarkable anti- diarrhoeal activity evidenced by the reduction in the rate of defecation and consistency of faeces in Wistar rats.

2.11.2 Anti-hypertensive effect of *Ocimum gratissimum*

The cardiovascular effects of intravenous treatment with the essential oil of *Ocimum gratissimum* and its main constituent, eugenol showed that intravenous treatment with *Ocimum gratissimum* dose-dependently decreased blood pressure in conscious DOCA-salt hypertensive rats. This enhancement appears related mainly to an increase in *Ocimum gratissimum*-induced vascular smooth relaxation in this hypertensive model (Interaminense *et al*., 2005; Interaminense *et al.,* 2007).

2.11.4 Antioxidant capacity of *Ocimum gratissimum*

The methanolic extract of *Ocimum gratissimum* had a DPPH scavenging activity of 84.6% at 250μg/mL and a reductive potential of 0.77 at 100 μg/mL. These values were comparable with those of gallic acid, 91.4% at 250 μg/ml and ascorbic acid, 0.79% at 60 μg/ml as standards for DPPH scavenging activity and reductive potential, respectively. These findings suggest the rich

phytochemical content of *Ocimum gratissimum* and its good antioxidant activity (Akinmoladun *et al.*, 2007).

2.11.5 Effect of *Ocimum gratissimum* on penile erection

Ocimum gratissimum was documented to improve male erectile and ejaculatory functions in mice (Pande and Pathak, 2009). Percentage ejaculatory performance increased while mount latency, intromission latency and post-ejaculatory latency reduced following *Ocimum gratissimum* administration in male mice (Pande and Pathak, 2009). However, the mechanism involved is yet to be reported.

2.12 Phytochemical constituents of Ocimum gratissimum leaf

Scientific publications have identified variability in the composition of leaves from the same plant specie, this was attributed to variation of climatic condition and soil content of the location where the plant is being cultivated (Martins *et al*., 1999; Burt, 2004). The constituent variability was found to be in the principal constituent which is either eugenol, thymol or geraniol (Vieira *et al*., 2001). *Ocimum gratissimum* harvested in Togo, West Africa majorly composed of thymol (Koba *et al.*, 2008) similar to what was reported from India and south asia (Prahbu *et al*., 2009) while the leaves harvested in Kenya, East Africa was rich in eugenol (Matasyoh *et al*, 2008) similar to what was obtained in Brazil (Madeira *et al*, 2005).

Over a few decades, thymol has been the major component identified in *Ocimum gratissimum* from Nigeria, West Africa with no traces of eugenol (Sofowora, 1970), however, recent study by Saliu *et al*., (2011) characterised the leaves of *Ocimum gratissimum* from Northern part of Nigeria to contain more than 61% of eugenol, showing the variability in the phytochemical constituent of the plant even within the same country. The phytochemical screening of the *Ocimum gratissimum* leaves used for this study using Gas Chromatography Mass Spectrometry (GC-MS) showed that thymol was the most abundant in the leaves, hence, the use of thymol in this study.

2.12.1 Chemistry and Physical Characteristics and Pharmacokinetics of thymol

Thymol is a crystalline white substance also known as 2-isopropyl-5-methylphenol. Its has a melting point of about 49°C to 51°C and 0.96 g/cm^3 density at 25°C (Jordan *et al*., 1991; Lide and Frederikse, 1996).

Oral administration of thymol showed that it is rapidly absorbed and reached maximum plasma concentration (T_{max}) within 30 minutes and eliminated in about 24 hours (Nieddu *et al*., 2014).

2.12.2 Pharmacological uses of thymol

Multiple therapeutic actions of thymol have been reported. These include antioxidant (by scavenging free radicals and enhancing the endogenous nonenzymatic and enzymatic antioxidants) (Venu *et al*., 2013), anti-inflammatory (by inhibiting the recruitment of cytokines and chemokines) (Ku and Lin, 2013), antihyperlipidemic (increasing the levels of high-density lipoprotein and decreasing the levels of low-density lipoprotein cholesterol and low-density lipoprotein cholesterol in the circulation) (Saravanan and Pari, 2015). The anti-hypertensive property of thymol in rats was reported through its vascular tissue relaxant activity and membrane potential stabilisation all of which are not dependent on vascular endothelium (Peixoto-Neves *et al*., 2010). It also reduced the activity of Ca^{2+}-pump canine cardiomyocyte (Szentandrassy *et al*., 2003).

CHAPTER THREE

Materials and Methods

3.1 Materials

The following materials and chemicals were used in this study.

Plain sample bottles (5 mL), Eppendorf bottle (2 mL), Pasteur pipette, micropipette, glass slides, needle and syringe (1 mL, 2 mL and 5 mL), gloves, cotton wool, dissecting set. Distilled water, estradiol benzoate, formalin, sucrose, xylene, ethanol, paraffin, eosin, haematoxylin, tween 80, N-(1-naphthyl) ethylenediamine, sulfanilic acid, phosphoric acid, sodium nitrite, sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulphate heptahydrate ($MgSO_4$. $7H_2O$), sodium bicarbonate ($NaHCO_3$), potassium dihydrogen phosphate (KH_2PO_4), glucose, phenylephrine chloride, acetylcholine chloride, Nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), Methylene blue, indomethacin, nifedipine, 5-(1,4-diazepan-1-ylsulfonyl)isoquinoline (Fasudil®), 1-((5-iodonaphthalen-1-yl)sulfonyl)-1,4-diazepane hydrochloride (ML-7®), Ethylene Glycol-bis(β-aminoethyl ether)-N,N,N′,N′-Tetraacetic Acid (EGTA).

3.2 Experimental Animals

A total of fifty-two adult Wistar rats comprising thirty-two males (170 to 190 g) and twenty females (150 to 170 g) were used for this study. They were obtained from the Central Animal House, College of Medicine, University of Ibadan and kept in well-ventilated rat cages under standard laboratory conditions. They were fed with standard rat pellets (Ladokun feeds®, Ibadan, Nigeria) and tap water *ad libitum*. The rats were acclimatized for two weeks prior to the start of the experiments. Animal handling was in compliance with the institutional ethics on the

use of animals for experiment as approved by the Animal Care and Use Research Committee (UI-ACUREC/2017/046), University of Ibadan.

3.3 *OCIMUM GRATISSIMUM* LEAF EXTRACT PREPARATION AND ANALYSIS OF ITS PHYTOCHEMICAL CONSTITUENTS

3.3.1 Plant Collection and Extraction

Ocimum gratissimum leaves were bought at Bodija market in Ibadan metropolis. The plant was authenticated at the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria, where voucher number FHI:110026 was assigned to the specimen.

The leaves were air dried and pulverized, then four kilogram (4 kg) of the pulverized *Ocimum gratissimum* leaf was soaked in distilled water in a glass container for twenty-four hours after which it was filtered. The filtrate was concentrated using a rotary evaporator and lyophilised. This yielded 328 g of powdery mass (8%) which was then stored and used for the study.

3.3.2 Identification of phytochemical constituents

The quantitative analysis of aqueous extract of *Ocimum gratissimum leaf* GC-MS analysis was performed at the Department of Chemical Engineering, University of Ilorin, Ilorin Nigeria. Two gram (2 g) of the lyophilised aqueous extract of *Ocimum gratissimum* leaf was dissolved in 4 mL of absolute methanol to form a solution. The solution was analysed using a Varian 450 Gas Chromatograph coupled to a Varian 240-MS ion-trap mass spectrometer (VF-5 MS Column) with injector and oven temperature at 250°C and 200°C respectively. The heating rate of equipment was programmed at 10 °C/minutes. The injection was performed in the split ratio of 200 and the volume was 10 µL. The flow of carrier gases was constant at 1.0 mL/minutes. The spectrum scan time was completed in 45 minute and the constituents were identified by comparing the retention time of each constituents with mass spectra data in the National Institute of Standards and Technology (NIST) library.

The most abundant component identified in aqueous extract of *Ocimum gratissimum* leaf after the analysis was thymol and the synthetic form was obtained from AK Scientific, Inc., California, USA.

3.4 Study Design

The study design was divided into two phases; *in-vivo* and *ex-vivo* studies

The *in-vivo* phase involved male and female (for mating procedures) Wistar rats while the *ex-vivo* phase involved penile tissues isolated from male Wistar rats.

3.4.1 *In vivo* Phase

Forty Wistar rats, 20 males and 20 females, were used for the study in the *in vivo* phase. The rats were grouped into 4 of 10 rats per group and were treated as shown in table 3.1.

Table 3.1 Animal grouping and treatment

Group	**Number of Wistar rats**	**Treatment**
Group 1	10 (5 males and 5 females kept in separate cages until mating experiment)	The male rats were administered 0.5 mL/kg distilled water for 28 days and served as control. The female rats were maintained on normal feed and their oestrous cycle was synchronized to make them receptive for the mating experiment.
Group 2	10 (5 males and 5 females kept in separate cages until mating experiment)	The male rats were administered 0.5 mL/kg of 1% corn oil which was the vehicle used to dissolve thymol. The administration was done daily for 28 days The female rats were maintained on normal feed and their oestrous cycle was synchronized to make them receptive for the mating experiment.
Group 3	10 (5 males and 5 females kept in separate cages until mating experiment)	The male rats were administered 300 mg/kg of aqueous extract of *Ocimum gratissimum* leaf distilled water for 28 days. The effect of this dose has been previously reported by Obianime *et al*. (2010). The female rats were maintained on normal feed and their oestrous cycle was synchronized to make them receptive for the mating experiment.
Group 4	10 (5 males and 5 females kept in separate cages until mating experiment)	The male rats were administered 7.5 mg/kg of thymol for 28 days (Nagoor-Meeran *et al*., 2012). The female rats were maintained on normal feed and their oestrous cycle was synchronized to make them receptive for the mating experiment.

3.4.2 Synchronization of oestrous cycle in female Wistar rats

The female Wistar rats were made receptive by artificially synchronizing them into estrus phase of the oestrous cycle as described by Dare *et al*. (2015). Estrus was induced by oral administration of estradiol benzoate (10 µg/100 g) 48 hours before the mating experiment and subcutaneous administration of progesterone (0.5 mg/100 g) 4 hours before the pairing.

Estrus was confirmed as described by Marcondes *et al.* (2002). Briefly, vaginal lavage was collected with Pasteur pipette filled with about 0.1 mL of normal saline (0.9 % NaCl) by gently inserting the tip of the pipette into the rat's vagina. The sampled vaginal content was placed on a glass slide and the smear was spread out evenly. The glass slide was examined using the x10 and x40 objective lens of the light microscope (Olympus, Japan). Presence of anucleated cornified cells in the smear confirmed successful induction of estrus.

3.4.3 Assessment of Mating behaviour in male Wistar rat

The procedure was conducted by pairing male and female rats in a rectangular cage (40 × 60 × 40 cm) made of plastic, covered with wire mesh and placed in a light and sound controlled environment marked as mating arena. The activities in the cages were remotely monitored and recorded by an indoor closed-circuit television camera mounted at a 280 cm height and 140^{o} angle of vision to clearly cover a 200 x 200 cm ground area of the mating arena. The camera had an in-built infrared LED lights which enables a clear recording at night (Figure 3.1).

Mating behaviour was assessed on day 28, the last day of treatments in the male Wistar rats as described by Zanoli *et al*. (2008). Pairing of male and female in ratio 1:1 was commenced at 6:00pm. Male rat was placed in the mating arena 5 minutes before introducing the female rat. The time interval between the introduction of the female and the first mount by the male was noted as mount latency while the number of mounts with intromission within 30 min was noted as the mounting frequency. Mount latency and mounting frequency were used as indices of mating behaviour.

The male rat was considered to have mounted when its hind limbs were on the ground while the fore limbs were firmly positioned on the rear two-third of the female rat’s abdomen. The

sequences of activities of the Wistar rats paired in the mating arena in this study is as shown in figure 3.1 (A-D)

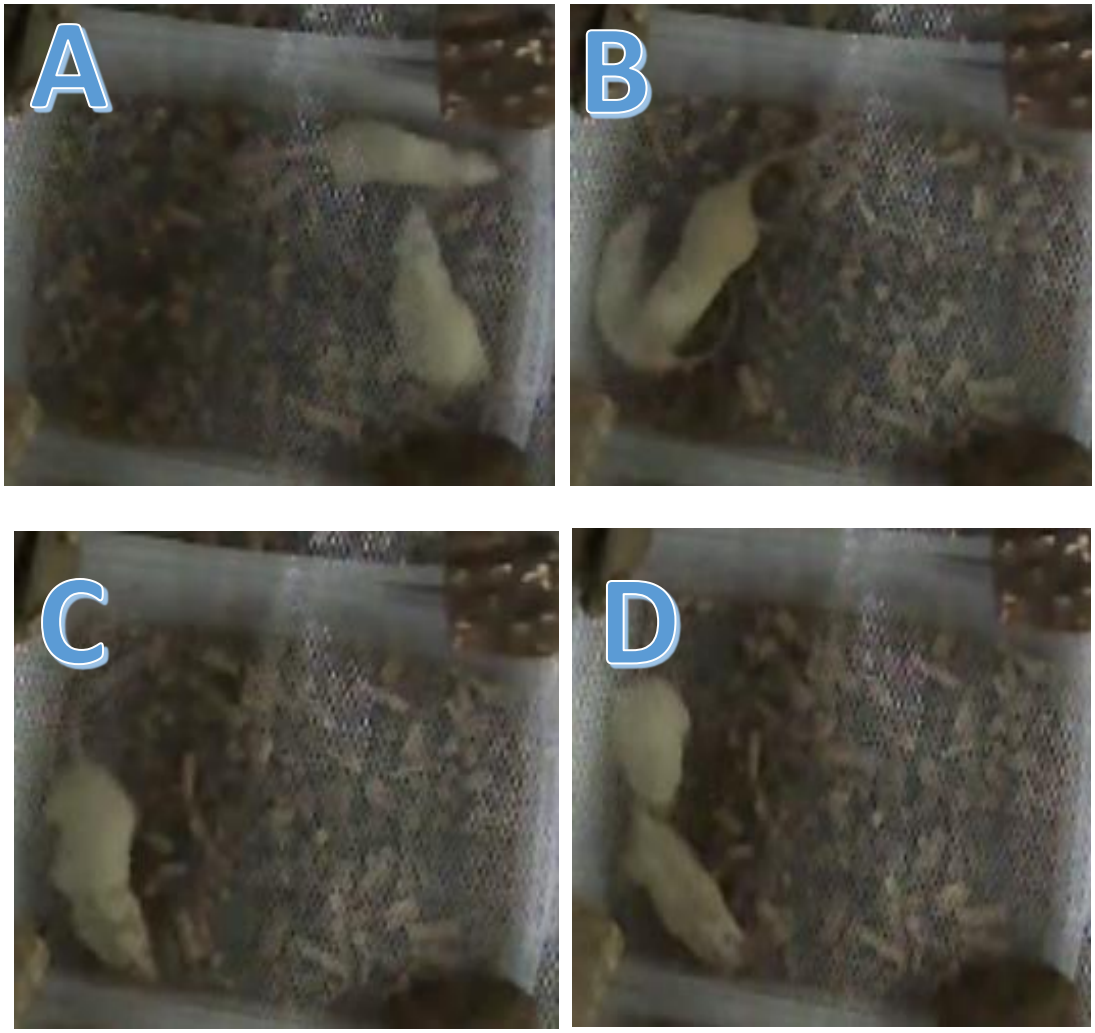


Figure 3.1: Pictures showing the sequence of activities of male and female rats in the mating arena. (A) Male and female rats newly paired. (B) Male rat approaching the female. (C) The male rat mounts the female (D) after a cycle of mounting.

3.4.4 Blood sampling and penile tissue harvesting

After the mating procedure on day 28, blood sample was collected *via* cardiac puncture into 5 mL plain sample bottle under anaesthesia induced by 50 mg/kg of sodium thiopental (i.p.). The bottle was placed on the bench for 20 minutes to allow adequate time for clotting at room temperature after which it was centrifuged at 3000 x g for 10 minutes to obtain the serum. The serum was separated into 2 mL Eppendorf bottle using Pasteur pipette and stored at -18℃ until it was used to determine the levels of luteinizing hormone, follicle stimulating hormone and testosterone.

Penile tissue was isolated by opening the penis up to the level of the crural attachment of the ischium, where it was detached. The harvested penile tissue was divided into two, one portion was fixed in 10% formalin for histopathology assessment while the remaining portion was homogenized in 0.32 M sucrose for assays of nitric oxide, cyclic guanylate monophosphate level, calcium ion level and calcium ATPase activity. Sucrose solution was used in the homogenization to eliminate phosphate contamination that may exaggerate the result if buffer solution containing phosphate was used.

3.4.5 Determination of serum reproductive hormone level

The serum levels of testosterone, follicle stimulating hormone and luteinizing hormone were determined using Enzyme-Linked Immunosorbent Assay (ELISA) kits.

3.4.6 Determination of Penile Nitric Oxide level

Nitric oxide in the penile tissue hormogenate was determined by the Griess reaction method. The Griess reaction analyses nitrate via its catalytic reduction to nitrite (Green *et al*., 1982). In the reaction, sulfanilic acid is quantitatively converted to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to N-(1-naphthyl) ethylenediamine, forming an azo dye that can be spectrophotometrically quantified at absorbance of 548 nm.

Equal volumes of N-(1-naphthyl) ethylenediamine (Component A) and sulfanilic acid (Component B) were freshly mixed together to form the Griess Reagent in a total volume sufficient for the assay. One hundred micoliters (100 µL) of Griess reagent, 300 µL of the

sample, and 2.6 mL of deionized water. The Photometric reference was prepared by mixing 100 µL of Griess reagent and 2.9 mL of deionized water. The standard was prepared by mixing 100 µL of Griess reagent, 300 µL of varied concentration of commponent C (1-100 µL) and 2.6 mL of deionized water. The mixtures were incubated for 30 minutes at room temperature and absorbances were read at 548 nm wavelenght relative to the reference.

The standard curve was generated by ploting the known nitrite concentration of the standard solution (x-axis) against their corresponding absorbance (y-axis). The nitrite concentrations in each tissue was then extrapolated from the standard curve.

3.4.7 Determination of penile cyclic Guanosine Mono Phosphate level (cGMP)

The cGMP level in penile tissue was determined using Enzyme-Linked Immunosorbent Assay (ELISA) kit.

Pre-coated ELISA plate, standard solution, standard diluent, streptavidin-HRP, stop solution, substrate solution A, substrate solution B and biotinylated rat cGMP antibody were used as supplied in the kit. Twenty milliliters (20 mL) of wash buffer concentrate were diluted with 480 mL distilled water as directed by the manufacturer.

Forty microliters (40µL) of the penile tissue homogenate and 10µL anti-cGMP antibody was added to sample wells while 50µL standard to standard well. Streptavidin-HRP (50µL) was then added to sample wells and standard wells, the wells were mixed and incubated for 60 minutes at 37°C. Three hundred and fifty microliters (350 µL) of wash buffer was added to the mixture and decanted to wash the unbound cGMP content and the washing procedure was repeated 3 time. Fifty microliters (50 µL) substrate solution A and fifty microliters (50 µL) substrate solution B were step wisely added to each well and incubated again for 10 minutes at 37°C. Fifty microliters (50 µL) of stop solution was added to each well to develop a yellow colour. The absorbance was immediately read using a microplate reader set to 450 nm within 10 minuets after adding the stop solution.

3.4.8 Determination of Penile calcium ion level

Calcium ion level was assayed using Calbiotech® assay kit and the procedure was as described in the kit. Reaction mixture containing standard (250 µL) or penile tissue homogenate (250 µL), Chromogenic Reagent (450 µL) and Calcium Assay Buffer (300 µL) was incubated at room temperature for 5-10 minutes and read within 30 minutes at 575 nm. Calcium level in each sample was extrapolated from the standard curve.

3.4.9 Penile calcium ATPase activity

Calcium ATPase activity in penile tissue was estimated using colorimetric assay kit by elabscience, Wuhan, China. The assay protocol was based on the estimation of amount of inorganic phosphate (Pi) liberated from ATP during the enzymatic reaction between the penile tissue homogenate and the reagents supplied in the kit.

The reagents supplied in the kit labelled reagents 1 to 7, Phosphorus standard solution, Phosphorus determination reagent and stop solution and they were used as directed by the manufacturer. The procedure for generation of inorganic phosphate and estimation of phosphate level is shown in table 3.2 and 3.3 respectively.

Table 3.2: Procedure for generation of inorganic phosphate

	Control tube	Sample tube
Reagent 1 (µL)	70	70
Reagent 2 (µL)	20	--
Reagent 3 (µL)	--	20
Reagent 4 (µL)	20	20
Reagent 5 (µL)	--	20
Reagent 6 (µL)	20	--
Penile tissue homogenate (µL)	--	100
The mixture was incubated for 10 minutes at at 37℃		
Reagent 7 (µL)	50	50
Penile tissue homogenate (µL)	100	--
The reaction mixture was centrifuged at 3500 x g for 10 minutes and the level of inorganic phosphate liberated from this reaction was determined.		

Table 3.3: Determination of inorganic phosphate level.

	Blank	Standard	Control	Sample
Double-Distilled water (µL)	150	--	--	--
Phosphorus standard solution (µL)	--	150	--	--
Supernatant (µL)	--	--	150	150
Phosphorus determination reagent (µL)	500	500	500	500
The mixture was incubated for 2 min at room temperature				
Stop solution (µL)	500	500	500	500
It was allowed to stand for 5 minutes the absorbance was read on a spectrophotometer at 636 nm with1cm cuvette.				

$$\text{Liberated Phosphate (Pi)} = \frac{\text{ODSample} - \text{ODControl}}{\text{ODStandard} - \text{ODBlank}} \times \text{Concentration of standard} \times 6 \times 2.8$$

$$\text{Calcium ATPase activity} = \frac{\text{Liberated Phosphate (Pi)}}{\text{Protein concentration of the sample}}$$

3.4.10 Determination of total protein

Total protein level was measured by spectrophotometric assay kit (Fortress Diagnostics, USA).
Test Principle: Copper ions react in alkaline solution, with protein peptide bonds to give a purple-coloured biuret complex. The amount of complex formed is directly proportional to the amount of protein in the specimen.
Biuret reagent and protein standard were used as supplied in the kit.
Twenty micro-liters of distilled water, standard and sample were added to the blank, standard and sample test tubes respectively. Biuret reagent (1000 µL) was added to all test tubes and incubated for 10 minutes at 37 °C. The absorbance of standard and samples were measured against the blank at 546 nm.

3.4.11 Penile tissue histology (Haematoxylin and Eosin Staining Technique)

The penile tissue of each rat was removed and quickly fixed in 10% formalin. The tissues were then dehydrated by passing through graded ethanol (70%, 95% and 100%) for 2 hours respectively so as to remove the inherent water content of the tissues.

The tissues were then cleared of ethanol by immersing them in xylene for 6 hours and this makes the cells transparent at the microscopic level. They were then infiltrated by placing them in molten paraffin wax, which served as support to the tissues for subsequent stages of sectioning. The tissues were thus embedded and moulded into blocks. The blocks were clamped and positioned for sectioning. Sectioning was carried out using a microtome to slice the block at a pre-set thickness of 4 µm, the sliced sections were floated with 20% alcohol on water at temperature of 50℃ and picked up with frosted edge microscope glass slides. The slides were then labelled with a pencil and arranged in a slide carrier and transferred to an oven to dewax the sections and also allow the sections to stick more to the slide. The sections were further dewaxed by passing it through xylene for 3 minutes after which they were rinsed in graded ethanol (95%, 90% and 85%) for two minutes each and rinsed in water to wash away the ethanol before they were dipped in haematoxylin for 10 minutes. The haematoxylin-stained slides were bleached with 1% hydrochloric acid in water to achieve light blue stained slides. The slides were counter stained with aqueous alcohol

The slides were then stained with haematoxylin and eosin and then dewaxed with xylene three times for 3 minutes each and then rehydrated in 100% ethanol, 95% ethanol and 70% ethanol and then water (all for 3 minutes each). The slides were then stained with haematoxylin for 15 minutes and excess stain was washed off and the slides were placed in 1% hydrochloric acid for 5 seconds and washed. The slides were counterstained in eosin for 3 minutes, dehydrated in graded ethanol (50%, 70%, 95% and 100%) and cover-slipped with DPX mountant for histological examination under microscope. Photomicrographs of the slides were then taken after examination under the microscope at ×400 magnification.

3.5 *EX-VIVO* STUDY

Twelve male Wistar rats were used for the *ex-vivo* study. The penile tissue of each animal was harvested and divided into two to make a total of 24 tissues in all. Six tissues each were used to study the contraction and relaxation responses in the presence or absence of *Ocimum gratissimum* or thymol. Thus, the *ex-vivo* study was also grouped into 4 (n = 6) as control, vehicle, *Ocimum gratissimum* or thymol as in the *in-vivo* study.

3.5.1 Preparation of the corpus cavernosa smooth muscle strips for *ex-vivo* experiment

The rats were sacrificed by cervical dislocation. The penis was identified and dissected to the level of the crural attachment of the ischium, where it was detached and immediately placed in Krebs Heinseleit solution with the composition shown in table 3.4 below. The corpus cavernosum was dissected from the tunica albuginea and two longitudinal strips were obtained. Each corpus cavernosum was suspended in a 50 mL chamber of an organ bath containing Krebs Heinseleit buffer solution.

Table 3.4: Composition of Krebs Heinseleit solution

Chemicals	Quantity (g/L)
Sodium chloride (NaCl)	6.9
Potassium chloride (KCl)	0.35
Calcium chloride ($CaCl_2$)	0.28
Magnesium sulphate ($MgSO_4$. $7H_2O$)	0.28
Sodium bicarbonate ($NaHCO_3$)	2.1
Potassium dihydrogen phosphate (KH_2PO_4)	0.16
Glucose	2.0

3.5.2 System Set-up and tissue mounting

An experimental set-up comprising of organ bath (model 4400; Ugo Basile, Varese, Italy), force transducer (model 7004; Ugo Basile, Varese, Italy) and a data acquisition system (model 17400; Ugo Basile, Varese, Italy) connected to a personal computer was used to monitor and record the contractile and relaxation responses of the isolated corpus cavernosum *ex-vivo*.

The organ bath filled with Krebs Heinseleit solution was turned on 15 minutes prior to the experiment to heat up the bath temperature to 37°C which was maintained by an in-built thermostat throughout the experiment. The air inlet was opened to allow constant aeration with 95% oxygen and 5% carbon dioxide air mixture. The corpus cavernosum was tied at the two ends by a tiny silk thread with the thread being looped on one end and connected to the organ chamber, while the thread on the other end was attached to the force transducer connected to the data acquisition system which was earlier launched on the computer. An initial tension of 2 g was applied to the corpus cavernosum strip then allowed to stabilise for 90 minutes in the set-up. During the stabilisation period, the tissues were stimulated thrice with 10^{-7} M phenylephrine for 5 minutes at an interval of 30 minutes to ascertain the integrity of the tissues for the experiment (Salahdeen *et al.*, 2014). The picture and schematic illustration of the experimental set-up for the *ex-vivo* study are shown in figure 3.2 and 3.3 respectively.

Figure 3.2: Picture showing the laboratory experimantal set-up for the *ex-vivo* study.

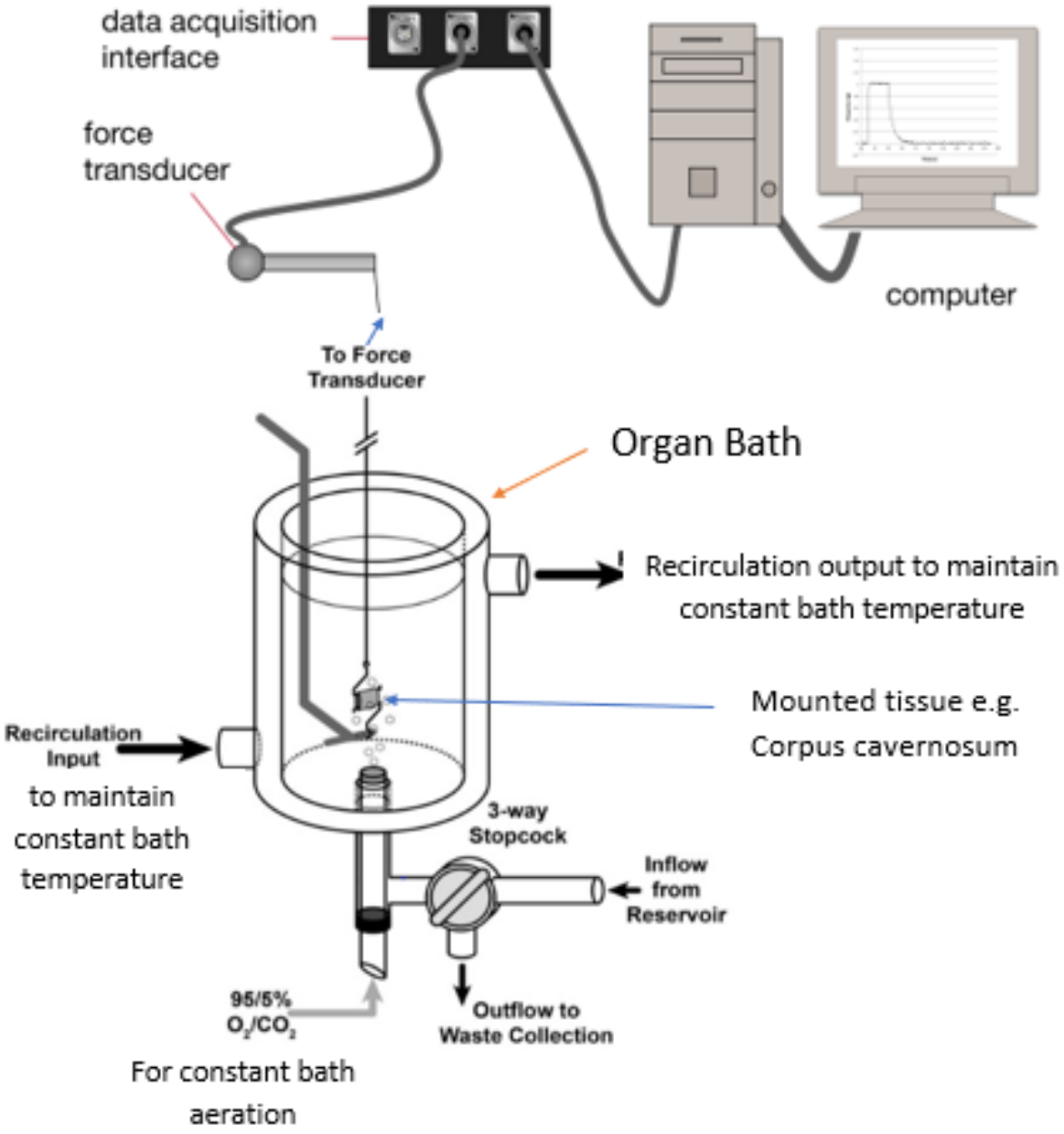


Figure 3.3: Schematic representation of the experimental set-up (Jespersen *et al.*, 2015; Salahdeen *et al.*, 2014)

3.5.3 Conversion of drug solutions from stock concentrations to bath concentrations

The concentration of extract, drugs and chemicals used in the *ex-vivo* experiments were reported as their concentration in the organ bath. To achieve this, 0.5 mL of extract, drug or chemical stock concentration was added to a 50 mL organ bath so that the final bath concentration of the extract or drugs were 100 times less than the stock concentration. Thus, a 10^{-4} M stock solution gave 10^{-2} M in the 50 mL organ bath (Jespersen *et al*., 2015)

3.5.4 *Ex-vivo* experimental design

The corpus cavernosa tissues used in this study were grouped into four based on the content of the Krebs' solution they were incubated in.

Group 1 tissues were (control) incubated in blank Krebs Heinseleit solution for 10 minutes.

Group 2 tissues were incubated in Krebs Heinseleit solution containing 60 µg/mL of *Ocimum gratissimum* leaf extract for 10 minutes (Interaminense *et al*., 2007).

Group 3 tissues were incubated in Krebs Heinseleit solution containing 1% (v/v) ethanol (the vehicle used in dissolving thymol) for 10 minutes (Castro *et al*., 1995)

Group 4 tissues were incubated in Krebs Heinseleit solution containing 0.06 µg/mL of thymol for 10 minutes (Peixoto-Neves *et al*., 2010)

3.5.5 Assessment of the effects of *Ocimum gratissimum* leaf or thymol on corpus cavernosum contraction response

The effects of aqueous extract of *Ocimum gratissimum* leaf or thymol on corpus cavernosum contractile response to phenylephrine and potassium chloride were studied.

3.5.5.1 Response to phenylephrine induced contraction

The tissues were incubated in media containing blank Krebs Heinseleit solution, aqueous extract of *Ocimum gratissimum* leaf, vehicle or thymol for 10 minutes, then graded (10^{-9} M to 10^{-5} M) doses of phenylephrine were added. The contraction patterns of the tissues in each incubation medium were recorded in tracings. The media were washed off of phenylephrine and the tissues were allowed to stabilised. The tension (contractile response) of the tissue at the various doses of phenylephrine were generated by the data acquisition system and used to calculate the percentage response according to Salahdeen *et al*. (2014) as follows.

$$\text{Response to Phenylephrine (\%)} = \frac{\text{Tension at final dose} - \text{Tension at the current dose}}{\text{Tension at final dose}} \times 100$$

3.5.5.2 Response to Potassium Chloride induced contraction

The stabilised tissues were incubated in media containing blank Krebs Heinseleit solution, aqueous extract of *Ocimum gratissimum* leaf, vehicle or thymol for 10 minutes, then graded (10 mM to 80 mM) doses of potassium chloride were added. The contraction patterns of the tissues in each incubation medium were recorded in tracings. The media were washed off of potassium chloride and the tissues were allowed to stabilise. The tension (contractile response) of the tissue at various doses of potassium chloride generated by the data acquisition system were used to calculate the percentage response according to Salahdeen *et al*. (2014) as shown below.

$$\text{Response to potassium chloride (\%)} = \frac{\text{Tension at final dose} - \text{Tension at the current dose}}{\text{Tension at final dose}} \times 100$$

3.5.6 Assessment of the effects of *Ocimum gratissimum* Leaf or thymol on corpus cavernosum relaxation response

The effects of *Ocimum gratissimum* leaf or thymol on the relaxation responses to acetylcholine were studied in phenylephrine- and potassium chloride- precontracted corpus cavernosum.

3.5.6.1 Response to acetylcholine in Phenylephrine pre-contracted tissue

The stabilised tissues were incubated in media containing blank Krebs Heinseleit solution, *Ocimum gratissimum* leaf, vehicle or thymol for 10 minutes. They were pre-contracted with phenylephrine (10^{-7} M) and relaxed with graded (10^{-9} M to 10^{-5} M) doses of acetylcholine. The relaxation pattern of the tissues in each incubation medium were recorded in tracings. The tissues were washed and allowed to stabilised. The tension on the tissue upon pre-contraction and the tension at each dose of acetylcholine generated by the data acquisition system were used to calculate the percentage relaxation response to acetylcholine (Zhang *et al.,* 2004) as shown below

$$\text{Response to acetylcholine (\%)} = \frac{\text{Tension at precontraction} - \text{Tension at the current Acetylcholine dose}}{\text{Tension at precontraction}} \times 100$$

3.5.6.2 Response to acetylcholine in potassium chloride pre-contracted tissue

The stabilised tissues were incubated in media containing blank Krebs Heinseleit solution, aqueous extract of *Ocimum gratissimum* leaf, vehicle or thymol for 10 minutes. They were pre-contracted with potassium chloride (60 mM) and relaxed with graded (10^{-9} M to 10^{-5} M) doses of acetylcholine. The relaxation pattern of the tissues in each incubation media were recorded in tracings. The tissues were washed and allowed to stabilise. The tension on the tissue upon pre-contraction and the tension at each dose of acetylcholine were generated by the data acquisition system and were used to calculate the percentage relaxation response to acetylcholine (Cartledge *et al*., 2001; Zhang *et al.,* 2004) as shown below:

$$\text{Response to acetylcholine (\%)} = \frac{\text{Tension at precontraction} - \text{Tension at the current Acetylcholine dose}}{\text{Tension at precontraction}} \times 100$$

3.6 Assessment of the mechanisms involved in the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response *ex-vivo*

The mechanism involved in the effects of aqueous extract of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response *ex-vivo* was investigated as described by Chamiot-Clerc *et al*. (2000). Pathway inhibitors acting at different stages of smooth muscle relaxation were included in the media used in the incubation of corpus cavernosum. The effects of *Ocimum gratissimum* leaf or thymol on the relaxation response to acetylcholine in the presence of

pathway inhibitors were then compared with their corresponding responses in the absence of the inhibitors.

3.6.1 The role of vascular endothelium in the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response *ex-vivo*

The role of the vascular endothelium in the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response was investigated by blocking endothelium releasing factors, Nitric oxide and prostaglandin.

3.6.1.1 Nitric oxide blockade experiment

The contribution of nitric oxide as a vascular endothelial releasing factor in the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response to acetylcholine was studied using Nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) in phenylephrine-precontracted and potassium chloride-precontracted tissues.

Stabilised tissues were incubated in media containing L-NAME, *Ocimum gratissimum* leaf with L-NAME or thymol with L-NAME for 10 minutes. They were then pre-contracted with phenylephrine (10^{-7} M) and relaxed with graded (10^{-9} M to 10^{-5} M) doses of acetylcholine. The relaxation pattern of the tissues in each incubation medium were recorded in tracings. The tissues were washed and allowed to stabilise. The procedure was repeated in KCl-precontracted tissues.

The tension on the tissue upon phenylephrine or KCl pre-contraction and the tension at each dose of acetylcholine generated respectively by the data acquisition system were used to calculate the percentage relaxation response to acetylcholine (Zhang *et al* 2004) as shown below:

$$\text{Response to acetylcholine (\%)} = \frac{\text{Tension at precontraction} - \text{Tension at the current Acetylcholine dose}}{\text{Tension at precontraction}} \times 100$$

3.6.1.2 Prostaglandin blockade experiment

The contribution of prostaglandin in the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response to acetylcholine was studied using indomethacin (10^{-4} M) in phenylephrine-precontracted and potassium chloride-precontracted tissues.

Stabilised tissues were incubated in media containing indomethacin, *Ocimum gratissimum* leaf with indomethacin or thymol with indomethacin for 10 minutes. They were then pre-contracted with phenylephrine (10^{-7} M) and relaxed with graded (10^{-9} M to 10^{-5} M) doses of acetylcholine. The relaxation pattern of the tissues in each incubation medium were recorded in tracings. The tissues were washed and allowed to stabilise. The procedure was repeated in KCl-precontracted tissues.

The tension on the tissue upon phenylephrine or KCl pre-contraction and the tension at each dose of acetylcholine generated respectively by the data acquisition system were used to calculate the percentage relaxation response to acetylcholine (Zhang *et al.,* 2004) as shown below:

$$\text{Response to acetylcholine (\%)} = \frac{\text{Tension at precontraction} - \text{Tension at the current Acetylcholine dose}}{\text{Tension at precontraction}} \times 100$$

3.6.2 The role of cyclic Guanosine Monophosphate (cGMP) in the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response *ex-vivo*

The role of cGMP in the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response was investigated by using a selective cGMP blocker, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10^{-4}M) and a non-selective cGMP blocker Methylene Blue (MB, 10^{-4} M).

3.6.2.1 Selective blockade of cGMP production

1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one is a chemical that specifically inhibits conversion of soluble guanylate cyclase to cyclic GMP, thus ODQ (10^{-4}M) was used in this experiment.

Stabilised tissues were incubated in media containing ODQ, *Ocimum gratissimum* leaf with ODQ or thymol with ODQ for 10 minutes. They were then pre-contracted with phenylephrine (10^{-7} M) and relaxed with graded (10^{-9} M to 10^{-5} M) doses of acetylcholine. The relaxation pattern of the tissues in each incubation medium were recorded in tracings. The tissues were washed and allowed to stabilised. The procedure was repeated in KCl-precontracted tissues.

The tension on the tissue upon phenylephrine or KCl pre-contraction and the tension at each dose of acetylcholine generated respectively by the data acquisition system were used to calculate the percentage relaxation response to acetylcholine (Zhang *et al.,* 2004) as shown below:

$$\text{Response to acetylcholine (\%)} = \frac{\text{Tension at precontraction} - \text{Tension at the current Acetylcholine dose}}{\text{Tension at precontraction}} \times 100$$

3.6.2.2 Non-selective blockade of cGMP production

Methylene Blue (MB) inhibits conversion of soluble Guanylate cyclase to cyclic GMP and at the same time mop up all the available nitric oxide in the tissue, thus MB (10^{-4}M) was used as a non-selective blocker of cGMP in this experiment.

Stabilised tissues were incubated in media containing MB, *Ocimum gratissimum* leaf with MB or thymol with MB for 10 minutes. They were then pre-contracted with phenylephrine (10^{-7} M) and relaxed with graded (10^{-9} M to 10^{-5} M) dose of acetylcholine. The relaxation pattern of the tissues in each incubation medium were recorded in tracings. The tissues were washed and allowed to stabilise. The procedure was repeated in KCl-precontracted tissues.

The tension on the tissue upon phenylephrine or KCl pre-contraction and the tension at each dose of acetylcholine generated respectively by the data acquisition system were used to calculate the percentage relaxation response to acetylcholine (Zhang *et al.,* 2004) as shown below:

$$\text{Response to acetylcholine (\%)} = \frac{\text{Tension at precontraction} - \text{Tension at the current Acetylcholine dose}}{\text{Tension at precontraction}} \times 100$$

3.6.3 The role of voltage gated calcium channel in the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response *ex-vivo*

The role of calcium channel in the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response was investigated by using a voltage gated calcium channel blocker, nifedipine (10^{-4}M).

The stabilised tissues were incubated in media containing nifedipine, *Ocimum gratissimum* leaf with nifedipine or thymol with nifedipine for 10 minutes. They were then pre-contracted with phenylephrine (10^{-7} M) and relaxed with graded (10^{-9} M to 10^{-5} M) dose of acetylcholine. The relaxation pattern of the tissues in each incubation medium were recorded in tracings. The tissues were washed and allowed to stabilised. The procedure was repeated in KCl-precontracted tissues.

The tension on the tissue upon phenylephrine or KCl pre-contraction and the tension at each dose of acetylcholine generated respectively by the data acquisition system were used to calculate the percentage relaxation response to acetylcholine (Zhang *et al.,* 2004) as shown below:

$$\text{Response to acetylcholine (\%)} = \frac{\text{Tension at precontraction} - \text{Tension at the current Acetylcholine dose}}{\text{Tension at precontraction}} \times 100$$

3.6.4 The role of myosin light chain kinase in the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response *ex-vivo*

The role of myosin light chain kinase in the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response was investigated by using a myosin light chain kinase inhibitor, 1-((5-iodonaphthalen-1-yl)sulfonyl)-1,4-diazepane hydrochloride (ML-7, 10^{-3}M).

Stabilised tissues were incubated in media containing ML-7, *Ocimum gratissimum* leaf with ML-7 or thymol with ML-7 for 10 minutes. They were then pre-contracted with phenylephrine (10^{-7} M) and relaxed with graded (10^{-9} M to 10^{-5} M) dose of acetylcholine. The relaxation pattern of the tissues in each incubation medium were recorded in tracings. The tissues were washed and allowed to stabilise. The procedure was repeated in KCl-precontracted tissues.

The tension on the tissue upon phenylephrine or KCl pre-contraction and the tension at each dose of acetylcholine generated respectively by the data acquisition system were used to calculate the percentage relaxation response to acetylcholine (Zhang *et al.,* 2004) as shown below:

$$\text{Response to acetylcholine (\%)} = \frac{\text{Tension at precontraction} - \text{Tension at the current Acetylcholine dose}}{\text{Tension at precontraction}} \times 100$$

3.6.5 The role of Rho-associated protein kinase in the effects of Ocimum *gratissimum* or thymol on corpus cavernosum relaxation response *ex-vivo*

The role of Rho-associated protein kinase in Rho-associated protein kinase the effects of *Ocimum gratissimum* or thymol on corpus cavernosum relaxation response was investigated by using 1-(5-Isoquinolinesulfonyl) homopiperazine hydrochloride (Fasudil®, 10^{-3}M)

Stabilised tissues were incubated in media containing fasudil, *Ocimum gratissimum* leaf with fasudil or thymol with fasudil for 10 minutes. They were then pre-contracted with phenylephrine (10^{-7} M) and relaxed with graded (10^{-9} M to 10^{-5} M) dose of acetylcholine. The relaxation pattern of the tissues in each incubation medium were recorded in tracings. The tissues were washed and allowed to stabilise. The procedure was repeated in KCl-precontracted tissues.

The tension on the tissue upon phenylephrine or KCl pre-contraction and the tension at each dose of acetylcholine generated respectively by the data acquisition system were used to calculate the percentage relaxation response to acetylcholine (Zhang *et al.,* 2004) as shown below:

$$\text{Response to acetylcholine (\%)} = \frac{\text{Tension at precontraction} - \text{Tension at the current Acetylcholine dose}}{\text{Tension at precontraction}} \times 100$$

3.6.6 Response to calcium chloride in calcium-free physiological solution

Calcium-free medium was prepared by excluding calcium chloride from kreb's Heinseleit solution preparation and addition of Ethylene Glycol-bis(β-aminoethyl ether)-N,N,N′,N′-Tetraacetic Acid (EGTA, 0.5 mM) to the physiological solution. The contraction responses of stabilised tissues to graded doses of calcium chloride (10 mM to 50 mM) were then recorded in

blank calcium-free medium and medium containing aqueous extract of *Ocimum gratissimum* leaf, vehicle or thymol in the presence or absence of nifedipine, ML-7 or Fasudil.

The tension on the tissue upon calcium chloride contraction generated by the data acquisition system was used to calculate the percentage contraction response to calcium chloride as shown below:

$$\text{Response to calcium chloride (\%)} = \frac{\text{Tension at final dose} - \text{Tension at the current dose}}{\text{Tension at final dose}} \times 100$$

3.7 Statistical Analysis

The results of the *in vivo* and the *ex-vivo* studies were presented as mean ± standard error of mean (SEM). In the *ex-vivo* study, the mean responses of penile smooth muscle to the varied doses of the contracting or relaxing agent were used to generate the tissue response curve. The response at the highest dose represented the maximum contraction or relaxation response while the area under the curve was calculated to show the cumulative response of the tissues to contracting or relaxing agent. The data were analysed using one-way analysis of variance and $p<0.05$ was considered to indicate statistical significance. All data computation and analysis were done using Graph Pad Prism® version 7.0.

CHAPTER FOUR

Results

4.1 Characterisation of aqueous extract of *Ocimum gratissimum* leaf using Gas Chromatography Mass Spectrometry

The chromatogram of the Gas Chromatography Mass Spectrometry (GC-MS) of the aqueous extract of *Ocimum gratissimum* leaf is shown in figure 4.1. Twelve peaks indicating the presence of twelve major chemical constituents were observed. On comparison of the mass spectra of the constituents with spectra in the National Institute of Standards and Technology (NIST) database, the list of these twelve compounds with their corresponding retention time (min) and percentage abundance (%) are shown in table 4.1. The 1st compound identified with the least retention time (20.80 min) was 1,2,4,5-Tetramethylbenzene, while 2-isopropyl-5-methyl-2-cyclohexen-1-one was the 12th compound with the highest retention time (37.50 min). Thymol (retention time = 21.89) is 47.32% in abundance and therefore was the major component of aqueous extract of *Ocimum gratissimum* used in this study.

