Enteric Neurotransmission is Inhibited by Pure Compounds Isolated From *Garcinia buchananii* Bark: (2R,3S,2″R,3″R)-GB-2, (2R,3S,2″S)-buchananiflavanone, and (2R,3S,2'' R,3''R)-manniflavanone

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Authorization to Submit Thesis

This thesis of Savannah Marie Patterson, submitted for the degree of Master of Science with a major in Neuroscience and titled “Enteric neurotransmission is inhibited by pure compounds isolated from *Garcinia buchananii* Bark: (2R,3S,2″R,3″R)-GB-2, (2R,3S,2″S)-buchananiflavanone, and (2R,3S,2’’ R,3’’R)-manniflavanone”, has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

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To my birth-giver and my grand-matriarch, your support and faith in me inspired me to attempt things no one else thought I could accomplish. You both have never doubted me and I am the woman I am today because of that. I hope to one day be like you both, and I will work hard every day to continue to make you proud.

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Dedication

I dedicate this thesis to my G-ma, Mary Ann, who said “Fine. If you publish a book you can get a *Calico Cat and Gingham Dog* tattoo”. Here’s the book, G-ma!

Abstract

Garcinia buchananii bark extract (GBB) is a traditional medicine used to treat diarrhea and gastrointestinal pain. Previous experiments from our lab have demonstrated GBB's anti-diarrheal, anti-propulsive motility, and anti-nociceptive effects. GBB is a non-opiod, neuroactive preparation that inhibits synaptic transmission. Some GBB derivatives retain the antimotility and anti-nociceptive effects, specifically the fractions PTLC1, PTLC5, M4, M5 and M7-4. The primary components of these fractions are the pure compounds (2R,3S,2″R,3″R)-GB-2 (denoted as TDS1, of fractions PTLC1, M4), (2R,3S,2″S)-buchananiflavanone (denoted as TDS2, of fractions PTLC1, M5), and TDS3 (the structure of this compound is not yet known, of fractions PTLC5, M7-4). MPLC fraction M3 does not retain anti-motility effects, but the primary component, (2R,3S,2'' R,3''R)-manniflavanone (denoted as TDS4), has been shown to inhibit L-type calcium channels. By using conventional intracellular microelectrode recording of porcine ileum smooth muscle inhibitory junction potentials (IJPs), and calcium imaging of post-synaptic calcium transients in guinea pig myenteric ganglia, we find that TDS1, TDS2 and TDS4 inhibit IJPs and post-synaptic calcium transients. Therefore, TDS1, TDS2 and TDS3 are likely the compounds within GBB that cause inhibition of neuromuscular transmission and synaptic transmission, and thus, inhibition of diarrhea.

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Chapter 1: Introduction and Literature Review

Introduction

Diarrheal diseases have a large global impact with more than 4 billion cases and 1.5 million fatalities annually—and killing more children than AIDS and malaria combined, (Figure 1.1) [1, 2, 3]. The majority of cases occur in developing countries, where 6-7 episodes per child per year are reported, in comparison with developed countries reporting 1-2 episodes per child per year [1, 4]. According to the World Health Organization, (WHO) and the United Nations Children's Emergency Fund (UNICEF), the significant disparity between the occurrences of diarrhea in these areas is chiefly because of poor water sanitation, which leads to contaminated drinking water [1, 5]. More than 12% of the world's population does not have access to safe drinking water, and diseases related to poor water sanitation cause 3.5 million deaths globally. Just over 40% of these deaths are due to diarrhea [2, 3, 4, 6].

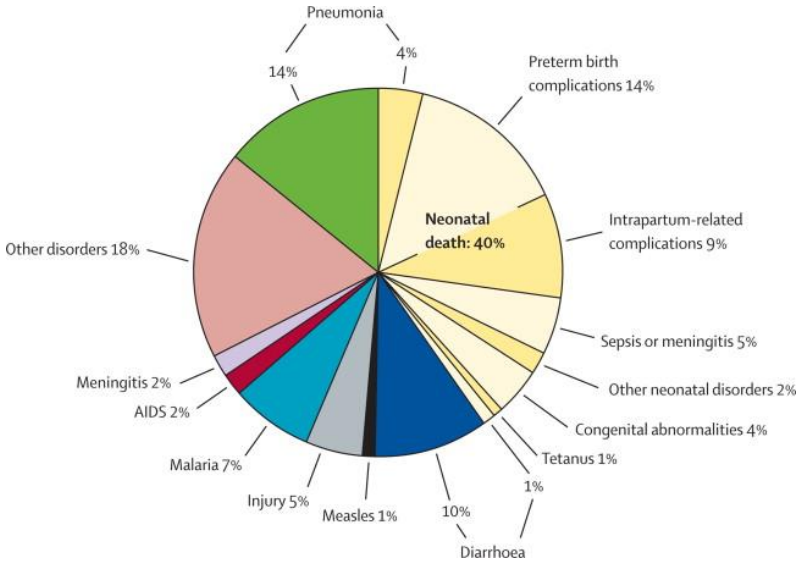

Figure 1.1. Global causes of childhood deaths in 2010 [6].

To combat the significant morbidity and mortality associated with diarrheal diseases, the WHO recommends supplementation with Zinc to improve immune system function

to better fight some diarrheal disease causes, in addition to vaccines for preventable diarrheal illnesses [1, 7, 8]. After a patient contracts diarrhea, WHO recommends a step-wise treatment regimen aimed at reducing the duration of the diarrheal episode, with oral rehydration solutions (ORS) first, then opiates, and finally, antibiotics or antivirals when necessary [1, 2]. ORS is a simple mixture of electrolytes dissolved in water to help prevent further water loss during the diarrheal episode. Opiates help reduce hypermotility and the pain associated with diarrheal diseases [1, 9]. However, opiates have numerous undesirable effects that often over-correct and cause constipation. In addition to constipation, opiates are addictive, expensive, controlled substances that cause paralytic ileus in children [9, 10]. Currently, this ORS and opiates combination is the only broadly-applicable anti-diarrheal treatment available [2, 9]. There is a clear need for an alternative, or additional, non-opioid therapy to help combat the duration and pain of diarrheal episodes. Because opiates act by modulating neurotransmission in the gut, the most appropriate alternative therapies to opiates should target neurotransmission. This idea is well supported by recent research advocating for the need of new, non-opioid drugs for treating diarrhea that work by inhibiting neurotransmission [8, 11].

The enteric nervous system and typical intestinal absorption and motility

The enteric nervous system (ENS) is the largest subdivision of the peripheral nervous system, containing 10^8 neurons, as many as the central nervous system (CNS) of a house cat [12, 13]. The ENS is capable of the coordinating functions of the gastrointestinal tract while also integrating signals from, and sending signals to, the CNS [12, 25, 21]. Interestingly, when inputs from the CNS are removed, the ENS continues to coordinate gastrointestinal function and reflexes normally [8,13, 22].

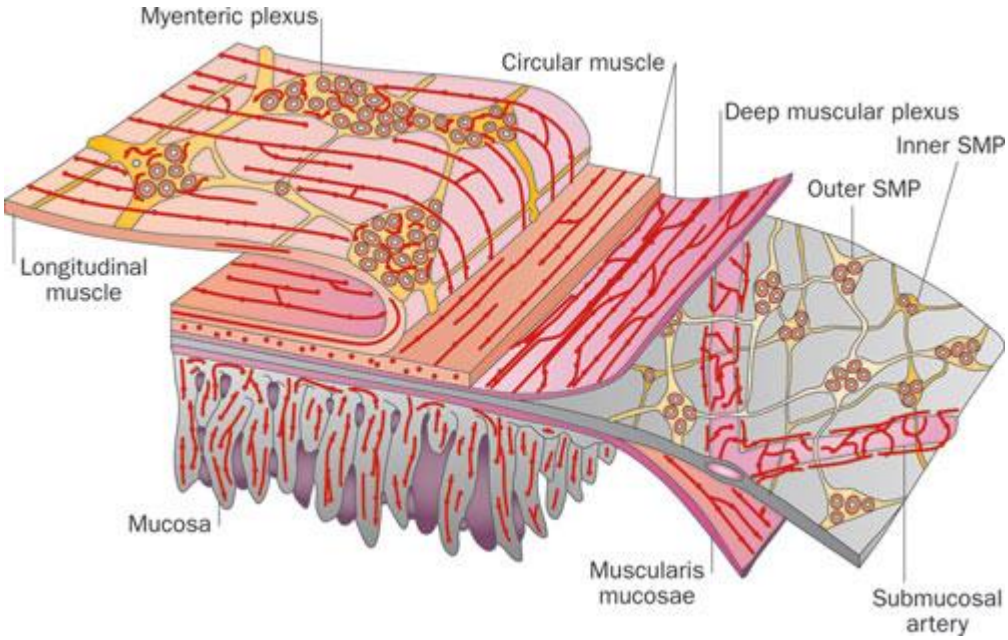

Figure 1.2. Organization of the alimentary canal. This demonstrates the location of the major plexuses of the ENS [20].

The ENS is organized into two major plexuses, like nets, made up of groupings of neuron cell bodies into small ganglia [13, 21]. The two major plexuses can be subdivided in larger animals, but for the purpose of this study, I will describe the two major plexuses as they are known in porcine and guinea pig ileums. The submucosal

plexus (SMP) lies deep to the mucosal layer of the alimentary canal (figure 1.2) [8, 13]. Deep to the SMP is the circular smooth muscle layer, followed by the myenteric plexus (MP), which is located between the inner circular and outer longitudinal smooth muscle layers [8, 13].

The primary function of the SMP is to control local absorption, secretion, and blood flow, whilst the MP function is to coordinate smooth muscle contractions, and subsequently, motor patterns of the gastrointestinal tract (GI) [8, 12, 13, 21]. The two muscular layers in the GI are coordinated by the MP to create peristaltic motion [8, 13]. The law of the gut states that stimulation anywhere along the alimentary canal will cause smooth muscle cells in the oral direction to contract, and smooth muscle cells in the aboral direction to relax [18, 3]. This motion, peristalsis, can be completed without any input from the central nervous system, and is entirely regulated by communication between the neurons and smooth muscle cells within the intestines [13, 21, 22]. The neurons with projections leading in the oral direction will release excitatory signals to the smooth muscle cells causing the smooth muscle to contract [8, 12, 13]. Neurons with aborally directed projections will release inhibitory signals that will cause relaxation in smooth muscle cells [8, 12, 13]. The coordinated contraction and relaxation causes the pressure gradient necessary for peristalsis [13, 20]. The functions of the SMP and MP are vital in regulating GI function, and all diarrhea illnesses disrupt normal ENS control of absorption, secretion and motility.

Diarrhea: an overview

Diarrhea is a symptom common to many illnesses and adverse reactions in the gut [13, 20]. Diarrhea includes hypersecretion, hypermotility, pain resulting from intestinal cramping, and is defined as three or more watery stools per day [1, 22]. Hypersecretion is the alteration of the intestines from a state of absorption to a state of secretion. Intestinal epithelial cells secrete mucous into the lumen of the intestines, causing a change in the osmotic potential. Water and electrolytes are then drawn into the lumen of the intestine. This increases the volume of fluid content in the intestines, and when expelled from the body by hypermotility, results in dehydration [5, 13, 22]. Hypermotility occurs when the rate of peristaltic motion is increased [22]. This alteration of the rate at which contents are propelled (propulsive velocity) affects nutrient and water absorption, and can cause debilitating pain. The increased intestinal secretion and rate of peristalsis experienced during diarrhea are driven largely by the enteric nervous system (ENS) [8, 12, 13, 22].

Diarrhea is classified as either infectious or non-infectious. Bacteria, viruses, and parasites cause infectious diarrhea worldwide, and are the leading cause of diarrhea in children and infants [1, 14, 22]. In addition, water-borne pathogens, spread by inadequate water sanitation techniques and availability, are the leading cause of diarrheal episodes for adults in developing countries [4, 22]. Non-infectious diarrhea arises from causes that cannot transfer to another person, such as celiac disease, food allergies and stress [M]. Further, both infectious and non-infectious diarrhea can be classified as either acute (resolved between 5 and 10 days) or persistent (lasting more

than 14 days). Disorders such as irritable bowel syndrome (IBS), and IBS with diarrhea, can be categorized as chronic, with frequent recurring episodes [12, 13, 20].

