INVESTIGATION OF THE IN-VIVO AND IN-VITRO ANTIDIARRHEAL ACTIVITY OF THE FREEZE-DRIED EXTRACTS OF *Tylosema fassoglense (Schweinf.)* Torre & Hillc. IN SELECTED ANIMAL MODELS OF DIARRHEA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT FOR THE AWARD OF DEGREE IN MASTERS OF SCIENCE IN MEDICAL PHYSIOLOGY OF THE UNIVERSITY OF NAIROBI.

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DECLARATION

I hereby declare that this thesis is my original work and to the best of my knowledge has not been presented elsewhere for approval and for the award of a degree, diploma, or certificate.

I further declare that all material cited in this thesis that is not my own work has been duly referenced.

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LIST OF ABBREVIATIONS AND ACRONYMS

CAMP-Cyclic adenine monophosphate

CFTR-cystic fibrosis transmembrane conductive regulator channels

CGMP-cyclic guanosine monophosphate

CMC-Carboxymethyl cellulose

CT-cholera toxin

EDTA- Ethylenediaminetetraacetic acid

EEC-enteroendocrine cells.

GIT-Gastrointestinal tract.

HIV-Human immunodeficiency virus

IPANs-intrinsic primary afferent neurons.

LC-MS-liquid chromatography-mass spectrometry.

L-NAME- L-Nitro-Arginine Methyl Ester

NHEs-sodium-proton exchanger

NO-nitrous oxide.

SCFAs- short-chain fatty acids.

SGC- Soluble guanylyl cyclase

STIM1-Stromal interactional molecule 1.

TFG-*Tylosema fassoglensis*

TGR5-Tekeda G-protein receptor 5

VIP-vasoactive intestinal peptide.

ICC-intestinal cell of Cajal

ABSTRACT

Diarrhea is a gastrointestinal disorder characterized by an increase in the frequency of bowel movements, liquidity of stool, and amount of stool with a high mortality rate, especially among under-five children. Current treatment approaches are etiology-based limited by financial constraints and side effects. This study investigated the antidiarrheal activities of the freeze-dried extracts of *Tylosema fassoglense* (Schweinf.) Torre & Hillc. (TFG) tubers in the selected animal models of diarrhea.

Tylosema fassoglense tubers extract were prepared by lyophilization and their antidiarrheal activity evaluated in both in-vivo and ex-vivo experimental models of diarrhea. The antidiarrheal effects of TFG were evaluated in the castor oil-induced diarrhea model. Twenty-five adult Sprague-Dawley rats (n=25) were randomized into the negative control (normal saline), low dose (200 mg/kg), medium dose (400 mg/kg), high dose (800 mg/kg), and positive control (5 mg/kg Loperamide) groups. The fecal diarrheic mass and count produced by experimental animals for the first four hours one after the administration of one ml of castor oil (administered to the respective groups one hour after administration of respective treatments by oral gavage) was determined.

The effects on luminal secretion were evaluated in the castor oil-induced enteropooling test. Twenty adult Sprague-Dawley rats were randomized into the negative control (normal saline), medium-dose test (400 mg/kg), high dose test (800 mg/kg), and positive control (5 mg/kg Loperamide) groups administering one ml of castor oil by oral gavage one hour after respective treatment following a 24-hours starvation period with ad libitum access to water. The respective weights of luminal content of the small intestine were determined thirty minutes after castor oil administration and recorded.

The effect of extracts on gastrointestinal motility was determined in Phenol red test. Twenty adult Sprague-Dawley rats (n=20) were randomized into the negative control (normal saline), medium-dose test (400 mg/kg), high dose test (800 mg/kg), and positive control (5 mg/kg Loperamide) groups. Neostigmine (0.05 mg/kg i.p.) was administered to each experimental animal thirty minutes after administration of the respective treatments. Phenol red meal (0.5 mg of phenol red dye per ml of 1.5% CMC) was then administered by oral gavage to each rat at a dose of (10 ml/kg) twenty minutes after administration of neostigmine. The amount of phenol red dye retained in the stomach after administration was assayed and determined by spectrophotometry.

The effects of different concentrations of TFG (0.5-3.5 mg/ml) on the spontaneous contraction of ten-centimeter jejunal segments isolated from adult New Zealand White Rabbits (n=15) as well as in the presence of 10^{-5} M acetylcholine, 80 mM KCl, and $CaCl_2$ (0.00003-0.03 M), prazosin, propranolol, methylene blue, L-NAME, and naloxone were evaluated.

The experimental data were expressed as Mean ± SEM and analyzed using, one-way ANOVA and Turkey's posthoc test in cases of significance which was set at $p \leq 0.05$ using GraphPad Prism™ suite of statistical software.

The TFG extract reduced the fecal mass and count in the castor oil-induced diarrhea ($p<0.05$). The extract also reduced luminal secretion in the enteropooling assay test ($p<0.05$). The extract also reduced both gastric emptying and peristaltic rate ($p<0.05$) possibly via the antagonistic effect on muscarinic receptors. The extracts dose-dependently diminished the spontaneous, acetylcholine,

KCl (80 mM), and $CaCl_2$ induced contractions. The spontaneous spasmolytic effects were significantly attenuated by naloxone, methylene blue, and L-NAME but not by adrenergic blockers.

The TFG extracts possess significant antidiarrheal activities that are possibly mediated via modulation of nitrous oxide pathway, voltage-gated calcium channels, muscarinic and opioid receptors. These findings, therefore, validate the traditional use of this plant as an antidiarrheal.

CHAPTER ONE: INTRODUCTION

1.1 Background

Diarrhea is a gastrointestinal disorder characterized by an increase in the frequency of bowel movements (≥ 3 times/day) (Levine et al., 2017), liquidity of stool (Shane et al., 2017), and amount of stool (≥ 200 gm/day) (Eads et al., 2020). Diarrhea accounts for 1 in 9 deaths of children under the age of 5 globally (Centers for Disease Control and Prevention, 2015). The death rate is eleven times higher for children with comorbidities such as HIV (Acácio et al., 2021).

The Global South bears an inordinately huge burden of diarrheal diseases largely attributable to infectious diseases such as cholera (Kotloff et al., 2017). Untreated, diarrhea leads to decreased brush boarder function, microbial dysbiosis which worsens malnutrition and increase risk of severe diarrhea in a vicious cycle (Vilander et al., 2022), electrolyte and acid-base imbalance where there is loss of minerals such as sodium as well as organ damage such as pre-renal kidney injury due to loss of sodium and water that contribute to needed renal blood flow (Seifter and Chang, 2017), all of which lower the quality of life, prolong hospital stays, and raise healthcare costs (Kotloff et al., 2017).

The current therapeutic approaches in diarrhea attempt to deal with the primary cause, that is, the use of antibiotics in bacterial infections, bile acid sequestrant for patients with bile salts malabsorption (Camilleri and Vijayvargiya, 2020), fecal microbiota transplant for patients with gut microbiota dysbiosis (Niccum et al., 2018), and rotavirus vaccines for children under the age of 5 years who are susceptible to rotavirus infection (Dennehy, 2008). These therapeutics agents are expensive There has been an increase in the adoption of alternative medicine for the management of diarrheal diseases (Patel et al., 2013). The present study aimed to investigate the

efficacy of the freeze-dried extracts of *Tylosema fassoglense*, whose tubers are traditionally used in the management of diarrhea in Western Kenya (Maundu et al., 1999)

1.2 Problem Statement and Study Justification

There are approximately 2 billion global cases of diarrhea each year with 1.9 million children under the age of 5 years dying from diarrheal diseases (World Health Organization, 2017). Developing countries in the Global South account for 78% of diarrhea-related deaths (World Health Organization, 2017). Kenya is ranked tenth in Africa in terms of the burden of diarrheal diseases and associated mortality (Mulatya and Ochieng, 2020). This picture has not changed much in the last decade despite ramped-up broad-spectrum interventional measures (World Health Organization, 2017). In Global South countries, financial constrain has led the adoption of alternative antidiarrheal therapies owing to their availability, accessibility, and affordability. There is a need to establish the efficacy of these remedies and help in their judicious utilization (George, 2011). *Tylosema fassoglense* is a natural herbal remedy that has been used in the treatment of diarrhea in Western Kenya. The safety, efficacy, and mechanism of action of *Tylosema fassoglense* have not been established.

1.3 Hypothesis

1.3.1 Null Hypothesis

Freeze-dried extracts of *Tylosema fassoglense* do not possess significant antidiarrheal activity.

1.3.2 Alternative Hypothesis

Freeze-dried extracts of *Tylosema fassoglense* possess significant antidiarrheal activity.

1.4 Objectives

1.4.1 Broad Objective

The broad objective of this study was to investigate the antidiarrheal activities of the freeze-dried extracts of *Tylosema fassoglense* in animal models of diarrhea.

1.4.2 Specific Objectives

The specific objectives of this study were:

1. To determine the antidiarrheal activities of the freeze-dried extracts of *Tylosema fassoglense* (Schweinf.) Torre & Hillc by measuring the fecal mass, diarrheic fecal count, enteropooling assay mass, gastric emptying rates, and peristaltic indices in the castor oil and neostigmine-induced diarrhea rodent models.
2. To evaluate the *ex-vivo* antidiarrheal activities of the freeze-dried extracts of *Tylosema fassoglense* (Schweinf.) Torre & Hillc by comparing extract's effect on pre and post-treatment measurements of the jejunal force of contraction on spontaneous as well as in acetylcholine, high potassium chloride, and calcium chloride-induced contraction of isolated jejunal segments of New Zealand white rabbits.
3. To investigate the possible spasmolytic mechanisms of action of the freeze-dried extracts of *Tylosema fassoglense* (Schweinf.) Torre & Hillc by comparing extract's effect on pre and post-treatment (with prazosin, propranolol, methylene blue, L-NAME, and naloxone)

measurements of the force of contractions of jejunal segments isolated from New Zealand white rabbits.

1.5 Research questions

1. What are the *in-vivo* antidiarrheal activities of the freeze-dried extracts of *Tylosema fassoglense* (Schweinf.) Torre & Hillc by measuring the fecal mass, diarrheic fecal count, enteropooling assay mass, gastric emptying rates, and peristaltic indices in the castor oil and neostigmine-induced diarrhea rodent models?
2. What are the *ex-vivo* antidiarrheal activities of the freeze-dried extracts of *Tylosema fassoglense* (Schweinf.) Torre & Hillc by comparing extract's effect on pre and post-treatment measurements of jejunal force on spontaneous as well as in acetylcholine, high potassium chloride, and calcium chloride-induced contraction of isolated jejunal segments of New Zealand white rabbits?
3. What are the possible spasmolytic mechanisms of action of the freeze-dried extracts of *Tylosema fassoglense* (Schweinf.) Torre & Hillc by comparing extract's effect on pre and post-treatment (with prazosin, propranolol, methylene blue, L-NAME, and naloxone) measurements of the force of contractions of jejunal segments isolated from New Zealand white rabbits?

CHAPTER TWO: LITERATURE REVIEW

2.1 *Tylosema fassoglense* (Schweinf.) Torre & Hillc

Tylosema fassoglense (Schweinf.) Torre & Hillc. is a perennial non-climbing shrub belonging to the Fabaceae family (Chingwaru et al., 2015). It grows in the tropical climates of Eastern and Central Africa (Quattrocchi, 2016). The species is leguminous and has underground tubers, axillary forked tendrils, and alternate heart-shaped bilobed leaves. It bears pods that are broad, flat, and leathery (Quattrocchi, 2016). An image of Tylosema fassoglense is shown in Figure 2.1 below.

Figure 2.1: The image of *Tylosema fassoglense* (Schweinf.) Torre & Hillc

2.1.1 Ethnobotanical Profile

Tylosema fassoglense, known as *imbasa* among Luhya community, has been traditionally used in the management of several conditions. The tubers are used in management of diarrhea, anemia, fever, and asthma. Solution of powdered flowers is used to treat hypertension and jaundice. The roots and flower decoctions are used to treat impotence and as an analgesia of postpartum uterine pain (Quattrocchi, 2016). The tubers of this plant have a dietary value in some communities in

semi-arid areas (Dubois et al., 1995). In traditional veterinary medicine, root decoctions are given to cows as galactagogue before calving. The leaves are use as paralytic agent for fishing and as insecticide (Quattrocchi, 2016)..

2.1.2 Phytochemistry

Phytochemical studies on *Tylosema fassoglense* extracts have demonstrated the presence of fatty acids such as linoleic, oleic, and palmitic acids (Dubois et al., 1995). It has amino acids such as tyrosine, lysine, and proline (Dubois et al., 1995) as well as phytates and trypsin inhibitors (Dubois et al., 1995) . It also has rotenone which is a lyophilic molecule used in fishing (Fern, 2018).

Table 1.1 Phytochemical constituents of *Tylosema fassoglense* and their physiological functions

Phytochemical Constituents	**Physiological Functions**
Tyrosine inhibitors	Skin care (Dubois et al., 1995)
Lysine	Enhance calcium absorption (Quattrocchi, 2016).
Rotenone	Lipophilic molecule that paralyses fish, used for fishing (Quattrocchi, 2016).
Proline	Enhance wound healing (Quattrocchi, 2016).
Trypsin	Promote cytokine production (Dubois et al., 1995)
Phytates	Reduce mineral absorption (Dubois et al., 1995)

2.2 Diarrhea

Diarrhea can be classified based on the cause (infectious vs non-infectious), the anatomic site affected (small vs large bowel diarrhea), duration of illness (acute vs chronic), or the pathophysiologic mechanism underlying it which are mostly interrelated (secretory, malabsorptive, osmotic, and motility diarrheas) (Marks, 2013).

2.2.1 Acute vs chronic diarrhea

Acute diarrhea lasts for less than 14 days (Marks, 2013). Acute diarrhea is mainly caused by infectious agents such as *E. coli*, *Vibrio cholerae*, and rotavirus (Hakomori, 1984). Infectious agents produce toxins such as cholera toxin that causes ADP rybolisation thus activating the Gsα and increase intracellular cAMP which in turn gates chloride channels causing secretory diarrhea. It is often self-limited although some cases warrant antibiotic treatment, fluid, and electrolyte replacement at times (Manatsathit et al., 2002).

Chronic diarrhea last on the other hand, lasts for more than four weeks (Headstrom and Surawicz, 2005) and it is mostly indicative of a non-infectious cause such as inflammatory bowel disease (Crohn's disease and ulcerative colitis), irritable bowel syndrome (Iwasaki et al., 2019), carcinoid tumors, congenital Na+ disorder, type 1 and 2 bile acid malabsorption (Pattni and Walters, 2009), and deficiency of brush border enzymes such as lactase (Binder, 2010). Treatment is based on the etiology.

2.2.2 Small- vs Large- bowel diarrhea

Small bowel diarrhea manifests with voluminous watery diarrhea since out of the ten liters of fluid that is presented in the small intestine, 90 % is normally absorbed alongside nutrients and electrolytes (Cheng et al., 2020). Common small bowel conditions that manifest with diarrhea include celiac, HIV enteropathy, and bacterial overgrowth (Murray, 2013). Large bowel diarrhea

manifests with low-volume semi-solid feces but with tenesmus due to constant stimulation of stretch receptors in the rectum (Marks, 2013). It is commonly caused by colonic inflammatory conditions such as ulcerative colitis and Crohn's disease (Uchiyama et al., 2018).

2.2.3 Secretory diarrhea

Secretory diarrheas commonly occur when certain viral and bacterial agents release bioactive enterotoxins that cause increased activity of luminal membrane Ca^{2+}-activated chloride ion channels (anoctamin 1) and cAMP-activated cystic fibrosis transmembrane conductive regulator channels (CFTR) (Yu et al., 2015). In Rotavirus infections, the virus's nonstructural protein 4 (NSP4) functions as viroporin (virus-encoded ion channel) that releases calcium from the endoplasmic reticulum (ER) into the cytosol to be used for its replication through the phospholipase C pathway (Crawford et al., 2017; Pham et al., 2017). The ER calcium reduction activates stromal interaction molecules 1 (STIM1), an ER Ca^{2+}-sensor, and consequently Ca^{2+} influx through the plasma membrane (Crawford et al., 2017; Pham et al., 2017). An increase in cytosolic Ca^{2+} gates anoctamin 1 leading to chloride ion hypersecretion and osmotic luminal water loss (Crawford et al., 2017; Pham et al., 2017). *Vibrio cholerae* on the other hand releases cholera toxin (CT), whose subunits cause the ADP-ribosylation of Gs alpha subunit ($G\alpha_s$), maintaining it in an activated state (Thiagarajah et al., 2015). This state of activation of $G\alpha_s$ is associated with increased adenylate cyclase activity resulting in increased production of cyclic adenosine monophosphate (cAMP). cAMP gates CFTR resulting in hypersecretion of chloride ions accompanied by luminal water loss via osmosis (Thiagarajah et al., 2015).

Some neoplasms are associated with diarrhea. Tumors cause diarrhea through the release of bioactive molecules e.g. vasoactive intestinal peptide (VIP) in the case of VIPoma (Geldre and Lefebvre, 2004). There are two VIP receptors, $VPAC_1$ and $VPAC_2$, which can be activated by

pituitary adenylate cyclase-activating peptides (Geldre and Lefebvre, 2004). VPAC1 receptors are expressed in epithelial cells, myenteric neurons, vascular and enteric smooth muscles while $VPAC_2$ receptors are predominantly expressed on the smooth muscles of the GIT (Geldre and Lefebvre, 2004). The binding of VIP onto VPAC1 causes the activation of adenylate cyclase, leading to increased cAMP levels with subsequent gating of CFTR and Cl^- secretion (Patel et al., 2013).

2.2.4 Motility disorder diarrhea

Motility disorder diarrhea is caused by the hormones and neurotransmitters released by the enteroendocrine cells (EEC) and enteric motor neurons respectively (Sternini et al., 2016). Serotonin (5HT) is a hormone secreted by enterochromaffin cells and also a neurotransmitter co-localized in some enteric neurons widely distributed in the gastrointestinal tract (De Ponti, 2004). Enterochromaffin cells act as transducers of stimuli such as stretch, chemicals associated with luminal nutrient content, hyperosmotic stimuli, and toxins to secrete paracrine serotonin (Browning, 2015).

The serotonergic contractile effects are mediated via the $5HT_3$ ionotropic receptors on the primary afferent neurons (IPANs) (Fung and Vanden Berghe, 2020) that in turn stimulate interneurons (ascending and descending) via the cholinergic pathway (De Ponti, 2004). The descending interneuron synapses stimulate inhibitory motor neurons which release NO, VIP, as well as ATP leading to descending relaxation reflex (De Ponti, 2004). The ascending interneurons stimulate excitatory enteric motor neurons leading to ascending contractions reflex via cholinergic stimulation of intestinal smooth muscle muscarinic receptors (De Ponti, 2004).

There are two main types of cholinergic muscarinic receptors in the gut: M2 and M3 in a ratio of 3:1 respectively (Uchiyama and Chess-Williams, 2004). The M2 receptors lower the cytosolic

cAMP hence an increase in calcium sensitization (Uchiyama and Chess-Williams, 2004). The M3 is the main receptor that mediates cholinergic signal transduction in the GIT (Furness et al., 2014). The binding of acetylcholine to the M3 receptor activates phospholipase C leading to the mobilization of cytoplasmic Ca^{2+} from sarcoplasmic reticulum stores (Furness et al., 2014). Elevated cytosolic Ca^{2+} causes the activation of the contractile elements leading to increased motility and reduced gut transit time (Li et al., 2018).

2.2.5 Malabsorptive diarrhea

Malabsorptive diarrhea has multiple etiologies including but not limited to congenital disorders (Camilleri et al., 2017), and inflammatory diseases (Montoro-Huguet et al., 2021). It can also be iatrogenically induced via terminal ileal resection (Camilleri et al., 2017). Congenital disorders such as an abnormally short small intestine (Spiller, 2006), deficiency of brush-border disaccharidases e.g., lactase, and absence of intestinal protein transporters, cause malabsorptive diarrhea by increasing the luminal bulk of undigested and unabsorbed material (H. J. Binder, 2010) (Binder, 2010). Colonic microbiota anaerobically breaks down the unabsorbed nutrients into short-chain fatty acids (SCFAs) which induce osmotically-mediated diarrhea (H. J. Binder, 2010). The bacteria also generate gases such as methane that distend the colon, stimulating stretch receptors leading to increased peristalsis and thus shortened transit time (Camilleri et al., 2017).

The congenital absence of apical sodium-dependent bile transporters (ASBT) that transport luminal bile salts into the cytoplasm causes type 1 bile salt malabsorption (Camilleri et al., 2017). Deconjugation of bile acids (Camilleri et al., 2017) and terminal ileal resection (Camilleri, 2015)affect the absorption of bile salt via ASBT (Camilleri, 2015). Unabsorbed bile salts increase mucosal permeability, induce mucus secretion by goblet cells (Camilleri et al., 2017), and reduce transit time, thus causing diarrhea (Spiller, 2006). Bile salts also activate the stimulatory Tekeda

G-protein receptor 5 (TGR5) which is coupled to the adenylyl cyclase system (Camilleri et al., 2017) and thus increases the secretion of chloride ions via CFTR (Field, 2003).

Congenital Na^+ disorder is a rare autosomal recessive disease that affects the sodium-proton exchanger (NHEs) (Yun et al., 1997). There are nine isoforms of NHEs where isoforms 2, 3, and 4 are present in the GIT (Cao et al., 2020). The brush border NHE3 is a key pathway for sodium and water absorption by acting in concert with a proton exchanger to mediate electroneutral sodium chloride absorption (Cao et al., 2020). The congenital absence of NHE3 leads to the loss of sodium and fluid in fecal matter that begins in utero and manifests as hyponatremia and metabolic acidosis (Cao et al., 2020). There is also associated chloride loss in the stool due to a defective Cl^-/HCO^-_3 antiporter (Turkia et al., 2018)

2.2.6 Diagnosis of Diarrhea

Diarrhea can be secondary to increased gastric emptying rate, reduced intestinal transit, or both. Increased gastric emptying rate can be diagnosed using a wireless pH and motility capsule while reduced intestinal transit can be diagnosed using the hydrogen breath test (Miller et al., 1997).

To differentiate secretory from osmotic diarrhea, the fecal osmotic gap $\{300 - 2 \times (Stool\ Sodium + Stool\ Pottasium)\}$ is determined (Duncan et al., 1992). The normal human fecal osmolality is about 300 mOsm/kg while the normal fecal osmotic gap is 50- 100 mosm/kg (Eads et al., 2020). Secretory diarrhea has a low fecal osmotic gap (less than 50 mosm/kg) because sodium and potassium make up a bigger proportion of the overall fecal osmolality (Kasırga, 2019). Osmotic diarrhea has a high stool osmotic gap (more than 100 mosm/kg) because it is caused by unabsorbed osmotically active food particles, and not increased sodium or potassium (Headstrom and Surawicz, 2005).

2.3 Rat Models of Diarrhea

Diarrhea in animal models is achieved by inducing a state of enteric cholinergic toxidrome or by irritating the enteric mucosal lining (Awouters et al., 1978). The enteric cholinergic toxidrome increases intestinal motility through increased cholinergic activity via muscarinic receptors (Sagar et al., 2005). In this model, neostigmine, a cholinesterase enzyme inhibitor, is used to increase levels of acetylcholine (Li et al., 2018).

The irritative method involves the use of castor oil which causes the inflammation of the enteric mucosal lining (Awouters et al., 1978). Castor oil contains ricinoleic acid which causes mucosal irritation within the first hour of oral administration (Awouters et al., 1978). The irritation induces inflammation and the release of inflammatory mediators such as prostaglandins which have a stimulatory effect on the enteric nervous system leading to increased motility (Uchiyama et al., 2018). The inflammation of the mucosal lining also interferes with water and electrolyte absorption (Uchiyama et al., 2018).

CHAPTER 3: MATERIAL AND METHODS

3.1 Plant Collection and Extract Preparation

Tubers of *T. fassoglense* were collected from Mumias, Western Kenya (Lusheya Lubinu ward Location GPS coordinates: 0°18'17.6"N 34°32'13.6"E.). The region has sunshine throughout the year, temperature ranges of 14 -18 ℃ with heavy rainfall between March to July (World Weather & Climate Information, 2022). The identity of the collected plant material was confirmed by the plant taxonomist at the University of Nairobi herbarium and a voucher specimen deposited therein (AMW 2019/001). The plant tubers were chopped into small pieces, air-dried for one week, and then milled to a fine powder. The resulting fine powder was then macerated in distilled water in a 1:10 ratio (weight/volume) for one (1) hour with intermittent shaking. The resulting suspensions were then sequentially filtered using cotton wool and by Whatman ™ filter paper (diameter, thickness, pore size: 125 mm, 0.26 mm, 30 μm respectively). The resulting filtrate was then lyophilized to obtain a freeze-dried extract. The freeze-dried extract was weighed and placed in amber-colored sample bottles and stored in a refrigerator at 4 °C.

3.2 Study Animals

Sixty-five (65) adult Sprague Dawley rats of both sexes (33 females and 32 males, ratio of 1.01) aged between 12 and 14 weeks weighing 300 ± 50g were used in the *in-vivo* studies. Fifteen (15) New Zealand White Rabbits of both sexes weighing 1.75 ± 0.25 kg were used in the *ex-vivo* studies. The animals were housed in the animal house located within the Department of Medical Physiology, University of Nairobi. The temperature within the animal house was maintained at 22-25 ℃. A 12-hour light/dark cycle (6am-6pm-6am) was maintained within the animal house. The animals were allowed access to water and standard animal chow ad libitum (UNGA Farm

Care Ltd). The animals were acclimatized for two weeks to the study environment prior to the commencement of the experiments.

3.3 Sample Size and Statistical Power Analysis

A priori power analysis was conducted using G*Power version 3.1 to test the differences between five experimental groups using one-way ANOVA test, a large effect size (d= 0.80), and an alpha of 0.05 which showed that a total sample of 25 Sprague Dawley rats with equal sized groups (n=5) was required to achieve a power of 0.82, a power of 82%.

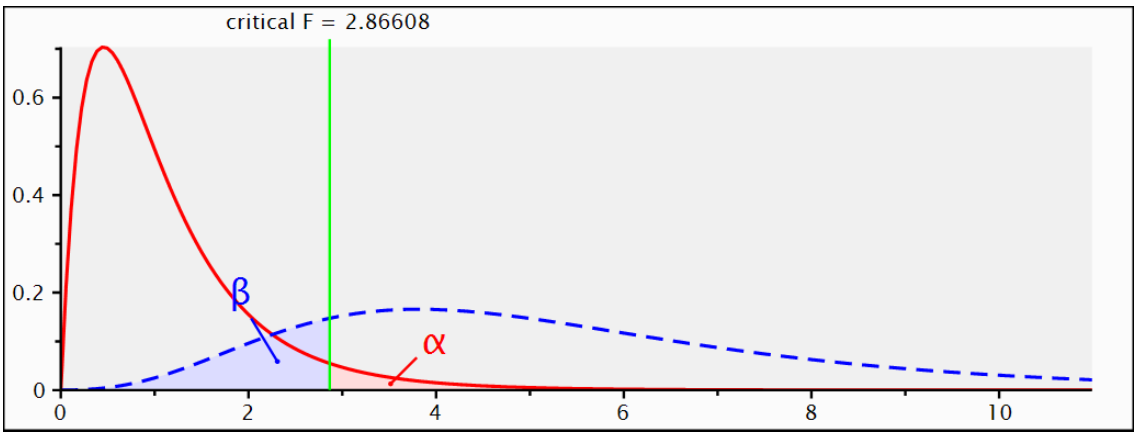


Figure 3. 1 graphic diagram showing central and non-central distribution

3.4 *In-vivo* antidiarrheal activities Studies

3.4.1 Castor Oil-Induced Diarrhea

The antidiarrheal activity of TFG extract in castor oil-induced diarrhea was investigated according to the method described by Awouters et al. (1978). Briefly, twenty-five (25) Sprague Dawley rats were randomly allocated into 5 groups (n = 5): the negative control (normal saline), positive control (5 mg/kg Loperamide), low dose test (200 mg/kg), medium-dose test (400 mg/kg), and high dose test (800 mg/kg) groups. The rats were fasted for 24 hours but with ad libitum access to water prior to the experiment. One (1) ml of castor oil (ARANDI Castor Seed Kenya Ltd) was administered one hour after administration of the respective treatments by oral gavage. The animals were observed for four (4) hours and the cages were examined for the presence of diarrheal

feces. The count and mass of the diarrheic feces were recorded. The mass of diarrheal feces was determined by subtracting the weight of the filter paper from the final weight of the paper in the individual cages. The data were then expressed as the percentage of the negative control (Awouters et al., 1978). The doses of TFG with significant antidiarrheal effect were used for the subsequent *in-vivo* studies.

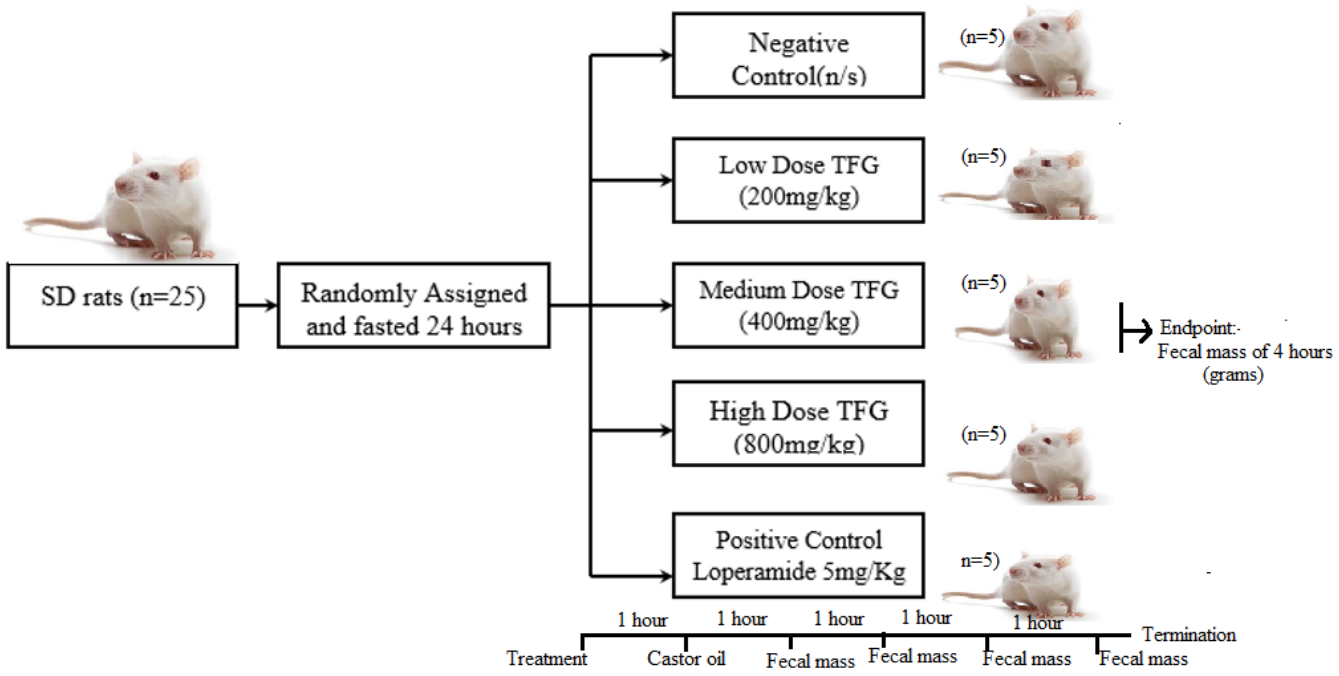


Figure 3. 2: Schematic diagram showing how the experimental rats were randomized into various groups.