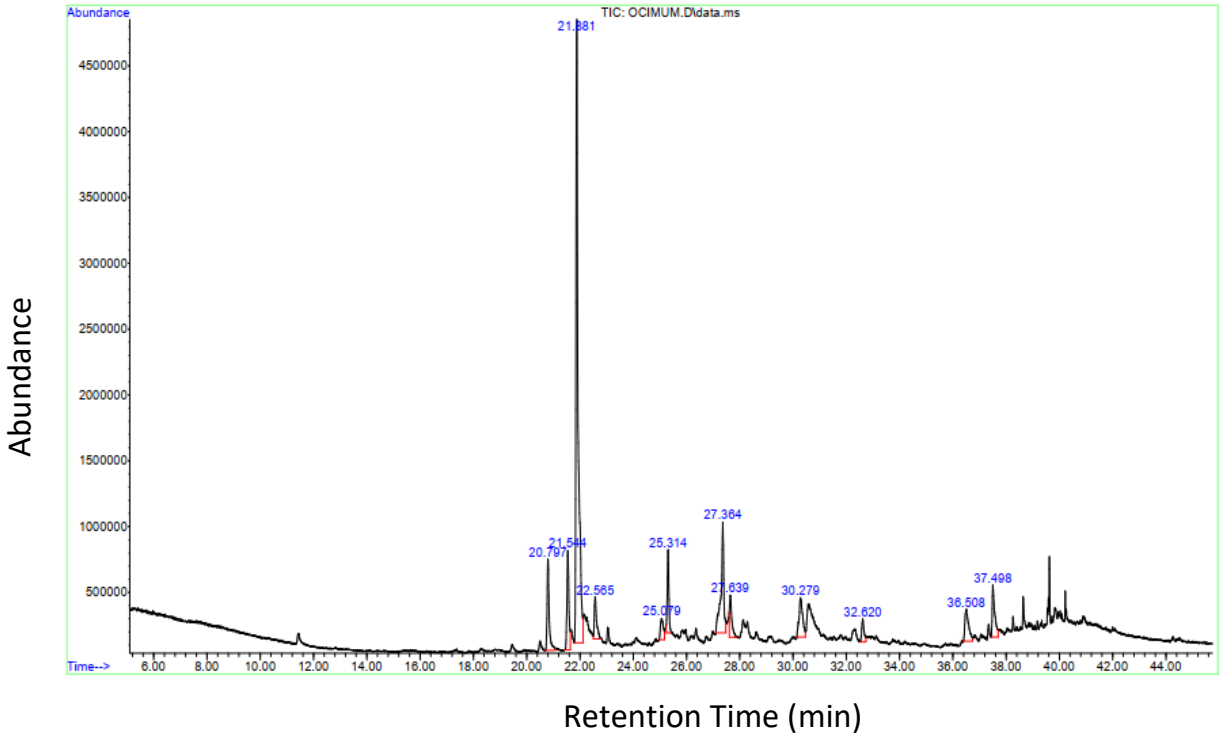


Figure 4.1: Gas chromatography mass spectrometry chromatogram of aqueous extract of *Ocimum gratissimum* leaf.

Table 4.1: Identified compounds in aqueous extract of *Ocimum gratissimum* leaf

S/N	Retention time (min)	Chemical Compound	percentage abundance **(%)**
1	20.80	1,2,4,5-Tetramethylbenzene	6.50%
2	21.54	1,3,8-p-Menthatriene	6.64%
3	21.88	Thymol	47.32%
4	22.57	p-Cymene	3.35%
5	25.08	*p*,α-Dimethylstyrene	2.13%
6	25.31	Caryophyllene	4.85%
7	27.36	beta-selinene	10.44%
8	27.64	1-(2-methylprop-2-enoxy)-4-tert-butyl-benzene	3.14%
9	30.28	5-Hydroxy-2(3*H*)-benzofuranone	5.02%
10	32.62	Cyclohexene	1.83%
11	36.51	1-(α-chloroacetyl)-1,2,5,6 tetrahydropyridine	4.17%
12	37.50	2-isopropyl-5-methyl-2-cyclohexen-1-one	4.61%

4.2 Effects of aqueous extract of *Ocimum gratissimum* and thymol on mounting latency and frequency in Wistar rats

The result of the mounting latency and frequency of *Ocimum gratissimum* or thymol treated rats are shown respectively in figure 4.2 and figure 4.3.

Mounting latency was significantly decreased ($p<0.05$) in the *Ocimum gratissimum* treated group compared with their respective day 0 value, while thymol produced no significant effect on the mounting frequency after the 28 days treatment (figure 4.2). As shown in figure 4.3. Both *Ocimum gratissimum* and thymol significantly increased ($p<0.05$) the mounting frequency in rats after treatments.

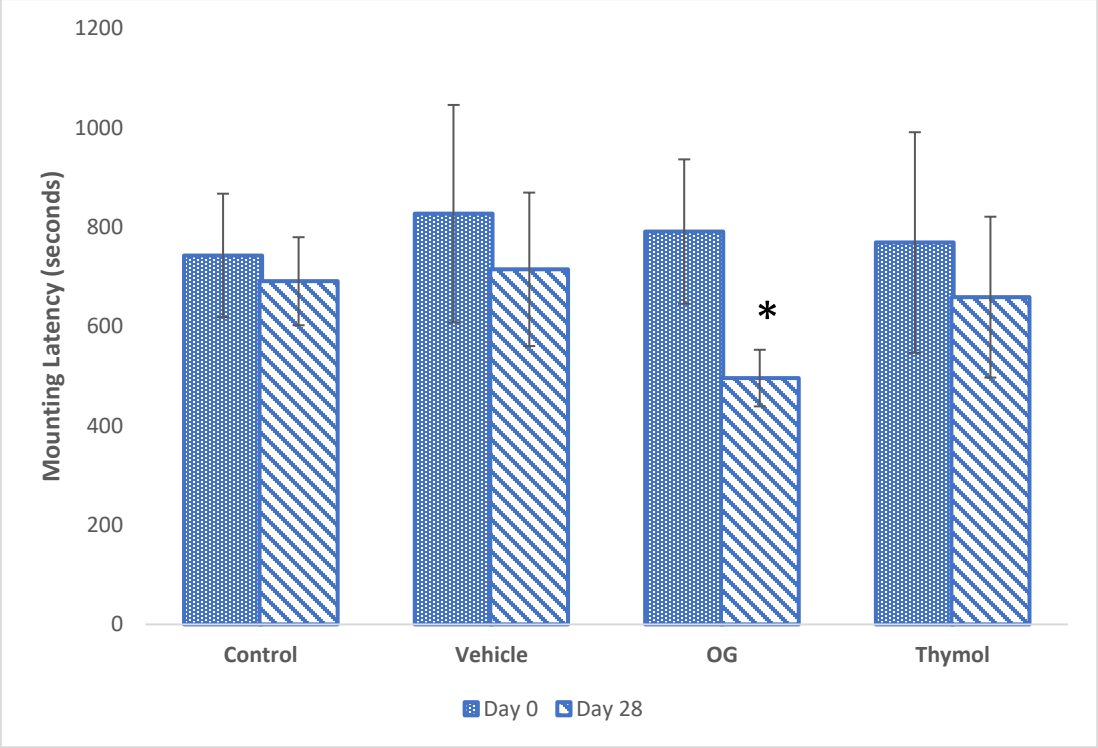


Figure 4.2: Graph showing the effects of aqueous extract of *Ocimum gratissimum* leaf and thymol on mounting latency in male Wistar rats.

Data represent mean ± SEM. *n* =5. * = $p<0.05$ compared with the Day 0.

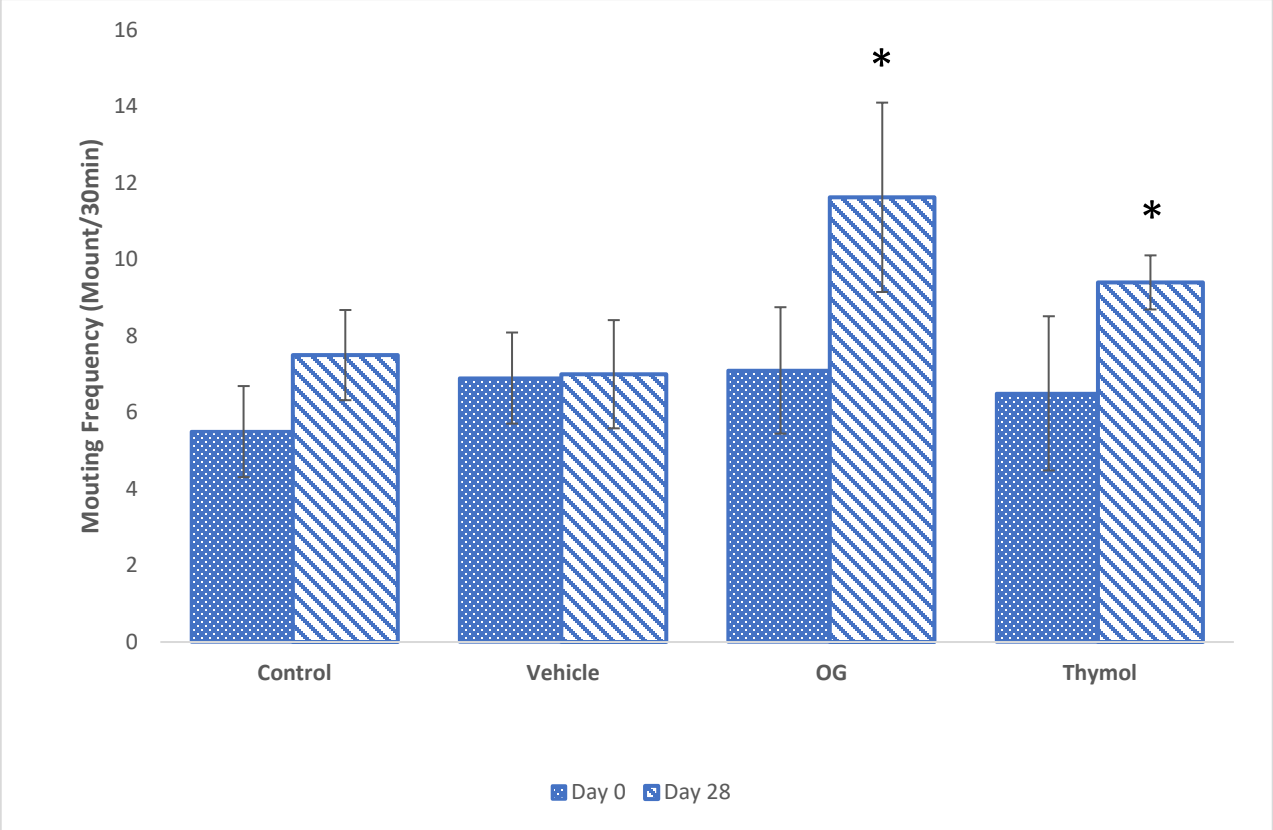


Figure 4.3: Graph showing the effects of aqueous extract of *Ocimum gratissimum* leaf (OG) and thymol on mounting frequency in male Wistar rats.

Data represent mean ± SEM. *n* =5. * = $p<0.05$ compared with the Day 0.

4.3 Effects of aqueous extract of *Ocimum gratissimum* leaf and thymol on reproductive hormone levels in male Wistar rats

The effects of aqueous extract of *Ocimum gratissimum* leaf and thymol on reproductive hormone levels in male Wistar rats are shown in figures 4.4 – 4.6.

Luteinizing hormone was significantly increased in rats treated with *Ocimum gratissimum* ($p<0.05$) while the apparent increase in the thymol rats was not significantly different when compared with the control (figure 4.4). The levels of follicle stimulating hormone (Figure 4.5) and testosterone (Figure 4.6) were not different among all groups.

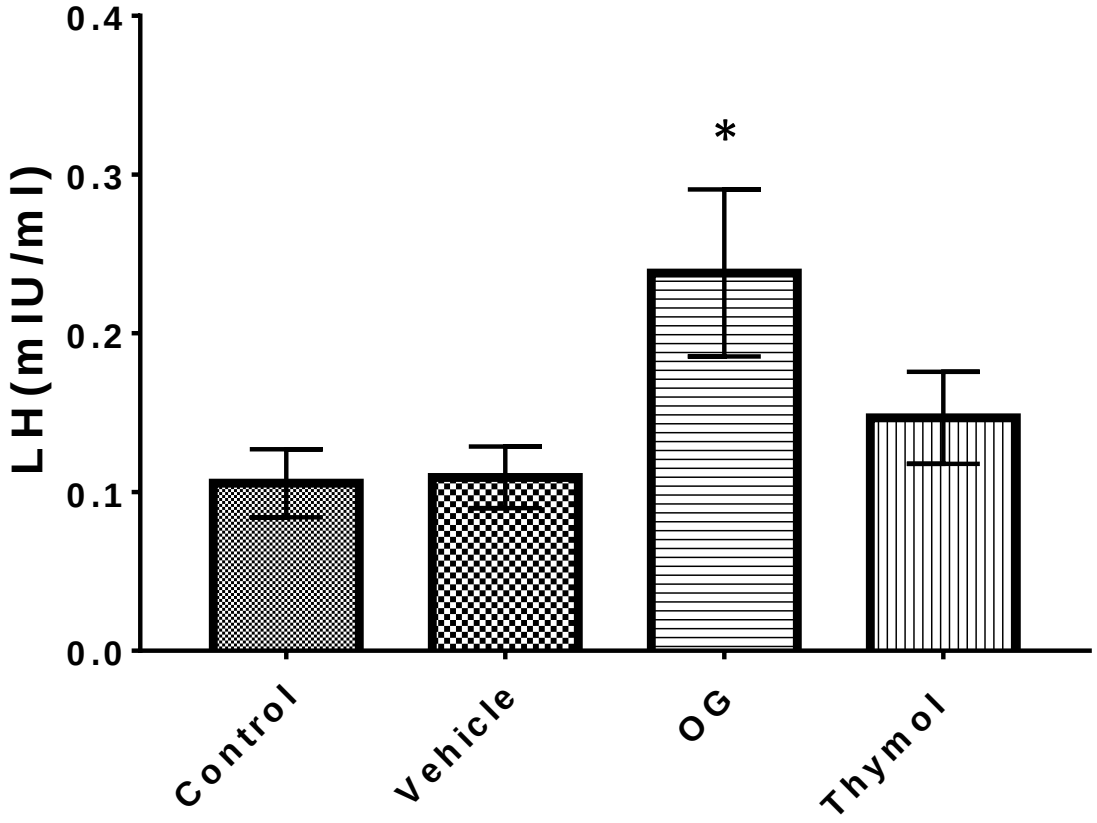


Figure 4.4: Graph showing the effects of aqueous extract of *Ocimum gratissimum* leaf (OG) and thymol on Serum Luteinizing Hormone level (LH) in male Wistar rats. Data represent mean ± SEM. *n =5.* * = $p<0.05$ compared with the control.

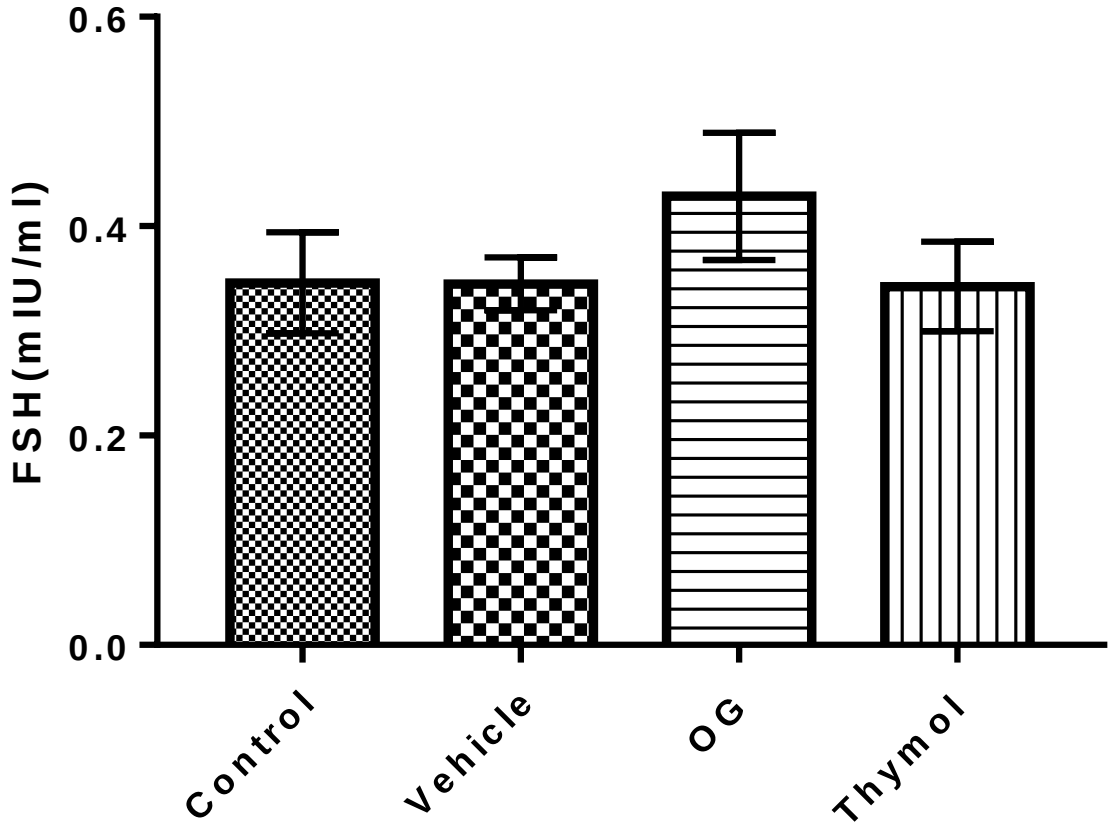


Figure 4.5: Graph showing the effects of aqueous extract of *Ocimum gratissimum* leaf (OG) and thymol on serum Follicle Stimulating Hormone level (FSH) in male Wistar rats. Data represent mean ± SEM. *n =5*

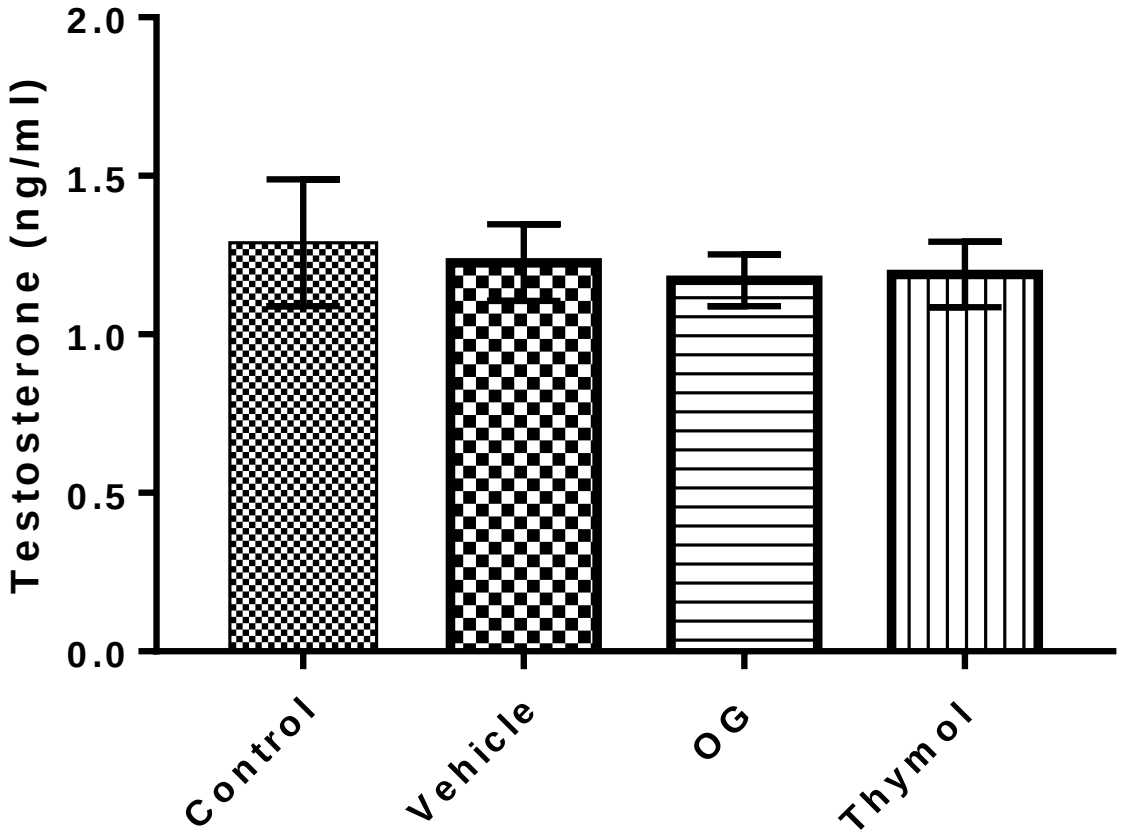


Figure 4.6: Graph showing the effects of aqueous extract of *Ocimum gratissimum* leaf (OG) and thymol on Serum Testosterone level in male Wistar rats. Data represent mean ± SEM. *n =5*.

4.4 Effects of *Ocimum gratissiumum* and thymol on Penile tissue histoarchitecture

Effects of *Ocimum gratissiumum* and thymol on corpus cavernosum and vascular tissue histoarchitecture are shown in plates 1 and 2, respectively.

There was no visible lesion in the corpus cavernosum histoarchitecture (Plates 1 A-D) while the vascular endothelium (Plate 2 A-D) was also intact in rats treated with *Ocimum gratissimum* or thymol compared with the control.

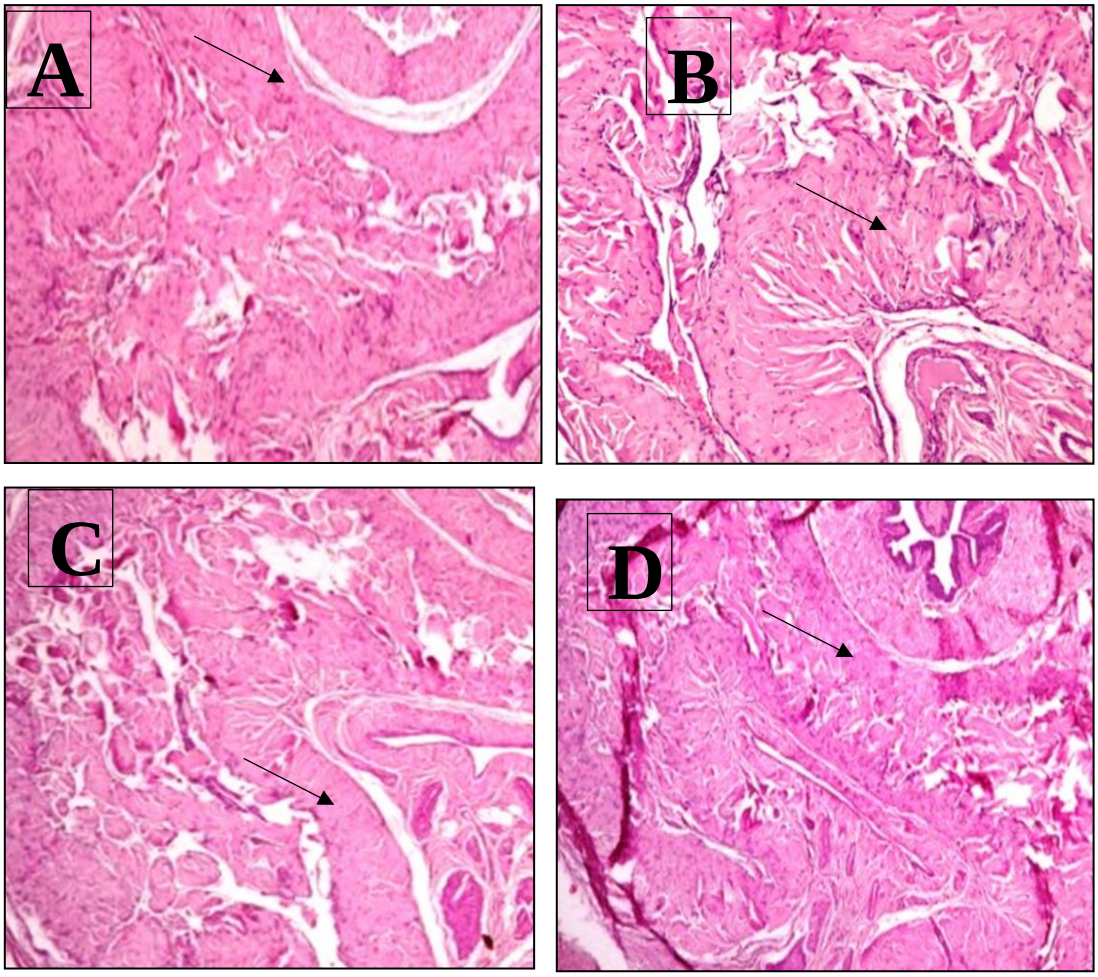


Plate 1: Effects of *Ocimum gratissimum* and thymol on the histoarchitecture of penile tissue corpus cavernosum.

A= Control B= Vehicle C= *Ocimum gratissimum* D= Thymol.

Black arrow= Corpus Cavernosum

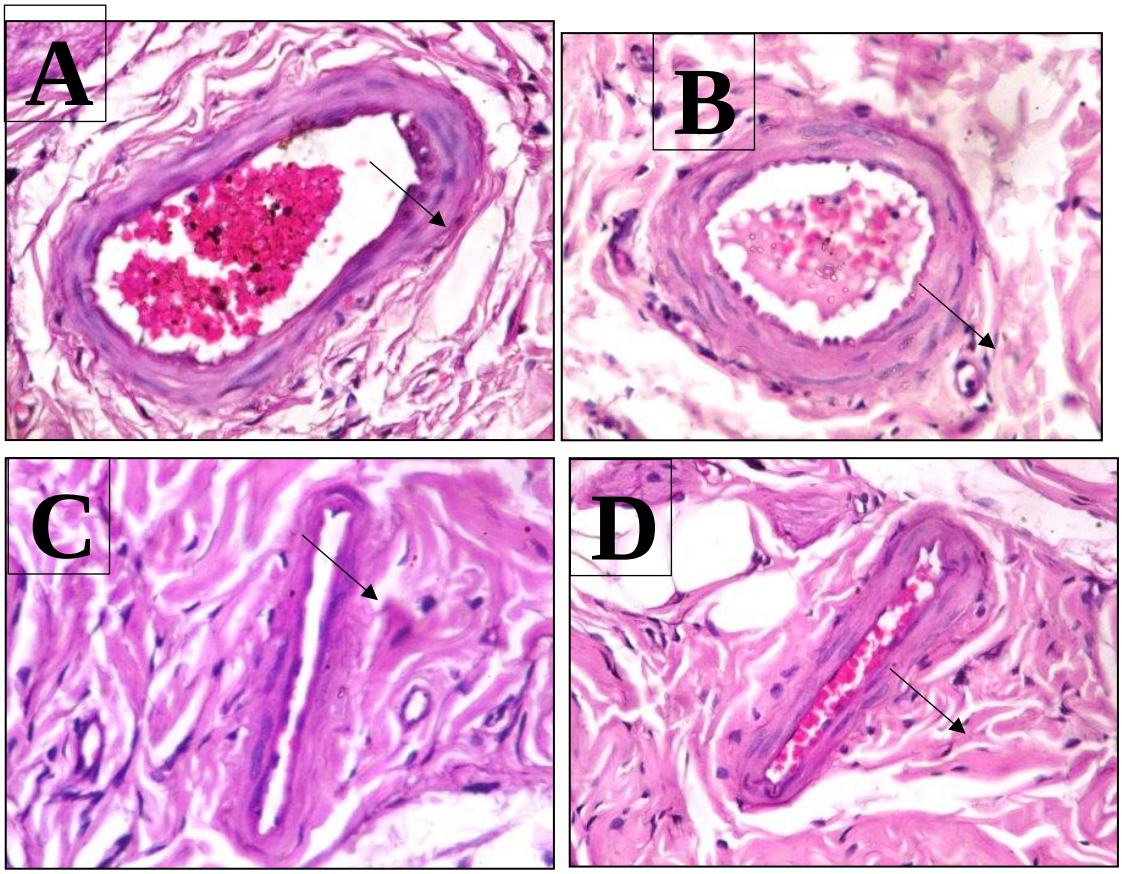


Plate 2: Effects of *Ocimum gratissimum* and thymol on the penile vescular histoarchitecture.

A= Control B= Vehicle C= *Ocimum gratissimum* D= Thymol.

Black arrow= vascular endothelium

4.5 Effects of *Ocimum gratissimum* or thymol on corpus cavernosum contraction and relaxation responses

The responses of corpus cavernosum to phenylephrine-induced contraction and potassium chloride-induced contraction respectively are shown in figure 4.7-4.9 and figure 4.10-4.12.

Figure 4.13-4.15 and figure 4.16-4.18 respectively showed the corpus cavernosum response to acetylcholine-induced relaxation in phenylephrine-precontracted tissue or potassium chloride-precontracted tissue *ex-vivo*.

Figure 4.19-4.21 and figure 4.22-4.24 respectively showed the corpus cavernosum response to sodium nitroprusside-induced relaxation in phenylephrine-precontracted tissue or potassium chloride-precontracted tissue *ex-vivo*.

The tissues incubated with *Ocimum gratissimum* and thymol responded to phenylephrine, potassium chloride, acetylcholine and sodium nitroprusside in a dose dependent manner similar to the control. The maximum contraction response (MCR) as well as the cumulative response to phenylephrine (figure 4.8 and 4.9 respectively) or potassium chloride (figure 4.11 and 4.12 respectively) were significantly reduced in *Ocimum gratissimum* incubated and thymol incubated tissues compared with the control ($p<0.05$).

In phenylephrine-precontracted tissues, the maximum relaxation (MRR, figure 4.14) response and the cumulative response (CR, figure 4.15) to acetylcholine was increased in tissues incubated with thymol ($p<0.05$) while there was no significant difference in the MRR and CR of tissues incubated with *Ocimum gratissimum* compared with control. The MRR (Figure 4.16) and CR (figure 4.18) to sodium nitroprusside were not significantly different in all the tissues.

Both the MRR and CR to acetylcholine (figure 4.20 and 4.21 respectively) or sodium nitroprusside (figure 4.23 and 4.24 respectively) significantly increased ($p<0.05$) in potassium chloride-precontracted tissue when they were incubated in *Ocimum gratissimum* or thymol, compared with the control.

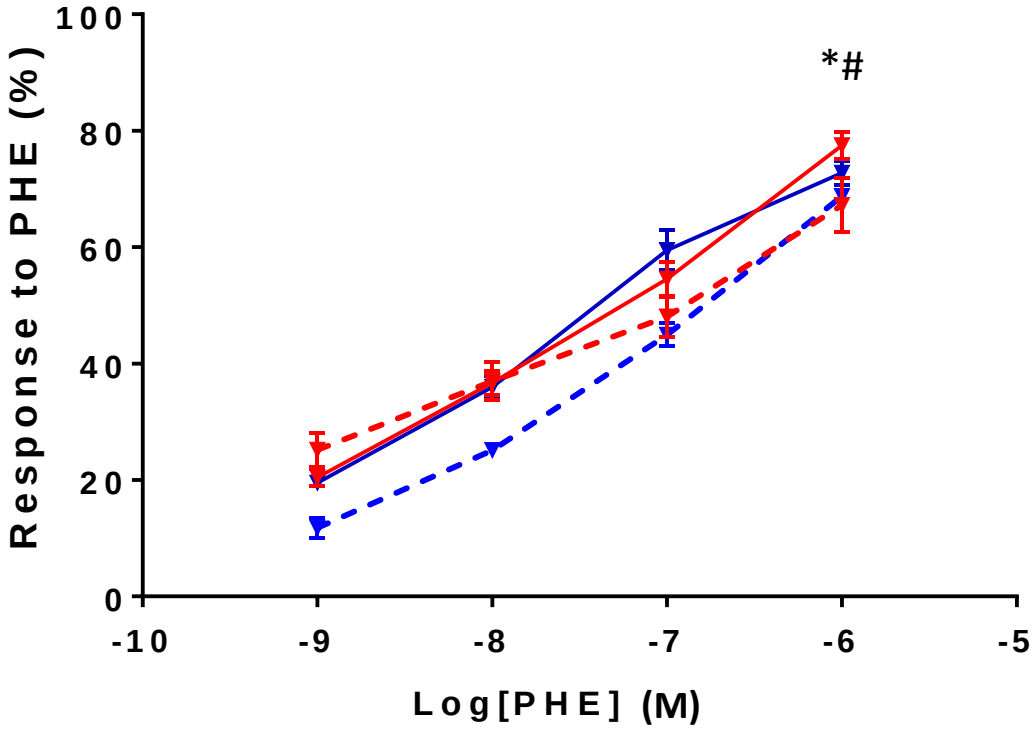


Figure 4.7: Graph showing the responses of corpus cavernosa smooth muscle to graded dose of Phenylephrine (PHE) when incubated in Blank Kreb's Solution (Red line), OG (Red dotted line), Vehicle (Blue Line), Thymol (Blue dotted line).

* = $p<0.05$ (OG vs Control), # = $p<0.05$ (Thymol vs control)

PHE = Phenylephrine OG = *Ocimum gratisimum*

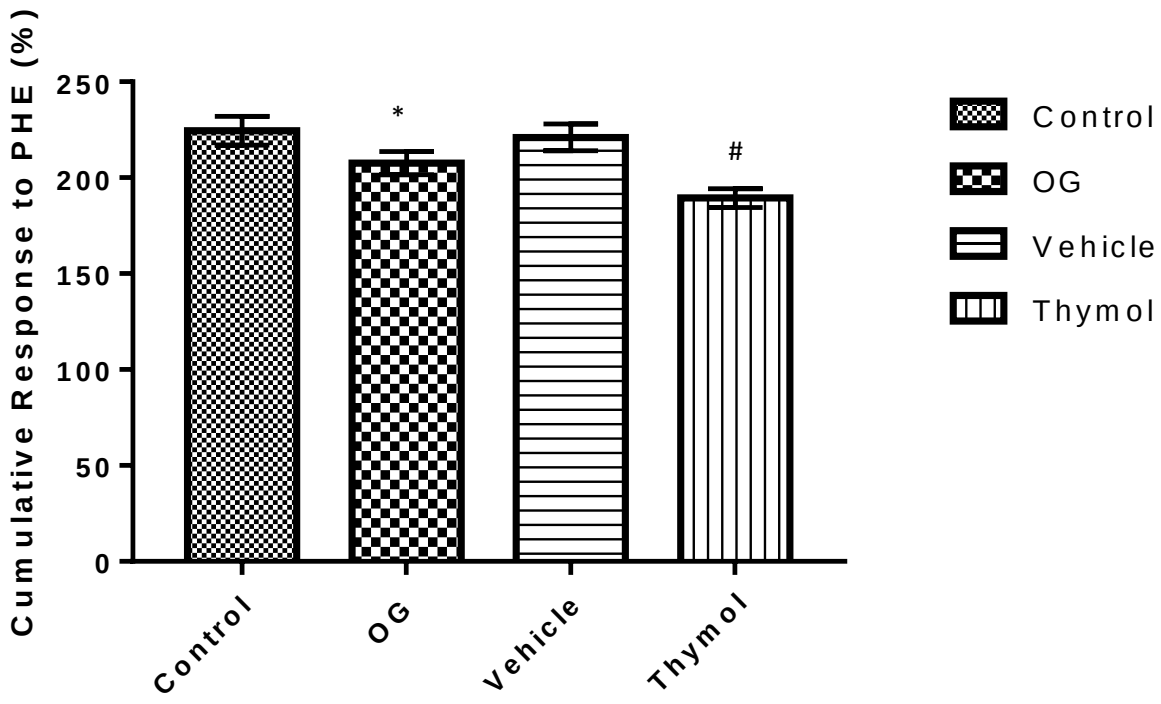


Figure 4.8: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative Response of corpus cavernosa smooth muscle to phenylephrine when incubated in Blank Kreb's Solution (Red line), *Ocimum gratissimum* (OG), Vehicle or Thymol.

* = $p<0.05$ (OG vs Control), # = $p<0.05$ (Thymol vs control)

PHE = Phenylephrine

OG = *Ocimum gratissimum*

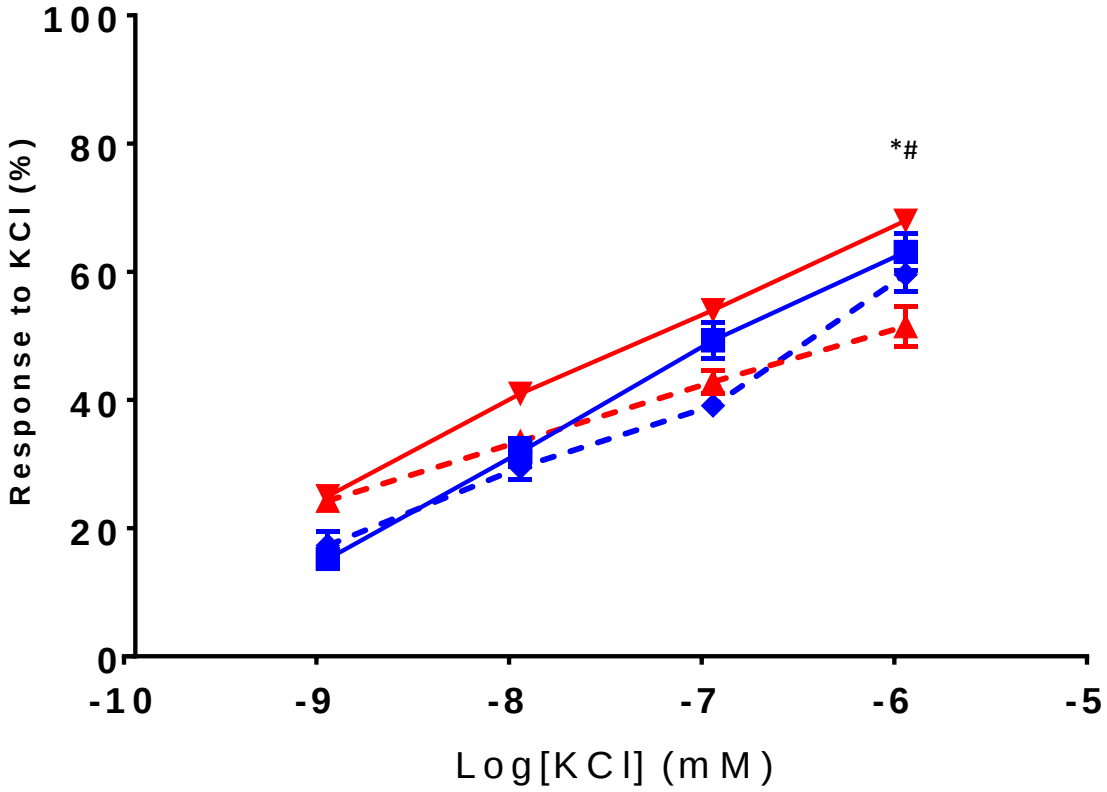


Figure 4.9: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of corpus cavernosa smooth muscle to graded dose of Potassium chloride when incubated in Blank Kreb's Solution (Red line), *Ocimum gratissimum* (OG) (Red dotted line), Vehicle (Blue Line), Thymol (Blue dotted line).

* = $p<0.05$ (OG vs Control), # = $p<0.05$ (Thymol vs Control)

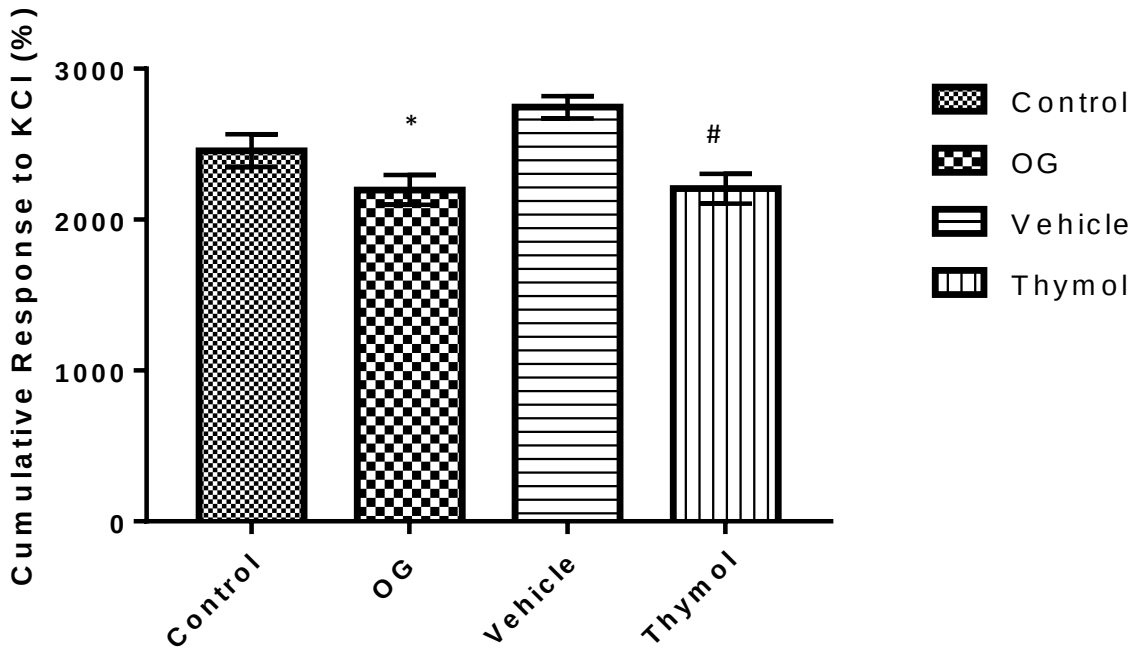


Figure 4.10: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative Response of corpus cavernosa smooth muscle to potassium chloride when incubated in Blank Kreb's Solution (Red line), *Ocimum gratissimum* (OG), Vehicle or Thymol.

* = $p<0.05$ (OG vs Control), # = $p<0.05$ (Thymol vs control)

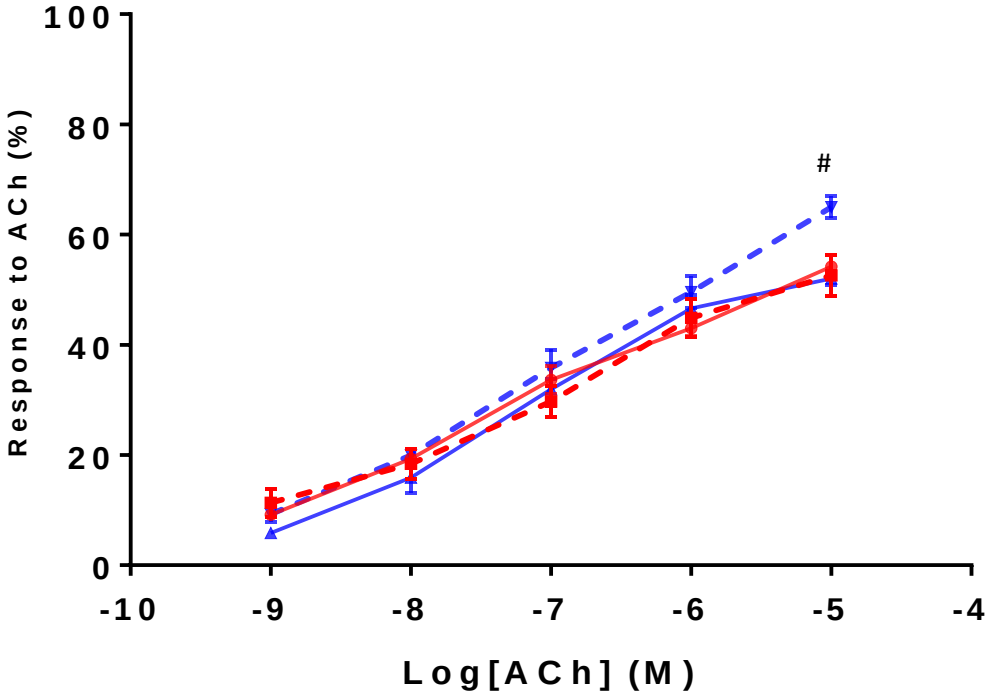


Figure 4.11: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative Responses of phenylephrine-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine (ACh) when incubated with Blank Kreb's Solution (Red line), OG (Red dot), Vehicle (Blue Line) or Thymol (Blue dot).