In combination with hypersecretion, hypermotility is an effective method for the body to neutralize and remove hazardous material from the intestinal lumen [13, 22]. In the case of both infectious and non-infectious diarrhea, these measures can help to remove the offending pathogens. Too often, however, this self-protective measure results in malnutrition, dehydration, debilitating pain, and health [13, 20, 22].

Treatment options for diarrheal illnesses

The WHO recommends treating diarrhea with oral rehydration solutions (ORS) therapy, opiates and antiviral or antibiotics as needed [1, 22]. The recommendation is to follow the treatment in a step-wise order [1, 2], with the ultimate goal being to mitigate loss of fluid and reduce distress, to reduce the duration of the diarrheal episode, and reduce hospitalization time [1, 22].

ORS is a water-salt mixture that helps to combat the secretion of water and electrolytes by making the intestinal contents isotonic to bodily fluids. Studies report that in developing regions, access to ORS is limited, and this makes ORS difficult to implement [1, 2, 4]. Additionally, ORS have no effect on the duration, hypermotility or pain associated with diarrhea [2, 5, 4]. This is likely why patient compliance with ORS has been traditionally poor [2, 5]. Therefore, the best approach is to combine ORS with adjunctive therapies, such as opiates, to shorten the duration of the diarrheal episode.

Opiates are prescribed to combat hypermotility and pain associated with diarrhea, primarily because they have a unique capacity to slow enteric motility [9, 10, 23]. The problem is that opiates powerfully inhibit neurotransmission and motility, often leading to constipation in adults, and can cause mortality in children through paralytic ileus [9, 24, 10]. Opiate effects on gastric secretion are controversial, but the majority of research shows a significant reduction in pancreatic secretion [23]. Inhibiting enzymatic secretion has a minute effect on luminal osmolarity, but a significant effect on digestive capabilities. In addition, opiates are expensive, addictive, controlled substances, with the primary purpose of inhibiting neurotransmission [9, 10]. Recent research specifically calls for the need for development of a new non-opioid, anti-nociceptive and anti-motility treatment for the shortening the duration of diarrhea [8, 26, 27].

Traditional plant therapies for diarrhea are potential sources of non-opiate drugs

Thirty-nine percent of the population of the world's developing regions does not have access to modern therapies and 80% of developing countries' populations depend on traditional remedies and phytotherapies to treat diarrheal illnesses [7, 15, 28, 29]. Extracts from Garcinia genus plant bark and seeds are among the most generally used preparations for treating acute, chronic and bloody diarrhea in tropical regions globally, including Asia, Africa and Brazil [28, 29, 30]. One such anti-diarrheal traditional remedy is the stem bark extract of *Garcinia Buchannanii* baker trees (GBB, figure. 1.3). This

preparation has been used in sub-Saharan Africa for generations to treat a variety of gastrointestinal illnesses, ascariasis, diarrhea and dysentery [17, 19, 31]. Ethnobotanists report widespread GBB use, and importantly, that it is regarded as safe enough for use on opportunistic infections in immunocompromised AIDS patients [25, 29, 30].

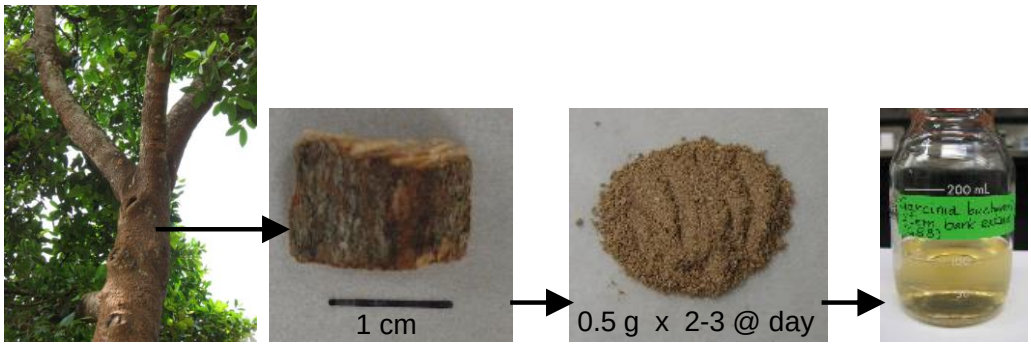

Figure 1.3. *G. buchananii* stem bark extract preparation for traditional therapies. The bark can be chewed, or ground to powder to make into tea.

Previous studies have shown GBB is a non-opiate and a non-adrenergic preparation, and it is an effective therapy for lactose-induced diarrhea [15, 16, 17, 31]. GBB has been demonstrated as an effective therapy for diarrhea through its ability to alleviate pain and hypersecretion, and to inhibit hypermotility [15, 16, 17, 41]. GBB reduces nociception (pain), and peristalsis by inhibiting neurotransmission [15, 17, 41]. Additional studies show that the anti-motility effects of GBB are likely a result of inhibition of neurotransmission by blocking 5-ht3 and 5-ht4 receptors [17] and by reducing smooth muscle excitability by blocking L-type calcium channels [41]. These findings suggest that GBB is a potential source of new non-opiate compounds, which can be used as adjunctive therapies with ORS to mitigate diarrheal illnesses.

Components/Fractions of GBB have anti-diarrheal and anti-nociceptive effects

Previous studies suggest that GBB contains natural compounds with potential for novel anti-diarrheal, anti-nociceptive drugs [15, 17, 18, 19]. Previous research in my lab has shown that GBB, in its entirety, has both anti-nociceptive and anti-diarrheal effects that inhibit neurotransmission in myenteric plexus [15, 16, 17, 41]. In the interest of identifying the specific compounds (or a combination of compounds) in GBB responsible for these effects, it was separated by preparative thin-layer chromatography (PTLC, Figure 1.4) into five fractions (PTCL 1- 5) and tested for effects on propulsive motility and for treating lactose-induced diarrhea. Results showed that, like GBB, PTLC1 and PTLC inhibit motility, and are effective anti-diarrheal treatment [19]. Two PTLC fractions (PTCL1 and PTCL5) retained GBB's anti-motility effects. Interestingly, PTLC 2, PTLC3 and PTLC4 had mostly pro-motility (increased propulsive motility) effects [15, 19].

Furthermore, Medium-Pressure Liquid Chromatography (MPLC) was used to separate GBB into eight fractions (M1-M8) [17]. Correlation of PTLC with MPLC fractions showed that PTLC1 corresponds with M4 and M5, and PTLC5 corresponds to M7 [40]. Preliminary data showed that three MPLC fractions (M4, M5, M7) also have anti-motility effects similar to GBB and their corresponding PTLC fractions [16, 19]. This suggests that these fractions have neuroactive compounds that inhibit neurotransmission in the ENS. Interestingly, M6 had pro-motility effects (increased

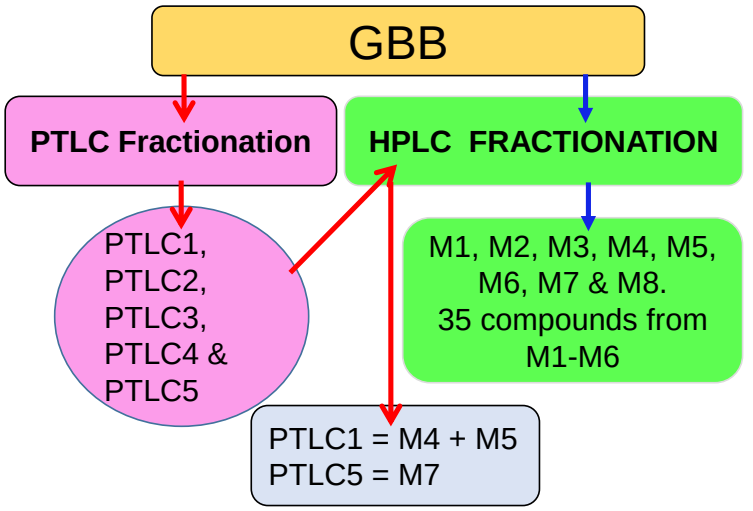

Figure 1.4. Separation of GBB by PTLC and MPLC**.** Correlations between MPLC and PTLC fractions are such that M4 and M5 correlate with PTLC 1, and M7 correlates with PTLC5.

propulsive velocity), and the remaining fractions had no effect on motility. In addition M4 and M5 demonstrated anti-nociceptive properties [31]. Further HPLC analysis of the MPLC fractions, HPLC LC-MS/MS analysis, 1D- and 2D-NMR, and circular dichroism (CD) spectroscopy were then used to identify the major compounds in M1-M8. This showed that the major compound in MPLC fractions that have anti-motility effects, and which likely inhibit neurotransmission and treat diarrhea, to be: (2R,3S,2″R,3″R)-GB-2 (here forth denoted as TDS1) is the major compound in M4, (2R,3S,2″S)-Buchananiflavanone (here forth denoted as TDS2) is the major compound in M5, and (2R,3S,2'' R,3''R)-manniflavanone, (here forth denoted as TDS4) is the major compound in M3 [17, 31]. A deeper HPLC separation of M7 into four subfractions revealed a single subfraction (M7-4) having anti-motility properties, and is from here forward denoted as TDS3. TDS1, TDS2 and TDS3 were further identified as biflavanones [15, 18].

Collectively, these results suggest that GBB in its entirety, or compounds found within the preparation, represent a novel anti-diarrheal treatment alone, or as an adjunctive therapy to ORS. The current knowledge gap is which specific compounds within GBB

are the bio-active, anti-motility components, and if they inhibit neurotransmission (figure 1.5). There is currently no known, active research on the effects of biflavanones on the function of the ENS, or, for potential novel therapeutic sources. The goal of the experiments in this project is to identify the neuroactive compounds within GBB. This is an important step, as it will enable us to move forward with testing their efficacy against diarrhea and pain in an effort to develop a novel treatment to curtail hypermotility, hypersecretion and pain associated with diarrhea.

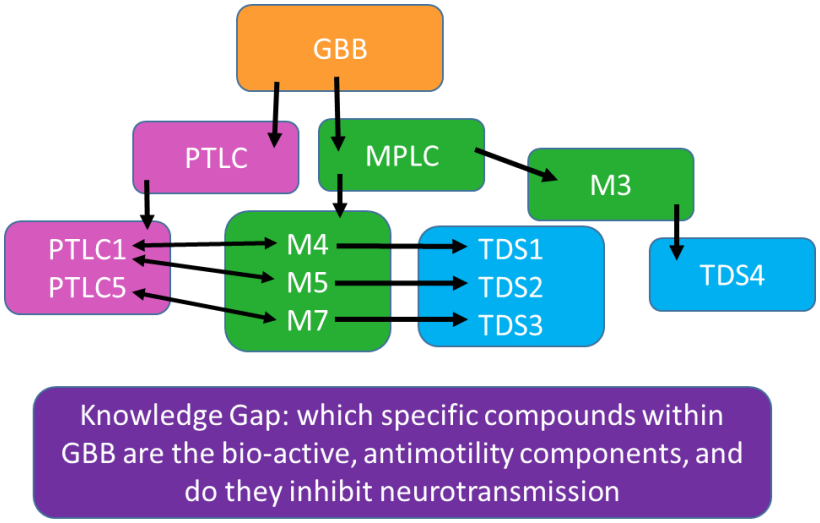

Figure 1.5. Bioactivity-guided identification of neuro-active compounds. Flowchart of breakdown of GBB and the knowledge gap it will address.