3.4.2 Enteropooling Test

The effect of TFG on castor oil-induced enteropooling was evaluated using the method described by Degu et al. (2016). Twenty (20) Sprague Dawley rats were randomized into the negative control (normal saline), medium dose TFG (400 mg/kg), high dose TFG (800 mg/kg), and positive control (Loperamide,5 mg/kg) groups. The rats were given 1 ml of castor oil by oral gavage 1 hour after respective treatment in each group following 24-hour starvation but with ad libitum access to water. The rats were terminated by stunning thirty (30) minutes after the administration of castor oil. An abdominal incision was made in each rat and the luminal content of the respective small intestines from the pylorus to the ileocecal junction weighed and recorded.

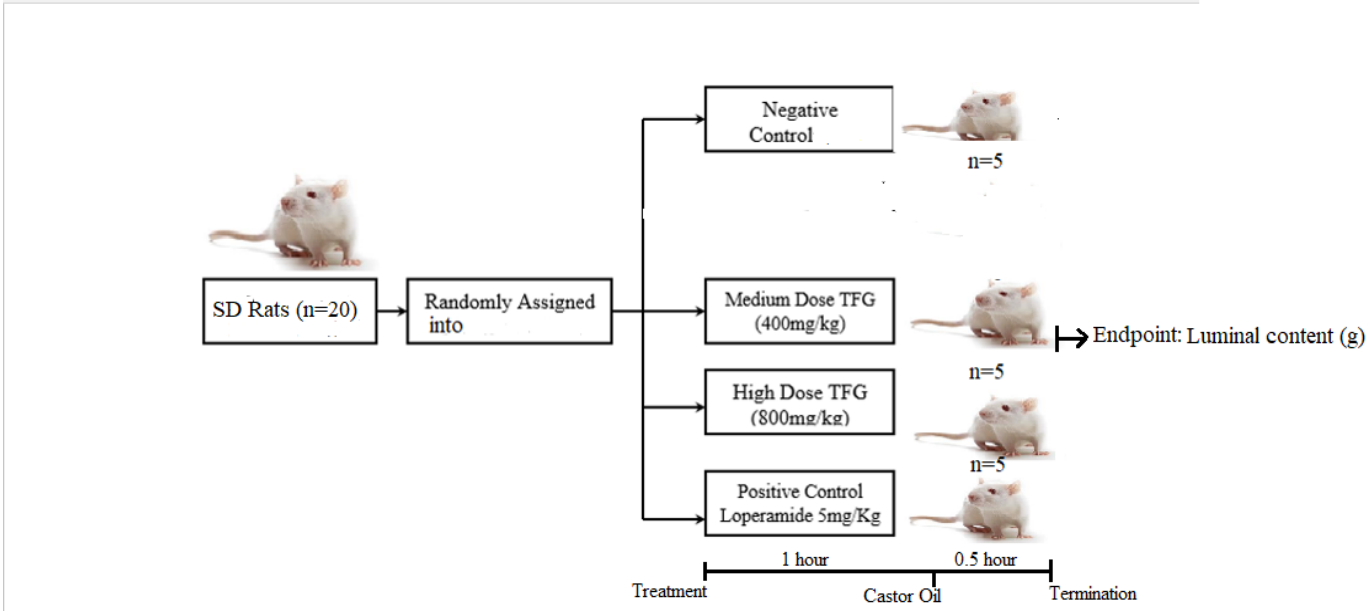


Figure 3. 3: Schematic diagram showing how the experimental rats were randomized into various groups in enteropooling assay test.

3.4.3 Phenol Red Meal Test

The effect of TFG extract on gastrointestinal motility was investigated using the phenol red method described by Qu et al. (2014). Twenty (20) Sprague Dawley rats were randomized into the control (1.5 % Carboxymethyl cellulose gel, CMC, 10 ml/kg), low test dose (400 mg/kg TFG extract), high test dose (800 mg/kg TFG extract), and positive control (Loperamide 5 mg/kg) groups. The respective treatments were administered to experimental animals via oral gavage after they had been starved for 24 hours but with ad libitum access to water.

Neostigmine (0.05 mg/kg, i.p) was administered to each experimental animal thirty (30) minutes after administration of the respective treatment. Phenol red meal (0.5 mg of phenol red dye per ml of 1.5% CMC) was then administered by oral gavage to each rat at a dose of (10 ml/kg) twenty (20) minutes after administration of neostigmine. The experimental animals were then terminated by stunning twenty (20) minutes after phenol red meal administration. An abdominal incision was made in each animal and the stomach and small intestines were isolated.

The amount of phenol red dye retained in the stomach was assayed and determined by the spectrophotometric method. Stomachs belonging to respective animals were each homogenized in 25 ml of 0.1M sodium hydroxide for 30 seconds and the resulting homogenate was allowed to settle for 1 hour. One (1) ml of trichloroacetic acid (33% w/v) was then added to 8 ml of supernatant fluid to deproteinize the supernatant which was then centrifuged for ten minutes at 3200 revolutions per minute. One (1) ml of 2M sodium hydroxide was then added to four (4) ml of the centrifuged supernatant and the sample's absorbency was determined at a wavelength of 560 nm and recorded. The absorbency of non-treated experimental animals (n=5) given phenol red meal and terminated immediately served as the standard reference of a stomach with 100% phenol red dye. The gastric emptying rate was determined using the formula:

Gastric Emptying Rate

$$= [1 - \left(\frac{\text{Absorbency of Test Sample at 560 after 20 minutes}}{\text{Absorbency of standard reference at 560}}\right)] \times 100$$

The distance covered by phenol red meal from pylorus was measured and expressed as a percentage of the total length of the small intestine to find the peristaltic index.

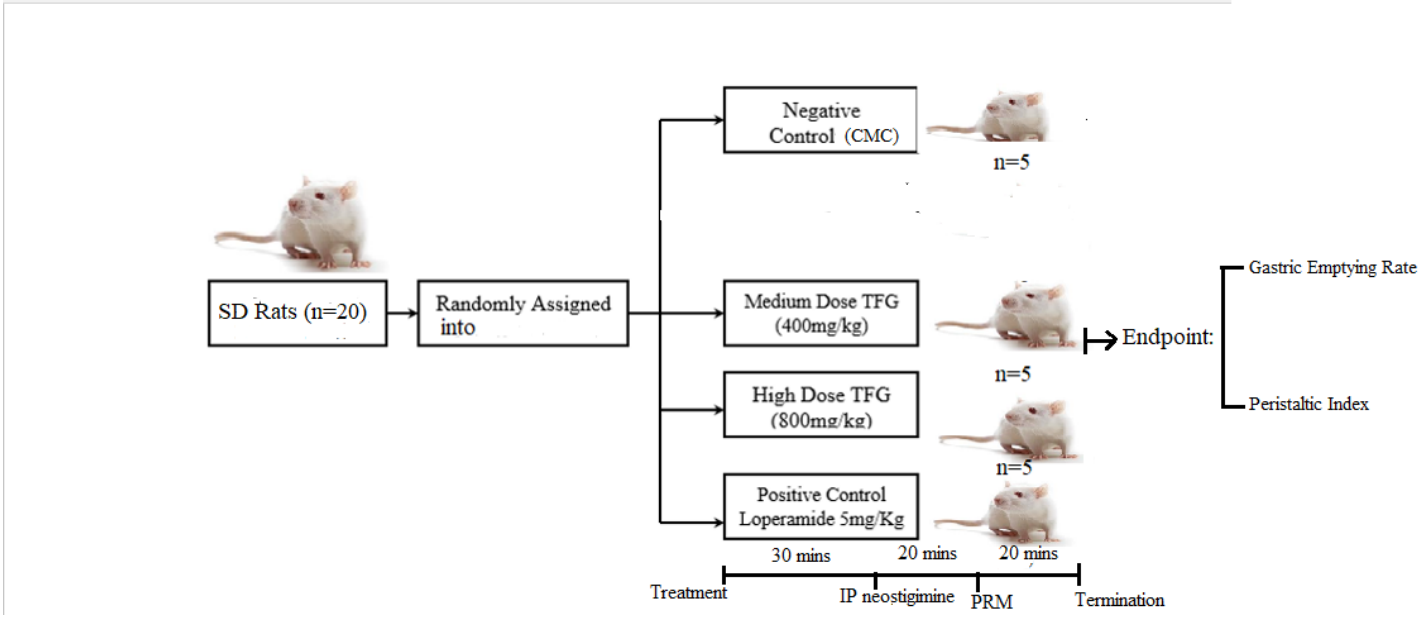


Figure 3. 4: Schematic diagram showing how the experimental rats were randomized into various groups phenol red meal test (IP intraperitoneal, PRM-phenol red meal gavage)

3.5 Ex-vivo experiments using Isolated Rabbit's Jejunum

The experimental animals were starved for 24 hours but with ad libitum access to water prior to the experiment. The rabbits were euthanized by stunning, and an abdominal incision performed after which 10-centimeter jejunal segments were isolated. The jejunum segments were dipped in beaker containing Tyrode's solution (8 g/l NaCl, 0.2 g/l KCl, 1 g/l $NaHCO_3$, 0.05 g/l $NaHPO_4$, 1 g/l $MgCl_2$, 0.2 g/l $CaCl_2$, and pH 7.4) which had been pre-aerated with carbogen (carbon dioxide: oxygen, 1:19) at 0 ºC. The individual jejunal strips contents were flushed out and the strips were transferred to a beaker containing Tyrode's solution at room temperature (25 ºC). Two (2) centimeter jejunal segments were then vertically suspended in an organ bath containing forty (40) ml of Tyrode's solution maintained at 37 ℃ and continuously aerated with carbogen. The proximal part of the respective jejunal segment was connected to an isometric force transducer (ML500/A^{TM}, AD instruments) connected to the Powerlab^{TM} data acquisition system (AD Instruments). The experimental setup is shown in Figure 3.1.

The respective suspended jejunal segments were equilibrated for 1-hour prior the evaluation of the effects of different concentrations of TFG (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 mg/ml) on jejunal contraction. Normal spontaneous jejunal contractions were recorded for 3 minutes followed by a 10-minute recording after the addition of each concentration of TFG. The organ bath was washed out and rinsed thrice prior to adding another forty (40) ml of fresh pre-warmed Tyrode's solution in the organ bath while ensuring full recovery of the jejunal segment before moving to the next concentration. The experiment started with lowest concentration (0.5 mg/ml) and ascended in gradations of 0.5 mg/ml to a maximum of 3.5 mg/ml of TFG. The respective contraction forces (uv) of each treatment were expressed as a percentage of control.

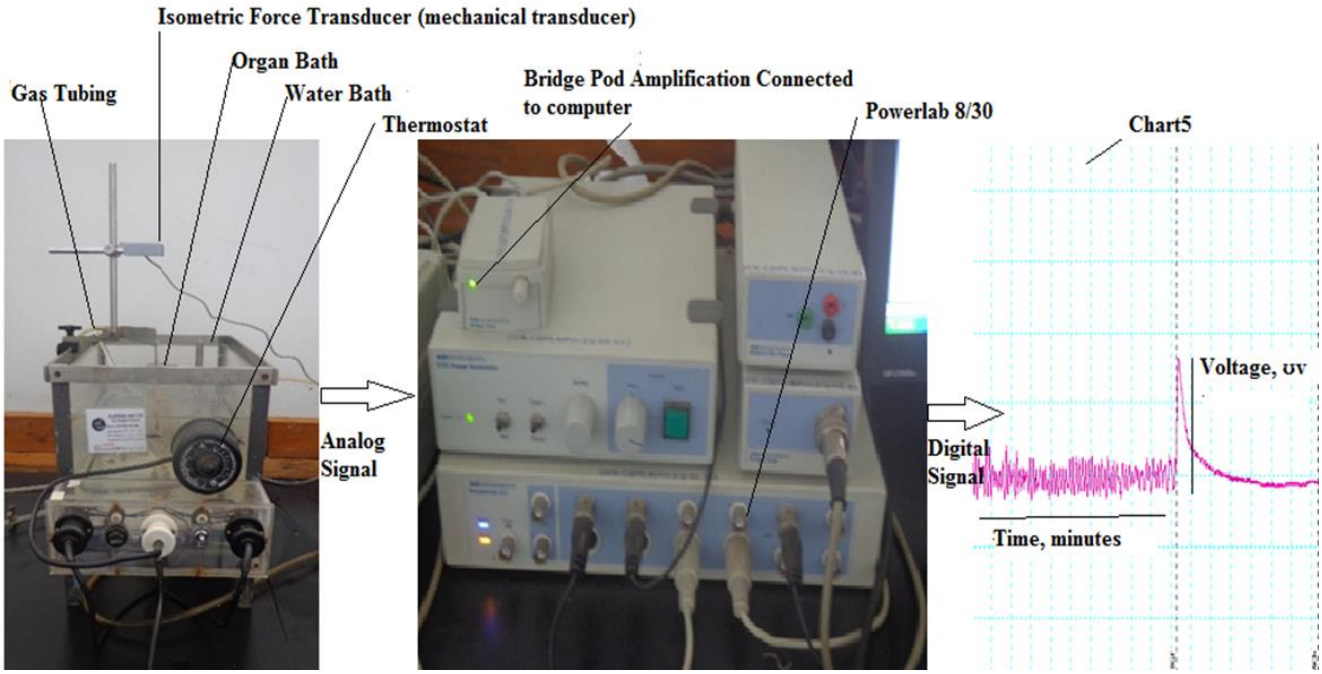


Figure 3.5: Powerlab data acquisition system

3.6 Mechanism of Action Experiments

The effect of various concentrations (0.5,1.0,1.5, 2.5 and 3.5 mg /ml) of TFG extract on spontaneous jejunal contraction were evaluated in the presence of standard receptor blockers (prazosin [a α1-adrenergic blocker], propranolol [a non-selective β- adrenergic blocker], and naloxone [a non-selective opioid blocker]) and enzyme inhibitors: L-NAME- (nitric oxide synthase inhibitor), and methylene blue (guanylyl cyclase inhibitor). The spasmogenic effect of acetylcholine and a Tyrode's solution enriched with 80mM potassium chloride were also evaluated in the presence of various TFG's concentrations. In addition, the effect of calcium chloride solutions of various concentration (0.00003 M, 0.000 3M, 0.003 M, 0.03 M) were evaluated in the presence and absence of various concentration of TFG (0.5 mg/ml, 2.5 mg/ml and 3.5 mg/ml) as well as verapamil (0.025 mM) using a modified Tyrode's solution i.e., KCl concentration was raised from 3 mM to 80 mM by equimolar replacement of NaCl, $CaCl_2$ was withdrawn, and the resulting solution enriched with EDTA.

The procedure in these mechanism of action experiments entailed an initial recording of the normal spontaneous contraction of respective jejunal strips for 3 minutes prior to the addition of receptor blockers and enzyme inhibitors. The jejunal segments were then incubated with receptor blockers and methylene blue for 2 minutes (20 minutes for L-NAME) as described by (Li et al., 2018) after which tracings of 3 minutes were recorded. The recordings of the extract's effect were performed for 10 minutes after the addition of the respective TFG concentration.

3.7 Phytochemical Analysis of T. fassoglense Extract by LC-MS

Phytochemical analysis was performed via liquid chromatography-mass spectrometry (LC-MS). One (1) gram of *T. fassoglense* extract was reconstituted in LC-MS water (1mg/1ml) and 20 µl from stoke volume injected in a quaternary LC pump (Model 1200) ESI-MS system coupled to Agilent MSD 6120-Triple quadruple MS with electrospray source (Palo Alto, CA). The sample was separated on a reverse-phase liquid chromatography on Agilent technologies 1200 infinite series, Zorbax SB C18 analytical column (2.1 mm x 50 mm, 1.8 um). The mobile phase consisted of water (eluent A) and acetonitrile (eluent B) for elution using a linear gradient program of: 0 min, 5 % B; 0-5 min, 5-50 % B; 5-10 min, 50-80 % B; 10-15 min, 80-100 % B; 15-25 min 100 % B; 25-30 min 5 % B; 30-35 min 5 % B. The flow rate was held constant at 1 mL/min and the injection volume was 1.0 uL and the data were acquired in full-scan negative at 100 to 1500 m/z scan. The dwell time for each ion was 50ms. Operating conditions were as follow for MS detection: capillary voltage, 3.0kV; cone voltage, 70 V; extract voltage 5 V; radio frequency (RF) voltage, 0.5 V; source temperature, 110 ℃; nitrogen gas temperature for desolvation, 380 ℃; nitrogen gas flow for desolvation, 400 L/h. Linear calibration equation curve of peak area vs. concentration using the following equation (y=6008.9x-5250.3 (R2=0.9987) was used for external quantitation of all peaks.

3.8 Ethical considerations

Ethical approval to perform the study was sought and obtained from the bio-safety animal use and ethics committee of the Department of Veterinary Physiology, University of Nairobi (permit number RVM BAUEC/2020/260). All the protocols used in this study were in adherence with the National Institute of Health Guidelines for care and use of laboratory animals (8^{th} Edition) and complied with 4Rs (reduction, replacement, refinement, and rehabilitation) tenets of ethical experimental design.

3.9 Statistical analysis

The experimental data were expressed as mean ± standard error of the mean (SEM) and analyzed using one-way ANOVA followed by Turkey's post hoc test in cases of significance which was set at $p < 0.05$. Analysis was performed using GraphPad Prism^{TM} version 8 suite of statistical software.

CHAPTER 4: RESULTS

4.1 Percentage yield

One thousand (1000) grams of dry, milled plant tubers yielded thirty (30) grams of freeze-dried extract. The percentage yield was therefore 3 %.

4.2 Castor oil-induced diarrhea model

4.2.1 Fecal mass

There were significant decreases in fecal mass between the experimental groups 60 minutes after castor treatment: [3.03 ± 0.0.70 g (negative control) vs. 2.54 ± 0.60 g (low dose test) vs. 0.80 ± 0.61 g (medium dose test) vs. 0.14 ± 0.68 g (high dose test) vs. 0.00 ± 0.00 g (positive control): ($F(4, 20) = 7.802$, $P= 0.0006$]. Post-hoc statistical analysis using Tukey's multiple comparisons test revealed that there were significant differences between the negative control and medium-dose test ($p = 0.0359$), the negative control and high dose test ($P < 0.0047$), and the negative control and the positive control ($p < 0.0030$) groups.

There were significant decreases in fecal mass between the experimental groups 120 minutes after castor treatment: [5.10 ± 0.66 g (negative control) vs. 4.16 ± 0.52 g (low dose test) vs. 1.30 ± 0.67 g (medium dose test) vs. 00.39 ± 0.12 g (high dose test) vs. 0.00 ± 0.00 g (positive control): ($F(4, 20) = 22.17$, $P= <0.0001$]. Post-hoc statistical analysis using Tukey's multiple comparisons test revealed that there were significant differences between the negative control and medium-dose test ($p = 0.0002$), the negative control and high dose test ($p< 0.0001$), and the negative control and the positive control ($p < 0.0001$), low dose test and medium test ($p=0.042$), low dose test and high dose test ($p<0.0002$), low dose test and positive control ($P<0.0001$) groups.

There were significant decreases in fecal mass between the experimental groups 180 minutes after castor treatment: [8.26 ± 1.12 g (negative control) vs. 4.60 ± 0.47 g (low dose test) vs. 2.91 ± 0.45

g (medium dose test) vs. 1.20 ± 0.45 g (high dose test) vs. 0.00 ± 0.00 g (positive control): (F (4, 20) = 27.57, P<0.0001]. Post-hoc statistical analysis using Tukey's multiple comparisons test revealed that there were significant differences between the negative control and low dose test (p=0.0035), negative control and medium-dose test (p <0.0001), the negative control and high dose test (P < 0.0001), and the negative control and the positive control (p < 0.0001), low dose test and high dose test (0.0069), low dose test and positive control (p=0.0069) groups.

There were significant decreases in fecal mass between the experimental groups 240 minutes after castor treatment: [10.042 ± 0.71g (negative control) vs. 5.25 ± 0.46 g (low dose test) vs. 5.15 ± 0.58 g (medium dose test) vs. 3.49 ± 1.23 g (high dose test) vs. 1.18 ± 0.26 g (positive control): (F (4, 20) = 18.69, P < 0.0001]. Post-hoc statistical analysis using Tukey's multiple comparisons test revealed significant differences between the negative control and low dose test (p = 0.0001), the negative control and medium-dose test (p = 0.0017), the negative control and high dose test (P < 0.0001), and the negative control and the positive control (p < 0.0001), low dose test and positive control (p=0.0023) groups as shown in Figure 4.1. TFG extract had a dose-dependent antidiarrheal effect on fecal output with ED50 of 310 mg/kg as shown in Figure 4.2.

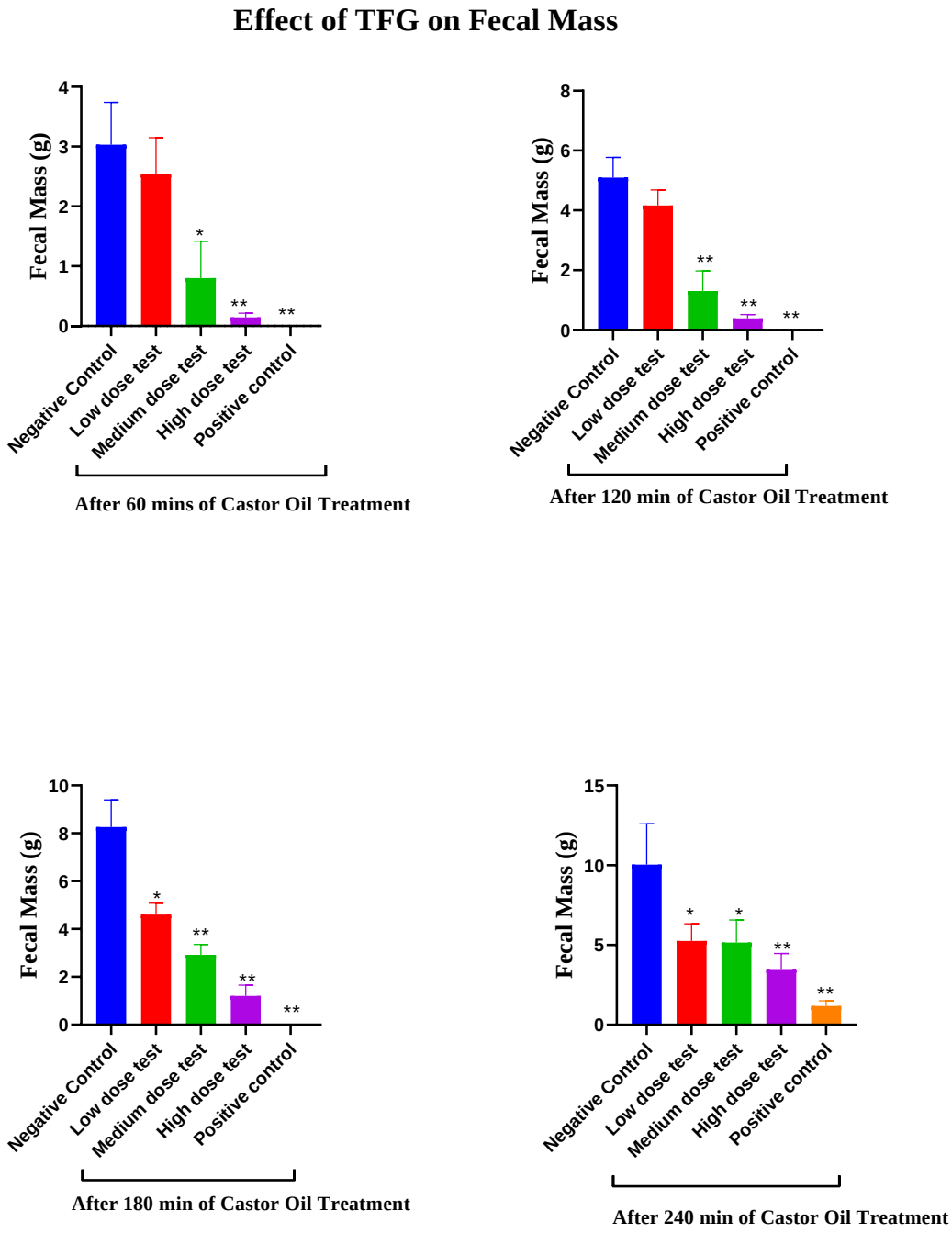


Figure 4.1:The effects of different dosages of freeze-dried extract of TFG on castor oil-induced on fecal mass for 60, 120, 180, and 240 minutes. Results are expressed as mean ± SEM. **p < 0.01, * p < 0.05 compared with negative control. ANOVA followed by Turkey's multiple comparison test

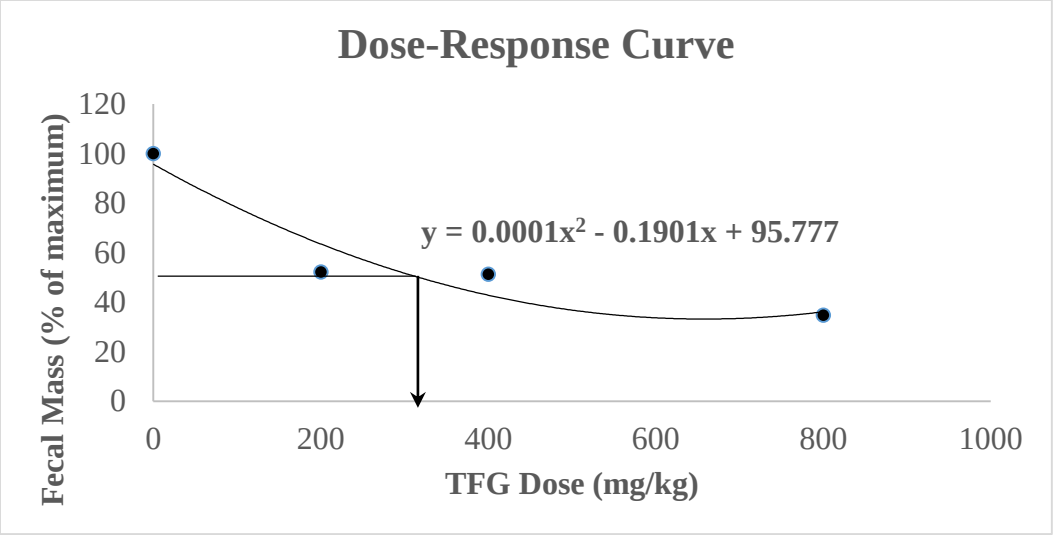


Figure 4.2: Dose–response curve of freeze-dried extracts of *Tylosema fassoglense* (TFG) in the castor oil-induced diarrhea model (n=5) with an ED 50 of 310 mg/kg

4.2.2 Diarrheic Fecal count

There were significant decreases in diarrheic fecal count between the experimental groups 60 minutes after castor oil treatment: [4.00 ± 0.00 (negative control) vs. 2.60 ± 0.0.40 (low dose test) vs. 1.20 ± 0.37 (medium dose test) vs. 0.60 ± 0.244 (high dose test) vs. 0.00 ± 0.0 (positive control): ($F (4, 20) = 36.28$, $P < 0.0001$]. Post-hoc statistical analysis with Tukey's multiple comparisons test revealed that there were significant differences between the negative control and low dose test ($p=0.0113$), the negative control and medium-dose test ($p < 0.0001$), negative control and high dose test ($P < 0.0001$), and the negative control and positive control ($p < 0.0001$), low dose test and high dose test ($p=0.0003$) groups.

There were significant decreases in diarrheic fecal count between the experimental groups 120 minutes after castor oil treatment: [7.60 ± 0.24 (negative control) vs. 4.60 ± 0.40 (low dose test) vs. 2.80 ± 0.58 (medium dose test) vs. 1.40 ± 0.40 (high dose test) vs. 0.00 ± 0.00 (positive control): ($F (4, 20) = 60.64$, $P < 0.0001$]. Post-hoc statistical analysis with Tukey's multiple comparisons test revealed that there were significant differences between the negative control and low dose test ($p < 0.0002$), the negative control and medium-dose test ($p < 0.0001$), negative control and high dose test ($P < 0.0001$), and the negative control and positive control ($p < 0.0001$), low dose test and medium dose test ($p=0.0234$), low dose test and high dose test ($p<0.0001$), low dose test and positive control ($p<0.0001$) groups.

There were significant decreases in diarrheic fecal count between the experimental groups 180 minutes after castor oil treatment: [11.40 ± 0.24 (negative control) vs. 6.60 ± 0.40 (low dose test) vs. 4.20 ± 0.37 (medium dose test) vs. 2.40 ± 0.67 (high dose test) vs. 0.00 ± 0.0 (positive control): ($F (4, 20) = 115.7$, $P < 0.0001$]. Post-hoc statistical analysis with Tukey's multiple comparisons test revealed that there were significant differences between the negative control and low dose test

($p < 0.0001$), the negative control and medium-dose test ($p < 0.0001$), negative control and high dose test ($P < 0.0001$), and the negative control and positive control ($p < 0.0001$), low dose test and medium dose test (0.0037), low dose test and high dose test ($p<0.0001$), low dose test and positive control ($p<0.0001$), medium dose and high dose test ($p<0.0366$) groups.

There were significant decreases in diarrheic fecal count between the experimental groups after 240 minutes of castor oil treatment: [14.40 ± 0.24 (negative control) vs. 8.60 ± 0.40 (low dose test) vs. 5.80 ± 0.24 (medium dose test) vs. 4.40 ± 0.67 (high dose test) vs. 1.00 ± 0.0 (positive control): ($F (4, 20) = 154.5$, $P < 0.0001$]. Post-hoc statistical analysis with Tukey's multiple comparisons test revealed that there were significant differences between the negative control and low dose test ($p < 0.0001$), the negative control and medium-dose test ($p < 0.0001$), negative control and high dose test ($P < 0.0001$), and the negative control and positive control ($p < 0.0001$), low dose test and medium dose test ($p=0.0008$), low dose test and high dose test ($p<0.0001$), low dose test and positive control ($p<0.0001$) groups as shown in Figure 4.3 below.