#p<0.05 (Thymol vs Control)

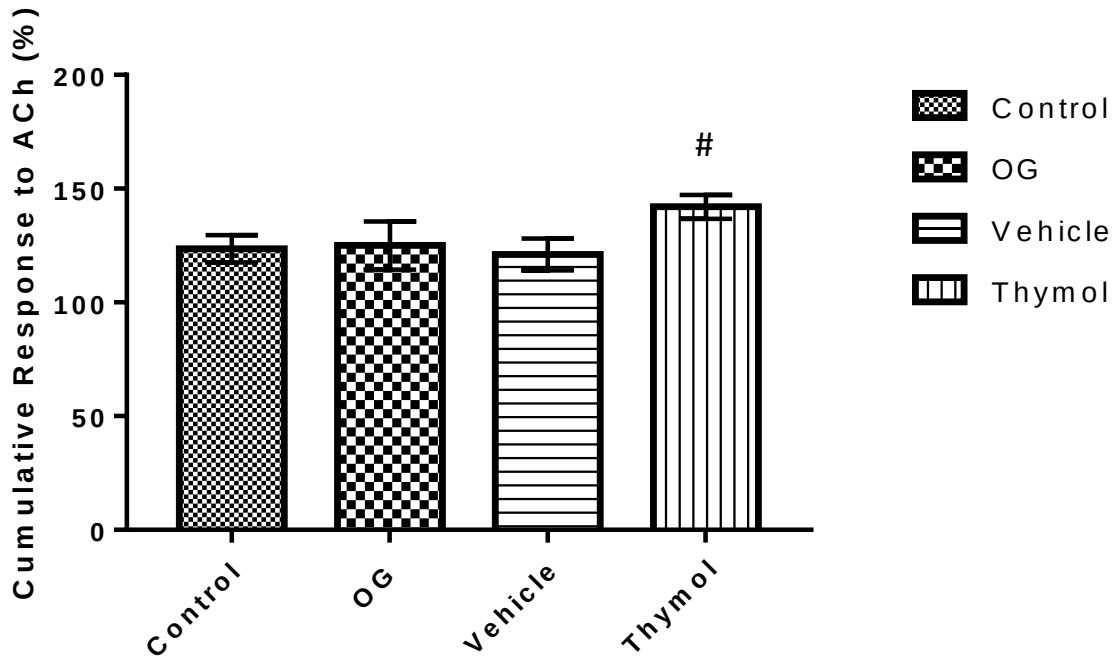


Figure 4.12: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative responses of phenylephrine-precontracted corpus cavernosa smooth muscle to acetylcholine (ACh) when incubated with Blank Kreb's Solution, *Ocimum gratissimum* (OG), Vehicle or Thymol. #$p<0.05$ (Thymol vs Control)

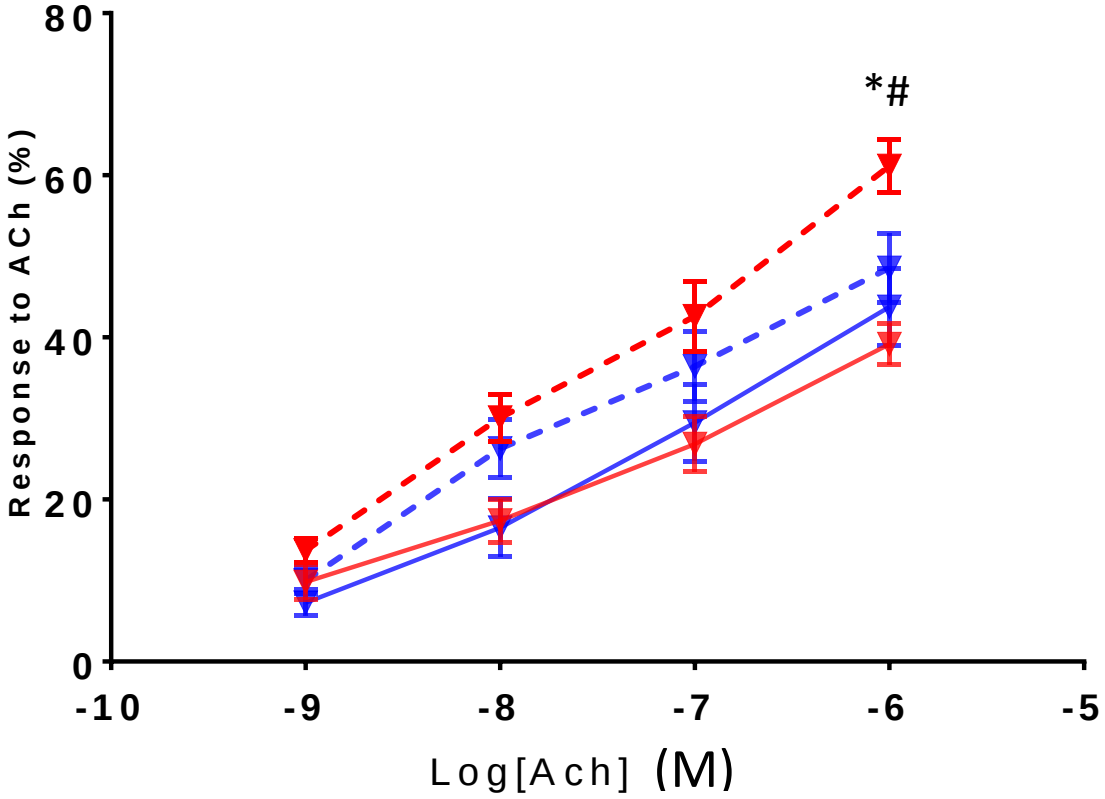


Figure 4.13: Graph showing the effects of *Ocimum gratissimum* and thymol on the Responses of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of Acetylcholine (Ach) when incubated with Blank Kreb's Solution (Control, Red line), *Ocimum gratissimum* (OG, Red dot), Vehicle (Blue Line) or Thymol (Blue dot). *$p<0.05$ (OG vs Control). #$p<0.05$ (Thymol vs Control)

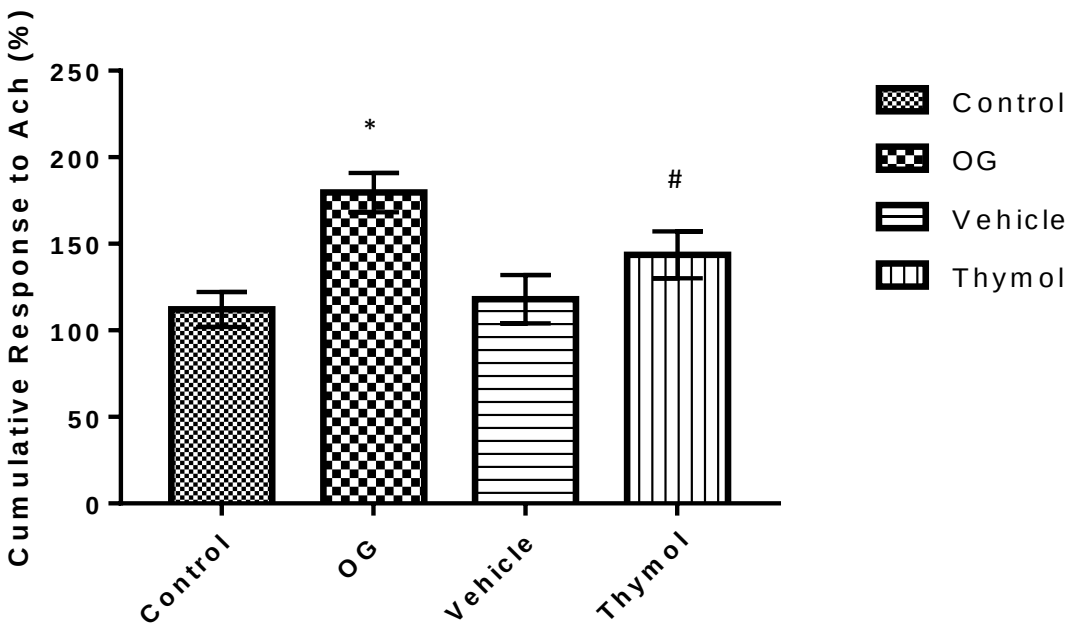


Figure 4.14: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of potassium chloride-precontracted corpus cavernosa smooth muscle to acetylcholine when incubated with Blank Kreb's Solution (Control), *Ocimum gratissimum* (OG), Vehicle or Thymol. *$p<0.05$ (OG vs Control), #$p<0.05$ (Thymol vs Control)

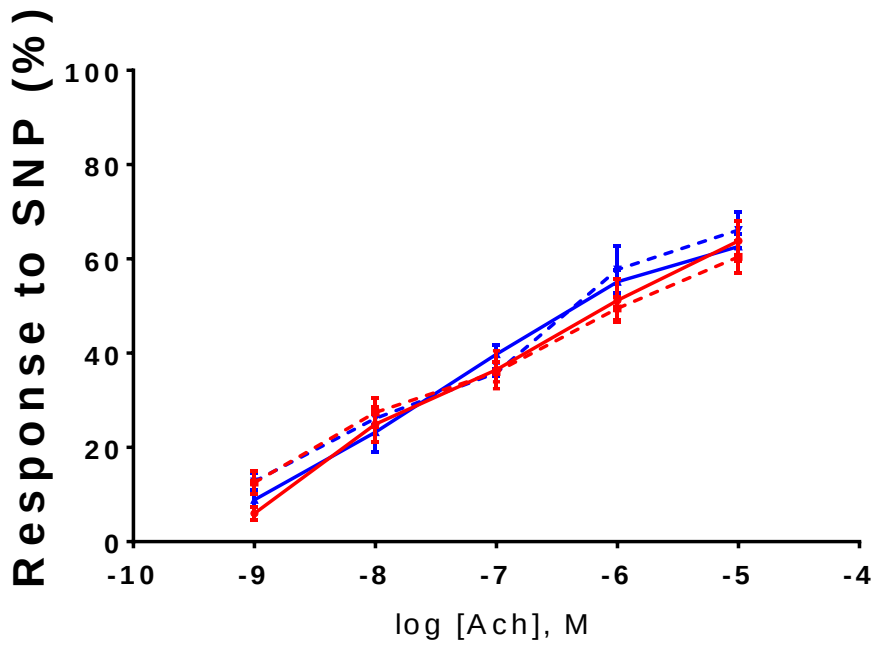


Figure 4.15: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of phenylephrine-precontracted corpus cavernosa smooth muscle to graded dose of sodium nitroprusside when incubated with Blank Kreb's Solution (Control, Red line), *Ocimum gratissimum* (Red dot), Vehicle (Blue Line) or Thymol (Blue dot),

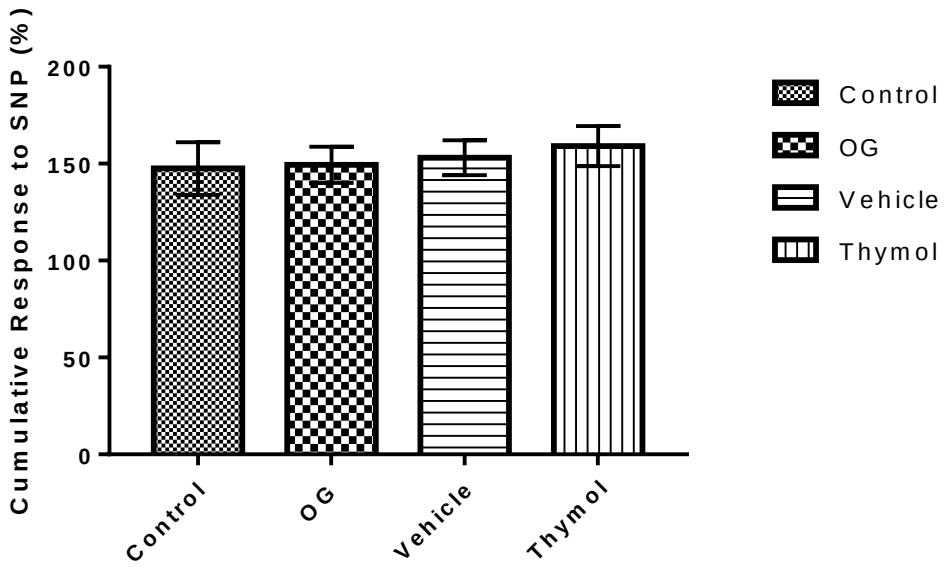


Figure 4.16: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of phenylephrine-precontracted corpus cavernosa smooth muscle to sodium nitroprusside when incubated with Blank Kreb's Solution, *Ocimum gratissimum* (OG), Vehicle or Thymol.

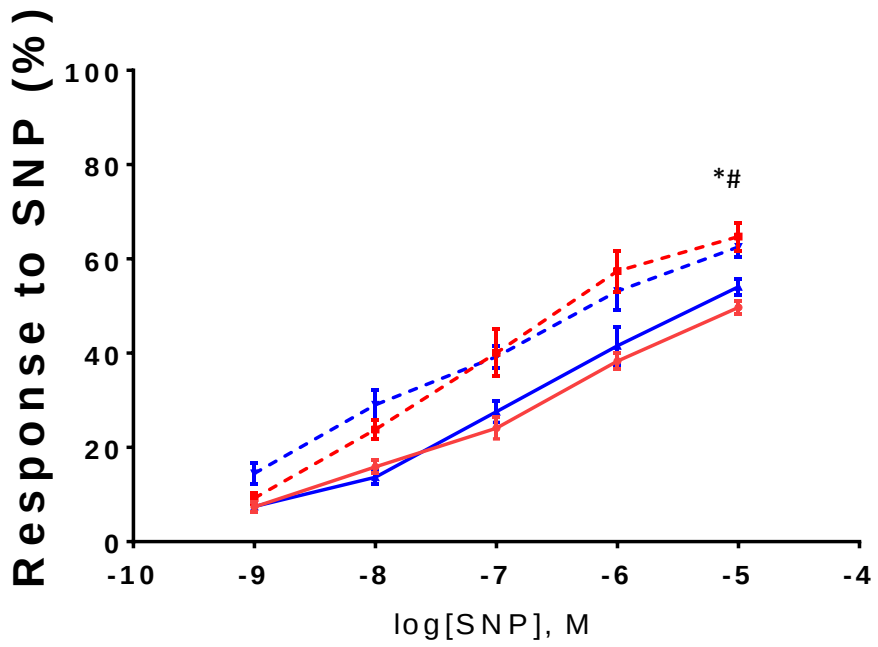


Figure 4.17: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of potassium chloride (KCl)-precontracted corpus cavernosa smooth muscle to sodium nitroprusside (SNP) when incubated with Blank Kreb's Solution (Control, Red line), *Ocimmum gratissimum* (OG, Red dot), Vehicle (Blue Line) or Thymol (Blue dot).

*$p<0.05$ (OG vs Control). #$p<0.05$ (Thymol vs Control)

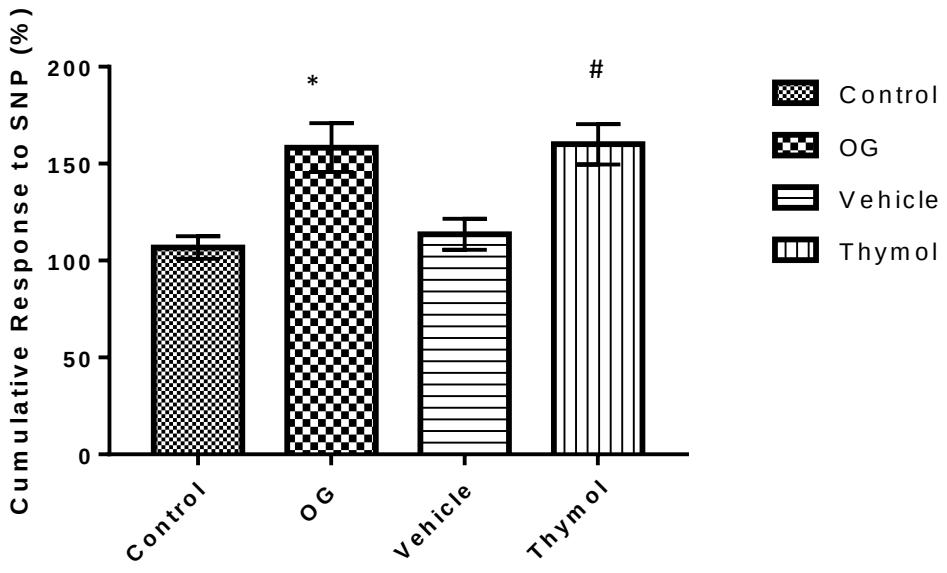


Figure 4.18: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of KCl-precontracted corpus cavernosa smooth muscle to sodium nitroprusside (SNP) when incubated with blank kreb's solution (Control), *Ocimum gratissimum* (OG), vehicle or thymol. *$p<0.05$ (OG vs Control), #$p<0.05$ (Thymol vs Control)

4.6 Effects of *Ocimum gratissimum* and thymol on penile tissue endothelial functions in Wistar rat

The effects of *Ocimum gratissimum* and thymol on penile vascular endothelial functions in Wistar rat are shown in figure 4.25 – 4.37.

Administration of *Ocimum gratissimum* or thymol to the rats had no significant effect on the level of nitric oxide in the penile tissue (figure 4.25) compared with the control.

In phenylephrine precontraction, the Maximum Relaxation Response (MRR) (figure 4.27) and the cumulative response (figure 4.28) to acetylcholine was significantly increased in tissues incubated with thymol + L-NAME but not in the tissues incubated with *Ocimum gratissimum* + L-NAME compared with thymol or *Ocimum gratissimum* incubated tissues respectively. Co-incubation of indomethacin with *Ocimum gratissimum* or with thymol had no significant effect on the tissue's MRR (figure 4.33) and CR (figure 4.34) to Acetylcholine compared with when indomethacin was absent.

In potassium chloride precontraction, MRR (figure 4.30) and CR (figure 4.31) to acetylcholine was significantly reduced when L-NAME was co-incubated with *Ocimum gratissimum* ($p<0.05$) while it increased when L-NAME was co-incubated with thymol compared to when the tissues were incubated in *Ocimum gratissimum* or thymol alone. The MRR but not CR was increased when Indomethacin was co-incubated with thymol while MRR and CR to acetylcholine (figure 4.36 and figure 4.37) was significantly reduced when indomethacin was co-incubated with *Ocimum gratissimum* compared with when they were incubated in thymol alone *or Ocimum gratissimum* respectively.

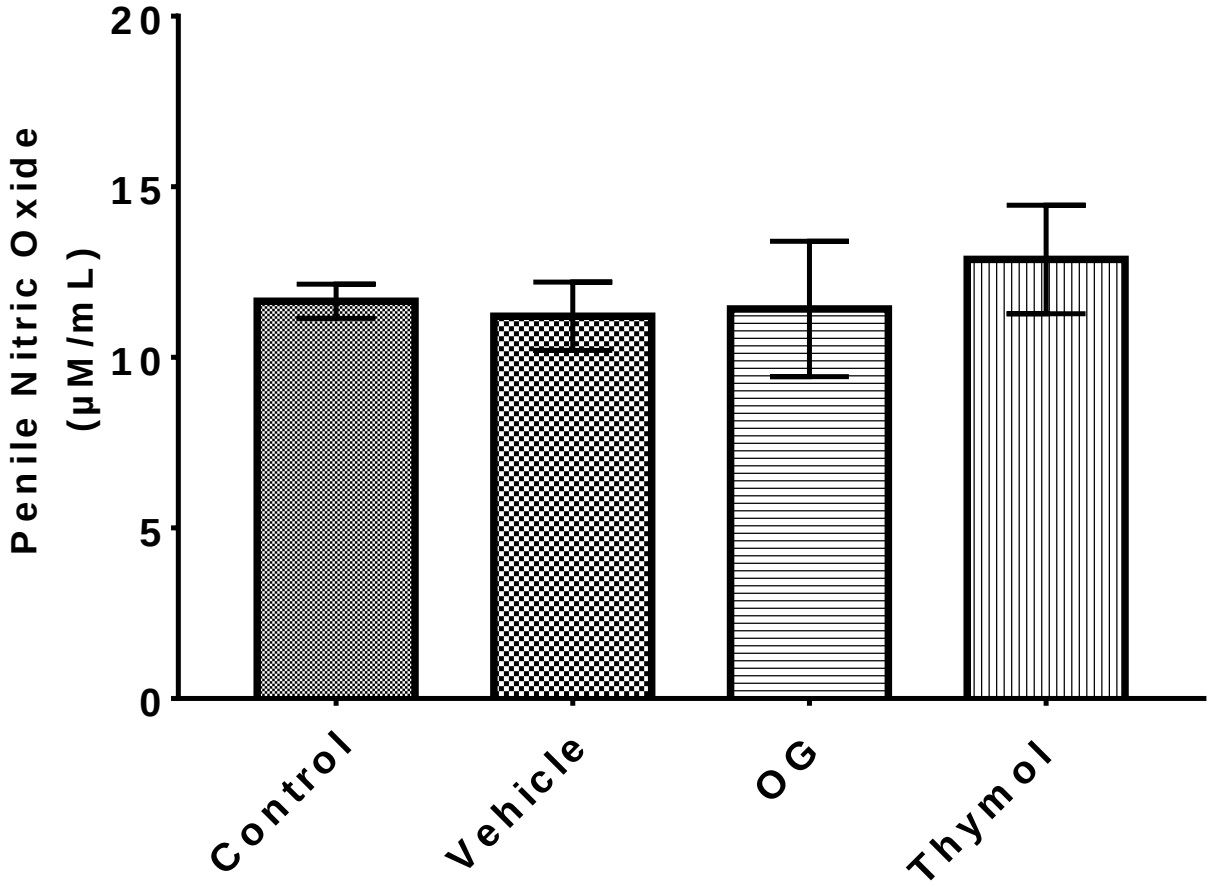


Figure 4.19: Graph showing the effects of *Ocimum gratissimum* (OG) and thymol on Penile Nitric Oxide of male rats. Data represent mean ± SEM. *n =5*.

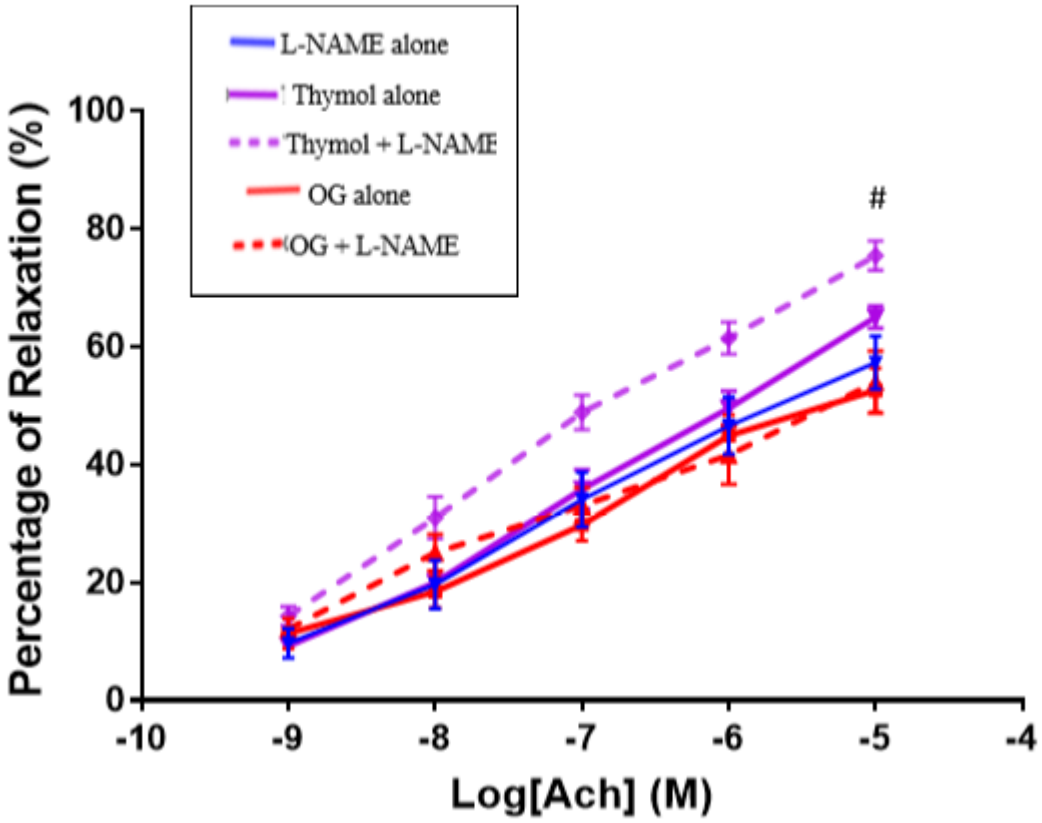


Figure 4.20: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of phenylephrine pre-contracted corpus cavernosa smooth muscle to acetylcholine in the presence of nitric oxide inhibitor.

#p<0.05 (Thymol alone vs Thymol + L-NAME)

OG = *Ocimum gratissimum*

L-NAME = Nitro-L-arginine methyl ester

Ach = Acetylcholine

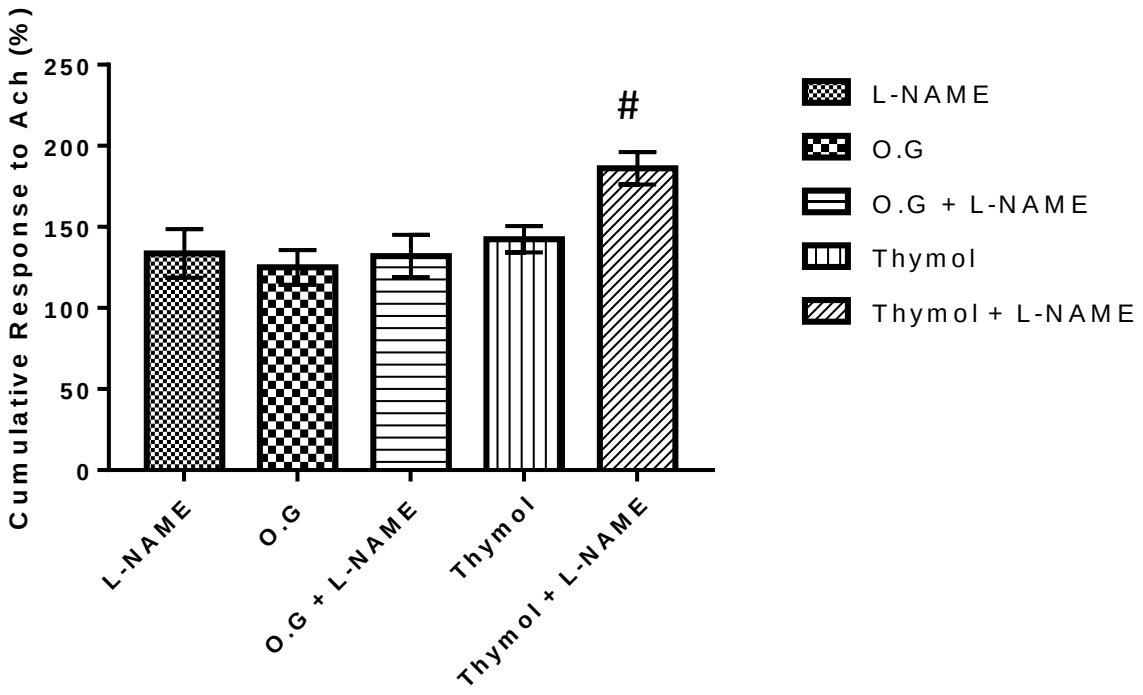


Figure 4.21: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of phenylephrine pre-contracted corpus cavernosa smooth muscle to Acetylcholine in the presence of nitric oxide inhibitor.

#p<0.05 (Thymol alone vs Thymol + L-NAME)

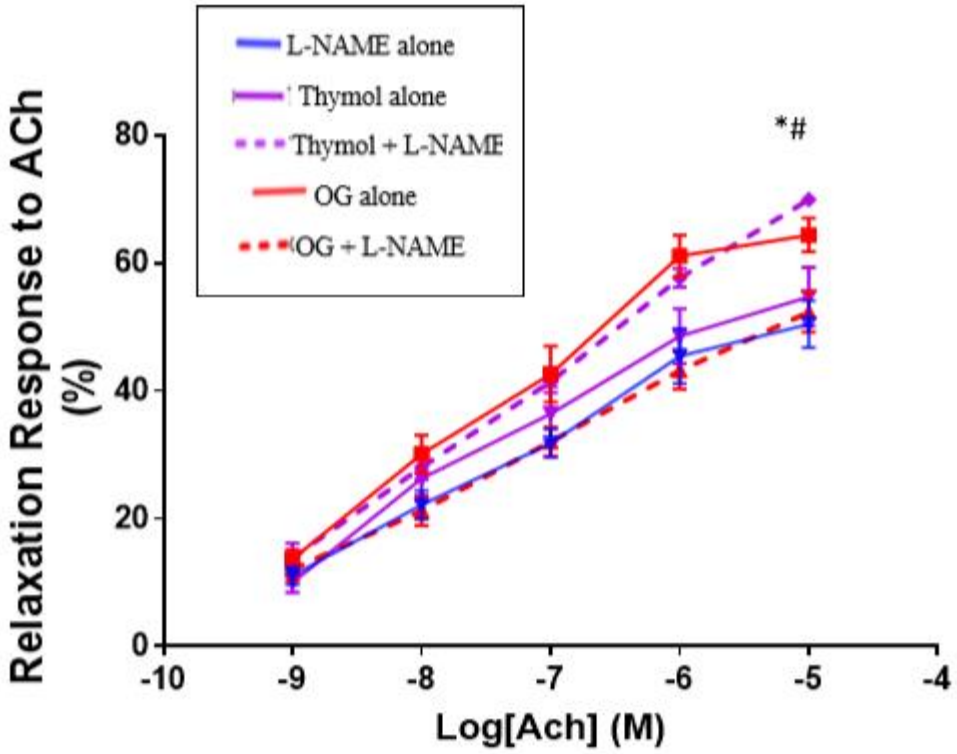


Figure 4.22: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of potassium chloride (KCl)-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of nitric oxide inhibitor.

*p<0.05 (OG alone vs OG + L-NAME); #p<0.05 (Thymol alone vs Thymol + L-NAME)

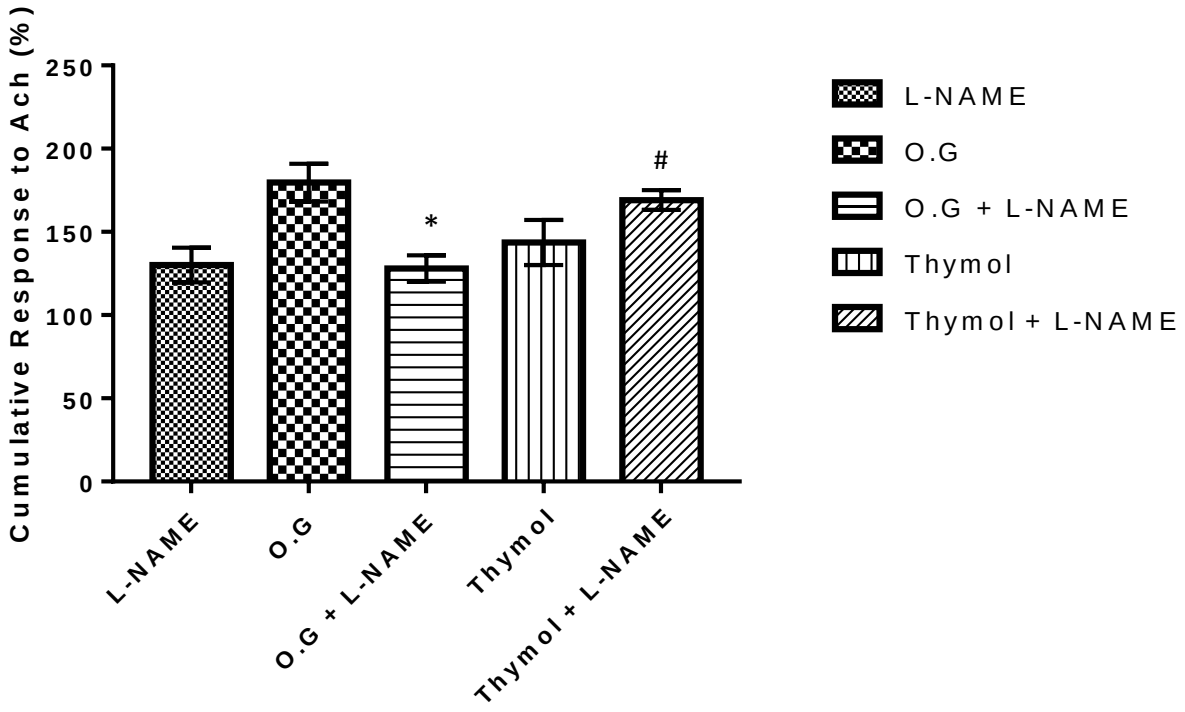


Figure 4.23: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of potassium chloride (KCl)-precontracted corpus cavernosa smooth muscle to acetylcholine in the presence of nitric oxide inhibitor.

*$p<0.05$ (OG alone vs OG + L-NAME); #$p<0.05$ (Thymol alone vs Thymol + L-NAME)

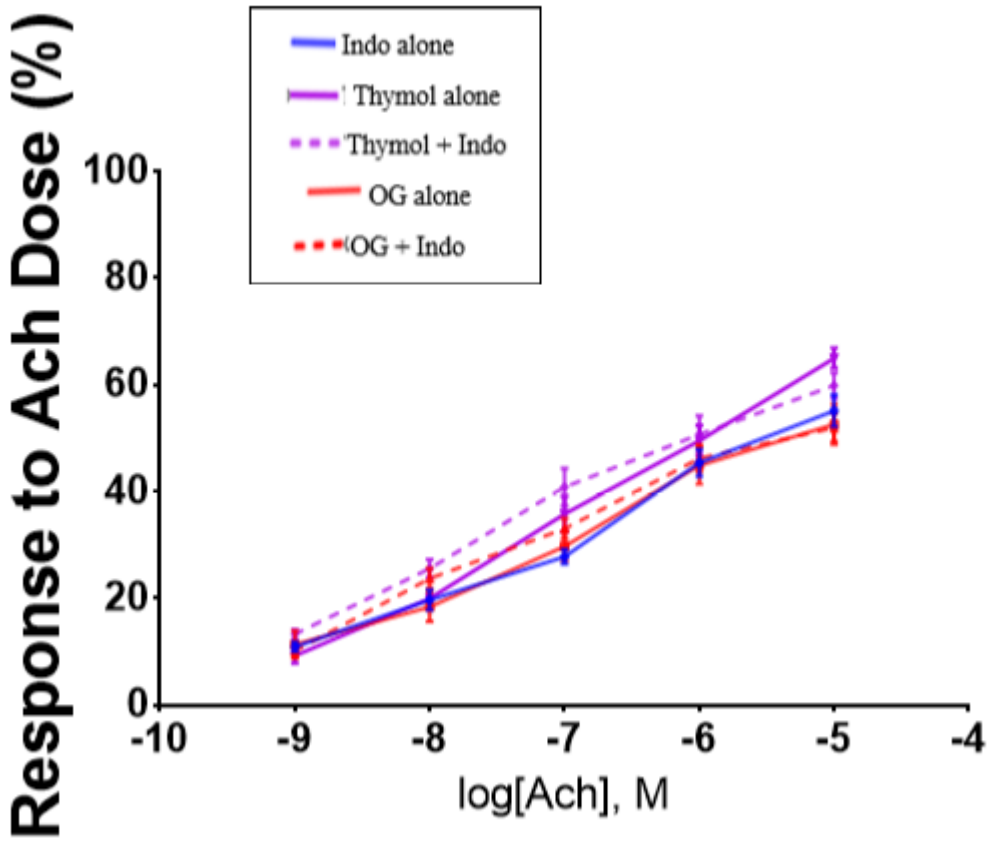


Figure 4.24: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of Phenylepinephrine-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of Indomethacin (Indo).

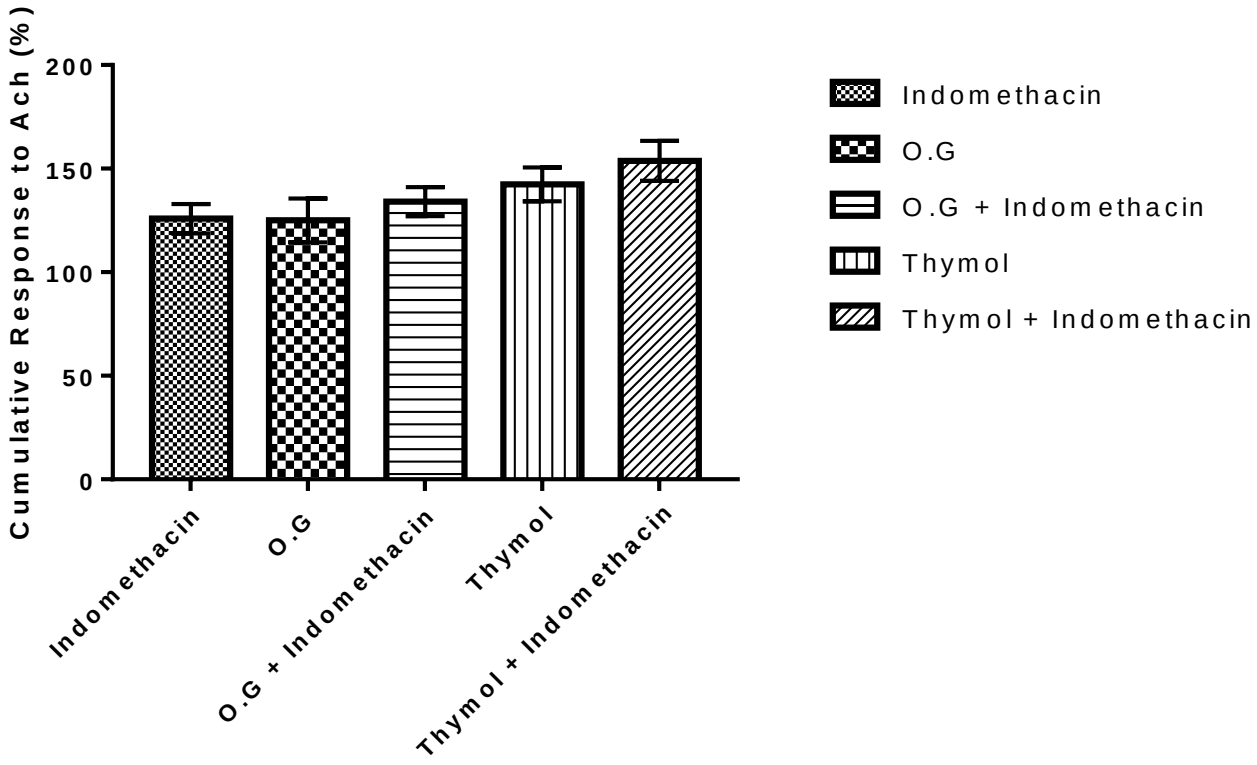


Figure 4.25: Graph showing the effects of *Ocimum gratissimum* (O.G) and thymol on the cumulative response of phenylephrine -precontracted corpus cavernosa smooth muscle to acetylcholine in the presence of prostaglandin inhibitor.

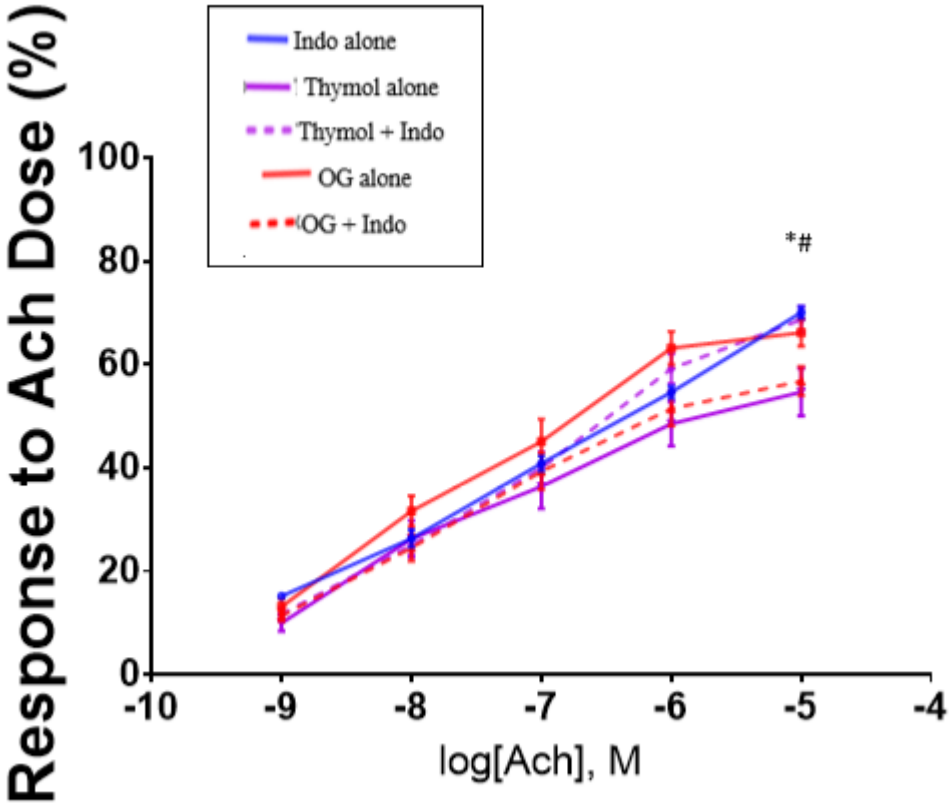


Figure 4.26: Graph showing the effects of *Ocimum gratissimum (OG)* and thymol on the responses of KCl-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of prostaglandin inhibitor.

*p<0.05 (OG alone vs OG + Indomethacin);

#p<0.05 (Thymol alone vs Thymol + Indomethacin)

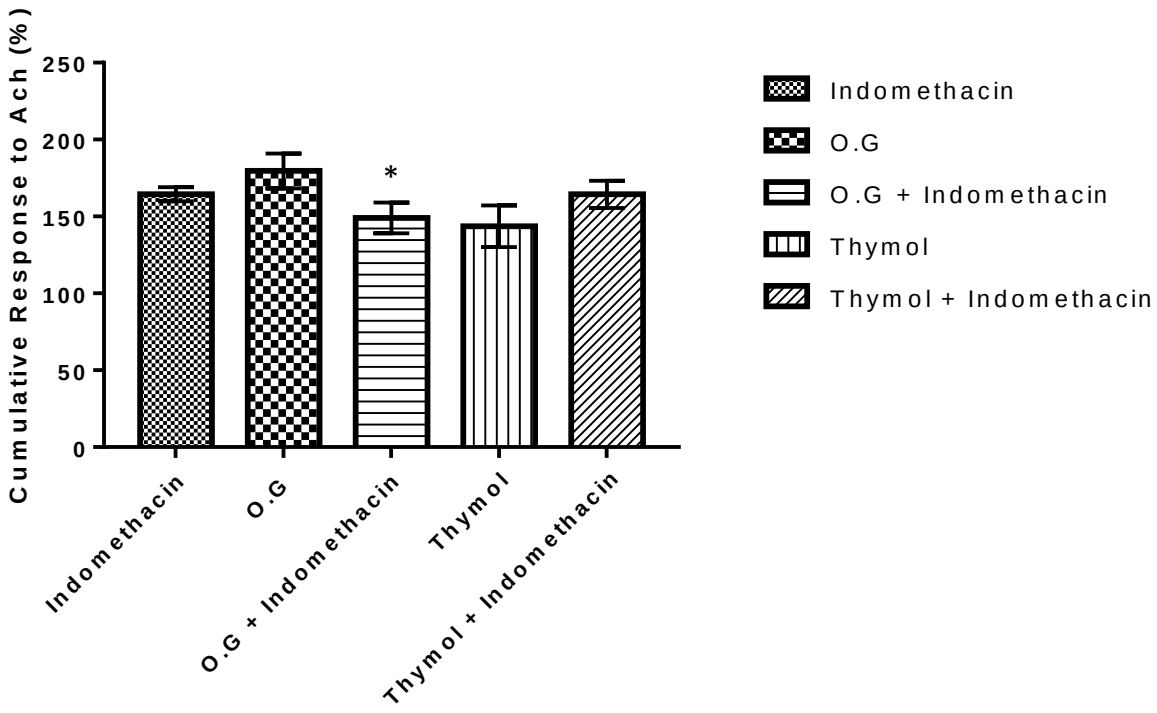


Figure 4.27: Graph showing the effects of *Ocimum gratissimum (O.G)* and thymol on the cumulative response of potassium chloride-precontracted corpus cavernosa smooth muscle to acetylcholine in the presence of prostaglandin inhibitor.

*$p<0.05$ (OG alone vs OG + Indomethacin)

4.7 Effects of *Ocimum gratissimum* and Thymol on cyclic guanosine monophosphate (cGMP) production in Wistar rat

The effects of *Ocimum gratissiumum* and thymol on penile cGMP level in Wistar rats' penile tissue are shown in figure 4.38 - 450

In vivo administration of *Ocimum gratissimum* or thymol had no significant effect on the level of cGMP compared with the control (figure 4.38).

The Maximum Relaxation Response (MRR) and Cumulative Response (CR) to acetylcholine was significantly reduced when *Ocimum gratissimum* was co-incubated with ODQ in the phenylephrine-precontracted (figures 4.40 and 4.41) and potassium chloride-precontracted (figures 4.43 and 4.44) tissues compared with the MRR in the tissues incubated with *Ocimum gratissimum* alone ($p<0.05$). There was, however, no significant difference in the MRR and CR to acetylcholine when ODQ was co-incubated with thymol both in phenylephrine-precontracted and potassium chloride-precontracted tissues compared with the tissues incubated with thymol alone.

Co-incubation of Methylene Blue (MB) with *Ocimum gratissimum* significantly ($p<0.05$) increased the MRR and CR to acetylcholine in phenylephrine-precontracted tissues (figure 4.46) while the MRR and CR were not changed in potassium chloride-precontracted tissues (figure 4.49) compared to incubation with *Ocimum gratissimum* alone. There was no significant difference in the MRR and CR to acetylcholine when MB was co-incubated with thymol both in phenylephrine-precontracted and potassium chloride (KCl)-precontracted tissues compared to incubation with thymol alone.

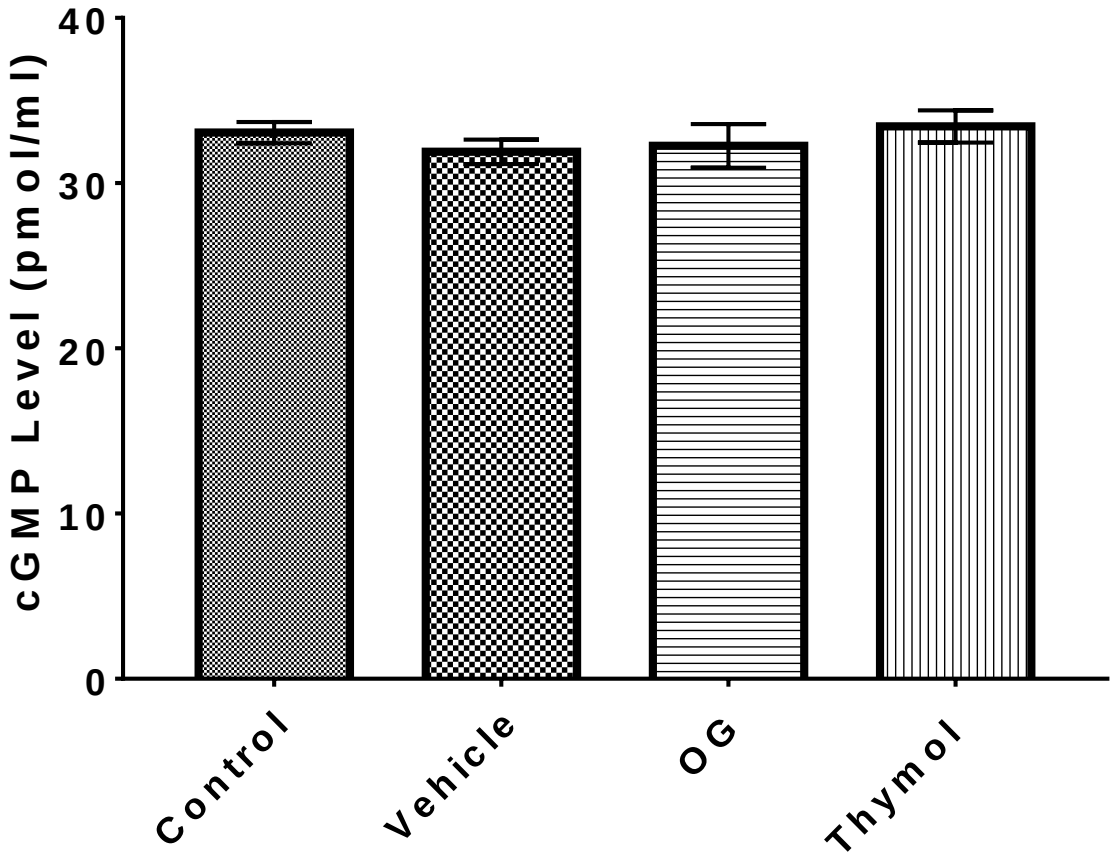


Figure 4.28: Graph showing the effects of *Ocimum gratissimum* and thymol on Penile cGMP level. Data represent mean ± SEM. *n =5*

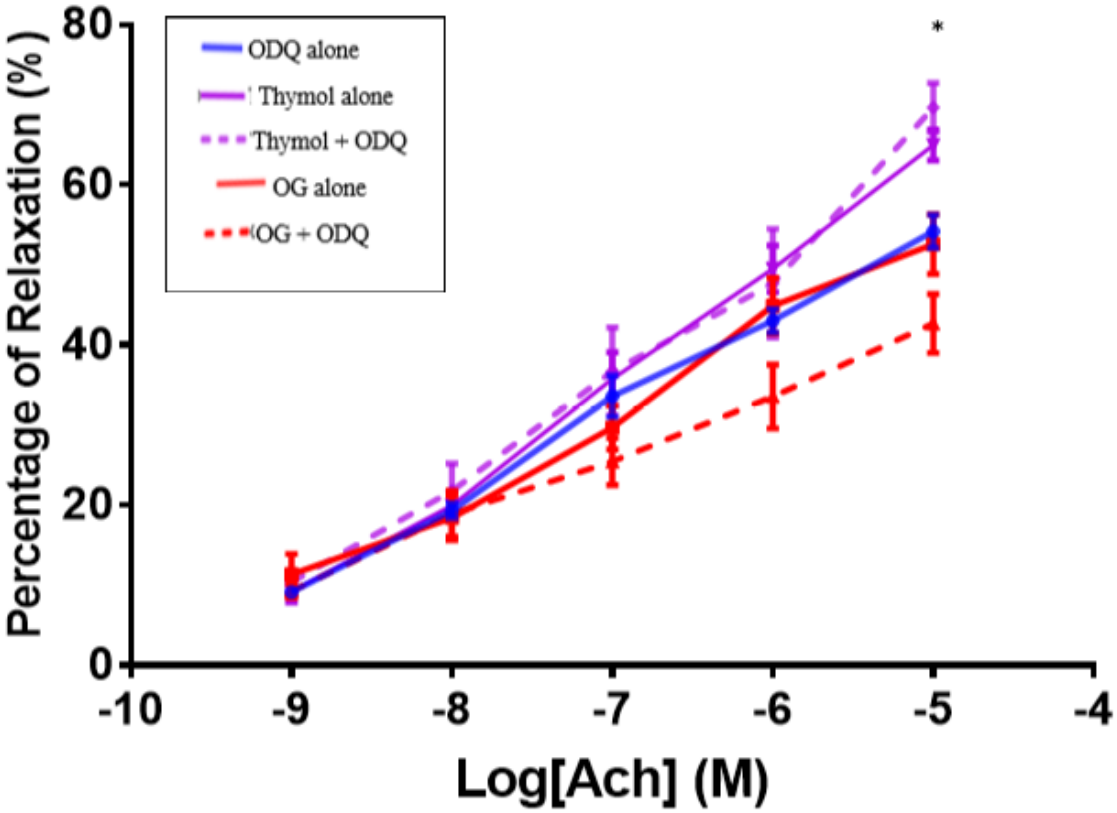


Figure 4.29: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of Phenylephrine-precontracted corpus cavernosa smooth muscle to acetylcholine in the presence of selective cGMP inhibitor. Each point represent mean ± SEM, n=6

*p<0.05 (OG vs OG + ODQ)

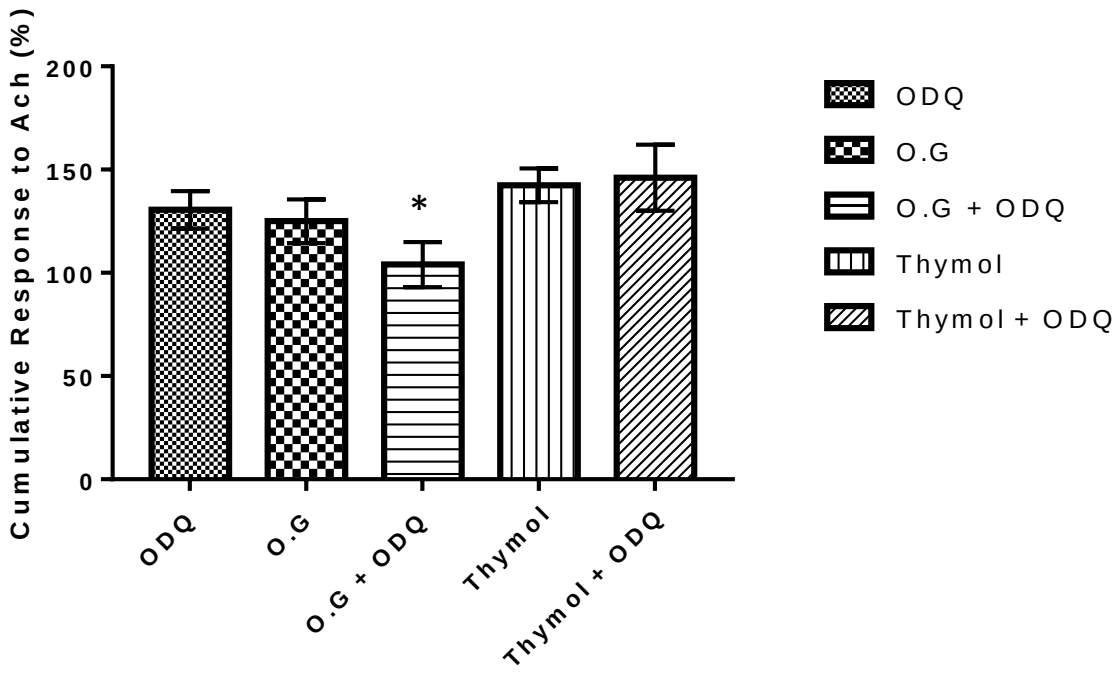


Figure 4.30: Graph showing the effects of *Ocimum gratissimum* (OG) and thymol on the cumulative response of phenylephrine-precontracted corpus cavernosa smooth muscle to acetylcholine in the presence of selective cGMP inhibitor.

