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Botanical Aphrodisiacs for Women's Health

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BOTANICAL APHRODISIACS FOR WOMEN'S HEALTH

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ABSTRACT

Female Sexual Dysfunction (FSD) affects nearly 40% of women in the United States. While males have five FDA approved drugs for ED, there is only one FDA-approved as-needed treatment for premenopausal women with acquired, generalized Hypoactive Sexual Desire Disorder (HSDD), the most prevalent FSD. The lack of approved drugs is largely due to the biopsychosocial complexity of HSDD; however, there are neurobiological underpinnings evident. Due to the lack of safe efficacious treatment options, we hypothesize that botanical species traditionally used as aphrodisiacs may be promising leads, and exert their effects via activation of melanocortin's, an excitatory circuit implicated in sexual function, specifically at MC4R, and later the MC3R. We conducted a review of the aphrodisiac products in the U.S. dietary supplement market to determine their levels of ethnobotanical and clinical evidence and narrow species selection. Utilizing market data, we found that 53 species were used for female specific sexual complaints; concluding that there is little to no clinical evidence from the literature to substantiate their use. We selected six species for further evaluation, *Corynanthe johimbe*, *Labisia pumila, Asparagus racemosus*, *Tribulus terrestris*, *Eurycoma longifolia*, and *Trigonella foenum-graecum*. Species were sequentially extracted, concentrated, dried, and tested to determine an MTC before subjection to melanocortin assays. While *T. foenum-graecum* initially demonstrated activation, replication experiments did not. *C. johimbe, L. pumila,* demonstrated activation of both the MC4R and MC3R over control ($p < .05$); however further fractionation of *L. pumila* resulted in a loss of activity. Select isolated and pure secondary metabolites of these species were also assessed, however no activation was observed. Both species were then assessed for their ability to secrete GnRH from a GT1-7 cell line, as well as ability to cause transcriptional changes in four genes of interest. Due to high secretion variability we cannot conclude the effects of our species on GnRH secretion; however, we observed significant transcriptional changes at GNRH1 and AGRP. This study is the first to evaluate botanical aphrodisiacs at the melanocortin receptors and provides preliminary insight into a mechanistic reasoning for their persistence as such.

DEDICATION

This dissertation is dedicated to all of the Hayley's on all timelines past present and future. Thank you for being strong and resilient, and getting it done even when you weren't sure you could continue on another day. I am so proud of you and the woman that you have become, and cannot wait to see where the future brings us.

LIST OF ABBREVIATIONS AND SYMBOLS

ACTH-adrenocorticotrophic hormone

α-MSH- alpha melanocyte stimulating hormone

ANOVA- analysis of variance

AP- Aphrodisiac Product

APA- American Psychiatric Association

ASEX- Arizona Sexual Experience Scale

BID- bis in die (2x a day)

BPH- Benign Prostatic Hyperplasia

D1/2- Dopamine receptor 1 and 2

DAS- Dyadic adjustment scale

DHEA- Dehydroepiandrosterone

DISF-SR- The Derogatis Interview for Sexual Functioning

DSM- The Diagnostic and Statistical Manual of Mental Disorders

EPOR- Excitement, Plateau, Orgasm, Resolution

FDA- Food and Drug Administration

FIEI- Female Intervention Efficacy Index

FSD- Female Sexual Dysfunction

FSDS- Female Sexual Distress Scale

FSFI- Female Sexual Function Index

FSAID- Female Sexual Arousal Interest Disorder

FSH- Follicle Stimulating Hormone

GBE- *Ginkgo biloba* extract

GnRH- Gonadotropin Releasing Hormone

HAM-D- Hamilton Depression Rating Scale

HAM-A- Hamilton Anxiety Rating Scale

HPLC- High Pressure Liquid Chromatography

HSDD-Hypoactive Sexual Desire Disorder

ITT- Intent to Treat

ISIF- Index of Female Sexual Function

K-BDI- Korean-Beck Depression Inventory

KI- Kupperman Index

KRG- Korean Red Ginseng

LH- Luteinizing Hormone

MC/R- Melanocortin, Melanocortin Receptor

MEN-QOL- Menopause-Specific Quality of Life

MFI-20-Multi-dimensional fatigue symptom inventory

MGH-FSQ- Massachusetts General Hospital Sexual Function Questionnaire

MTC- Minimum Toxic Concentration

NO- Nitric Oxide

PDR- Physicians' Desk Reference

PGART- Patient's Global Assessment of Response to Therapy

PGATT- Patients Global Assessment of Tolerability to Therapy

PSS- Perceived Stress Scale

QoL- Quality of Life

QS-F- Quality of Sexual Function

SAR- Sexual Activity Record

SNRI- Serotonin-Norepinephrine Reuptake Inhibitor

SHBG- sex hormone-binding globulin

SQ-F- Sexual Quotient-Female

SSRI-Selective serotonin reuptake inhibitor

SSS-W: Sexual Satisfaction Scale-Women

STAI- The State-Trait Anxiety Inventory

TCM- Traditional Chinese Medicine

TID- Three times daily

WHO- World Health Organization

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To my universal tribe that has stuck with me no matter where in the world I have ended up, thank you for showing me that friendship can transcend all time and distance.

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1.1 APHRODISIACS

The etymology of the word aphrodisiac originates in 1719 from the Latinized form of the Greek word *aphrodisiakos* meaning inducing sexual desire, the origin pertaining to the Greek goddess of love and beauty, Aphrodite. While aphrodisiacs by modern definition are agents that arouse or held to arouse sexual desire, the use of such products dates back thousands of years across several cultures. These substances often appear in a variety of forms including plants, animals and minerals. The focus of our research is specifically on botanical aphrodisiacs of which some are rooted in deep lore of their efficacy with inconclusive or unavailable at times clinical validation. Although several species have been evaluated extensively in male models, the mechanisms vary significantly for the umbrella terminology. For instance, while *C. johimbe* may work to block alpha adrenergic receptors which increase blood vessel dilation, *Phoenix dactylifera* L. (Arecacaeae) is reported to have a positive effect on sperm quality (Kotta 2013). In females the paucity of research is coupled by the complexity of having subjective as well as genital arousal considered and while largely used as an excuse for the lack of progression, we believe the wealth of knowledge provided by centuries use of botanicals can further guide us towards clinically relevant answers. (Meston, Stanton 2019).

1.2 FEMALE SEXUAL FUNCTIONING

1.2.1 THE FEMALE SEXUAL RESPONSE

The female sexual response cycle has been postulated about in the modern world since the early 1950's when the Kinsey Reports first made headlines (Reumann 2005). Over the next forty years, the female sexual response cycle would undergo modifications starting with Masters and Johnson who conducted a comprehensive study of human sexuality, paving the way for current research on sexual disorders and

dysfunctions (Masters, Johnson et al. 1966). In a clinical lab setting, they observed volunteers to understand the psychology and physiology of sexual behavior resulting in a linear four-stage model of sexual response. This model, EPOR, excitement, plateau, orgasm, and resolution, respectively, stood as a model in the 1960s and has persisted to today (Fig. 1A(Masters, Johnson et al. 1966)).

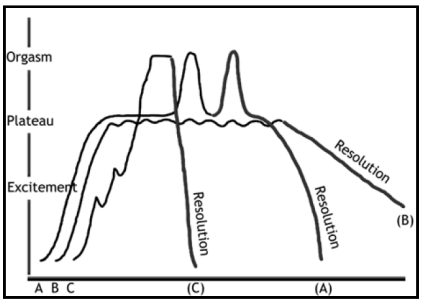

Fig. 1A Masters & Johnson Female Sexual Response Cycle

The first stage of the human sexual response cycle is the excitement phase (arousal phase), which in females can last from minutes to hrs. Generally, increases in heart rate, respiration, and blood pressure occur as well as vaso-congestion in which the female sex organs engorge and produce a lubricant to prepare for intercourse. Following comes the plateau phase, which is the period of sexual excitement before orgasm. Physiologically females continue to experience the responses of the E phase along with increased sensitivity and lubrication. The "E phase" can be considered the peak of sexual excitement for those who cannot move to the next phase of orgasm. As the third phase, orgasm is the pinnacle of the sexual experience with increased lubrication, contraction of the vagina and uterus, and overall pleasure. Following orgasm exists a resolution phase in which the body can wind down from an excited state; however, women can experience a refractory period in which they can orgasm multiple times with prolonged stimulation. These four phases happened in a consecutive linear manner and were seen as completed when all four phases had occurred.

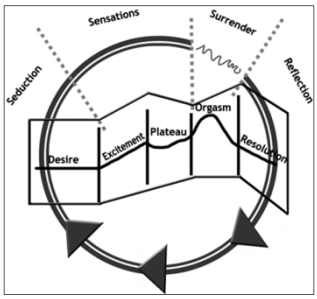

Fig. 1B Kaplan Female Sexual Response

Kaplan edited Masters and Johnsons' four phase linear model strictly based on physiology and added in the psychological component of desire. This new model although still linear was now tri-phasic (Desire, Excitement, Orgasm (Kaplan 1979)). Similarly, Whipple, and Brash-McGreer added desire into their model but recognized that all women do not fit into a linear response. Their four phase circular model is outlined as seduction (encompassing desire), sensations (excitement and plateau), surrender (orgasm) and reflection (resolution) (Fig 1B (Whipple B 1997)). This new model also portrayed the idea that prior satisfying sexual events may lead to future ones. Basson was the latest to propose a modified non-linear model which incorporated the importance of intimacy, sexual stimuli, and satisfaction within the relationship. She also challenged that desire does not need to be present for arousal to happen (Fig 1C(Jabs and Brotto 2018)).

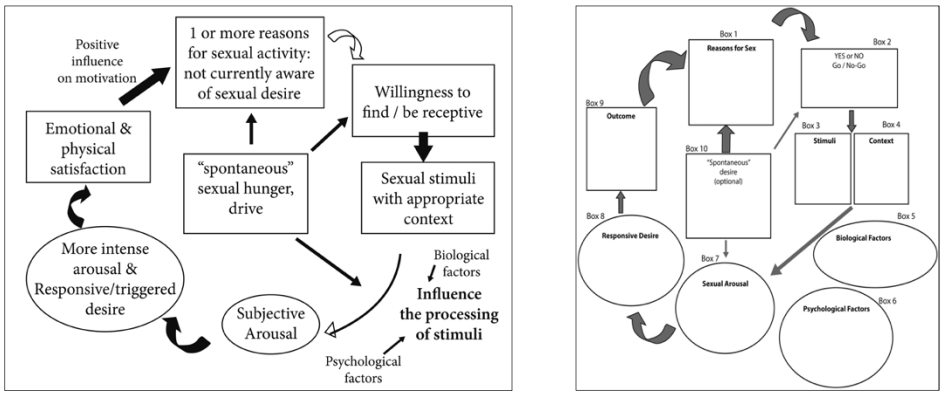

Fig 1C Whipple & Brash-McGreer Female Sexual Response Cycle; Fig 1D Adaption of Basson's Female Sexual Response Cycle

Although several models have been proposed, there is not one that is accepted as the standard for all women's sexual responses. Four studies have been conducted to evaluate women's endorsements of a model of sexual response. The results show that in general women support each model equally, but those with FSD were more likely to endorse the Basson model, while those without favored linear models (Sand and Fisher 2007, Giraldi, Kristensen et al. 2015, Nowosielski, Wrobel et al. 2016).