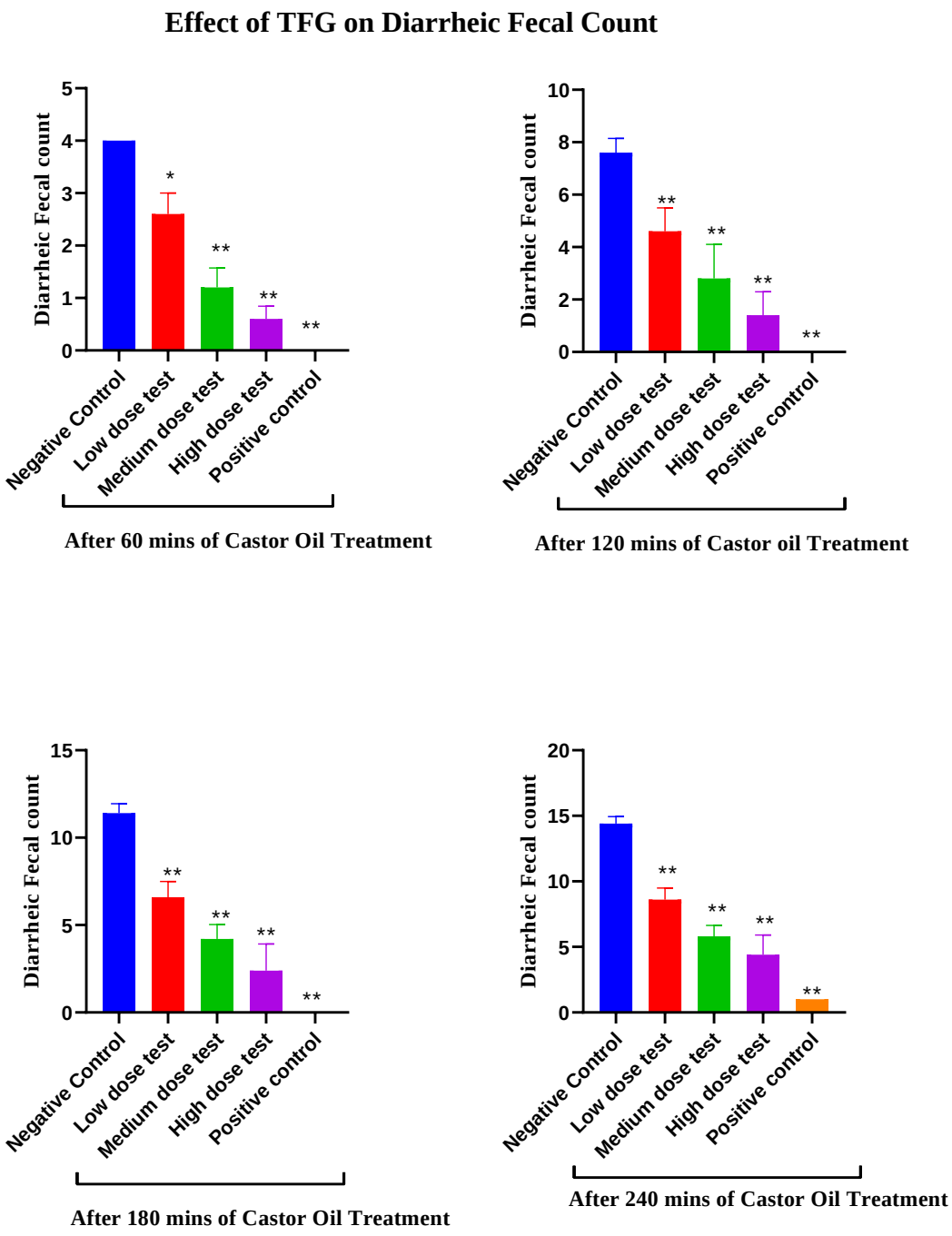


Figure 4.3: The effects of different dosages of freeze-dried extract of TFG on the fecal count in castor oil-induced bowel movement for 60, 120, 180, and 240 minutes. Results are expressed as mean ± SEM. **p < 0.01, * p < 0.05. ANOVA followed by Turkeys' multiple comparison test

4.3 Phenol red meal transit test

4.3.1 Effect on gastric emptying rate

There were significant decreases in gastric emptying rates between the experimental groups: [85.95 ± 3.39 % (negative control) vs 84.02 ± 0.65 % (medium dose test) vs. 52.47 ± 7.76 % (high dose test) vs. 45.22 ± 3.98 % (positive control): ($F(3, 16) = 20.21$, $P < 0.0001$)]. Post-hoc statistical analysis with Tukey's multiple comparisons test revealed that there were significant differences between the negative control and high dose test ($P = 0.0006$), and the negative control and positive control ($P < 0.0001$). These results are shown in the Table 4.1below.

Table 4.1: Effect of different *Tylosema fassoglense* (TFG) doses on the gastric emptying rate in the neostigmine-induced gastrointestinal motility model

Study Groups	Dose (mg/kg)	Absorbency of Gastric Phenol Red at 560 nm	Gastric Emptying
Negative control	10	0.331	85.95 ± 3.39%
TFG	400	0.377	84.02 ± 0.65%
	800	1.121	52.47± 7.76%**
Loperamide	5	1.292	45.22± 3.98%**
Reference	5	2.509	0

Results are expressed as mean ± SEM of 5 Sprague Dawley Rats. **p<0.01 compared with negative control. ANOVA followed by Turkey's multiple comparison test.

4.3.2 Effect on peristaltic Index

There were significant decreases in peristaltic indices between the experimental groups: [83.37 ± 2.63 % (negative control) vs. 59.82 ± 2.14 % (medium dose test) vs. 55.64 ± 1.40 % (high dose test) vs. 45.58 ± 1.94 % (positive control): ($F(3, 16) = 59.94$, $P < 0.0001$)]. Post-hoc analysis with Tukey's multiple comparisons test revealed significant differences between the negative control and medium dose test ($P < 0.0001$), negative control and high dose test ($P < 0.0001$), and the negative control and positive control ($P < 0.0001$) groups. These results are illustrated in Table 4.2.

Table 4.2: Effect of different *Tylosema fassoglense* (TFG) doses on intestinal transit rate in neostigmine-induced gastrointestinal motility

		Mean		
Study Groups	Dose	Small intestine length	Phenol red meal distance traveled	%Intestinal transit
Negative control	10 mls/kg	125.38 cm	104.54 cm	83.37 ± 2.63 %
TFG	400 mg/kg	119.44 cm	71.46 cm	59.82± 2.14 % **
TFG	800 mg/kg	120.54 cm	67.08 cm	55.64± 1.40 % **
Loperamide	5 mg/kg	130.32 cm	59.40 cm	45.58± 1.94 % **

Results are expressed as mean ± SEM (n=5). **p<0.01 compared with negative control. ANOVA followed by Turkey's multiple comparison test.

4.4 Castor oil-induced enteropooling test

There were significant decreases in mass of luminal content between the experimental groups: [3.79 ± 0.05 g (negative control) vs. 3.23 ± 0.14 g (medium dose test) vs. 2.82 ± 0.32 g (high dose test) vs. 2.16 ± 0.20 g (positive control): ((F (3, 16) = 11.00, P = 0.0004).]. Post-hoc statistical analysis with Tukey's multiple comparisons test revealed that there were significant differences between the negative control and high dose test groups (P = 0.0203) and the negative control and positive control groups (P = 0.0002). These results are illustrated in Figure 4.4 below.

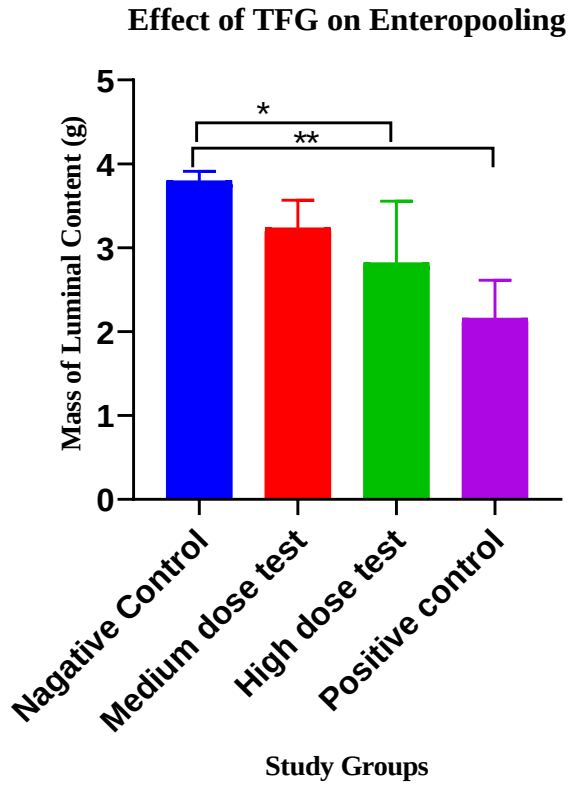


Figure 4.4: The effects of different dosages of freeze-dried extract of TFG on castor oil-induced enteropooling assays in grams. Results are expressed as mean ± SEM. **p < 0.01, * p < 0.05. ANOVA followed by Turkey's multiple comparison test

4.5 *Ex-vivo* experiments using Isolated Rabbit's Jejunum

4.5.1 Effect of freeze-dried extract of TFG on spontaneous contraction

There were significant decreases in percentage inhibition of spontaneous jejunal force of contraction between different TFG dose study-groups: [8.44 ± 4.45 % (0.5 mg/ml TFG) vs. (35.15 ± 5.93% (1.0 mg/ml TFG) vs. (45.98 ± 4.28 % (1.5 mg/ml TFG) vs. (59.17 ± 6.63 % (2.0 mg/ml TFG) vs. (58.19 ± 5.82 % (2.5 mg/ml TFG) vs. (68.01 ± 5.34 % (3.0 mg/ml TFG) vs. (74.80 ± 2.11% (3.5 mg/ml TFG): ($F(6, 28) = 19.92$): $P < 0.0001$)]. Post-hoc statistical analysis with Tukey's multiple comparisons test revealed that there were significant differences between the following groups: 0.5 mg TFG vs. 1.0 mg TFG ($p = 0.0129$), 0.5 mg TFG vs. 1.5 mg TFG ($p = 0.0001$), 0.5 mg TFG vs. 2.0 mg/ml TFG ($p < 0.0001$), 0.5 mg TFG vs. 2.5 mg/ml TFG ($p < 0.0001$), 0.5 mg TFG vs. 3.0 mg/ml TFG ($p < 0.0001$), 0.5 mg TFG vs. 3.5 mg/ml TFG ($p < 0.0001$), 1.0 mg TFG vs. 2.0 mg/ml TFG ($p = 0.0319$), 1.0 mg TFG vs. 2.5 mg/ml TFG ($p=0.0460$), 1.0 mg TFG vs. 3.0 mg/ml TFG ($p = 0.0014$), 1.0 mg TFG vs. 3.5 mg/ml TFG ($p=0.0001$), and 1.5 mg TFG vs. 3.5 mg/ml TFG ($p = 0.0119$) as shown in Figure 5 below. Figure 4.6 illustrate dose-response curve with an ED of 1.09 mg/ml.

Effect of TFG on Spontaneous Contraction

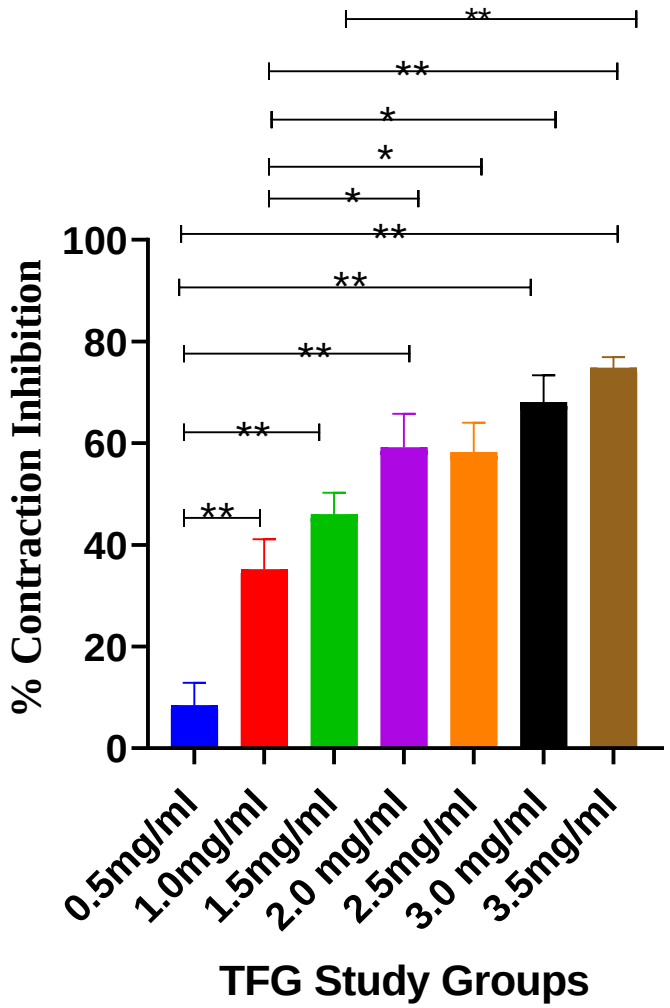


Figure 4.5: Effect of different concentrations of TFG on spontaneous contraction of an isolated rabbit jejunum. *p<0.05, **p<0.01. ANOVA followed by Turkey's multiple comparison test

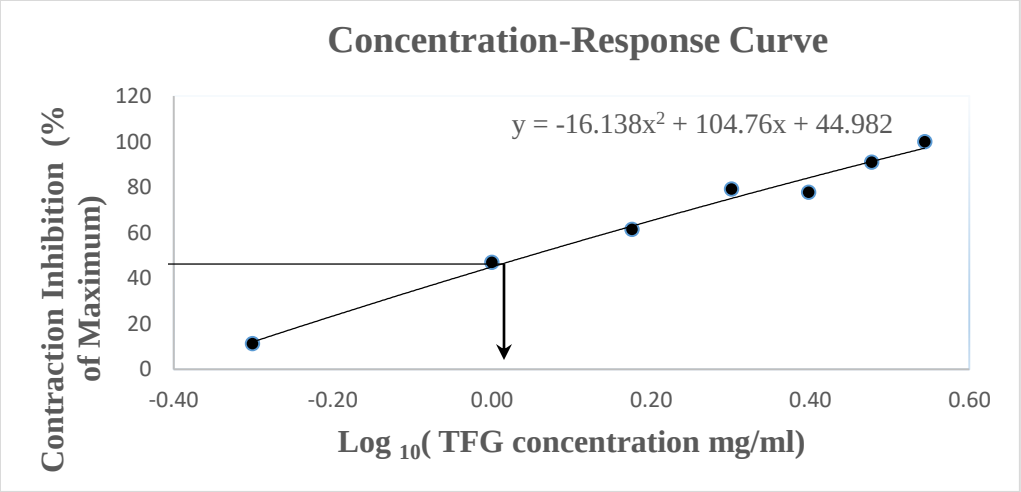


Figure 4.6: Dose–response curve of freeze-dried extracts of TFG on the spontaneous contraction of isolated jejunum with an ED 50 of 1.09 mg/ml

4.5.2 Effect of freeze-dried extract of TFG on Acetylcholine-induced contraction

There were significant increases in percentage inhibition of jejunal force of acetylcholine-induced contraction between different TFG dose study-groups: [7.19 ± 3.464 % (0.5 mg/ml TFG) vs. (39.48 ± 5.56 % (1.0 mg/ml TFG) vs. (67.56 ± 11.57 % (1.5 mg/ml TFG) vs. (80.42 ± 6.66 % (2.0 mg/ml TFG) vs. (75.71 ± 7.526 % (2.5 mg/ml TFG) vs. (70.86 ± 10.66 % (3.0 mg/ml TFG) vs. (74.44 ± 6.587 % (3.5 mg/ml TFG): ($F(6, 28) = 11.59$): $P < 0.0001$).]. Post-hoc statistical analysis with Tukey's multiple comparisons test revealed that there were significant differences between the following groups: 0.5 mg TFG vs. 1.5 mg TFG ($p = 0.0002$), 0.5 mg TFG vs. 2.0 mg TFG ($p < 0.0001$), 0.5 mg TFG vs. 2.5 mg/ml TFG ($p < 0.0001$), 0.5 mg TFG vs. 3.0 mg/ml TFG ($p < 0.0001$), 0.5 mg TFG vs. 3.5 mg/ml TFG ($p < 0.0001$), 1.0 mg TFG vs. 2.0 mg/ml TFG ($p = 0.0154$), and 1.0 mg TFG vs 2.5 mg/ml TFG ($p = 0.0273$) as shown in Figure 4.7 while Figure 4.8 shows a dose-response curve with an ED 50 of 0.83 mg/ml. Figure 4.9 on the other hand shows a

representative Powerlab tracings of the effect of TFG on spontaneous and acetylcholine-induced contraction of isolated rabbit's jejunum.

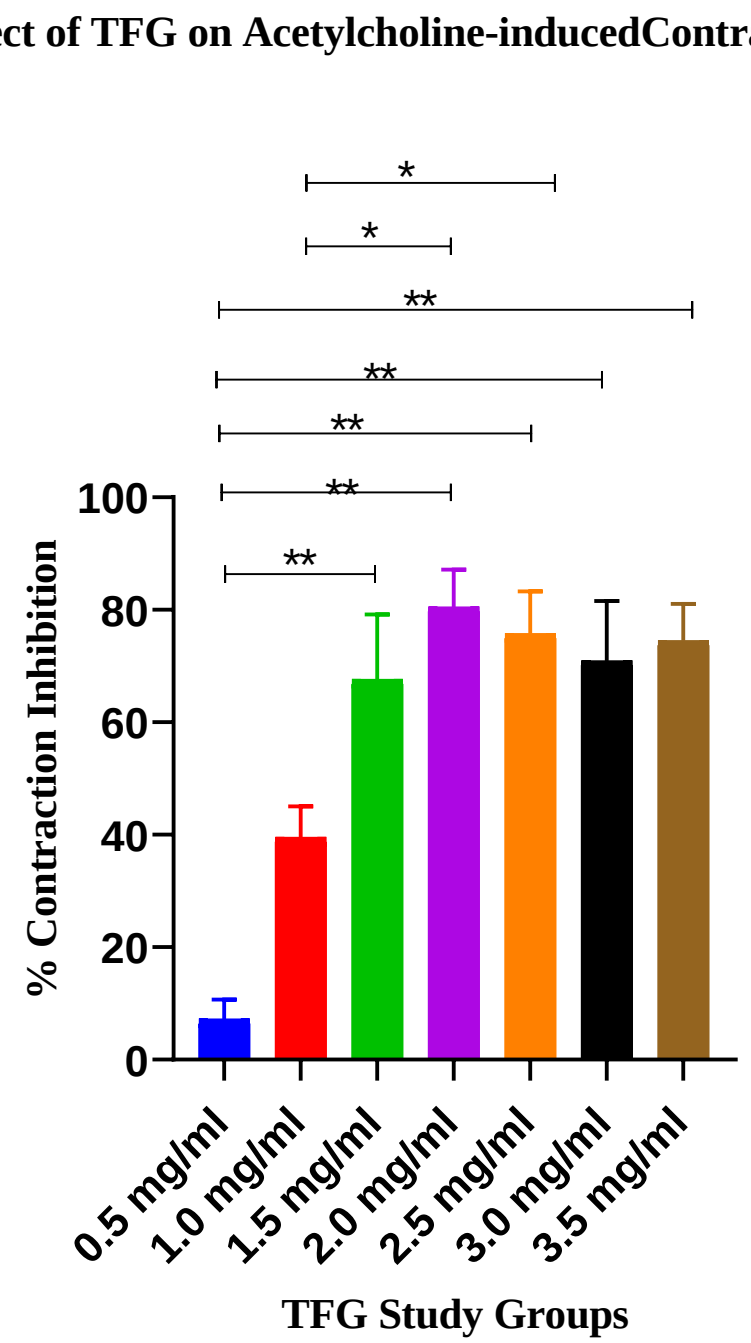


Figure 4.7: Effect of different concentrations of TFG on acetylcholine-induced contraction of an isolated rabbit jejunum. *p<0.05, **p<0.01. ANOVA followed by Turkey's multiple comparison test

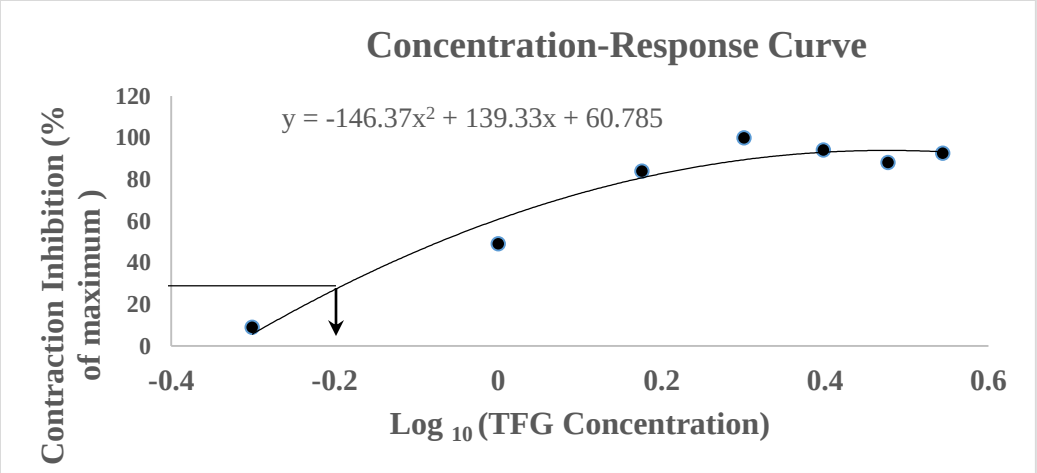


Figure 4.8: Dose–response curve of freeze-dried extracts of TFG on the acetylcholine-induced contraction of isolated jejunum with an ED 50 of 0.83 mg/ml

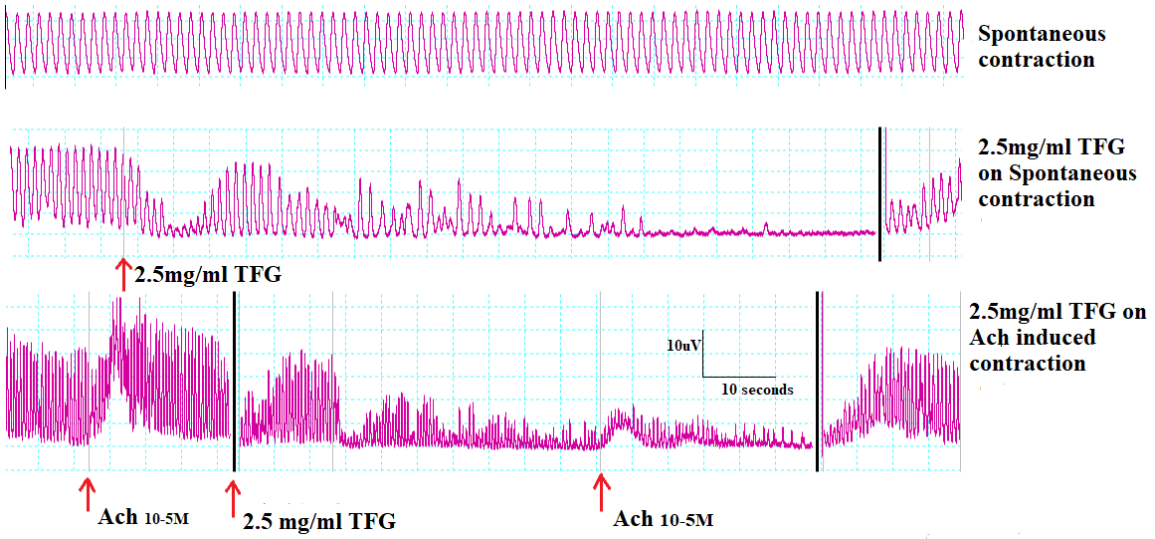


Figure 4.9: Representative Powerlab tracings showing spontaneous contractions and the effect of freeze-dried extract of TFG on spontaneous and acetylcholine (10-5 M)-induced contraction of isolated rabbit's jejunum

4.5.3 Effect TFG extracts on 80mM KCl-induced contraction.

There were significant increases in percentage inhibition of 80mM KCl-induced jejunal force of contraction between different TFG dose study-groups: [17.19 ± 7.484 % (0.5 mg/ml TFG test group) vs. (46.80 ± 10.26 % (2.5 mg/ml TFG test group) vs. (61.94 ± 4.712% (3.5 mg/ml TFG test group): (F (3, 12) = 7.147, P = 0.0052)]. Post-hoc statistical analysis using Tukey's multiple comparisons test revealed that there were significant differences between 0.5 mg TFG vs. 2.5 mg TFG (p = 0.0489) and 0.5mg TFG test group vs. 3.5 mg TFG (p = 0.0043) groups. The dose-response curve is as shown in Figure 4.10. Figure 4.11 below shows a Powerlab tracings illustrating the effect of TFG extracts on 80 mM KCl-induced jejunal contraction.

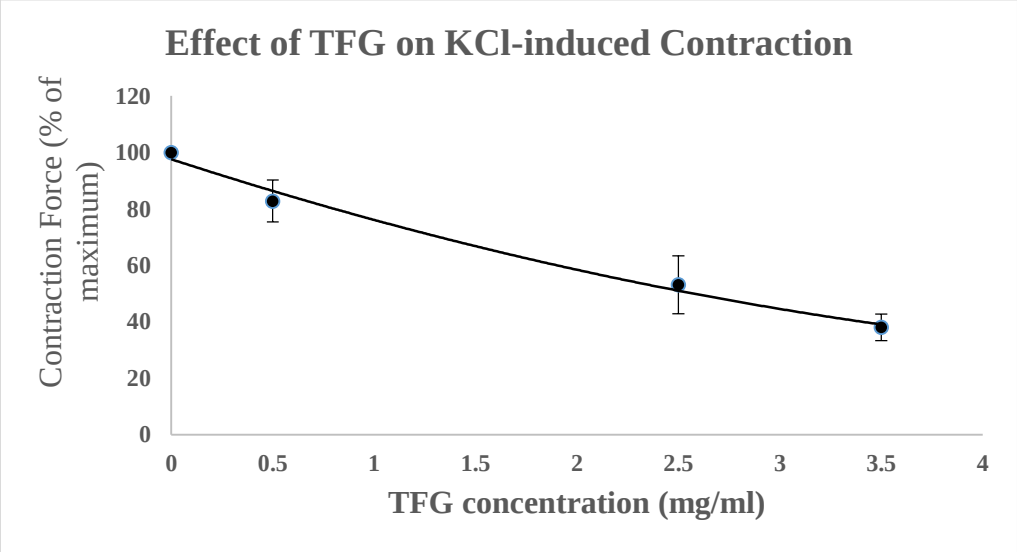


Figure 4.10: Dose-response curve of freeze-dried extract of TFG on 80mM KCl-induced contraction of isolated rabbit's jejunum

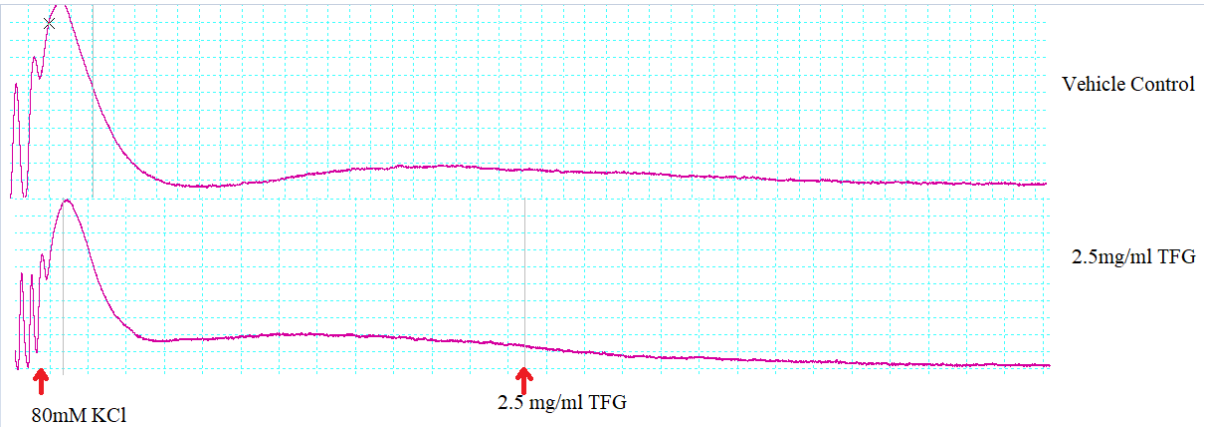


Figure 4.11: Representative Powerlab Tracings showing sustained contraction in vehicle control and spasmolytic effect of 2.5mg/ml of freeze-dried extract of TFG in a 80mM KCl-induced jejunal contraction

4.5.4 Effect of freeze-dried extract of TFG on $CaCl_2$-induced contraction

There were significant decreases in percentage contraction induced by 0.0003M calcium chloride between different experimental study groups: [24.61 ± 2.01 % (negative control) vs. (13.89 ± 2.22% (2.5 mg/ml TFG test group) vs. (12.02 ± 0.9% (3.5 mg/ml TFG test group) vs. (15.20 ± 2.12 % (positive control, 0.025 mM Verapamil): (F (4, 20) = 9.127, P = 0.0002).]. Post-hoc statistical analysis using Tukey's multiple comparisons test revealed significant differences between negative control vs 2.5 mg/ml TFG test group (P = 0.0146), negative control vs 3.5mg/ml TFG test group (P = 0.0037), and negative control vs positive control (P = 0.00372).

There were significant decreases in percentage contraction induced by 0.003M calcium chloride between different experimental study groups: [84.49 ± 2.01 % (negative control) vs. (48.30 ± 9.36% (2.5 mg/ml TFG test group) vs. (15.88 ± 2.39% (positive control, 0.025 mM Verapamil): (F (4, 20) = 5.413, P = 0.004).]. Post-hoc statistical analysis using Tukey's multiple comparisons

test revealed that there were significant differences between negative control vs 2.5 mg/ml TFG test group ($P = 0.0383$), and negative control vs positive control ($P < 0.0001$) groups.