*$p<0.05$ (OG vs OG + ODQ)

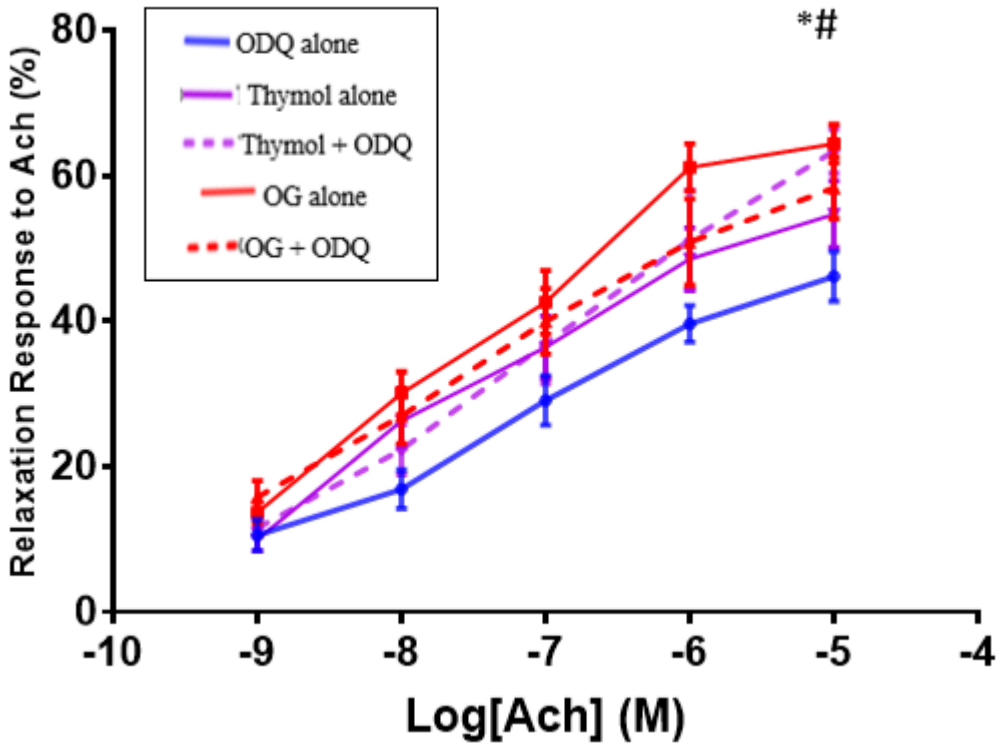


Figure 4.31: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of potassium chloride (KCl)-precontracted corpus cavernosa smooth muscle to Acetylcholine in the presence of selective cGMP inhibitor. Each point represent mean ± SEM, n=6

*p<0.05 (OG alone vs OG + ODQ)

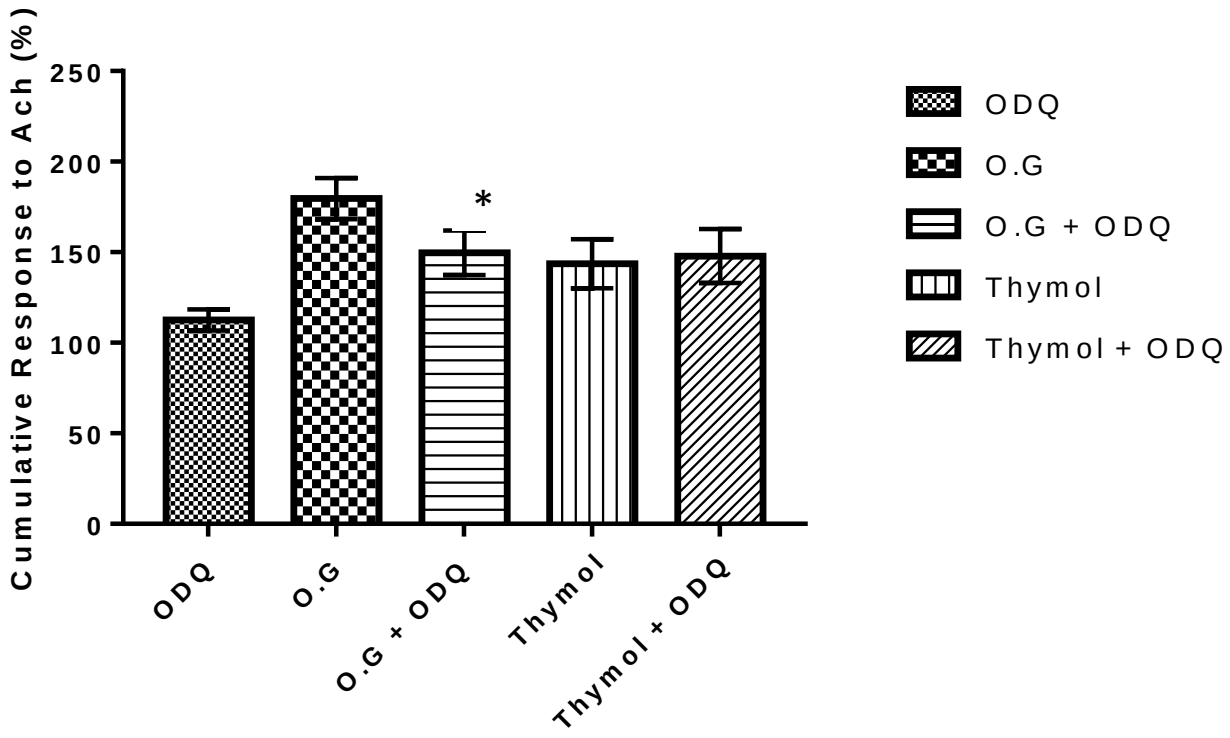


Figure 4.32: Graph showing the Effects of *Ocimum gratissimum* and thymol on the cumulative response of potassium chloride (KCl)-precontracted corpus cavernosa smooth muscle to acetylcholine in the presence of selective cGMP inhibitor.

*$p<0.05$ (OG vs OG + ODQ)

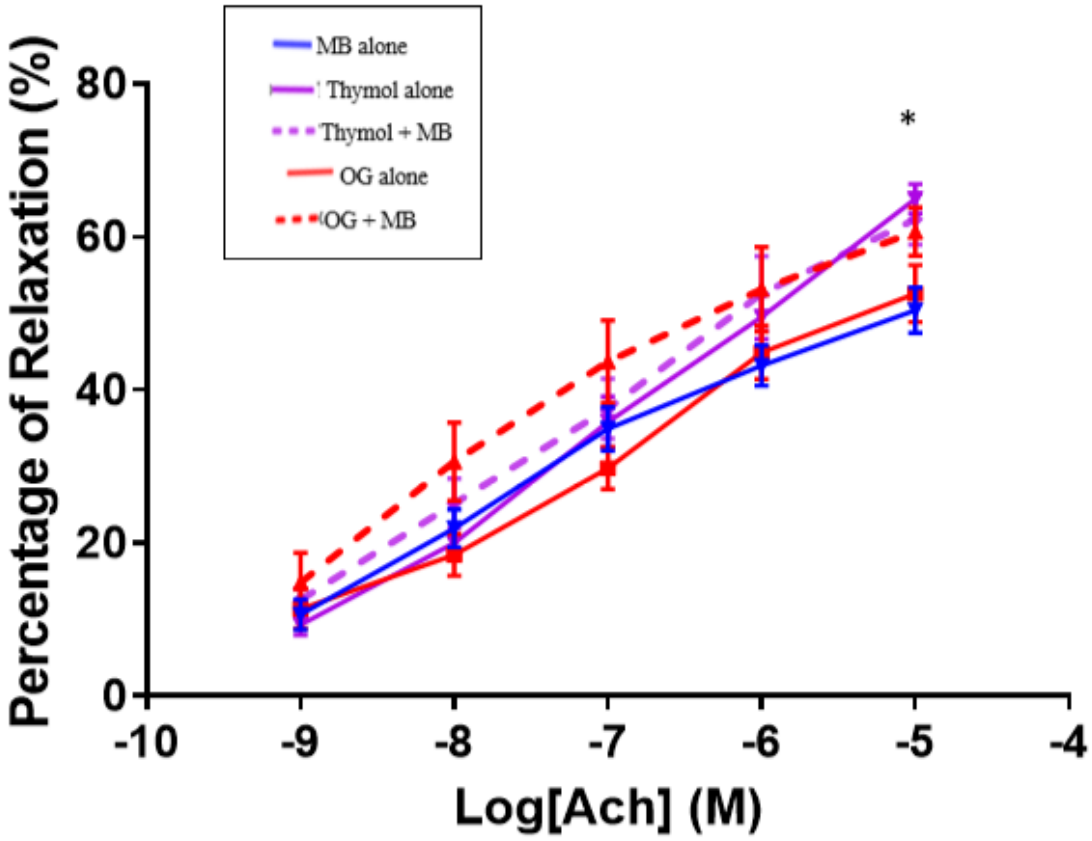


Figure 4.33: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of phenylephrine-precontracted corpus cavernosa smooth muscle to Acetylcholine in the presence of non-selective cGMP inhibitor. Each point represent mean ± SEM, n=6

*p<0.05 (OG vs OG + MB)

OG= *Ocimum gratissimum*

MB= Methylene Blue

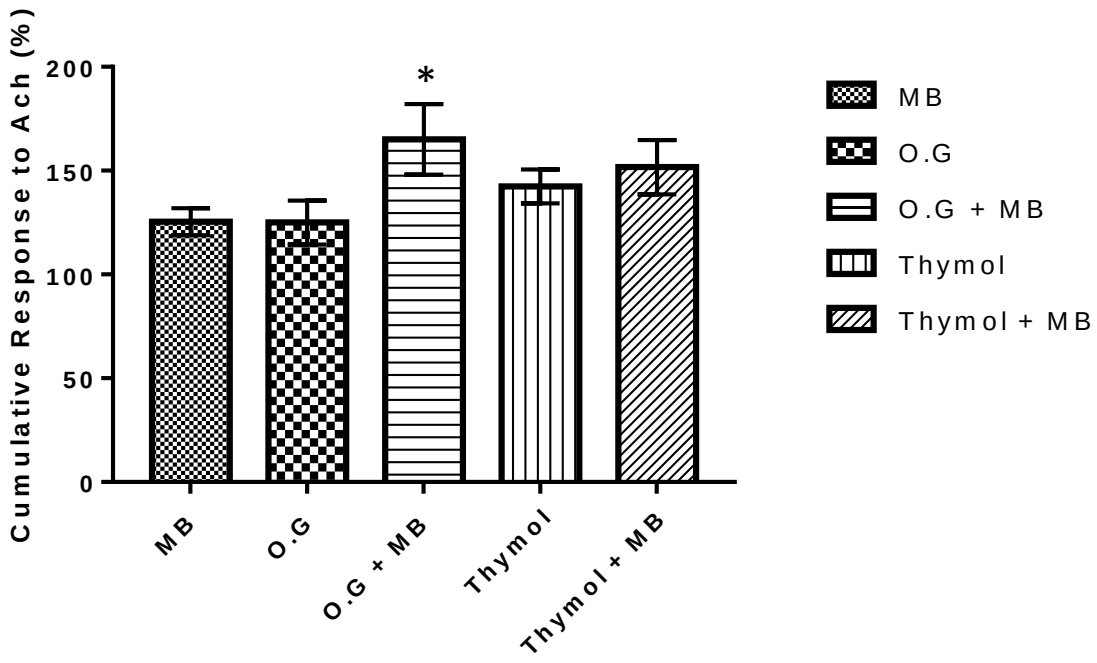


Figure 4.34: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of phenylephrine-precontracted corpus cavernosa smooth muscle to acetylcholine in the presence of non-selective cGMP inhibitor.

*$p<0.05$ (OG vs OG + MB)

OG= *Ocimum gratissimum*

MB= Methylene Blue

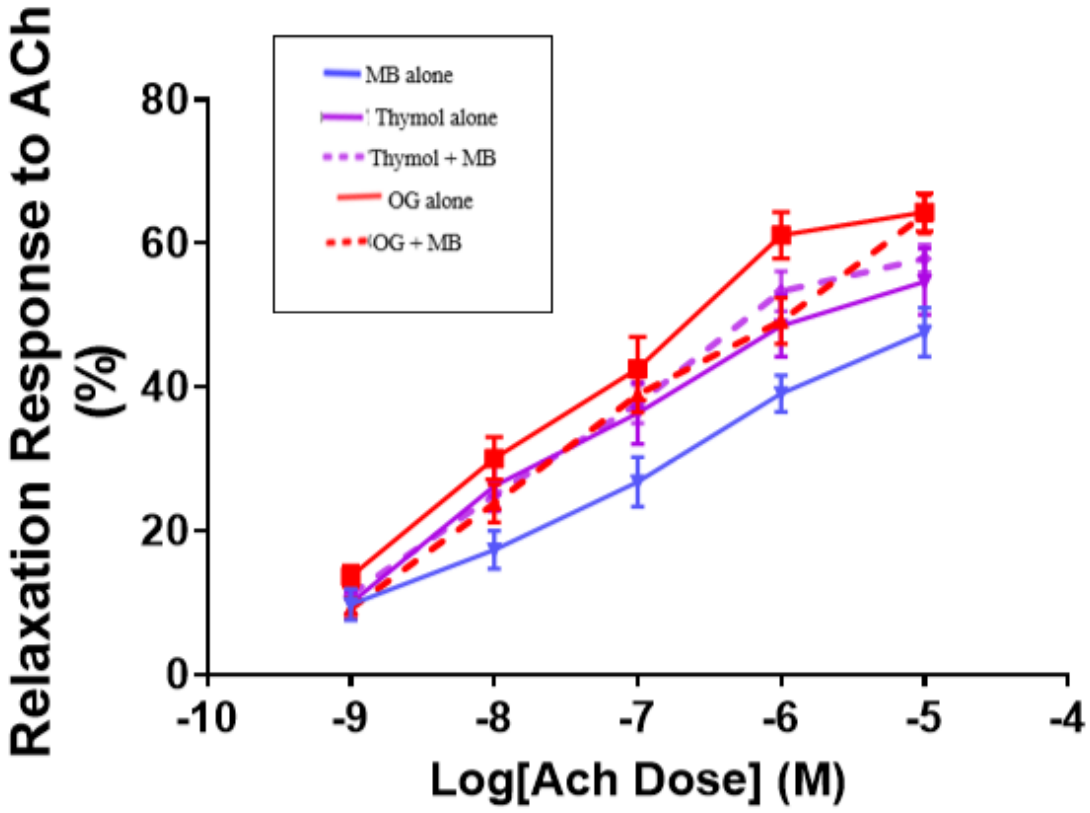


Figure 4.35: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of potassium chloride (KCl)-precontracted corpus cavernosa smooth muscle to graded dose of Acetylcholine in the presence of non-selective cGMP inhibitor. Each point represent mean ± SEM, n=6

OG= *Ocimum gratissimum*

MB= Methylene Blue

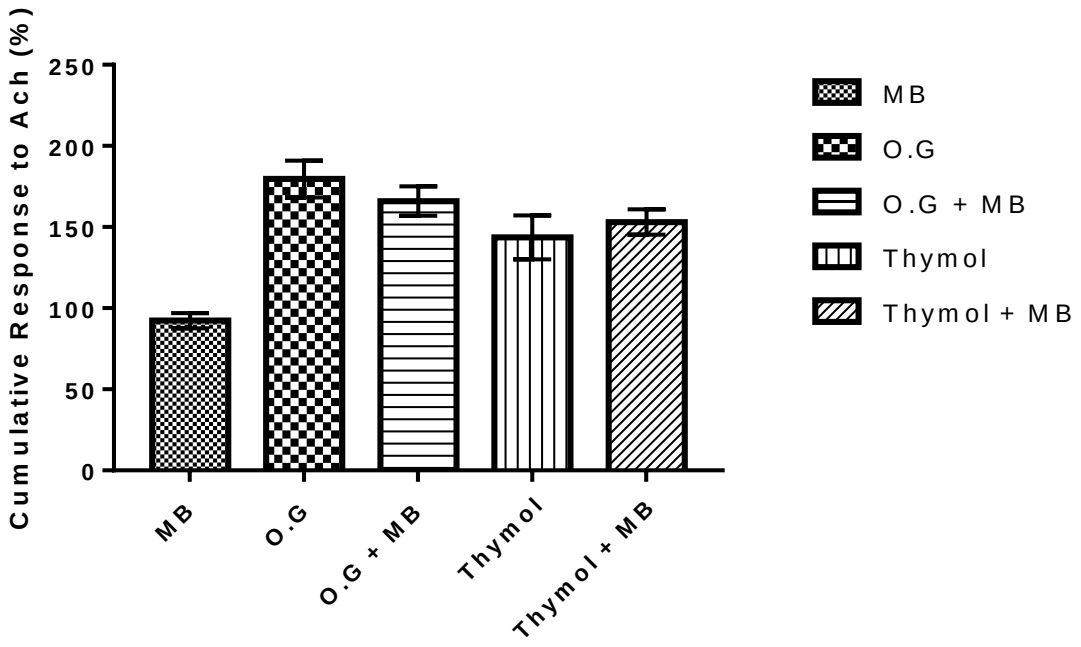


Figure 4.36: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of potassium chloride-precontracted corpus cavernosa smooth muscle to acetylcholine in the presence of non-selective cGMP.

OG= *Ocimum gratissimum*

MB= Methylene Blue

4.8 Effects of *Ocimum gratissimum* and thymol on penile calcium ion modulation in Wistar rat

The effects of *Ocimum gratissimum* and thymol on penile calcium ion modulation in Wistar rat are shown in figures 4.51 – 4.61.

While calcium ATPase activity was significantly ($p<0.05$) increased in *Ocimum gratissimum* treated group compared to the control (figure 4.51), there was no significant difference in calcium ion levels of all groups (figure 4.52).

In phenylephrine-precontraction and KCl-precontracted tissues, the response to acetylcholine in tissues incubated with *Ocimum gratissimum* and those incubated with thymol were not different from the response observed in tissues incubated in nifedipine alone. Co-incubation of nifedipine with *Ocimum gratissimum* significantly decreased the maximum response to acetylcholine both in phenylephrine-precontracted and potassium chloride-precontracted tissues compare the *Ocimum gratissimum* incubated tissue ($p<0.05$). There was no significant difference in the maximum relaxation response to acetylcholine when nifedipine was co-incubated with thymol both in phenylephrine-precontracted and potassium chloride (KCl)-precontracted tissues compared the tissues incubated with thymol only.

Co-incubation of nifedipine with *Ocimum gratissimum* had no effect on the response to calcium chloride in calcium free medium compared with the tissues incubated in *Ocimum gratissimum* only. Co-incubation of nifedipine with thymol significantly reduced the response to calcium chloride in calcium free medium compared with the tissues incubated in thymol only (figure 4.54).

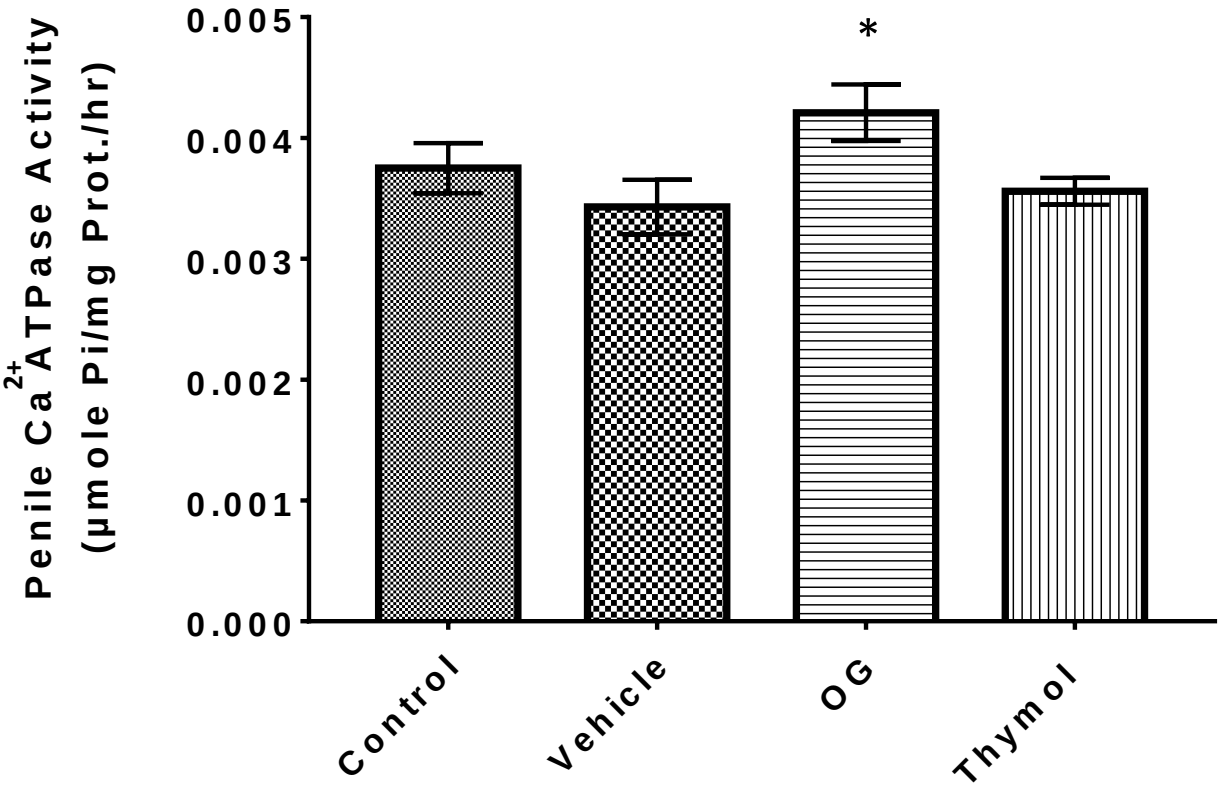


Figure 4.37: Graph showing the effects of *Ocimum gratissimum* and thymol on Penile Calcium-ATPase activity in Wistar rats. Data represent mean ± SEM. *n =5* * = $p<0.05$ compared with the control.

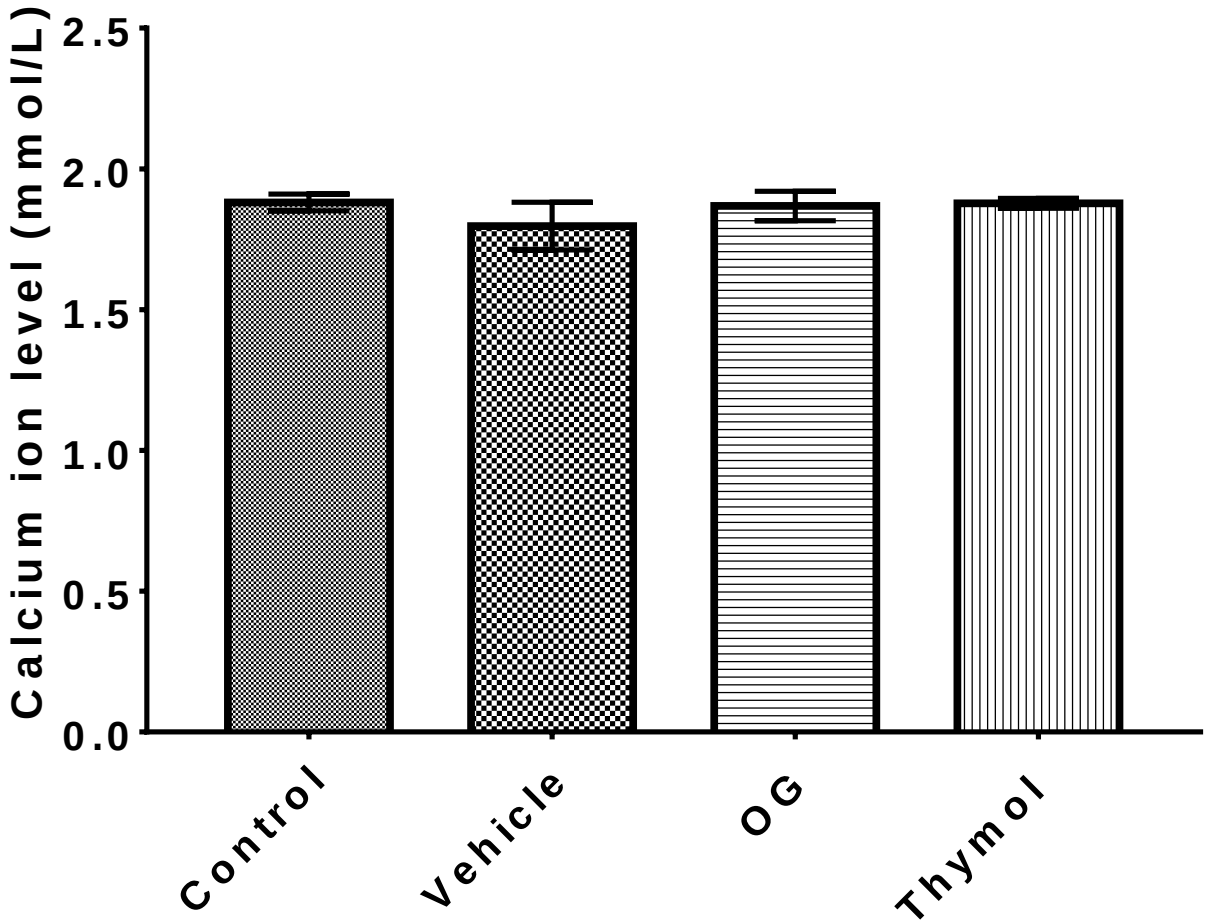


Figure 4.38: Graph showing the effects of *Ocimum gratissimum* and thymol on Penile Calcium level in Wistar rat. Data represent mean ± SEM. *n =5*

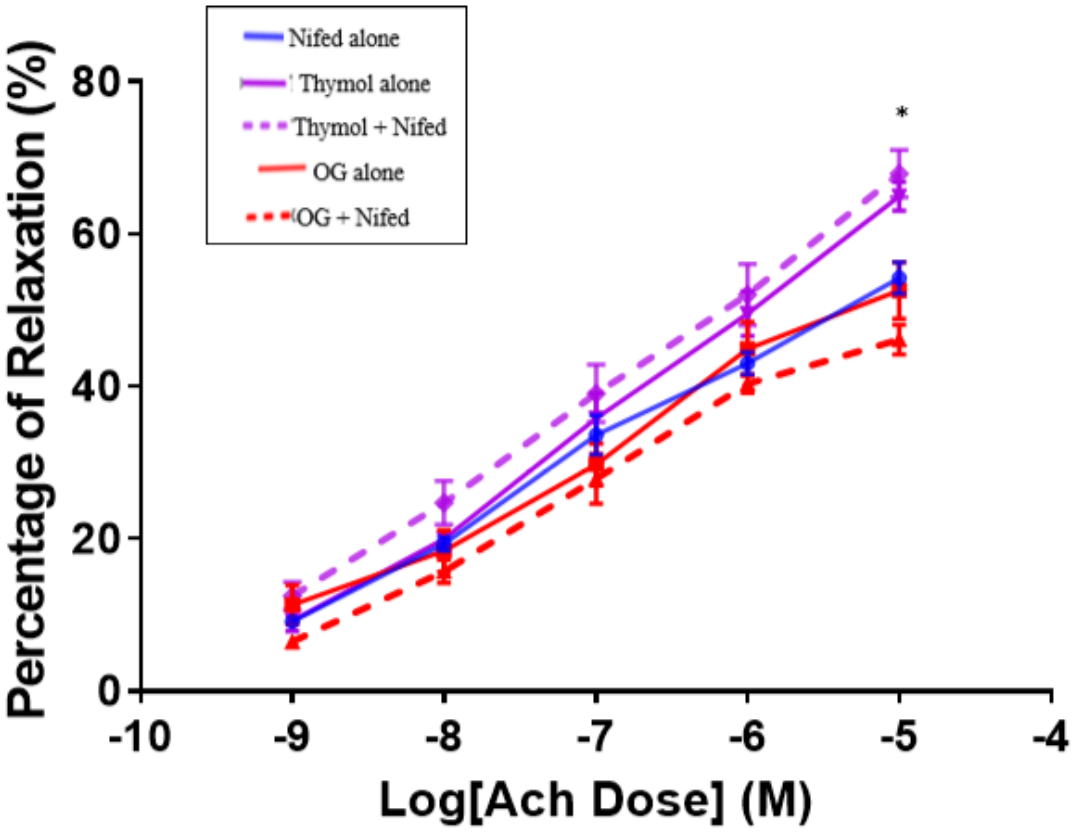


Figure 4.39: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of phenylephrine-precontracted corpus cavernosa smooth muscle to Acetylcholine in the presence of calcium channel blocker. Each point represent mean ± SEM, n=6

*p<0.05 (OG vs OG + Nifedipine)

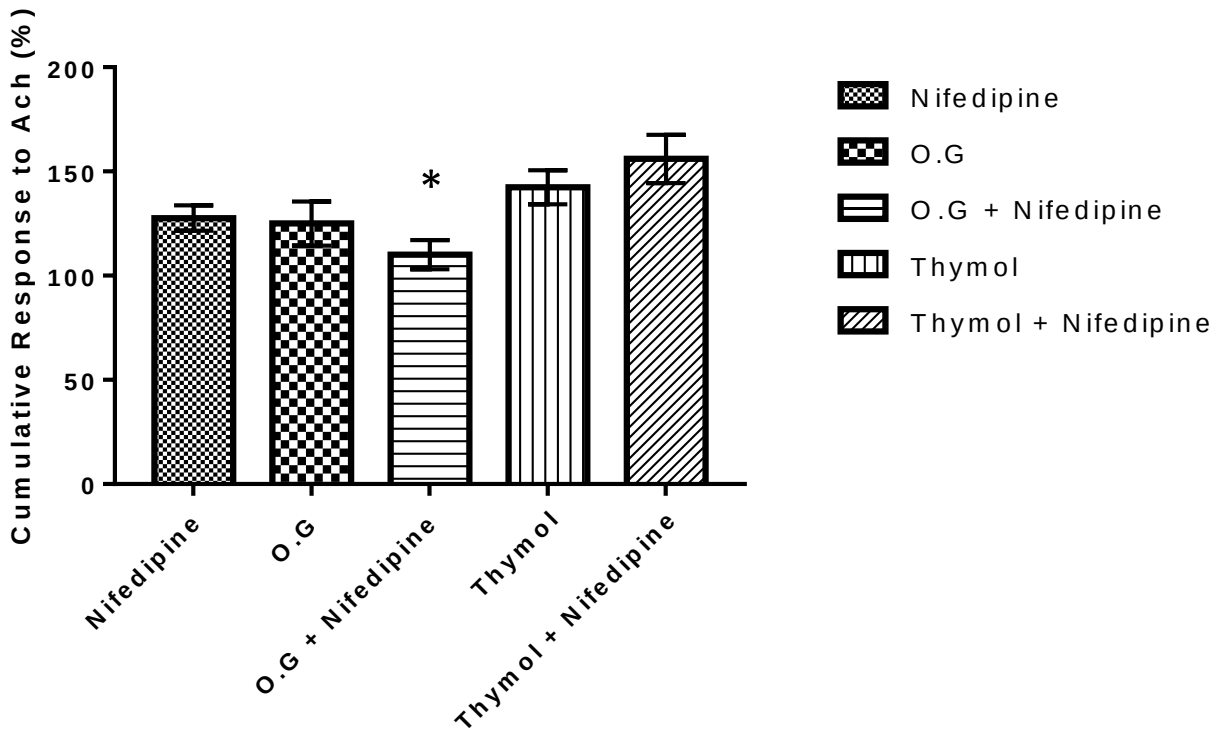


Figure 4.40: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of potassium chloride-precontracted corpus cavernosa smooth muscle to acetylcholine in the presence of calcium channel blocker.

*$p<0.05$ (OG vs OG + Nifedipine)

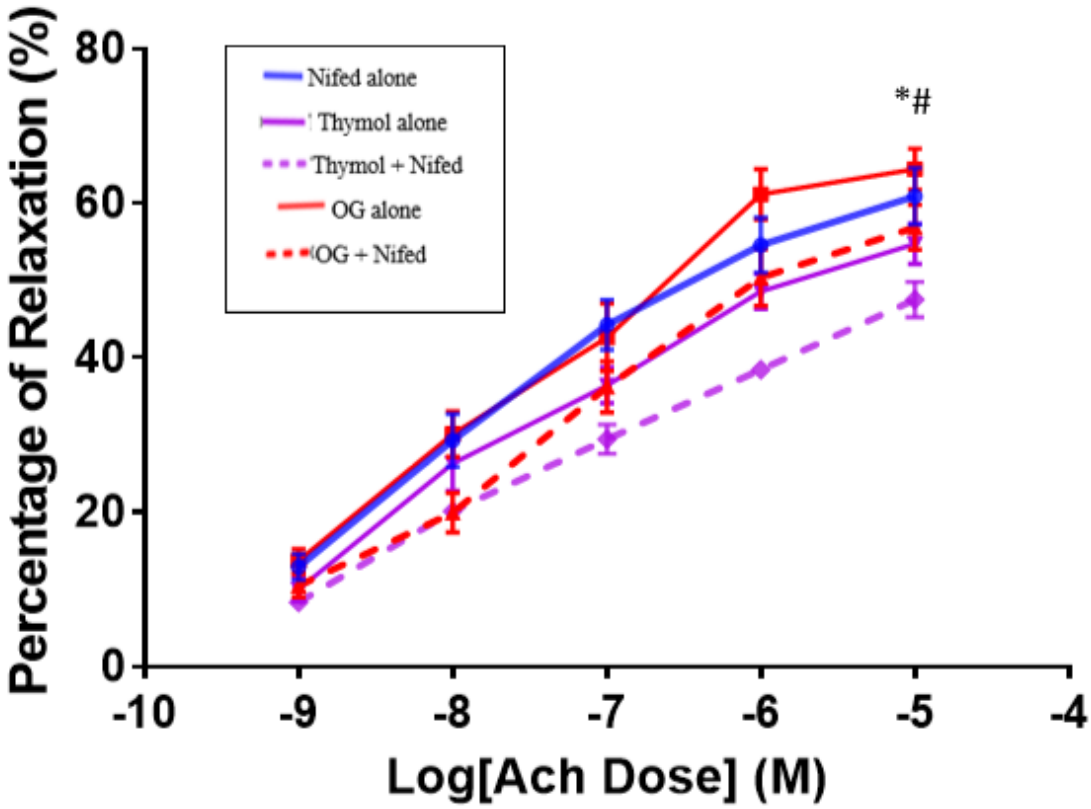


Figure 4.41: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of potassium chloride (KCl)-precontracted corpus cavernosa smooth muscle to Acetylcholine in the presence of calcium channel blocker. Each point represent mean ± SEM, n=6.

*p<0.05 (OG vs OG + Nifedipine), #p<0.05 (Thymol vs Thymol + Nifedipine)

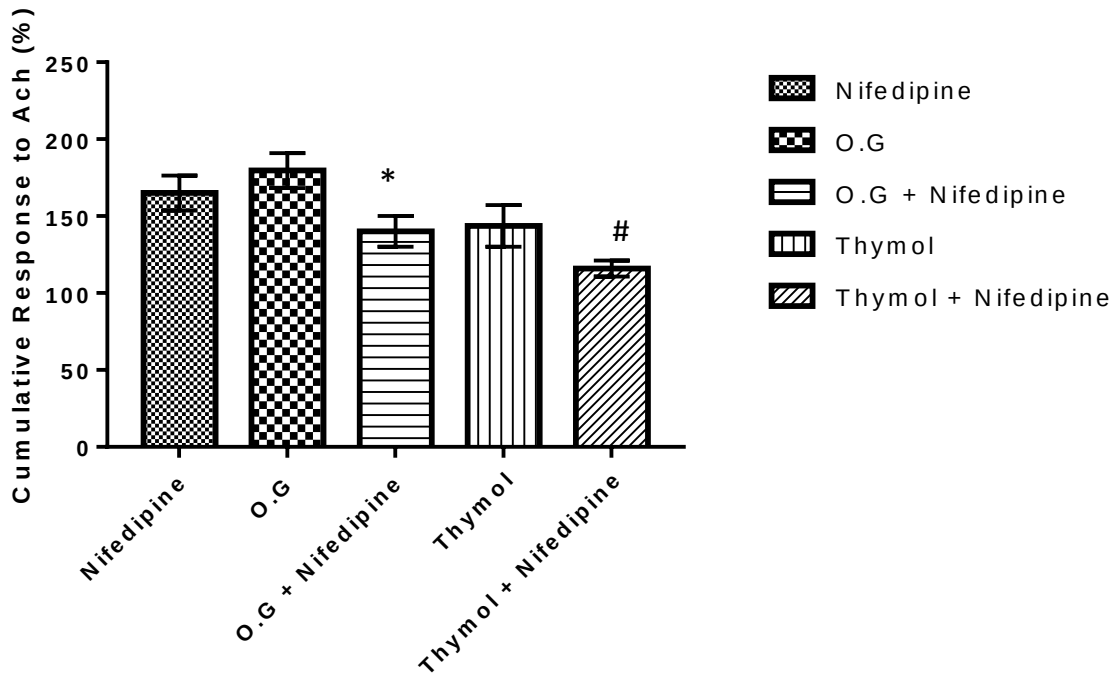


Figure 4.42: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of potassium chloride-precontracted corpus cavernosa smooth muscle to acetylcholine in the presence of calcium channel blocker.

*p<0.05 (OG vs OG + Nifedipine), #p<0.05 (Thymol vs Thymol + Nifedipine)

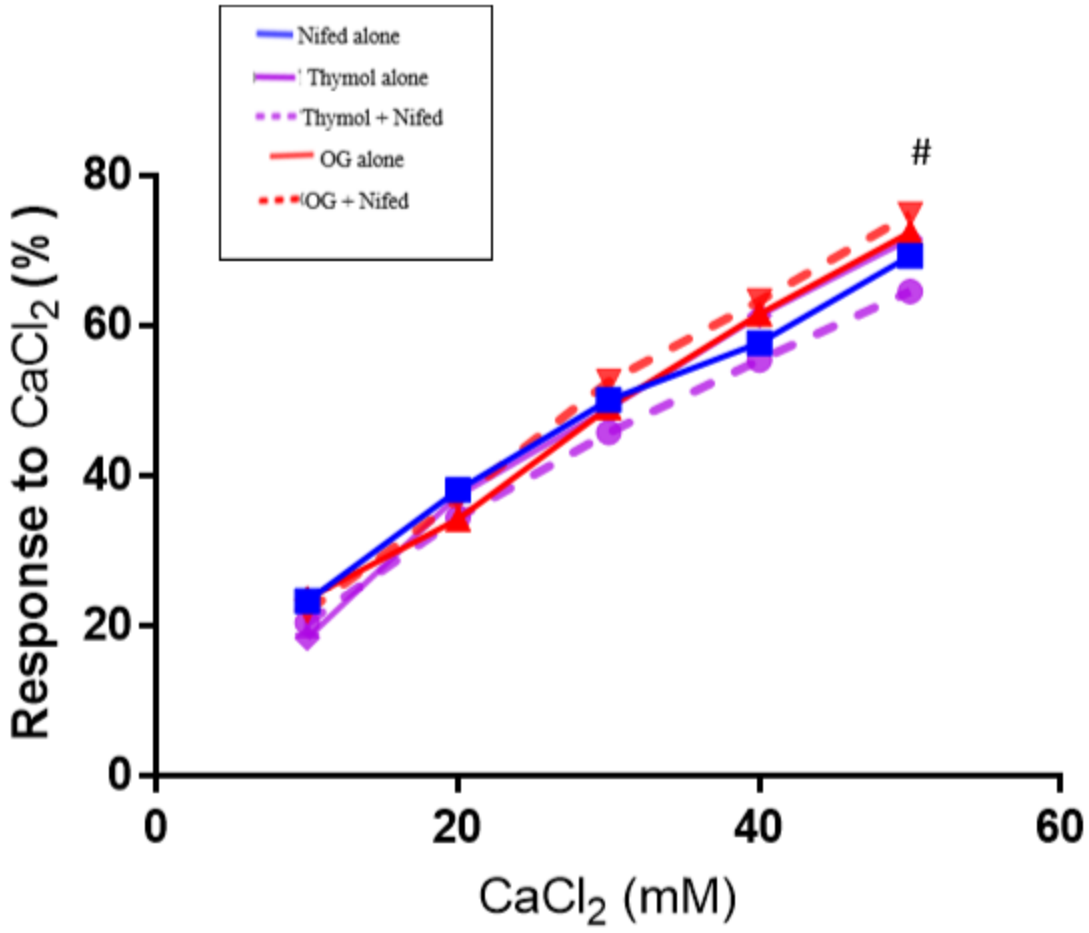


Figure 4.43: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of corpus cavernosa smooth muscle to calcium chloride in the presence of calcium channel blocker in calcium free medium.

#p<0.05 (Thymol vs Thymol + Nifedipine)

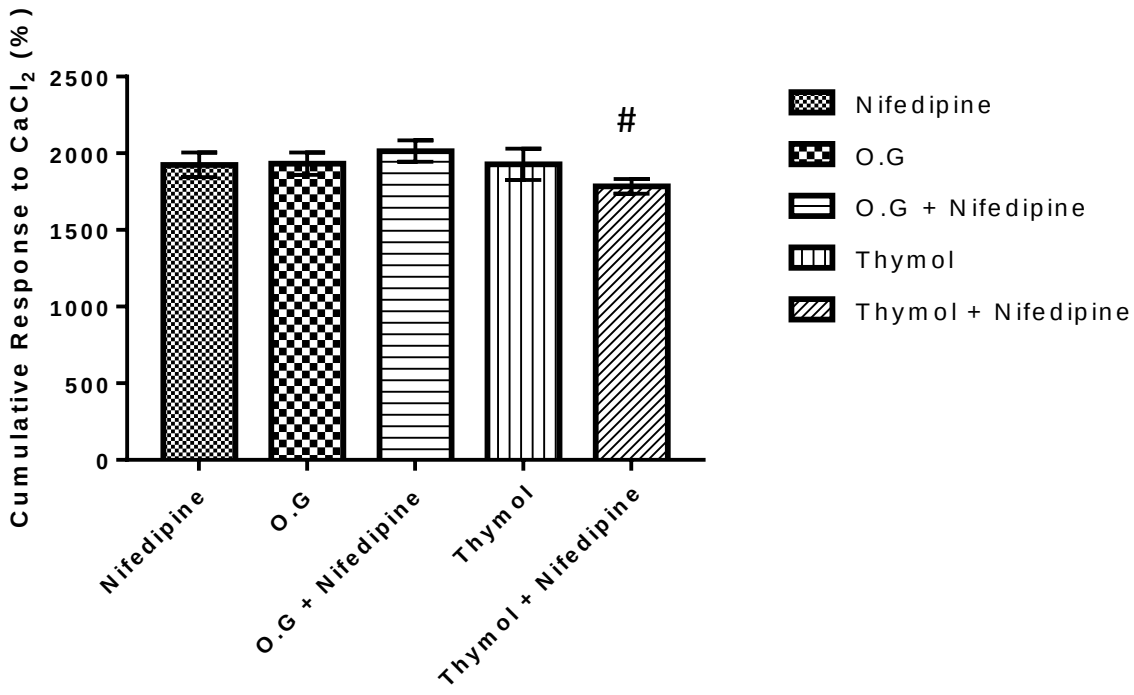


Figure 4.44: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of corpus cavernosa smooth muscle graded dose of calcium chloride in the presence of calcium channel blocker in calcium free medium.

#p<0.05 (Thymol vs Thymol + Nifedipine)

4.9 Effects of *Ocimum gratissimum* and Thymol on penile tissue calcium ion sensitization in Wistar rats

The effects of *Ocimum gratissimum* and thymol on penile tissue calcium ion sensitization in Wistar rats are shown in figures 4.62 – 4.79.

There was no significant difference in the maximum relaxation response (MRR) and cumulative response (CR) to acetylcholine in phenylephrine-induced contraction (figures 4.63 and 4.64) or potassium chloride-induced contraction (figures 4.67 and 4.68) when ML-7 was co-incubated with *Ocimum gratissimum* compared to *Ocimum gratissimum* alone. Similarly, the MRR response to calcium chloride in calcium free medium was not significantly different in tissues co-incubated with ML-7 and *Ocimum gratissimum* compare with *Ocimum gratissimum* alone (figures 4.69 and 4.70). Co-incubation of ML-7 with thymol significantly increased the response to acetylcholine in phenylephrine-induced and potassium chloride-induced contraction while the response to calcium chloride in calcium free medium containing EGTA was significantly reduced when ML-7 was co-incubated with thymol compared to thymol alone.

The response to acetylcholine was not significantly different in phenylephrine-induced contraction (figures 4.72 and 4.73) while it significantly reduced in potassium chloride-induced contraction (figures 4.75 and 4.76) when *Ocimum gratissimum* or thymol was co-incubated with fasudil compared to *Ocimum gratissimum* or thymol alone. While co-incubation of thymol with fasudil in calcium free medium containing EGTA no significant effect on the response to calcium chloride, the response to calcium chloride was significantly reduced in tissue co-incubated with *Ocimum gratissimum* and fasudil compared with *Ocimum gratissiumum* alone (figures 4.78 and 4.79).