Although several studies have aimed to understand women's endorsement of models of sexual response, only one has focused on disruptions in the sexual response of women with FSIAD. Jabs and Brotto conducted a study utilizing a worksheet depicting a circular model of sexual response adapted from Basson's model (fig 1D(Jabs and Brotto 2018)). The aim of the study was to use Basson's model to identify where "breaks" (negative responses or absence of positive responses) occurred in the sexual response

cycle of women with female sexual interest arousal disorder (FSIAD) to determine model applicability. They concluded out of 11 possible places in the cycle to break, women on average experienced 5.1 due to biological factors such as pain or discomfort or psychological factors such as being distracted or memories of past experiences as hindrances (Jabs and Brotto 2018).

While these studies reinforce the known heterogeneity of women's sexual experiences, they share general commonalities that women without FSD were more likely to identify with linear models compared to those with FSD identifying with more circular models. A consensus needs to be made on a model to be used in clinical trials so that researchers can further identify factors in FSIAD to target and/or test before/after treatment. Identifying breaks in the cycle may also be useful in psychotherapy clinical settings as the origins of low libido/desire have psychological and sociocultural factors as well.

1.2.2 FEMALE SEXUAL DYSFUNCTIONS

These dysfunctions are complex conditions with biopsychosocial origins that contribute negatively to overall sexual health and quality of life of those women afflicted, which has been explicitly demonstrated in HSDD (Clayton, Kingsberg et al. 2018). Although an increased incidence of FSD is associated with aging and the decline of estrogen, it can affect all women of reproductive age (Mazaro-Costa, Andersen et al. 2010). Sexual disorders have existed in the American Psychiatric Associations' Diagnostic and Statistical Manual of Mental Disorders (DSM) since 1980. As stated previously, only one FDA approved treatment for FSD exists on the market today for Hypoactive Sexual Desire Disorder (HSDD) which is the most prevalent form of FSD, and is estimated to affect 5.4%-13.6% of women according to one study, while another estimated prevalence of 8.3% (Segraves and Woodard 2006, West, D'Aloisio et al. 2008). Exact prevalence may not be accurate as patients underreport their low desire and distress due to reasons such as discomfort and embarrassment while health practitioners cite reasons like a perceived lack of effective therapies (Shifren, Johannes et al. 2009, Kingsberg 2014). The latest overhaul to the DSM combines HSDD with an arousal disorder into an all-encompassing Female Sexual Interest/Arousal Disorder (FSIAD)(American Psychiatric Association 2013). Due to the lack of research under the current diagnostic classification of FSIAD, this paper will speak of evidence found under the DSM IV name HSDD or general sexual function/dysfunction.

Table 1: Classifications of Female Sexual Dysfunctions according to the Diagnostic and Statistical Manual of Mental Disorders	
DSM-IV-TR (American Psychiatric Association 2000)	DSM-V-TR (American Psychiatric Association 2013)
Desire disorders • Hypoactive Sexual Desire Disorder (HSDD) • Sexual Aversion Disorder	Desire/Arousal disorders • Female Sexual Interest/Arousal Disorder (FSIAD)
Arousal disorders	
Orgasm disorders	Female Orgasmic Disorder
Pain Disorders • Dyspareunia • Vaginismus	Genito-pelvic pain/penetration disorder

1.2.3 NEUROBIOLOGICAL REGULATION OF THE FEMALE SEXUAL RESPONSE

The pathophysiology of arousal/desire disorders has not been fully elucidated, though the literature currently accepts that there are strong neurobiological underpinnings. The Dual Control System summarizes sexual response as a result of interaction between excitatory and inhibitory processes (Bancroft, Graham et al. 2009). It is an interplay of these neuromodulatory processes through hyperactive inhibition, hypoactive excitation, or a combination of both that causes dysfunction (Pfaus 2009).

The inhibitory circuit is believed to be primarily affected by serotonin and opioids, while endocannabinoids have differing effects depending on sex. The effect of serotonins on sexual functioning was discovered through psychiatric medications such as SSRI's, which are known to reduce sexual motivation and desire. In general, serotonin regulates satiety, and in animal models, it has been shown that inhibition through 5-HT1A agonism inhibits lordosis but stimulate solicitations, while inhibition through 5-HT2A antagonism promotes lordosis and inhibits solicitations. Opioids are believed to also act as inhibitory neurotransmitters mediating sexual rewards upon activation of their receptors post orgasm. This mediation has been demonstrated in the long-term use of opioid addicts having a severely diminished capacity for sexual arousal that upon withdrawal of the opiate sexual functioning gradually returns (Pfaus and Gorzalka 1987). However, while opioid agonists can inhibit lordosis via the μ receptor, lordosis can be facilitated through the agonists at the δ receptor. Some current literature suggests that endocannabinoids function as inhibitory to sexual behavior, in comparison to males, endocannabinoids seem to facilitate sexual behavior in females perhaps through elevation of adrenal androgens which have been linked for decades to sexual arousal in women (Gorzalka and Hill 2006).

The excitatory circuit is believed to be modulated by dopamine, norepinephrine, melanocortin's, and oxytocin. Dopamine (DA) is crucial for regulating behavioral reward signaling and processing the aspects of arousal and desire. The regulation has been demonstrated in drug's known to increase dopamine acutely, thereby enhancing sexual desire and arousal. Distinct dopaminergic pathways are responsible for various aspects of the sexual response, and although shown in male's, further studies need to be conducted to fully understand to role of DA in female sexual behavior (Brom, Both et al. 2014). Graham and Pfaus set out to understand the role of DA and the medial preoptic area in female sexual behavior using a DA agonist. A nonselective agonist at D1 and D2 receptors produced a significant increase in hops and darts while trending towards a significance in solicitations suggesting that DA in the medial preoptic area is essential for control of precopulatory sexual behaviors in the female rat. However, using selective agonist activity at D2 increases hops, darts, and solicitations while at D1 reduces them (Graham and Pfaus 2010). A second study was conducted that reinforced D1/2 receptor activity within the medial preoptic area as critical of expression of precopulatory sexual behaviors which can be altered by progesterone. Further studies in female animals should be conducted to confirm the role of different dopaminergic pathways in pre and copulatory behaviors (Graham and Pfaus 2012).

Norepinephrine is an excitatory component that has been demonstrated in animals to reduce sexual desire when signaling has been inhibited and to promote it when stimulated. An increase in peripheral norepinephrine is seen during sexual activity, peaking at orgasm. Oxytocin is a nano-peptide involved in the excitatory circuit of the female sexual response where it exists as a critical factor (Dębiec 2007). Its' involvement has been demonstrated by the positive correlation of rising plasma oxytocin levels with orgasm (Dębiec 2007). In animal studies, it has also been shown to mediate female sexual receptivity indicated by lordosis (Dębiec 2007). Melanocortin's were first observed to be involved in sexual functioning in the male animal models. It was only reported that α-MSH influenced female sexual behavior in rats, but it depended on receptivity whether this was inhibitory or stimulatory concluding that α-MSH had no specific effect on lordosis but may work through arousal (Gantz and Fong 2003). Until a study on a melanocortin analog that acted as a melanocortin receptor agonist was conducted, the role of melanocortin's in women was still relatively unclear. This study provided evidence that female rats sexual behavior was enhanced

upon administration with selective binding to MC Type 3/4 receptors and high affinity to the MC type 1 receptor (Pfaus, Shadiack et al. 2004).

1.2.4 HORMONES INVOLVED IN THE FEMALE SEXUAL RESPONSE

Testosterone and Estrogen (most notably Estradiol) are the most well-studied hormones involved in sexual response. The importance of testosterone may be best understood in those with lower levels such as aging women, or those with androgen insufficiencies having a reduction in sexual desire/arousal, receptivity, and pleasure. The data on androgen levels corresponding to sexual dysfunction are conflicting; however, in postmenopausal women, several trials testing exogenous testosterone in the treatment of HSDD have been conducted. A review of these studies suggests that testosterone, in combination with hormone therapy, improves sexual function scores and the number of total satisfying sexual events (Somboonporn, Davis et al. 2005). Estrogen plays an integral role in vaginal epithelium, and the vagina is mostly estrogen-dependent while the clitoris is androgen dependent. This direct role in the peripheral sexual response aids in responding to stimuli via lubrication and vasoconstriction (Kingsberg, Clayton et al. 2015, Bitzer 2016).

1.2.5 THE MELANOCORTIN RECEPTORS AND SEXUAL FUNCTIONING

The melanocortin system is a family of the rhodopsin family of 7-transmembrane G protein-coupled receptors. The family is comprised of five known receptors with endogenous agonists and two antagonists (agouti signaling protein, and agouti related peptide) arising from the proopiomelanocortin (POMC) gene which is primarily expressed in the central nervous system (Gantz 2003). The melanocortin receptor (MCR) signaling peptides are cleaved from the post-translational POMC prohormone and consist of adrenocorticotrophic hormone (ACTH) which is specific to the melanocortin 2 receptor, and three melanocyte-stimulate hormones (alpha, beta, gamma) which can stimulate the other melanocortin receptors. These peptide hormones are essential for maintaining energy homeostasis and in the last few decades, MC4R and to a lesser extent MC3R and MC1R have been increasingly implicated in both male and female sexual functioning (Tao 2010).

The endogenous agonist of the MC4R, alpha-MSH has been shown in female rats to have the ability to facilitate or inhibit lordosis depending on the hormonal status of the animals. Interestingly, estradiol

increases alpha-MSH levels in the hypothalamus which would suggest that alpha-MSH release may be one of the several intermediaries of estrogen action. Knockout mouse models of this receptor have shown that female animals remain fertile; however, they demonstrate advanced reproductive aging by a decreased ovulation rate (Sandrock et al 2009).

Although previously hypothesized that the melanocortin's were involved in regulating the sexual functioning of some animals during experimentation in the 60's, the discovery of melanocortin agonists resulting in favorable sexual stimulation was discovered by chance. After an early collaboration produced highly potent melanotropic peptides, Mac Hadley consumed a substituted analog of what was believed to be a tanning agent and discovered the unusual side effect of an erection which led to the subsequent licensing and development of the compound (Hadley 2005). This discovery in particular led to the current drug on the market for generalized acquired HSDD, Bremelanotide, a cyclic heptapeptide which is well tolerated, binds to MC3R and MC4R, and has no cardiovascular liabilities (Molinoff 2003).