Summary of thesis

My central hypothesis is that *GBB and derivative compounds are an alternative, effective, novel, non-opiate, anti-diarrheal therapy or adjunctive therapy to ORS that mitigates diarrhea and pain by inhibiting the enteric nervous system's (ENS) synaptic transmission and neuromuscular transmission.* The objective of this project is to fill t*he current knowledge gap on whether the pure compounds, TDS1 and TDS2 and TDS3 isolated from M4, M5, and M7, respectively, are the neuroactive compounds within GBB that inhibit neurotransmission*. To achieve this objective, I will test two hypotheses:

Aim 1. Test the hypothesis that TDS1, TDS2, and TDS3 inhibit neuromuscular excitation (neuron-muscle). I used conventional intracellular micro-electrode recording to measure the effect of TDS1 and TDS2 and TDS3 on IJPs, the electrical signals triggered in response to inhibitory neurotransmitters in smooth muscle cells. Results were compared with baseline recordings, TDS4, GBB and parent HPLC fractions, tetrodotoxin, hesperetin, and quercetin.

Aim 2. Test the hypothesis that TDS1, TDS2, and TDS3 isolated from GBB inhibit synaptic transmission (neuron-neuron). I used calcium imaging to visualize calcium transients elicited in myenteric neurons by fiber tract stimulations to trigger synaptic transmission through neurotransmitter release. To test for inhibition by the compounds, I compared fluorescent baseline signals with signals obtained after application of the pure compounds, TDS4, GBB, hesperetin, and quercetin.

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Chapter 2: GBB Derivative Compounds Inhibit Propulsive Motility by Decreasing Inhibitory Junction Potentials

Introduction

Garcinia buchananii stem bark extract (GBB) mitigates lactose-induced diarrhea and nociception [1, 2, 3, 4]. It is believed that GBB exerts these effects by inhibiting neurotransmission in the ENS, and by inhibiting propulsive motility (peristalsis) and secretion [1, 3, 4, 5]. This suggests that GBB could be a source of compounds for treating diarrhea and associated pain. To begin to identify these compounds, GBB was separated using Preparative Thin-Layer Chromatography (PTLC) into five fractions (PTCL 1- 5), which were then analyzed using *in vitro* guinea pig colon motility assays. Two fractions, PTLC3 and PTLC4, had no effect. PTLC2 increased motility, while two PTLC fractions (PTCL1 and PTCL5) retained GBB's anti-motility effects [2]. This suggests that PTCL1 and PTCL5 are likely the components containing anti-motility compounds, which act by inhibiting neurotransmission [6].

Further separation of GBB by liquid chromatography was necessary to identify the bioactive compounds, thus medium-pressure liquid chromatography (MPLC) was used to separate GBB into eight fractions, M1-M8 [7]. MPLC analysis of the PTLC fractions showed that PTLC1 corresponds with M4 and M5, and PTLC5 corresponds to M7 [7, 8]. Using HPLC LC-MS/MS analysis, 1D- and 2D-NMR, and circular dichroism (CD) spectroscopy, TDS1 (naringenin-C-3/C-8''dihydroquercetin linked biflavanone;

previously called GB-2) was identified as the major component of M4, TDS2 ((2R,3S,2″S)-buchananiflavanone, previously called buchananiflavanone), was identified as the major component of M5, and TDS3 (the structure of this compound is not yet fully characterized) is the major component of M7 [8, 9]. A fourth compound, (2R,3S,2'' R,3''R)-manniflavanone, (here forth denoted as TDS4) is the single compound that makes up the largest proportion of GBB [10, 11]. The parent MPLC fraction of TDS4, M3, did not correlate with any PTLC fractions shown to inhibit motility or neurotransmission. Taken together, the results of PTLC analysis and identification of major compounds suggests that the bioactive, anti-motility and anti-diarrheal components of GBB are the MPLC fractions M4, M5, and M7, and the active compounds within these fractions are TDS1, TDS2, and TDS3.

Motility assays are a high-throughput and informative screening tool for identifying components of GBB that inhibit neurotransmission and motility [2, 12]. Based on the law of the gut, an increase or decrease in peristaltic velocity suggests a direct effect of a test substance on the enteric nervous system, the smooth muscle cells or a combination of these components [12, 13, 14]. In initial motility assay trials of GBB, PTLC1, and PTLC5, ectopic contractions were observed aboral to the pellets, suggesting that neuroactive compounds in the components of GBB inhibit smooth muscle relaxation [2, 14].

Relaxation and contraction of intestinal smooth muscle is regulated by inhibitory neurotransmitters from the ENS [13, 14, 15]. Release of inhibitory and excitatory neurotransmitters from inhibitory motor neurons and excitatory motor neurons, respectively, causes electrical events in intestinal smooth muscle cells called inhibitory

junction potentials and excitatory junction potentials (IJP and EJP, respectively) [1, 13, 15, 16, 17, 18]. EJPs trigger smooth muscle contraction oral to the site of stimulation (typically a bolus), and IJPs elicit relaxation aboral to the site of stimulation, which creates the pressure gradient that generates gastrointestinal motility (peristalsis) [1, 14, 15]. An IJP is a rapid smooth muscle cell membrane hyperpolarization, followed by depolarization to the resting membrane potential [15, 16]. The membrane hyperpolarization dampens the excitability of smooth muscle, which causes relaxation [15]. An inhibition of the IJP would disable smooth muscle relaxation aboral to the bolus, and could be the root cause of anti-motility effects of GBB and derived fractions. Therefore, measurement of the effects of MPLC fractions and their major compounds on IJPs would identify the fractions and or compounds which inhibit neurotransmission and diminish smooth muscle relaxation.

The purpose of this study is to test the central hypothesis that by reducing inhibitory junction potentials, MPLC fractions (and therefore their primary compounds) prevent aboral relaxation by triggering ectopic contractions, thereby inhibiting propulsive motility and increasing overall transit time. To investigate these ideas, MPLC fractions (M3, M4, M5 and M7) were tested for effects on propulsive motility in isolated guinea pig colon motility assays. In addition, M4, M5 and M7as well as TDS1 and TDS2 were analyzed by using intracellular micro-electrode recording to measure their effects on IJPs. Their effects were compared to GBB and control flavanones, hesperetin, quercetin, and TDS4. The results suggest that M4, M5, and M7, and pure compounds TDS1 and TDS2 are the neuroactive compounds within GBB that inhibit neurotransmission.

Materials and Methods

Animals and solutions

The University of Idaho Animal Care and Use Committee approved of all studies. Six-month-old male and female guinea pigs were maintained at 23–24 °C on a 12/12 hour light-dark cycle with access to food and water ad libitum in plastic cages with soft bedding. Colon sections of approximately 10-12 cm in length were collected via midline laparotomy following isofluorane anesthesia and exsanguination. After dissection and before use, colons were kept in ice-chilled Kreb's solution (mmol L^{-1}: NaCl, 121; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; and glucose 8; all from Sigma, St. Louis, MO, USA; aerated with 95% O_2/5% CO_2).

Colon Motility Assays

The dissected segments of distal colon were pinned in a 50 mL silicone resin (Silgard) lined tissue bath, with continuously-cycling Kreb's solution (cycling rate: 10 mL min^{-1}) maintained at 36 ˚C, Figure. 2. Propulsive velocities were determined using Gastrointestinal Motility Monitoring system (GIMM, Med-Associates Inc., Saint Albans, VT, USA) to film nail-polish-coated guinea pig pellets (similar in size to the naturally-occurring pellets in that particular animal). Motility assays were performed as described previously by our lab [2]. Pellet velocity, the time it takes for a pellet to traverse the pinned segment, was calculated using the GIMM software, as in previous studies [2].

Pellets were inserted in the oral end of the colon sections every five minutes throughout the thirty-minute equilibration period, the baseline recording period, and after the drug application. Six to eight baseline recordings preceded any treatment application. Drugs were delivered via superfusion of the compounds onto the tissue by bath application. Treatment velocities were compared to baseline recordings. Each guinea pig colon produced three sections, making each section either oral, middle or aboral. We randomized the treatments such that each drug was tested on an oral, middle and aboral section.

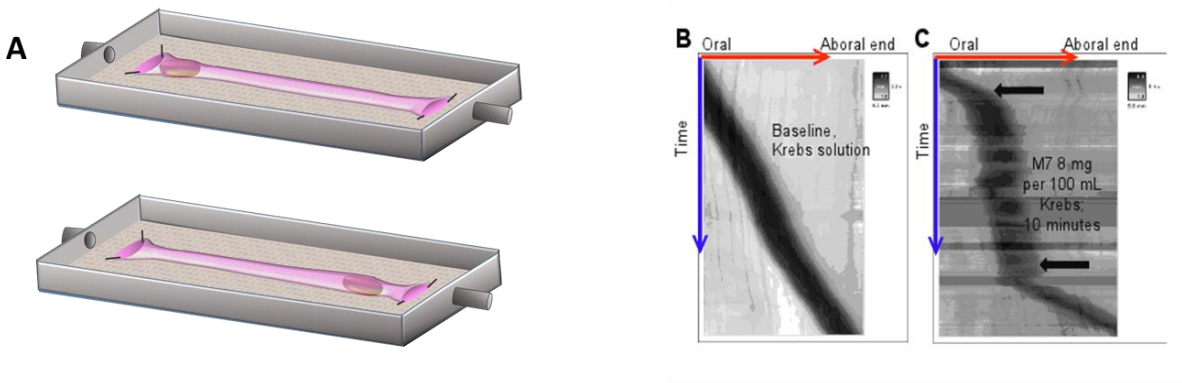

Figure 2.1. Motility assay organ bath and spatiotemporal map depiction. **A**, Represents the physical set-up for motility assay recording as a pellet travels through the pinned colon section. **B and C** show a spatiotemporal map that represents pellet transit from oral to aboral end during the recording period. **B** is a baseline trial and **C** is with M7 applied by bath application. The black arrows indicate where the pellet temporarily stopped moving forward.

Drugs

All compounds were first dissolved in ETOH, except hesperetin, which was solubilized in DMSO, to prepare stock solutions for mixing in 100 mL oxygenated Kreb's, bathing solution, the concentration of M4 (6mg), M5 (10 mg), M7 (8mg) in mg/100 mL Kreb's solution was determined based on how many milligrams each fraction contributes 100 mg of M1-M8 combined [9]. In previous studies, vehicles (ETOH and DMSO) were shown to have no effect on motility at >1:1000 dilution, which is the concentration used in this study [1, 2].

Intracellular Recording

Conventional microelectrode recording was used, as described by Belmonte and Gallego, and Eccles [18, 22]. Porcine ileum samples, donated by C&L Meat Locker in Moscow, Idaho, were collected five to ten minutes after animals were killed by gunshot to the head, followed by exsanguination. Samples were transported (5-8 min) to the lab in ice-chilled HEPES buffer (20 mM HEPES (pH 7.4), 115 mM NaCl, 5.4 mM KC1, 2.2 mM CaCl and 0.8 mM MgC12, 13.8 mM glucose, all purchased from Sigma, St. Louis, MO, USA). Samples were dissected in HEPES to remove the mucosa and submucosa, to expose the inner circular muscle. Dissection and recording were performed using nisodipine (1 µM, purchased from Sigma, St. Louis, MO, USA) in the circulating Kreb's solution, or HEPES to block muscle contractions and EJPs.

Muscularis externa preparations (1.5 x 1.0 cm; Figure. 2.2) were transferred into a recording chamber containing ice-chilled HEPES solution and were pinned-stretched.