There were significant decreases in percentage contraction induced by 0.03 M calcium chloride between different experimental study groups: [100 ± 0.00 % (negative control) vs. (55.62 ± 6.20% (1.0 mg/ml TFG test group) vs. (59.48 ± 9.36 % (2.5 mg/ml TFG test group) vs. (65.51 ± 8.78% (3.5 mg/ml TFG test group) vs. (17.11 ± 2.45% (positive control, 0.025 mM Verapamil): ($F (4, 20) = 27.94$, $P < 0.0001$).]. Post-hoc statistical analysis using Tukey's multiple comparisons test revealed that there were significant differences between negative control vs 1 mg/ml TFG test group ($P = 0.0002$), negative control vs 2.5 mg/ml TFG test group ($P = 0.0005$), negative control vs 3.5 mg/ml TFG test group ($P = 0.0033$), negative control vs verapamil positive control ($P < 0.0001$) groups as shown in Figure 4.12. The representative Powerlab tracings illustrating the inhibitory effect of these blockers are shown in Figure 4.13.

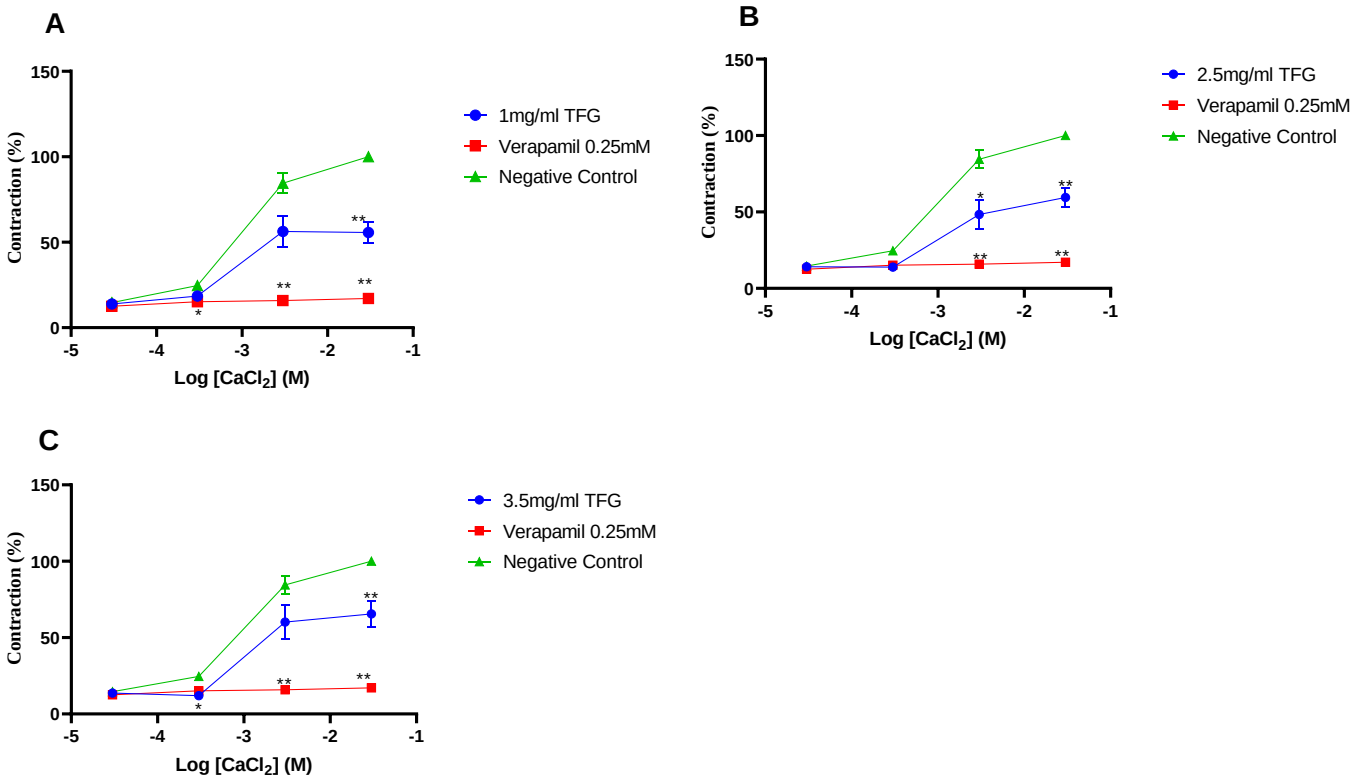


Figure 4.12: Effect of 1mg/ml (A), 2.5 mg/ml (B), 3.5 mg/ml (C) of TFG and verapamil on CaCl2 induced contraction of an isolated rabbit jejunum. Compared with negative control group: *p<0.05, **p<0.01. ANOVA followed by Turkey's multiple comparison test

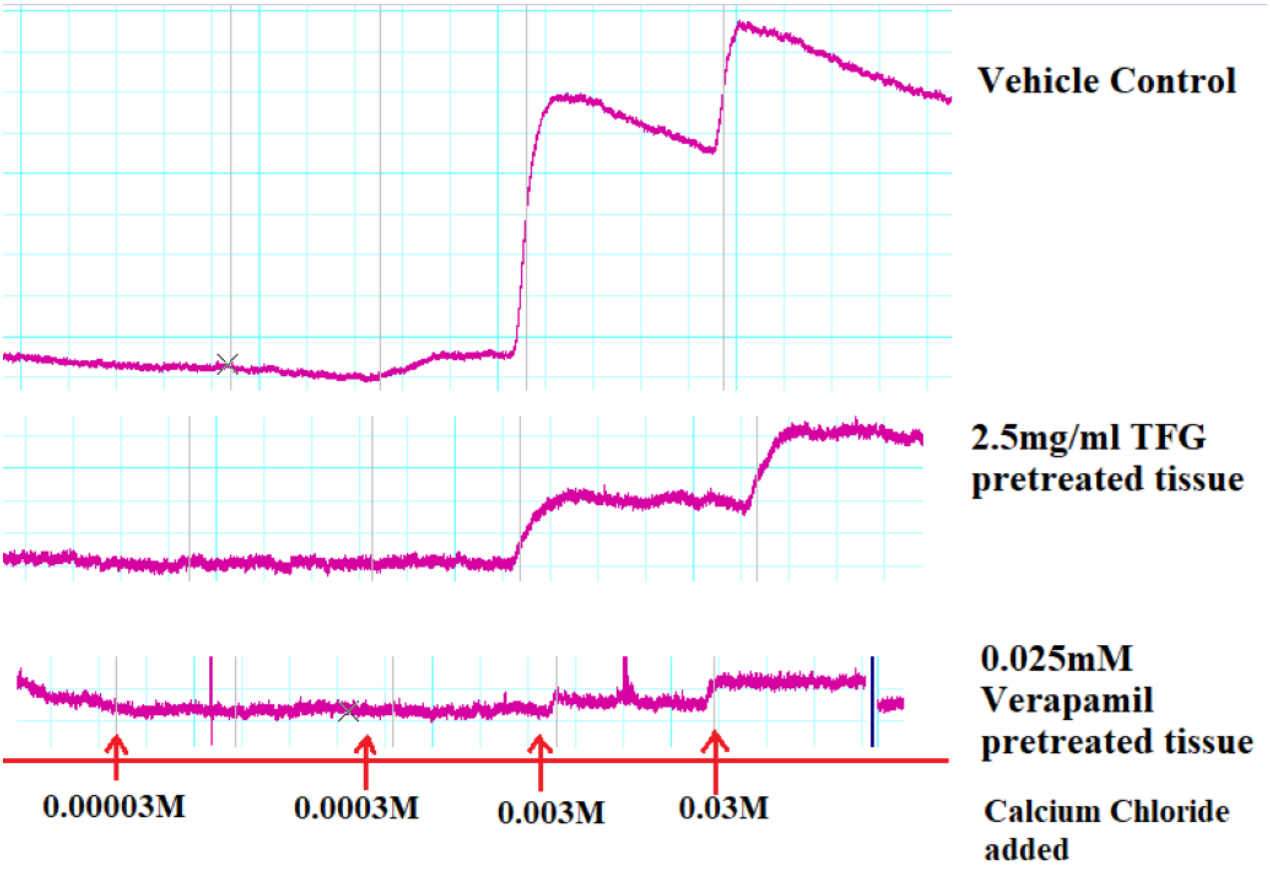


Figure 4.13: Representative Powerlab tracing showing attenuation effect of freeze-dried extract of TFG and 0.025 mM Verapamil on various concentrations of CaCl2-induced contraction in a depolarized isolated rabbit's jejunum using 80 mM KCl in calcium-free Tyrodes solution containing EDTA

4.5.5 Effect of various blockers on TFG-mediated relaxation of isolated rabbit's jejunum

There were no significant differences in percentage contraction inhibition caused by 0.5 mg/ml TFG between the negative control segments and those pretreated with various antagonists: [8.44 ± 4.45 % (negative control) vs 12.35 ± 4.17 % (naloxone) vs. 6.29 ± 2.14% (methylene blue) vs. 2.52 ± 1.23 % (L-NAME) vs 7.30 ± 4.09 % (prazosin) vs. 23.64 ± 5.25 % (propranolol) ($P > 0.05$)].

There were significant decreases in percentage contraction inhibition caused by 1.0 mg/ml TFG between the negative control segments and those pretreated with various antagonists: [35.15 ± 5.93% (negative control) vs. (42.27 ± 9.92 % (naloxone) vs. (7.31 ± 8.92 (methylene blue) vs. (2.09 ± 1.23 % (L-NAME) vs. (27.64 ± 9.048 % (prazosin) vs. (29.82 ± 3.33 % (propranolol) ($F(5,24) = 4.968$, $P = 0.0029$)]. Post hoc statistical analysis using Tukey's multiple comparisons test revealed that there were significant differences between the negative control and the L-NAME group ($p = 0.0341$).

There were significant decreases in percentage contraction inhibition caused by 1.5 mg/ml TFG between the negative control segments and those pretreated with various antagonists: [45.98 ± 4.28 % (negative control) vs. (22.10 ± 3.44 % (naloxone) vs. (11.03 ± 4.66 % (methylene blue) vs. (8.78 ± 7.20 % (L-NAME) vs. (39.50 ± 6.97 % (prazosin) vs. (45.32 ± 3.69 % (propranolol) ($F(5,24) = 10.28$, $P < 0.0001$)]. Post hoc statistical analysis using Tukey's multiple comparisons test revealed that there were significant differences between the negative control vs. L-NAME group ($p < 0.0005$) and the negative control vs. methylene blue ($p = 0.0011$).

There were significant decreases in percentage contraction inhibition caused by 2.5 mg/ml TFG between the negative control segments and those pretreated with various antagonists: [59.17 ± 6.63 % (negative control) vs. (25.81 ± 3.84 % (naloxone) vs. (24.70 ± 6.29 % (methylene blue) vs. (14.15 ± 2.63 % (L-NAME) vs. 57.26 ± 5.35 % (prazosin) vs. (55.22 ± 2.59 % (propranolol) (F

(5,24) =18.01, P = 0.0001)]. Post hoc statistical analysis using Tukey's multiple comparisons test revealed that there were significant differences between negative control vs. L-NAME ($p < 0.0001$), negative control vs. methylene blue ($p=0.0004$), and the negative control vs. naloxone ($P = 0.0007$).

There were significant decreases in percentage contraction inhibition caused by 3.5 mg/ml TFG between the negative control segments and those pretreated with various antagonists: [74.80 ± 2.11% (negative control) vs. (40.73 ± 5.00% (naloxone) vs. (25.50 ± 9.34% (methylene blue) vs. (28.67 ± 12.44% (L-NAME) vs. (58.90 ± 2.32% (prazosin) vs. (57.55 ± 10.87% (propranolol) ($F(5,24) = 5.656$, $P = 0.0014$)]. Post hoc statistical analysis using Tukey's multiple comparisons test revealed that there were significant differences between negative control vs L-NAME ($p = 0.0059$), and the negative control vs methylene blue ($p = 0.003$). These results are shown in Figure 4.14. The power lab representation of these results is shown in Figure 4.15.

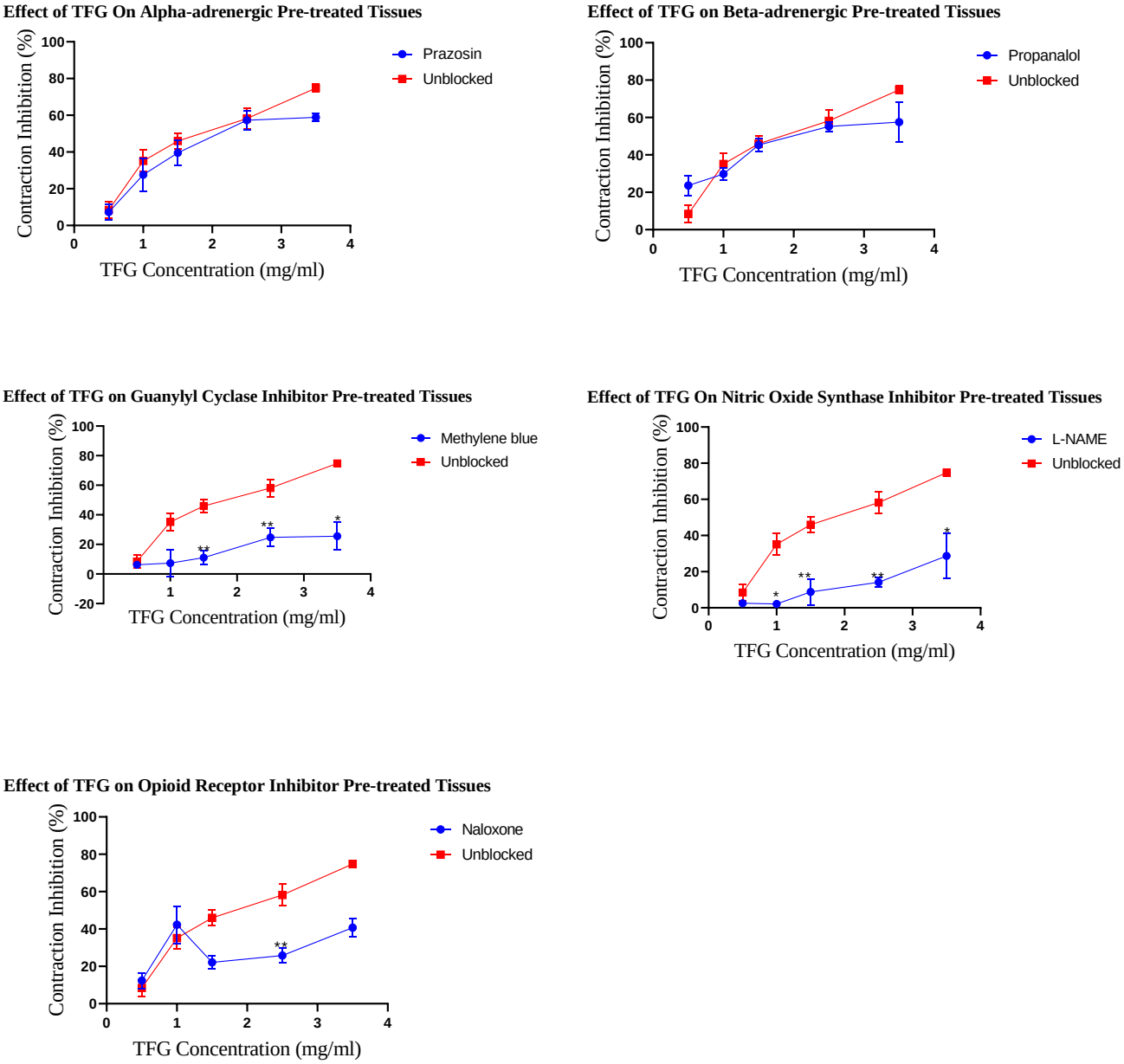


Figure 4.14: Effects prazosin, propranolol, methylene blue, L-NAME, and naloxone pretreated tissue to the freeze-dried extract *Tylosema fassoglense* on the contractile effect. Compared with vehicle control group: *p<0.05 **p<0.01. ANOVA followed by Turkey's multiple comparison test.

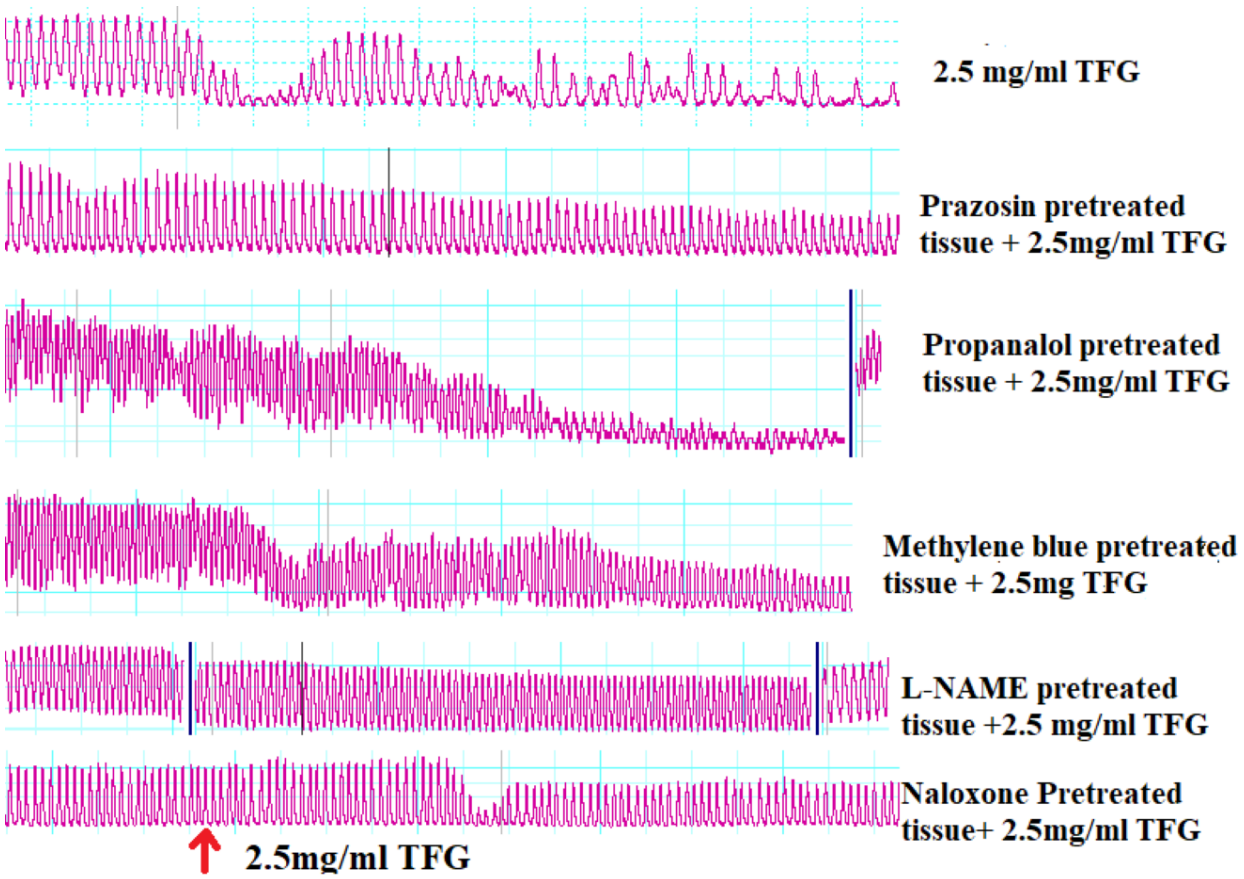


Figure 4.15: Representative Powerlab tracing showing the effect of freeze-dried extract of *Tylosema fassoglense* (TFG) on spontaneous contraction, prazosin, propranolol, methylene blue, L-NAME, and naloxone pretreated tissue

4.6 Phytochemical profile of *Tylosema fassoglense*

Chemical constituents in freeze-dried extracts of *Tylosema fassoglense* were characterized using Liquid chromatography-mass spectrometry (LC-MS). A total of 30 compounds were identified and characterized as shown in Table 4.3: twelve phenolic compounds (1-11, 16), fourteen flavonoids (12-15, 17-24, 27-28), and four alkaloids (25-26, 29-30). The chromatogram is shown in Figure 4.16.

Table 4.3: Characterization of compound of *Tylosema fassoglensis* extract by LC-MS

Number	RT	Name	Ion Mode	MH	% abundance
1	4.79	Gallic acid	Neg	169.1	1.2734
2	5.02	Protocatechuic acid	Neg	353	2.0882
3	6.33	4-Hydroxybenzoic acid	Neg	153	6.1958
4	7.03	Syringic acid	Neg	183	7.3075
5	7.41	Caffeic acid	Neg	179	1.0076
6	7.88	P-Coumaric acid	Neg	151	0.9068
7	8.92	Sinapic acid	Neg	163	4.2437
8	9.97	M-Coumaric acid	Neg	164	3.4253
9	12.9	Ferulic acid	Neg	609	1.7985
10	13.4	Cinnamic acid	Neg	611	5.1324
11	14.2	Catechin	Neg	463.1	3.1143
12	14.3	Rutin	Neg	137	2.2965
13	14.4	Naringin	Neg	138	0.7547
14	14.5	Hesperidin	Neg	317	0.5197
15	18.7	Fisetin	Neg	285	1.5329
16	22.3	Coumarin	Neg	147	1.3976
17	22.9	Quercetin	Neg	300.9	2.1639
18	23.2	Naringenin	Neg	271	1.1987
19	31.4	Hesperitin	Neg	301	3.9876
20	33.5	Luteolin	Neg	285	4.0843
21	33.7	Kaempferol	Neg	285.2	3.5478
22	33.8	Epigenin	Neg	269	2.6432
23	34.2	Rhamnetin	Neg	315.3	1.5435
24	37.5	Chrysin	Neg	253	2.5423
25	38.02	Chelerythine	Neg	537.2	3.6432
26	38.4	Piperine	Neg	653	1.5423
27	40.2	Proathocyanidin	Neg	753	11.5423
28	43.3	Quercitanin	Neg	553	1.5423
29	43.8	Hygrine	Neg	453	0.5423
30	45.1	Conine	Neg	372	0.2423

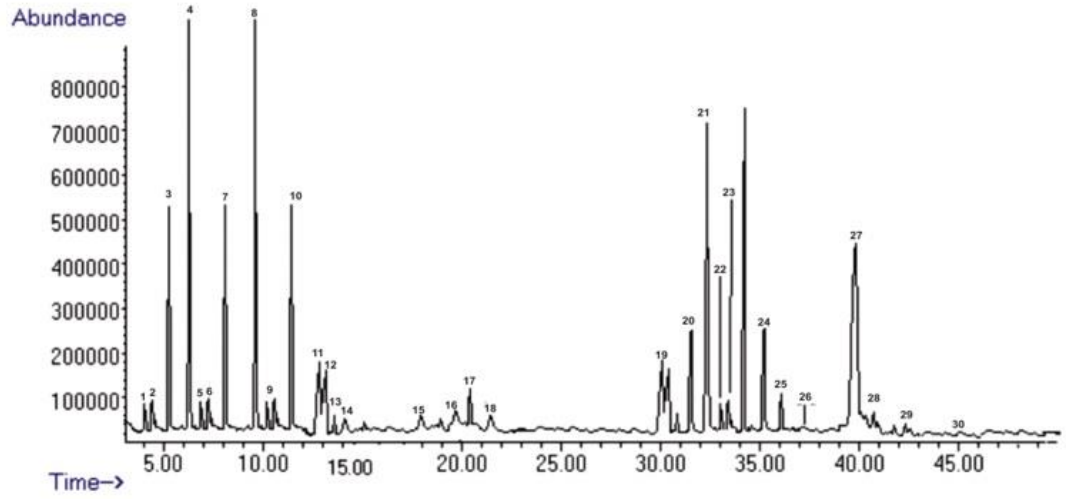


Figure 4.16: The LC-MS chromatogram of freeze-dried extract of *Tylosema fassoglense*

CHAPTER 5: DISCUSSION

5.1 Introduction

Diarrheal diseases account for 1 in 9 deaths of children under the age of 5 globally (CDC, 2015). The mortality rate is eleven-fold higher in children with comorbidities such as HIV (CDC, 2015). Despite advancements in therapeutics approaches such as the use of antibiotics in infectious diarrhea, bile acid sequestrants in patients with bile acid diarrhea(Camilleri and Vijayvargiya, 2020), fecal microbiota transplant for patients with gut dysbiosis, and rotavirus vaccines for children under the age of 5 years, mortality rates in developing countries have remained relatively high (Dennehy, 2008). This highlights the need for the introduction of cheaper, easily accessible, and efficacious antidiarrheal agents. The present study aimed to investigate the efficacy of the aqueous extracts of *Tylosema fassoglense* whose tubers have been used traditionally in Western Kenya for the treatment of diarrhea (Maundu et al., 1999). The study also evaluated the possible antidiarrheal mechanisms of actions of these extracts.

5.2 Efficacy studies

The freeze-dried extract of TFG caused significant reductions in fecal mass, number of diarrheic wet stools, and luminal enteropooling in the castor oil models of diarrhea. This indicated significant reductions in stool volume, frequency of defecation, and secretory activity, respectively. Orally administered castor oil undergoes digestion by pancreatic lipase in the small intestine resulting in the liberation of ricinoleic acid (Awouters et al., 1978) which acts via activation of G-protein-coupled E3 and E4 prostanoid receptors (Tunaru et al., 2012). The E3 Prostanoid receptors are predominantly expressed in intestinal smooth muscles (Bos et al., 2004). The binding to the prostanoid receptor E3 lead to the G_i -mediated inactivation of adenylyl cyclase and reduction in cytosolic cAMP levels (Bos et al., 2004). High concentrations of cAMP in the cytosol have been shown to cause calcium desensitization (Uchiyama and Chess-Williams, 2004).

The reduction of cAMP concentration therefore leads to increased force of contraction of the intestinal smooth muscle (Fukata et al., 2001). Ricinoleic acid has also been shown to bind onto the prostanoid receptor E4 that is variably distributed on the intestinal epithelial mucosa (Bos et al., 2004). Activation of these receptors leads to elevation in cAMP via activation of adenylyl cyclase which causes chloride hypersecretion via CFTR channels (Bos et al., 2004). Luminal fluid accumulation provides an additional propulsive drive that shortens the transit time leading to diarrhea (Awouters et al., 1978).

All the three doses of the extract tested had a significant effect on fecal mass and the number of wet stools but only the high dose of TFG (800mg/kg) had a significant effect on enteropooling. The extract's significant effect in reducing fecal mass output and the number of diarrheic wet stool in all the doses tested but exhibiting antisecretory activity only at the highest dose tested in the study is probably because it mainly mediates its effect via blockade of the E3 prostanoid receptors due to their greater levels of expression.

Extracts of TFG possessed significant effects on the peristaltic index at all the doses tested but only exhibited a significant effect on gastric emptying rate at the highest dose in the neostigmine-induced gastrointestinal motility experiment. Neostigmine induces a mild enteric cholinergic toxidrome at a dose of 0.01-0.05 mg/kg by inhibiting the activity of the acetylcholinesterase enzyme thus increasing the levels of endogenous acetylcholine (Li et al., 2018). Acetylcholine acts on M2 and M3 receptors on the intestinal smooth muscles causing an increase in the force of contraction thus enhancing gastrointestinal motility (Fukata et al., 2001). Attenuation of neostigmine-induced diarrhea points to the possible effects of the extracts on cholinergic signaling. While diarrhea mainly results from intestinal hyperactivity, rapid gastric emptying has been shown to amplify intestinal motility thus increasing diarrhea (Li et al., 2018).

There were seemingly contradictory differences in responsiveness to the extract where all doses had a significant reduction on intestinal transit rate but only the high dose had a significant effect on gastric emptying rate. The possible explanation of these observations is anchored on the premise that pre-prandial and post-prandial gastric contractions are qualitatively different from those of small intestines (Gill et al., 1989; Surjanhata et al., 2018) . It should be noted that the experimental animals were fasted for 24 hours before the commencement of the study. The pre-prandial gastric motility is characterized with quiescent contractions with temporal relations to coordinated migrating motor complex (MMC) (Gill et al., 1989). The MMC originate from the proximal stomach toward the small intestine with a frequency of 3 to 4 contractions per minute in the antral stomach and 11 cycles per minute in the duodenum (Deloose et al., 2012). Feeding abolishes the periodic MMC of the proximal and distal stomach and initiates random proximal and distal motility. Feeding also reduces the tone of the antral stomach which allows accommodation that serves to minimize intragastric pressure when gastric luminal content increase (Deloose et al., 2012). The distal stomach tends to have tonic contraction postprandially to enhance emptying of gastric content (Gill et al., 1989). The presence of luminal content in the duodenum also has been shown to reproduce the prandial effect (Gill et al., 1989). The prandial response of the small intestine on the other hand is mostly uniform and hence the expected differences in the degree of pharmacological stimulation (Surjanhata et al., 2018). These differences in both interdigestive and fed state motility most likely underlie the observed anatomical dose differences in terms of responsiveness to the extract.

5.3 Possible antidiarrheal mechanisms of action of the TFG Extracts

Ex-vivo studies using isolated rabbit's jejunum were performed to elucidate possible mechanisms of action of the extracts. The freeze-dried extract of TFG possessed a significant dose-dependent inhibitory effect on the spontaneous contraction of the isolated jejunal segments. The *in-vitro*

spasmolytic effects of TFG are an indication that the phytochemical constituents are intrinsically active and do not necessarily need microbiotic or metabolic biotransformation into active molecules to exert their actions.

The TFG extracts had dose-dependent inhibition of acetylcholine pre-contracted isolated jejunal segments. This demonstrates the possible presence of anticholinergic compounds within the extracts. The extracts may also possess alternative compounds that cause blockade of an influx of Ca^{2+} ions, the final common pathway in smooth muscle contraction (Jiang and Stephens, 1994).

The extracts significantly inhibited the 80mM KCl-induced contraction of the isolated jejunal segment. Substances possessing spasmolytic effects on smooth muscle contraction induced by high potassium chloride concentration solutions (KCl > 30mM) are considered calcium channel blockers (Li et al., 2018; Vogalis, 2000). This is because high potassium chloride concentration solutions cause membrane depolarization with the subsequent opening of voltage-gated calcium channels and resultant calcium-induced tonic contraction (Vogalis, 2000). The freeze-dried extracts of TFG had significant effects on calcium chloride-induced contraction and the effects paralleled those of verapamil, a use-dependent dihydropyridine channel blocker (Vogalis, 2000). The observed effect of TFG on calcium chloride-induced contraction is therefore confirmatory of extract's blocking activity on voltage-gated calcium channels.

Naloxone, a nonspecific opioid receptor blocker, significantly attenuated the spasmolytic effect of the freeze-dried extracts of TFG. The mu opioid receptors (MORs) are predominant in the gastrointestinal tract especially the interstitial cell of Cajal (ICC) and the enteric neurons (Feng, 2020). The binding of opioid agonists to MORs reduces tissue excitability thus causing hypomotility (Vogalis, 2000) and also inhibits secretory activity (Galligan and Akbarali, 2014).

Attenuation of the activity of the extracts through opioid blockade points to the possible activity of some extract components on the MORs.