Bremelanotide has been studied extensively for drug approval purposes. Subcutaneous injections of the drug dramatically increase solicitation behaviors such as hops and darts in ovariectomized rats primed with estradiol or in combination with progesterone with no corresponding decrease in pacing or effects on lordosis. Due to the lack of effects on general locomotion it was concluded that the effects were behaviorally specific. Identical effects were demonstrated following infusions to the lateral ventricles or medial preoptic area but not the ventromedial nucleus of the hypothalamus, which were reversed by the addition of an MC4R antagonist. The peripheral administration of the peptide in particular is clearly acting in the brain to alter behavior suggesting that agonist activity at the MC4R in the mPOA is an important component of sexual solicitation. Looking further the subcutaneous injections also increased dopamine release only in the mPOA, seeming to stimulate hormone-mediated neurochemical pathways involved in translating desire into action without making females less discriminative in copulatory preference. In human female clinical trials intranasal administration enhanced sexual desire, trended towards enhancing feelings of genital arousal, and females reported greater satisfaction with levels of sexual arousal (Diamond 2006). In women exhibiting female sexual arousal disorder, the treatment group showed an increase in the mean arousal score of the FSFI, as well as greater intercourse satisfaction over placebo. A larger double-blind

placebo-controlled study evaluating potency showed the highest dose administered significantly improved over placebo in response to all domains of the FSFI (Krychman et al 2014)

There is currently not enough evidence in females to rule out that it is hitting MCR in the spinal cord or periphery which has been experimented further in male animals showing that the brain and spinal cord participate in translating melanocortin's binding into behavior (Van der Ploeg et al. 2002).

CHAPTER 2 ASSESSMENT OF BOTANICAL APHRODISIACS MARKETED FOR WOMEN IN THE UNITED STATES

2.1 OVERVIEW

The American Sexual Health Association states, “an important part of overall sexual health is healthy sexual functioning—being able to experience sexual pleasure and satisfaction when desired." Female sexual dysfunction (FSD) affects around 40% of women and encompasses a wide variety of disorders, including those relating to sexual pain, arousal, desire, interest, and orgasm. Nonvitamin, nonmineral dietary supplements are the most commonly used complementary health approach among adults in the United States (Clarke, Black et al. 2015). Surveys conducted by the NIH show that women are more likely to use herbs or supplements than men; however, only 33.4% tell their conventional health provider about their use (Kennedy 2005). A survey of 3,239 women with self-reported sexual problems and associated distress found that only 34.5% of women had ever formally discussed their sexual issues with a healthcare provider, and 66% had never received any form of treatment for their distressing sexual problem (Shifren, Johannes et al. 2009). There are several off-label treatment options for FSD, but only two FDA-approved-drugs on the market; therefore, many women have resorted to using “libido enhancing” dietary supplements. These supplements are comprised of plants considered to be aphrodisiacs as well as vitamins and minerals. According to the FDA, aphrodisiac products (AP) are "any product that bears labeling claims that it will arouse or increase sexual desire, or that it will improve sexual performance." Aphrodisiacs have been used for centuries across several civilizations to enhance sexual desire and/or function. The majority of anecdotal information of aphrodisiacs pertain directly to men, and with the advent of PDE5 inhibitors and a billion-dollar industry, treatment options for women are severely lacking and necessary.

While there are assays to evaluate the aphrodisiac potential of plants in men, there is currently no standard for their evaluation in women. This review will cover the background of FSD, and highlight the

medicinal plants claimed on the label of female-specific AP currently on the market in the United States. These AP have claims to increase libido and desire as well as improve sexual functioning. Each plant will be covered in section 4 detailing traditional use as well as clinical evidence that would support its appearance in these supplements. Traditional use of nearly all of these plants expand beyond the scope of what is focused on within this paper; therefore, the ethnobotanical use included focuses on applications only in women's health that allow some correlation to be drawn to its appearance in AP on the market. An attempt to review this topic was undertaken by Mazaro-Costa et al 2010 in the Journal of Sexual Medicine; however, the focus on their paper was climacteric women while this paper focuses on a broader application (Mazaro-Costa, Andersen et al. 2010). Previously, Rowland and Tai in 2003 also reviewed plant derived and herbal approaches for the treatment of sexual dysfunction including both men, and women (Rowland and Tai 2003).

Few plants had Latin binomials listed in the ingredients causing concern as common names can sometimes be the same for two distinct species or even genii. For those plants without a Latin binomial attached, it was assigned referencing Herbs of Commerce. Some of the supplements were standardized for constituent content such as withanolide content of Ashwagandha, or L-dopa content of Mucuna although these constituents have also not been explicitly evaluated for FSD or aphrodisiac potential.

2.2 METHODS

For this review, 27 AP's were selected from the three most common large drug store retailers as well as the largest online marketplace. These places were chosen to most accurately represent the products that women would be purchasing in the United States. The only exclusion criteria for products was that it could not contain cannabis, and only one product fit this criterion. An ingredients list was recorded for each supplement, and from that 53 species were found to be included across formulations. This number includes all potential species names if a standardized common name was provided on the label. It does not include nomenclature denoting multiple species i.e. *Schisandra* spp, pine bark, flower pollen, tongkat ali due to the inability to match a botanical synonym. Formulations listing a common name without a Latin binomial were cross referenced against the second edition of Herbs of Commerce, and assigned the botanical synonym. Ingredients such as vitamins and minerals were not included in this review, as well as the ingredient "Pine

Bark" found on an AP label due to the inability to pair with a Latin binomial. The aphrodisiac species *Corynanthe johimbe* K. Schum. (Yohimbe) will not be covered in this paper due its' active constituent Yohimbine being well established in studies within the literature (Ernst and Pittler 1998).

Clinical evidence was selected from a PubMed search with the term's "plant" (common name/Latin binomial of the plant) + female sexual function OR dysfunction). Utilizing a common search engine, several plants appear as female aphrodisiacs; however, looking into the literature some of these plants have only been evaluated in male animal models. Therefore, only those studies utilizing female animal models were included. Partial *in vivo* studies of females and males were included with only the female data being discussed. Ethnobotanical use of the herbs was obtained from published monographs, ethnobotanical surveys, and comprehensive reviews of such information.

Studies were evaluated for level of evidence utilizing four categories including length of study, validated tool of measurement, use of control, and botanical verification. Due to the products being located in the United States, this method of evaluation was determined from the FDA's Draft Guidance for Industry published in 2016 titled "Low Sexual Interest, Desire, and/or Arousal in Women: Developing Drugs for Treatment"(FDA 2008).

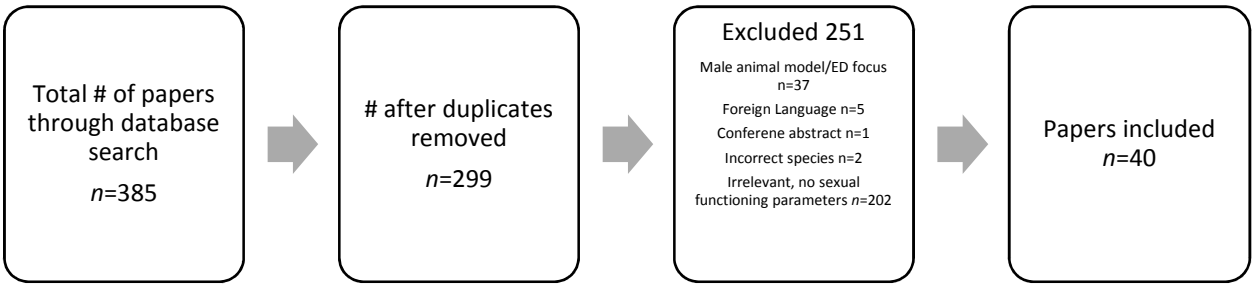

Fig. 2 Process for Inclusion/Exclusion of Papers in Review Manuscript

Table 2: Plants found in female AP on the market in the United States			
Frequency in AP (n=27)	Latin Binomial	Name on Label	Traditional Use as aphrodisiac in women
3	*Actaea racemosa* L. Ranunculaceae	Black Cohosh SCN	Y
6	*Angelica sinensis* (Oliv.) Diels Apiaceae	Dong quai (3) SCN *Angelica sinensis* (3)	Y
1	*Asparagus racemosus* Willd.	Shatavari SCN	Y

	Asparagaceae		
2	*Asparagus officinalis L.* *Asparagaceae*	Asparagus [SCN]	N
3	*Avena sativa* L. Poaceae	Oat Straw [SCN]	Y
4	*Capsicum annuum* L. Solanaceae	Cayenne (2 standardized to 30,000 SHU) [SCN]	N
3	*Tetragastris catuaba* Soares da Cunha Burseraceae	*Erythroxylum catuaba (1)* Catuaba (2) [SCN]	N
4	*Cnidium monnieri* (L.) Cusson Apiaceae	*Cnidium monnieri (3)* Cnidium (1) [SCN]	N
1	*Crataegus laevigata* (Poir.) DC. *Crataegus monogyna* Jacq. *Crataegus piperi* Britton *Crataegus rivularis* Nutt. Rosaceae	Hawthorne [SCN] berry	N
1	*Curcuma zedoaria* (Christm.) Roscoe Zingiberaceae	*Curcuma zedoaria* (White Turmeric)	N
3	*Dioscorea villosa* L. Dioscoreaceae	Wild Yam [SCN]	N
9	*Epimedium sagittatum* (Siebold & Zucc) Maxim. Berberidaceae	*Epimedium sagittatum (9)* (3 standardized for 10% icariins, 1 standardized for 40% icariins, 1 standardized for 1% icariin)	N
2	*Epimedium grandiflorum* C. Morren Berberidaceae	*Epimedium grandiflorum (2)*	Y
4	"Epimedium" *Epimedium brevicornu* Maxim *Epimedium koreanum* Nakai *Epimedium pubescens* Maxim *Epimedium wushanense* T.S. Ying Berberidaceae	Epimedium (4) [SCN]	N
3	*Eurycoma longifolia* Jack Simaroubaceae	*Eurycoma longifolia*	N
2	*?*	Tongkat ali	N
1	?	As flower pollen	N
9	*Ginkgo biloba* L. Ginkgoaceae	*Ginkgo biloba* (1 standardized to 24% ginkgosides and 6% terpene lactones)	N
	Eleutherococcus senticosus (Rupr. & Maxim) Maxim. Araliaceae *Panax quinquefolius* L. (American) Araliaceae *Panax ginseng* C.A. Mey (Asian, Korean) Araliaceae Korean red?	21 *Eleutherococcus senticosus*-1 standardized for .8% eleuthrosides E and B *Panax ginseng*-11 (1 standardized to 7%ginsenosides, 1 at 4%ginsenosides) Asian ginseng-3 (1 80% ginsenosides, 1 at 4% ginsenosides), Ginseng-1 American ginseng-2 Korean red ginseng-2 Korean ginseng-1	Y