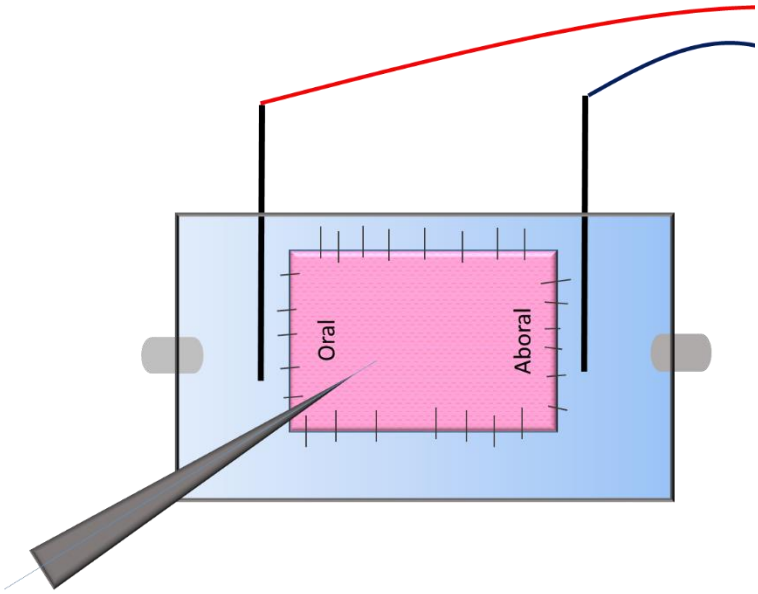

Figure 2.2. Physical set-up for Intracellular micro-electrode recording**.** Oral and aboral ends of tissue sample were oriented toward the positive and negative electrodes, respectively.

The tissues then were transferred onto a stage of an inverted microscope and superfused with circulating, oxygenated Kreb's solution (36 ˚C) and equilibrated for three hours. Steel stimulating electrodes were placed in the bathing solution, oral and aboral to the muscularis externa sample. IJPs were elicited by delivering single transmural stimuli of 100 volts for 200 and 500 milliseconds to simulate release of neurotransmitters from the myenteric plexus. Tissue stimulation was repeated at five-minute intervals to allow for re-equilibration. Measurement of membrane voltage changes was accomplished by impaling smooth muscle cells with a fine glass electrode filled to the shoulder with 1.0 mol L^{-1} KCl and filled the rest of the way with 2.0 mol L^{-1} KCl. Stimulations were accomplished using an amplifier, GRASS S1U5 stimulus isolation unit (Grass Instruments Co., Quincy, MA, USA) and the electrical signals were acquired and analyzed using a PowerLab Chart version 5.01 data acquisition device

(ADInstruments, Colorado Springs, CO, USA). We actively sought out smooth muscle cells exhibiting a resting membrane potential of -50 millivolts that demonstrated slow-wave activity (Figure. 2.4).

Drugs

Drugs used included: M4 6mg, M5 10 mg, M7 8mg, TDS1 200 µM, TDS2 200µM, TDS3 200µM, TDS4200 µM, 0.05 g GBB, quercetin 200 µM, and hesperetin 200 µM (all GBB derivatives were dissolved in ETOH, while hesperetin and quercetin were dissolved in DMSO to make stock solutions first). In addition, TTX 1 µM, L-name 200 µM and MRS 2179 1µM were used to characterize junction potentials. All drugs were applied to tissues in the recording chamber via re-circulating Kreb's solution (100 mL) for 30 minutes following roughly 20 minutes of baseline recordings.

Statistical Analysis

GraphPad Prism 5.1 software was used to perform all statistical analyses. A one-way ANOVA was used to compare the treatments and controls to the baseline measurements. We considered differences as statistically significant at $P < .05$, and is indicated by *, where ** is $P< .010$, *** $P< .005$, and **** $P<.001$. Numerical values given for treatments, baselines and controls are means followed by the standard deviation.

Results

M4, M5 and M7 inhibit colon motility

In the present study, pellet velocities were expressed as the distance the pellet was propelled through the segment, and the amount of time taken, in millimeters per second. We recorded 5-6 baseline pellet velocities prior to treating tissues with any drug, and the average baseline velocity was 2.6 +/- 0.5 mm/sec (Figure 2.3). After 10 minutes of exposure M4 reduced pellet velocity (M4: 0.86 +/- 0.37 mm/sec, *n*= 4; *P< 0.001*), M5 reduced pellet velocity (M5: 1.01 +/- 0.14 mm/sec, *n* = 2; *P< 0.005*) and M7 reduced pellet velocity (M7: 0.77 +/- .03 mm/sec, *n* = 2; *P< 0.005*). M3 did not affect pellet velocity (M3: 2.708 +/- 0.273 mm/sec, *n*= 5). Interestingly, hesperetin inhibited propulsive motility (hesperetin: 0.49 +/- 0.4 mm/sec, *n*= 4; *P< 0.001*). Furthermore, as with GBB, PTLC1, and PTLC5, MPLC fractions (M4, M5, and M7) caused aboral ectopic contractions.

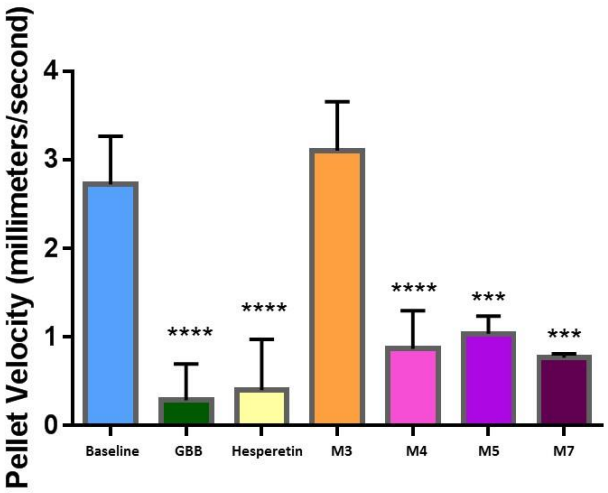

Figure 2.3. M4, M5 and M7 significantly reduced pellet propulsive velocity. Bar graph showing that compared to average baseline pellet propulsion velocity, GBB (*P < 0.001,*), M4 (*P < 0.001*), M5 (*P < 0.005*), M7 (*P < 0.005*) as well as hesperetin (P<*.001*) significantly inhibited pellet velocity. M3 had no effect on velocity.

Characteristics of IJPS in porcine ileum

Intracellular micro-electrode recording of circular smooth muscle cells in muscularis externa from porcine ileum produced characteristic traces of rhythmic electrical membrane potential fluctuations, called slow waves (Figure 2.4). As indicated by Figure 2.4 A, transmural field electrical stimulations were triggered during the end of a slow wave, as the smooth muscle cell membrane potential returned to resting potential. It is known that IJPs are biphasic electrical events consisting of a fast and a slow phase, the fast phase is caused by the purines acting at P2Y1 receptors (purinergic signaling), and the slow phase by nitric oxide interacting with guanylate cyclase (niteregic signaling, Figure 2.5.) [15, 19, 20]. IJP amplitudes are measured by the difference in voltage between their peak hyperpolarization during the event and the resting membrane potential.

To characterize whether IJPs are elicited by neurotransmitter release from the myenteric plexus, we recorded baseline IJPS and then tested the effect of Tetrodotoxin (TTX), a neurotoxin known to block most voltage-gated sodium channels [23], on the discharge of IJPs. TTX abolished IJPs after 3-5 minutes (Figure 2.6 C). We then evaluated if nitric oxide (NO) contributes to the discharge of IJPs in porcine ileum by blocking the synthesis of NO using 10 µM L-name, a nitric oxide synthase inhibitor [24]. We found that application of L-name for up to 30 minutes did not affect IJP amplitude. To test if purines play a role in the discharge of IJPs in porcine ileum by activating P2Y1 receptors, we used MRS 2179, a P2Y1 receptor inhibitor [25]. Our results show that MRS 2179 inhibits IJPs after 3-7 minutes (Figure 2.6 B). Taken all together, our results suggests that in porcine ileum, IJPs are triggered by release of purines from

the myenteric plexus; these purines communicate with smooth muscle via P2Y1 receptors.

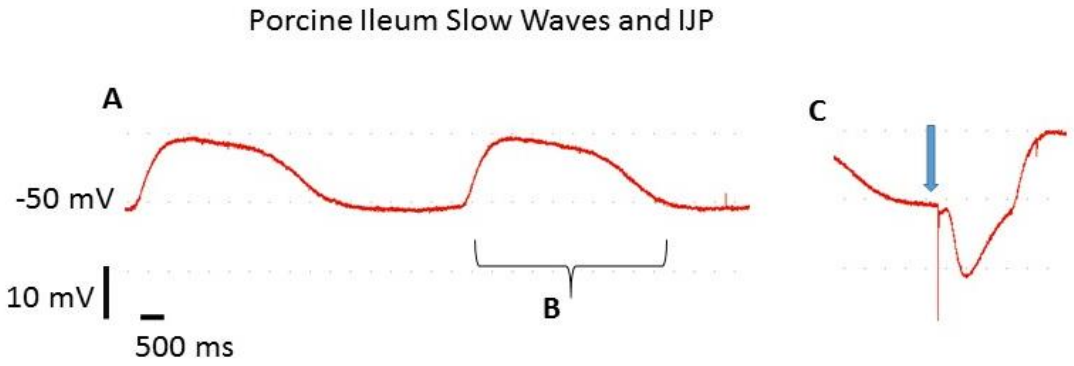

Figure 2.4. Traces from porcine ileum circular muscle. **A** Depiction shows slow waves (indicated by region **B**), in porcine ileum circular smooth muscle, with a resting membrane potential of -50 mV. **C** shows IJP in same trial, and (blue arrow) shows timing of 100 volts stimulus triggering IJP.

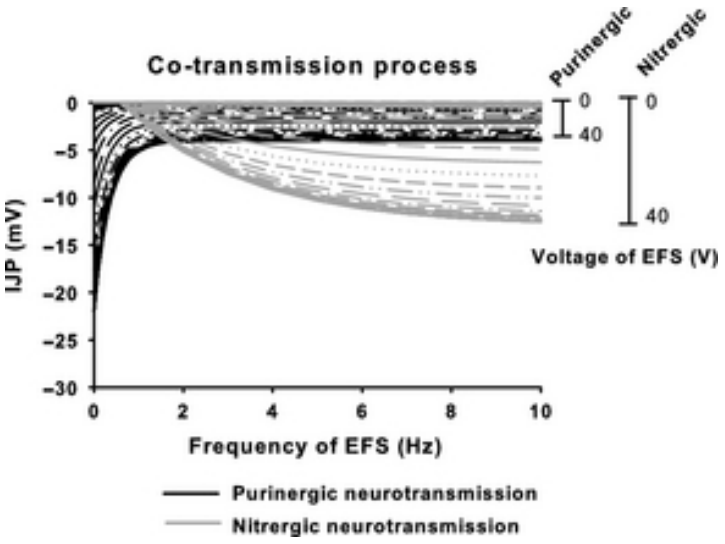

Figure 2.5. Roles of purinergic and nitrergic signaling in human colon relaxation. As voltage of stimulation increases co-transmission and response increases [47].