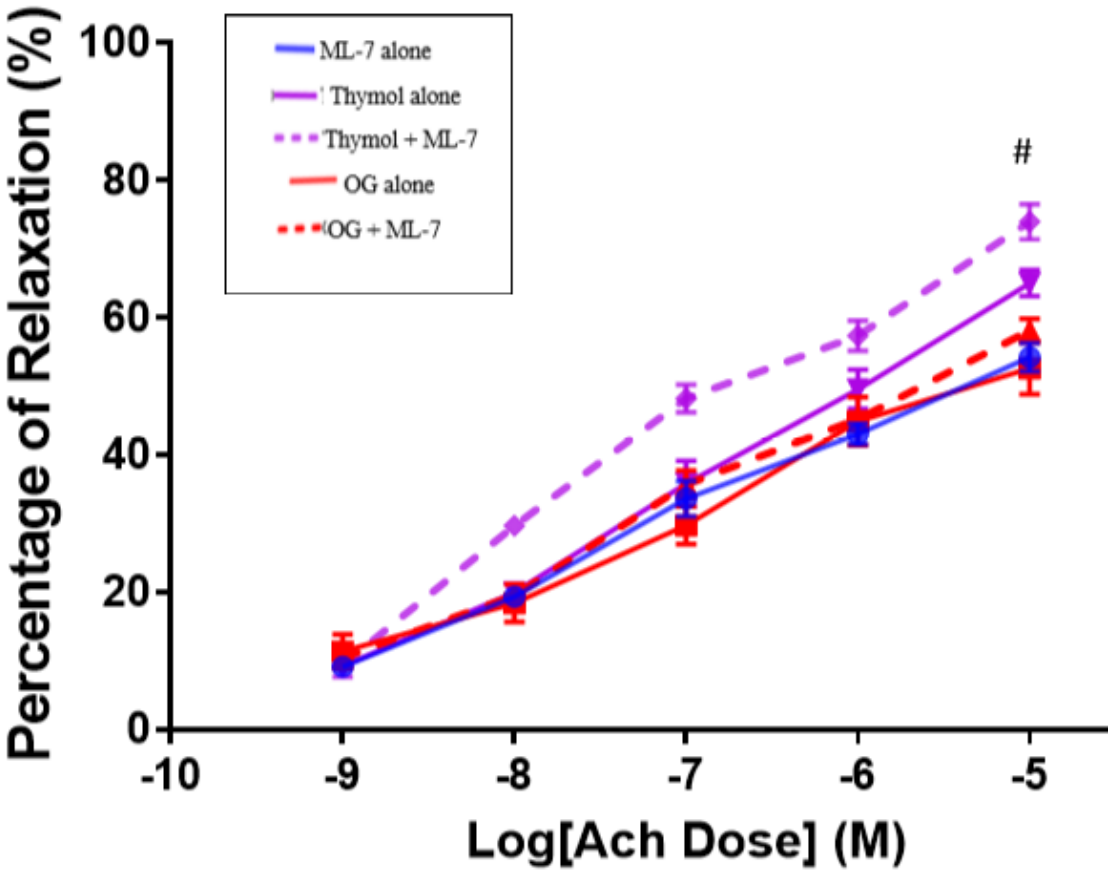


Figure 4.45: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of phenylephrine-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of myosin light chain kinase

inhibitor. Each point represent mean ± SEM, n=6

#p<0.05 (Thymol vs Thymol + ML-7)

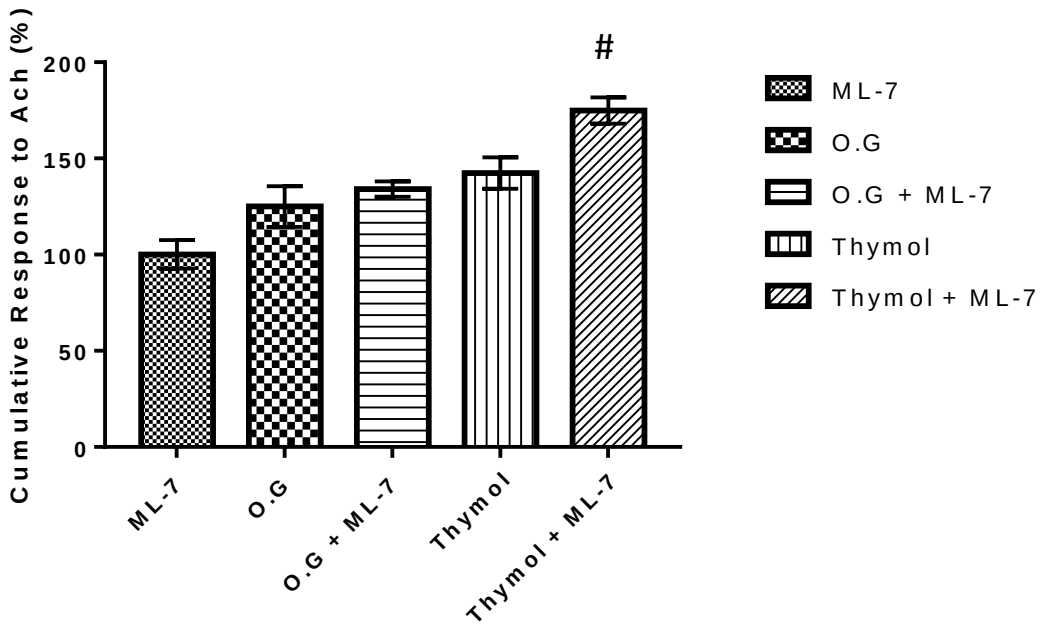


Figure 4.46: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of phenylephrine-precontracted corpus cavernosa smooth

muscle to graded dose of acetylcholine in the presence of myosin light chain

kinase inhibitor

#p<0.05 (Thymol vs Thymol + ML-7)

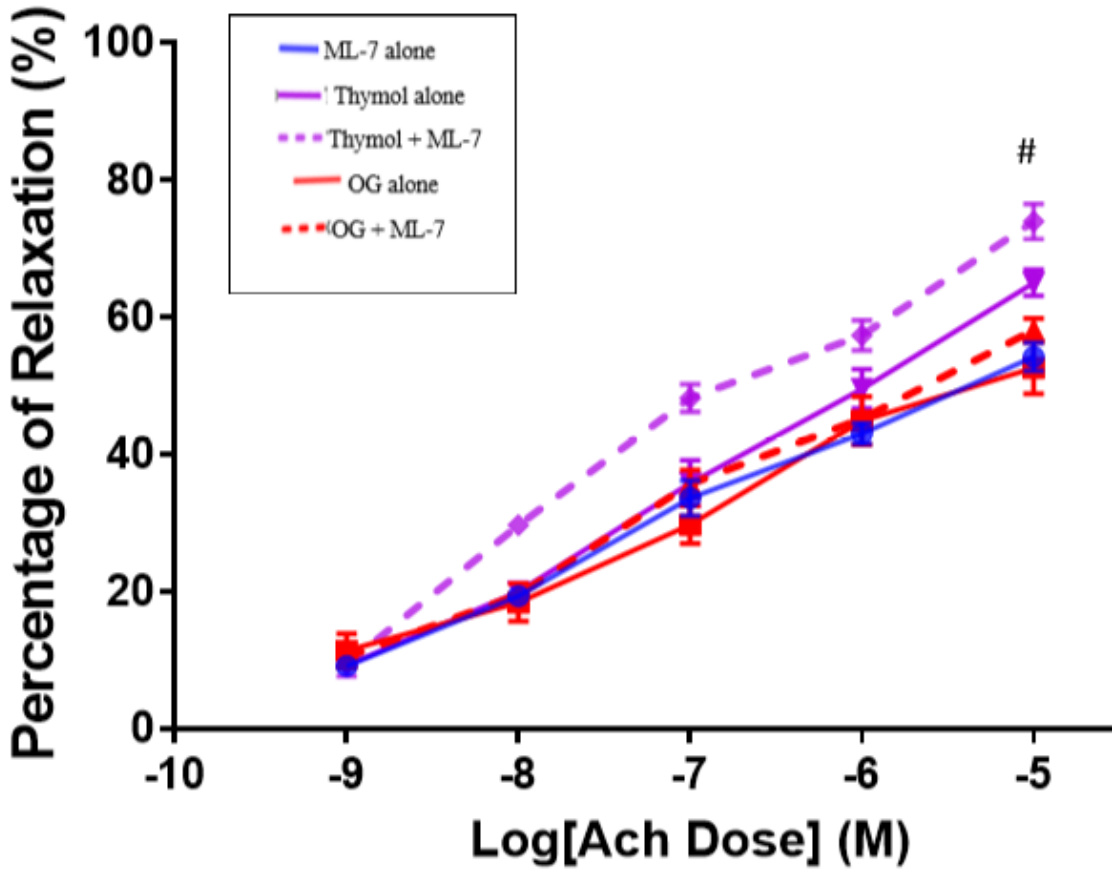


Figure 4.46: Graph showing the effects of *Ocimum gratissimum* and thymol on the response of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of myosin light chain kinase inhibitor. Each point represent mean ± SEM, n=6

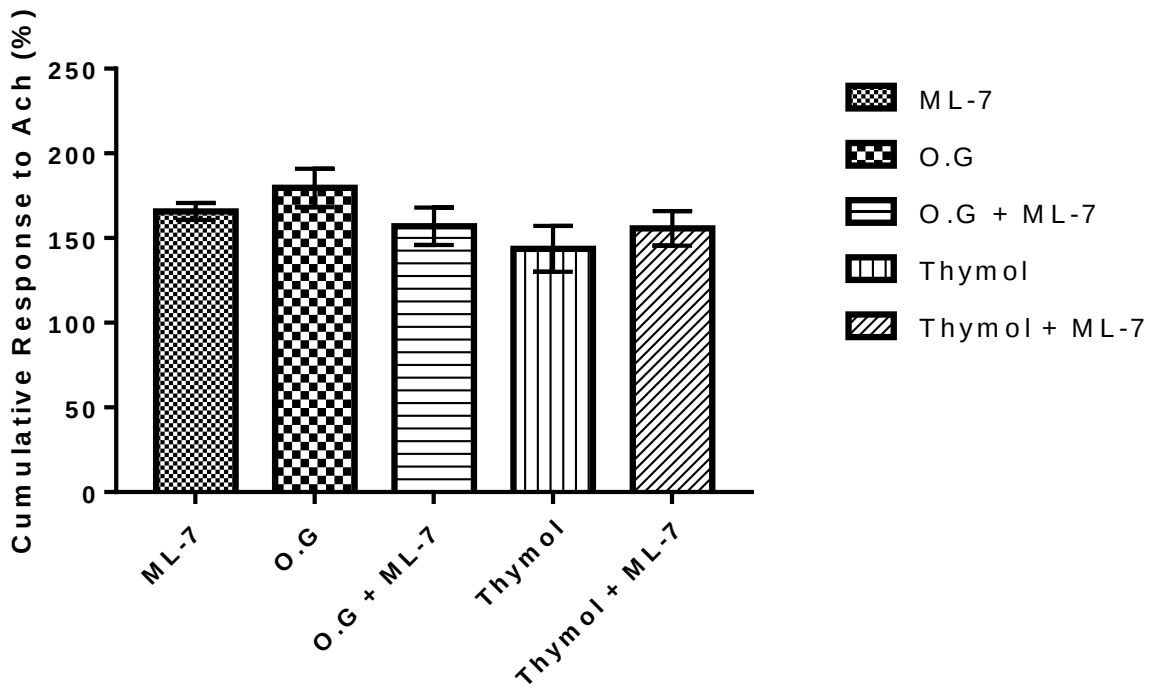


Figure 4.47: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of myosin light chain kinase inhibitor.

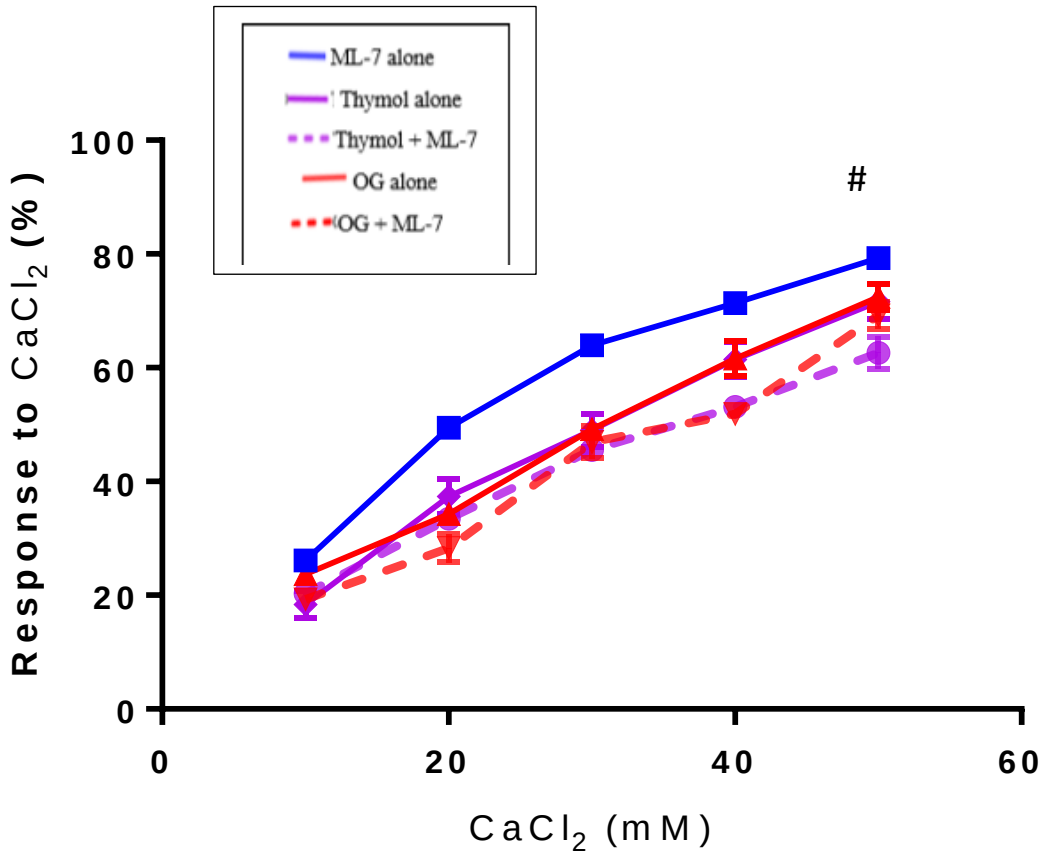


Figure 4.48: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of corpus cavernosa smooth muscle to calcium chloride in the presence of myosin light chain kinase inhibitor in corpus cavernosa tissue.

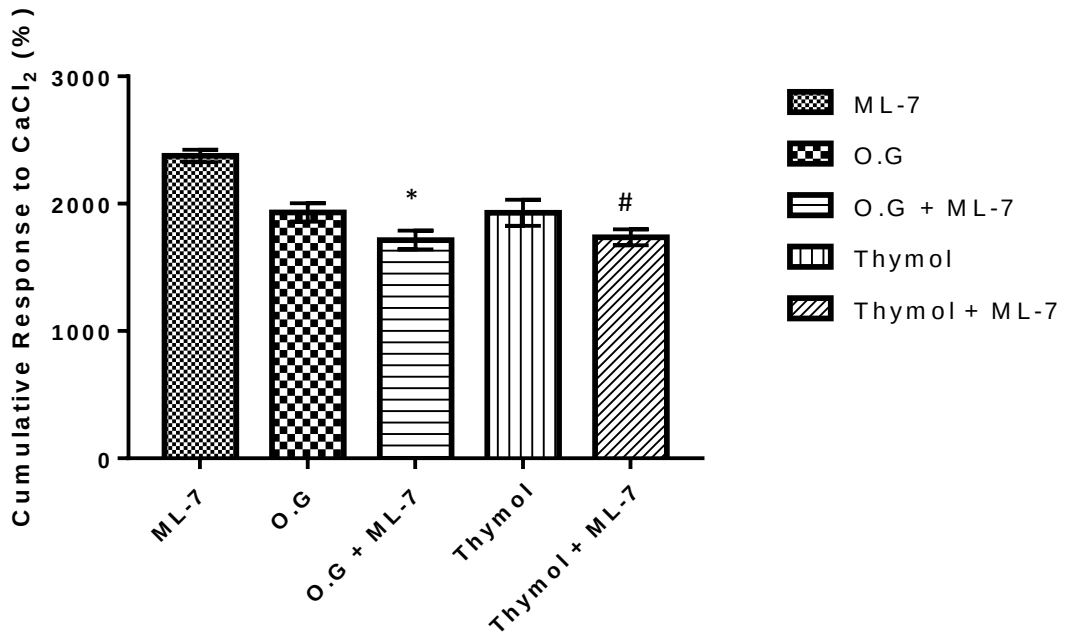


Figure 4.49: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of corpus cavernosa smooth muscle graded dose of calcium

chloride in the presence of myosin light chain kinase inhibitor.

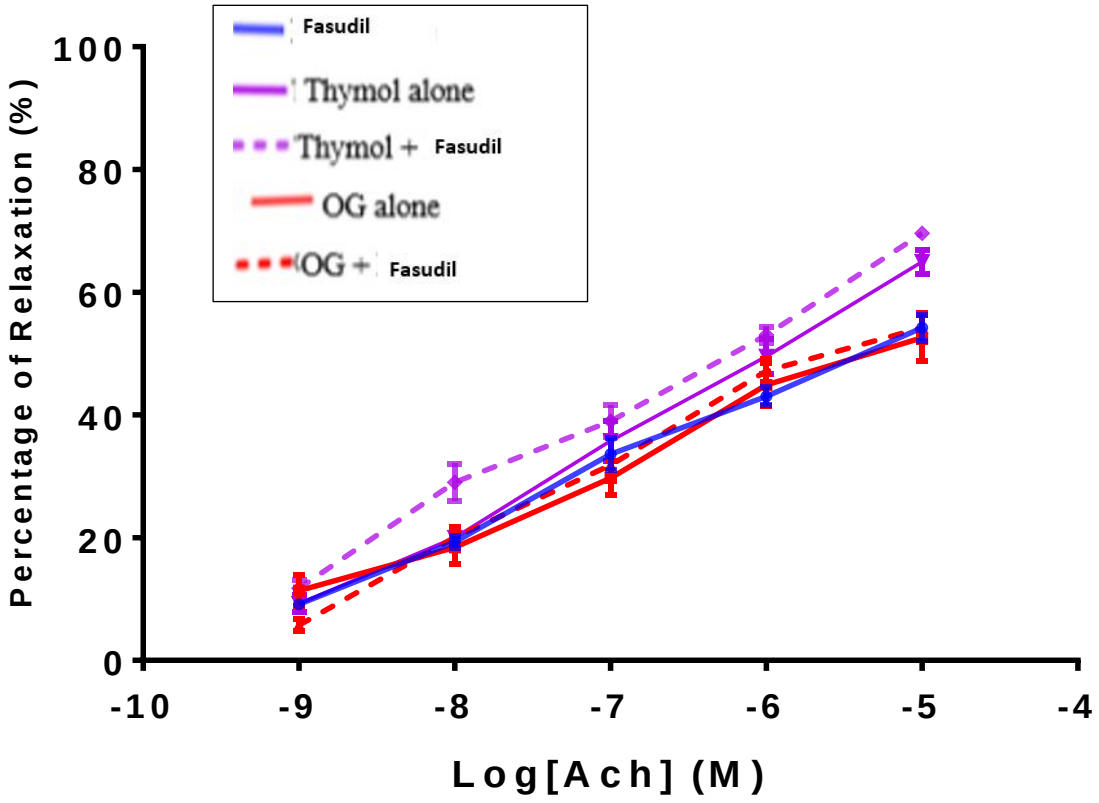


Figure 4.50: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of phenylephrine-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of rho-kinase inhibitor . Each point represent mean ± SEM, n=6

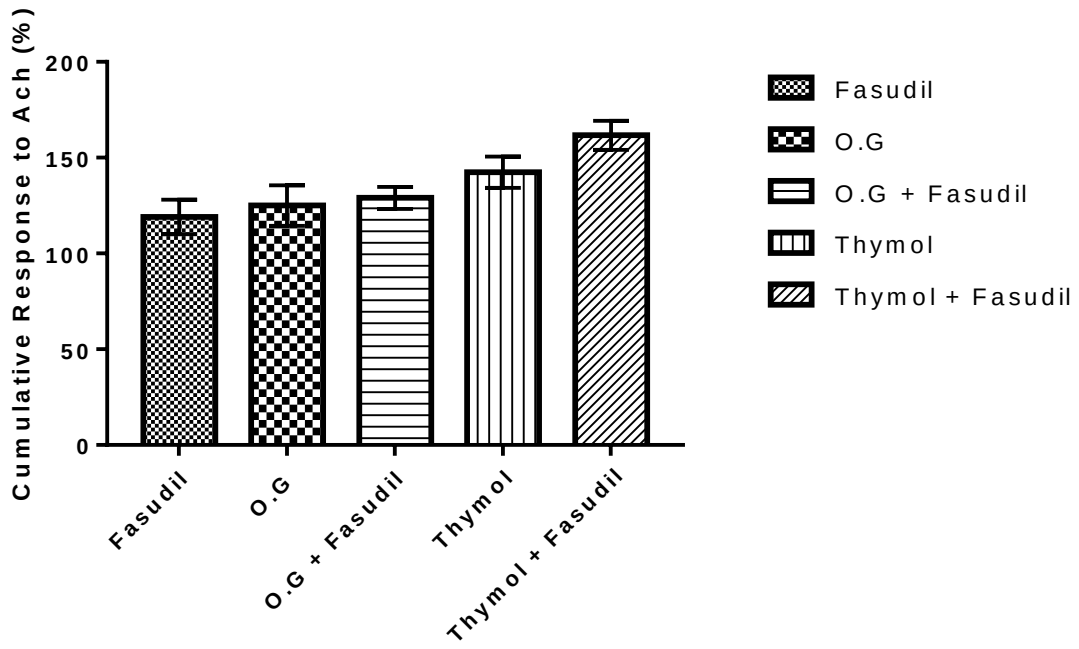


Figure 4.51: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of phenylephrine-precontracted corpus cavernosa smooth

muscle to graded dose of acetylcholine in the presence rho-kinase inhibitor.

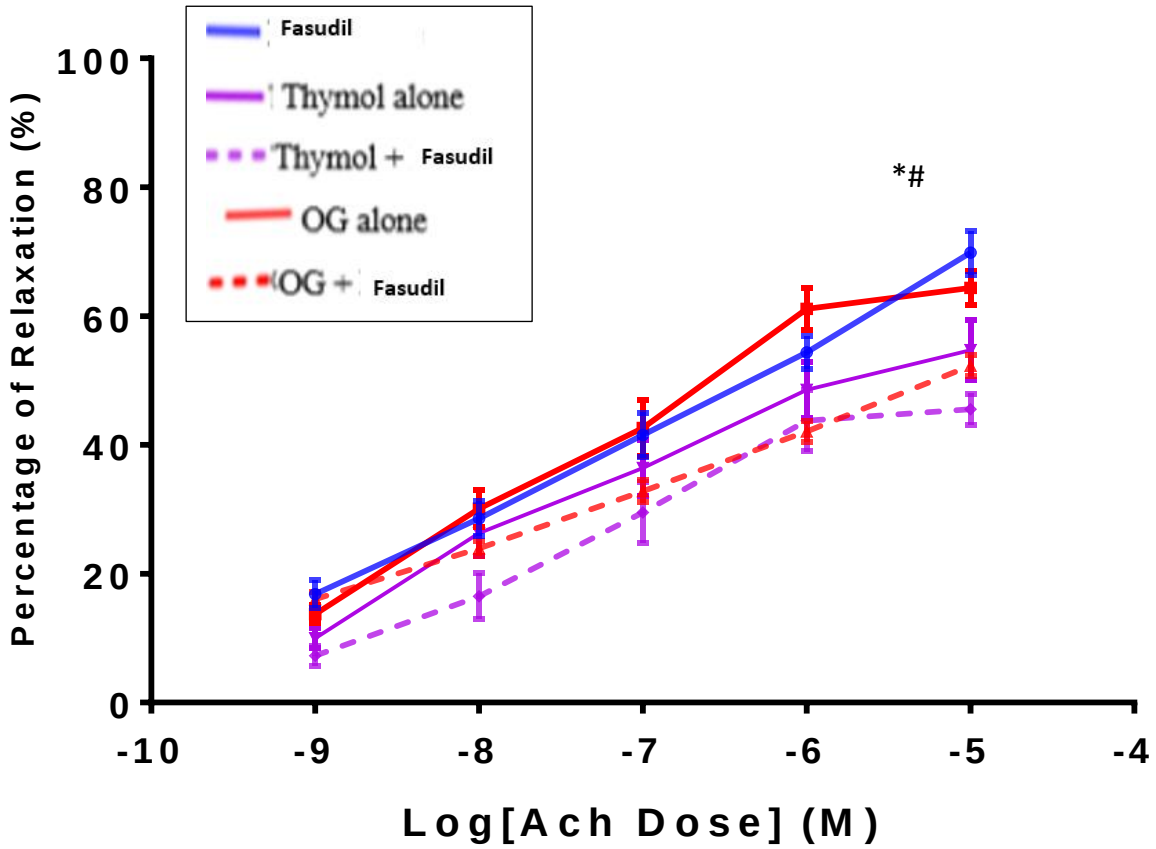


Figure 4.52: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of potassium chloride (KCl)-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence rho-kinase inhibitor. Each point represent mean ± SEM, n=6

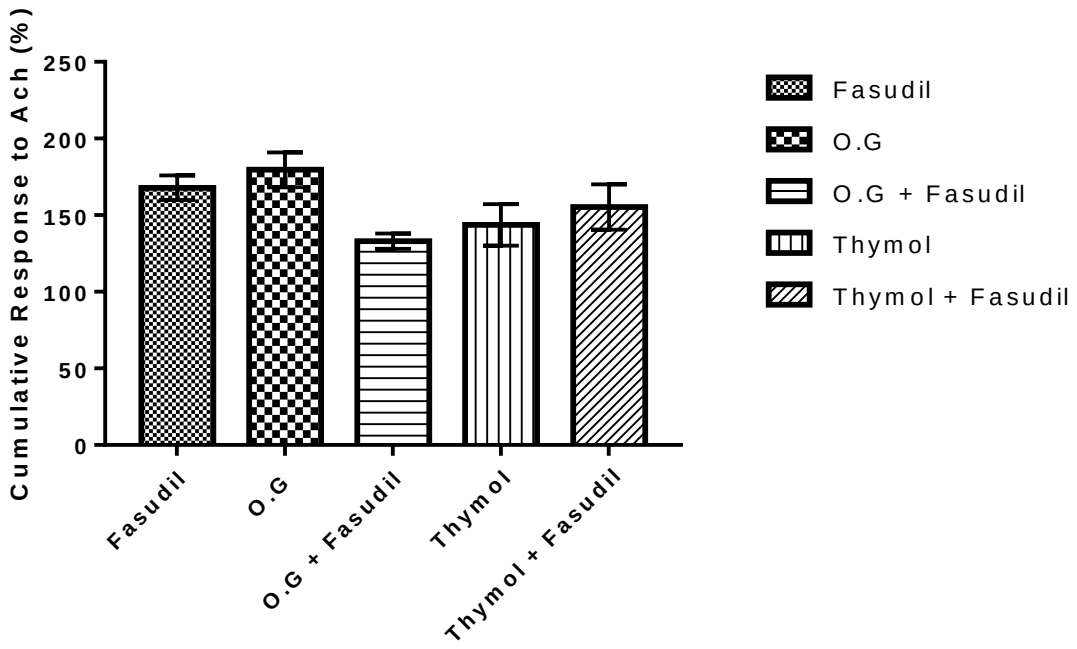


Figure 4.52: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence rho-kinase inhibitor

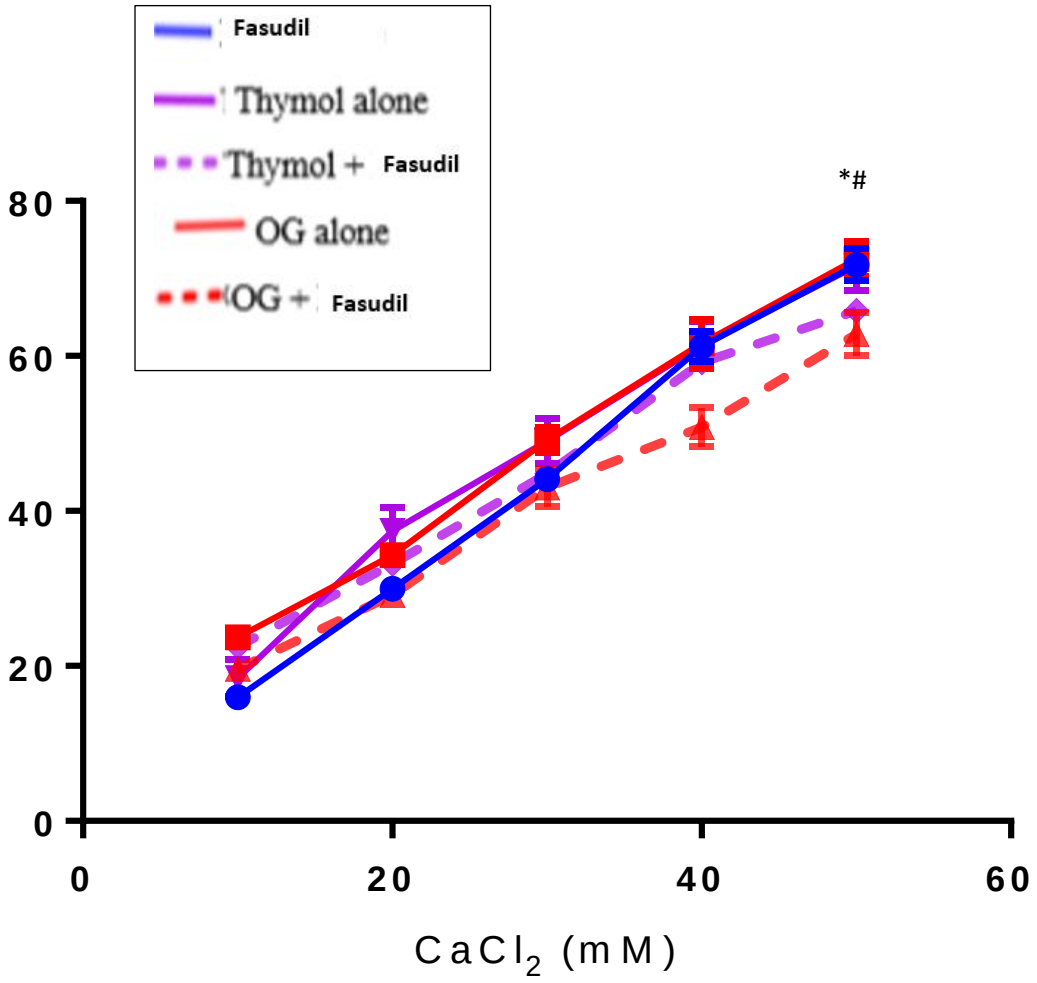


Figure 4.54: Graph showing the effects of *Ocimum gratissimum* and thymol on the responses of corpus cavernosa smooth muscle graded dose of calcium chloride in the presence of rho-kinase inhibitor in calcium free medium

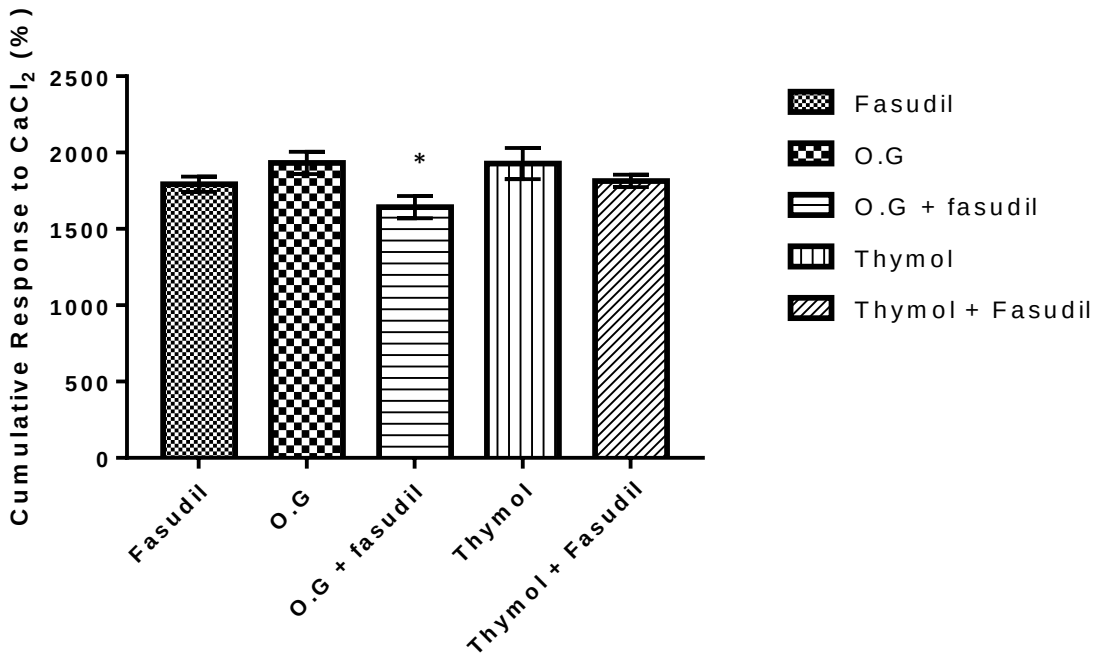


Figure 4.55: Graph showing the effects of *Ocimum gratissimum* and thymol on the cumulative response of corpus cavernosa smooth muscle graded dose of calcium chloride in the presence of rho-kinase inhibitor in calcium free medium

CHAPTER FIVE

Discusion

5.1 Characterisation of aqueous extract of *Ocimum gratissimum* leaf using Gas Chromatography Mass Spectrometry

The observed distribution of the components of *Ocimum gratissimum* is closely related to the report of Pandey *et al.* (2014). Thymol is a terpenoid phenol that has been widely used in food industry as a preservative and in dentistry as analgesic agent (Ozen *et al.*, 2011). It has calcium modulatory activity (Leal-Cardoso and Fonteles, 1999) and promotes relaxation of canine ventricular cardiomyocyte (Magyar *et al.*, 2002) and isolated rat aorta (Peixoto-Neves *et al.*, 2010) by inhibiting calcium channel activities. It was also reported to reduce intracellular calcium ion by inhibiting Ca^{2+}-ATPase activity in isoproterenol-induced myocardial necrosis in male Wistar rat (Nagoor-Meeran *et al.*, 2015).

5.2 Effects of aqueous extract of *Ocimum gratissimum* and thymol on mounting latency and frequency in Wistar rats

Mounting latency is the time difference between the introduction of female rat to the male rat compartment and first mount while mounting frequency is the number of mounts with intromission within 30 minutes observatory period (Dare *et al.*, 2015). The observed decrease in mounting latency in the *Ocimum gratissimum* treated group is an indication that *Ocimum gratissimum* promotes sexual desire and motivation. Extracts and drugs that reduce mounting latency and increase mounting frequency have been postulated to improve sexual arousal and libido in male (Dau *et al.*, 2020). The event of arousal and penile erection depend on the

hormonal signal from the pituitary gonadal axis, penile tissue integrity and signal transduction within the penile tissue.

5.3 Effects of aqueous extract of *Ocimum gratissimum* leaf and thymol on reproductive hormone levels in male Wistar rats

The expression of luteinizing hormone receptor in the penile tissue was reported by Kokk *et al.* (2011) to explain non-gonadal activities of luteinizing hormone in the control of penile erection and the need for its consideration in hypo-androgenic patients exhibiting difficulties in penile erection (Foresta *et al.*, 2003). Evans and Distiller, (1979) showed that luteinizing hormone level and sexual arousal increased in patient administered with luteinizing hormone-releasing hormone without changes in the testosterone level. The increased luteinizing hormone level and decreased mounting latency without any change in the testosterone level observed in this study may suggest that *Ocimum gratissimum* could mimic the luteinizing hormone-releasing hormone effect earlier reported by Evans and Distiller *et al.* (1979).

5.4 Effects of *Ocimum gratissiumum* and thymol on Penile tissue histoarchitecture

Intact penile tissue and functional vascular endothelium contribute to a successful penile erection (Zhang *et al*., 2004). The observed intact corpus cavernosum and penile vascular endothelium in this study showed that *Ocimum gratissiumum* and thymol are not deleterious to penile tissue.

5.5 Effects of *Ocimum gratissimum* or thymol on corpus cavernosum contraction and relaxation responses

The ability of *Ocimum gratissimum* and thymol to reduce the responses to phenylephrine as well as potassium chloride showed that they may slow down the signals that lead to penile flaccidity irrespective of being an agonist- or electrochemical-induced signal. Phenylephrine is an adrenergic agonist that stimulates G-protein coupled receptor leading to generation of multiple cell signals which results in decreased nitric oxide level, cyclic guanosine monophosphate level and increased cytosolic calcium ion level to activate myosin light chain kinase (Somlyo and Somlyo 2000). It also activates RhoA kinase activity which increases corpus cavernosum sensitivity to calcium ion (Zewdie *et al.* 2020). On the other hand, potassium chloride induces tissue contraction by hyperpolarizing the membrane potential, thereby causing the influx of

calcium without changing the levels of nitric oxide nor cyclic guanosine monophosphate (Karaki *et al.*, 1990; Hirano 2007).

The transition of penile tissue from flaccid to erected state during sexual arousal is initiated by acetylcholine released by cholinergic parasympathetic nerves (Webb *et al* 2003) and nitric oxide release by non-cholinergic parasympathetic nerves (nitergenic nitric oxide) (Mas, 2010). Both the relaxation induced by acetylcholine and the one induced by nitric oxide was simulated in this experiment using acetylcholine chloride and sodium nitroprusside. Sodium nitroprusside is a chemical that generate nitric oxide in physiological solutions (Salahdeen *et al*., 20014).

The observed dose dependent response of corpus cavernosum to acetylcholine and sodium nitroprusside in the presence or absence of *Ocimum gratissimum* or thymol showed that *Ocimum gratissimum* and thymol did not disrupt the relaxant activity of corpus cavernosum. This corroborates the earlier observation in this study that *Ocimum gratissimum* or thymol had no deleterious effect on penile tissue.

Unlike the nitergenic nitric oxide which bypasses the penile vascular endothelium to stimulate penile erection, acetylcholine stimulates the penile vascular endothelium to synthesize more nitric oxide which reverses cascade of events in penile tissue from contraction to relaxation (Chen 1988; Zhang *et al.*, 2004). Acetylcholine was therefore adopted in this study to investigate if the activities *Ocimum gratissimum* and thymol on penile tissue relaxation is dependent or independent of penile vascular endothelium.

5.6 Effects of *Ocimum gratissimum* and thymol on penile tissue endothelial functions in Wistar rat

The role of vascular endothelium in penile erection involves the production of Endothelium Derived Relaxing Factor (EDRF), Nitric oxide and prostaglandin E1 to initiate cascade of activities leading to corpus cavernosum relaxation then penile erection (Bauer and Sotnikova, 2010). In smooth muscle experiments, L-NAME is used to inhibit the activation of L-arginine, a precursor for nitric oxide production within the vascular endothelium while indomethacin is used to inhibit prostaglandin activity (Xavier *et al.*, 2014).

The unchanged relaxation responses to acetylcholine by phenylephrine-precontracted tissues incubated with *Ocimum gratissimum* in the presence of L-NAME or indomethacin compared with absence of L-NAME or indomethacin showed that *Ocimum gratissimum* plays no role in

nitric oxide nor prostaglandin synthesis. The graded response to acetylcholine despite nitric oxide or prostaglandin inhibition is in line with the report of Cohen *et al*. (1999) and Xavier *et al*. (2014) that acetylcholine may still stimulate smooth muscle relaxation in the presence of nitric oxide blocker, L-NAME or prostaglandin blocker, indomethacin. They suggested that this may be due to the ability of the endothelium to produce endothelial dependent hyperpolarizing factor (EDHF) which stabilises membrane potential of smooth muscle through calcium-induced potassium efflux (Hoepfl *et al.*, 2002) or reduce tissue sensitivity to calcium ion through inhibition of rhoA kinase activity (Chitaley *et al.*, 2001). The increased relaxation response to sodium nitroprusside, nitric oxide donor in *Ocimum gratissimum* or thymol earlier reported in potassium chloride-precontracted tissue in this study (figures 4.23 and 4.24) indicated that the activities of *Ocimum gratissimum* and thymol is not via the release of EDHF. In the work of Xavier *et al*. (2014), it was documented that for a compound to be attributed to the modulation of EDHF release, the response to nitric oxide donor is usually abolished in potassium chloride challenged tissue.

Therefore, further investigation of the activities of *Ocimum gratisimum* and thymol in the downstream stage of penile smooth muscle relaxation becomes important.

5.7 Effects of *Ocimum gratissimum* and Thymol on cyclic guanosine monophosphate (cGMP) production in Wistar rat

The ODQ is a selective inhibitor of cGMP production while MB is a non-selective inhibitor of cGMP (Hwang *et al.*, 1998). They are chemicals used in differentiating agents acting via cGMP dependent from cGMP-independent pathway (Feelisch *et al.* 1999). While ODQ only inhibits nitric oxide stimulated cGMP production, MB inhibits production of cGMP and at the same time mop up the available nitric oxide through generation of superoxide anions (Wolin *et al.*, 1990; Marczin *et al.*, 1992; Kontos and Wei, 1993).

Although, the downstream effect of nitric oxide is to induce smooth muscle relaxation, it may also function to prepare the smooth muscle for subsequent contraction, hence the crosstalk between NO/cGMP/calcium dependent pathway and rho-kinase modulated calcium sensitization pathway (Sopko *et al.*, 2014). When nitric oxide is synthesized, it simultaneously increases the rhoA protein expression as it increases cGMP production, however, the expressed rhoA protein is continuously phosphorylated by cGMP, hence smooth muscle relaxation becomes the dominant effect of nitric oxide (Sasaki *et al.*, 1993; Narumiya, 1996; Amano *et al.*, 2000; Priviero *et al.*, 2010). The observed decreased MRR in tissues co-incubated with ODQ and *Ocimum*

gratissimum showed that *Ocimum gratissimum* is not involved in the production of cGMP and that a component of *Ocimum gratissimum* may facilitate the expression of rhoA protein in preparation for the next cycle of contraction. This may be an advantage over some aphrodisiac drugs like phosphodiesterase inhibitor which are associated with priapism (Aoyagi *et al.*, 1999; Sharma *et al.*, 2009). Priapism is a prolonged and painful penile erection which does not subside after sexual intercourse (Burnett, 2003); It results from excessive blockade of penile detuminence process or disruption of the intrinsic mechanisms that balances the penile erection-flaccidity processes (Horst *et al.*, 2003). The role of methylene blue in the treatment of pharmacologically induced priapism has been previously reported by Martínez *et al.* (2001). The increased MRR in tissues co-incubated with *Ocimum gratissimum* and MB as well as the insignificant difference in the effect of thymol on smooth muscle relaxation in the presence of MB showed that the influence of *Ocimum gratissimum* or thymol on acetylcholine induced relaxation is independent of nitric oxide or cGMP. This observation suggests that *Ocimum gratissimum* may be useful in the prevention of priapism while its erectogenic property is still maintained.

5.8 Effects of *Ocimum gratissimum* and thymol on penile calcium ion modulation in Wistar rat

Reduced cytosolic calcium ion level is required for smooth muscle relaxation (Arii *et al* 1999). This may be achieved either by inhibition of extracellular calcium ion influx through voltage operated calcium channel, extrusion of calcium ion from the cytosol to the extracellular space through plasma membrane calcium ATPase (PMCA) or sequestration of cytosolic calcium ion into the sarcoplasmic reticulum through sarcoplasmic reticulum calcium ATPase (SERCA) (Fill and Copello, 2002).

Nifedipine is a calcium channel blocker that inhibits influx of calcium ion through the voltage operated calcium channel (Raicu and Florea, 2001) and it has been reported to have a relaxant activity on smooth muscle, similar to the observed effect in this study. The insignificant difference in the relaxation response to acetylcholine observed between the tissues incubated in nifedipine compared with the tissues incubated in OG alone or thymol alone showed that both OG and thymol can play a role in the restriction calcium ion entry through L-type voltage gated calcium channel similar to nifedipine. This corroborates the report of other researchers that *Ocimum gratissimum* (Interaminense *et al*., 2007) and thymol (Peixoto-nerve *et al*., 2010) inhibits the transport of calcium ion through voltage operated calcium channel. Evidenced from

the observed increase in calcium ATPase activity in OG treated rats in-vivo, it is speculated that *Ocimum gratissimum* may enhance modulation of intracellular calcium ion by enhancing its outflow from the cytosol through Plasma Membrane Calcium ATPase (PMCA) or uptake from cytosol into the sarcoplasmic reticulum through Sarcoplasmic Reticulum Calcium ATPase (SERCA).

Decreased relaxation response to acetylcholine observed in the tissues co-incubated in nifedipine and OG showed that the OG does not inhibit the intrinsic property of corpus cavernosum to activate myogenic contraction usually initiated when the relaxation tension of corpus cavernosum is at its maximum (Hill and Meininger, 2012). In this instance, mechano-receptors within the cavernosa tissues senses the threshold where the smooth relaxation becomes excessive and activates muscle contraction via two distinct mechanisms (Ferrier *et al*., 2000). As documented by Ferrier *et al*., (2000) the first mechanism may involve generation of membrane potential by cavernosa pace-maker cell to induce voltage sensitive calcium release. The presence of pace-make cell activity in the penile smooth muscle similar to Interstitial Cell of Cajal responsible for myogenic tone in the intestine was reported by Shafik (2007) and Doyle *et al.* (2012). According to Ferrier *et al*. (2000), the pace-maker cell generated membrane potential activates the extrusion of calcium ion from the sarcoplasmic reticulum back into the cytosol through the ryanodine and inositol triphosphate (IP_3) regulated calcium channel which bring about phasic contraction which culminate to tonic contraction when it is sustained. It has been documented that the type of contraction initiated by these mechanisms is resistant to nifedipine because it occurs independent of L-type voltage gated calcium channel (Schubert and Brayden, 2005). This raised the inquisitiveness on the role of *Ocimum gratissimum or thymol* on the intrinsic electrical activity of penile smooth muscle. This was done by observing the responses of penile tissue to calcium induced contraction in membrane potential stabilised tissue in calcium free medium. It has been reported that inclusion of EGTA in physiological solution facilitates the stabilisation of tissues membrane potential in smooth muscle experiments (Laugier and Petersen, 1980; Harris and Hanrahan, 1994).

The observed reduction in response corpus cavernosa smooth muscle to calcium chloride induced contraction in EGTA containing- calcium-free medium when nifedipine was co-incubated with thymol and the insignificant difference in the response to calcium chloride when nifedipine was co-incubated with *Ocimum gratissimum* compared with thymol of *Ocimum gratissimum* clarified that the activities of *Ocimum gratissimum* and thymol in facilitating smooth muscle

relaxation do not inhibit the membrane potential required to initiate a tonic contraction by cavernosa pace-maker even at the highest dose of calcium chloride.

In line with the report of Sahin *et al*., (2018), that modulators of calcium ion sensitization especially through rho kinase pathway does not inhibit the intrinsic tonic contraction of smooth muscle, it then becomes pertinent to investigate the activity of *Ocimum gratissimum* or thymol on penile tissue sensitivity to calcium ion relative to standard modulators calcium sensitization.

5.9 Effects of *Ocimum gratissimum* and Thymol on penile tissue calcium ion sensitization in Wistar rats

Phosphorylation of myosin light chain is the prerequisite for the initiation smooth muscle contraction and the enzyme responsible for this is myosin light chain kinase (MLCK) (Gallagher *et al.,* 1997; Kamm and Stull, 2001). Indicating that calcium ion does not directly initiate smooth muscle contraction as applicable in skeletal and cardiac muscle, rather, it serves as a second messenger in the process of smooth muscle contraction. calcium ion binds to calmodulin to form a calcium-calmodulin complex (Gerthoffer, 1991; Karaki *et al.*, 1997). This complex activates MLCK to induce the phosphorylation of myosin light chain which is then followed by the onset of the smooth muscle contraction (Murthy, 2006). The phosphorylated myosin light chain is spontaneously dephosphorylated by myosin light chain phosphatase (MLCP) (Hirano, 2007), hence the initiated contraction is sustained by rho-kinase, an enzyme that blocks the activity of MLCP. Therefore, inhibition MLCK and rho-kinase activity in penile smooth muscle is require for erection.