Methylene blue and L-NAME significantly and independently attenuated the spasmolytic activity of the freeze-dried extracts of TFG. L-NAME is a nitric oxide synthase inhibitor (Qu et al., 2014). Nitric oxide synthase catalyzes the cleavage of endothelial L-arginine into citrulline with the liberation of nitric oxide gas in oxygen and NADPH-dependent reaction (Cartledge et al., 2001). Methylene blue on the other hand inhibits soluble guanylyl cyclase (Ragy and Elbassuoni, 2012). Nitric oxide (NO) activates soluble guanylate cyclase by binding on its heme component (Cartledge et al., 2001). Guanylyl cyclase hydrolyzes GTP into cGMP which activates protein kinase G (PKG) that in turn phosphorylates myosin light chain kinase (MLCK), reducing its activity (Khromov et al., 2006). This leads to calcium desensitization and hence smooth muscle relaxation (Khromov et al., 2006). The observed attenuation of the extract's spasmolytic effect in L-NAME and methylene blue pre-treated jejunal segments indicates that the spasmolytic effects of TFG extracts may be partially mediated by modulation of the NO-cGMP pathway.

There were no significant differences in the presence and absence of adrenergic blockers (Prazosin and propanol) in the TFG-mediated spasmolytic effect. This indicates that the spasmolytic activities of the freeze-dried extracts of TFG are possibly not mediated via modulation of adrenergic receptors.

5.4 Phytochemical components of TFG and their effects on diarrheal pathophysiology

It is observable from the foregoing discussion of ex-*vivo* findings that TFG extract possesses multiple spasmolytic signaling pathways. The possible explanation of these observations that plant extracts are multicomponent mixtures of bioactive compounds, some of which may have potentiation or antagonistic effects on each other. Indeed, the LC-MS characterized 30 compounds (phenolic, flavonoids, and alkaloids) which possibly act via different signaling pathways. This may

partially explain these multiple pathways some of which are seemingly contradictory to what is expected.

The LC-MS phytochemical profile of TFG indicated the presence of sizeable amounts of Proathocyanidin and Luteolin. Luteolin has been reported to possess a significant *in-vivo* antidiarrheal effect in mice (Sadraei et al., 2019). Proathocyanidin on the other hand has been shown to activate the NO-sGC-cGMP pathway in *ex-vivo* studies using isolated aorta of a rat (DalBó et al., 2008). The presence of these substances may therefore partly explain the significant effect of TFG extracts observed in this study.

5.5 Limitations of the study

The current study utilized a mixed animal model. Due to resource limitations, the only available force transducer in the Langendorff organ bath available for use was more sensitive to jejunal contractions of rabbits than those of rats. Therefore, the study utilized a mixed animal model i.e. Sprague Dawley rats in the *in-vivo* and isolated rabbit's jejunum in the *ex-vivo* studies. Consequently, the *in-vivo* results should be interpreted with caution alongside the *ex-vivo* findings since biological differences exist between animal species.

The phenol red experiments were conducted by pretreating the animals with neostigmine. Therefore, the question as to whether the extracts can cause gastroparesis remains unanswered.

5.6 Conclusion

The freeze-dried extracts of TFG significantly reduced the secretory effect of castor oil in enteropooling assay test, blocked the effect endogenous acetylcholine in neostigmine-induced diarrhea therefore significantly reducing the gastrointestinal motility, exerted inhibitory effect on the voltage-gated calcium channels in high potassium induced membrane depolarization thus significantly reducing *ex-vivo* contractile strength as well as enhancing signaling through the

nitrous oxide-cGMP pathway since presence of methylene blue and L-NAME significantly attenuated its spasmolytic effect on jejunal segments. These findings seem to validate the traditional use of the plant as an antidiarrheal medicine. These tubers would potentially be effective in multiple types of diarrhea since they possess both antisecretory and spasmolytic activities. Future studies should aim at trying to determine which of the major chemical moieties found in the tubers are responsible for mediating the antidiarrheal effects. In addition, although the results of this study indicate that extract derived from tubers of *Tylosema fassoglense* possess significant antidiarrheal activity, exploitation of this species should only take place after more rigorous acute and chronic toxicities have been done. It should be noted however that no signs of toxicities were observed while conducting these studies even at the highest doses used i.e. 800 mg/kg. Also, there need of establishing a commercial growing program to ensure sustainability.

5.7 Recommendation

The multiple-pathway physiological effect displayed by freeze-dried extracts of TFG provide promising future prospects. Due to the observed signaling effect on nitrous oxide-cGMP pathway, we recommend future studies to investigate the capacity TFG extract to inhibit platelets activation with subsequent inhibition of surface activation of clotting factors which is a leading thrombogenic event causing strokes. More so, we recommend future studies to focus on extract's inhibitory effect on voltage-gated calcium channels and possibility to manage hypertensive diseases as well as to reduce vasculature remodeling due to high blood flow and high blood pressure in hypertensive patients, a complication that causes high morbidity and mortality in these patients.

REFERENCES

Acácio, S., Nhampossa, T., Quintò, L., Vubil, D., Garrine, M., Bassat, Q., Farag, T., Panchalingam, S., Nataro, J.P., Kotloff, K.L., Levine, M.M., Tennant, S.M., Alonso, P.L., Mandomando, I., 2021. Rotavirus disease burden pre-vaccine introduction in young children in Rural Southern Mozambique, an area of high HIV prevalence. PLOS ONE 16, e0249714. https://doi.org/10.1371/journal.pone.0249714

Awouters, F., Niemegeers, C.J.E., Lenaerts, F.M., Janssen, P. a. J., 1978. Delay of castor oil diarrhoea in rats: a new way to evaluate inhibitors of prostaglandin biosynthesis. J J. Pharm. Pharmacol. 30, 41–45. https://doi.org/10.1111/j.2042-7158.1978.tb13150.x

Binder, 2010. Role of colonic short-chain fatty acid transport in diarrhea. - PubMed - NCBI. URL https://www.ncbi.nlm.nih.gov/pubmed/20148677.

Binder, H.J., 2010. Role of Colonic Short-Chain Fatty Acid Transport in Diarrhea. Annu. Rev. Physiol. 72, 297–313. https://doi.org/10.1146/annurev-physiol-021909-135817

Bos, C.L., Richel, D.J., Ritsema, T., Peppelenbosch, M.P., Versteeg, H.H., 2004. Prostanoids and prostanoid receptors in signal transduction. Int. J. Biochem. Cell Biol. . 36, 1187–1205.

Browning, K.N., 2015. Role of central vagal 5-HT3 receptors in gastrointestinal physiology and pathophysiology. Front Neurosci 9, 413. https://doi.org/10.3389/fnins.2015.00413

Camilleri, M., 2015. Bile Acid Diarrhea: Prevalence, Pathogenesis, and Therapy. Gut Liver 9, 332–339. https://doi.org/10.5009/gnl14397

Camilleri, M., Sellin, J.H., Barrett, K.E., 2017. Pathophysiology, Evaluation, and Management of Chronic Watery Diarrhea. Gastroenterology 152, 515-532.e2. https://doi.org/10.1053/j.gastro.2016.10.014

Camilleri, M., Vijayvargiya, P., 2020. The Role of Bile Acids in Chronic Diarrhea. Am J Gastroenterol | ACG 115, 1596–1603. https://doi.org/10.14309/ajg.0000000000000696

Cao, L., Yuan, Z., Liu, M., Stock, C., 2020. (Patho-)Physiology of Na+/H+ Exchangers (NHEs) in the Digestive System. Front. Physiol. 10. https://doi.org/10.3389/fphys.2019.01566

Cartledge, J., Minhas, S., Eardley, I., 2001. The role of nitric oxide in penile erection. Expert Opinion on Pharmacotherapy 2, 95–107. https://doi.org/10.1517/14656566.2.1.95

CDC, 2015. Global Diarrhea Burden | Global Water, Sanitation and Hygiene | Healthy Water | CDC. URL https://www.cdc.gov/healthywater/global/diarrhea-burden.html.

Cheng, H.M., Mah, K.K., Seluakumaran, K., 2020. Intestinal Fluid Handling: Absorption, in: Cheng, H.M., Mah, K.K., Seluakumaran, K. (Eds.), Defining Physiology: Principles, Themes, Concepts. Volume 2: Neurophysiology and Gastrointestinal Systems. Springer International Publishing, Cham, pp. 47–49. https://doi.org/10.1007/978-3-030-62285-5_13

Chingwaru, Vidmar, Kapewangolo, Mazimba, 2015. Therapeutic and Prophylactic Potential of Morama (Tylosema esculentum).

Crawford, S.E., Ramani, S., Tate, J.E., Parashar, U.D., Svensson, L., Hagbom, M., Franco, M.A., Greenberg, H.B., O'Ryan, M., Kang, G., Desselberger, U., Estes, M.K., 2017. Rotavirus infection. Nat. Rev. Dis. Primers 3, 1–16. https://doi.org/10.1038/nrdp.2017.83

DalBó, S., Goulart, S., Horst, H., Pizzolatti, M.G., Ribeiro-do-Valle, R.M., 2008. Activation of endothelial nitric oxide synthase by proanthocyanidin-rich fraction from Croton celtidifolius (Euphorbiaceae): involvement of extracellular calcium influx in rat thoracic aorta. J. Pharmacol. Sci. 107, 181–189. https://doi.org/10.1254/jphs.fp0072385
De Ponti, F., 2004. Pharmacology of serotonin: what a clinician should know. Gut 53, 1520–1535. https://doi.org/10.1136/gut.2003.035568
Degu, A., Engidawork, E., Shibeshi, W., 2016. Evaluation of the anti-diarrheal activity of the leaf extract of Croton macrostachyus Hocsht. ex Del. (Euphorbiaceae) in mice model. BMC Complement Altern. Med. 16, 379. https://doi.org/10.1186/s12906-016-1357-9
Deloose, E., Janssen, P., Depoortere, I., Tack, J., 2012. The migrating motor complex: control mechanisms and its role in health and disease. Nat Rev Gastroenterol Hepatol 9, 271–285. https://doi.org/10.1038/nrgastro.2012.57
Dennehy, P.H., 2008. Rotavirus Vaccines: an Overview. Clinical Microbiology Reviews 21, 198–208. https://doi.org/10.1128/CMR.00029-07
Dubois, M., Lognay, G., Baudart, E., Marlier, M., Severin, M., Dardenne, G., Malaisse, F., 1995. Chemical characterisation of Tylosema fassoglensis (Kotschy) Torre & Hillc oilseed. J. Sci. Food Agric. 67, 163–167. https://doi.org/10.1002/jsfa.2740670204
Duncan, A., Robertson, C., Russell, R.I., 1992. The fecal osmotic gap: Technical aspects regarding its calculation. The J. Lab. Med.. 119, 359–363. https://doi.org/10.5555/uri:pii:002221439290203W
Eads, J.R., Reidy-Lagunes, D., Soares, H.P., Chan, J.A., Anthony, L.B., Halfdanarson, T.R., Naraev, B.G., Wolin, E.M., Halperin, D.M., Li, D., Pommier, R.F., Zacks, J.S., Morse, M.A., Metz, D.C., Collaborative, from the C.S.C., 2020. Differential Diagnosis of Diarrhea in Patients With Neuroendocrine Tumors. Pancreas 49, 1123–1130. https://doi.org/10.1097/MPA.0000000000001658
Feng, B., 2020. New Insights on Expression and Function of Mu and Delta Opioid Receptors in Mouse Gastrointestinal Tract. Cell. Mol. Gastroenterol. Hepatol 9, 553–554. https://doi.org/10.1016/j.jcmgh.2020.01.001
Fern, K., 2018. Tylosema fassoglense - Useful Tropical Plants. http://tropical.theferns.info/viewtropical.php?id=Tylosema+fassoglense (accessed 5.6.19).
Field, M., 2003. Intestinal ion transport and the pathophysiology of diarrhea. J Clin Invest 111, 931–943. https://doi.org/10.1172/JCI200318326
Fukata, Y., Kaibuchi, K., Amano, M., Kaibuchi, K., 2001. Rho–Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. Trends in Pharmacological Sciences 22, 32–39. https://doi.org/10.1016/S0165-6147(00)01596-0
Fung, C., Vanden Berghe, P., 2020. Functional circuits and signal processing in the enteric nervous system. Cell. Mol. Life Sci. 77, 4505–4522. https://doi.org/10.1007/s00018-020-03543-6
Furness, J.B., Callaghan, B.P., Rivera, L.R., Cho, H.-J., 2014. The enteric nervous system and gastrointestinal innervation: integrated local and central control, in: Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease. Springer, pp. 39–71.
Galligan, J.J., Akbarali, H.I., 2014. Molecular Physiology of Enteric Opioid Receptors. Am J Gastroenterol Suppl 2, 17–21. https://doi.org/10.1038/ajgsup.2014.5

Geldre, V., Lefebvre, 2004. Interaction of NO and VIP in gastrointestinal smooth muscle relaxation. - PubMed - NCBI. URL https://www.ncbi.nlm.nih.gov/pubmed/15320758 (accessed 5.21.19).
George, P., 2011. Concerns regarding the safety and toxicity of medicinal plants-An overview. J. Appl. Pharm. Sci. 1, 40–44.
Gill, R.C., Pilot, M.-A., Thomas, P.A., Wingate, D.L., 1989. Effect of feeding on motor activity of Canine Stomach. Digest Dis Sci 34, 865–872. https://doi.org/10.1007/BF01540271
Hakomori, S., 1984. Ganglioside Receptors: A Brief Overview and Introductory Remarks, in: Ledeen, R.W., Yu, R.K., Rapport, M.M., Suzuki, K. (Eds.), Ganglioside Structure, Function, and Biomedical Potential, Advances in Experimental Medicine and Biology. Springer US, Boston, MA, pp. 333–339. https://doi.org/10.1007/978-1-4684-1200-0_28
Headstrom, P.D., Surawicz, C.M., 2005. Chronic Diarrhea. Clin. Gastroenterol. Hepatol. 3, 734–737. https://doi.org/10.1016/S1542-3565(05)00298-3
Iwasaki, M., Akiba, Y., Kaunitz, J.D., 2019. Recent advances in vasoactive intestinal peptide physiology and pathophysiology: focus on the gastrointestinal system. F1000Res 8. https://doi.org/10.12688/f1000research.18039.1
Jiang, H., Stephens, N.L., 1994. Calcium and smooth muscle contraction. Mol Cell Biochem 135, 1–9. https://doi.org/10.1007/BF00925956
Kasırga, E., 2019. The importance of stool tests in diagnosis and follow-up of gastrointestinal disorders in children. Turk Pediatri Ars 54, 141–148. https://doi.org/10.14744/TurkPediatriArs.2018.00483
Khromov, A.S., Wang, H., Choudhury, N., McDuffie, M., Herring, B.P., Nakamoto, R., Owens, G.K., Somlyo, A.P., Somlyo, A.V., 2006. Smooth muscle of telokin-deficient mice exhibits increased sensitivity to Ca2+ and decreased cGMP-induced relaxation. PNAS 103, 2440–2445. https://doi.org/10.1073/pnas.0508566103
Kotloff, K.L., Platts-Mills, J.A., Nasrin, D., Roose, A., Blackwelder, W.C., Levine, M.M., 2017. Global burden of diarrheal diseases among children in developing countries: Incidence, etiology, and insights from new molecular diagnostic techniques. Vaccine, The 2016 Vaccines Against and Enterotoxigenic (VASE) Conference 35, 6783–6789. https://doi.org/10.1016/j.vaccine.2017.07.036
Levine, G.A., Walson, J.L., Atlas, H.E., Lamberti, L.M., Pavlinac, P.B., 2017. Defining Pediatric Diarrhea in Low-Resource Settings. J Pediatric Infect Dis Soc 6, 289–293. https://doi.org/10.1093/jpids/pix024
Li, H., Qu, Y., Zhang, Jiawei, Zhang, Jingze, Gao, W., 2018. Spasmolytic activity of Aquilariae Lignum Resinatum extract on gastrointestinal motility involves muscarinic receptors, calcium channels and NO release. Pharmaceutical Biology 56, 559–566. https://doi.org/10.1080/13880209.2018.1492000
Manatsathit, S., Dupont, H.L., Farthing, M., Kositchaiwat, C., Leelakusolvong, S., Ramakrishna, B., Sabra, A., Speelman, P., Surangsrirat, S., 2002. Guideline for the management of acute diarrhea in adults. J. Gastroenterol. Hepatol 17, S54–S71. https://doi.org/10.1046/j.1440-1746.17.s1.11.x
Marks, S.L., 2013. Diarrhea. Canine and Feline Gastroenterology 99–108. https://doi.org/10.1016/B978-1-4160-3661-6.00011-0

Miller, M.A., Parkman, H.P., Urbain, J.-L.C., Brown, K.L., Donahue, D.J., Knight, L.C., Maurer, A.H., Fisher, R.S., 1997. Comparison of Scintigraphy and Lactulose Breath Hydrogen Test for Assessment of Orocecal Transit (Lactulose Accelerates Small Bowel Transit). Dig Dis Sci 42, 10–18. https://doi.org/10.1023/A:1018864400566
Montoro-Huguet, M.A., Belloc, B., Domínguez-Cajal, M., 2021. Small and Large Intestine (I): Malabsorption of Nutrients. Nutrients 13, 1254. https://doi.org/10.3390/nu13041254
Mulatya, D.M., Ochieng, C., 2020. Disease burden and risk factors of diarrhoea in children under five years: Evidence from Kenya's demographic health survey 2014. Int. J. Infect. Dis. 93, 359–366. https://doi.org/10.1016/j.ijid.2020.02.003
Murray, J., 2013. Diarrhoea due to small bowel diseases. URL https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3621726/ (accessed 5.20.19).
Niccum, B.A., Stein, D.J., Behm, B.W., Hays, R.A., 2018. Zinc Deficiency and the Recurrence of Clostridium difficile Infection after Fecal Microbiota Transplant: A Retrospective Cohort Study. J Nutr Metab 2018, 9682975. https://doi.org/10.1155/2018/9682975
Patel, Crutchley, Tucker, Cottreau, Garey, 2013. Crofelemer for the treatment of chronic diarrhea in patients living with HIV/AIDS. - PubMed - NCBI. PubMed. URL https://www.ncbi.nlm.nih.gov/pubmed/23888120 (accessed 5.1.19).
Pattni, S., Walters, J.R.F., 2009. Recent advances in the understanding of bile acid malabsorption. British Medical Bulletin 92, 79–93. https://doi.org/10.1093/bmb/ldp032
Pham, T., Perry, J.L., Dosey, T.L., Delcour, A.H., Hyser, J.M., 2017. The Rotavirus NSP4 Viroporin Domain is a Calcium-conducting Ion Channel. Scientific Reports 7, 43487. https://doi.org/10.1038/srep43487
Qu, Z., Zhang, J., Gao, W., Guo, H., Liu, C., 2014. Bidirectional effects of methanol extract of Wei-Chang-An pill on gastrointestinal transit and the spasmolytic activity on isolated rat jejunum. J Ethnopharmacol 155, 203–212. https://doi.org/10.1016/j.jep.2014.05.017
Quattrocchi, U., 2016. CRC World Dictionary of Medicinal and Poisonous Plants: Common Names, Scientific Names, Eponyms, Synonyms, and Etymology (5 Volume Set). CRC Press.
Ragy, M., Elbassuoni, E., 2012. The role of nitric oxide and l-type calcium channel blocker in the contractility of rabbit ileum in vitro. J Physiol Biochem 68, 521–528. https://doi.org/10.1007/s13105-012-0167-x
Sadraei, H., Ghanadian, S.M., Moazeni, S., 2019. Inhibitory effect of hydroalcoholic and flavonoids extracts of Dracocephalum kotschyi, and its components luteolin, apigenin and apigenin-4'-galactoside on intestinal transit in mice. J Herbmed Pharmacol 8, 8–13. https://doi.org/10.15171/jhp.2019.02
Sagar, L., Sehgal, R., Ojha, S., 2005. Evaluation of antimotility effect of Lantana camara L. var. acuelata constituents on neostigmine induced gastrointestinal transit in mice. BMC Complement Altern. Med. 5, 18. https://doi.org/10.1186/1472-6882-5-18
Schäfer, M.-H., Eiden, L.E., Weihe, E., 1998. Cholinergic neurons and terminal fields revealed by immunohistochemistry for the vesicular acetylcholine transporter. II. The peripheral nervous system. Neuroscience 84, 361–376.
Seifter, J.L., Chang, H.-Y., 2017. Disorders of Acid-Base Balance: New Perspectives. Kidney Dis (Basel) 2, 170–186. https://doi.org/10.1159/000453028

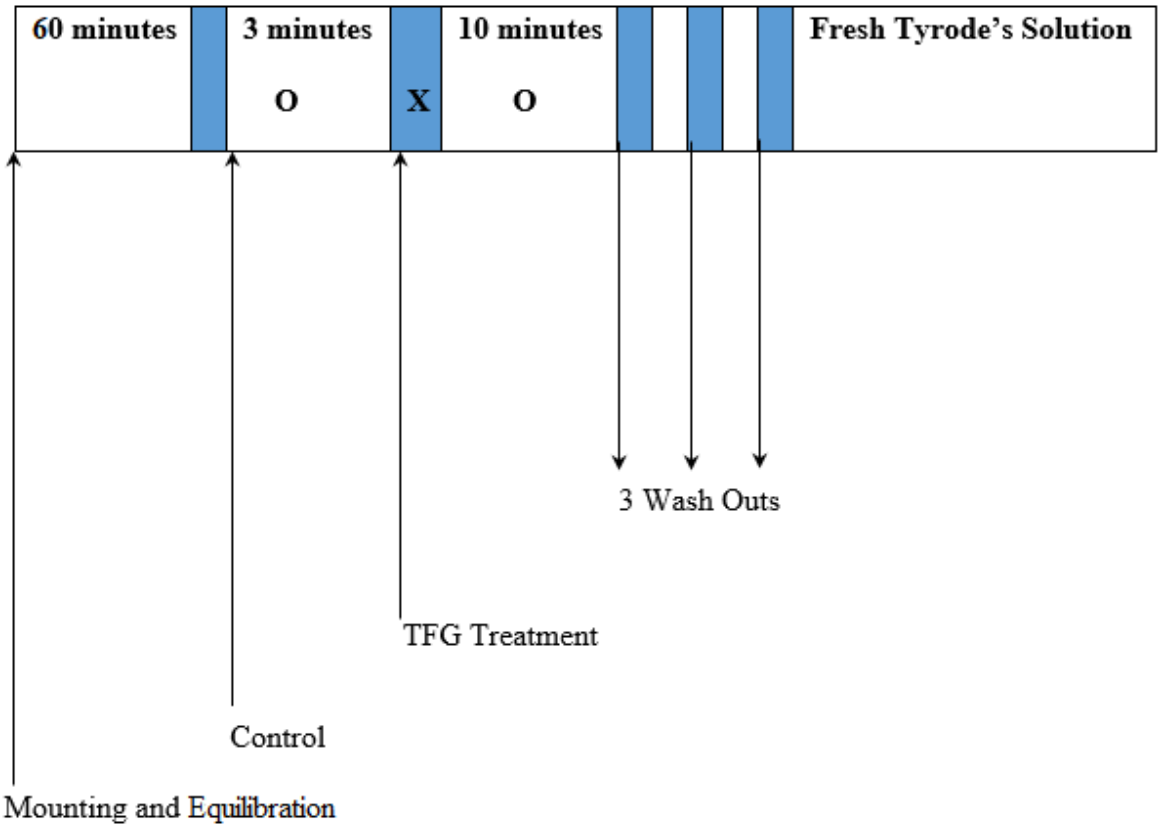
Shane, A.L., Mody, R.K., Crump, J.A., Tarr, P.I., Steiner, T.S., Kotloff, K., Langley, J.M., Wanke, C., Warren, C.A., Cheng, A.C., Cantey, J., Pickering, L.K., 2017. 2017 Infectious Diseases Society of America Clinical Practice Guidelines for the Diagnosis and Management of Infectious Diarrhea. Clin Infect Dis 65, e45–e80. https://doi.org/10.1093/cid/cix669
Spiller, R., 2006. Role of motility in chronic diarrhoea. Neurogastroenterol. Motil. 18, 1045–1055. https://doi.org/10.1111/j.1365-2982.2006.00836.x
Sternini, L., De Giorgio, Greenwood-Van Meerveld, 2016. Enteroendocrine Cells: A Review of Their Role In Brain-Gut Communication. URL https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4842178/.
Surjanhata, B., Brun, R., Wilding, G., Semler, J., Kuo, B., 2018. Small bowel fed response as measured by wireless motility capsule: Comparative analysis in healthy, gastroparetic, and constipated subjects. Neurogastroenterol Motil 30, e13268. https://doi.org/10.1111/nmo.13268
Thiagarajah, J., Donowitz, M., Verkman, A., 2015. Secretory diarrhoea: mechanisms and emerging therapies. URL https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4786374/.
Tunaru, S., Althoff, T.F., Nüsing, R.M., Diener, M., Offermanns, S., 2012. Castor oil induces laxation and uterus contraction via ricinoleic acid activating prostaglandin EP3 receptors. Proceedings of the National Academy of Sciences 109, 9179–9184.
Turkia, H.B., Ahmed, Y.H., Bucheeri, M.A., Eldssouky, H.A., 2018. Congenital Chloride Diarrhea in Two Yemeni Siblings. Bahrain Medical Bulletin 40.
Uchiyama, K., Kishi, H., Komatsu, W., Nagao, M., Ohhira, S., Kobashi, G., 2018. Lipid and Bile Acid Dysmetabolism in Crohn's Disease. J. Immunol. Res. 2018, 1–6. https://doi.org/10.1155/2018/7270486
Uchiyama, T., Chess-Williams, R., 2004. Muscarinic receptor subtypes of the bladder and gastrointestinal tract. J. Smooth Muscle Res. 40, 237–247. https://doi.org/10.1540/jsmr.40.237
Vilander, A.C., Hess, A., Abdo, Z., Ibrahim, H., Doumbia, L., Douyon, S., Koné, K., Boré, A., Zambrana, L.E., Vilchez, S., Koita, O., Ryan, E.P., 2022. A Randomized Controlled Trial of Dietary Rice Bran Intake on Microbiota Diversity, Enteric Dysfunction, and Fecal Secretory IgA in Malian and Nicaraguan Infants. *J Nutr* nxac087. https://doi.org/10.1093/jn/nxac087
Vogalis, F., 2000. Potassium channels in gastrointestinal smooth muscle. J. Auton. Pharmacol. 20, 207–219. https://doi.org/10.1046/j.1365-2680.2000.00183.x
World Health Organization, 2017. Diarrhoeal disease. URL https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease.
World Weather & Climate Information, 2022. Climate and average weather in Kenya. World Weather & Climate Information. URL https://weather-and-climate.com:80/average-monthly-Rainfall-Temperature-Sunshine-in-Kenya.
Yu, B., Jiang, Y., Liu, Y., Ma, T., Yang, H., 2015. Prevention of secretory diarrhea by ethanol extract of Bistortae rhizoma through inhibition of chloride channel. Bangladesh J. Pharmacol. 10, 533–542. https://doi.org/10.3329/bjp.v10i3.23260
Yun, C., Oh, S., Zizak, M., Steplock, D., Yun, C., Tsao, S., Ming, C., Weinman, E., Donowitz, M., 1997. cAMP-mediated inhibition of the epithelial brush border Na+/H+ exchanger, NHE3, requires an
associated regulatory protein.https://www.ncbi.nlm.nih.gov/pmc/articles/PMC20313

APPENDICES

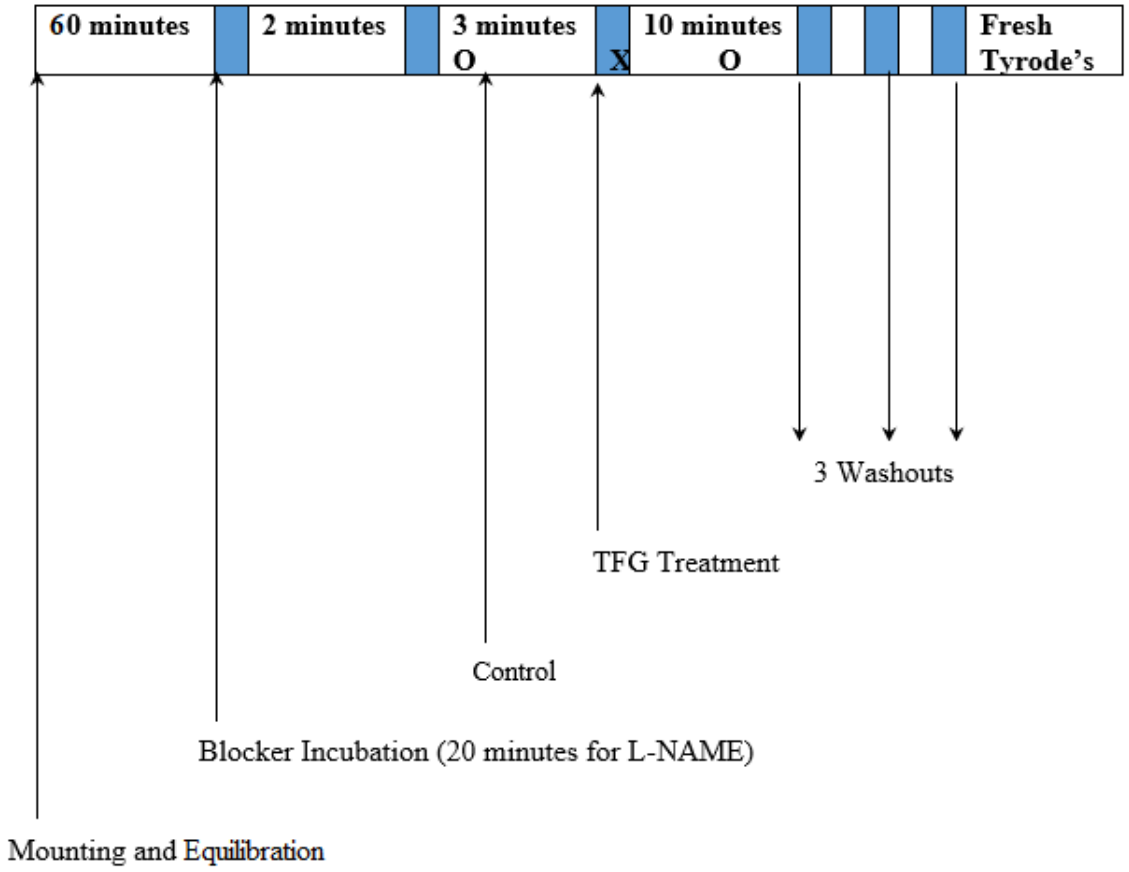
Appendix 1: Tyrodes Solution

	Grams/liter		
SALT	Normal Tyrodes	Calcium Free Tyrodes	80 mM KCl Tyrode's
NaCl	8	3.325	3.325
KCl	0.2	5.964	5.964
NaHCO3	1	1	1
NaHPO4	0.05	0.05	0.05
Glucose	1	2	2
MgCl	0.1	0.1	0.1
CaCl2	0.2	0	0.2
EDTA	0	0.029	0