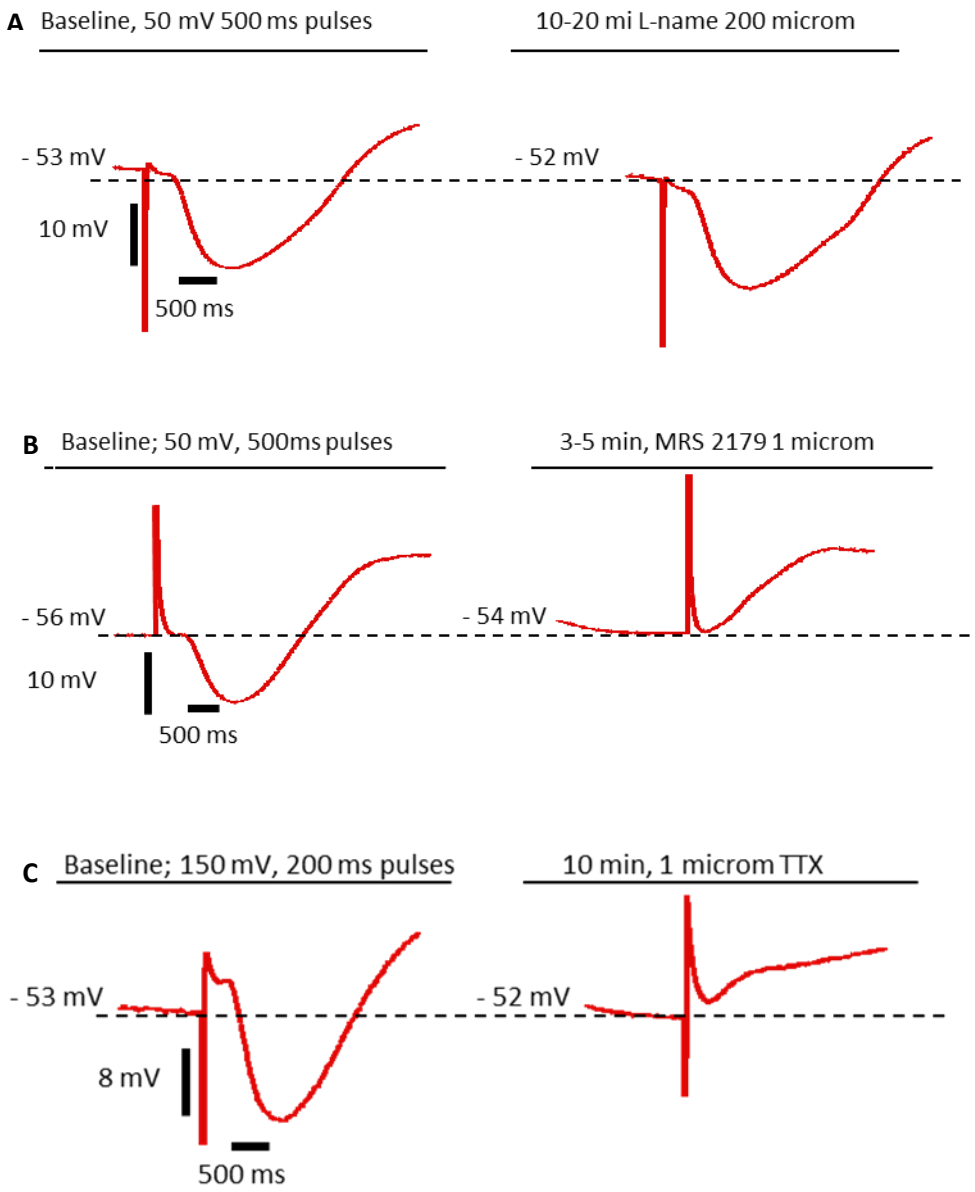

Figure 2.6. Recordings of the effects on IJP in porcine ileum circular muscle by L-name, MRS 2179 and tetrodotoxin. **A** shows the recorded traces before and after bath application of MRS 2179, **B** shows the recorded traces before and after bath application of L-name, and **C** shows the recorded traces before and after bath application of TTX.

GBB, M4, M5 and M7 inhibit IJPs

We investigated the effect of GBB, M4, M5, and M7 on the discharge of IJPs in smooth muscle cells of porcine ileum. GBB significantly reduced IJP amplitudes after 10 minutes and IJPs were almost abolished after 20 minutes (Baseline: 13.1 +/- 1.2 mV, *n* = 5, GBB 10 minute: 1.9 +/- 1.3 mV, *n*=3; *P< 0.001;* GBB 20 minute: 0.003 +/- 0.001 mV, *n* = 3, *P < 0.001*) (Figure 2.7). We found that M4 significantly inhibited IJPs after both 10 and 20 minutes (Baseline 13.1 +/- 1.2 mV, *n*= 5, M4 10 minute: 10.37 +/- 0.01 mV, *n*=2; *P< 0.05;* M4 20 minute: 9.62 +/- 0.03 mV, *n* = 3, *P < 0.005*), and that M5 inhibited discharge of IJPs after 20 minutes (Baseline 13.1 +/- 1.2 mV, *n*= 5, M5 20 minute: 10.77 +/- 1.1 mV, *n*=2; *P< 0.01*). The control compound TTX completely abolished IJPs (Baseline 13.1 +/- 1.2 mV, *n*= 5, TTX 10 minute: 0 +/- 0 mV, *n*=2; *P< 0.001;* TTX 20 minute 0 +/- 0 mV, *n* = 3, *P < 0.001*). Compared to baseline measurements, M7 did not have a significant effect on IJPs even after prolonged exposure (Baseline 13.1 +/- 1.2 mV, *n*= 5, M7 20 minute: 13.43 +/- 0.5 mV, *n*=2). Collectively, these results suggest that M4 and M5 are the active components of GBB that contain compounds which inhibit neurotransmission.

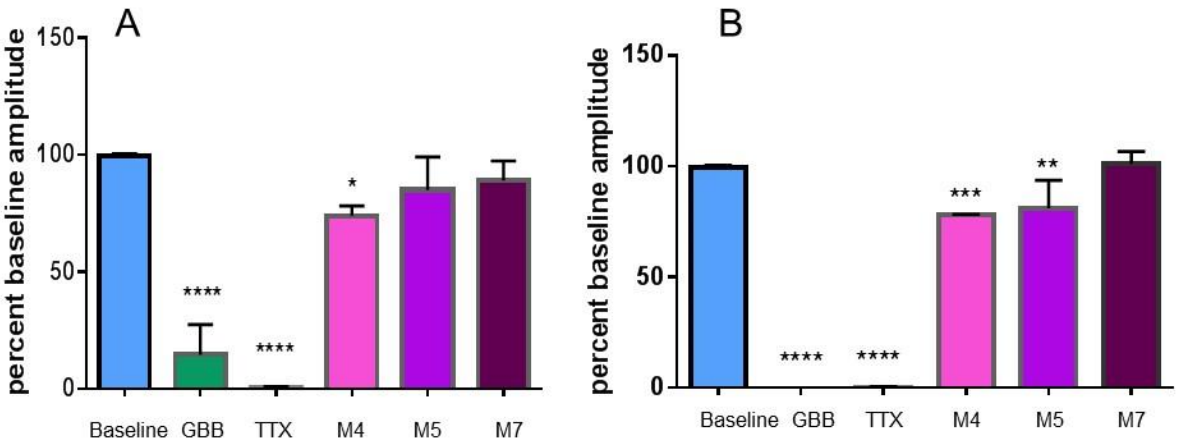

Figure 2.7. IJP results for MPLC fractions. GBB (**** *P< .001)*, M4 (10 minutes: * *P< .05; 20 minutes:* *** *P< .005)* and M5 (** *P< .01)* significantly reduce IJP amplitude. IJP amplitude measurement results for **A** 10 and **B** 20 minutes after bath application of HPLC fractions in comparison with tetrodotoxin (TTX, (**** *P< .001*), GBB, and baseline recordings. Results shown as a percentage of baseline IJP amplitude.

TDS1 and TDS2 inhibit IJPs

In light of the findings that the active compounds in M4 and M5 are the biflavanones TDS1 and TDS2 respectively, we investigated the effects of these pure compounds on IJPs. We found that TDS1 significantly reduced IJP amplitude after 10 minutes and its inhibitory effect on IJPs increased with the exposure time (Baseline 13.1 +/- 1.2 mV, *n*= 5, TDS1 10 minute: 6.3 +/- 2.96 mV, *n*=4; *P< 0.005;* TDS1 20 minute: 6.38 +/- 1.98 mV, *n* = 4, *P < 0.001*), Figure. 2.8 B). Similarly, we found that TDS2 inhibited IJPs after 10 minutes and the effect increased after prolonged exposure (Baseline 13.1 +/- 1.2 mV, *n*= 5, TDS2 10 minute: 8.1 +/- .09 mV, *n*=; *P< 0.01;* TDS2 20 minute: 5.98 +/- 0.66 mV, *n* = 3, *P < 0.001*). We wanted to know if other biflavanones, flavanones and flavonoid compounds inhibit IJPs. Therefore, TDS4 (a biflavanone) [26], hesperetin, a flavanone monomer [27], and quercetin, a flavonoid, [27, 28] were used in control

experiments. The results showed that TDS4 inhibited IJPs after 20 minutes (Baseline 13.1 +/- 1.2 mV, *n*= 5; TDS4 20 minute: 8.24 +/- 1.46 mV, *n* = 3, *P < 0.001)* and similarly, that hesperetin inhibited IJPs after 20 minutes (Baseline 13.1 +/- 1.2 mV, *n*= 5; hesperetin 20 minute: 8.11 +/- 1.06 mV, *n* = 2, *P < 0.001)*. Quercetin did not have any measureable effect. These results show for the first time that GBB inhibits IJPs and that TDS1, TDS2 and TDS4 are neuroactive compounds that inhibit IJPs. However, TDS1 and TDS2 are more potent than TDS4.

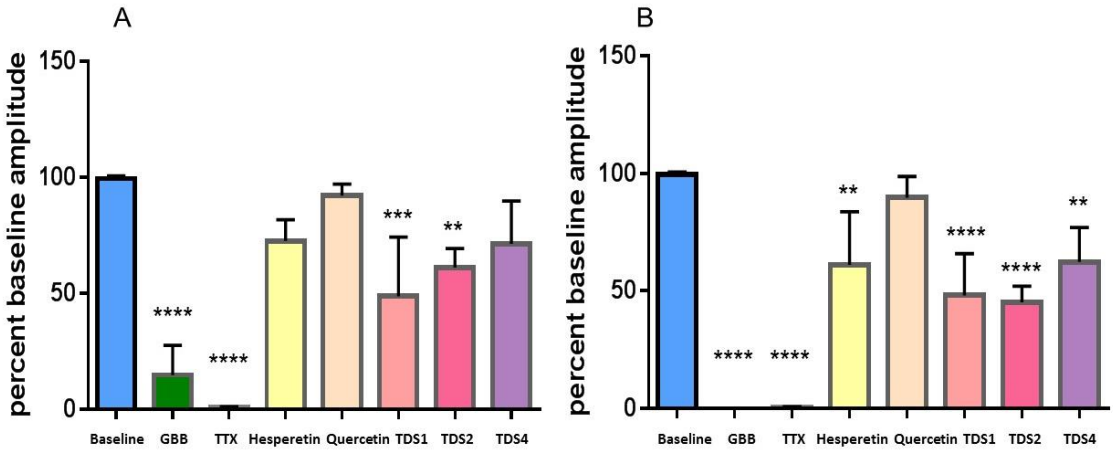

Figure 2.8. IJP results for 10 and 20 minutes of bath application of pure compounds. TDS1 (10 minutes: *** *P< 0.005;* 20 minutes: **** *P< 0.001)*, TDS2 (10 minutes: ** *P< 0.01;* 20 minutes **** *P< 0.001)*, hesperetin (** *P< .01)*, quercetin and TDS4 (** *P< 0.01)* inhibited IJPs and is compared with tetrodotoxin (TTX, **** *P< 0.001*), GBB (**** *P< 0.001)* and baseline recordings. Results shown as a percentage of baseline IJP amplitude. **A** depicts after ten minutes of bath application, and **B** after 20 minutes bath application.

Discussion

The goal of this study was to test the central hypothesis that MPLC fractions (and therefore primary compounds in these fractions) decrease propulsive motility and increase overall transit time by triggering aboral ectopic contractions, which prevent aboral smooth muscle relaxation, and that this occurs by inhibition of IJPS. The main findings of this study showed that MPLC fractions M4, M5 and M7 significantly inhibited motility. Furthermore, that M4 and M5, as well as the main bioactive compounds of M4 and M5, TDS1 and TDS2 respectively, significantly inhibited IJPs. Additional new findings were the observations that GBB, hesperetin, and TDS4 significantly inhibited IJPs. Taken together, these findings strongly suggest that as the main components of HPLC fractions M4 and M5, TDS1 and TDS2, slow motility by inhibiting aboral relaxation through the inhibition of inhibitory junction potentials. Given that in porcine ileum IJPs were purely purinergic as shown previously, this suggests that M4, M5, TDS1, and TDS2 inhibit purinergic IJPs.