		1 as Angelica in parenthesis of Panax Ginseng which is incorrect*	
1	*Glycyrrhiza glabra* L. Fabaceae	Licorice [SCN]	Y
1	*Hydrastis canadensis* L. Ranunculaceae	Goldenseal [SCN]	Y
21	*Lepidium meyenii* Walp. Brassicaceae	*Lepidium meyenii (3)* Maca (18) (1 standardized to 0.6% macaenes and macamides, 1 standardized to 6% macamides) [SCN]	Y
10	*Mucuna pruriens* (L.) DC.	*Mucuna pruriens (8)* (1 standardized to 25% L-dopa, 1 standardized to 15% L-dopa, and 2 standardized as 15% seed extract) Velvet Bean (2) (standardized to 15% L Dopa) [SCN]	Y
1	*?*	*Passiflora*	?
4	*Corynanthe johimbe* K. Schum. Rubiaceae	Yohimbe (3) [SCN] Yohimbine bark extract (1)	N
1	*?*	Pine bark	?
11	*Piper nigrum*	7 as Bioperine Black Pepper extract (95% Piperine), 4 as Bioperine complex (Bioperine + Ginger extract (5% gingerols)	
4	*Polypodium vulgare* L. Polypodiaceae	*Polypodium vulgare*	N
9	*Ptychopetalum olacoides* Benth. Olacaceae	Muira Puama [SCN]	N
1	*Pueraria mirifica* Airy Shaw & Suvat Fabaceae	*Pueraria mirifica* (standardized to 40%isoflavones)	Y
2	*Pueraria montana var. lobata* (Willd.) Maesen & S.M. Almeida ex Sanjappa & Predeep Fabaceae	Kudzu (1 standardized to 40% isoflavones) [SCN]	N
2	*Rehmannia glutinosa* (Gaertn.) DC. Orobanchaceae	*Rehmannia glutinosa* (Di huang)	Y
2	*Sedum rosea* (L.) Scop. Crassulaceae	*Rhodiola rosea* (1 standardized for 3%rosavins and 1%salidroside, 1 standardized for 3% salidrosides)	N
1	*Rubus idaeus* L. Rosaceae	Red Raspberry [OCN]	N
1	*Schisandra chinensis* (Turcz.) Baill. Schisandraceae	*Schisandra chinensis*	N
1	*?*	Schisandra spp.	N
4	*Serenoa repens* (W. Bartram) Small Arecaceae	Saw Palmetto [SCN]	Y
1	*Smilax ornata* Lem. *Smilax purhampuy* Ruiz *Smilax aristolochiifolia* Mill. Smilacaceae	Sarsaparilla [SCN]	N
3	*Theobroma cacao* L.	3	Y

	Malvaceae	1 as Cocoa extract (6-8% theobromine) and 2 theobromine	
14	*Tribulus terrestris* L. Zygophyllaceae	12 as *Tribulus terrestris* (3 standardized to 60%saponins, 1 standardized to 20% saponins) 2 as Tribulus (1 standardized to 40% saponins) [SCN]	Y
1	*Trigonella foenum-graecum* L. Fabaceae	*Trigonella foenum-graecum*	Y
12	*Turnera diffusa* Willd. Ex Schult. Passifloraceae	*Turnera diffusa* (3) Damiana (9) [SCN]	Y
1	*Urtica dioica* L. Urticaceae	Stinging Nettle [SCN]	N
1	*Valeriana officinalis* L. Caprifoliaceae	Valerian [SCN]	Y
3	*Vitex agnus-castus* L. Lamiaceae	*Vitex agnus-castus* (1) Chaste-berry (2) [SCN]	?
9	*Withania somnifera* (L.) Dunal Solanaceae	*Withania somnifera* (4) (1 standardized to 8% withanolides, 1 to 4% withanolides 1 to 10% withanolides) Ashwagandha (5) [SCN]	Y
3	*Xanthoparmelia scabrosa* (Taylor) Hale Parmeliaceae	*Xanthoparmelia scabrosa*	N
10	*Zingiber officinale* Roscoe Zingiberaceae	Ginger (6) (1 standardized to 5% gingerols) [SCN] 4 as Bioperine Complex ((Bioperine + Ginger extract (5% gingerols))	Y

2.3 RESULTS AND DISCUSSION

2.3.1 Ashwagandha (*Withania somnifera*)

Withania somnifera (Solanaceae) commonly known as Ashwagandha, is a woody herb or shrub of which the roots are used medicinally (Royal Botanic Gardens 2019). It is first mentioned in Ancient Assyria for use as an aphrodisiac and is speculated to have been used in ancient Arabia and Mesopotamia. Acharya Sushruta, the co-founder of Ayurveda, stated that it is an unsurpassable vajikarana (aphrodisiac) and it remains in the Ayurvedic Pharmacopoeia of India as such (Rätsch 2013). It is currently used in modern Ayurveda for rejuvenation and treatment of sexual impotence.

In the Hafizabad district of Punjab-Pakistan, Ashwagandha is used in topical, oral, and snuff applications and includes aiding menstrual flow amongst various other irrelevant medicinal applications (Umair, Altaf et al. 2017).

An *in vivo*, double blind, placebo controlled, randomized pilot study in 50 women tested the efficacy and safety of a root extract of *W. somnifera* in improving sexual function in women over eight weeks. The study was done in combination with a counseling program which included two seminars and an individualized consulting session on addressing FSD. The extract utilized was water based, and the highest concentration extract as assessed by withanolide fraction available on the market at 300mg. The dose was determined based on withanolide concentration of 5% whereas a traditional dosage of raw root powder is 3000mg BID. The placebo was starch powder, and both were put into identical gelatin capsules. The primary efficacy outcome was evaluated by the Female Sexual Function Index (FSFI) by a change from baseline at four weeks, and termination at eight weeks. The secondary efficacy outcomes were the Female Sexual Distress Scale (FSDS), the Sexuality Activity Record (SAR), the Patient's Global Assessment of Response to Therapy (PGART) as well as the Patients Global Assessment of Tolerability to Therapy (PGATT). After treatment, *W. somnifera* extract significantly increased the FSFI total score at both time points evaluated. In the five individual domains (desire, arousal, lubrication, orgasm, satisfaction) of the FSFI, there was a significant increase in score relative to baseline at both endpoints. However, in the pain domain of the FSFI, treatment did not show significance over placebo. The mean score of the FSDS increased significantly over placebo at both endpoints. In the SAR, there was no significant difference between groups in the total number of sexual encounters; however, the number of successful sexual encounters significantly improved over placebo at the termination of the study. In the PGART, 60% of patients (15/25) scored their therapy response as excellent, 36% as "good" (9/25), and only (1/25) as moderate. In the PGATT, no adverse effects were observed, and all patients showed excellent tolerability (Dongre, Langade et al. 2015). The mechanism of action that may contribute to its aphrodisiac qualities is the extracts ability to stimulate gonadotrophin-releasing hormone cells to release GnRH, as demonstrated *in vitro* (Kataria, Gupta et al. 2015).

2.3.2 Black Cohosh (*Actaea racemosa* L.)

Actaea racemosa (Ranunculaceae) is an herbaceous perennial indigenous to North America(Kew 2019). It grows as a forb/herb, and the root and rhizome are used in herbal medicine. One of the earliest listings of its medicinal use for "female debility" came from Carol von Linne in 1749. Throughout Appalachia and Native American ethnobotany, the plant was used for menstrual issues as it was believed to have a high affinity for the uterus (Clymer 1905, Millspaugh 1974, Crellin 1990, Moerman 1998). While no traditional literature explicitly claims aphrodisiac usage, in modern phytotherapy for women's health, the herb is considered to be hormone regulating, oxytocic, tonifying for the uterus as well as a uterine antispasmodic (Romm 2010). Although our search criteria did not yield any papers, the use of *A. racemosa* for climacteric women specifically has evidence based on selective estrogen receptor modulation, and interaction with the dopaminergic and serotonergic pathway, and anxiolytic effects. Further studies in the specific climacteric subpopulation of women affected by FSD may lead to a better understanding of the hormones and pathways involved.

2.3.3 Cacao (*Theobroma cacao*)

Theobroma cacao (Malvaceae) is a perennial dicot that grows as a tree indigenous to Mexico, Central America, and northern South America (Kew 2019). Commonly known as Cacao, the fruit of the plant is most widely used in medicine and food applications. Prepared as a paste or butter, Cacao is known to have been used as an aphrodisiac to increase sexual appetite, desire, or pleasure since 1577. Ethnobotanical use includes being employed as an aphrodisiac by the Mexican Indians, while the artwork of the pre-Colombian Mayan, and the Aztecs depict it as such (Rätsch 2013). In the supplement industry, the isolated bitter alkaloid Theobromine is used in AP. Theobromine has not been studied in FSD but may potentially be included in formulas due to it being a cardiac stimulant and smooth muscle relaxant. As a molecule in the class of methylxanthines, which caffeine also belongs to, it has been suggested to compete with adenosine blocking its inhibitory effect and causing arousal (Bruinsma and Taren 1999, Dillinger, Barriga et al. 2000).

A convenience sample of 163 women completed an anonymous interview to assess whether an association exists between daily chocolate intake and sexual function. Tools used to assess sexual

function, distress, and depression were conducted with three domains of the FSFI (desire, arousal, satisfaction), the FSDS, Beck Depression Inventory Scale and Center for Epidemiological Survey Depression Scale. The women were divided into two groups for analysis, those who consume chocolate on a daily basis, and those who do not. Out of the 152 complete responses available for analysis, the results show that the total FSFI score as well as the desire domain within the FSFI were significantly higher in women who consume chocolate; however, when adjusted for age and body mass index, no significance is evident. There was no difference between each group in the remaining questionnaires evaluated (Salonia, Fabbri et al. 2006). While the idea for the study is interesting, convenience sampling has a high risk of bias and is unreliable to draw sound conclusions from. If any future work is to be conducted on Cacao, it should be standardized to Theobromine which is found in AP formulations, and conducted in a randomized double-blind fashion to reduce any chance of bias.

2.3.4 Catuaba (*Tetragastris catuaba* Soares da Cunha)

The originating Latin binomial of *Erythroxylum catuaba* (now accepted as *Tetragastris catuaba*) had been first described in 1904 by A. Jose da Silva (Da Silva 1904). However, with no botanical material to reference and a common name of Catuaba, it was determined that several barks native to the Brazilian Amazon contributed to the preparation most notably *E. vaccinifolium* and *Trichilia catigua* (Kew 2019). However, further confusion exists because the Brazilian Pharmacopeia recommends *Anemopaegma arvense* (Vell.) Stellfeld ex De Souza as Catuaba (Brazilian Pharmacopeia 1926). Due to these inconsistencies it is hard to say what plant material was included in the AP formulations discovered.

2.3.5 Cayenne (*Capsicum annuum* L.)

Capsicum annuum (Solanaceae) is a fruiting pepper commonly called Cayenne with ancestors that evolved in Bolivia and Southern Brazil (Kew 2019). Traditionally Cayenne has been employed as a food and medicine throughout North America, Europe, and throughout most of the world (Palevitch 1995). Its' medicinal properties of local stimulation have been employed throughout Ayurvedic formulations and appear in modern day approval for topical analgesic products. Anecdotal use as an aphrodisiac is due to the belief that spicy foods are thought to stimulate sexual desire and arousal perhaps due to the warming

effects. While no clinical trials have demonstrated such use as beneficial in FSD, this idea seems borrowed from Ayurvedic medicine and the idea of a driver similar to how *P. nigrum* is used in formulas in the belief that it will increase bioavailability of other compounds.