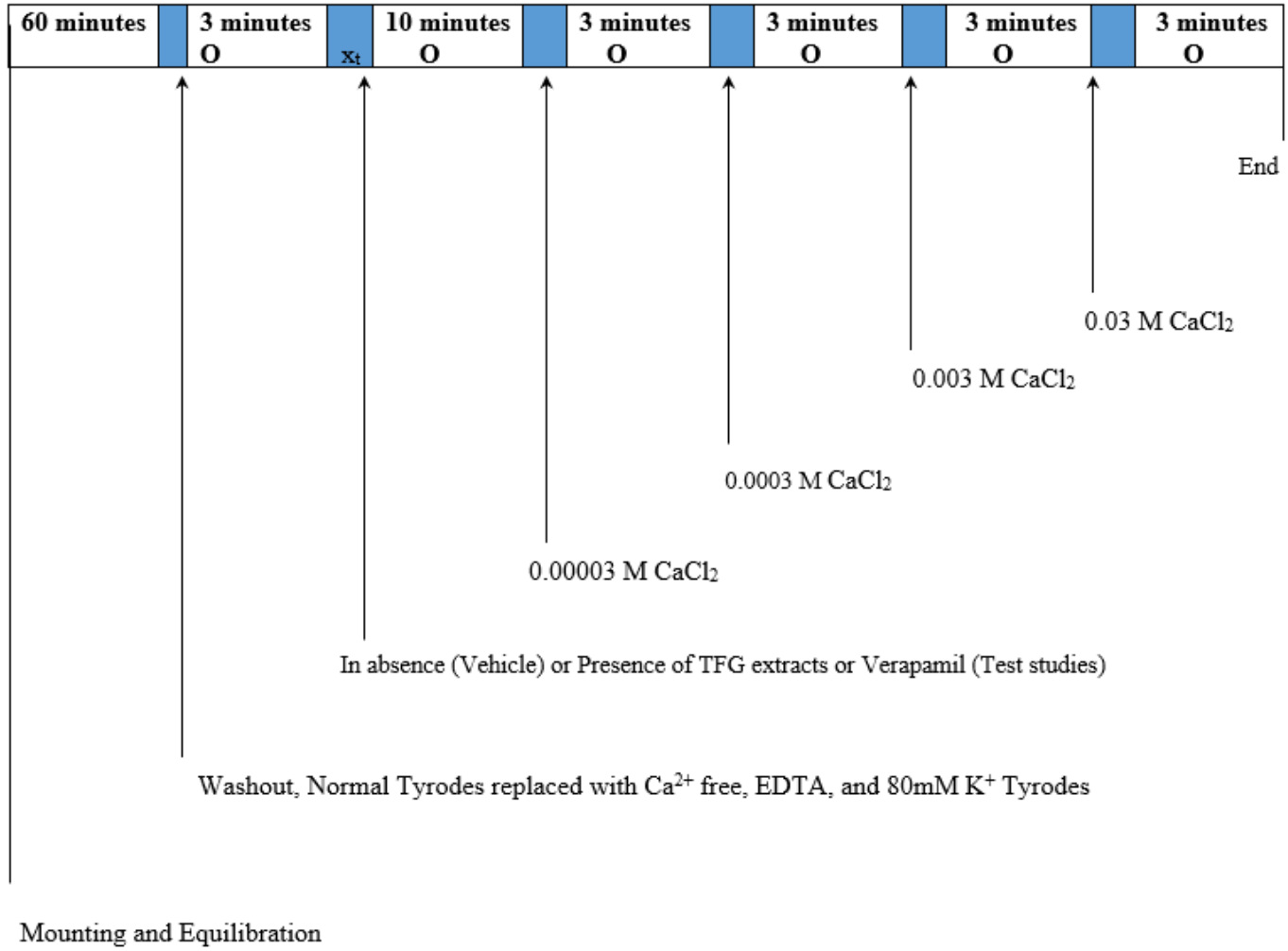
Appendix 2: The pre-test post-test design in the determination of the effect of TFG extracts on the spontaneous contraction of isolated rabbits jejunal segment [observation (O), experimental treatment (X), observation (O)]



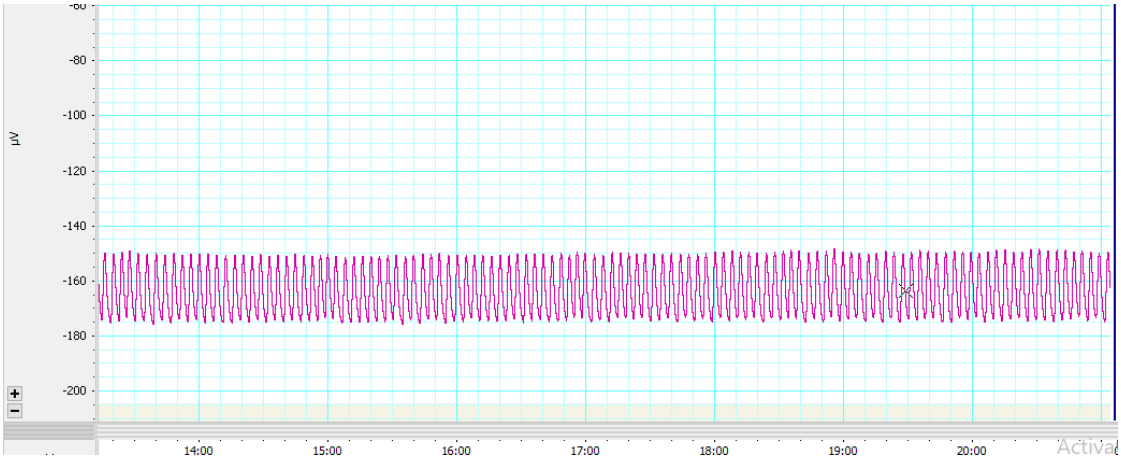
Appendix 3: The pre-test post-test design for determination of effect various blockers (prazosin, propranolol, methylene blue, L-NAME, and naloxone) on the spasmolytic effect of TFG on the contraction of isolated rabbit's jejunal segment (O-X-O)



Appendix 4: The pre-test post-test control study design for determination of effect various concentrations of TFG and verapamil on the spasmogenic effect of various concentrations of calcium chloride.

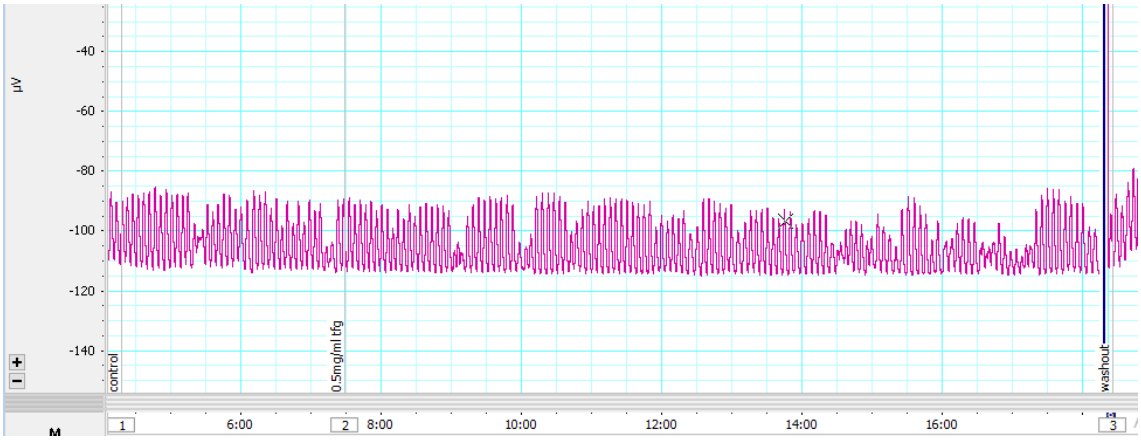


Appendix 5: Representative Powerlab tracing of spontaneous jejunal Contractions

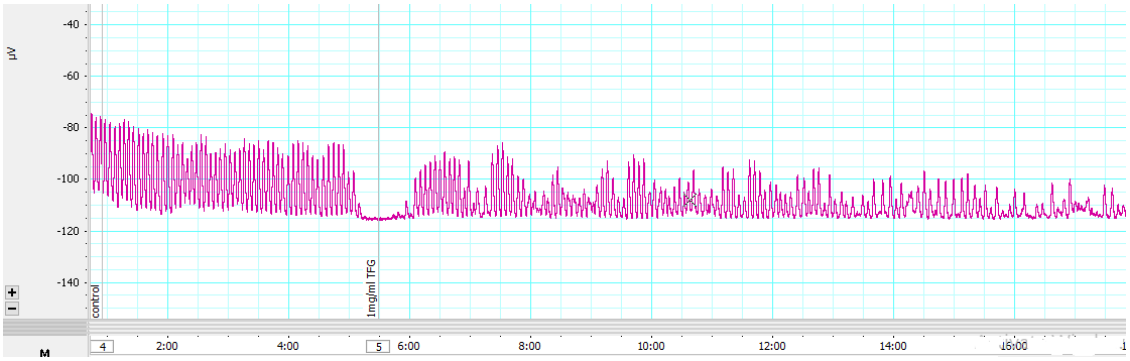


Appendix 6: Representative Powerlab tracings of the spasmolytic effect of various TFG concentrations on the spontaneous contraction of isolated jejunal segments.

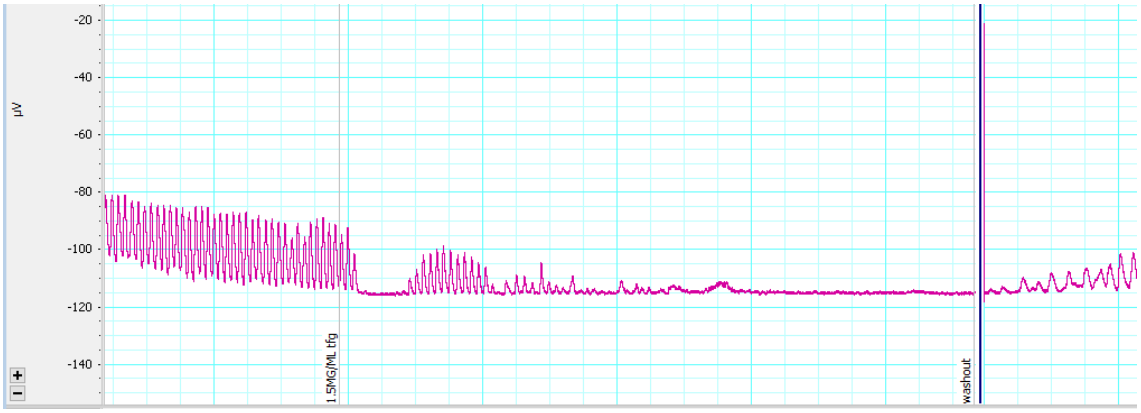
Spasmolytic effect of 0.5 mg of TFG



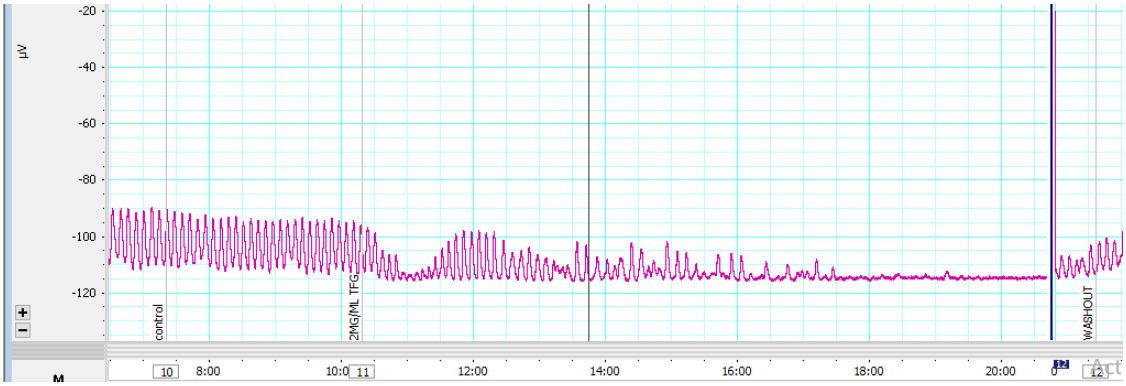
Spasmolytic effect of 1.0 mg of TFG



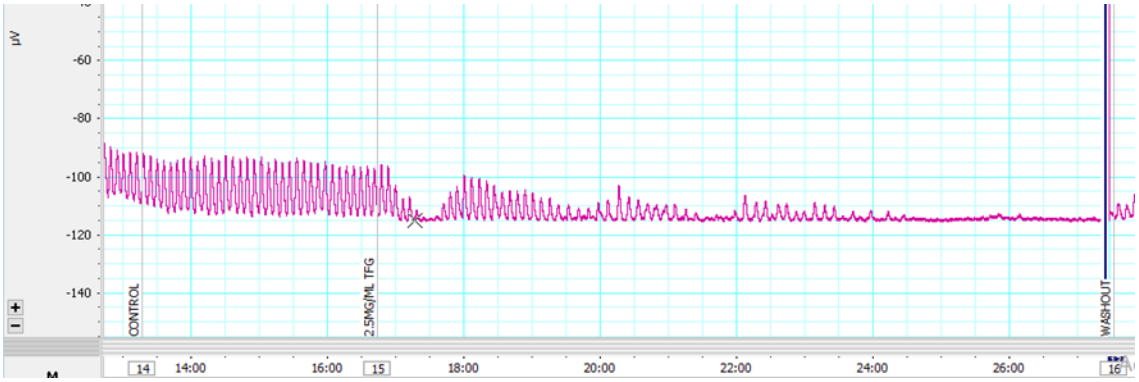
Spasmolytic effect of 1.5mg/ml of TFG



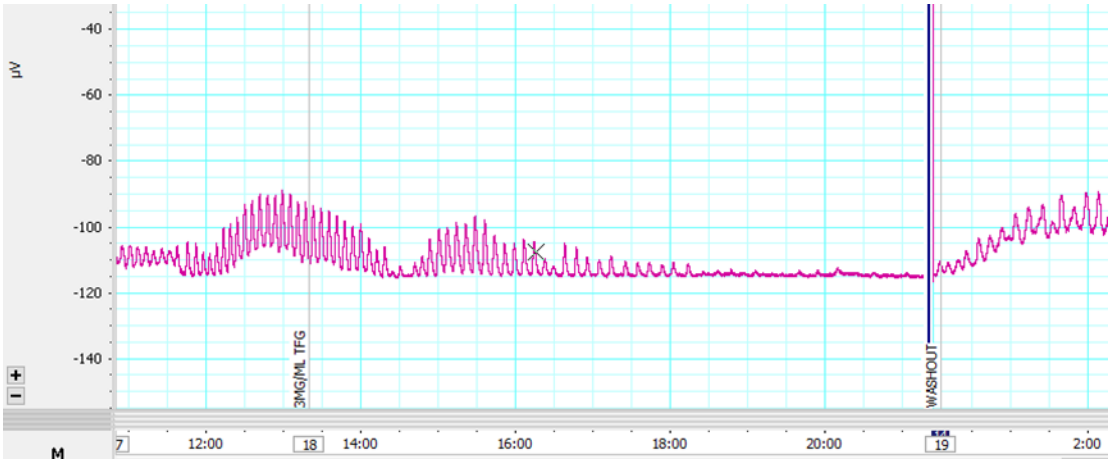
Spasmolytic effect of 2.0 mg/ml of TFG



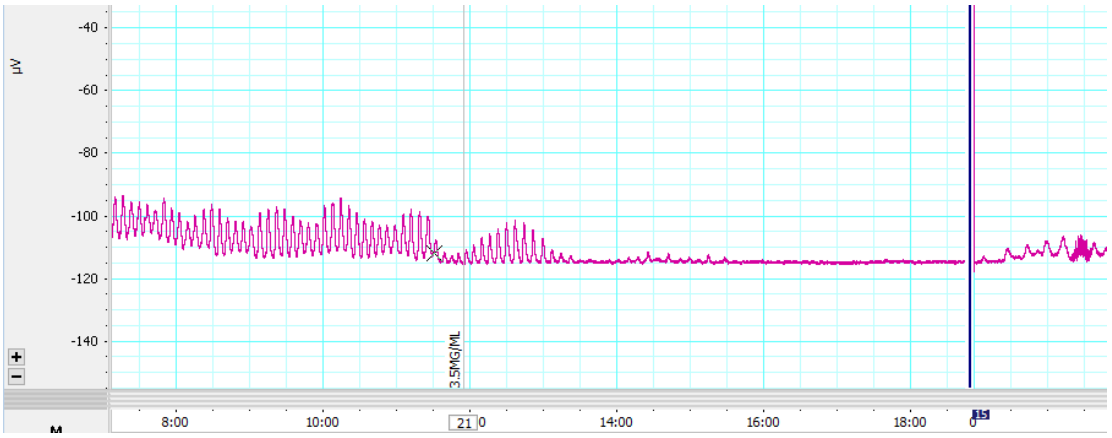
Spasmolytic effect of 2.5 mg/ml TFG



Spasmolytic effect of 3.0 mg/ml TFG

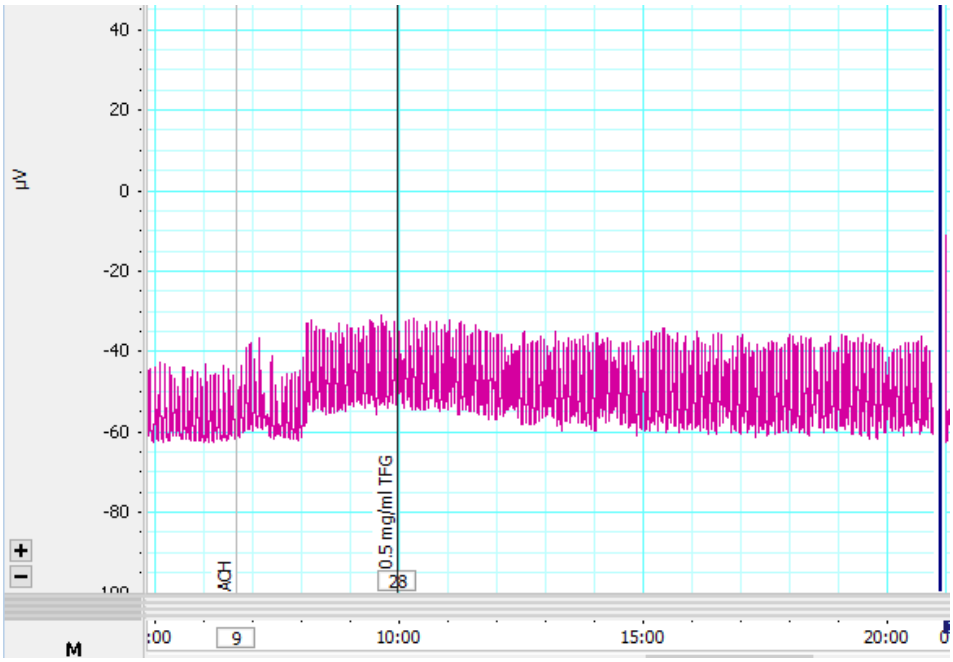


Spasmolytic effect of 3.5 mg/ml TFG

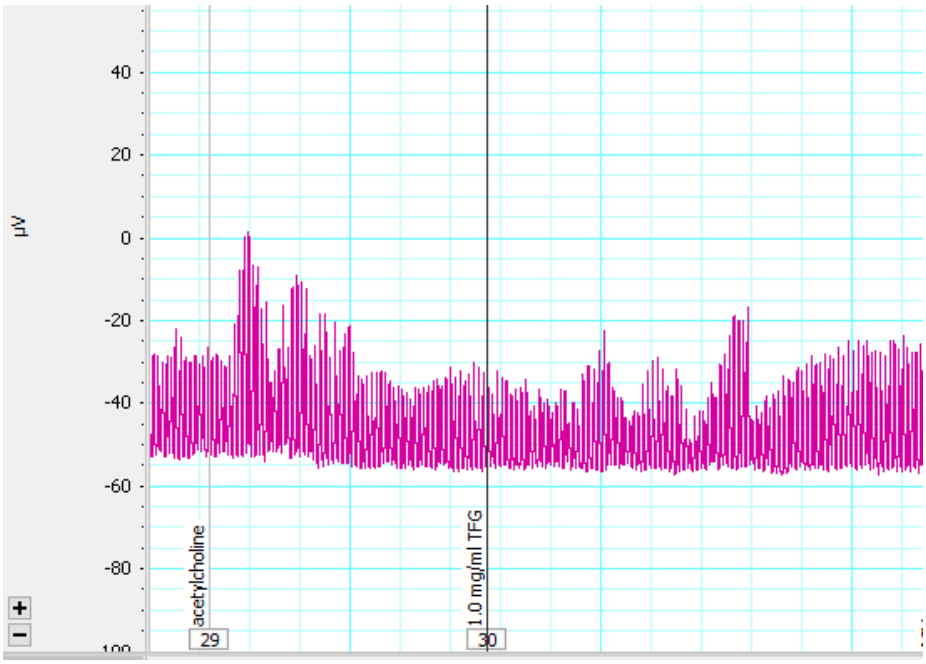


Appendix 7: Representative Powerlab tracings of the effect of TFG on the acetylcholine-induced contraction of isolated jejunal segments.

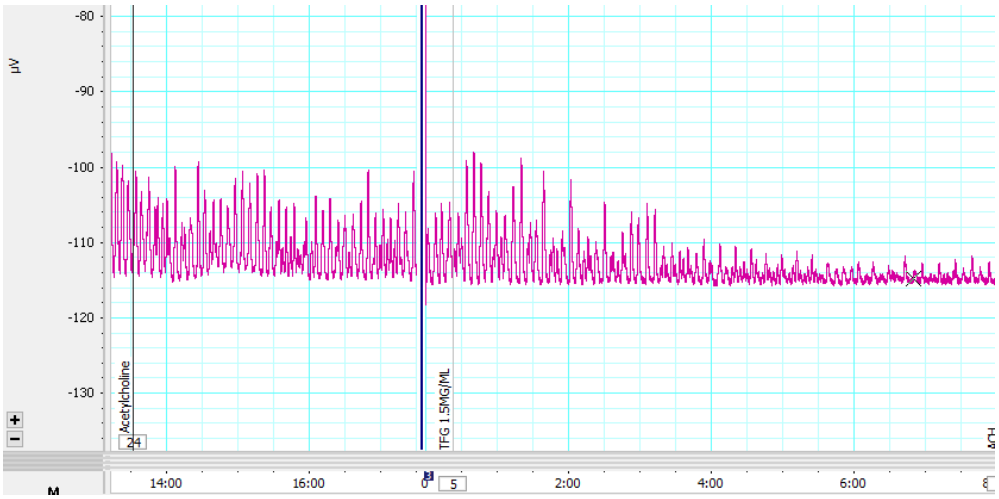
Effect of 0.5 mg/ml on acetylcholine-induced contraction



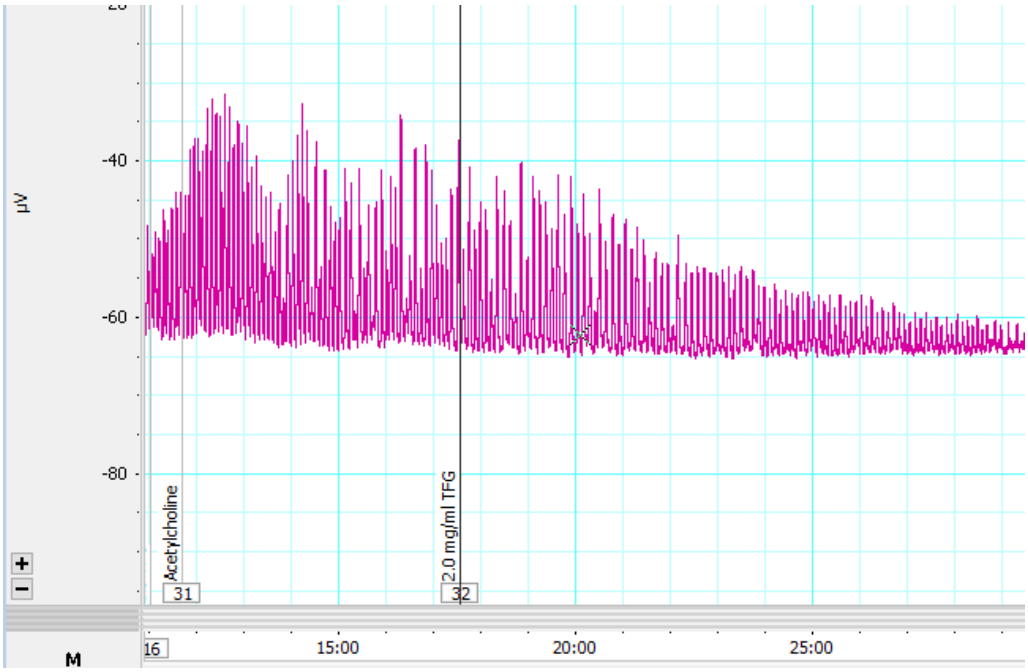
Effect of 1.0 mg/ml TFG on acetylcholine-induced contraction



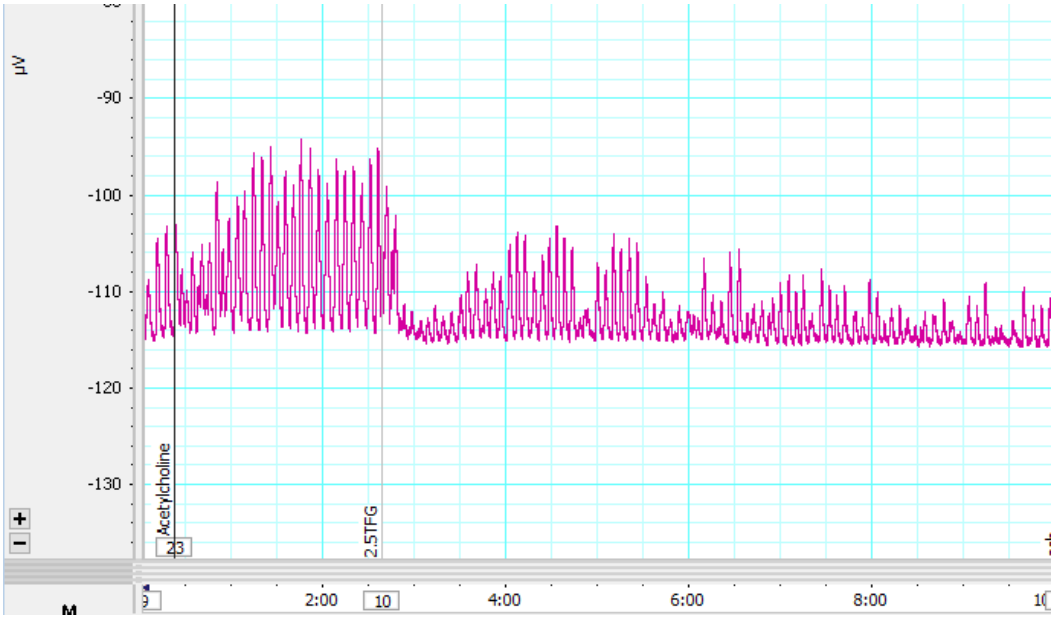
Effect of 1.5 mg/ml TFG on acetylcholine-induced contraction



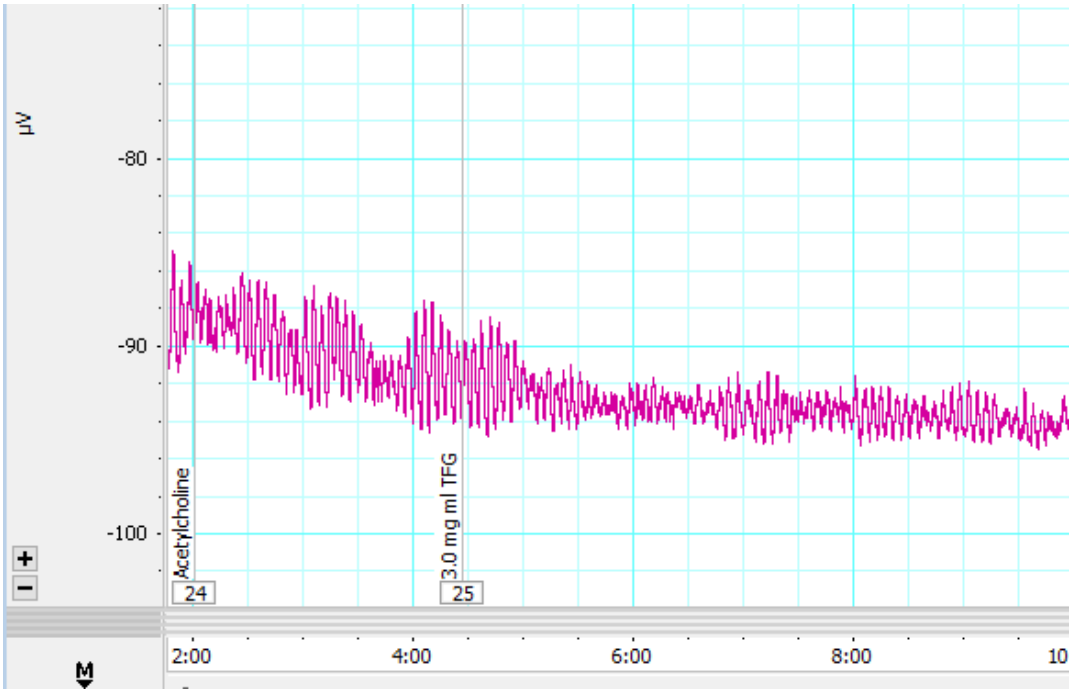
Effect of 2.0 mg/ml TFG on acetylcholine-induced contraction