In the present study, we discovered that MPLC fractions M4, M5, and M7 are the components of GBB that significantly inhibit motility, suggesting that the anti-motility compounds within GBB reside in these fractions. Motility assays are a high-throughput screening tool that can be used to detect substances that modulate enteric neurotransmission and motility [2, 12]. GI motility requires complex integration of neurochemical signaling within the ENS as well as ENS signaling to smooth muscle to coordinate rhythmic contraction and relaxation [13, 14, 17, 20]. To exert the anti-motility effects, a substance must effect ENS signaling, or inhibit enteric neuromuscular transmission, or inhibit smooth muscle contraction or relaxation [12, 14, 17]. These

results were the first to show that post-separation from GBB, M4, M5, and M7 retain anti-motility effects. Previous research showed that PTLC1 and PTLC5 treated diarrhea, and correlate with M4 M5, and M7 [7]. This suggests that M4, M5, and M7 have anti-diarrheal effects. These results strongly suggest that M4, M5, and M7 contain the compounds causing GBB's anti-diarrheal effects and that likely the active compounds could reduce hypermotility, which is associated with diarrheal illnesses.

The most important discovery was that GBB, M4, and M5 inhibited motility by significantly inhibiting IJPs, and that TDS1 and TDS2 are the main neuroactive compounds within M4 and M5. Our results support the hypothesis that GBB, M4, M5 inhibit aboral smooth muscle relaxation, which is necessary for peristalsis, by causing ectopic contractions via inhibition of IJPs. Noteworthy, GBB, inhibited IJPs more dramatically than M4, and M5. The main reasons for the difference are not apparent at this time. TDS1 and TDS2 significantly inhibited IJPs suggesting that they are the main neuroactive compounds of M4 and M5, and therefore GBB, and that these compounds inhibit neurotransmission. We have previously shown that GBB inhibits synaptic transmission [1], mechanosensory neurons [4], and nociceptive signaling in mesenteric afferents [3]. In addition, M4, and M5 inhibit mechanosensory neurons and nociceptive signaling in mesenteric afferents [4, 5]. Therefore, while our findings suggest that GBB, M4, M5, TDS1 and TDS2 inhibit neuromuscular transmission, it is likely that TDS1 and TDS2 inhibit synaptic transmission, mechanosensory neurons and nociception. Both nitric oxide and purines act as inhibitory neurotransmitters from motor neurons to intestinal smooth muscle cells in human and in some animal species [14, 21, 20]. In porcine ileum smooth muscle cells, however, purines, which act via P2Y1 receptor, are

the predominant inhibitory neurotransmitters [21, 20]. Our results show that GBB, M4, and M5 reduced IJPs in a way similar to the results of MRS 2179, a P2Y1 receptor antagonist [21], suggesting that GBB derived fractions and active compounds either inhibit purinergic neurotransmitter release, or possibly inhibit P2Y1 receptors. However, the observation that IJPs were blocked by TTX, suggest that GBB derived fractions and active compounds act via a neuromechanism. Determining whether this blockade is pre-synaptic, or post-synaptic however, requires tests on enteric neurons as well P2Y1 receptor expressing cells as shown by Liñán-Rico and colleagues [21].

Because M7 does inhibit motility but not IJPs it must be acting through a different mechanism than M4 and M5. The effect on muscle function but not on IJPs suggests a mechanism other than inhibiting relaxation.

For the first time our results showed that TDS4 (a characterized biflavanone) and hesperetin (a known flavanone) significantly inhibit IJPs. TDS4 is the primary component (by concentration) of GBB, and is main component of the MPLC fraction M3 [13, 26]. Because previous studies showed that M3 and TDS4 do not affect motility [10], we assumed that TDS4 would not affect IJPs, and as such used it as negative control. TDS4 unexpectedly inhibited IJPs. Likewise, hesperetin inhibits IJPs, however, hesperetin also inhibits motility. Clearly, TDS4 and hesperetin have significant effects on neurotransmission. Taken together, our results of suggests that biflavanones (TDS1, TDS3, TDS4) and flavanone monomers (hesperetin) and compounds of related chemical structures could affect motility and neuromuscular transmission each potentially with different mechanisms.

TDS4, hesperetin and quercetin have been the subject of extensive testing for their anti-oxidative capacities [10, 26, 27, 28]. Until this study, no research has been conducted on their effects on enteric motility or IJPs. Quercetin, a flavonoid, and hesperetin, a flavanone, are found in citrus fruits but are not readily absorbed [27, 28]. We selected these two characterized compounds to test if inhibition of motility and IJPs was a characteristic common to flavanones and flavonoids. Although quercetin has been shown to inhibit synaptic transmission [27, 28], it did not inhibit IJPs in our testing. This suggests a specific blockade of signaling that does not affect the transmission of signals between final inhibitory motor neurons to smooth muscle [13, 17, 19].

Conclusions. Collectively, we have discovered that GBB derived compounds, TDS1 and TDS2 inhibit motility and inhibitory neuromuscular transmission. Our findings strongly suggest that TDS1 and TDS2 are anti-diarrheal, neuroactive compounds of GBB that inhibit motility, neurotransmission and pain. These results strongly support the view that GBB is a potential source for new, neuroactive, anti-motility compounds, and that further work is needed to elucidate the derivative compounds' mechanistic actions. Further testing on biflavanones, flavanones and flavonoids is necessary to elucidate how these commonly ingested compounds effect enteric motility, and their effects on the ENS.

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Chapter 3: GBB and Derivative Compounds, (2R,3S,2″R,3″R)-GB-2, and (2R,3S,2″S)-buchananiflavanone Inhibit Synaptic Transmission

Introduction

Garcinia buchananii stem bark extract (GBB) is a traditional phytotherapy that has been used in sub-Saharan Africa for generations to treat a variety of gastrointestinal illnesses, diarrhea, dysentery and pain [1, 2, 3, 4]. Research shows that GBB is an effective treatment for lactose-induced diarrhea and is anti-nociceptive [3]. Currently, oral rehydration solutions (ORS, to combat dehydration due to intestinal hypersecretion) and opiates (to slow enteric motility and curtail pain) taken together represent the only broadly applicable combination therapy that mitigates diarrhea and pain [5, 6, 7]. However, opiates are controlled, addictive substances with 15.5 million opioid-dependent adults worldwide, which can cause constipation, fatal overdoses and fatal paralytic ileus in children [5, 8, 9, 10, 11]. ORS is effective in reducing dehydration during diarrheal episodes, but has no effect on motility or the duration of the diarrheal episode, and has low patient compliance due to these deficits [6, 12, 13]. The medical and scientific communities show critical need for novel, antidiarrheal treatments as alternatives to opiates that act by inhibiting neurotransmission in the enteric nervous system (ENS), and thus can inhibit hypermotility and pain associated with diarrheal illnesses [14, 15].

A growing body of research suggests that GBB exerts anti-diarrheal effects by reducing intestinal fluid secretion [3] and by reducing propulsive motility (peristalsis) and nociception, through inhibition of synaptic transmission within the ENS [1, 16, 17]. However, the neuroactive compounds that inhibit synaptic transmission are not known.

Identification of compounds that cause the antidiarrheal effects in GBB required separation of GBB into fractions and bioactivity-guided purification of the active compounds [18]. Separation of GBB using preparative thin-layer chromatography (PTLC) and medium-pressure liquid chromatography (MPLC) showed that PTLC and MPLC separation of GBB yielded several fractions (two PTLC: PTLC1, PTLC5 and three MPLC fractions: M4, M5 and M7) that retained GBB's antidiarrheal and antimotility and effects [3, 19, 20; Patterson et al., unpublished; chapter 2]. Comparison of the chemical composition of the PTLC and HPLC fractions showed correlation between the antimotility fractions, such that, PTLC1 corresponds with M4 and M5, and PTLC 5 corresponds with M7 [18, 21]. Further, the primary pure compounds in M4, M5 and M7 were identified as TDS1 (naringenin-C-3/C-8''dihydroquercetin linked biflavanone; previously called GB-2), TDS2 ((2R,3S,2″S)-buchananiflavanone, previously called buchananiflavanone), TDS3 (the structure of this compound is not yet fully characterized), respectively [18, 20]. Noteworthy, a compound that constitutes about 43% of the mass of known and unknown compounds in the ethanolic extract of GBB is found in MPLC fraction 3, also known as M3. This compound is a biflavanone known as (2R,3S,2'' R,3''R)-manniflavanone, here forth denoted as TDS4. Interestingly, while TDS4 did not affect colon motility in isolated guinea pig ileum, which suggests that it does not affect synaptic transmission [Patterson et al., unpublished,

chapter 2], it has been shown to inhibit L-type calcium channels and neurotransmission [22, 23; Patterson et al., unpublished; chapter 2].

To screen for neuroactive compounds having antimotility effects that act by inhibiting neurotransmission, we employed gastrointestinal motility assays, [3, 24] in addition to micro-electrode intracellular recording of junction potentials [25, 26, 27]. The results show that M4, M5, and M7 all significantly inhibited propulsive velocity in guinea pig distal colons, and that M4 and M5 inhibited inhibitory junction potentials in the circular smooth muscle of porcine ileum, while M7 did not. In light of the discovery that TDS1 and TDS2 are the primary components of M4 and M5 [19, 21], the pure compounds were tested as well, and also showed significant inhibition of IJPs [Patterson et al., unpublished; chapter 2]. Collectively, these results suggest that TDS1 and TDS2 are the neuroactive compounds of GBB that inhibit neuromuscular transmission and likely inhibit synaptic transmission and mitigate diarrhea and pain.

Synaptic transmission in the ENS is the signaling via neurotransmitters between ENS neurons (sensory neurons, interneurons and motor neurons) and from ENS neurons to effector cells [23, 28, 29]. The ENS coordinates intestinal secretion and motility, therefore hypersecretion and hypermotility associated with diarrhea must be the result of modulation of synaptic transmission within the ENS [25, 28, 30]. The classic way to test the effects of a drug thought to inhibit synaptic transmission in the ENS is to use fiber tract stimulation (FTS) of internodal strands to trigger neurotransmitter release and measure postsynaptic effect by intracellular microelectrode recording in neurons, but this method is very laborious and only allows for individual cellular recording at a time [1, 26]. Effects on synaptic transmission can be measured with calcium imaging,

as it allows for visualization of signaling between neurons, and can be used to look at networks of neurons in ganglia (conglomerations of neuron cell bodies), and therefore measure the effects in multiple neurons simultaneously [21, 28, 30]. In calcium imaging, calcium indicator dye binds free intracellular calcium to emit fluorescence and is used as a reporter of neural signaling [21]. Fiber tract stimulation (FTS) of internodal strands between myenteric ganglia in combination with calcium imaging allows for testing of post-synaptic responses in neurons within a ganglion, to measure their responses to stimulation before and after drug application [11, 21, 30].

The main goal of this study was to test the hypothesis that TDS1, TDS2, and TDS3 inhibit synaptic transmission. This was accomplished by visualizing calcium transients in response to FTS of internodal stands between myenteric ganglia of guinea pig distal colon, before and after drug application. Our results show that GBB and TDS2 inhibited post-synaptic calcium transients in guinea pig colon myenteric ganglia. TDS2 is likely the primary component of GBB that inhibits synaptic transmission.

Materials and Methods

Animals and solutions

The University of Idaho Animal Care and Use Committee approved of all studies. Six-month-old male and female guinea pigs were maintained at 23–24 °C on a 12/12 hour light-dark cycle with access to food and water ad libitum in plastic cages with soft bedding. Distal colon sections of approximately 10-12 cm in length were collected via midline laparotomy following isofluorane anesthesia and exsanguination. After dissection and before use, colon sections were kept in ice-chilled HEPES buffer (20 mM HEPES (pH 7.4), 115 mM NaCl, 5.4 mM KC1, 2.2 mM CaCl, 0.8 mM MgC12, 1 µM probenecid, and 13.8 mM glucose, all purchased all from Sigma, St. Louis, MO, USA). Imaging tissues were perfused with oxygenated, circulating Kreb's solution (mmol L^{-1}: NaCl, 121; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; .001 probenecid; and glucose 8; all from Sigma, St. Louis, MO, USA; aerated with 95% O_2/5% CO_2). Dye dilution was 1 vial fluo-4-AM filled (Life Technologies, Eugene, Oregon, USA) with DMSO to make 2.5 mM stock solution (purchased from AMRESCO, Cleveland, OH, USA). Loading dye (2mL HEPES, .01% Koliphor, Sigma, St. Louis, MO, USA and 20 µM, fluo-4-AM dilution).