The increase response to acetylcholine in phenylephrine pre-contraction when *Ocimum gratissimum* or thymol was co-incubated with myosin light chain kinase inhibitor coupled with the insignificant difference when *Ocimum gratissimum* or thymol was co-incubated with rhoA kinase inhibitor showed that *Ocimum gratissimum* and thymol plays no role in the regulation of myosin light chain kinase activity. This is more evidenced in the insignificant difference in the relaxation response to acetylcholine when *Ocimum gratissimum* was co-incubated with myosin light chain kinase, unlike the reduced responses to acetylcholine in potassium chloride-precontraction when *Ocimum gratissmum* or thymol was co-incubated with other pro-relaxing agents, nifedipine and rho-kinase inhibitor. Furthermore, the significantly reduced sensitivity to calcium chloride in calcium-free krebs' solution when *Ocimum gratissimum* was co-incubated rho-kinase inhibitor showed that *Ocimum gratissimum* may continuously promote penile tissue

relaxation activity in as much as the membrane potential is kept constant or below the threshold to generate a myogenic contractile response. Undisrupted corpus cavernosa myogenic contraction is an advantage for penile ejaculatory process and transition into the post-ejaculatory penile resolution period (Sumylo and Sumylo, 2003) during sexual intercourse. However, the time interval to regain erectile potential depend on the extent to which rho-kinase activity is inhibited (Sopko *et al.*, 2014) given that the sexual arousal stimulus is kept constant.

CHAPTER SIX

Summary and Conclusion

6.1 Findings

The result of this study showed that

1. Thymol is the most abundant constituent of *Ocimum gratissimum linn*
2. Both *Ocimum gratissimum* and thymol promoted mounting frequency in Wistar rat.
3. *Ocimum gratissimum* but not thymol had a significant effect on luteinizing hormone level which explains the reduced mounting latency observed only in *Ocimum gratissimum* treated rats.
4. Both *Ocimum gratissimum* extract and thymol had no deleterious effect a penile tissue integrity and vascular endothelial function in wistar rat indicating their safety on sexual function.
5. Both *Ocimum gratissimum* and thymol reduced the maximum contraction response to phenylephrine and potassium chloride and promoted the relaxation response to Acetylcholine. Meaning both that *Ocimum gratissimum* and thymol enhanced the relaxation by process of penile tissue, hence favours penile erection over flaccidity.
6. Blockade of nitric oxide and cyclic GMP production did not reduce the effect of *Ocimum gratissimum* and thymol on penile tissue relaxation. This that the activity of *Ocimum gratissimum* and thymol in penile erection is independent of Nitric and cyclic GMP hence, the role may be within the rho-kinase pathway.
7. Co-incubation of *Ocimum gratissimum* or thymol with either calcium channel blocker or rho-kinase inhibitor significantly reduced the effect of *Ocimum gratissimum linn* and thymol on penile tissue relaxation. This give an inference that *Ocimum gratissimum linn* and thymol modulates calcium ion sensitivity similar to rho-kinase inhibitor.
8. Ocimum gratissium does not inhibit the intrinsic myogenic contraction response, a feedback mechanism to prevent a prolong and painful penile erection.

5.2 Conclusion

The findings from this study corroborate the erectogenic property of aqueous extract of *Ocimum gratissimum linn*. This study has shown that the mechanism of action involved in the erectogenic activity of *Ocimum gratissimum* in elicited via the rho-kinase pathway. This study has also shown that *Ocimum gratissimum* in non-toxic to penile tissue.

5.3 Contribution to knowledge

The findings from this study have satisfy the inquisitiveness earlier raised on the erectogenic property and mechanisms of actions of aqueous extract of *Ocimum gratissimum linn*. it has shown that the activity of *Ocimum gratissimum* in elicited via the rho-kinase pathway of calcium ion sensitization. This study has also shown that *Ocimum gratissimum* in non-toxic to side effect on penile tissue.

5.4 Further studies

1. To compare the erectogenic activity of *Ocimum gratissimum linn* and thymol with Rho-kinase inhibitor and some other aphrodisiac drugs *in vivo.*

2. To assess the effect of *Ocimum gratissimum* membrane potential and voltage gated ion channels

REFERENCES

Adebusoye L., Olapade-Olaopa E., Ladipo M. and Owoaje E. (2012). Prevalence and Correlates of Erectile Dysfunction among Primary Care Clinic Attendees in Nigeria. *Global Journal of Health Science,* 4: 107-117.

Adimoelja A., and Adaikan P.G. (1997). Protodioscin from herbal plant *Tribulus terrestris* L. improves male sexual functions possibly via DHEA. *International Journal of Impotence Research*, 9(1):64-74.

Akinmoladun A. C., Ibukun E.O., Afor E. , Obuotor E. M. and Farombi E. O. (2007). Phytochemical constituent and antioxidant activity of extract from the leaves of *Ocimum gratissimum. Scientific Research and Essay*. 2, 163-166.

Akinmoladun, A.O, Efere M and Farombi O. (2010). Evaluation of antioxidant and free radical scavenging capacities of some nigerian indigenous medicinal plants. *Journal of Medicinal Food*, 13: 444-451.

Akinyemi K.O., Oladapo O., Okwara C.E., Ibe C.C. and Fasure K.A. (2005). Screening of crude extracts of six medicinal plants used in South- West Nigerian unorthodox medicine for anti-methicillin resistant Staphylococcus aureus activity. *BMC Complementary and Alternative Medicine,* 5: 6-10.

Ali S.T. and Rakkah N.I. (2008). Probable neuro sexual mode of action of Casimiroa edulis seed extract versus [correction of verses] sildenafil citrate (Viagra) on mating behavior in normal male rats**.** *Pakistan Journal of Pharmaceutical Sciences*. 21(1):1-6.

Allen, M. S. and Emma E. W. (2019). Erectile Dysfunction: An Umbrella Review of Meta-Analyses of Risk-Factors, Treatment, and Prevalence Outcomes. *Journal of Sexual Medicine,* 16 (4): 531–41.

Allen, M.S. (2019). Physical activity as an adjunct treatment for erectile dysfunction. *Nature Reviews Urology*, 16(9): 553–562.

Amano, M., Fukata, Y. and Kaibuchi, K. (2000). Regulation and functions of Rho-associated kinase. *Experimental Cell Research*, 261: 44–51

Andersson K.E. (2003). Erectile physiological and pathophysiological pathways involved in erectile dysfunction. *Journal of Urology,* 170**:** 6–13.

Andersson K.E. and Wagner G. (1995). Physiology of penile erection. *Physiological Reviews***,** 75:191–236.

Ang, H.H. and Cheang, H.S. (2001). Effects of Eurycoma longifolia Jack on laevator ani muscle in both uncastrated and Testosterone-Stimulated castrated intact male rats. *Archives of Pharmacal Research*, 24(5): 437–440.

Ang, H.H. and Ngai, T.H. (2001). Aphrodisiac evaluation in non-copulator male rats after chronic administration of Eurycoma longifolia Jack. *Fundamental and Clinical Pharmacology*, 15(4): 265–268.

Ang, H.H., Ikeda, S. and Gan, E.K. (2001). Evaluation of the potency activity of aphrodisiac in Eurycoma longifolia Jack. *Phytotherapy Research*, 15(5), pp.435–436.

Aoyagi, T., Hayakawa, K., Miyaji, K., Ishikawa, H. and Hata, M. (1999). Sildenafil Induced Priapism. *The Bulletin of Tokyo Dental College*, 40(4): 215–217.

Arii T., Ohyanagi M, Shibuya J. and Iwasaki T (1999). Increased function of the voltage-dependent calcium channels, without increase of Ca^{2+} release from the sarcoplasmic reticulum in the arterioles of spontaneous hypertensive rats. *American Journal of Hypertension,* 12(12):1236-1242.

Azadzoi, K.M., Yang, J. and Siroky, M.B. (2013). Neural regulation of sexual function in men. *World Journal of Clinical Urology*, 2(3): 32.

Aziba, P.I., Bass, D. and Elegbe, Y. (1999). Pharmacological investigation of Ocimum gratissimum in rodents. *Phytotherapy Research*, 13(5): 427–429.

Bauer, V. and Sotnikova, R. (2010). Nitric oxide – the endothelium-derived relaxing factor and its role in endothelial functions. *General Physiology and Biophysics*. *29*(4), 319–340.

Bauer, V., and Sotnikova, R. (2015). Endothelium and the effect of activated neutrophils on arterial smooth muscle. *Interdisciplinary toxicology*, *8*(1), 44-47.

Belew, D., Klaassen, Z. and Lewis, R.W. (2015). Intracavernosal Injection for the Diagnosis, Evaluation, and Treatment of Erectile Dysfunction: A Review. *Sexual Medicine Reviews*, 3(1): 11-23.

Berridge M. J. (2008) Smooth muscle cell calcium activation mechanisms. *Journal of Physiology.* 586:5047–5061

Bryan J. R. M., You J., Golding E. M., Marrelli S. P. (2005) Endothelium-derived hyperpolarizing factor: a cousin to nitric oxide and prostacyclin. *Anesthesiology* 102:1261–77.

Burnett, A. L. (2003). Pathophysiology of Priapism: Dysregulatory Erection Physiology Thesis. *Journal of Urology*, *170*(1), 26–34.

Burnett, A.L., Nelson, R.J., Calvin, D.C., Liu, J.-X., Demas, G.E., Klein, S.L., Kriegsfeld, L.J., Dawso, V.L., Dawson, T.M. and Snyder, S.H. (1996). Nitric Oxide-Dependent Penile Erection in Mice Lacking Neuronal Nitric Oxide Synthase. *Molecular Medicine*, 2(3): 288–296.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, 94(3): 223–253.

Campbell, J. and Burnett, A. (2017). Neuroprotective and Nerve Regenerative Approaches for Treatment of Erectile Dysfunction after Cavernous Nerve Injury. *International Journal of Molecular Sciences*, 18(8):1794.

Campbell, J.D., Milenkovic, U., Albersen, M. and Bivalacqua, T.J. (2018). What Is the Future of Erectile Dysfunction Therapy? *Current Sexual Health Reports*, 10(3):169–176.

Carro-Juárez, M., Cervantes, E., Cervantes-Méndez, M. and Rodríguez-Manzo G. (2004). Aphrodisiac properties of Montanoa tomentosa aqueous crude extract in male rats. *Pharmacology Biochemistry and Behaviour*, 78(1): 129–134.

Carro-Juárez, M., Lobatón, I., Benítez, O. and Espíritu, A. (2006). Pro-ejaculatory effect of the aqueous crude extract of cihuapatli (Montanoa tomentosa) in spinal male rats. *Journal of Ethnopharmacology*, 106(1): 111–116.

Cartledge, J. J., Eardley, I., & Morrison, J. F. B. (2001). Impairment of corpus cavernosal smooth muscle relaxation by glycosylated human haemoglobin. *British Journal of Urology International*, *85*(6), 735–741.

Castro, C. A., Hogan, J. B., Benson, K. A., Shehata, C. W., and Landauer, M. R. (1995). Behavioral effects of vehicles: DMSO, ethanol, Tween-20, Tween-80, and emulphor-620. *Pharmacology Biochemistry and Behavior*, *50*(4), 521–526.

Cazzaniga W., Capogrosso P., Boeri L., Montorsi F., Salonia A. (2020). Pharmacology of Male Sexual Function. In: Chapple C., Steers W., Evans C. (eds) Urologic Principles and Practice. *Springer Specialist Surgery Series. Springer*, Cham

Chamiot-Clerc, .P., Choukri, .N., Legrand, .M., Droy-Lefaix, .M., Safar, .M., & Renaud, .J. (2000). Relaxation of vascular smooth muscle by cicletanine in aged Wistar aorta under stress conditions: importance of nitric oxide. *American Journal of Hypertension*, 13(2), 208-213.

Chen, G., Suzuki, H. and Weston, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *British journal of pharmacology*, 95(4): 1165–74.

Chitaley, K. and Webb, R.C. (2002). Nitric Oxide induces Dilation of rat aorta via inhibition of Rho-Kinase Signaling. *Hypertension*, 39(2): 438–442.

Chitaley, K., Wingard, C.J., Clinton Webb, R., Branam, H., Stopper, V.S., Lewis, R.W. and Mills, T.M. (2001). Antagonism of Rho-kinase stimulates rat penile erection via a nitric oxide-independent pathway. *Nature Medicine*, 7(1): 119–122.

Cicero, A.F.G., Bandieri, E. and Arletti, R. (2001). Lepidium meyenii Walp. improves sexual behaviour in male rats independently from its action on spontaneous locomotor activity. *Journal of Ethnopharmacology*, 75(2-3): 225–229.

Cohen, R.A., Weisbrod, R.M., Gericke, M., Yaghoubi, M., Bierl, C. and Bolotina, V.M. (1999). Mechanism of Nitric Oxide–Induced Vasodilatation. *Circulation Research*, 84(2): 210–219.

Colson, M.H., Cuzin, B., Faix, A., Grellet, L. and Huyghes, E. (2018). Current epidemiology of erectile dysfunction, an update. *Sexologies*, 27(1): 7-13.

Corona, G., Isidori, A.M., Buvat, J., Aversa, A., Rastrelli, G., Hackett, G., Rochira, V., Sforza, A., Lenzi, A., Mannucci, E. and Maggi, M. (2014). Testosterone Supplementation and Sexual Function: A Meta-Analysis Study. *The Journal of Sexual Medicine*, 11(6): 1577–1592.

Corona, G., Rastrelli, G., Burri, A., Serra, E., Gianfrilli, D., Mannucci, E., Jannini, E. A., and Maggi, M. (2016). First-generation phosphodiesterase type 5 inhibitors dropout: a comprehensive review and meta-analysis. *Andrology*, *4*(6), 1002–1009.

Costa, P. and Potempa, A.J. (2012). Intraurethral Alprostadil for Erectile Dysfunction: a review of the literature. *Drugs*, 72(17): 2243–2254.

Dare, A., Salami, S., Tolulope, O., Akindele, O. and Raji, Y. (2015). Comparative Evaluation of the Aphrodisiac efficacy of Sildenafil and Carpolobia lutea Root Extract in Male Rabbits. *Journal of Intercultural Ethnopharmacology*, 4(4): 302.

Dau, T.D., Le, M.H., Nguyen, T.T.G.H., Dang, T.N.M., Do, T.N.Q. and Tran, Q.T. (2020). Effects of Cnidium monnieri (L.) Cuss. fruit extract on sexual behaviors in male rats. *Clinical Phytoscience*, 6(1): 1-6

Davis, M.J. and Hill, M.A. (1999). Signaling Mechanisms Underlying the Vascular Myogenic Response. *Physiological Reviews*, 79(2): 387–423.

Dean, R.C. and Lue, T.F. (2005). Physiology of Penile Erection and Pathophysiology of Erectile Dysfunction. *Urologic Clinics of North America*, 32(4): 379–395.

DeLay, K.J., Haney, N. and Hellstrom, W.J. (2016). Modifying Risk Factors in the Management of Erectile Dysfunction: A Review. *The World Journal of Men's Health*, 34(2): 89–100.

Dong, J.Y., Zhang, Y.H. and Qin, L.Q. (2011). Erectile Dysfunction and Risk of Cardiovascular Disease. *Journal of the American College of Cardiology*, 58(13): 1378–1385.

Doyle, C., Sergeant, G. P., Hollywood, M. A., McHale, N. G., & Thornbury, K. D. (2012). Effects of Phenylephrine on Spontaneous Activity and L-Type Ca2+ Current in Isolated Corpus Cavernosum Myocytes. *The Journal of Sexual Medicine*, *9*(11), 2795–2805.

Duca, Y., Calogero, A.E., Cannarella, R., Giacone, F., Mongioi, L.M., Condorelli, R.A. and La Vignera, S. (2019). Erectile dysfunction, physical activity and physical exercise: Recommendations for clinical practice. *Andrologia*, 51(5): 13264.

Effraim, K., Jacks, T. and Sadipo, O. (2010). Histopathological studies on the toxicity of *Ocimum gratissimum*leave extract on some organs of rabbit. *African Journal of Biomedical Research*, 6(1): 21-25.

Enema, O. J., Umoh, U. F., Umoh, R. A., Ekpo, E. G., Adesina, S. K. and Eseyimo, O. A. (2018). Chemistry and pharmacology of aphrodisiac plants: A review. *Journal of Chemistry and Pharmaceutical Research*, (7), 70- 98.

Essin, K. and Gollasch, M. (2009). Role of Ryanodine Receptor Subtypes in Initiation and Formation of Calcium Sparks in Arterial Smooth Muscle: Comparison with Striated Muscle. *Journal of Biomedicine and Biotechnology*, 2009: 1–15.

Estrada-Reyes, R., Ortiz-López, P., Gutiérrez-Ortíz, J., and Martínez-Mota, L. (2009). Turnera diffusa Wild (Turneraceae) recovers sexual behavior in sexually exhausted males. *Journal of Ethnopharmacology*, *123*(3), 423–429.

Evans, I.M. and Distiller, L.A. (1979). Effects of luteinizing hormone-releasing hormone on sexual arousal in normal men. *Archives of Sexual Behavior*, 8(5): 385–395.

Feelisch, M., Kotsonis, P., Siebe, J., Clement, B. and Schmidt, H.H.H.W. (1999). The Soluble Guanylyl Cyclase Inhibitor 1H-[1,2,4]Oxadiazolo[4,3,-a]quinoxalin-1-one is a nonselective heme protein inhibitor of nitric oxide synthase and other cytochrome P-450 enzymes involved in nitric oxide donor bioactivation. *Molecular Pharmacology*, 56(2): 243–253.

Ferrier, L., Segain, J.P., Pacaud, P., Cherbut, C., Loirand, G., Galmiche, J.P., Blottière, H.M. (2000). Pathways and receptors involved in peptide YY induced contraction of rat proximal colonic muscle in vitro. *Gut*, 46(3):370-375

Filippi, S., Vignozzi, L., Morelli, A., Chavalmane, A.K., Sarchielli, E., Fibbi, B., Saad, F., Sandner, P., Ruggiano, P., Vannelli, G.B., Mannucci, E. and Maggi, M. (2009). Testosterone Partially Ameliorates Metabolic Profile and Erectile Responsiveness to PDE5 Inhibitors in an Animal Model of Male Metabolic Syndrome. *The Journal of Sexual Medicine*, 6(12): 3274–3288.

Fill, M. and Copello, J.A. (2002). Ryanodine Receptor Calcium Release Channels. *Physiological Reviews*, 82(4): 893–922.

Foresta, C., Caretta, N., Garolla, A. and Rossato, M. (2003). Erectile function in elderly: Role of androgens. *Journal of endocrinological investigation*. 26(3): 77-81.

Francis, S.H., Zoraghi, R., Kotera, J., Ke, H., Bessay, E.P. and Blount, M.A. (2006). Phosphodiesterase 5: molecular characteristics relating to structure, function, and regulation. In: Beavo JA, Francis SH, Houslay MD (ed). Cyclic Nucleotide Phosphodiesterases in Health and Disease. CRC Press: Boca Raton, pp 131–164.

Gallagher, P.J., Paul Herring, B. And Stull, J.T. (1997). Myosin light chain kinases. *Journal of Muscle Research and Cell Motility*, 18(1): 1–16.

Gauthaman, K., Adaikan, P.G. and Prasad, R.N.V. (2002). Aphrodisiac properties of Tribulus Terrestris extract (Protodioscin) in normal and castrated rats. *Life Sciences*, 71(12): 1385–1396.

Gerthoffer, W.T., Murphey, K.A., Mangini, J., Boman, S. and Lattanzio, F.A. (1991). Myosin phosphorylation and calcium in tonic and phasic contractions of colonic smooth muscle. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 260(6): 958-964.

Ghalayini, I.F. (2004). Nitric oxide–cyclic GMP pathway with some emphasis on cavernosal contractility. *International Journal of Impotence Research*, 16(6): 459-469.

Giuliano, F., Jackson, G., Montorsi, F., Martin-Morales, A. and Raillard, P. (2010). Safety of sildenafil citrate: review of 67 double-blind placebo-controlled trials and the postmarketing safety database. *International Journal of Clinical Practice*, 64(2): 240–255.

Giuliano, F., Rampin, O., Bernabé, J. and Rousseau, J.P. (1995). Neural control of penile erection in the rat. *Journal of the Autonomic Nervous System*, 55(1-2): 36-44.

Goldstein, I., Goren, A., Li, V. W., Tang, W. Y., and Hassan, T. A. (2020). Epidemiology Update of Erectile Dysfunction in Eight Countries with High Burden. *Sexual Medicine Reviews*, *8*(1), 48–58.

Goldstein, I., Goren, A., Li, V., Tang, W.Y. and Hassan, T.A. (2017). Erectile Dysfunction Prevalence, Patient Characteristics, and Health Outcomes Globally. *The Journal of Sexual Medicine*, 14(5): 298.

Gonzales, G., Cordova, A., Vega, K., Chung, A., Villena, A. and Gonez, C. (2003). Effect of Lepidium meyenii (Maca), a root with aphrodisiac and fertility-enhancing properties, on serum reproductive hormone levels in adult healthy men. *Journal of Endocrinology*, 176(1): 163–168.

Gratzke, C., Strong, T.D., Gebska, M.A., Champion, H.C., Stief, C.G., Burnett, A.L. and Bivalacqua, T.J. (2010). Activated RhoA/Rho Kinase impairs erectile function after cavernous nerve injury in rats. *Journal of Urology*, 184(5): 2197–2204.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Analytical biochemistry*, *126*(1), 131–138.

Hannan, J.L., Albersen, M., Kutlu, O., Gratzke, C., Stief, C.G., Burnett, A.L., Lysiak, J.J., Hedlund, P. and Bivalacqua, T.J. (2013). Inhibition of Rho-Kinase improves erectile function, increases nitric oxide signaling and decreases penile apoptosis in a rat model of cavernous nerve injury. *Journal of Urology*, 189(3): 1155-1161.

Harris, R.A. and Hanrahan, J.W. (1994). Effects of EGTA on calcium signaling in airway epithelial cells. *The American Journal of Physiology*. 267(5):1426-1434.

Hatzimouratidis, K. and Hatzichristou, D.G. (2005). A Comparative Review of the Options for Treatment of Erectile Dysfunction. *Drugs*, 65(12): 1621–1650.

Hatzimouratidis, K., Salonia, A., Adaikan, G., Buvat, J., Carrier, S., El-Meliegy, A., McCullough, A., Torres, L.O. and Khera, M. (2016). Pharmacotherapy for erectile

dysfunction: recommendations from the fourth International Consultation for Sexual Medicine (ICSM 2015). *The Journal of Sexual Medicine*, 13(4): 465-488.

Hill, M.A. and Meininger, G.A. (2012). Arteriolar vascular smooth muscle cells: Mechanotransducers in a complex environment. *The International Journal of Biochemistry & Cell Biology*, 44(9), pp.1505–1510.

Hirano, K. (2007). Current Topics in the Regulatory Mechanism Underlying the Ca^{2+} Sensitization of the Contractile Apparatus in Vascular Smooth Muscle. *Journal of Pharmacological Sciences*, 104(2): 109–115.

Hoepfl, B., Rodenwaldt, B., Pohl, U. and de Wit, C. (2002). EDHF, but not NO or prostaglandins, is critical to evoke a conducted dilation upon ACh in hamster arterioles. *American Journal of Physiology-Heart and Circulatory Physiology*, 283(3): 996-1004.

Horst, C., Stuebinger, H., Seif, C., Melchior, D., Martínez-Portillo, F.J. and Juenemann, K.P. (2003). Priapism: etiology, pathophysiology and management. *International braz j urol*, 29(5), pp.391–400.

House, S.J., Potier, M., Bisaillon, J., Singer, H.A. and Trebak, M. (2008). The non-excitable smooth muscle: Calcium signaling and phenotypic switching during vascular disease. *Pflugers Archive: European journal of physiology*, 456(5), 769-785.

Hsu, G. L., Hsieh, C. H., Wen, H. S., Hsu, W. L., Wu, C. H., Fong, T. H., Chen, S. C. and Tseng, G. F. (2004). Anatomy of the Human Penis: The Relationship of the Architecture Between Skeletal and Smooth Muscles. *Journal of Andrology*, 25(3), 426–431.

Hwang, T. L., Wu, C. C. and Teng, C. M. (1998). Comparison of two soluble guanylyl cyclase inhibitors, methylene blue and ODQ, on sodium nitroprusside-induced relaxation in guinea-pig trachea. *British Journal of Pharmacology*, *125*(6), 1158-1163.

Ibrahim, E., Brackett, N. L., and Lynne, C. M. (2016). Advances in the management of infertility in men with spinal cord injury. *Asian journal of andrology*, *18*(3): 382-390.

Interaminense, L. F., Leal-Cardoso, J. H., Magalhães, P. J., Duarte, G. P. and Lahlou, S. (2005). Enhanced hypotensive effects of the essential oil of Ocimum gratissimum leaves and its main constituent, eugenol, in DOCA-salt hypertensive conscious rats. *Planta medica*, *71*(4), 376–378.

Interaminense, L.F.L., Jucá, D.M., Magalhães, P.J.C., Leal-Cardoso, J.H., Duarte, G.P. and Lahlou, S. (2007). Pharmacological evidence of calcium-channel blockade by essential oil

of Ocimum gratissimum and its main constituent, eugenol, in isolated aortic rings from DOCA-salt hypertensive rats. *Fundamental and Clinical Pharmacology*, 21(5): 497–506.

Jamshidi, N. and Cohen, M.M. (2017). The Clinical Efficacy and Safety of Tulsi in Humans: A Systematic Review of the Literature. *Evidence-based Complementary and Alternative Medicine : eCAM*, [online] 2017.

Jespersen, B., Tykocki, N. R., Watts, S. W., and Cobbett, P. J. (2015). Measurement of Smooth Muscle Function in the Isolated Tissue Bath-applications to Pharmacology Research. *Journal of Visualized Experiments*, *95*: 1-9

Jin, L., Liu, T., Lagoda, G.A., Champion, H.C., Bivalacqua, T.J. and Burnett, A.L. (2006). Elevated RhoA/Rho-kinase activity in the aged rat penis: mechanism for age-associated erectile dysfunction. *The FASEB Journal*, 20(3): 536–538.

Johannes C.B., Araujo A.B., Feldman H.A., Derby C.A., Kleinman K.P., and McKinlay J.B. (2000) Incidence of erectile dysfunction in men 40 to 69 years old: longitudinal results from the Massachusetts male aging study. *Journal of Urology* 163(2):460–463

Jordan, W., Van Barneveld, H., Gerlich, O., Kleine-Boymann, M., and Ullrich, J. (1991). "Phenol," in *Ullmanns Encyclopedia of Industrial Chemistry*, 5th Edn, Vol. A 19, eds B. Elvers, S. Hawkins, and G. Schulz (Weinheim: Wiley-VCH Verlag GmbH and Co. KGaA), 299–312.

Kalter-Leibovici, O., Wainstein, J., Ziv, A., Harman-Bohem, I., Murad, H. and Raz, I. (2005). Clinical, socioeconomic, and lifestyle parameters associated with erectile dysfunction among diabetic men. *Diabetes Care*, 28(7): 1739–1744.

Kamm, K.E. and Stull, J.T. (2001). Dedicated Myosin Light Chain Kinases with Diverse Cellular Functions. *Journal of Biological Chemistry*, 276(7): 4527–4530.

Kandeel, F.R., Koussa, V.K.T. and Swerdloff, R.S. (2001). Male Sexual Function and Its Disorders: Physiology, Pathophysiology, Clinical Investigation, and Treatment. *Endocrine Reviews*, 22(3): 342–388.

Kankara, S.S., Ibrahim, M.H., Mustafa, M. and Go, R. (2015). Ethnobotanical survey of medicinal plants used for traditional maternal healthcare in Katsina state, Nigeria. *South African Journal of Botany*, 97:165–175.

Karaki, H. (1990). The Intracellular Calcium-Force Relationship in Vascular Smooth Muscle: Time- and Stimulus-dependent Dissociation. *American Journal of Hypertension*, 3(8): 253-256.

Karaki, H., Ozaki, H., Hori, M., Mitsui-Saito, M., Amano, K., Harada, K., Miyamoto, S., Nakazawa, H., Won, K.J. and Sato, K. (1997). Calcium movements, distribution, and functions in smooth muscle. *Pharmacological Reviews*, 49(2):157-230.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996). Regulation of Myosin Phosphatase by Rho and Rho-Associated Kinase (Rho-Kinase). *Science*. 273(5272): 245-248.

Koba, K., Poutouli, P.W., Raynaud, C. and Sanda, K. (2008). Antifungal Activity of the Essential Oils from Ocimum gratissimum L. Grown in Togo. *Journal of Scientific Research*, 1(1): 164-171.

Kokk K, Kuuslahti M, Keisala T, Purmonen S, Kaipia A, Tammela T, Orro H, Simovart HE, and Pöllänen P. (2011). Expression of luteinizing hormone receptors in the mouse penis. *Journal of Andrology*, 32(1): 49-54.

Kontos, H.A. and Wei, E.P. (1993). Hydroxyl radical-dependent inactivation of guanylate cyclase in cerebral arterioles by methylene blue and by LY83583. *Stroke*, 24: 427-434.

Ku, C. M., and Lin, J. Y. (2013). Anti-inflammatory effects of 27 selected terpenoid compounds tested through modulating Th1/Th2 cytokine secretion profiles using murine primary splenocytes. *Food Chemistry.* 141: 1104–1113.

Laugier, R. and Petersen, O.H. (1980). Effects of intracellular EGTA injection on stimulant-evoked membrane potential and resistance changes in pancreatic acinar cells. *Pflugers Archive: European Journal of Physiology*, 386(2), pp.147–152.

Leal-Cardoso, J.H.and Fonteles, M.C.(1999). Pharmacological effects of essential oils of plants of the northeast of Brazil. *Annals of the Brazilian Academy of Sciences*, *71*: 207–213.

Lee, M. and Sharifi, R. (2018). Non-invasive management options for erectile dysfunction when a phosphodiesterase type 5 inhibitor fails. *Drugs and Aging*. 35: 175-87.

Lee, N.P.Y. and Cheng, C.Y. (2003). Regulation of Sertoli cell tight junction in the rat testis via the nitric oxide synthase/soluble guanylate cyclase/ cGMP/protein kinase G signaling pathway: an *ex-vivo* study. *Endocrinology*. 144:3114–3129.

Leite, R., Fernanda R. C. Giachini, Fernando S. Carneiro, Kenia P. Nunes, Rita C. Tostes, and Robert C. Webb. (2007). Targets for the Treatment of Erectile Dysfunction: Is NO/cGMP Still the Answer? *Recent Patents on Cardiovascular Drug Discovery*, *2*(2), 119–132.

Levine, L.A. and Dimitriou, R.J. (2001). Vacuum constriction and external erection devices in erectile dysfunction. *The Urologic Clinics of North America*. 28(2):335-341.

Levine, L.A., Becher, E., Bella, A., Brant, W., Kohler, T., Martinez-Salamanca, J.I., Trost, L. and Morey, A. (2016). Penile Prosthesis Surgery: Current recommendations from the international consultation on sexual medicine. *The Journal of Sexual Medicine*, 13(4), 489–518.

Lide, D. R., and Frederikse, H. P. R. (1996). CRC Handbook of Chemistry and Physics. Boca Raton, NY: CRC Press, 3–258.

Liu, C., Lopez, D.S., Chen, M. and Wang, R. (2017). Penile Rehabilitation Therapy Following Radical Prostatectomy: A Meta-Analysis. *The Journal of Sexual Medicine*, 14(12): 1496–1503.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193(1): 265–275.

Madeira, S.V., Rabelo, M., Soares, P.M., Souza, E.P., Meireles, A.V., Montenegro, C.M., Lima, R.F., Assreuy, A.M., and Criddle, D.N. (2005). Temporal variation of chemical composition and relaxant action of the essential oil of Ocimum gratissimum L. (Labiatae) on guinea-pig ileum. *Phytomedicine : international journal of phytotherapy and phytopharmacology, 12 (6-7)*, 506-509

Madeira, S.V.F., Matos, F.J.A., Leal-Cardoso, J.H. and Criddle, D.N. (2002). Relaxant effects of the essential oil of Ocimum gratissimum on isolated ileum of the guinea pig. *Journal of Ethnopharmacology*, 81(1): 1–4.

Magyar, J., Szentandrássy, N., Bányász, T., Fülöp, L., Varró, A. and Nánási, P.P. (2002). Effects of thymol on calcium and potassium currents in canine and human ventricular cardiomyocytes. *British Journal of Pharmacology*, 136(2): 330–338.

Marcondes, F.K., Bianchi, F.J. and Tanno, A.P. (2002). Determination of the estrous cycle phases of rats: some helpful considerations. *Brazilian Journal of Biology*, 62(4A): 609–614.

Marczin, N., Ryan, U.S. and Catravas, D.J. (1992). Methylene blue inhibits nitrovasodilator- and endothelium-derived relaxing factor-induced cyclic GMP accumulation in cultured pulmonary arterial smooth muscle cells via generation of superoxide anion. Journal of Pharmacology and Experimental Therapeutics, 263: 170-179.

Martínez Portillo, F.J., Martínez Portillo, F.J., Hoang-Boehm, J., Hoang-Boehm, J., Weiss, J., Weiss, J., Alken, P., Alken, P., Jünemann, K.P. and Jünemann, K.P. (2001). Methylene Blue as a Successful Treatment Alternative for Pharmacologically Induced Priapism. *European Urology*, 39(1), pp.20–23.

Martins, A.P., Salgueiro, L.R., Vila, R., Tomi, F., Canigueral, S., Casanova, J., Proenc¸a da Cunha, A., Adzet, T., (1999). Composition of the essentials oils of Ocimum canum, Ocimum gratissimum and Ocimum minimum. *Planta Medica*, 65: 187–189.

Mas M. (2010). Molecular mechanisms of penile erection. *Archivos Espanoles de Urologia*; 63(8):589-98.

Masuku, N. P., Unuofin, J. O., and Lebelo, S. L. (2020). Promising role of medicinal plants in the regulation and management of male erectile dysfunction. *Biomedicine & Pharmacotherapy*, *130*, 110555.

Matasyoh, L., Matasyoh, J., Wachira, F., Kinyua, M., Muigai, A., and Mukiama, T. (2008). Antimicrobial activity of essential oils of *Ocimum gratissimum* l. from different populations of Kenya. *African Journal of Traditional, Complementary and Alternative Medicines*, *5*(2).

McCullough, A.R., Hellstrom, W.G., Wang, R., Lepor, H., Wagner, K.R. and Engel, J.D. (2010). Recovery of erectile function after nerve sparing radical prostatectomy and penile rehabilitation with nightly intraurethral alprostadil versus sildenafil citrate. *Journal of Urology*, 183(6): 2451–2456.

Mohammed A., Tanko Y., Okasha M.A., Magaji R.A., Yaro A.H. (2007). Effects of aqueous leaves extract of *Ocimum gratissimum* on blood glucose levels of streptozotocin induced diabetic Wistar rats. *African Journal of Biotechnology; 6: 2087-90.*

Montorsi, F. and McCullough, A. (2005). Efficacy of Sildenafil Citrate in Men with Erectile Dysfunction Following Radical Prostatectomy: A Systematic Review of Clinical Data. *The Journal of Sexual Medicine*, 2(5): 658–667.

Moreland, R.B., Kim, N., Nehra, A., Goldstein, I. and Traish, A. (2003). Functional prostaglandin E (EP) receptors in human penile corpus cavernosum. *International Journal of Impotence Research*, 15(5): 362–368.

Morelli, A., Filippi, S., Mancina, R., Luconi, M., Vignozzi, L., Marini, M., Orlando, C., Vannelli, G.B., Aversa, A., Natali, A., Forti, G., Giorgi, M., Jannini, E.A., Ledda, F. and

Maggi, M. (2004). Androgens Regulate Phosphodiesterase Type 5 Expression and Functional Activity in Corpora Cavernosa. *Endocrinology*, 145(5): 2253–2263.

Mulhall, J.P., Jahoda, A.E., Cairney, M., Goldstein, B., Leitzes, R., Woods, J., Payton, T., Krane, R.J. And Goldstein, I. (1999). The causes of patient dropout from penile self-injection therapy for impotence. *Journal of Urology*, 162(4): 1291–1294.

Murthy, K.S. (2006). Signaling for contraction and relaxation in smooth muscle of the gut. *Annual Review of Physiology*, 68(1): 345–374.

Nagoor-Meeran, M.F. and Stanely Mainzen Prince, P. (2012). Protective effects of thymol on altered plasma lipid peroxidation and nonenzymic antioxidants in isoproterenol-induced myocardial infarcted rats. *Journal of Biochemical and Molecular Toxicology*, 26(9): 368–373.

Nagoor-Meeran, M.F., Jagadeesh, G.S. and Selvaraj, P. (2015). Thymol attenuates inflammation in isoproterenol induced myocardial infarcted rats by inhibiting the release of lysosomal enzymes and downregulating the expressions of proinflammatory cytokines. *European Journal of Pharmacology*, 754: 153–161.

Nakamura, C.V., Ueda-Nakamura, T., Bando, E., Melo, A.F.N., Cortez, D.A.G. and Dias Filho, B.P. (1999). Antibacterial activity of Ocimum gratissimum L. essential oil. *Memórias do Instituto Oswaldo Cruz*, 94(5): 675–678.

Narumiya, S. (1996). The Small GTPase Rho: Cellular Functions and Signal Transduction. *Journal of Biochemistry*, 120(2): 215–228.

Nelson, C.J., Hsiao, W., Balk, E., Narus, J., Tal, R., Bennett, N.E. and Mulhall, J.P. (2013). Injection anxiety and pain in men using intracavernosal injection therapy after radical pelvic surgery. *The Journal of Sexual Medicine*, 10(10): 2559–2565.

Nieddu, M., Rassu, G., Boatto, G., Bosi, P., Trevisi, P., Giunchedi, P., Carta, A., and Gavini, E. (2014). Improvement of thymol properties by complexation with cyclodextrins: In vitro and in vivo studies. *Carbohydrate Polymers*, *102*, 393–399.

Obaseiki-Ebor, E.E., Odukoya, K., Telikepalli, H., Mitscher, L.A. and Shankel, D.M. (1993). Antimutagenic activity of extracts of leaves of four common edible vegetable plants in Nigeria (West Africa). *Mutation Research Letters*, 302(2): 109–117.

Obianime, A.W., Aprioku, J.S. and Esomonu, C. (2010). The Effects of Aqueous Ocimum gratissimum Leaf Extract on Some Biochemical and Hematological Parameters in Male Mice. *Asian Journal of Biological Sciences*, 4(1): 44–52.

Offiah, V.N. and Chikwendu, U.A. (1999). Antidiarrhoeal effects of Ocimum gratissimum leaf extract in experimental animals. *Journal of Ethnopharmacology*, 68(1-3): 327–330.

Oladunmoye M.K. (2006). Immunostimulatory Activity of Ethanolic Leaf Extract from Ocimum gratissimum in Albino Rat Orogastrically Dosed with Escherichia coli (NCIB 86). *Journal of Pharmacology and Toxicology*, 1(4): 389–394.

Onajobi, F.D. (1986) Smooth Muscle Contracting Lipid-Soluble Principles in Chromatographic Fractions of Ocimum gratissimum. *Journal of Ethnopharmacology*, 18, 3-11.

Oyelade B.O., Jemilohun A.O. and Aderibigbe S.A. (2016) Prevalence of erectile dysfunction and possible risk factors among men of South-Western Nigeria: a population based study. *Pan African Medical Journal*, 24: 124.

Ozen, T., Demirtas, I. and Aksit, H. (2011). Determination of antioxidant activities of various extracts and essential oil compositions of Thymus praecox subsp. skorpilii var. skorpilii. *Food Chemistry*, 124(1): 58–64

Pande M., Pathak A. (2009). Effect of Ethanolic Extract of Ocimum gratissimum (ram tulsi) on Sexual Behaviour in Male Mice. *International Journal of Pharmtech Research*. 1: 468-473.

Pandey, A.K., Singh, P. and Tripathi, N.N. (2014). Chemistry and bioactivities of essential oils of some Ocimum species: an overview. *Asian Pacific Journal of Tropical Biomedicine*, 4(9): 682–694.

Peixoto-Neves, D., Silva-Alves, K.S., Gomes, M.D.M., Lima, F.C., Lahlou, S., Magalhães, P.J.C., Ceccatto, V.M., Coelho-de-Souza, A.N. and Leal-Cardoso, J.H. (2010). Vasorelaxant effects of the monoterpenic phenol isomers, carvacrol and thymol, on rat isolated aorta. *Fundamental & Clinical Pharmacology*, 24(3): 341–350.

Pfitzer, G. And Arner, A. (1998). Involvement of small GTPases in the regulation of smooth muscle contraction. *Acta Physiologica Scandinavica*, 164(4): 449–456.

Prabhu K.S., Lobo R., Shirwaikar A.A. and Shirwaikar A. (2009). *Ocimum gratissimum* : A Review of its Chemical, Pharmacological and Ethnomedicinal Properties. *The Open Complementary Medicine Journal, 1: 1-15.*

Pritchard, T.J., Bowman, P.S., Jefferson, A., Tosun, M., Lynch, R.M. and Paul, R.J. (2010). Na^+-K^+-ATPase and Ca^{2+} clearance proteins in smooth muscle: a functional unit. *American Journal of Physiology-Heart and Circulatory Physiology*, 299(2): 548–556.

Priviero, F. B., Jin, L. M., Ying, Z., Teixeira, C. E. and Webb, R. C. (2010). Up-regulation of the RhoA/Rho-kinase signaling pathway in corpus cavernosum from endothelial nitric-

oxide synthase (NOS), but not neuronal NOS, null mice. *The Journal of pharmacology and experimental therapeutics*, *333*(1): 184–192.

Quartey J.K.M. (1997). Microcirculation of penile and scrotal skin, In: Jordan GH (ed) Reconstruction for urethral stricture, Atlas of the Urologic Clinics of North America 5(1).

Raicu, M. and Florea, S. (2001). Deleterious effects of nifedipine on smooth muscle cells implies alterations of intracellular calcium signaling. *Fundamental and Clinical Pharmacology*, 15(6): 387–392.

Saenz de Tejada, I., Gonzalez-Cadavid, N., Heaton, J., Hedlund, H., Nehra, A., Pickard, R.S., (2000). anatomy, physiology and pathophysiology of erectile function. In: Jardin A, Wagner G, Khoury S, Giuliano F, Padma-Nathan H, Rosen R, editors. Erectile dysfunction. Plymouth, UK7 Health Publication, Plymbridge Distributors. 67–102.

Sahin, L., Cevik, O.S., Koyuncu, D.D. and Buyukafsar, K. (2018). Role of rho-kinase (ROCK) in tonic but not phasic contraction in the frog stomach smooth muscle. *Life Sciences*, 198: 46–55.

Sahouo G.B., Tonzibo Z.F., Boti B., Chopard C., Mahy J.P., N'guessan Y.T. (2003). Anti-inflammatory and analgesic activities: Chemical constituents of essential oils of *Ocimum gratissimum* , Eucalyptus citriodora and Cymbopogon giganteus inhibited lipoxygenase L-1 and cyclooxygenase of PGHS. *Bulletin of the Chemical Society of Ethiopia*, 17(2) 191-197.

Salahdeen H., Idowu G., Yemitan O., Murtala B., Alada A. (2014). The relaxant actions of ethanolic extract of Tridax procumbens on rat corpus cavernosum smooth muscle contraction. *Journal Of Basic and Clinical Physiology and Pharmacology*. 26(2):1-5.

Saliu, B.K., Usman, L.A., Sani, A., Muhammad, N.O., Akolade, J.O; (2011). Chemical composition and anti-bacterial (oral isolates) activity of leaf essential oil of Ocimum gratissimum L. grown in north central Nigeria. *International Journal of Current Research 33 (3): 022-028.*

Salonia, A., Castagna, G., Saccà, A., Ferrari, M., Capitanio, U., Castiglione, F., Rocchini, L., Briganti, A., Rigatti, P., & Montorsi, F. (2012). Is Erectile Dysfunction a Reliable Proxy of General Male Health Status? The Case for the International Index of Erectile Function—Erectile Function Domain. *The Journal of Sexual Medicine*, *9*(10), 2708–2715.

Saravanan, S., and Pari, L. (2015). Role of thymol on hyperglycaemia and hyperlipidemia in high fat diet-induced type 2 diabetic C57BL/6J mice. *Europian Journal of Pharmacology*. 761, 279–287.

Sasaki, T., Kato, M. and Takai, Y. (1993). Consequences of weak interaction of rho GDI with the GTP-bound forms of rho p21 and rac p21. *Journal of Biological Chemistry*, 268(32): 23959–23963.

Sauzeau, V., Le Jeune, H., Cario-Toumaniantz, C., Smolenski, A., Lohmann, S.M., Bertoglio, J., Chardin, P., Pacaud, P. and Loirand, G. (2000). Cyclic GMP-dependent Protein Kinase Signaling Pathway Inhibits RhoA-induced Ca^{2+} Sensitization of Contraction in Vascular Smooth Muscle. *Journal of Biological Chemistry*, 275(28): 21722–21729.

Sawada, N., Itoh, H., Yamashita, J., Doi, K., Inoue, M., Masatsugu, K., Fukunaga, Y., Sakaguchi, S., Sone, M., Yamahara, K., Yurugi, T. and Nakao, K. (2001). cGMP-Dependent Protein Kinase Phosphorylates and Inactivates RhoA. *Biochemical and Biophysical Research Communications*, 280(3): 798–805.

Scherzer, N. D., Dick, B., Gabrielson, A. T., Alzweri, L. M., & Hellstrom, W. J. G. (2019). Penile Prosthesis Complications: Planning, Prevention, and Decision Making. *Sexual Medicine Reviews*, *7*(2), 349–359.

Schouten, B.W.V., Bosch, J.L.H.R., Bernsen, R.M.D., Blanker, M.H., Thomas, S. and Bohnen, A.M. (2004). Incidence rates of erectile dysfunction in the Dutch general population. Effects of definition, clinical relevance and duration of follow-up in the Krimpen Study. *International Journal of Impotence Research*, 17(1): 58–62.

Schubert R, Brayden JE. (2005) Stretch-activated Cation Channels and the Myogenic Response of Small Arteries. In: Kamkin A, Kiseleva I, editors. Mechano-sensitivity in Cells and Tissues. Moscow: Academia.

Segal R. L., Tajkarimi K. and Burnett A. L., (2013). Viberect penile vibratory stimulation system: evaluation of its erectogenic efficacy. *Canadian Journal of Urology*. 20(4):6844-6847.

Shabsigh, R., Padma-Nathan, H., Gittleman, M., McMurray, J., Kaufman, J. and Goldstein, I. (2000). Intracavernous alprostadil alfadex is more efficacious, better tolerated, and preferred over intraurethral alprostadil plus optional ACTIS: a comparative, randomized, crossover, multicenter study. *Urology*, 55(1): 109–113.

Shabsigh, R., Perelman, M.A., Laumann, E.O. and Lockhart, D.C. (2004). Drivers and barriers to seeking treatment for erectile dysfunction: a comparison of six countries. *BJU international*, 94(7),1055–1065.

Shafik, A. (2007). Study of Interstitial Cells in the Penis: Human Study. *The Journal of Sexual Medicine*, *4*(1), 66–71.

Sharma, M. and Kumar, A. (2017). *Male Sexual Function. Basics of Human Andrology*, *251–281*.

Sharma, S., Panda, S., Sharma, S., Singh, S., Seth, A. and Gupta, N. (2009). Prolonged priapism following single dose administration of sildenafil: A rare case report. *Urology Annals*, 1(2): 67.

Shaw, H.M., Wu, J.L. and Wang, M.S. (2017). Antihypertensive effects of Ocimum gratissimum extract: Angiotensin-converting enzyme inhibitor in vitro and in vivo investigation. *Journal of Functional Foods*, 35:68–73.