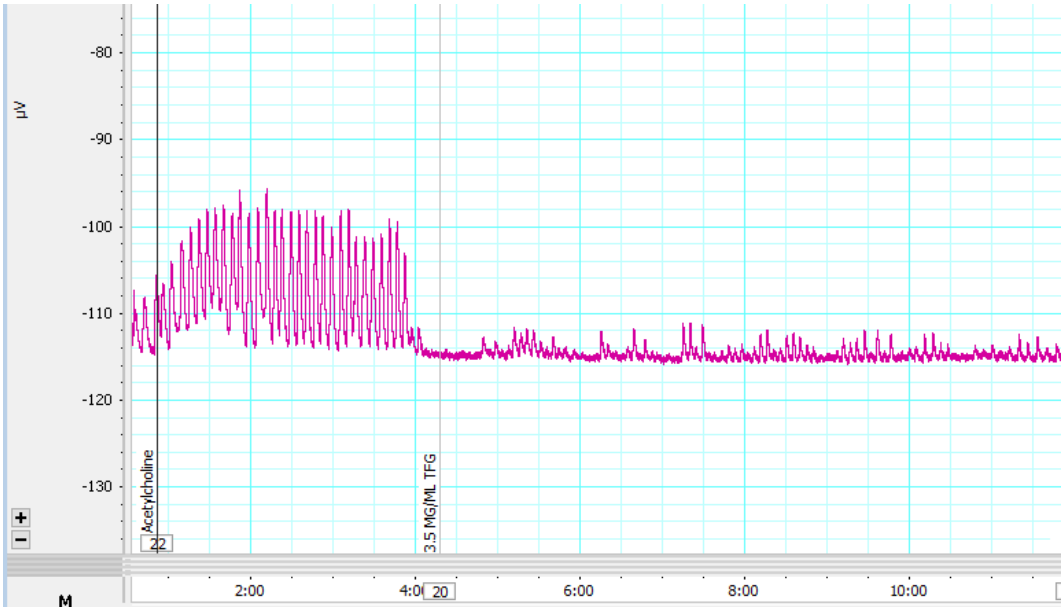
Effect of 2.5 mg/ml TFG on acetylcholine-induced contraction



Effect of 3.0 mg/ ml TFG on acetylcholine-induced contraction

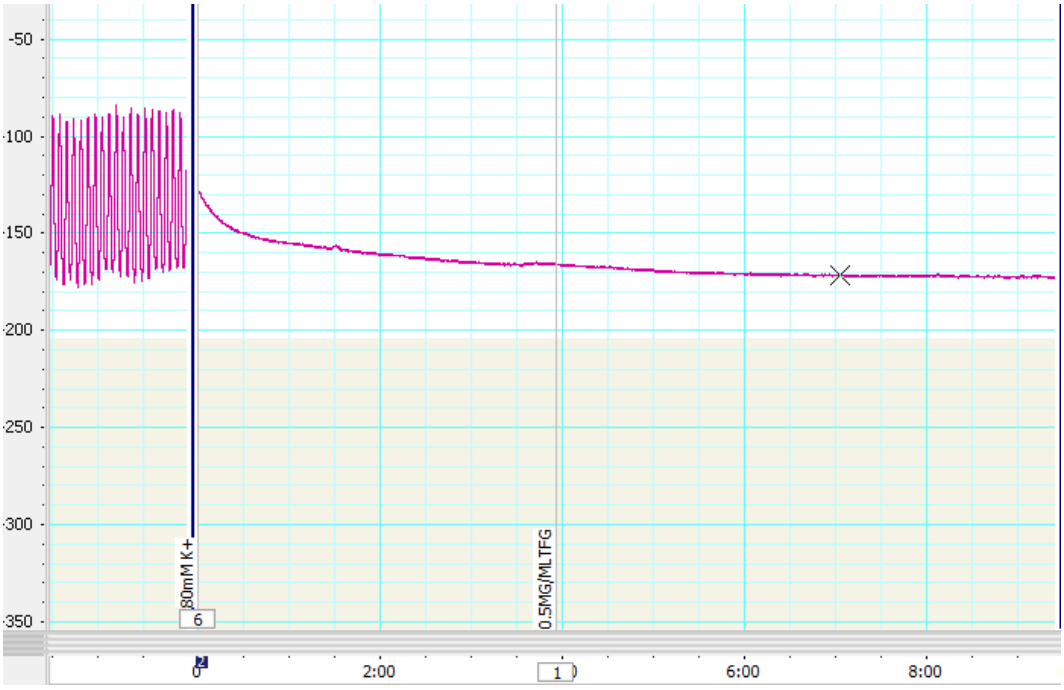


Effect of 3.5 mg/ml TFG on Acetylcholine-induced contraction

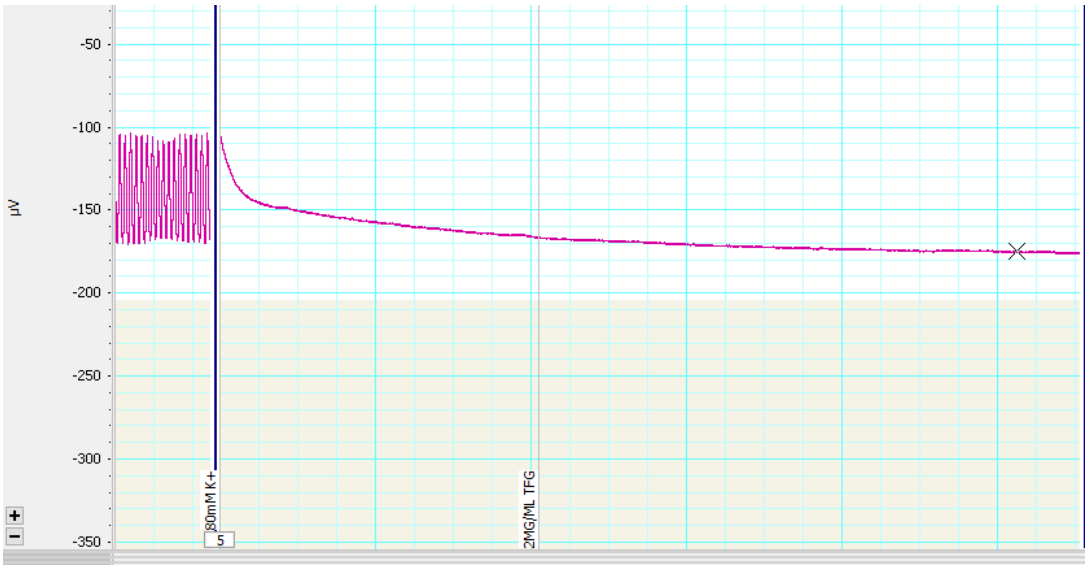


Appendix 8: Representative Powerlab tracing showing the effect of various TFG concentrations on 80mM K+ -induced contraction of isolated jejunal segments.

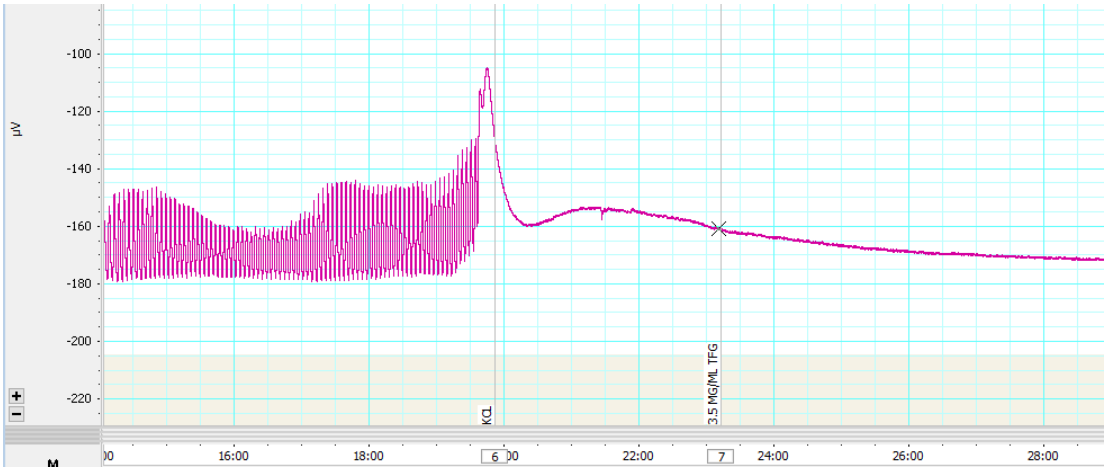
Effect of 0.5 mg/ml TFG on high potassium-induced contraction



Effect of 2.5 mg/ml TFG on high potassium-induced contraction

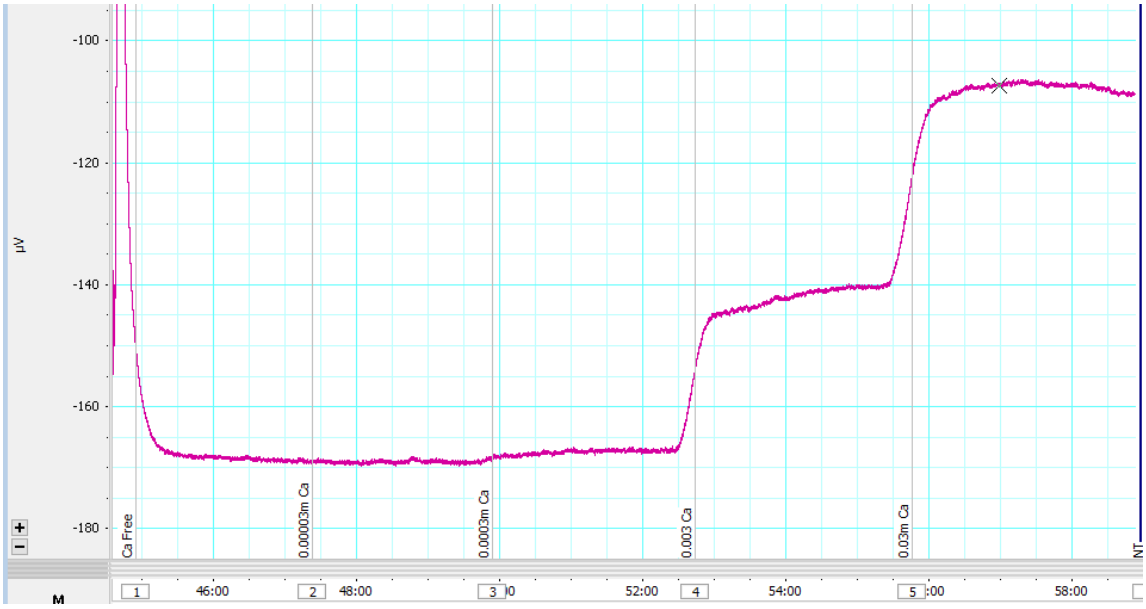


Effect of 3.5 mg/ml TFG on high potassium-induced contraction

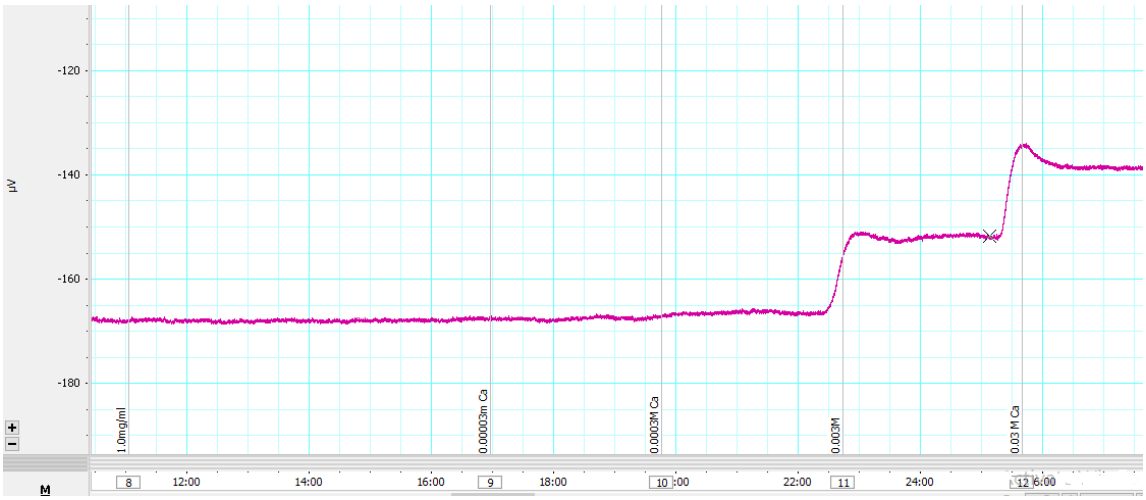


Appendix 9: Representative Powerlab tracings showing the effect of various TFG concentrations and verapamil on Calcium Chloride induced contraction on isolated jejunal segments.

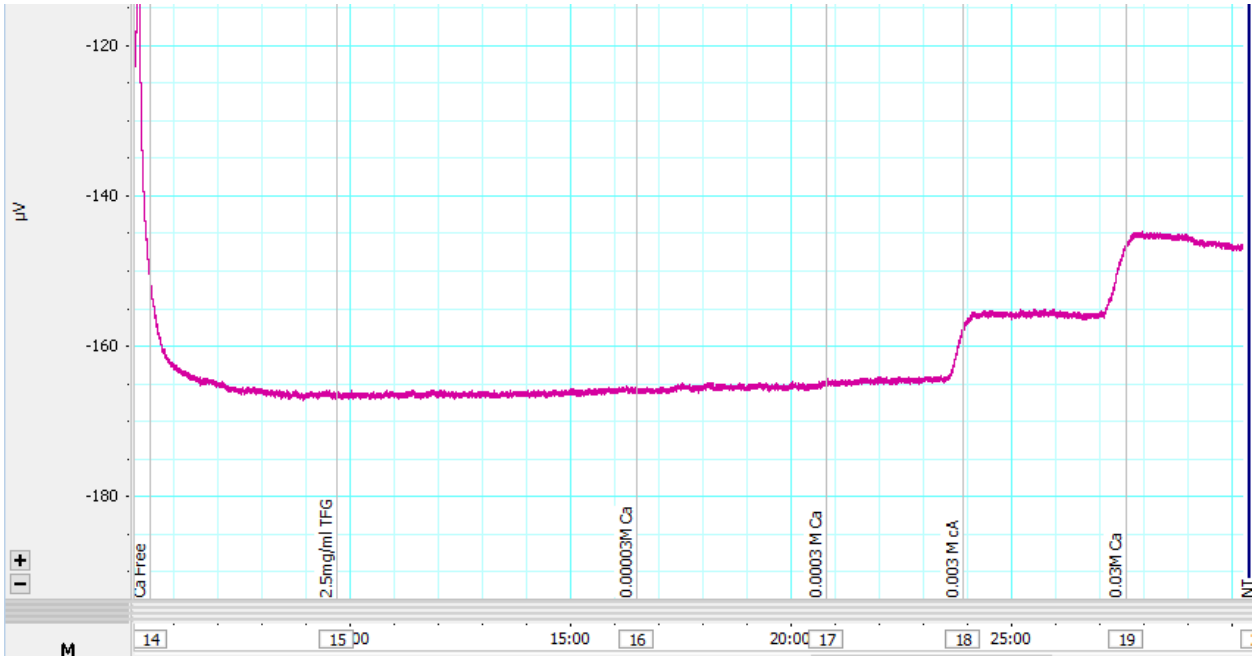
Effect of calcium chloride (0.00003, 0.0003, 0.003, 0.03 M) in absence of TFG and verapamil



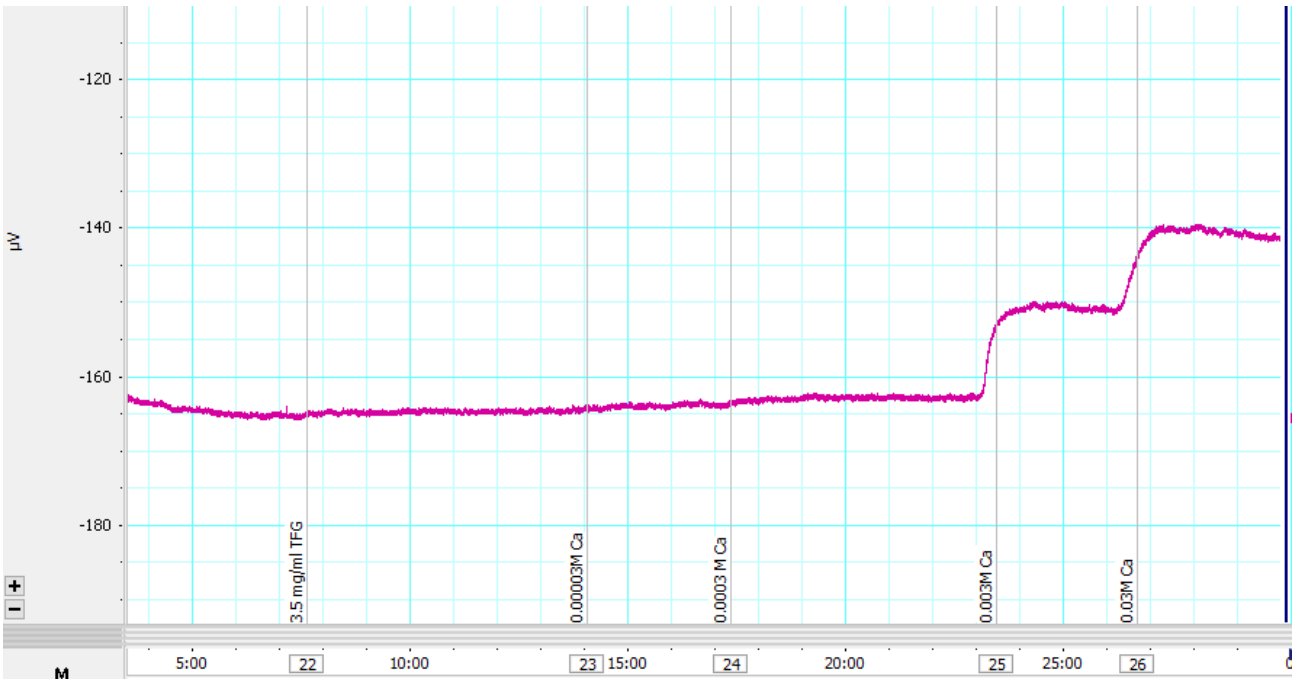
Effect of 1.0 mg/ml TFG on the spasmogenic effect of Calcium chloride



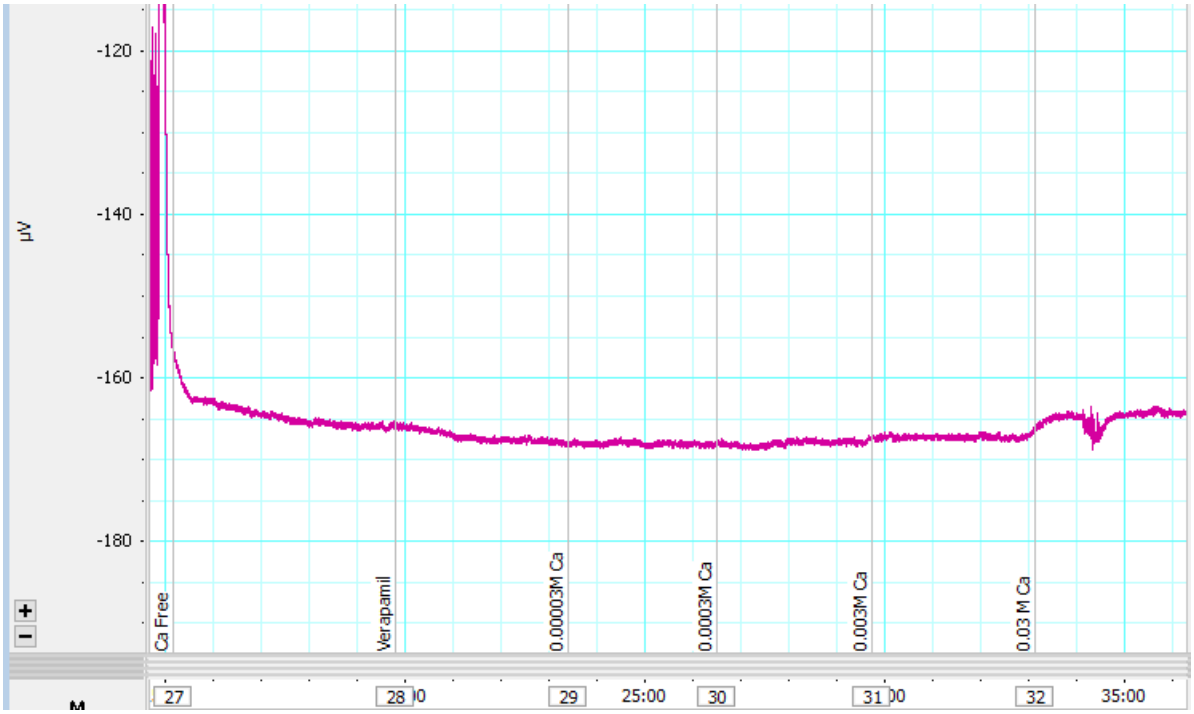
Effect of 2.5 mg/ ml TFG on the spasmogenic effect of Calcium chloride



Effect of 3.5 mg/ml TFG on the spasmogenic effect of Calcium chloride

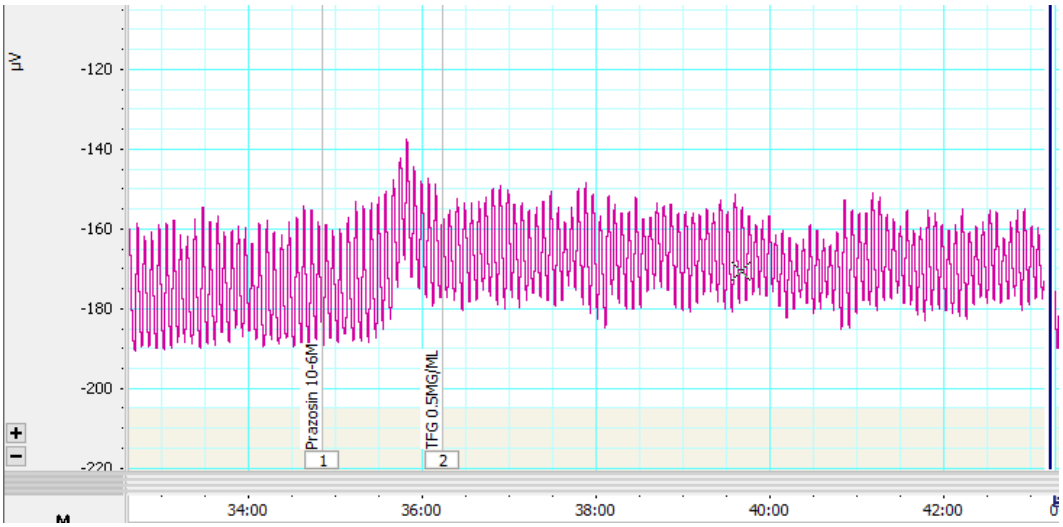


Effect of verapamil on the spasmogenic effect of calcium chloride

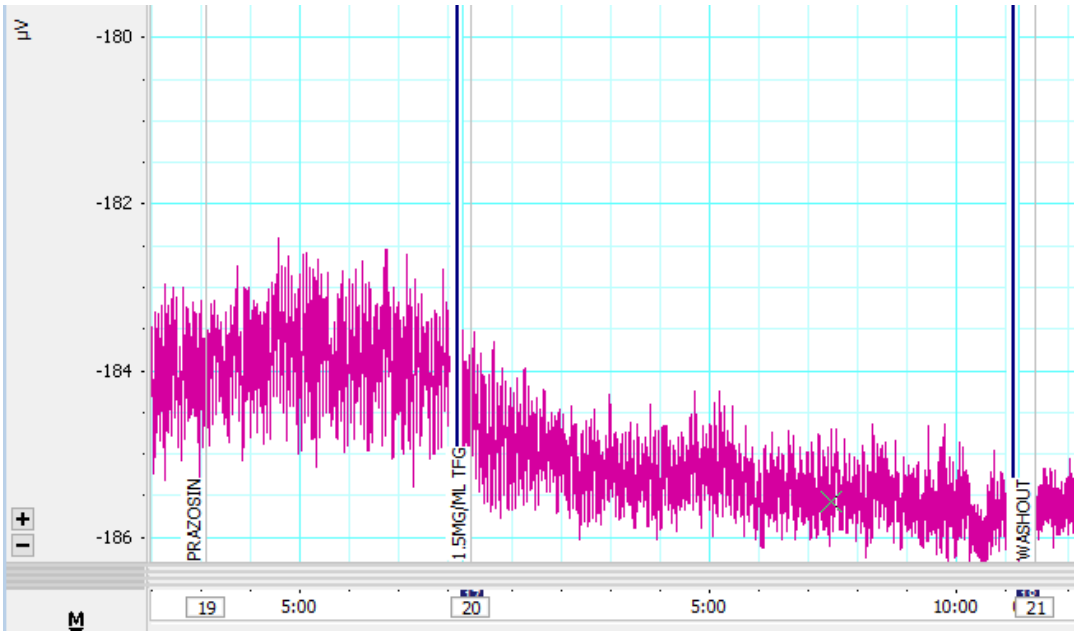


Appendix 10: Representative Powerlab tracings showing the effect of Prazosin on the spasmolytic effect of various TFG concentrations using isolated jejunal segments.

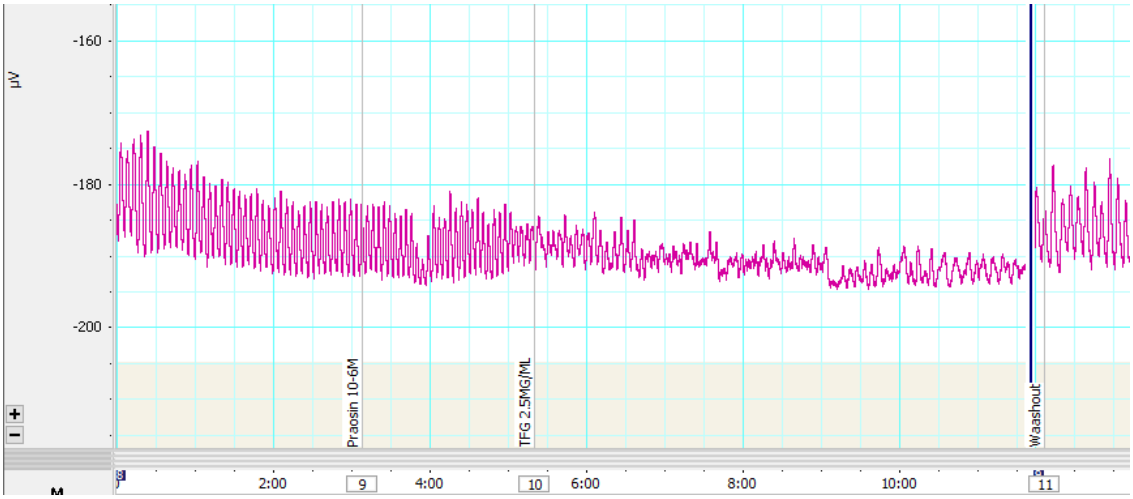
Effect of prazosin on the spasmolytic effect of 0.5 mg/ml TFG



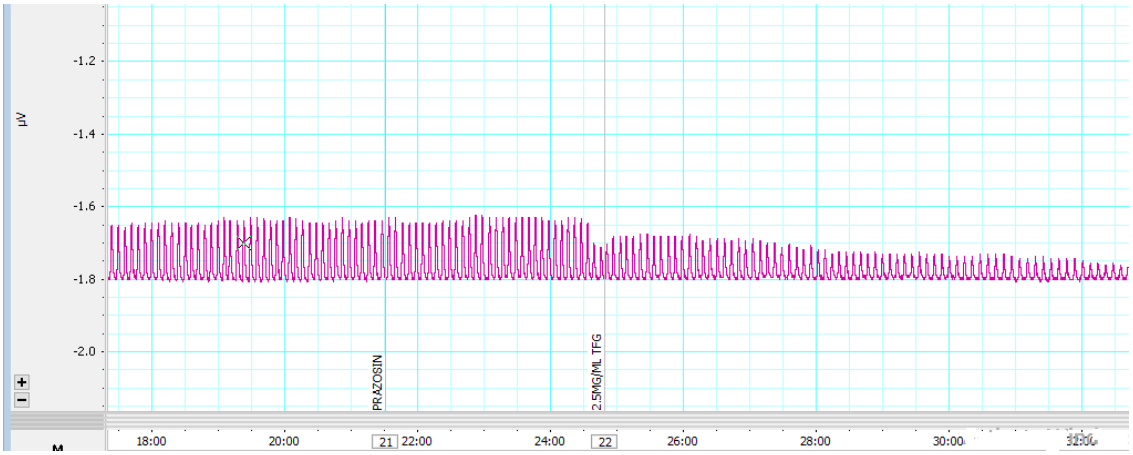
Effect of Prazosin on the spasmolytic effect of 1.0 mg/ml TFG



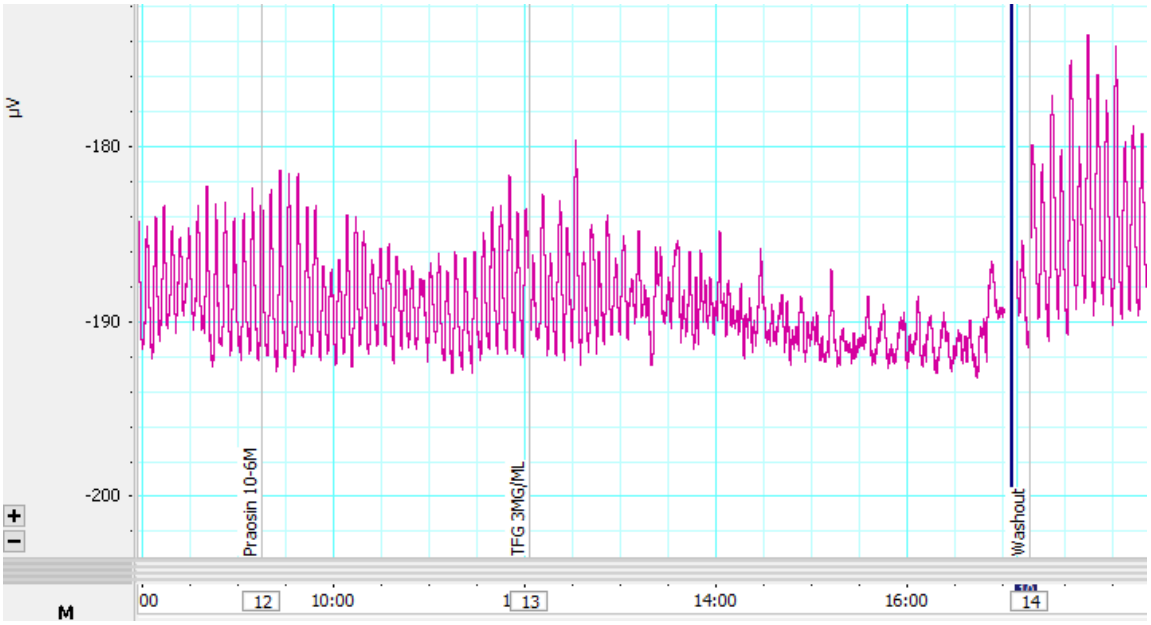
Effect of Prazosin on the spasmolytic effect of 2.5 mg/ml TFG



Effect of Prazosin on the spasmolytic effect of 2.5 mg/ml TFG

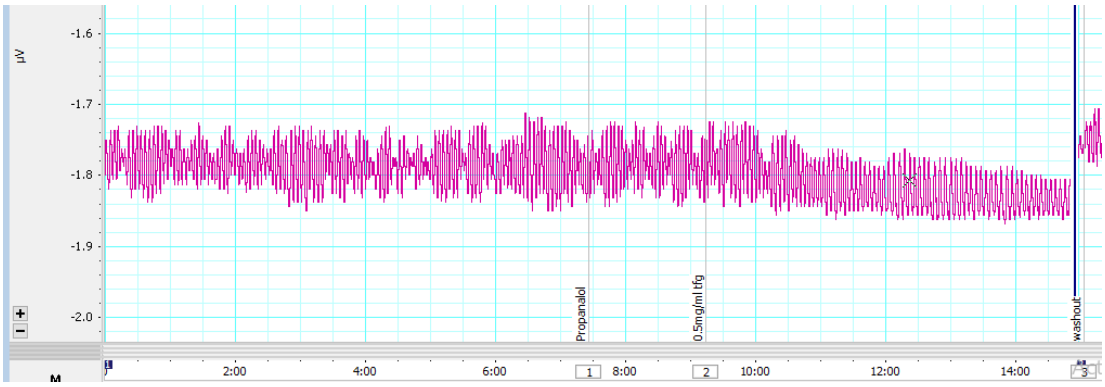


Effect of Prazosin on the spasmolytic effect of 3.5 mg/ml TFG

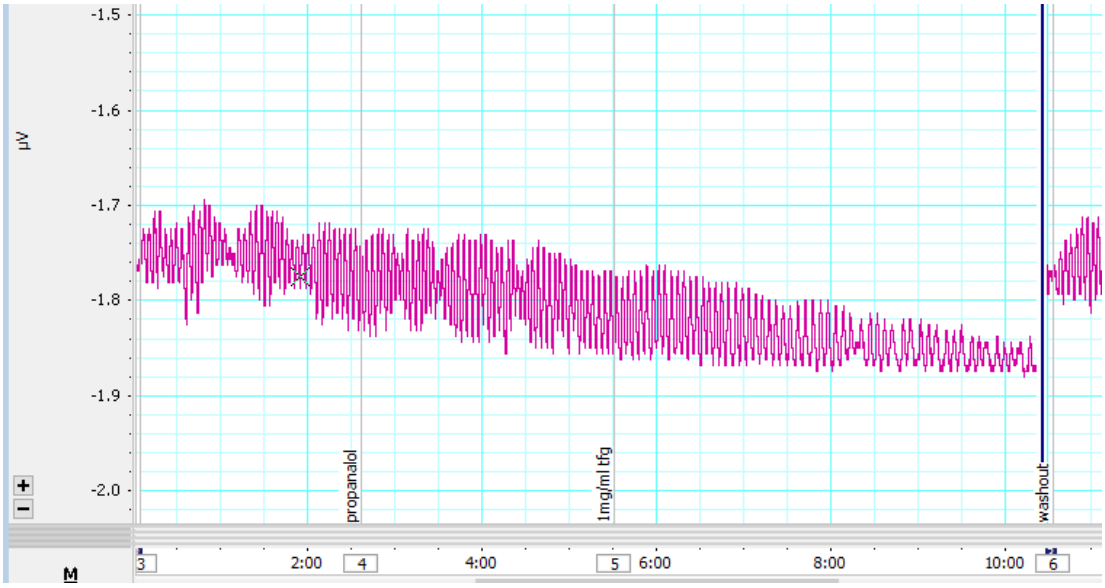


Appendix 11: Representative Powerlab tracings showing the effect of Propranolol on the spasmolytic effect of various TFG concentrations using isolated jejunal segments.