Calcium Imaging

Guinea pig distal colon samples were dissected in HEPES to remove the mucosa and submucosa and inner circular muscle to expose the longitudinal smooth muscle-myenteric plexus (LMMP). Dissection and recording were performed using HEPES containing nisodipine (1 µM, purchased from Sigma, St. Louis, MO, USA) and atropine (1 µM, purchased from Sigma, St. Louis, MO, USA)to block muscle contractions. LMMP preparations were trimmed (1.5 x1.5 cm) and pinned-stretched on custom made silicone resin (Silgard) blocks (0.8 cm x 1.1 cm) as described previously [31]. Tissues were then set into chambers with 2mL of loading dye for 3 hours, with gentle agitation every 20 minutes. A washing period followed for 30 minutes, in 2 mL HEPES, for de-esterification of intracellular dye. Loading and washing were done at room temperature, protected from light. For imaging, tissues were then placed on a cover slip at the bottom of a chamber and mounted onto the stage of a Nikon-Andor spinning disk confocal microscope (Nikon instruments, Seattle, WA, USA). Tissues were continuously superfused with recycling, oxygenated Kreb's solution and maintained at 36 ˚C, containing nisodipine (1 µM, purchased from Sigma, St. Louis, MO, USA) and atropine (1 µM, purchased from Sigma, St. Louis, MO, USA) to block muscle contractions. After an initial 30-minute equilibration period, we placed a Teflon-coated platinum electrode, connected to GRASS stimulator via a GRASS S1U5 stimulus isolation unit (Grass Instruments Co., Quincy, MA, USA), on top of internodal strands of the myenteric ganglia for fiber tract stimulation (FTS) and allowed to equilibrate for an additional 20 minutes (Figure. 2.1). We applied 3-second FTS of 100 Hz (at 50V, for 4 µs duration) at 5-minute intervals for two baseline recordings. We stimulated after

five minutes, 10 minutes and 20 minutes during drug application trials. Imaging was done using Zeiss Ar-Kr laser (Carl Zeiss Meditech INC., Dublin CA) to excite nerve cells at 488 nm and Nikon Elements software to capture and record images. We analyzed Calcium imaging results using measurements of the amplitude of the calcium transient spikes in ABUs (arbitrary units, Figure 2.2) and comparing the amplitude of spikes generated to the spikes generated during the baseline recordings. ABUs are the unit of measurement Nikon Elements Software uses to indicate relative changes in intensity of fluorescence.

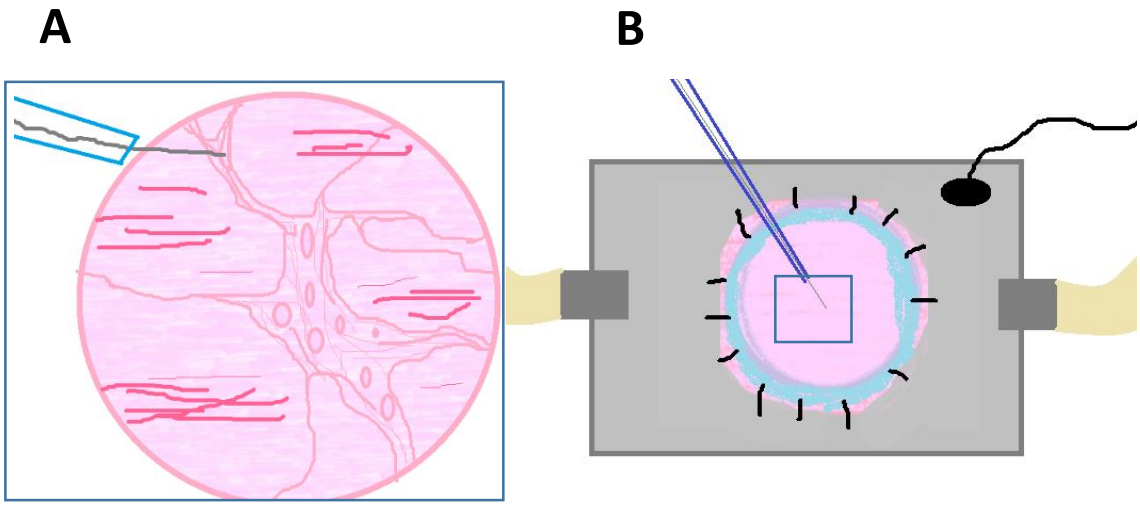

Figure 3.1. Calcium imaging and fiber tract stimulation tissue set up. **A,** Depiction of how imaging of muscularis externa preparations (of guinea pig distal colon myenteric ganglia) calcium transients was conducted. **B,** zoomed in depiction (from **A**) of Teflon-coated wire placed on the internodal fiber-tract of the myenteric plexus in guinea pig colon longitudinal smooth muscle-myenteric plexus (LMMP) preparation.

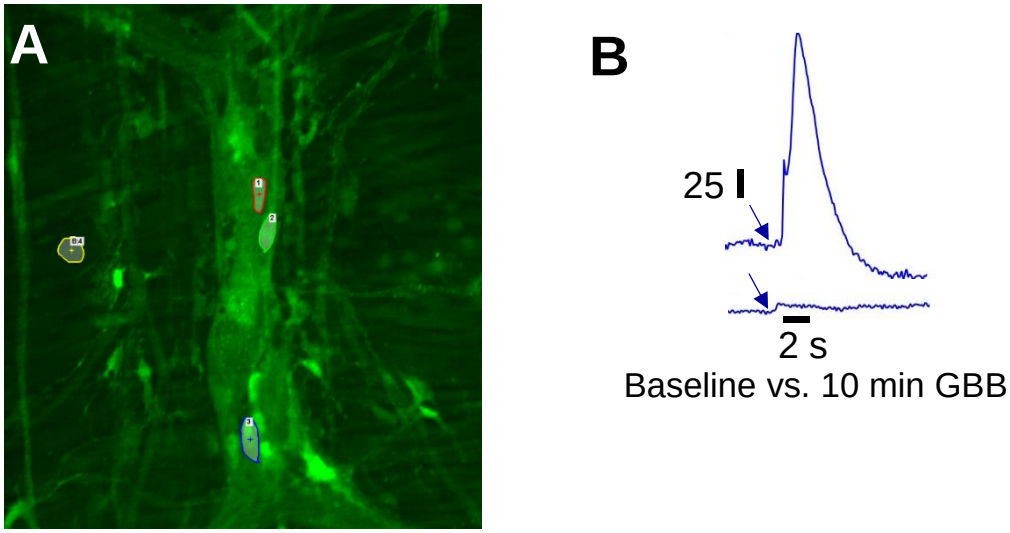

Figure 3.2. Demonstration of the LMMP preparation and a myenteric ganglion neurons (with ROIs) used to measure calcium transients (**A**). **B,** traces of post synaptic calcium transients triggered by FTS in ROI3 (see **A**) showing baseline amplitude (larger trace) and the effect GBB after 10 minute exposure (smaller trace). FTS indicated by arrows, scale of 25 indicates ABUs. ROIs = regions of interest.

Drugs

GBB, TDS1, TDS2, TDS3, TDS4 and quercetin were first dissolved into absolute ethanol to make stock solutions. Hesperetin was dissolved into DMSO. Drugs were then added to 100 mL re-cycling Kreb's solution for superfusion of the tissue via bath application. All drugs, except GBB, were applied in a 200 µL concentration, and GBB in .05 g/100mL Kreb's. The concentration of DMSO and ethanol was < 1:1000.

Statistical analysis

GraphPad Prism 5.1 software was used to perform all statistical analyses. A one-way ANOVA was used to compare the treatments and controls to the baseline measurements. We considered differences as statistically significant at $P < .05$, and is indicated by *, where ** is $P< .010$, *** $P< .005$, and **** $P<.001$. Numerical values given for treatments, baselines and controls are means followed by the standard deviation.

Results

GBB, TDS1, and TDS2 inhibit synaptic transmission

To determine if repetition of FTS of tissues alone caused reduction in subsequent calcium transient amplitudes, we measured calcium transient amplitudes at 0, 10, and 20 minutes on untreated tissues. To measure calcium transient amplitudes we first selected neuron cell bodies within myenteric ganglia. Neurons, and not glia, were chosen based on their large cell body size, visible nucleus, and by residence within a ganglion. Intensities of the fluorescent signal, depicted as a spikes (Figure 3.5), triggered by FTS before drug application were recorded as the baseline signal in an average of four neurons. Spikes recorded in the same neurons at ten and twenty minute time-points were subtracted from their baseline spikes, thus giving the

difference in calcium transient amplitude in each neuron. Results of measured neurons within a ganglion were averaged to give a collective percentage of baseline intensity per drug trial. For the untreated tissue signals, our results showed no significant reduction in amplitudes (*n* = 3), therefore reduction in amplitudes seen post-drug application are not due to photo-bleaching or dye loss. To determine whether a drug effected affect the amplitude of calcium transients, the amplitude measured at a given time point was divided by the averaged baseline amplitudes of transients measured in the same cells before drug application. Therefore, date is shown as percentage of baseline amplitude.

We investigated the effect of GBB, and derivative pure compounds TDS1, TDS2, and TDS3 on synaptic transmission. Compared to baseline, after 20 minutes of exposure, GBB, TDS1, and TDS2 significantly reduced the amplitudes of the intensity of postsynaptic calcium transients in myenteric neurons (Baseline: 101 +/- 4, *n* = 3; GBB: 13.5 +/- 12, *n* = 4; *P< 0.001*), (Baseline: 98 +/- 6, *n* = 3; TDS1: 45 +/- 30, *n* = 5; *P< 0.05*), (Baseline: 99 +/- 4 *n* = 4; TDS2: 20 +/- 9.8, *n*= 4; *P< 0.01*). Interestingly, TDS3 did not significantly affect post synaptic calcium transients (Baseline: 101 +/- 7, *n*= 3; TDS3: 95 +/- 26, *n* = 3) (Figure 3.3).

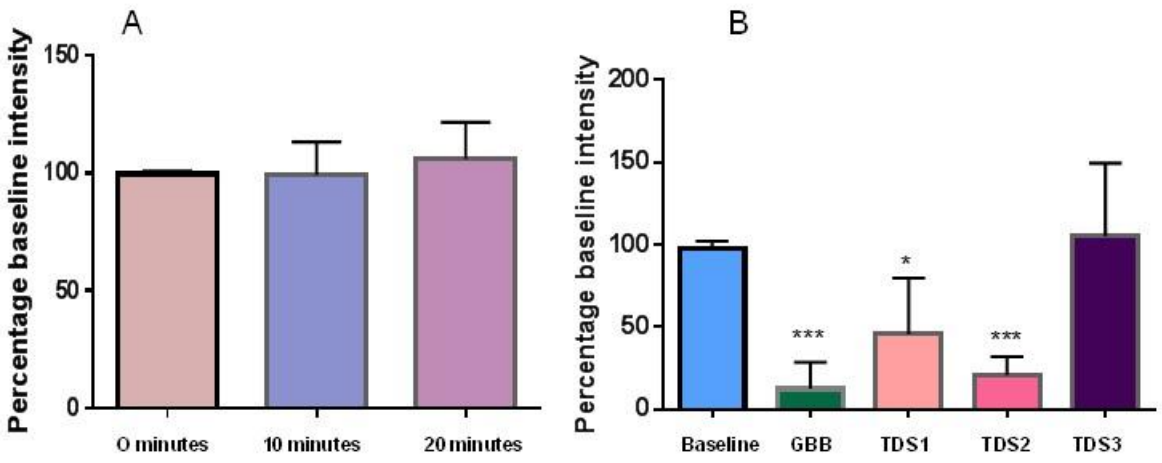

Figure 3.3. Calcium transient amplitude results from pure compounds. **A**, calcium transient amplitudes in untreated tissues. **B,** Calcium transients in tissues after 20 minutes of drug application. GBB (*** *P< 0.005)*, TDS1 (* *P< 0.05)* and TDS2 (** *P< 0.01),* significantly inhibited post synaptic calcium transients in the myenteric neurons. As measured by relative intensity in ABUs, Y-axis represents percentage of baseline intensity amplitude after drug application. The increase of intracellular calcium used as an indicator of post-synaptic neural activity after FTS.