2.3.6 Chaste Tree (*Vitex agnus-castus* L.)

Vitex agnus-castus (Lamiaceae) is a dicot perennial that grows as a shrub or small tree native from the Mediterranean to Pakistan, of which the fruit is used in herbal medicine (Kew 2019). The first mention of the plants potential as an anaphrodisiac is from Ancient Assyria, which is corroborated by Plato's writings (Rätsch 2013). However, the WHO monographs list *V. agnus-castus* as an aphrodisiac, and claim that homeopathically, it is applied in frigidity and sexual weakness (WHO 2009). Traditional women's herbal medicine regards the plant as a hormone regulating herb and an ovarian tonic for applications such as PMS, and polymenorrhea (AHP 2001, Romm 2010). Historically similar use has been identified helping the onset of menstruation and dysmenorrhea (Mills 1985). Conflicting information on whether it is an aphrodisiac or anaphrodisiac has persisted throughout history. One clinical trial has been conducted to evaluate the combination of *Nigella sativa* L. (Ranunculaceae), Vitex and a pharmaceutical SSRI on healthy menopausal women with hot flashes. The study was randomized, double-blind, and placebo controlled as a pilot study, and the only evaluation of sexual symptoms was within the Menopause-Specific Quality of Life questionnaire (Molaie, Darvishi et al. 2018). While no significance from baseline was determined in this study, due to endpoints focused elsewhere we cannot rely on this data to support inclusion or exclusion of Vitex in any AP.

2.3.7 Cnidium (*Cnidium monnieri* L. Cusson)

Cnidium monnieri (Apiaceae) is an annual dicot that grows as a forb/herb native from Temp. Asia to Indo-China, of which the seed is used(Kew 2019). Cnidium has a long history of traditional use in Chinese medicine appearing in the countries' Pharmacopeia. Applications regarding aphrodisiac use tend to focus on male impotence, while female applications range from broad TCM diagnoses such as a cold uterus, to vulvar pain and itching to menopausal tonics (Rätsch 2013, Li, Jia et al. 2015). Li et al provides a comprehensive overview of the traditional use as well as phytochemistry and pharmacology of

compounds present in the plant. Four coumarins isolated from Cnidium have been studied for their ability to have vasorelaxant effects on pre-contracted rabbit corpus cavernosum (Chiou, Huang et al. 2001). Although the study focuses on an application in regards to erectile dysfunction, the clitoris also contains the corpus cavernosum. *C. monnieri* may potentially increase blood flow to the clitoris, but further studies need to be conducted to validate reasoning for inclusion in these AP.

2.3.8 Damiana (*Turnera diffusa* Willd. Ex Shult.)

Turnera diffusa (Passifloraceae) is a perennial dicot that grows as a shrub/subshrub native to S. Texas, Central America, the Caribbean, and E. Brazil (Kew 2019). The leaf is commonly used in herbal medicine, and while this species is claimed to be an aphrodisiac, *T. aphrodisiaca* is as well (Romm 2010). According to Kew Gardens, *T. aphrodisiaca* is currently recognized as *T. diffusa (Kew 2019)*. The first mention of Damiana as an aphrodisiac in the European literature comes from the missionary Jesus Maria de Salvatiera in 1699 (Rätsch 2013). It continued to appear as such in the 19th-century pharmacopeias of the US and Mexico as a tonic and aphrodisiac with ethnobotanical use throughout Northern Mexico and Mayan regions. Aqueous, alcoholic, and dried smoking preparations are all said to produce the desired effect. As recent as the 1900s, European pharmacies used the species as an aphrodisiac and cure for impotence. The use of Damiana persists into modern day Mexico where a Damiana liquor alleged to be an aphrodisiac is manufactured and distributed. The claimed effects are due to the species claimed ability to encourage circulation in the lower abdomen, alleviate cramping, and ease inhibition. “Cyanogenic glycosides and alkaloids may be the most promising constituents to look at due to their bioactive effects and interaction with the NO pathway (Szewczyk and Zidorn 2014).” Szewczyk and Zidorn compiled an extensive review which details the traditional use of *T. diffusa.*

An *in vivo* randomized, double-blind, placebo-controlled study of a polyherbal formula containing Damiana (*T. aphrodisiaca*), as well as Ginkgo (*G. Biloba* leaf extract 50mg), and Korean Ginseng (*Panax ginseng* root aerial part and root 100 mg) was performed with 77 women (ages 22-71 yrs.) who were enrolled solely on interest in improving their sexual function. The supplement is listed to contain 50 mg of *T. aphrodisiaca,* and there is no information on the placebo. The study lasted four weeks and Index of Female Sexual Function (ISIF) scores, medical history, and blood pressure were taken at baseline and

termination. At termination a significant number of women improved in satisfaction with their overall sex life, as well as level of sexual desire compared to placebo. Significant improvements over placebo were also demonstrated in the frequency of intercourse, as well as reduced dryness, improvement in sexual relationship with partner, and decrease in discomfort during intercourse. Improvements were also seen in clitoral sensation and frequency of orgasm though barely insignificant ($p < 0.6$, 0.7, respectively)(Ito, Trant et al. 2001).

An *in vivo* double-blind, placebo-controlled study of the same polyherbal formula above was performed with 108 women (ages 22-73) reporting lack of sexual desire. The women self-reported menopausal status (pre-, peri- and post-) based on provided definitions. The study had the same design as discussed previously. Pre-menopausal women showed a significant improvement in attempts of sexual intercourse, feeling of sexual desire, level of sexual desire, and satisfaction with overall sex life. Peri-menopausal women showed a significant improvement in dryness during intercourse, attempts of intercourse, and satisfaction of sexual relationship with partner. Post-menopausal women showed significant improvement in rate of sexual desire (Ito, Polan et al. 2006).

A third study of this polyherbal formula was conducted to determine the effect on sexual functioning and quality of life among female cancer survivors. A 12-week, randomized, placebo-controlled trial was completed with 186 women and the outcomes were primarily assessed via the FSFI, while secondary outcomes were assessed using the Functional Assessment of Cancer Therapy- General questionnaire (FACT-G)(Cella, Tulsky et al. 1993). The treatment group took three capsules BID for a total of six capsules of the polyherbal formula which was independently analyzed for ingredients prior to distribution. There is no information on the placebo other than it was matching. Sexual functioning and quality, as well as the FSFI scores were determined at baseline, only FSFI scores were taken at the four-week intervals. The results show that at the end of the study, the polyherbal formula demonstrated no significant benefit over placebo to improved sexual functioning, although it did improve quality of life shown in the FACT-G's physical and functional wellbeing domain (Greven, Case et al. 2015).

Although the polyherbal formula including Damiana had demonstrated efficacy in two of the three studies, it is difficult to claim responsibility on one herb. The first two studies are flawed by short duration, and in the future should be extended by at least eight more weeks to demonstrate effects seen as reliable.

In order to narrow down the possible botanical responsible for the effects, studies with multiple arms, or concurrent studies on just the herb alone may then allow conclusions of synergism or potentiation regarding these formulas. Also the first two studies misreport the tool of measurement in assessing these parameters as the FSFI, the gold standard for studies, when it is actually the ISIF as per the reference (Kaplan, Reis et al. 1999). This scale had been newly developed by Kaplan et al at the time and has not undergone the scrutiny and validation that the FSFI has; therefore, no valid conclusions can be made from the results. The third study does use the validated FSFI as reported, but the timeline could be extended further to strengthen the conclusions.

An *in vitro* experiment was conducted on this polyherbal formula and the *Panax ginseng* it contains in order to determine whether the formulation included any estrogenic components. The Ishikawa cell line, a human endometrial adenocarcinoma line containing an alkaline phosphatase enzyme sensitive to estrogen stimulation was utilized. The maximum tested concentration of the polyherbal formula was 185 µg/mL and *P.ginseng* 25 µg/mL exceeding the minimally effective positive control dose of estradiol by a factor of 10^6. While estradiol showed significant stimulation via induction of formation of alkaline phosphatase, neither the polyherbal or singular herb stimulated alkaline phosphatase at any of the concentrations tested (Polan, Hochberg et al. 2004).

The use of these specific cell lines has been well demonstrated as a model for studying hormonally regulated events in human endometrial epithelium; however, due to its' high specificity for estrogens, the ability of other steroid hormones to produce a response in assays with less specificity may warrant further exploration(Littlefield, Gurpide et al. 1990).

Please refer to the following section on Ginkgo (4.11) for a reference to a polyherbal formula containing Damiana.

2.3.9 Dong quai (*Angelica sinensis* Oliv. Diels)

Angelica sinensis (Apiaceae) is an herbaceous perennial native to China whose root is used in TCM as a general blood tonic as well as for a host of gynecological indications(Kew 2019). Historically it appears in Lei Gong's Treatise on Preparation of Materia Medica, as well as the Ben Cao Gang Mu, the 22nd edition of the United States Dispensatory, and the Pharmacopeia of the Peoples Republic of China

(Rätsch 2013). Western herbalism recognizes it as a female tonic with use as an aphrodisiac (Romm 2010). In Ayurvedic medicines, women use the species to nourish the female genital system supposedly enhancing function. The Physicians' Desk Reference references a study done in the late eighties where an increase in sexual activity in female animals was reported though no mechanisms were proposed. These same researchers also pointed out dong quai's uterine effects "volatile oil content may produce a uterine depressant effect; however, a uterine stimulant effect is also attributed to the aqueous extract, some animal experiments have shown increased uterine excitability. Irregular, fast contractions are said to decrease and increase in regularity"(Zhu 1987). The PDR also states that the vasodilating actions of the species are thought to be due to various coumarins(WHO 2007). Another source claims that the constituents associated with the bioactivity are believed to be alkylphythalides, polysaccharides, and ferulic acid (WHO 2003). The review conducted by Mazaro-Costa et al determined that *A. sinensis* is indicated in FSD arousal/desire studies mainly in climacteric women due to its estrogenic effect, and reduction of vasomotor symptoms(Mazaro-Costa, Andersen et al. 2010). Due to the absence of clinical data returned from our methodology and specificity of the species studied in climacteric women, it may warrant further investigation into menopausal women with FSD.

2.3.10 Fenugreek (*Trigonella foenum-graecum* L.)

Trigonella foenum-graecum (Fabaceae) commonly known as Fenugreek, is an annual dicot forb/herb native from Iraq to Afghanistan whose seed is used in herbal medicine(Kew 2019). It appears as an aphrodisiac in the WHO monographs, and earlier, it was used in Arabic medicines for "all female issues" (WHO 2007). In TCM, the seeds are used in polyherbal formulas to treat diminished sexual function resulting from kidney failure. Avicenna mentions that both the species and its seeds are stimulants for sexual desire in his herbal, and the Duke handbook of medicinal herbs lists it as an aphrodisiac, while another ethnobotanical use has it in love potions(Duke, Bogenschutz-Godwin et al. 1929, Rätsch 2013).