Shittu S.T., Oyeyemi W.A., Shittu S.A. and Lasisi T.J (2018). Ocimum Gratissimum Inhibits Glycogen Phosphorylase Activity without Changes in Hepatic Nuclear Factor Kappa B (NF-kB) and Inducible Nitric Oxide Synthase (iNOS) in Streptozotocin-induced Diabetic Rats. *The Nigerian Medical Practitioner*. 73(1-2)10-17.

Shittu, S.T., Oyeyemi, W.A., Lasisi, T.J., Shittu, S.A., Lawal, T.T.,and Olujobi S.T. (2016). Aqueous leaf extract of Ocimum gratissimum improves hematological parameters in alloxan-induced diabetic rats via its antioxidant properties *International Journal of Applied and Basic Medical Research,* 6(2):96-100

Sofowora, E.A. (1970). A study of the variations in the essential oils of cultivated *Ocimum gratissimum. Planta Medica* 18, 173-176.

Somlyo, A.P. and Somlyo, A.V (1998). From pharmacomechanical coupling to G-proteins and myosin phosphatase. *Acta Physiologica Scandinavica*, 164(4): 437–448.

Somlyo, A.P. and Somlyo, A.V. (2000). Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *The Journal of Physiology*, 522(2): 177–185.

Somlyo, A.P. and Somlyo, A.V. (2003). Ca^{2+} Sensitivity of Smooth Muscle and Nonmuscle Myosin II: Modulated by G Proteins, Kinases, and Myosin Phosphatase. *Physiological Reviews*, 83(4): 1325–1358.

Sopko, N.A., Hannan, J.L. and Bivalacqua, T.J. (2014). Understanding and targeting the Rho kinase pathway in erectile dysfunction. *Nature Reviews Urology*, 11(11), 622–628.

Steers, W.D. (2000). Neural pathways and central sites involved in penile erection: neuroanatomy and clinical implications. *Neuroscience & Biobehavioral Reviews*, 24(5):507–516.

Szentandrassy, N., Szentesi, P., Magyar, J., Nanasi, P. P., and Csernoch, L. (2003). Effect of thymol on kinetic properties of Ca and K currents in rat skeletal muscle. *BMC Pharmacology*. 3:9.

Tajuddin, Ahmad, S., Latif, A. and Qasmi, I.A. (2003). Aphrodisiac activity of 50% ethanolic extracts of Myristica fragrans Houtt. (nutmeg) and Syzygium aromaticum (L) Merr. & Perry. (clove) in male mice: a comparative study. *BMC Complementary and Alternative Medicine*, 3(1): 1-5.

Takahashi, R., Nishimura, J., Hirano, K., Naito, S. And Kanaide, H. (2003). Modulation of Ca2+Sensitivity Regulates Contractility of Rabbit Corpus Cavernosum Smooth Muscle. *Journal of Urology*, 169(6): 2412–2416.

Terezinha J.F, Rafael S.F, Yassumoto L., Pinto de souza, J., Ishikawa, N.B., Aneli D.M. (2006). Antifungal activity of essential oil isolated from *Ocimum gratissimum* L. (eugenol chemotype) against phytopathogenic fungi. *Brazilian Archives of Biology and Technology*, 49(6): 867–871.

Trigo-Rocha, F., Hsu, G. L., Donatucci, C. F., & Lue, T. F. (1993). The Role of Cyclic Adenosine Monophosphate, Cyclic Guanosine Monophosphate, Endothelium and Nonadrenergic, Noncholinergic Neurotransmission in Canine Penile Erection. *The Journal of Urology*, 149(4): 872–877.

Uddin S.M.I., Mirbolouk M., Dardari Z., Feldman D.I., Cainzos-Achirica M., DeFilippis A.P., Greenland P., Blankstein R., Billups K.L., Miner M.M., Nasir K, and Blaha M.J., (2018). Erectile Dysfunction as an Independent Predictor of Future Cardiovascular Events: The Multi-Ethnic Study of Atherosclerosis. *Circulation* 138(5): 540–542.

Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M. and Narumiya, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature*, 389: 990–994.

Venu, S., Naik, D. B., Sarkar, S. K., Usha, K., Aravind, U. K., Nijamudheen, A., et al. (2013). Oxidation reactions of thymol: a pulse radiolysis and theoretical study. *Joirnal of Physical Chemistry.* 117: 291–299.

Vieira, R.F., Grayer, R.J., Paton, A. and Simon, J.E. (2001). Genetic diversity of Ocimum gratissimum L. based on volatile oil constituents, flavonoids and RAPD markers. *Biochemical Systematics and Ecology*, 29(3): 287–304.

Viigimaa, M. (2014). Management of erectile dysfunction in hypertension: Tips and tricks. *World Journal of Cardiology*, 6(9): 908.

Vlachopoulos C., Jackson G., Stefanadis C. and Montorsi P. (2013). Erectile dysfunction in the cardiovascular patient. European Heart Journal. 34(27): 2034-46.

Waldkirch, E., Ückert, S., Yildirim, H., Sohn, M., Jonas, U., Stief, C.G., Andersson, K.-E. and Hedlund, P. (2005). Cyclic AMP-specific and cyclic GMP-specific phosphodiesterase isoenzymes in human cavernous arteries—immunohistochemical distribution and functional significance. *World Journal of Urology*, 23(6): 405-410.

Wang, H., Eto, M., Steers, W.D., Somlyo, A.P. and Somlyo, A.V. (2002). RhoA-mediated Ca^{2+} Sensitization in Erectile Function. *Journal of Biological Chemistry*, 277(34): 30614–30621.

Watcho P, Fotsing D, Zelefack F, Nguelefack TB, Kamtchouing P, Tsamo E. (2006) Aphrodisiac effect of MH2 and MH6 isolated from Hexane extract of *Mondia whitei* in sexually inexperienced male rats. *Journal of Sex Medicine*; 5:382-452.

Webb, R.C. (2003). Smooth muscle contraction and relaxation. *Advances in Physiology Education*, 27(4): 201–206.

Wespes, E., Nogueira, M.C., Herbaut, A.G., Caufriez, M. and Schulman, C.C. (1990). Role of the Bulbocavernosus Muscles on the Mechanism of Human Erection. *European Urology*, 18(1): 45–48.

Wolin, M.S., Cherry, P.D., Rodenburg, J.M., Messina, E.J. and Kaley, G. (1990). Methylene blue inhibits vasodilation of skeletal muscle arterioles to acetylcholine and nitric oxide via the extracellular generation of superoxide anion. Journal of Pharmacology and Experimental Therapeutics. 254: 872-876.

Xavier, F. E., Blanco-Rivero, J., Sastre, E., Caracuel, L., Callejo, M. and Balfagón, G. (2014). Tranilast Increases Vasodilator Response to Acetylcholine in Rat Mesenteric Resistance Arteries through Increased EDHF Participation. *PLoS ONE, 9*(7), e100356.

Yang C.C. and Bradley WE. (1998). Peripheral distribution of the human dorsal nerve of the penis. *Journal of Urology*. 159:1912-1917.

Yuan, J., Zhang, R., Yang, Z., Lee, J., Liu, Y., Tian, J., Qin, X., Ren, Z., Ding, H., Chen, Q., Mao, C. and Tang, J. (2013). Comparative Effectiveness and Safety of Oral Phosphodiesterase Type 5 Inhibitors for Erectile Dysfunction: A Systematic Review and Network Meta-analysis. *European Urology*, 63(5): 902–912.

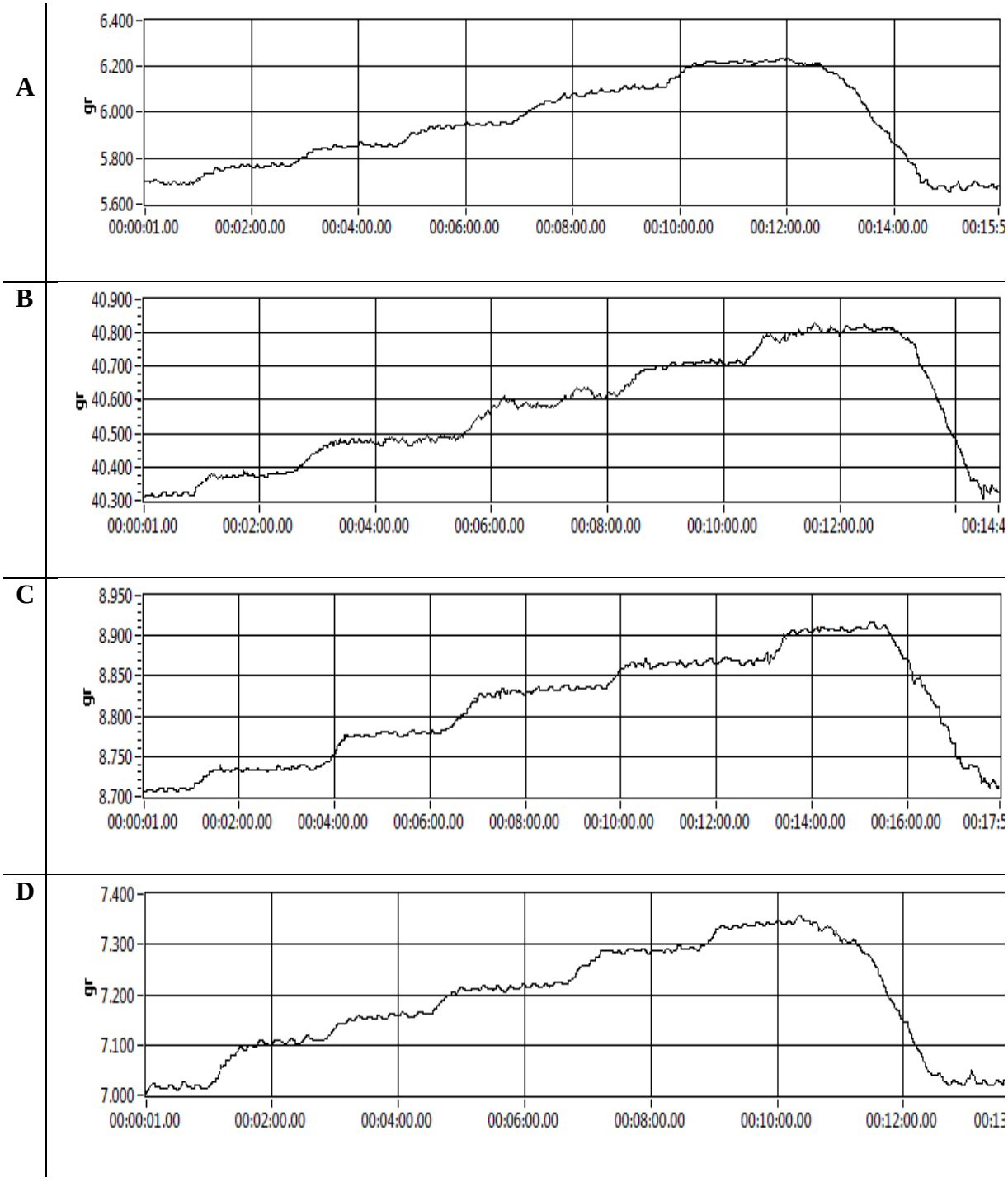
Yuill, K.H., McNeish, A.J., Kansui, Y., Garland, C.J. and Dora, K.A. (2009). Nitric Oxide Suppresses Cerebral Vasomotion by sGC-Independent Effects on Ryanodine Receptors and Voltage-Gated Calcium Channels. *Journal of Vascular Research*, 47(2): 93–107.

Zanoli, P., Benelli, A., Zavatti, M., Rivasi, M., Baraldi, C. and Baraldi, M. (2009). Improved sexual behavior in male rats treated with a Chinese herbal extract: hormonal and neuronal implications. *Asian Journal of Andrology*, 11(1): 139–139.

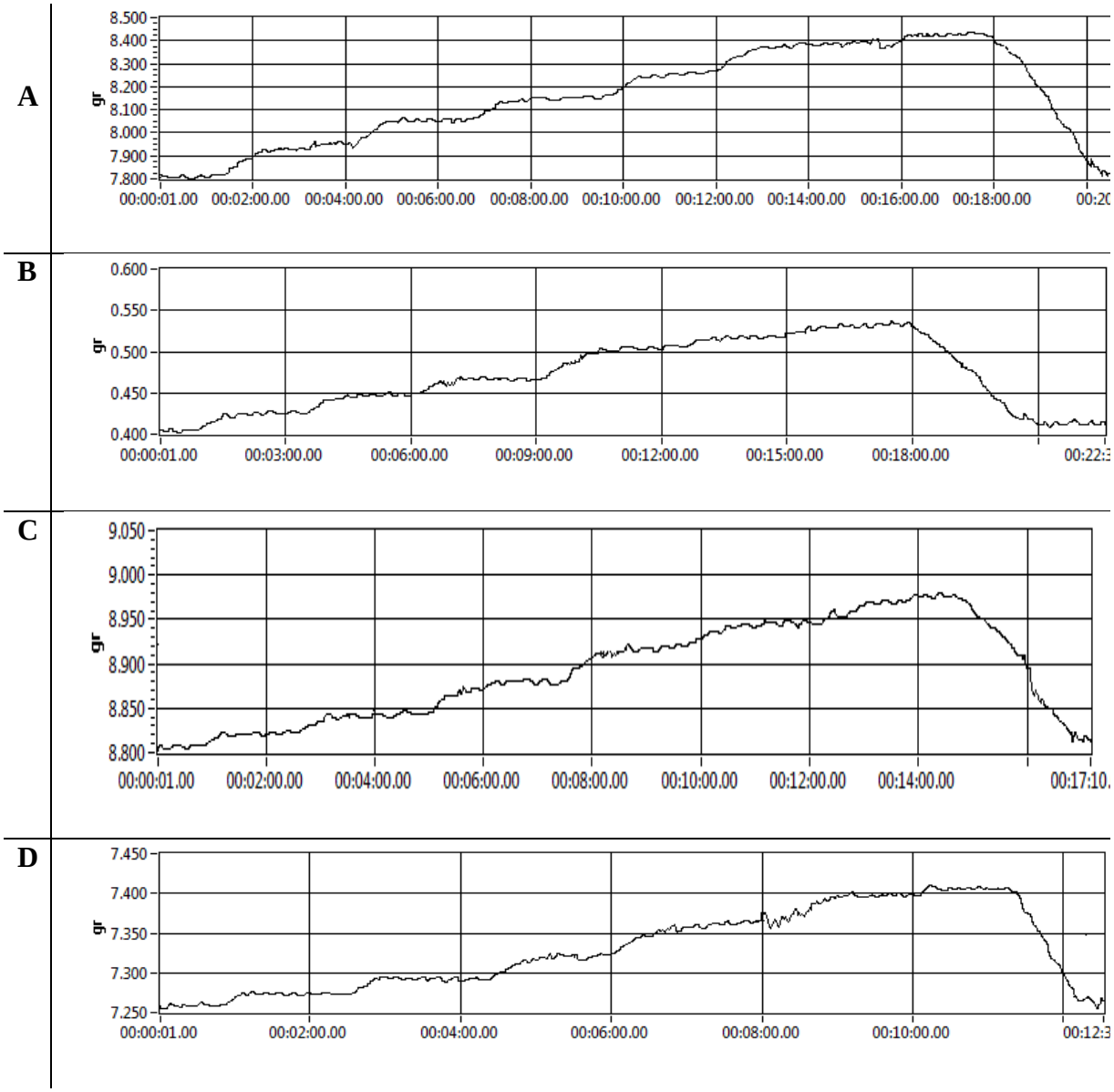
Zeng, X., Keyser, B., Li, M. and Sikka, S.C. (2005). T-Type (α1G) Low Voltage-Activated Calcium Channel Interactions with Nitric Oxide-Cyclic Guanosine Monophosphate Pathway and Regulation of Calcium Homeostasis in Human Cavernosal Cells. *The Journal of Sexual Medicine*, 2(5): 620-633.

Zewdie, K. A., Ayza, M. A., Tesfaye, B. A., Wondafrash, D. Z., & Berhe, D. F. (2020). A Systematic Review on Rho-Kinase as a Potential Therapeutic Target for the Treatment of Erectile Dysfunction. *Research and reports in urology*, *12*, 261–272.

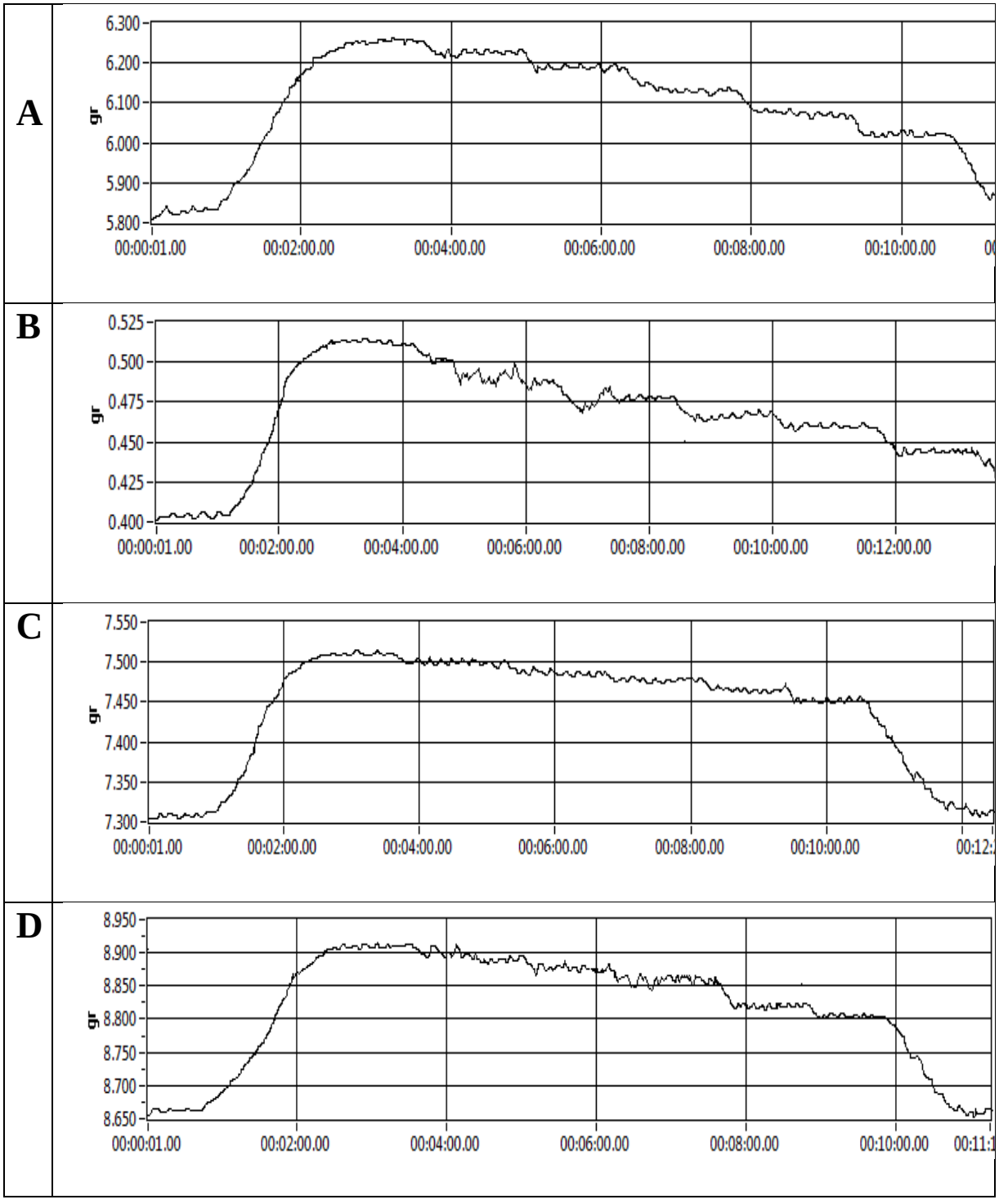
Zhang, D.X., Gauthier, K.M. and Campbell, W.B. (2004). Acetylcholine-Induced Relaxation and Hyperpolarization in Small Bovine Adrenal Cortical Arteries: Role of Cytochrome P450 Metabolites. *Endocrinology*, 145(10): 4532–4539.



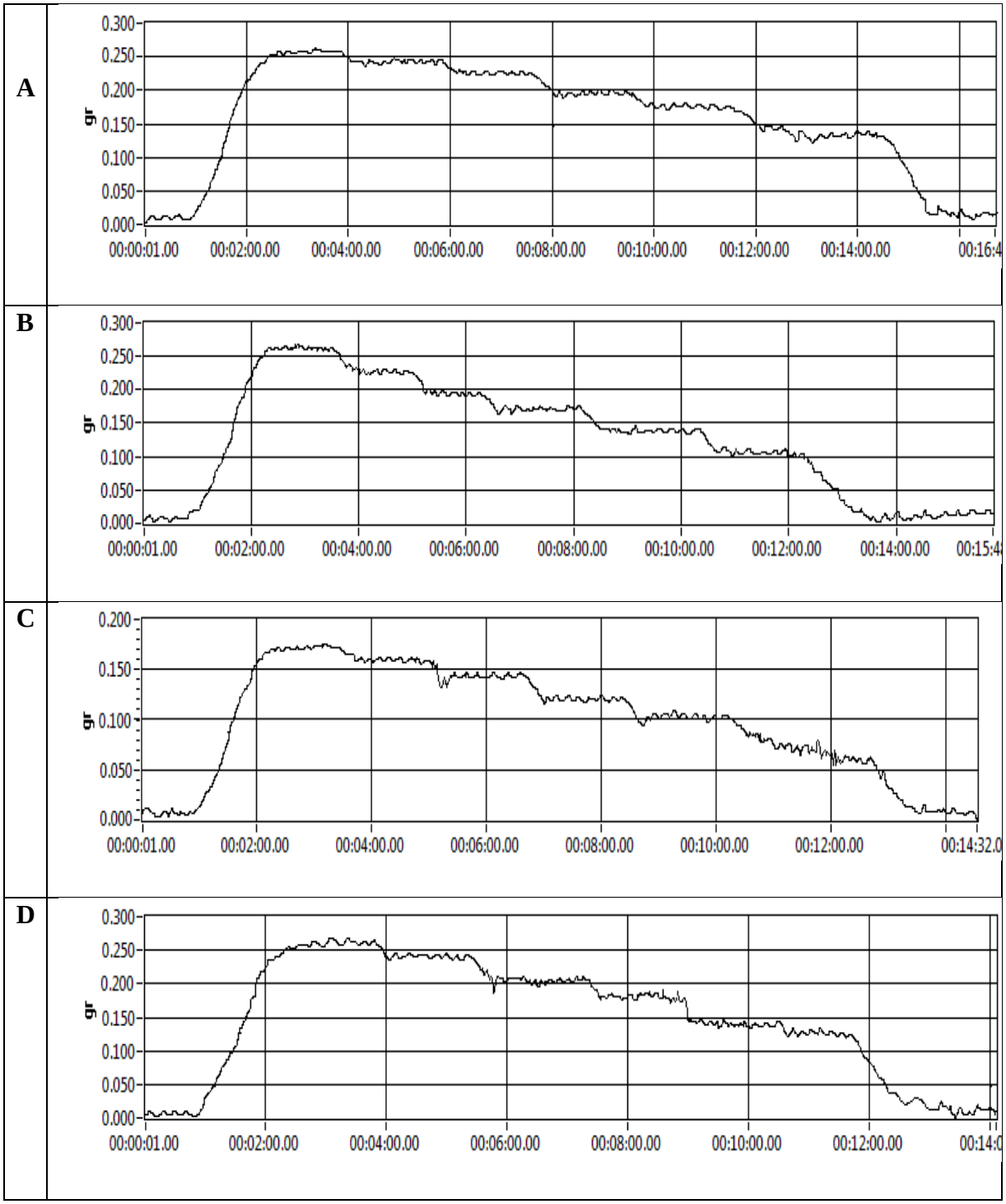
Appendix 1: Tracing of the corpus cavernosa smooth muscle response to phenylephrine when incubated in (A) Blank Kreb's Solution, (B) Vehicle, (C) *Ocimum gratissimum,* (D) Thymol.



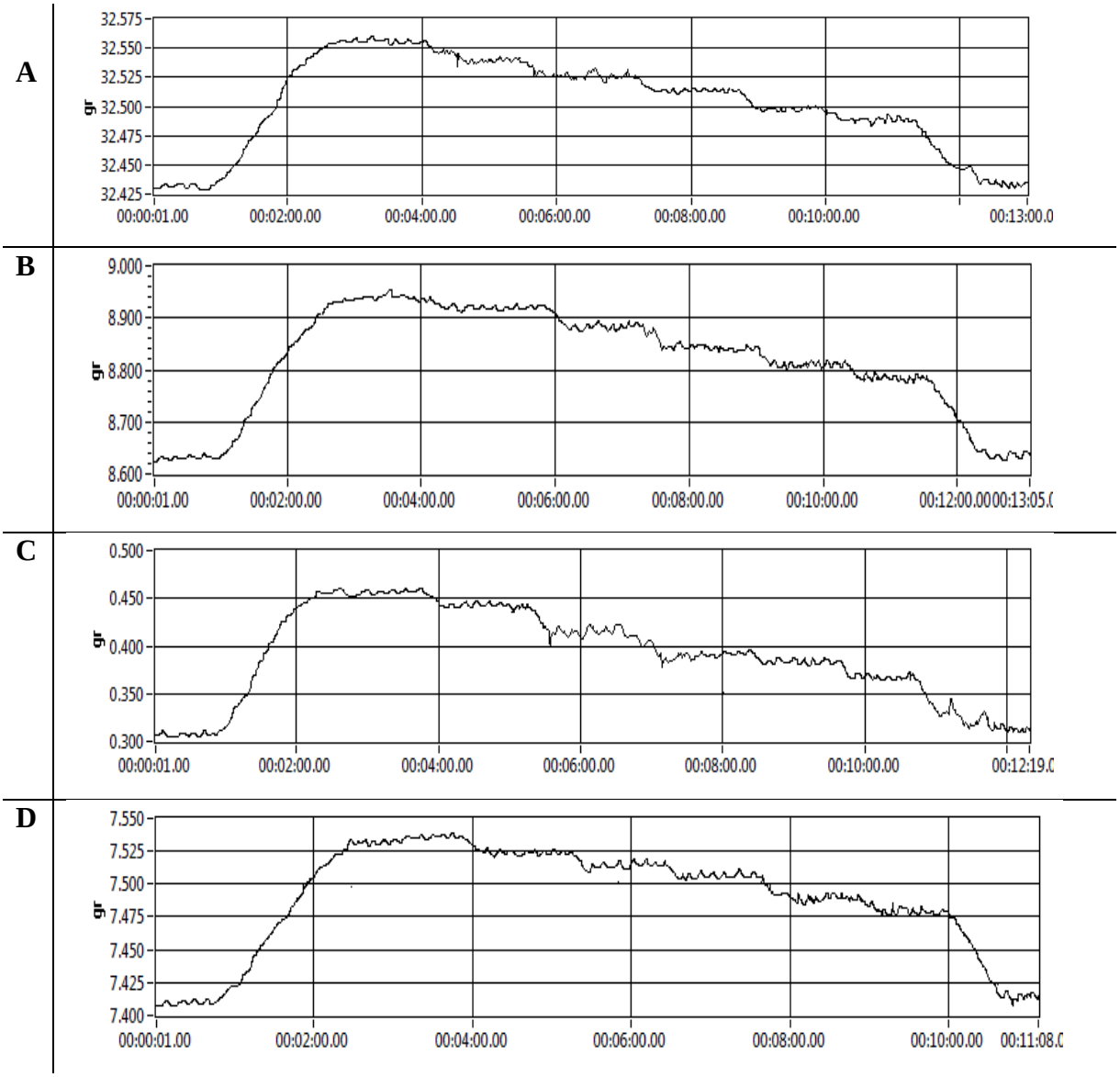
Appendix 2: Tracing of the Responses of corpus cavernosa smooth muscle to graded dose of potassium chloride (KCl) when incubated in (A) Blank Kreb's Solution, (B) Vehicle, (C) *Ocimum gratissimum*, (D) Thymol.



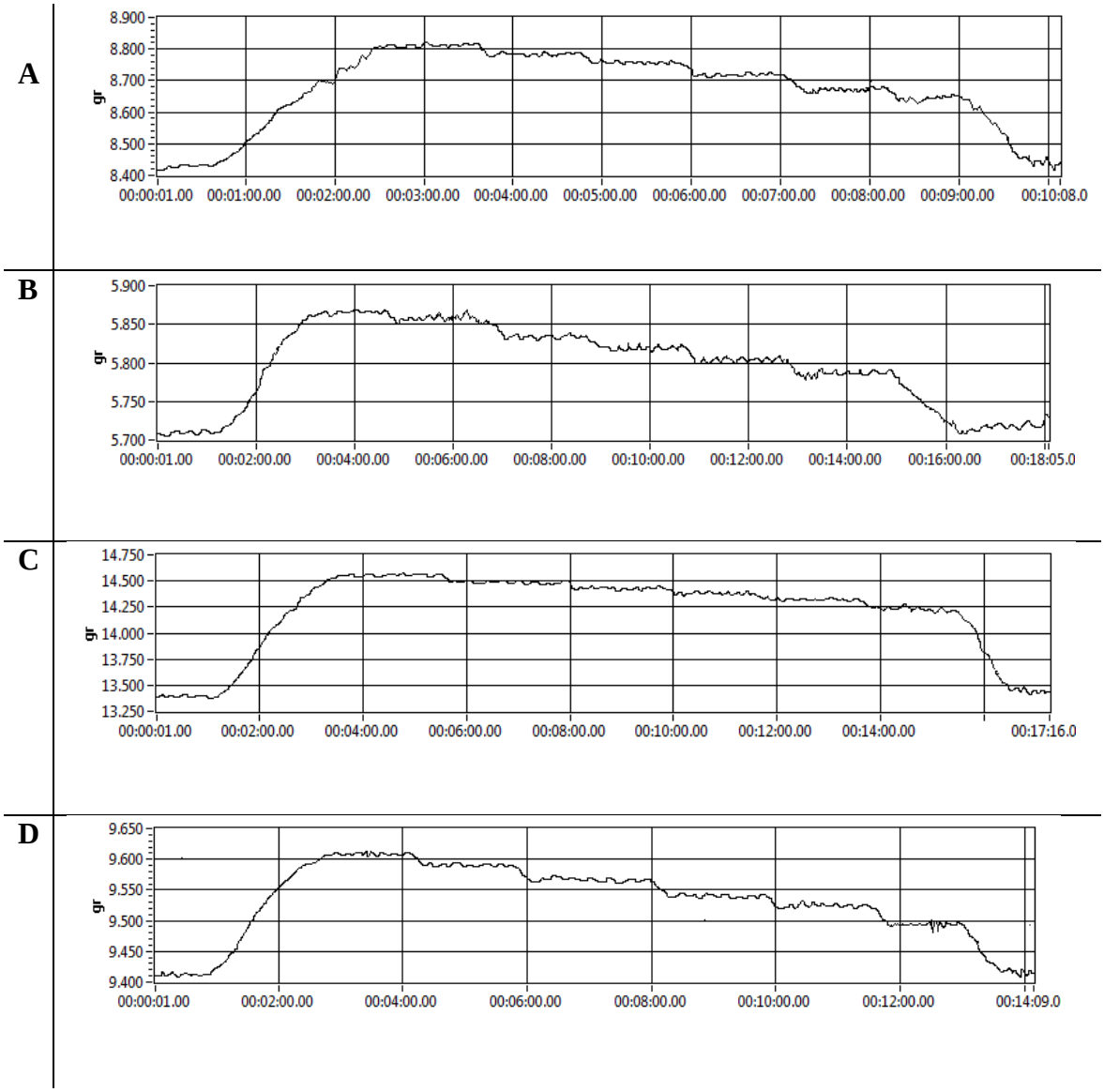
Appendix 3: Tracing of the responses of phenylephrine-precontracted corpus cavernosa smooth muscle to acetylcholine when incubated in (A) Blank Kreb's Solution, (B) Vehicle, (C) *Ocimum gratissimum,* (D) Thymol.



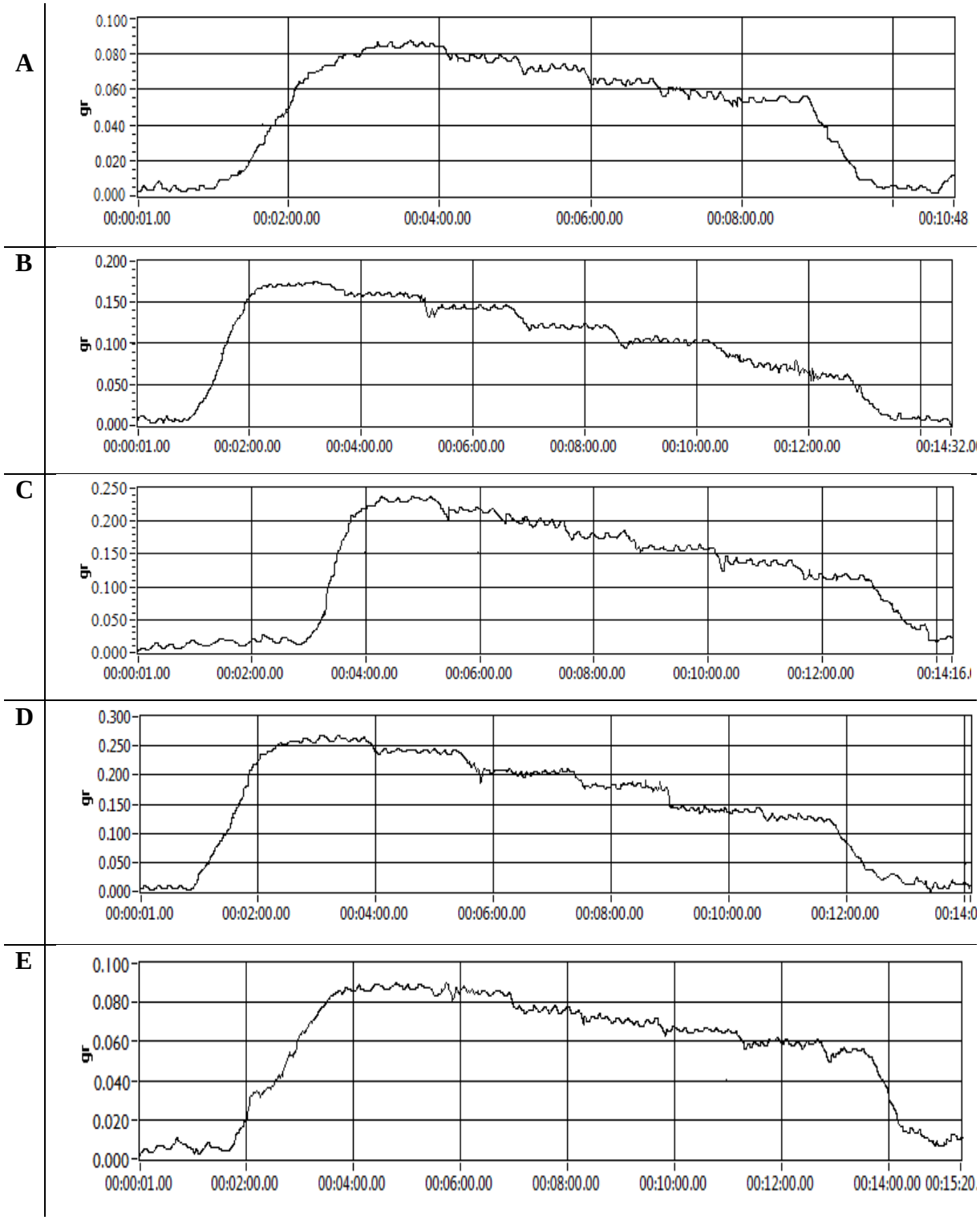
Appendix 4: Tracing of the responses of potassium chloride-precontracted corpus cavernosa smooth muscle to acetylcholine (Ach) when incubated in (A) Blank Kreb's Solution, (B) Vehicle, (C) *Ocimum gratissimum*, (D) Thymol.



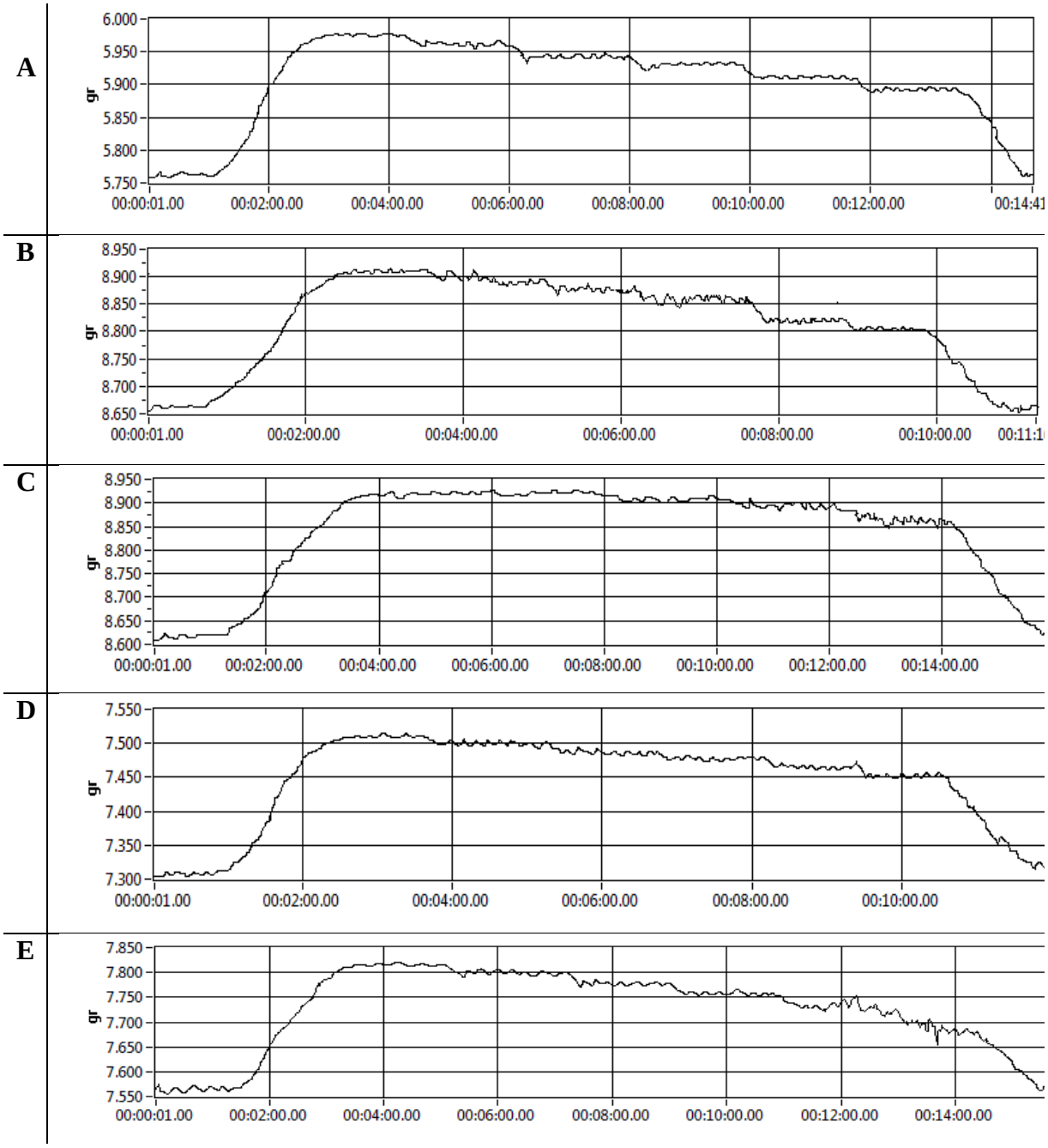
Appendix 5: Tracing of the responses of phenylephrine-precontracted corpus cavernosa smooth muscle to sodium nitroprusside when incubated in (A) Blank Kreb's Solution, (B) Vehicle, (C) *Ocimum gratissimum,* (D) Thymol.



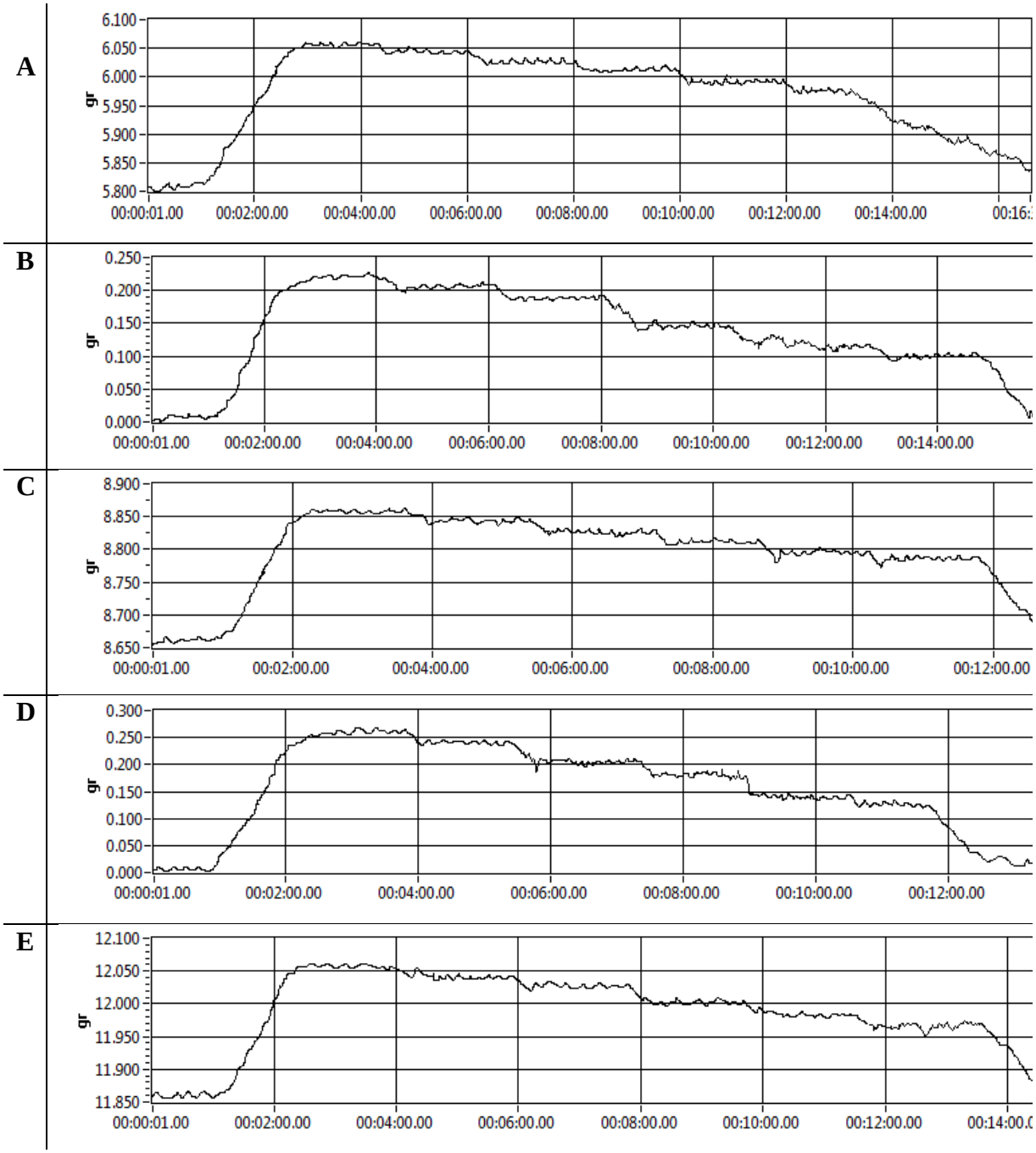
Appendix 6: tracing of the responses of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of sodium nitroprusside when incubated in (A) blank kreb's solution, (B) vehicle, (C) *Ocimum gratissimum*, (D) Thymol.



Appendix 7: Tracing of the responses of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in presence of nitric oxide when incubated in (A) L-NAME alone (B) OG alone (C) OG + L-NAME (D) Thymol alone (E) Thymol + L-NAME.

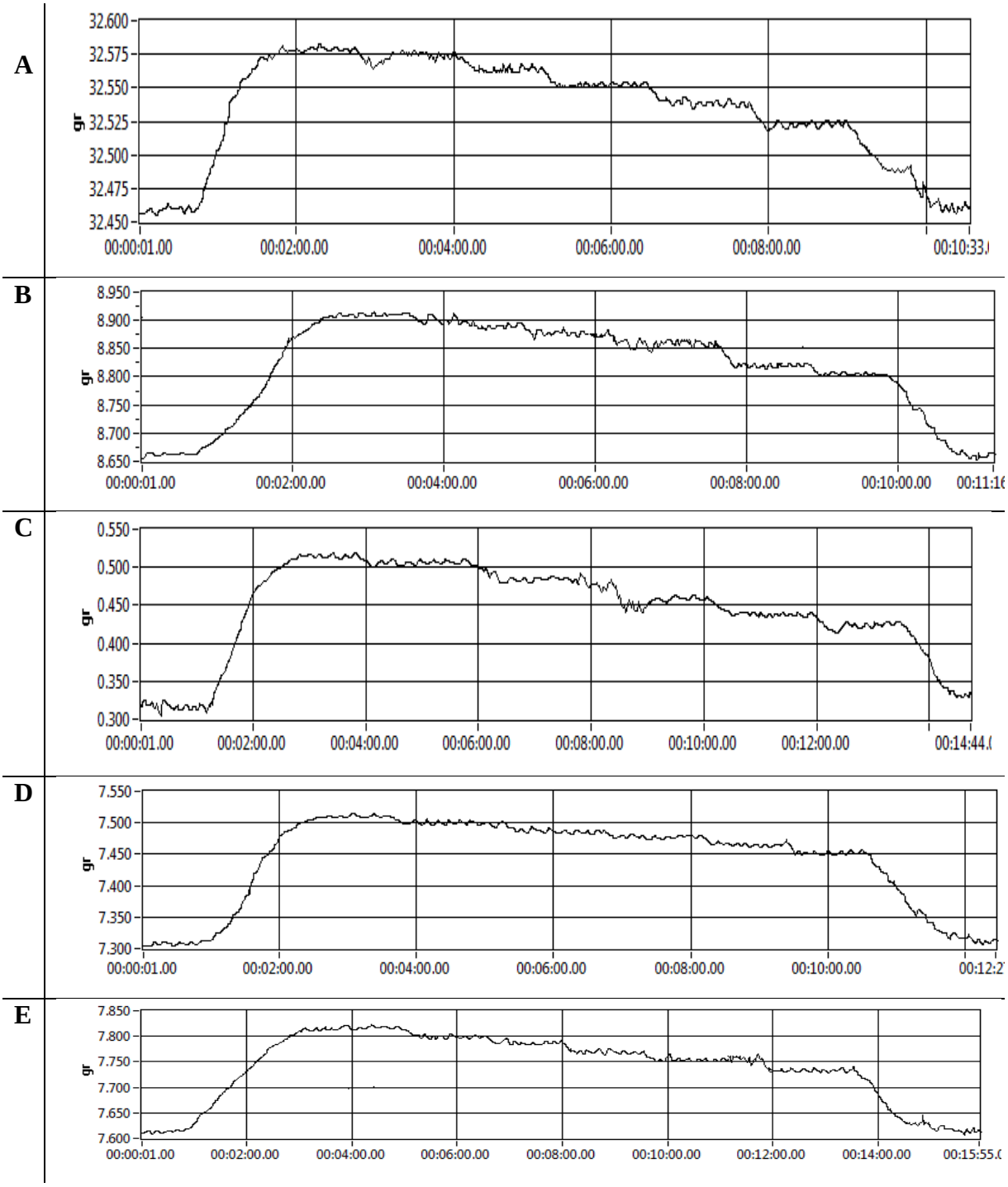


Appendix 8: Tracing of the responses of phenylephrine-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of prostaglandin inhibitor when incubated in (A) Indomethacin alone (B) OG alone (C) OG + Indomethacin (D) Thymol alone (E) Thymol + Indomethacin.