Hesperetin, quercetin and TDS4 inhibit synaptic transmission

In the light of the responses of GBB and derivative compounds, we tested if flavanones and biflavanones, in general, inhibit synaptic transmission. Quercetin is a flavonoid that has been shown to inhibit synaptic transmission [32], hesperetin is a flavanone monomer that has not been shown to inhibit synaptic transmission [32], and similar to TDS1 and TDS2, TDS4 is a biflavanone not expected to affect synaptic transmission because in previous studies TDS4 did not affect enteric motility [Patterson et al., unpublished; chapter 2]. Therefore these compounds were selected for control experiments. Surprisingly, After 20 minutes of exposure, hesperetin (Baseline: 103 +/- 9 n= 4; hesperetin: 19 +/- 5, *n* = 4; *P< 0.005*) quercetin (Baseline: 101 +/- 5, *n* =3,

Quercetin: 23 +/- 17, *n*= 3; *P< 0.001*) and TDS4 (Baseline: 96 +/- 6 *n*= 4; TDS4: 48 +/- 5, n= 2; *P< 0.01*) all inhibited synaptic transmission (Figure 3.4). When compared with pure compounds derived from GBB, quercetin (*** *P< 0.005)*, hesperetin (** *P< 0.01),* and TDS4 (* *P< 0.05)* maintain significant inhibition (Figure 3.5). Quercetin remains comparable to results seen in pure compound testing.

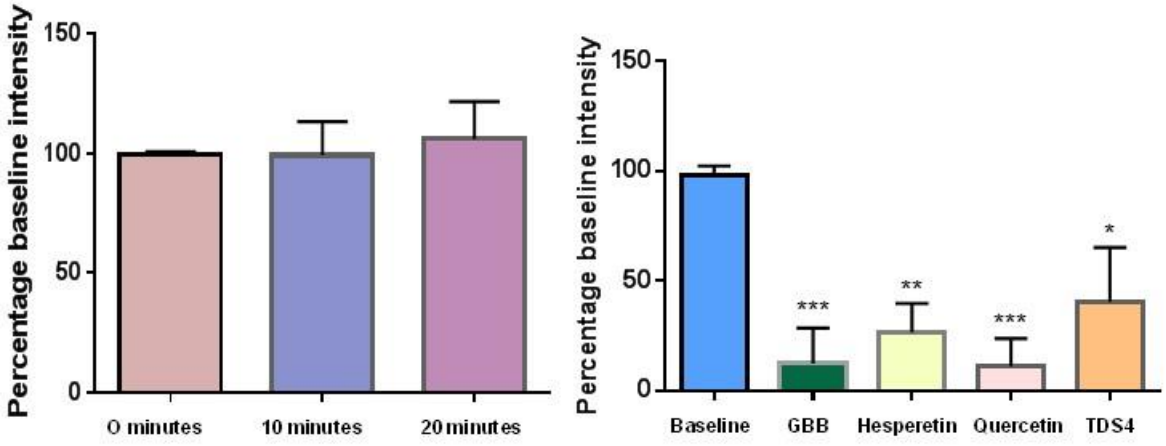

Figure 3.4. Calcium transient amplitude results from control compounds**. A**, calcium transient amplitudes in untreated tissues. **B,** Calcium transients in tissues after 20 minutes of drug application. GBB (**** *P< 0.001)*, hesperetin (**** *P< 0.001),* quercetin *(****P< 0.001)* and TDS4 (** *P< 0.01),* significantly inhibited post synaptic calcium transients in the myenteric neurons. As measured by relative intensity in ABUs, Y-axis represents percentage of baseline intensity amplitude after drug application. The increase of intracellular calcium used as an indicator of post-synaptic neural activity after FTS.

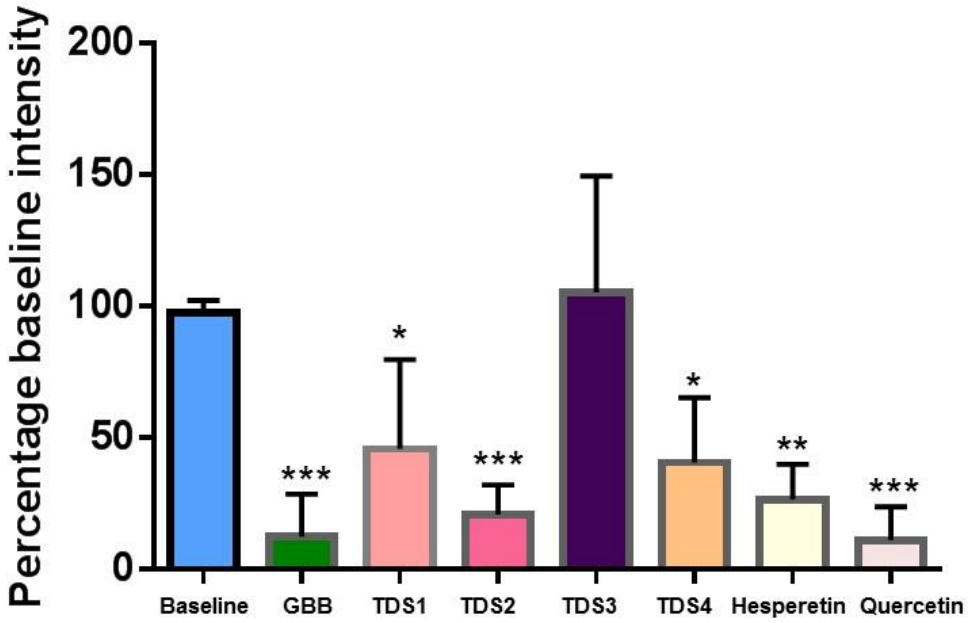

Figure 3.5. Comparison of calcium transient amplitudes in tissues after 20 minutes of drug application of all drugs tested in this study. GBB (*** *P< 0.005),* TDS1 (* *P< 0.05),* TDS2 (** *P< 0.01),* hesperetin (** *P< 0.01),* quercetin *(***P< 0.005)* and TDS4 (* *P< 0.05)* significantly inhibited post synaptic calcium transients in the myenteric neurons. As measured by relative intensity in ABUs, Y-axis represents percentage of baseline intensity amplitude after drug application. The increase of intracellular calcium used as an indicator of post-

Discussion

The goal of this study was to test the hypothesis that TDS1 and TDS2 inhibit synaptic transmission. The key results suggest that, similar to GBB, TDS1 and TDS2 significantly inhibited synaptic transmission in the myenteric plexus in guinea pig distal colon. In addition, TDS4, quercetin, and hesperetin, compounds selected for control experiments, also inhibited synaptic transmission.

The most important finding was that GBB, TDS1, and TDS2 significantly reduced the amplitudes of postsynaptic calcium transients in myenteric neurons. These results support our hypothesis that TDS1 and TDS2 inhibit synaptic transmission. It has been previously shown that GBB inhibits synaptic transmission in S-type interneurons in the myenteric plexus [1], and that it inhibits myenteric mechanosensory neurons [18] as well as nociceptive signaling in mesenteric afferents [16]. Therefore, our results suggest that TDS1 and TDS2 are likely the neuroactive compounds of GBB that inhibit neurotransmission in the myenteric plexus and nociceptive signaling. In the current study, GBB inhibited synaptic transmission more dramatically than TDS1 or TDS2, but the causes for this difference are not yet understood. It is possible that the overall effect of GBB is due to the presence of more than one neuroactive compounds in the extract. This indicates the need to test a combination of compounds isolated from GBB, most importantly the compounds that inhibit motility and junction potentials [Patterson et al., unpublished; chapter 2]. To summarize, this study identified TDS1 and TDS2 as likely being the primary neuroactive compounds in GBB that inhibit enteric neurotransmission and nociception, and as such, sets the stage for mechanistic studies.

Observations from this study suggest that some, but not all neurons, within a ganglion were inhibited by TDS1. In studying the effect of TDS1 and TDS2 on neuromuscular transmission both TDS1 and TDS2 as well as their parent GBB fractions (M4 and M5, respectively) significantly inhibited IJPs [Patterson et al., unpublished; chapter 2]. It has been shown that M4 and M5 inhibit nociceptive signaling in mesenteric afferents and mechanosensory neurons [1, 18]. Therefore, when taken with the current findings,

our studies strongly suggest that both TDS1 and TDS2 are main neuroactive compounds of GBB. However, this study suggest that effects from TDS2 are more significant than TDS1, and that TDS1 inhibits a specific phenotype of myenteric neurons. Our preliminary data suggest that TDS1 and TDS2 inhibit neuromuscular transmission in porcine ileum by inhibiting P2Y1 receptors [30]. This suggests that TDS1 and TDS2 could inhibit the excitation of P2Y1 receptor-expressing myenteric neurons, and thus TDS1 might only inhibit synaptic transmission in a specific subset of myenteric neurons while TDS2 inhibits a broader spectrum of neuron types [20, 25]. Further testing using immunohistochemistry analysis of neurons inhibited by these compounds would allow for identification of affected neurons, and if either of these drugs inhibit specific neuron types selectively.

A notable and new finding was that TDS4 significantly inhibited post-synaptic calcium transients in myenteric neurons, as well. TDS4 is the primary component of GBB, and is the pure compound isolated from M3 [13, 33]. Based on previous research, TDS4 does not significantly inhibit motility [Patterson et al., unpublished; chapter 2], but it does inhibit L-type calcium channels in smooth muscle [23]. The finding that TDS4 inhibited synaptic transmission is contrary to our expectations. However, these results correspond with the findings that TDS4 inhibits neuromuscular transmission [Patterson et al., unpublished; chapter 2]. Overall, these findings strongly implicate TDS4 as a neuroactive compound in GBB, and spurs the interest for further tests on synaptic transmission to identify the mechanisms by which TDS4 inhibits neurotransmission.

Hesperetin and quercetin have far surpassed our hypotheses regarding effects on signaling within the ENS. Quercetin is a flavonoid and hesperetin is a flavanone, and

world-wide both are commonly ingested in citrus fruits and drinks [32]. In an effort to test if antimotility and anti-neurotransmission effects were common to flavanone and flavonoid compounds, we included hesperetin and quercetin in our research. Hesperetin and quercetin both inhibited synaptic transmission. Other publications also report that quercetin inhibits synaptic transmission [32, 34]. In previous studies in our lab, Hesperetin, but not quercetin, inhibited IJPs [Patterson et al., unpublished; chapter 2]. The possible explanation for this discrepancy is that likely quercetin inhibits synaptic transmission between interneurons without affecting motor neurons. Therefore, motor neurons were still able to trigger IJPs. This reinforces a hypothesis made previously [Patterson et al., unpublished; chapter 2] as well, that these compounds likely affect different neuron types, different neurotransmitters, or different neurotransmitter receptors [1, 23, 26, 35].

Conclusions. The results of this study bolster the already-robust body of research suggesting that GBB is a potential source for novel anti-diarrheal therapies. We showed that TDS1, TDS2 and TDS4 are likely the neuroactive compounds within GBB that cause inhibition of synaptic transmission. Taken together, our results suggest that TDS1, TDS2 and TDS4 could contribute to GBB's anti-diarrheal and anti-nociceptive effects by inhibiting enteric neurotransmission and nociception. The effect of TDS4 on intestinal motility needs to be studied further. Additionally, our investigation into the effects of biflavanones, flavanones, and flavonoids on the ENS necessitates further studies, as the results suggest that these common compounds have significant effects on synaptic transmission.

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