An *in vivo* randomized placebo-controlled study was performed with 80 healthy menstruating women aged 20 to 49 years self-reporting low sexual function. The product contained a standardized 300mg *T. foenum-graecum* seed extract with a minimum of 50% saponins glycosides equivalent to 9.9g of dry herb, along with 30 mg of maltodextrin. The placebo product contained 330 mg of maltodextrin, and

both were in size 0 white hard gelatin non-marked capsules. The study capsules were administered BID with food one before breakfast and one before dinner for two menstrual cycles. The aims of the trial were to document the relationship between sexual function and circulating hormones in the study population, as well as the efficacy and safety of the botanical extract. The main outcomes measured at baseline and month two included a variety of hormones such as Testosterone and Estradiol, sexual functioning was evaluated using the FSFI and the Derogatis Interview for Sexual Functioning (DISF-SR), and stress fatigue and relationship quality were evaluated by the perceived stress scale (PSS), multi-dimensional fatigue symptom inventory (MFI-20), and dyadic adjustment scale (DAS). Safety and tolerability were also determined by various methods. Results showed that the treatment group had significantly increased levels of estradiol and free testosterone compared with placebo. A significant inter group difference was shown in the sexual arousal domain of the FSFI, and all domains of the DISF-SR showed significant improvements over placebo(Rao, Steels et al. 2015).

A second double blind randomized placebo-controlled study was conducted using the same product in the study above BID for 12 weeks. The trial recruited 115 women and primarily assessed the reduction of menopausal symptoms in healthy women via the Menopause-Specific Quality of Life questionnaire which contains a sexual domain score. The results demonstrated significant improvement in sexual functioning (Steels, Steele et al. 2017)

As the only two clinical study to evaluate the effects of *T. foenum-graecum* in women, future studies should be conducted to confirm its efficacy and safety. The expanded Commission E monographs detail a dosage equivalent of 6 g per day, making the studies' medicine higher than what would be traditionally utilized, it would serve to do a dose finding study in the future with this species. Longer treatment duration may also provide more clues to those hormones that remained unchanged. There is current controversy over the measuring of free testosterone as it applies to study outcomes due to the fact that measured testosterone levels in particular are impacted by various factors including lifestyle and age as well as a daily variability (Trost 2016). To combat the age variance, the first study would be strengthened by stratification of age amongst the treatment and control group to minimize these confounding variables. Although well tolerated, the evidence is insufficient and as stands too weak to support use in AP formulations. The second study does very little to add evidence as the purpose focused on reduction of menopausal symptoms with

sexual functioning comprising only a few questions. Please refer to the following section on Ginkgo (2.3.11) for a reference to a polyherbal formula containing fenugreek.

2.3.11 Ginkgo (*Ginkgo biloba* L.)

Ginkgo biloba (Ginkgoaceae) is a perennial deciduous tree native to eastern China of which the leaves are used as medicine(Kew 2019). In Japan, however, it is the seeds that are considered an effective tonic for the enhancement of physical and spiritual vitality and also libido (Rätsch 2013). In TCM it has been employed for impotence, but no specifics on traditional use as an aphrodisiac emerges. In the Commission E monographs, the leaf is listed as unapproved for the strengthening of reduced sexual activity, decreased libido, premature ejaculation, and sexual neurasthenia(Blumenthal and Busse 1998).

An *in vivo* triple-blind, randomized, placebo-controlled trial of a commercial Ginkgo product was carried out with 24 patients (10 women) with sexual impairment due to antidepressant drugs. The study was conducted for 12 weeks after an initial 1-week control period without treatment, and a non-blinded follow-up was continued for six more weeks. The initial dosage started at 125 mg daily during one week only to check for gastric irritation. Outcomes were measured using a developed sexual dysfunction questionnaire, a five-point memory and alertness scale conducted at each consultation, the Hamilton depression and anxiety rating scales (HAM-D, HAM-A) at week 0,6, and 12, and a gastric irritation question at each consultation. There were no significant differences from baseline to any week of the trail other than week 6 in the Ginkgo group, this same trend was continued during the follow-up week 18 compared with baseline or termination of blinding. Memory improved significantly in the placebo group but not in the Ginkgo group, while alertness significantly improved in both. The mean HAM-A score in the placebo group fell significantly, but was not seen in the Ginkgo group, while the mean HAM-D score decreased significantly in both groups. Side effects causing patients to be omitted from the study although low were severe enough in 1 placebo group patient, and 3 ginkgo patients (Wheatley 2004).

An *in vivo* second placebo-controlled, randomized, double-blind trial of *G. biloba* was conducted in 37 patients (10 women) with antidepressant-induced sexual dysfunction for one month and 25 patients (5 female) who continued for one month further. The botanical extract administered was standardized and claimed to contain flavonoid glycosides (mainly kaempferol), quercetin glucorhamnoside esters and

characteristic terpenes of ginkgolides and bilobalides. The dose followed the same given to elderly patients with cognitive dysfunction: 120 mg daily for two weeks and 160 mg for second two weeks (Alastair and Robert, 1998). Patients who continued the treatment received 240 mg for the following four weeks. Outcomes were assessed by a sexual function questionnaire comprised of 5 items for females at baseline, and weeks 2,4, and 8. Psychiatric symptoms were assessed at baseline using a Korean version of the Beck Depression Inventory (K-BDI), and trait and state anxiety were evaluated using the Spielberger state and trait anxiety inventory (STAI) (Beck et al.,1961, Spielberger et al., 1970). The results show no significant difference in any item at baseline, or weeks 2 and 4. The only item showing a significant difference appeared at week 8 relating to orgasm in the control group. Regarding baseline sexual functions in the *G. biloba* group it was reported that five items showed significant improvement at some period however only three of these items apply to female patients. In the placebo group all nine items showed significant improvement however only five of these apply to female patients (Kang, Lee et al. 2002).

The condition of FSD is characterized by not being caused by any concomitant medicinal use such as antidepressants. The first study lasted an adequate amount of time to draw conclusions from; however, the dosage claimed when compared to the Commission E monographs is too low. A recommended dose for a standardized extract falls between 120-240 mg BID or TID while a native dry extract falls between 120-160 mg. The developed sexual dysfunction scale could also be seen as unreliable as it has not been thoroughly validated; therefore, no conclusions on the effect of Ginkgo on sexual dysfunction can be made from this study. The HAM-A and HAM-D scales are well validated tools in psychology, and from the study results it may be inferred with low reliability due to the same placebo response, that Ginkgo can alleviate antidepressant induced sexual dysfunction due to mitigation of depression. In the second study the dose adjustment over the treatment duration is more consistent with the monograph dose previously described; however again the issue of a developed sexual function questionnaire that has not been validated is present. In general, both studies utilized an insignificant study population, and are not reliable to draw any conclusions from regarding the use of *G. biloba* in antidepressant induced sexual dysfunction.

An *in vitro* study was conducted to determine the short and long-term effects of *G. biloba* extract (GBE) on subjective and physiological measures of sexual function in women with Sexual Arousal Disorder with or without desire and/or orgasm concerns. The GBE used for the study was a standardized commercial

extract of *G. biloba* leaves containing concentrated (50:1) *G. biloba* leaf extract standardized to 24% ginkgo flavone glycosides and 6% “terpen lactose” (believed to be mistyped terpene lactones). The extract was selected due to prevalence in clinical trials, and was dosed at 300mg. The placebo pill and GBE pill were identical in appearance and texture. Patients ages 18-65 (*n*=99) were enrolled short term and 68 patients for the long-term study were randomly assigned either placebo (*n*=16), GBE (*n*=19), sex therapy (*n*=19) or sex therapy in combination with GBE (*n*=14) for a period of 8 weeks. Outcomes for sexual arousal were determined via vaginal photoplethysmorgraphy during a neutral and erotic video to measure vaginal pulse amplitude, an index of physiological sexual arousal in response to sexual stimuli. Subjective arousal was measured with a computer mouse fit on a wooden track with intervals from “0” to “7”. Sexual function was measured in a semi-structured clinical interview using the FSFI and SSS-W as well as an event log that was created following the FDA guidance document on guidelines for clinical trials for sexual treatments(FDA 2008). The sex therapy administered was a standardized 8 (55 min) session protocol with a 20-minute exercise to be completed at home every two days, and the same in combination with GBE. The short-term effects of a single dose (*n*=99) demonstrated a significant faciliatory effect on physiological but not subjective sexual arousal. Long-term effects (*n*=68) over a treatment period of 8 weeks, found that *G. biloba*, in combination with sex therapy significantly increased sexual desire and contentment over placebo but not alone (Meston, Rellini et al. 2008). This study in particular highlights the biopsychosocial nature of FSD and how the medical community can approach different treatment options. Prior to the advent of medications like Viagra, therapeutic approaches such as cognitive behavioral therapy and senate focus were common. Although this study did report a significant improvement of desire in for the combination of sex therapy and GBE, the lack of a placebo arm receiving therapy renders the results speculative due to the known placebo response evident in management of sexual function. The dose evalauted in this study is at the low end for a standardized dose due to administration only being once per day, and clinical implications of a short duration of treatment are unknown. A larger sample size and duration of treamtnet would strengthen the clinical implications of this study.

A preliminary *in vivo* study was conducted to investigate the self-reported efficacy of an herbal formula containing *G. biloba* as well as *Muira puama* (sec 4.19). The tablets utilized for treatment were 1,000 mg containing 175 mg of *Muira puama* extract and 16 mg of GBE. Healthy women (*n*=202) enrolled

complaining of low sex drive. Participants received a one-month supply of the AP, and 61% followed a low dose protocol of 2 tablets per day for thirty days, while 39% took a high dose protocol of 6 tablets over the first ten days as per the male formulations dosing protocol. Outcomes were self-reported using a provided questionnaire at beginning and end of protocol period. The results show that out of six parameters rated before and after treatment (capacity as lover, satisfaction with sex life, intensity of sexual desire, excitement of fantasies, ability to reach orgasm, and intensity of orgasm) all but the first significantly improved. Over half of the entire sample recorded improvements in all six aspects of sex life questioned after treatment. Postmenopausal women reported a significantly higher satisfaction with sex life, ability to reach orgasm, and intensity of orgasm at the end of inquiry though both groups maintained positive outcomes (Waynberg and Brewer 2000). Polyherbal formulations are difficult to assess due to the inability to distinguish what is causing the effect witnessed. Even at the high dose protocol, the amount of GBE taken is well below the dose outlined in the German Commission E monographs. Along with a dosing flaw, and no botanical validation, the endpoints are measured using an invalidated self-reporting questionnaire. No implications should be drawn from this study regarding the effectiveness of Ginkgo on sexual functioning.

Another polyherbal formula containing *G. biloba, T. foenum-graecum*, *T. terrestris* and *T. diffusa*, was studied in an exploratory, prospective, non-controlled observational study. The study was conducted in 30 postmenopausal women presenting with a low risk of FSD as assessed by the FSFI who were given a dose of this AP BID for a treatment period of 9 weeks. The primary endpoint assessed was the change from baseline in FSFI score, while secondary endpoints included change in free testosterone levels, sex hormone-binding globulin levels (SHBG), and tolerability. The AP is manufactured and distributed in another country but information from the manufacturer's website states the product consists of a dry extract of *T. foenum-graecum* seeds (600 mg; 50% saponins), dry extract of *G. biloba* leaves (50 mg; 25% ginkgoflavonoids), a dry extract of *T. terrestris* fruit (40 mg 90% saponins), and a dry extract of *T. diffusa* (4:1, 150 mg). The results show a significant increase in all domains except for the pain domain of the FSFI, as well as a significant increase in overall FSFI score. Free testosterone increased significantly from baseline to end of treatment and a significant decrease in SHBG was seen from baseline to end of treatment as well. The study has strengths in the exclusion and inclusion criteria for the patients undergoing treatment; however, the population is rather small. While the primary endpoint of the FSFI is considered to

be a gold standard, the supporting endpoints are weak. The controversy around the reliability of measuring free testosterone levels is discussed in section 2.3.10. It is also difficult to assess whether or not these extracts correlate to a published monographs' dosage information. As a member on the editorial board of the open access journal this article is published in, there exists a bias. The graphical representations of results within the article are blatantly mislabeled, and without a proper control the concluded results stand very weak (Palacios, Soler et al. 2019).