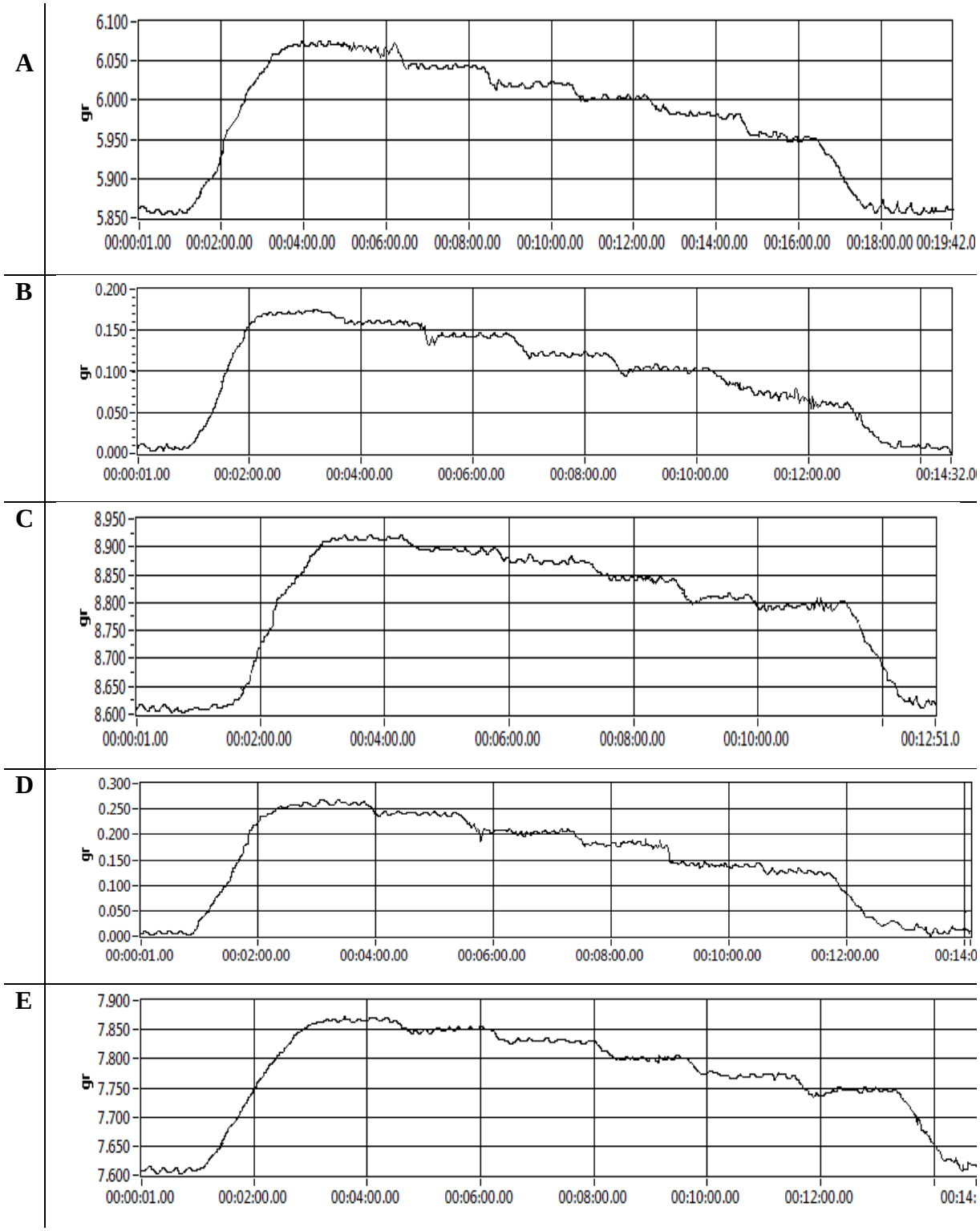
Appendix 9: Tracing of the responses of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine when incubated in (A) Indomethacin alone (B) OG alone (C) OG + Indomethacin (D) Thymol alone (E) Thymol + Indomethacin.

OG=*Ocimum gratissimum*

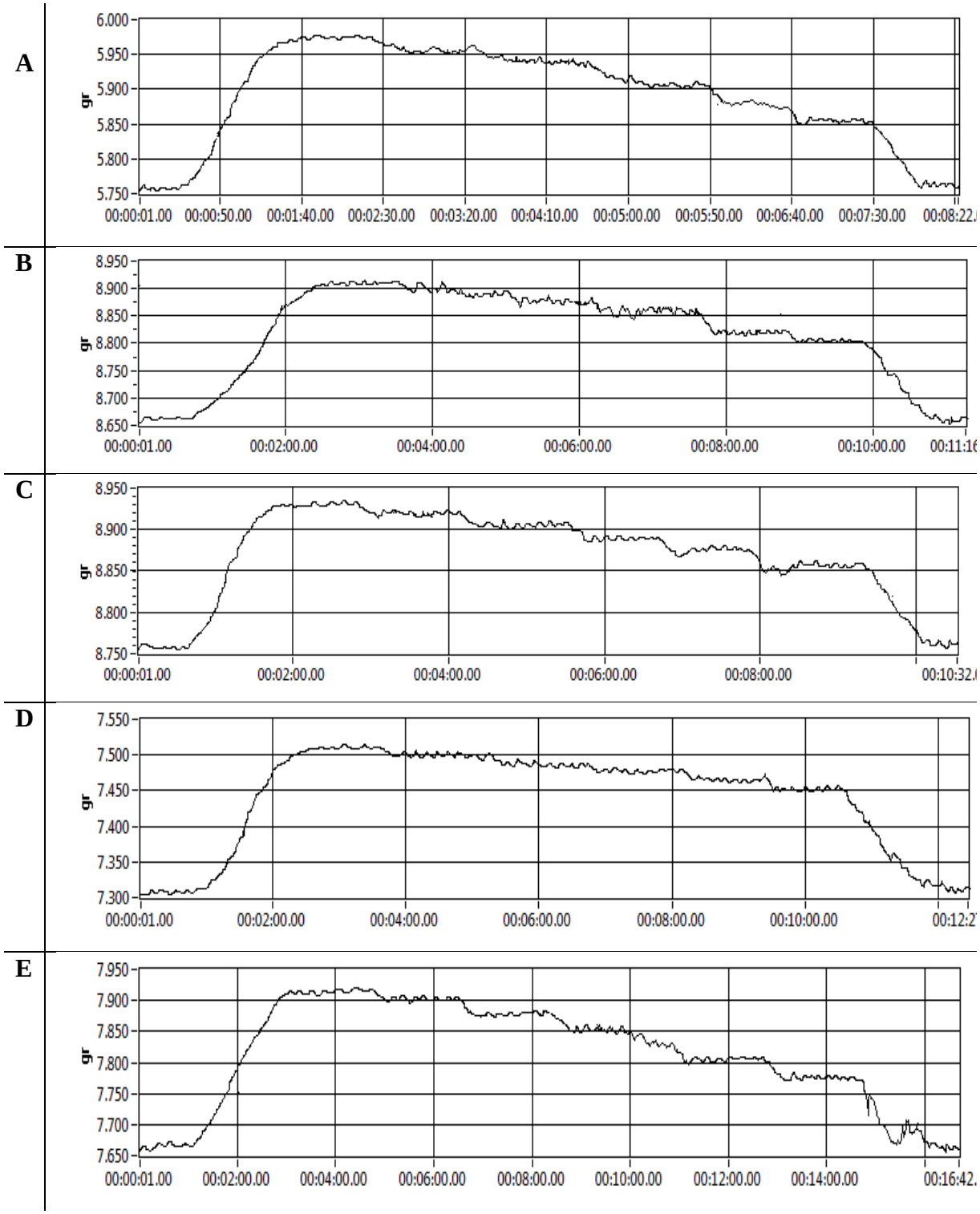


Appendix 10: Tracing of the responses of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of selective cGMP inhibitor when incubated in (A) ODQ alone (B) OG alone (C) OG + ODQ (D) Thymol alone (E) Thymol + ODQ.

OG= *Ocimum gratissimum*

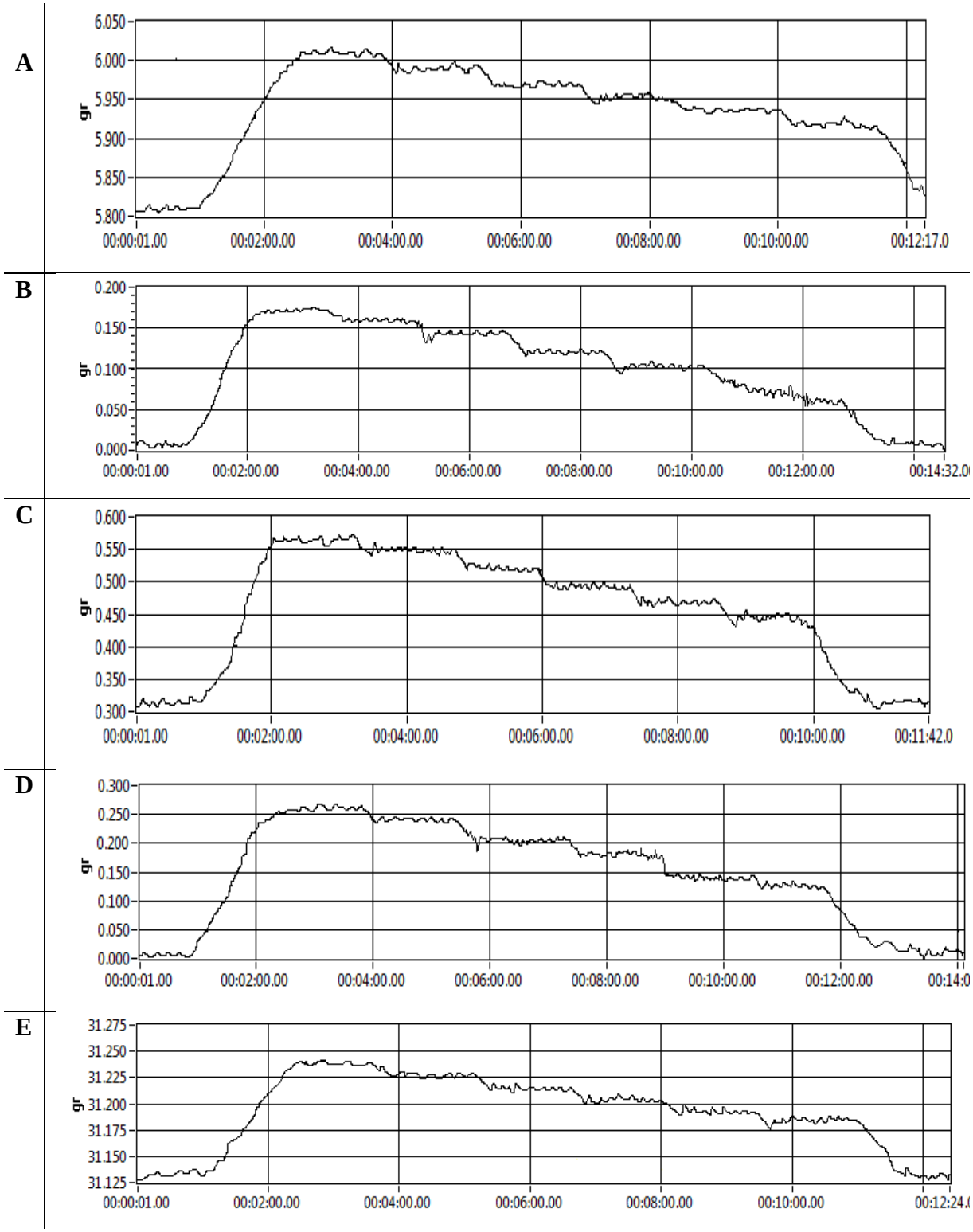


Appendix 11: Tracing of the responses of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of selective cGMP inhibitor when incubated in (A) ODQ alone (B) OG alone (C) OG + ODQ (D) Thymol alone (E) Thymol + ODQ.

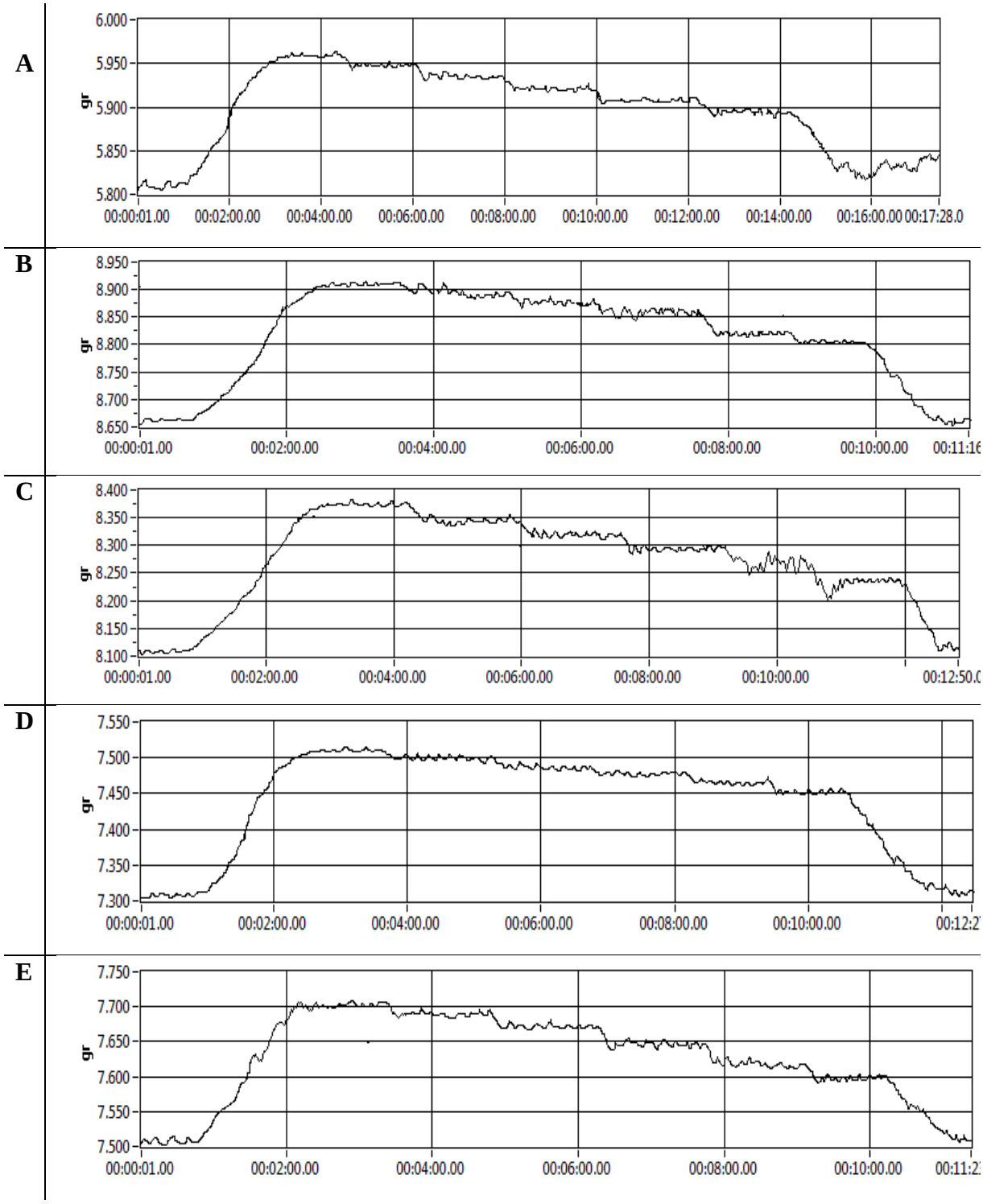


Appendix 12: Tracing of the responses of phenylephrine-precontracted corpus cavernosa smooth muscle to acetylcholine in the presence of non-selective cGMP Inhibitor when incubated in (A) MB alone (B) OG alone (C) OG + MB (D) Thymol alone (E) Thymol + MB.

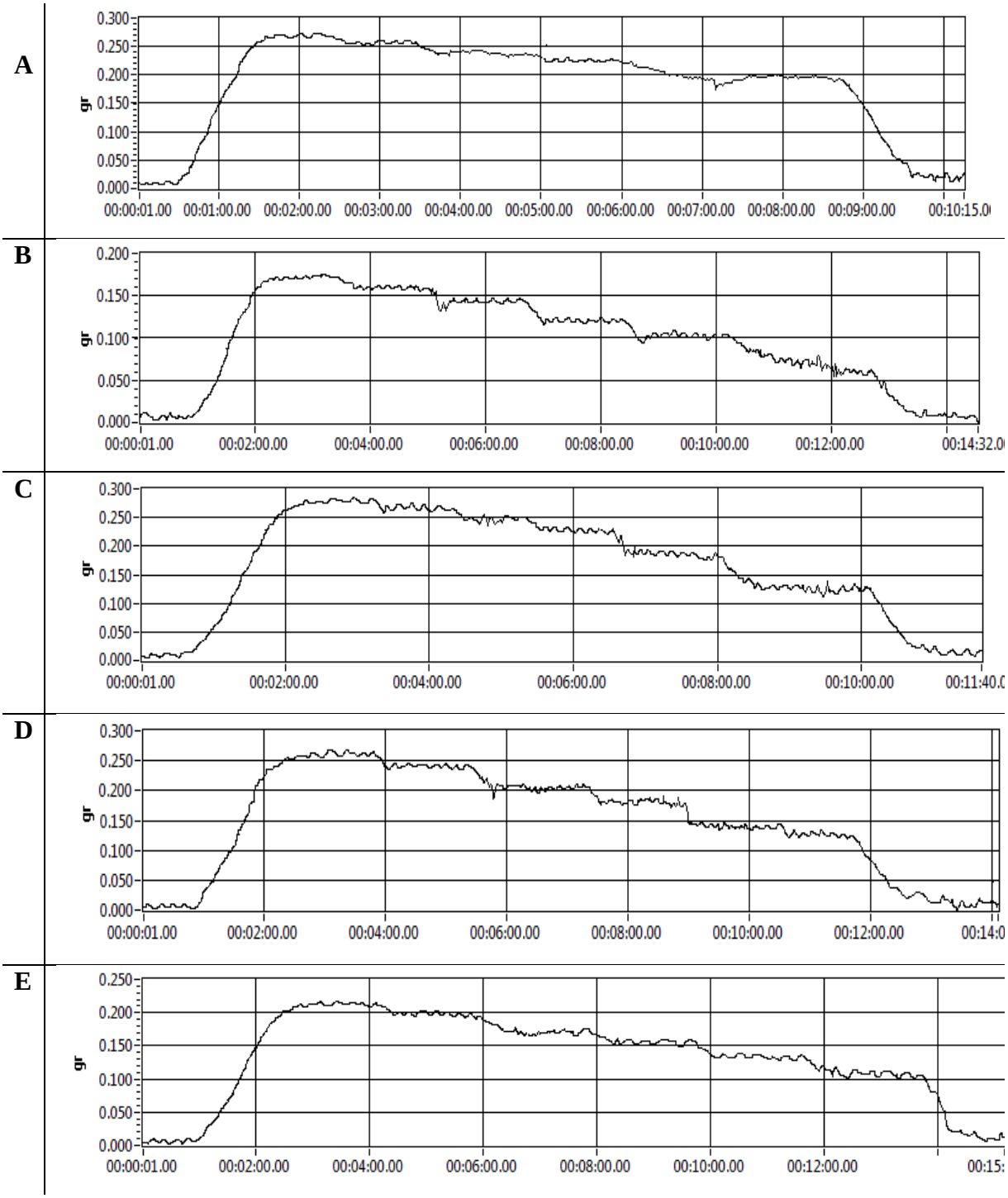
OG= *Ocimum gratissimum* MB= Methylene Blue



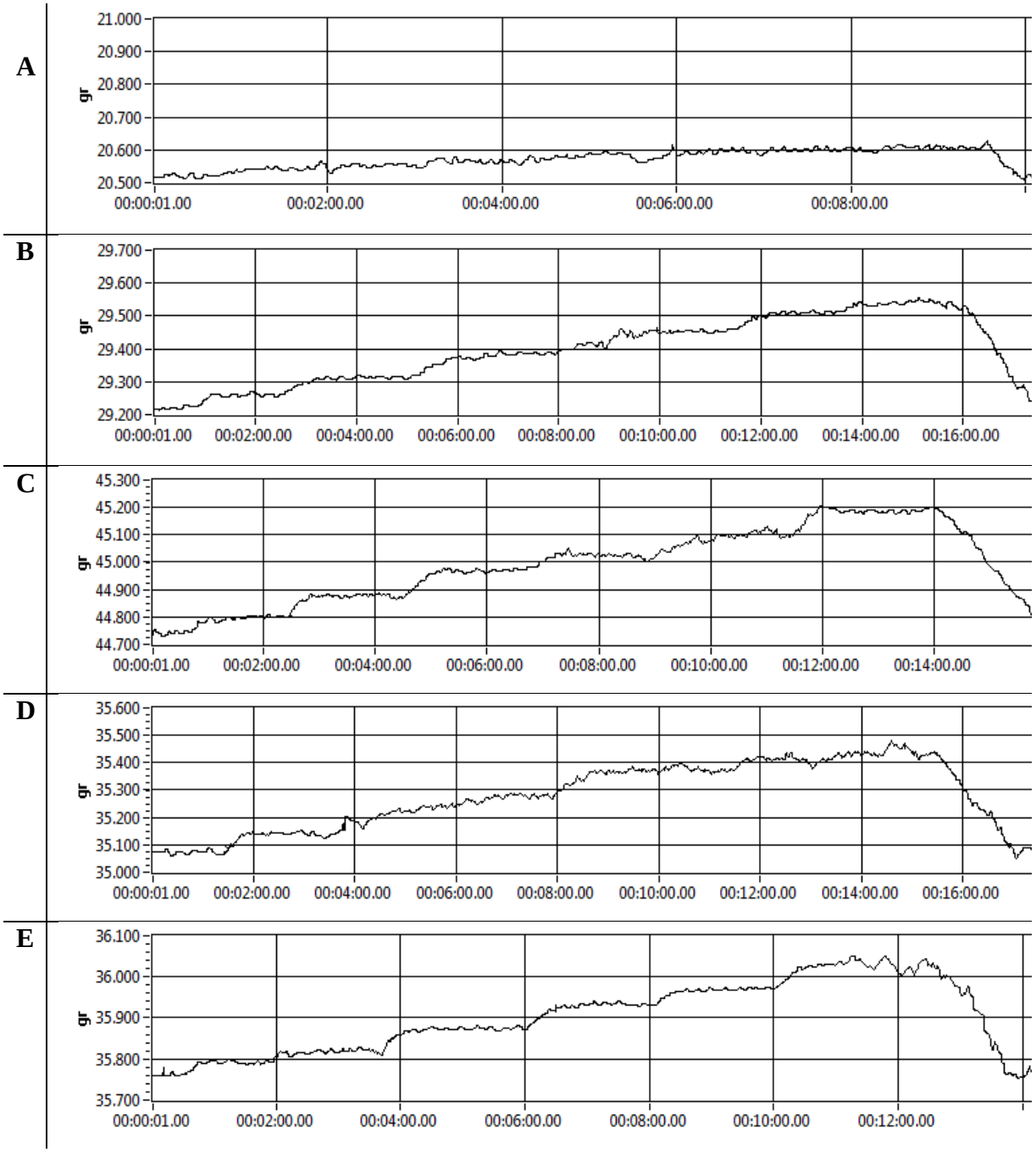
Appendix 13: Tracing of the responses of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of non-selective cGMP inhibitor when incubated in (A) MB alone (B) OG alone (C) OG + MB (D) Thymol alone (E) Thymol + MB. OG= *Ocimum gratissimum* MB= Methylene Blue



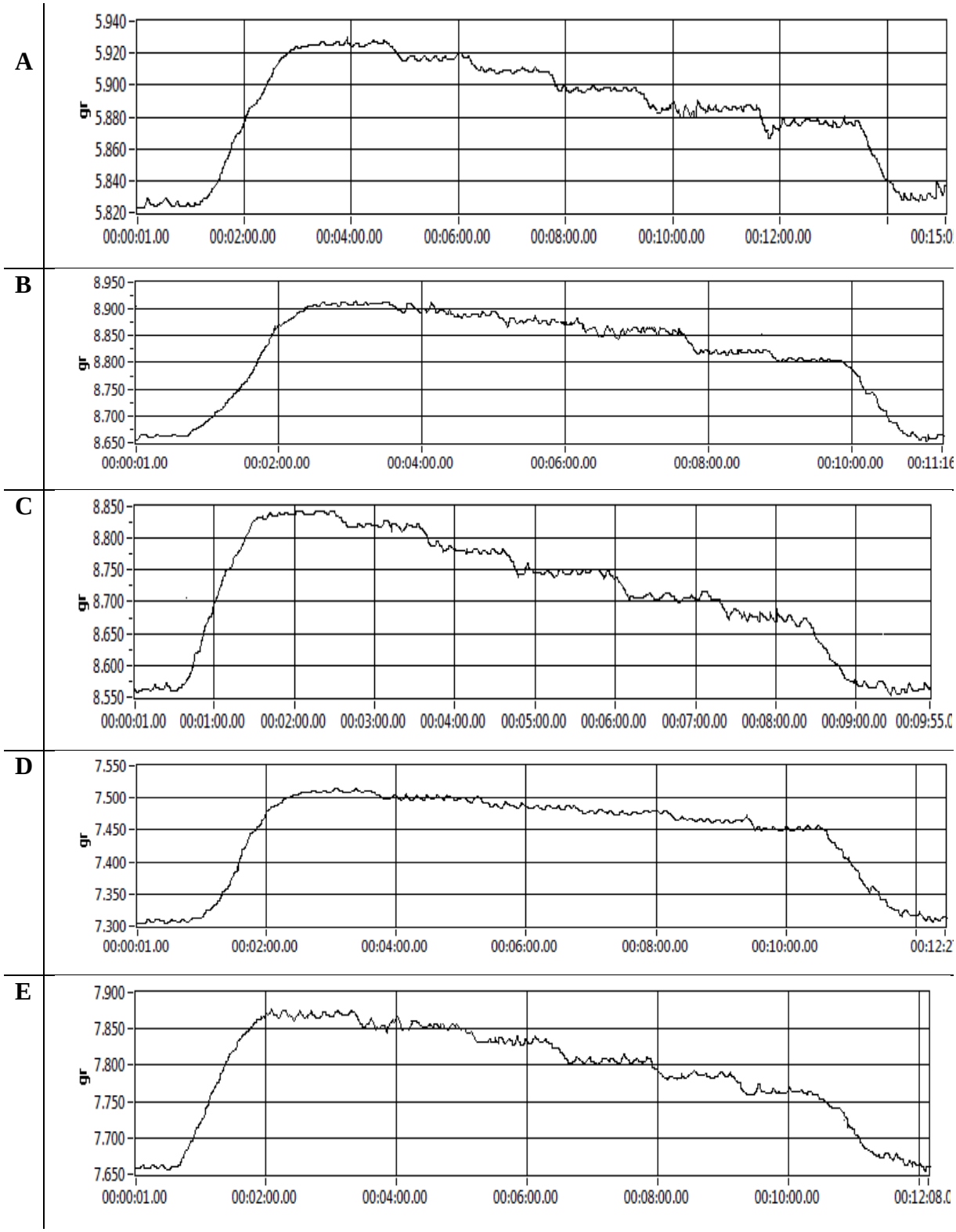
Appendix 14: Tracing of the responses of Phenylephrine-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of calcium channel blocker when incubated in (A) Nifedipine alone (B) OG alone (C) OG + Nifedipine (D) Thymol alone (E) Thymol + Nifedipine.



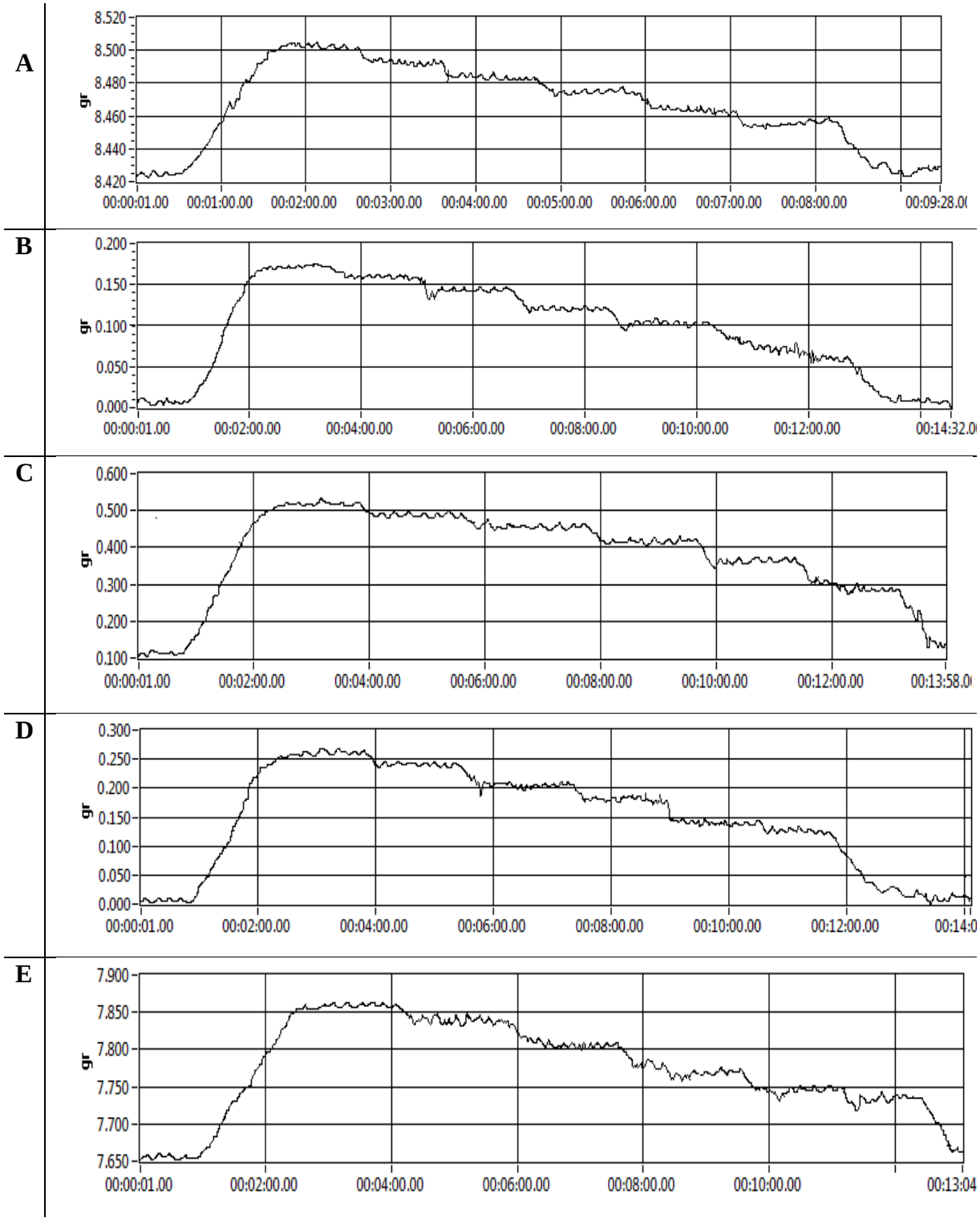
Appendix 15: Tracing of the responses of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine calcium channel blocker when incubated in (A) Nifedipine alone (B) OG alone (C) OG + Nifedipine (D) Thymol alone (E) Thymol + Nifedipine.



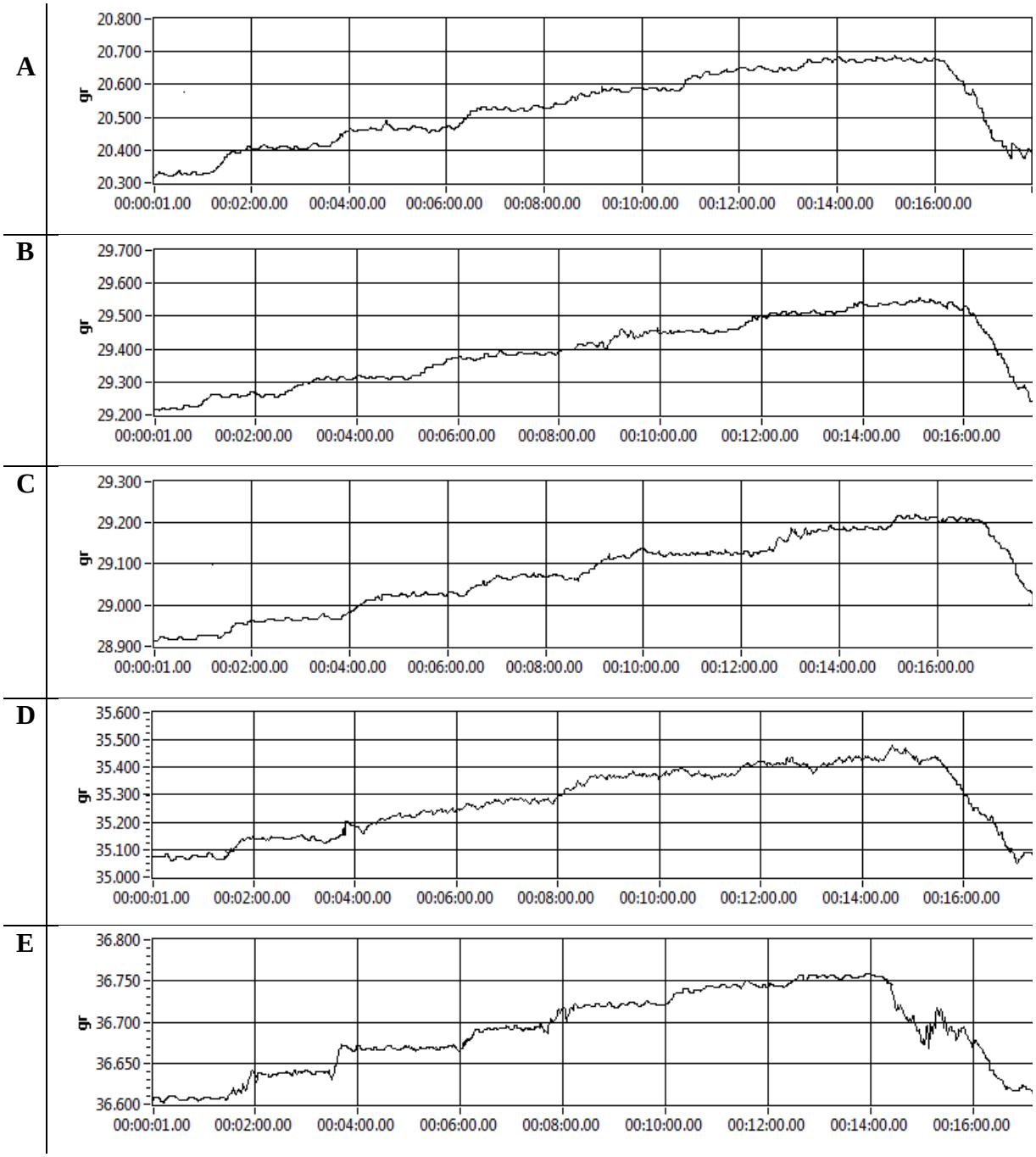
Appendix 16: Tracing of the responses of corpus cavernosa smooth muscle to calcium chloride in the presence of calcium channel blocker when incubated in calcium free medium with (A) Nifedipine (B) OG alone (C) OG + Nifedipine (D) Thymol alone (E) Thymol + Nifedipine.



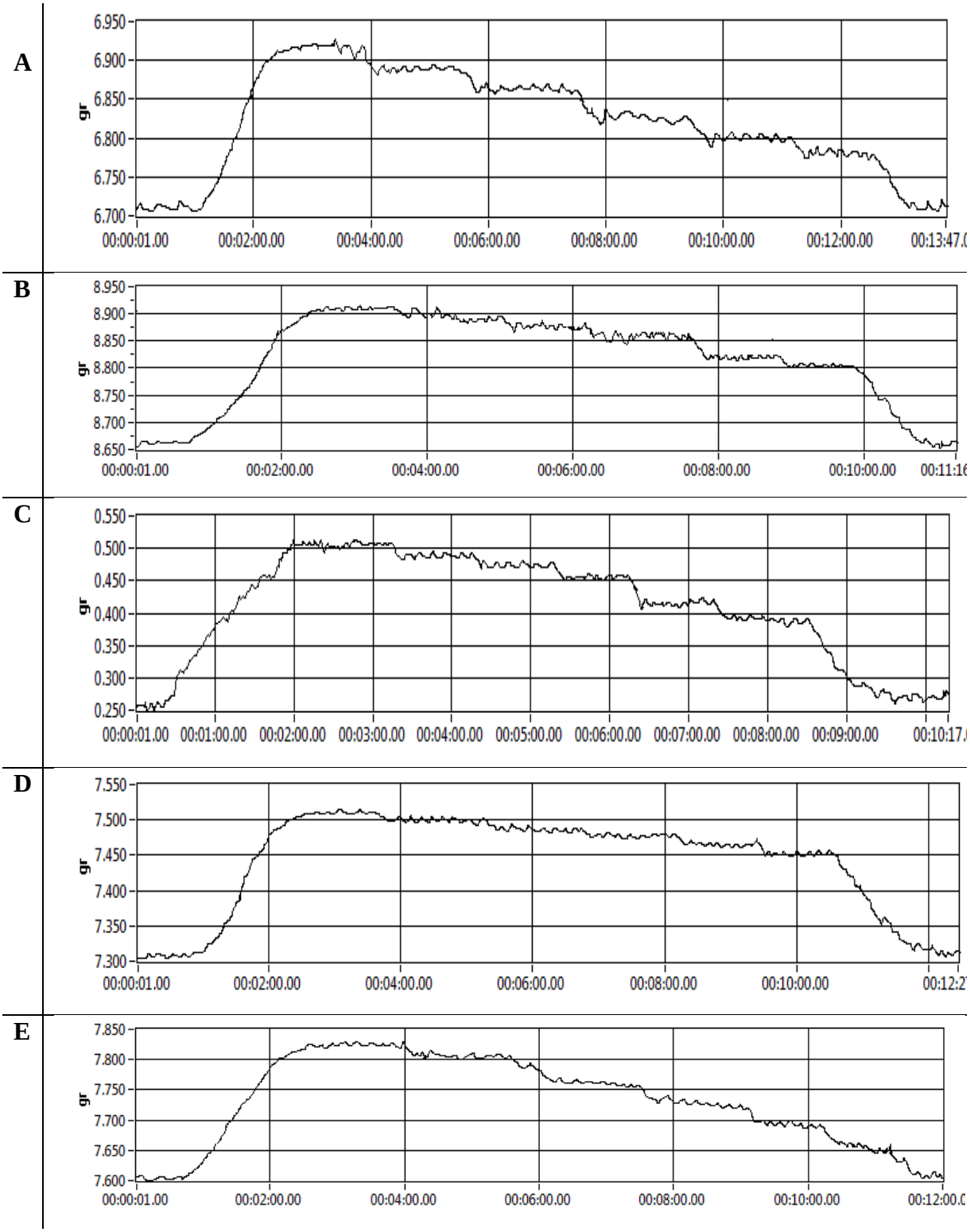
Appendix 17: Tracing of the responses of phenylephrine-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of myosin light chain kinase inhibitor when incubated (A) ML-7 alone (B) OG alone (C) OG + ML-7 (D) Thymol alone (E) Thymol + ML-7.



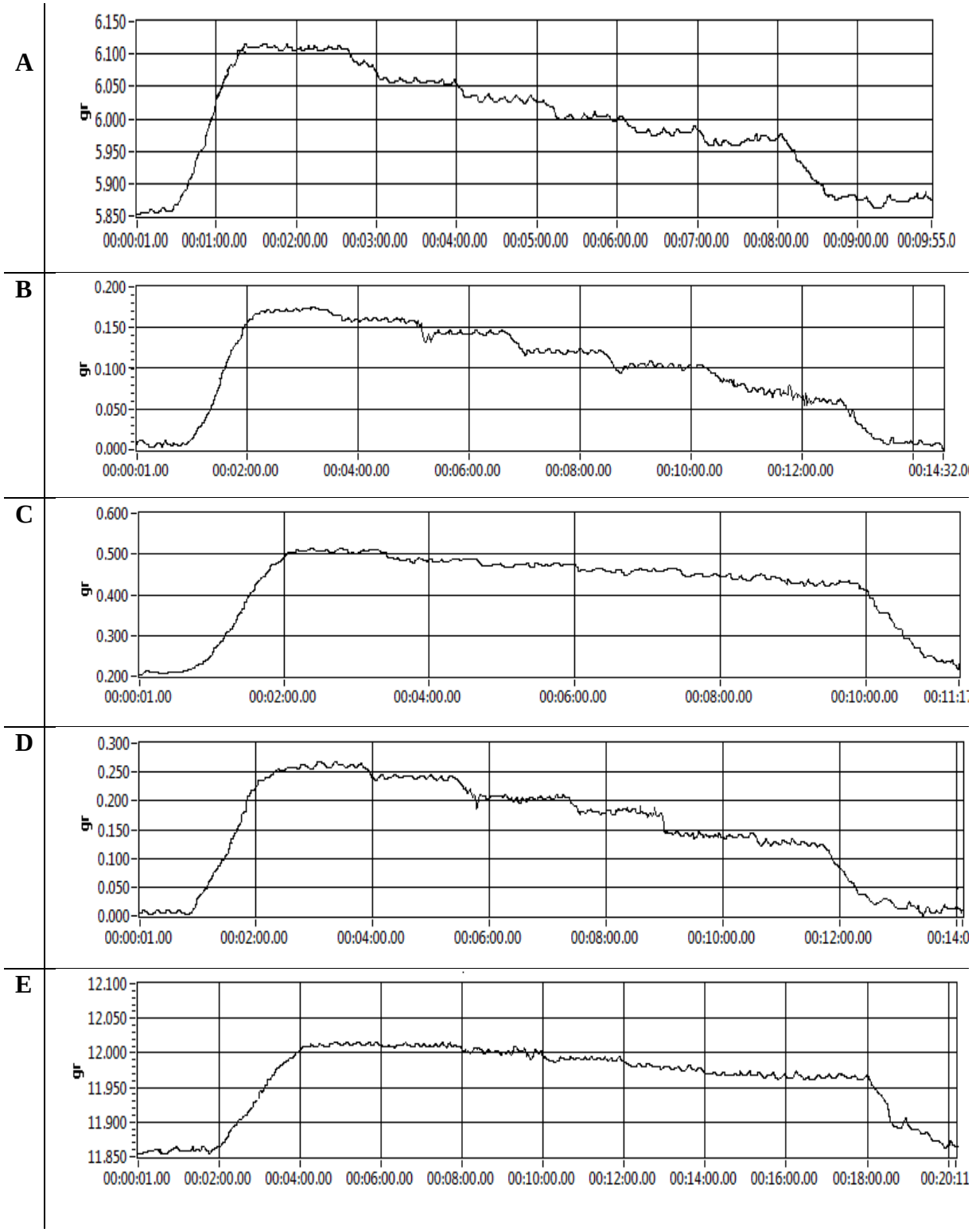
Appendix 18: Tracing of the responses of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of myosin light chain kinase inhibitor when incubated in (A) ML-7 alone (B) OG alone (C) OG + ML-7 (D) Thymol alone (E) Thymol + ML-7.



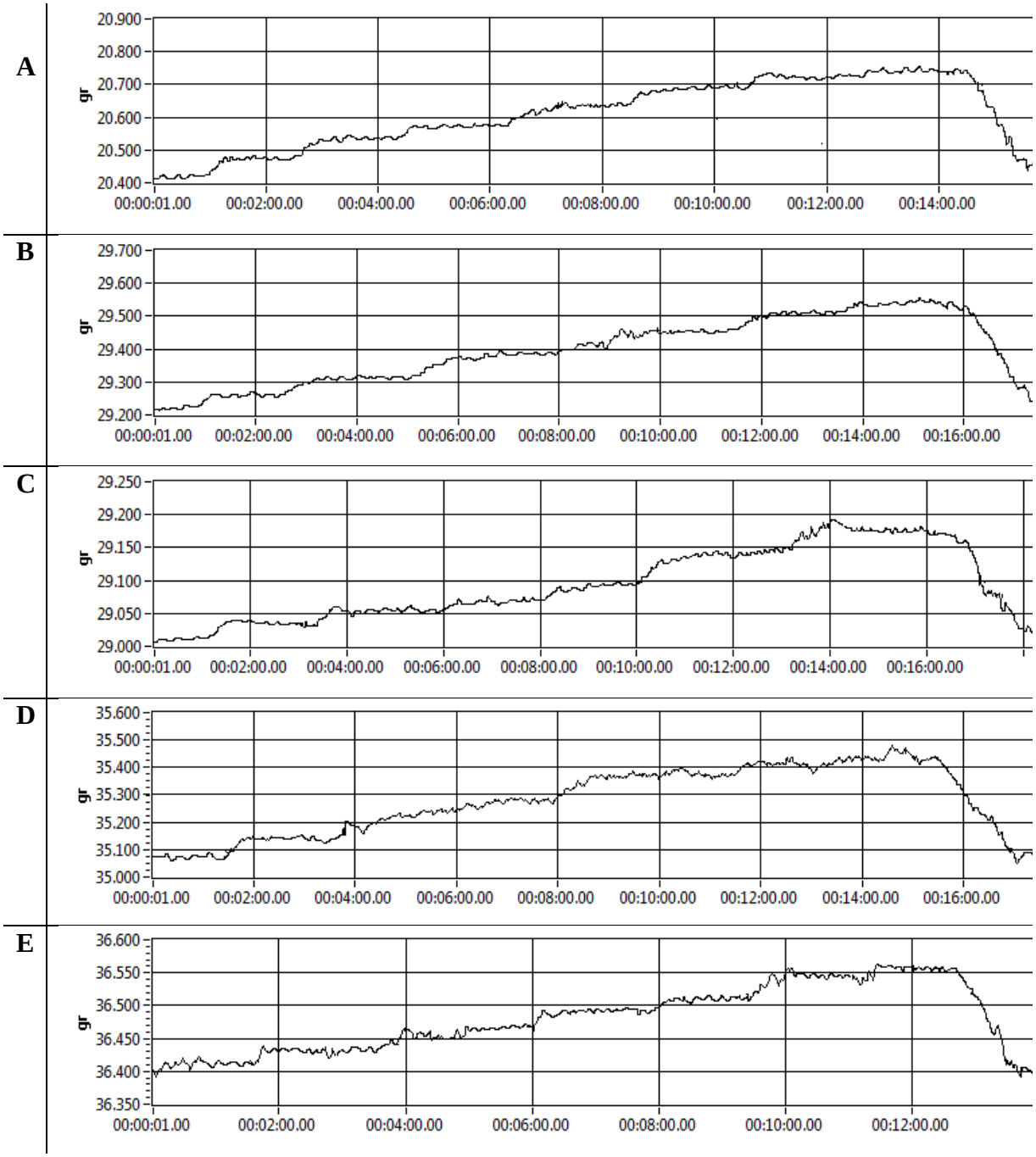
Appendix 19: Tracing of the responses of corpus cavernosa smooth muscle graded dose of calcium chloride in the presence of myosin light chain kinase inhibitor (A) ML-7 alone (B) OG alone (C) OG + ML-7 (D) Thymol alone (E) Thymol + ML-7.



Appendix 20: Tracing of the responses of phenylephrine-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of rho-kinase inhibitor when incubated in (A) Fasudil alone (B) OG alone (C) OG + Fasudil (D) Thymol alone (E) Thymol + Fasudil.



Appendix 21: Tracing of the responses of potassium chloride-precontracted corpus cavernosa smooth muscle to graded dose of acetylcholine in the presence of rho-kinase inhibitor when incubated in (A) Fasudil alone (B) OG alone (C) OG + Fasudil (D) Thymol alone (E) Thymol + Fasudil.



Appendix 22: Tracing of the responses of corpus cavernosa smooth muscle graded dose of calcium chloride in the presence of rho-kinase inhibitor in calcium free medium when incubated with (A) Fasudil alone (B) OG alone (C) OG + Fasudil (D) Thymol alone (E) Thymol + Fasudil.