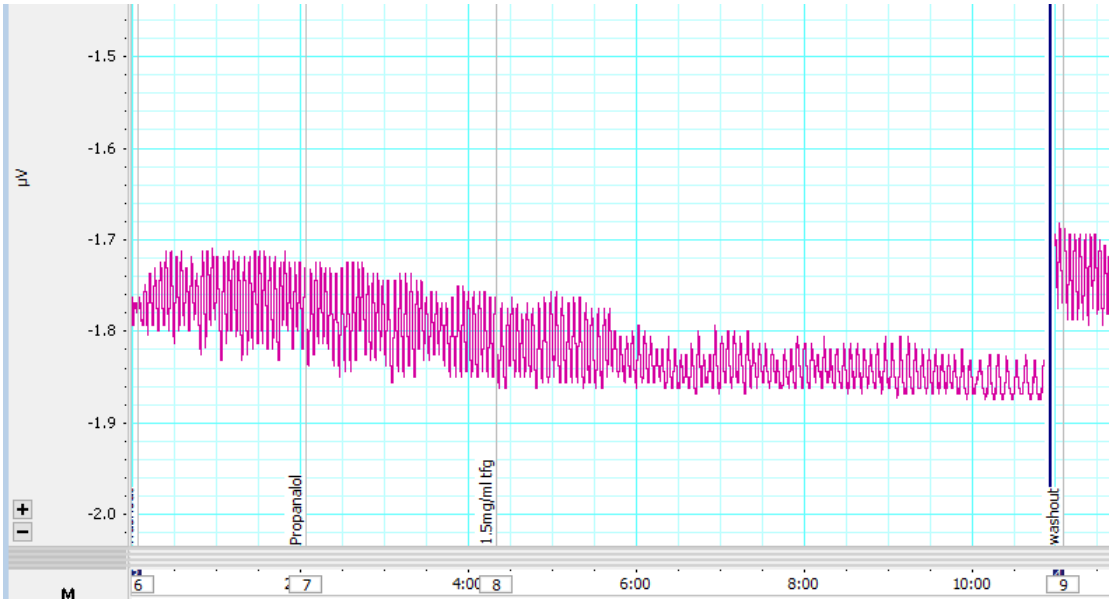
Effect of propranolol on spasmolytic effect 0.5 mg/ml TFG



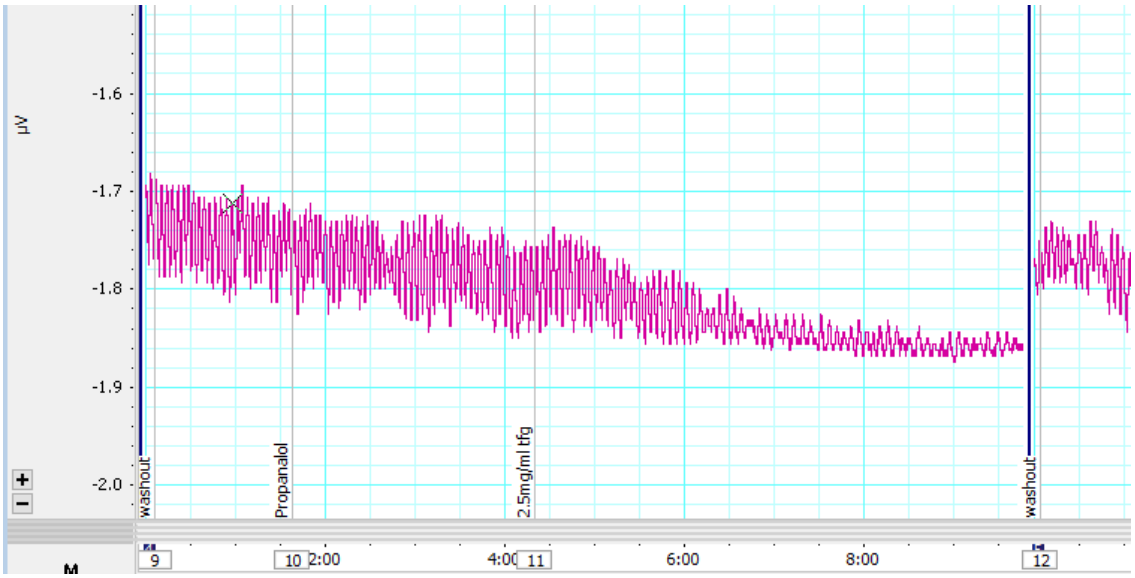
Effect of propranolol on the spasmolytic effect of 1.0 mg/ml TFG



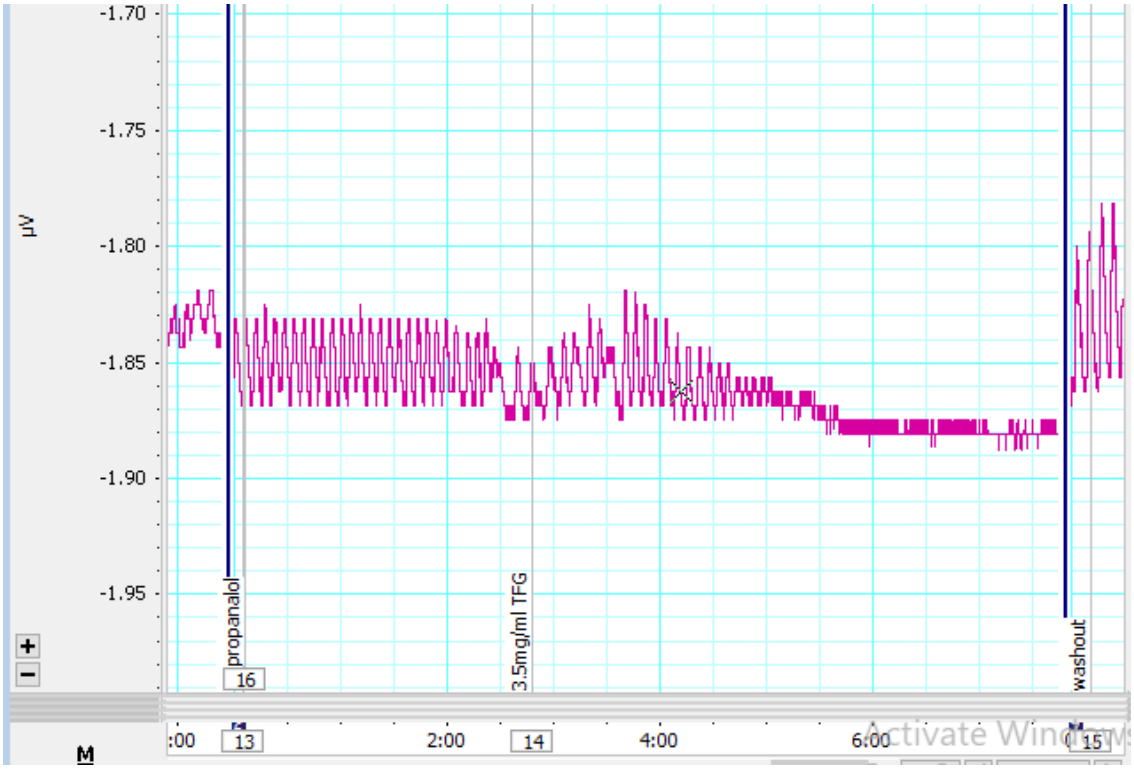
Effect of propranolol on the spasmolytic effect of 1.5 mg/ml TFG



Effect of propranolol on the spasmolytic effect of 2.5 mg/ml TFG

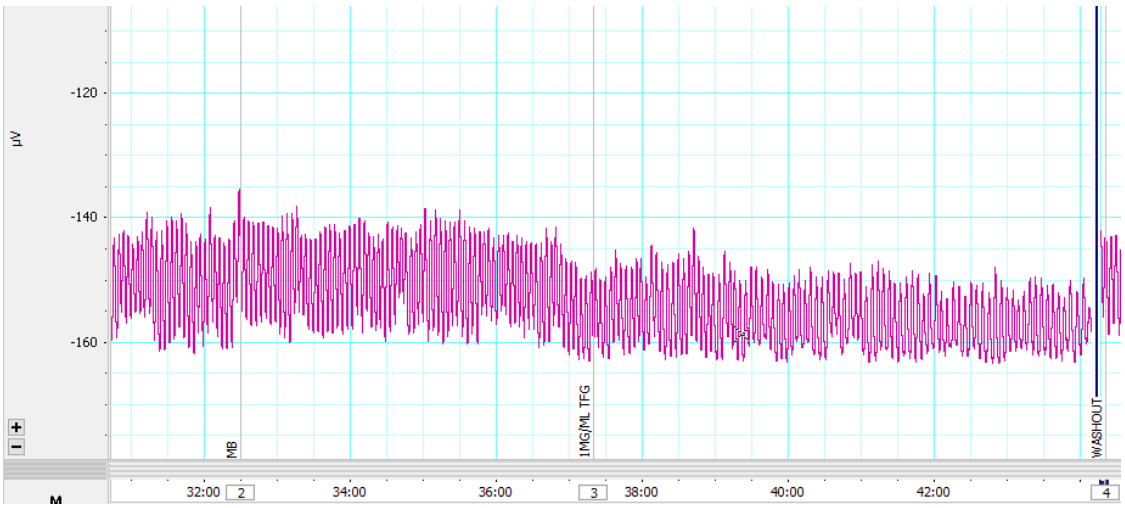


Effect of propranolol on the spasmolytic effect of 3.5 mg/ml TFG

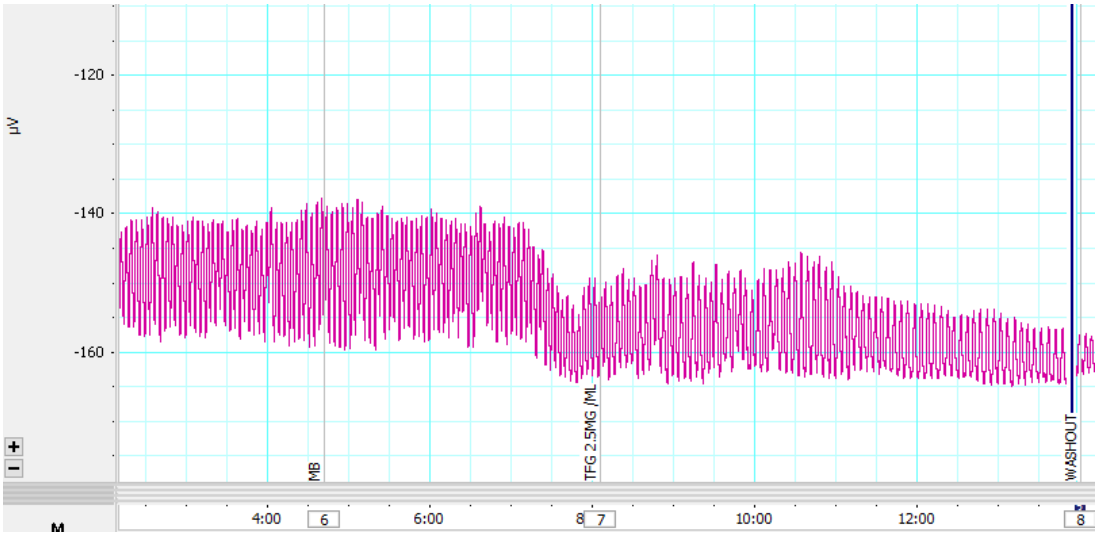


Appendix 12: Representative Powerlab tracings showing the effect of methylene blue on the spasmolytic effect of various TFG concentrations using isolated jejunal segments.

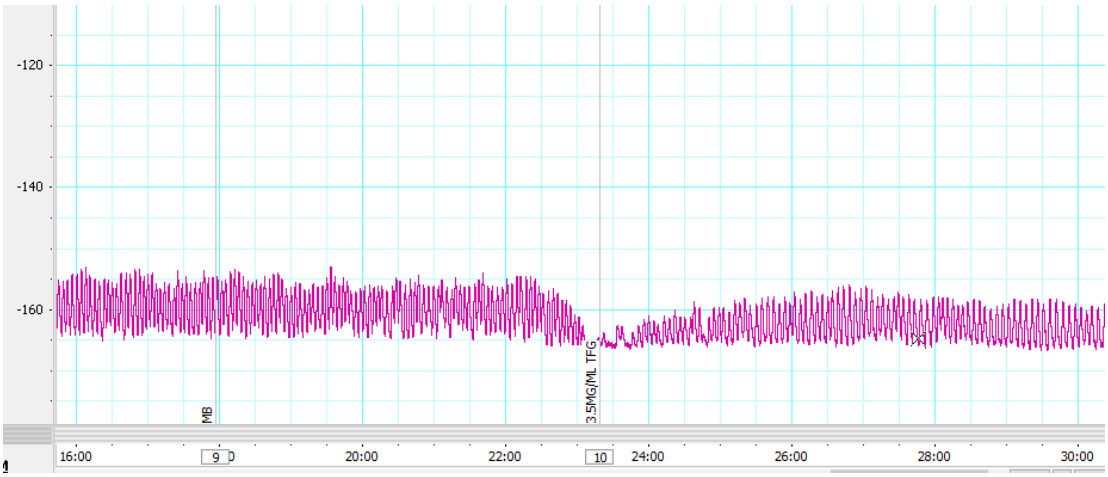
Effect of methylene blue on the spasmolytic effect of 1.0 mg/ml TFG



Effect of methylene blue on the spasmolytic effect of 2.5 mg/ml TFG

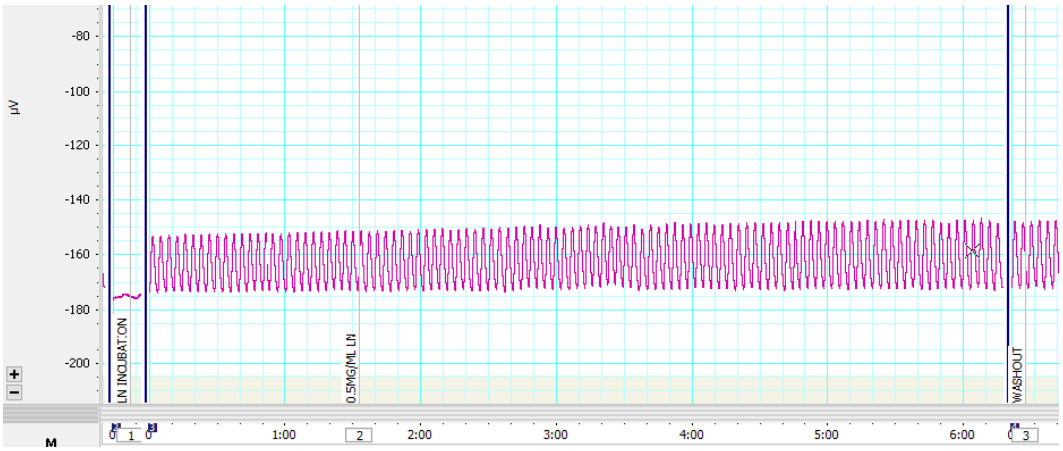


Effect of methylene blue on the spasmolytic effect of 3.5 mg/ml TFG

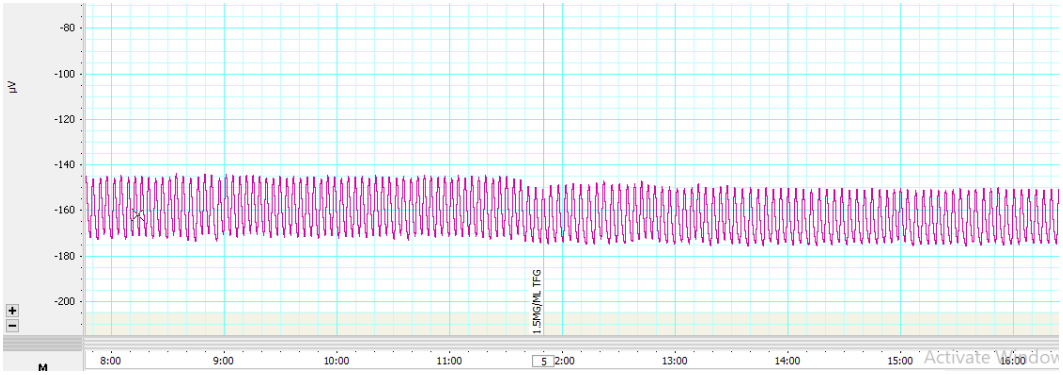


Appendix 13: Representative Powerlab tracings showing the effect of L-NAME on the spasmolytic effect of various TFG concentrations using isolated jejunal segments.

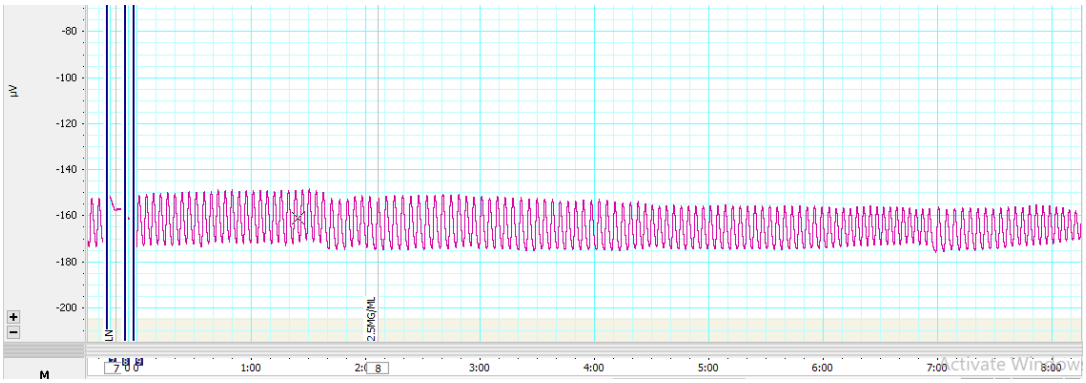
Effect of L-NAME on the spasmolytic effect of 0.5 mg/ml TFG



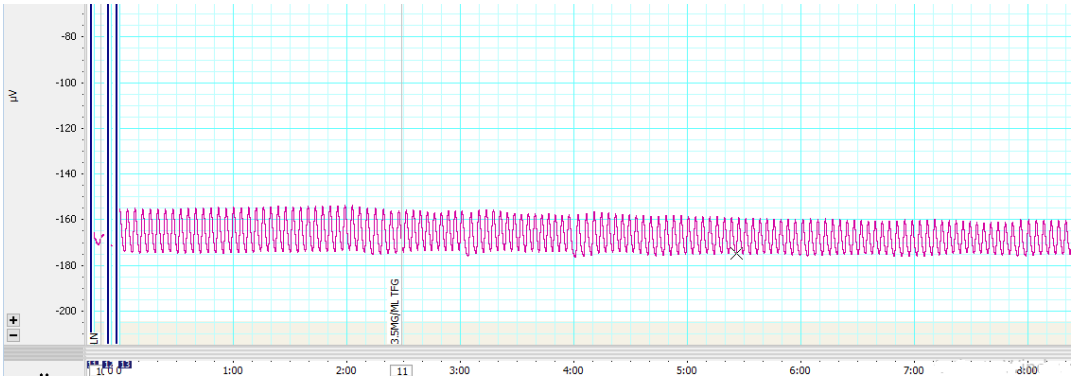
Effect of L-NAME on the spasmolytic effect of 1.5 mg/ml TFG



Effect of L-NAME on the spasmolytic effect of 2.5 mg/ml TFG

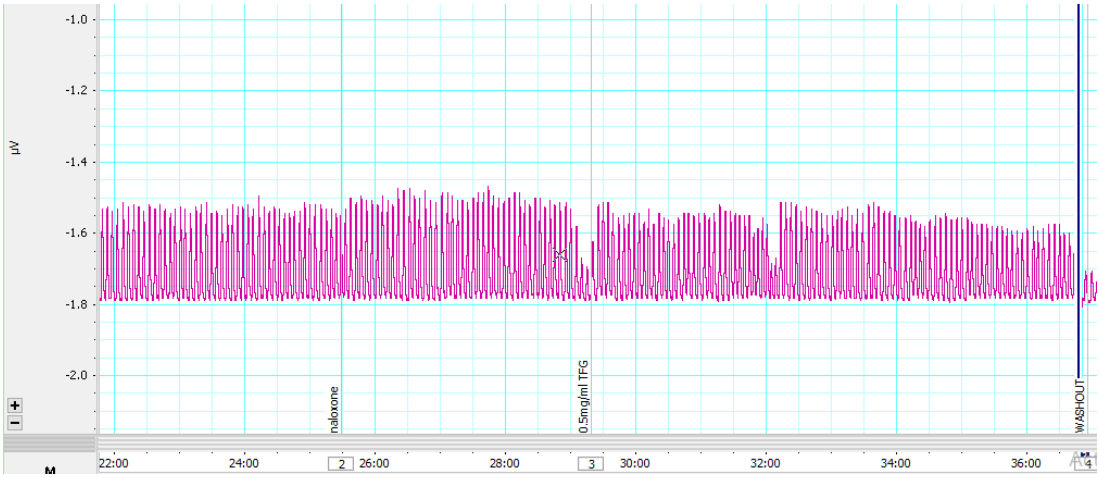


Effect of L-NAME on the spasmolytic effect of 3.5 mg/ml TFG

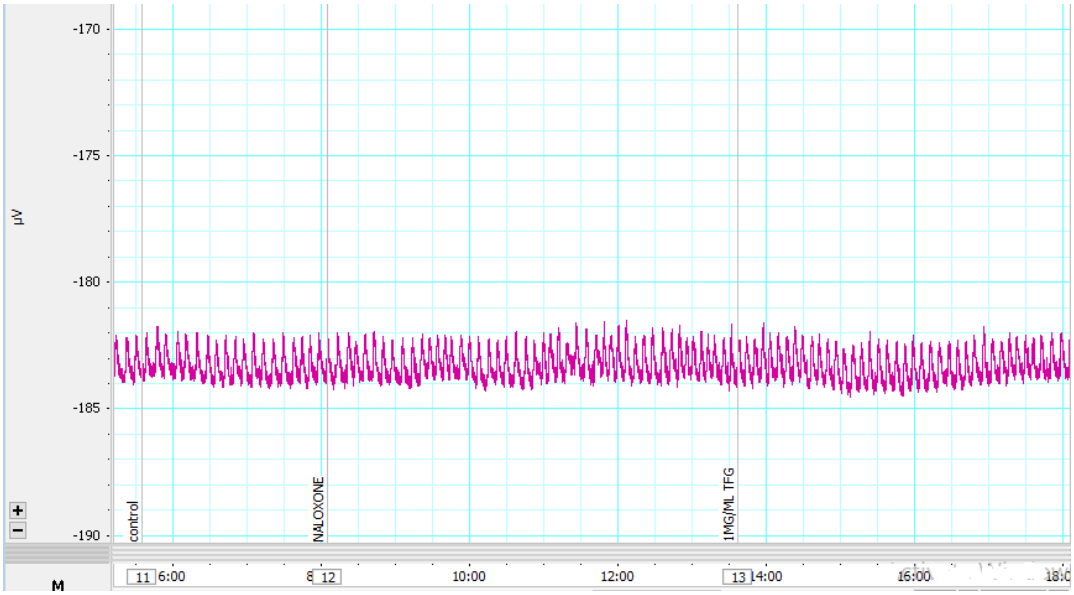


Appendix 14: Representative Powerlab tracings showing the effect of Naloxone on the spasmolytic effect of various TFG concentrations using isolated jejunal segments.

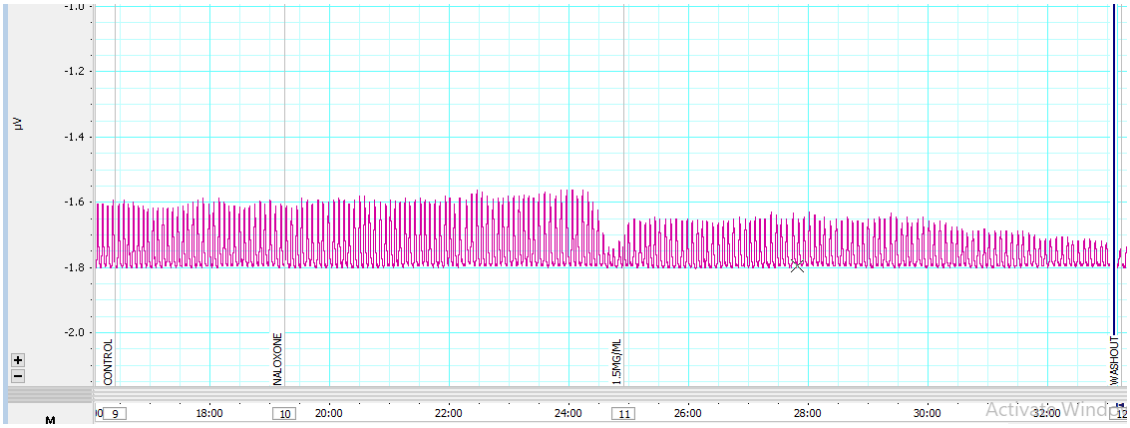
Effect of Naloxone on the spasmolytic effect of 0.5 mg/ml TFG



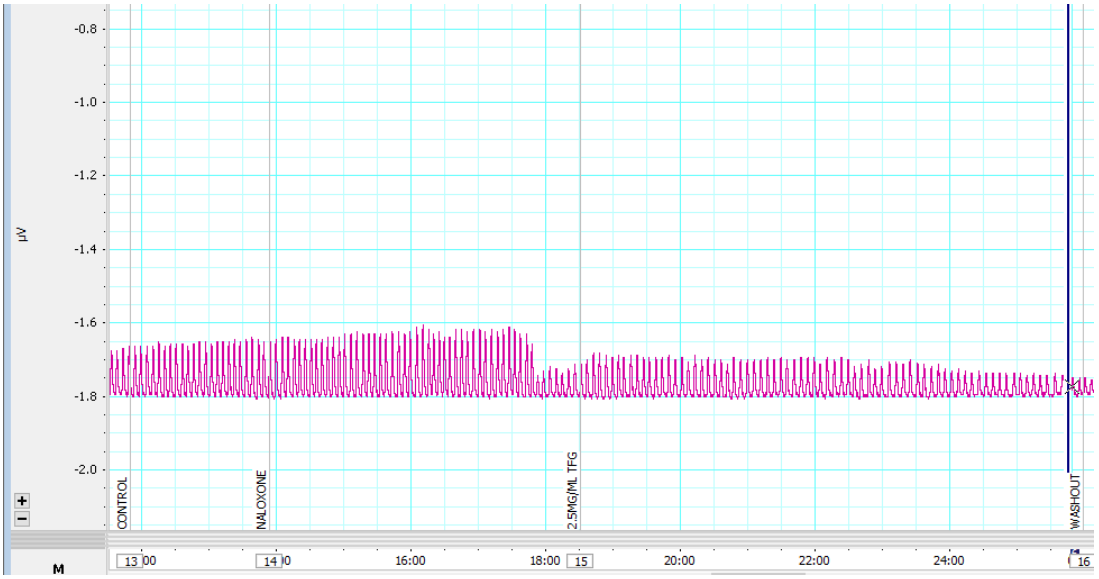
Effect of Naloxone on the spasmolytic effect of 1.0 mg/ml TFG



Effect of Naloxone on the spasmolytic effect of 1.5 mg/ml TFG



Effect of Naloxone on the spasmolytic effect of 2.5 mg/ml TFG



Effect of Naloxone on the spasmolytic effect of 3.5 mg/ml TFG