Refer to section 2.3.8 Damiana for information regarding studies on a separate polyherbal formula containing *G. biloba.*

2.3.12 Ginger (*Zingiber officinale* Roscoe)

Zingiber officinale (Zingiberaceae) is a monocot perennial forb/herb that appears in the traditional everyday use of its rhizome as the pungent aromatic spice, Ginger. It is possibly native to India and grown as a commercial crop worldwide (Kew 2019). Herbalists regard ginger as a uterine circulatory stimulant in women's health (Romm 2010). Religious texts such as the Quran contain it for strengthening sexual activities, specifically the Sahih al-Bukhari available in Arabic, and Avicenna writes in his herbal that the preserved and unpreserved forms of dried ginger stimulate sexual desire (Gruner 1930, Al-Hilali 1985, al-Jawziyya 1993). The rhizome is a vital ingredient in tonics and aphrodisiacs of South Africa, and the Lodha (Lodhi, Lodh) of India employ it as a remedy for increasing sexual appetite (Rätsch 2013). The use of ginger is likely similar to what is explained in section 4.5 regarding the use of a driver in formulations.

2.3.13 Ginseng

Ginseng (Araliaceae) is one of the most regarded herbs for energy and stamina, so it is no wonder that over 75% of the AP for women contain some form of ginseng in them. The WHO monographs only list *Panax ginseng* as useful for the male libido; however, there is plenty of traditional use of all types (1999). The Chinese believe that ginseng kindles the inner fire, and they have a male and female version. When they discovered American ginseng as the aphrodisiac, Xi Yang Shen, and they found it to be less potent than *P. ginseng*. The Tao aphrodisiac Yao Jiou employs ginseng as an ingredient. Native American's use of ginseng was as a women's agent, tonic, and aphrodisiac, while the Meskawaki Indians prepared a love

potion from the species (WHO 2012). It is approved in the PDR for lack of stamina and appears in the Duke Handbook of Medicinal Herbs as an aphrodisiac as well as anodyne, carminative, estrogenic, and for use in dysmenorrhea and pregnancy (Duke, Bogenschutz-Godwin et al. 1929, PDR 2007). In the United States, the Appalachian have folk use of the species as a tonic and aphrodisiac. The forms of ginseng appearing on the market in AP given by Latin binomial are *Eleutherococcus senticosus* and *Panax ginseng,* while common name ingredient labels include Asian ginseng, American ginseng, Korean red ginseng, Korean ginseng, and ginseng alone. This is a prime example of when Latin binomials are essential and necessary to distinguish what the ingredients are.

A placebo-controlled, double-blind, crossover clinical trial was conducted testing the effect of Korean Red Ginseng (KRG) (*Panax Ginseng*) extract on sexual function in forty-one premenopausal women, 23 subjects completed the study. The KRG capsule provided by a commercial company contained 500mg of dried ginseng powder from the root of a six-year-old *Panax ginseng* and was taken 6x per day. Details are written about the phytochemical composition of the plant though no specification on analytical methods, and the placebo pill contained starch. Two weeks were spent for collecting baseline data, while eight weeks were designated for treatment arms with a washout period in between. Outcomes were assessed by the Korean version of the FSFI which has been examined for reliability and internal consistency (Lee, Lim et al. 2014), as well as hormone levels and side effects. At the termination of treatment, total FSFI scores significantly increased when analyzing treatment and placebo groups independently; however, when compared against each other the scores were not significant. Specific domains of the FSFI including sexual desire, arousal, orgasm, and satisfaction were significantly improved with KRG; however, again when compared with the placebo response, no significant treatment effect was observed. This effect was also seen with hormone levels during the study. No significant side effects were seen with treatment of KRG (Chung, Hwang et al. 2015).

A second placebo-controlled, double-blinded, crossover clinical study was conducted to assess whether KRG extracts would improve sexual function in 32 menopausal women, 28 subjects completed the study. The study design was identical to the study conducted above; however, the KRG capsules were produced by a different commercial entity and contained only one gram of KRG/capsule to be taken twice a day. After treatment the KRG extract demonstrated significant improvement of scores on the FSFI in only

the sexual arousal domain. The global assessment questionnaire indicating whether sexual function improved after each clinical trial arm also showed significance over placebo (Oh, Chae et al. 2010).

A third study using *P. ginseng* was conducted in a double blind, randomized placebo-controlled fashion with 62 women in Iran. The title claims the subjects were menopausal women but the manuscript itself denotes the women as postmenopausal. The ginseng was commercially obtained and only information on two ginsenosides provided. The 500 mg capsules were taken BID for treatment period of four weeks. Outcomes were assessed by the FSFI, Menopause-Specific Quality of Life (MEN-QOL), and Greene Menopausal Symptoms Scale. Following the termination of treatment, the total FSFI scores as well as individual domain scores all significantly increased after adjusting for baseline. This observation held consistent with the MEN-QOL scores, and a reduction in menopausal symptoms was also significant. (Ghorbani, Mirghafourvand et al. 2019).

The results of the three studies presented suggest a safe and effective potential treatment option for women with FSD. However, they are conducted in different populations of women, at two different dose levels. A dose finding study would need to be conducted in order to move forward with evaluating KRG's potential in improving sexual functioning as the dose was justified based on KRG for treatment of erectile dysfunction (Jang, Lee et al. 2008). Future studies extending treatment time, and supplementing more patient reported outcomes are warranted.

A study was performed to evaluate the effect and mechanism of KRG extract on the relaxation response in isolated rabbit vaginal tissue. Results showed that the extract relaxed the vaginal tissue in a dose-dependent manner, and inhibited contraction induced by depolarization. The authors concluded that the relaxant effect of KRG on rabbit vaginal smooth muscle tissue might be mediated partly through the NO pathway and hyperpolarization via Ca^{2+} activated K^{+} channels (Kim, Kim et al. 2008). Although KRG was demonstrated to effect relaxation of rabbit vaginal tissue, further *in vivo* studies should be conducted to confirm the results.

Refer to section 2.3.8 Damiana for information regarding studies on the same polyherbal formula containing *P. ginseng.*

2.3.14 Horny Goat Weed (*Epimedium* spp.)

Epimedium spp. (Berberidaceae), commonly referred to as Horny Goat Weed encompasses the species listed in Table 2 above. In general Horny Goat Weed is traditionally employed as an aphrodisiac native to Japan (Kew 2019). Its anecdotal use ranges from Australia to China, and it is extensively used in TCM for male sexual dysfunctions (Rätsch 2013), one record of use as an aphrodisiac is listed for women's health however the species *E. aceranthus* is cited which is not a currently accepted species name (Romm 2010). No clinical studies are evaluating the aphrodisiac potential or use of these species in females for sexual dysfunction. However, one study returned from our search criteria evaluated the estrogenic effects of a prenylated flavonoid extract of *E. brevicornum*, which demonstrated selective estrogenic activity that is tissue specific and dose dependent (Indran, Zhang et al. 2014). Without repetitive studies, there is small amount of evidence to make conclusions from, but it may be possible that the use of Epimedium in AP formulas for women has to do with modulation of estrogen. Epimedium does have extensive studies conducted in males for sexual functioning (Ma, He et al. 2011, Li, Li et al. 2015).

2.3.15 Licorice (*Glycyrrhiza glabra* L.)

Glycyrrhiza glabra (Fabaceae) is a perennial forb/herb or subshrub with a wide native distribution from the NE Mediterranean to Eastern Asia, and reaching down as far as Saudi Arabia (Kew 2019). The history of Licorice as an aphrodisiac comes as early as 1650 BC where Hittite cuneiforms detail a powerful aphrodisiac from a beer containing the plant (Rätsch 2013). Afterwards, the Ratirahasya, an Indian sex manual written in Sanskrit, also claims Licorice as an aphrodisiac. Although traditional use of Licorice has been cited for use in males, while modern clinical herbalism regards it as an aphrodisiac for females (Nisteswar and Murthy 1989, Romm 2010).

2.3.16 Maca (*Lepidium meyenii* Walp.)

Lepidium meyenii (Brassicaceae) is perhaps one of the most popular species when thinking of female aphrodisiacs. Native from the high Andes of Peru to NW Argentina, it first appears in the dictionary of Assyrian botany as an aphrodisiac (*L. sativuum*) and has been traditionally employed as a legendary

strengthening agent and aphrodisiac (Romm 2010, Rätsch 2013, Kew 2019). It was once sold in the United States as natures Viagra but has come up short in investigations for clinical use.

An *in vivo* double-blind, randomized, parallel group pilot dose-finding study was conducted to determine whether maca is effective for selective-serotonin reuptake inhibitor (SSRI) induced sexual dysfunction. Remitted depressed outpatients with SSRI-induced FSD (*n*=20, 17 females) were stratified and randomized to a low dose (1.5 g/day) or high dose (3.0 g/day) treatment group. The maca was procured commercially and no testing was done on the researcher's end to verify the material. Routine clinical appointments occurred every two weeks over a period of 12 weeks of treatment. Only ten subjects completed the study, and sixteen subjects met the criteria for intent to treat analysis (ITT) (14 females). Utilizing the Massachusetts General Hospital Sexual Function Questionnaire (MGH-FSQ) and Arizona Sexual Experience Scale (ASEX), improvement in sexual function scores before and after treatment were significant from baseline to final visit in the ITT group. However, there was only significance in the ASEX score of the completers group. When separated to evaluate the effects of low vs high dose, the high dose ITT group showed significant improvement in both measurement tools, while the completers group showed no significance. Improvement in libido was noticed in the combined treatment groups ASEX and MGH-SFQ score although only the ASEX score was significant in the ITT and completers group. When comparing doses improvement was seen in each dosing group for ITT and completers but no significance was reached. When measured sexual attempts, satisfaction, and ability to achieve orgasm over a two-week period, the ITT high dose group showed significance in number of sexual attempts and satisfaction, while no other significance was determined throughout the rest of the ITT group, or completers (Dording, Fisher et al. 2008).

An *in vivo* double-blind, placebo-controlled follow-up study was conducted by the same researchers in a larger sample size of 45 women with SSRI/SNRI-induced sexual dysfunction whose depression remitted. The maca product used was dosed at 1500 mg BID for a period of 12 weeks. The commercial preparation and sample of the source plant was analyzed and vouchered by botanists at the Harvard University Herbaria as well as the Massachusetts College of Pharmacy and Health Sciences using LC-MS and standard macamides. Patients were assessed biweekly using the MGH-SFQ and ASEX, as well as degree and improvement of Antidepressant induced Sexual Dysfunction by the Clinical Global Impression

Severity and Clinical Global Impression Improvement Scales. Treatment after 12 weeks showed higher remission rates with maca treatment demonstrated by the ASEX and the MGH-SFQ (Dording, Schettler et al. 2015).

A potential mechanistic study was also conducted in which the estrogenic and androgenic activity of *L. meyenii* and its effect on the hormonal profile and symptoms were assessed in postmenopausal women. The study was a randomized, double blinded, placebo controlled, crossover study in fourteen women which lasted for twelve weeks. Baseline, six, and twelve-week samples of blood were collected and analyzed for estradiol, FSH, LH, SHBG while subjects completed the Greene Climacteric Scale to assess menopausal symptoms. Extracts of *L. meyenii* were also tested for estrogenic and androgenic activity using a yeast-based hormone-dependent reporter assay. The results show that maca does not exert an estrogenic effect in postmenopausal women; however, it does reduce psychological symptoms, including anxiety, depression, and sexual dysfunction associated with menopause (Brooks, Wilcox et al. 2008).

Although clinical diagnosis of FSD is not due to a psychiatric medication, these two studies serve to represent populations of women who have a medically induced sexual dysfunction due to use of psychiatric medications. The follow-up study corrected some of the omissions of the previous one including the validation and analysis of the product that was being dosed, as well as an increase in sample size. Due to the demonstrated safety of ingesting maca, it would strengthen the studies to extend the treatment period, and although the patient reported outcome tools such as the ASEX have been validated, use of the FSFI would enable comparison between studies utilizing other species.

2.3.17 Oats (*Avena sativa* L.)

Avena sativa (Poaceae) is an annual monocot graminoid descended from a wild oat native to the Fertile Crescent (modern Israel to western Iran)(Kew 2019). The straw and seeds are commonly used in herbal medicine, and herbalists employ it as the most significant grain as an aphrodisiac (Romm 2010). Traditional use has oats appearing in wedding rituals encouraging fertility, but they also appear in the Commission E unapproved herbs for sexual disorders (Blumenthal and Busse 1998). The traditional use

that may tie into use as an aphrodisiac is that Oats are believed to be a nervine restorative helping to strengthen those weakened by stress or anxiety (King 1905, Mills 1985, 1988).

2.3.18 Mucuna (*Mucuna pruriens* L. DC.)

Mucuna pruriens (Fabaceae) is an annual forb/herb vine native to the tropics and subtropics of the old world (Kew 2019). The seeds are most commonly used as medicine due to their reported content of L-dopa (Chaudhri 1996). Traditional use spreads across Tantric practices involving love magic, or as an amulet to increase sex appeal, while prevalence in traditional Ayurvedic medicine as a rasayana (a restorative) has existed for specific conditions such as Parkinson's (Singh G 1998). It is worth noting that the Ayurvedic aphrodisiac use pertains to men and specifically in terms of increasing quantity of semen or stimulating its' production, as well as delaying ejaculation (Chauhan 2014). In Mexico, the seeds are powdered to yield a potent aphrodisiac, and in Brazil, it also functions as such (Rätsch 2013).

2.3.19 Muira Puama (*Ptychopetalum olacoides* Benth.)

Ptychopetalum olacoides (Olacaceae), commonly referred to as potency wood, or Muira Puama, is native to South America and the Amazon rainforest (Kew 2019). It is in the Commission E monographs as an unapproved herb for the prevention of sexual disorders and as an aphrodisiac (Blumenthal and Busse 1998). The entire Amazon region employs the species as an aphrodisiac, with supplemental use in menstrual irregularities, it has appeared in the Brazilian Pharmacopeia since the 1950's. Modern day use dates to the 19th century, where it was employed in Europe as an aphrodisiac tonic and agent for impotence with multiple aphrodisiac pills listed from first and second world war (Rätsch 2013). Traditional use says that it is only useful as an alcoholic extract likely due to the constituent profile available. In general, the traditional use detailed applies only to males with studies conducted for impotence and erectile dysfunction specifically. Clearly the use in AP formulations on the US market is based on the information available for the species utilization in only males. Due to the lack of traditional evidence and absence of modern clinical evidence we would not recommend this species to be studied in women.

See section 2.3.11 for the study including *P. olacoides* in an herbal formula with *G. biloba*.

2.3.20 Passionflower (*Passiflora incarnata* L.)

Passiflora incarnata (Passifloraceae), commonly known as passionflower, is a perennial forb/herb vine native to Central and E. USA as well as Bermuda (Kew 2019). The medicinal plant is traditionally used for treatment of sleep disorders and anxiety though certain species have been used for menopausal symptoms (Dhawan, Dhawan et al. 2004). Its use in the Caribbean as an aphrodisiacal tea which is said to be calming and erotically stimulating while in North America it is consumed via smoking (Rätsch 2013). These effects may pave the way for erotic readiness in the mind as it is mildly sedating and relaxing.

An *in vivo* clinical study of a nutraceutical formula containing Passionflower was conducted in 72 perimenopausal women to evaluate the effect of such formula on quality of life and sexual function. The Italian formula is listed to contain "Passiflora dry extract (3.5% isovitexina) at 178 mg" and further investigation of the products website specifies *P. incarnata*. Endpoints were measured by the Kupperman Index (KI), a widely used instrument for evaluating menopausal symptoms, the Short Form-36 used to assess quality of life, as well as the FSDS and FSFI over a period of six months with intake at baseline, three months, and termination at six months. The results show a significant decrease at both time points compared to baseline in the KI score which demonstrates an improvement in vasomotor symptoms. Participant QoL improved significantly compared to baseline in all but mental health and vitality categories at the three-month time period, but significantly improved in all categories at termination of the study. While the FSFI individual and total domains did not show significance at three months compared to baseline, they did at the sixth month mark. This is the same for the total FSDS score (Caruso, Cianci et al. 2017).

Although significance in FSFI scores were determined at the studies termination, the study itself is highly flawed with no controls or randomization, a small sample size, and no botanical validation. Even though the outcome measurements tools were sufficient poor study design outweighs the results. Therefore, there is currently a very low level of evidence to support *P. incarnata* inclusion in AP.

2.3.21 Pueraria (*Pueraria mirifica* Airy Shaw & Suvat)

Pueraria “white kwao krua” (Fabaceae) may be the same species under the common name of Kudzu in a separate formula that is native to Thailand (Kew 2019). This example stresses the importance of using correct Latin binomials in ingredient labels. In Thailand, mainly, the white version is used for women as a tonic aphrodisiac and rejuvenating agent from menopausal problems to increasing sex drive and stabilizing hormonal disturbances (Rätsch 2013). The PDR lists Kudzu individually as having probable efficacy in menopausal symptoms (PDR 2007). There is no clinical evidence on this species from our search criteria to support its’ inclusion in an AP.

2.3.22 Raspberry Leaf (*Rubus spp*)

Rubus spp. (Rosaceae) commonly raspberry leaf is a perennial subshrub that is native from temperature and subtropical, to tropical mountains (Kew 2019). It appears in the Commission E monographs as unapproved for menstrual problems (Blumenthal and Busse 1998), and is included in the European Medicine’s Agency monograph as having well established traditional use associated with relief of menstrual cramps (EMA 2014). The Carrier people of northcentral BC document it’s use for weak blood or anemia in combination with another plant (Ritch-Krc, Thomas et al. 1996). In general herbal medicine, *Rubus idaeus* (the red species) is considered a uterine astringent and antihemorrhagic, as well as an overall uterine tonic although the AP formula does not have a species listed (Romm 2010). Although there is a widely documented traditional use of Rubus for its affinity to the female reproductive system, there is nothing specifically on its use as an aphrodisiac (Hummer 2010). The uterine affinity may lead future studies in the menopausal subpopulation of FSD; however, we would recommend other species detailed in this paper with a higher level of evidence first.

2.3.23 Di Huang (*Rehmannia glutinosa* Gaertn. DC.)

Rehmannia glutinosa (Orobanchaceae) commonly known by its genus name Rehmannia, or Di Huang, is native to China and a widely use traditional Chinese herb (Zhang, Li et al. 2008, Kew 2019) . In women's health, it is listed as an aphrodisiac, but no other traditional use found validates this claim (Romm 2010). The PDR lists its use in Chinese medicine distinguishing fresh root tuber for irregular menstruation,

and dried root tuber for metrorrhagia (PDR 2007). The understanding of Rehmannia's use as a Chinese herb is limited to one's understanding the principles of *Yin* and *Yang*, "two opposite, complementary, interdependent and exchangeable aspects of nature" (Tang, Liu et al. 2008). In general, the body is believed to have a constant energy circulation referred to as Qi, as well as blood, and disease occurs when this circulation or the *Yin-Yang* balance is altered. Rehmannia serves to nourish this Yin energy aiding in circulation via supplementing the blood, and promoting production of body fluids. In western medicine the framework and terminology to translate some of these TCM concepts into a clinical trial do not exist. Therefore, in order to study an herb like this for FSD it would be important to know the correlation between stagnation or imbalance and the symptomology that appears. For a review on the traditional use correlated with modern use see Zhang paper above.

2.3.24 Saw Palmetto (*Serenoa repens* W. Bartram)

Serenoa repens (Arecaceae) is a perennial shrub/tree native to the Southeast United States, that is listed in the WHO monographs as an aphrodisiac (WHO 2002, Kew 2019). The Commission E monographs state that the plant is antiandrogenic and the PDR confirms this while also saying it produces antiestrogen effects as well as an unproven use for the improvement of libido (Blumenthal and Busse 1998, PDR 2007). The modern herbal use of saw palmetto is also listed as an aphrodisiac for females (Romm 2010). The Seminole Native Americans utilized the berries of the species for urological disorders, and today it is frequently seen in formulations for men addressing symptoms of benign prostatic hyperplasia (BPH) (Chua, Eise et al. 2014). Future studies on the mechanism of saw palmetto specifically in BPH may help to understand how it has developed any reputation for the use of libido as an aphrodisiac; however, there is no current information to suggest it should appear in AP formulas, especially for women.

2.3.25 Schisandra (*Schisandra chinensis* Turcz. Baill*)*

Schisandra chinensis (Schisandraceae) is a deciduous woody vine native from the Russian Far East to N. China and Central Japan(Kew 2019). Five flavor berry is believed to exert effects on body systems ranging from the heart and stomach to the liver (Lee et al 2015). Traditional use in the aphrodisiac realm pertains to men and the species ability to prevent premature ejaculation, and it appears in one of the

earliest materia medicas, the Shen Nong Ben Cao Jing, as such (Kimura 1996, Rätsch 2013). The traditional effects of schisandra in modern herbalism focus instead on the species' action on the CNS, which may allude to how it effects sexual functioning. The deficiency of female related history in sexual functioning in particular suggests that this species may not be worth looking into further; however, as the knowledge surrounding the neurobiological basis of FSD increases, species which exhibit specific effects on the CNS may be useful.

2.3.26 Shatavari (*Asparagus racemosus* Willd.)

Asparagus racemosus (Asparagaceae) is a climbing shrub native from tropical Africa to N. Australia (Kew 2019). It is commonly referred to as Shatavari coming from a Sanskrit word roughly translated to "that which possesses one hundred men "alluding to its aphrodisiac properties. It appears in the ancient Indian Sanskrit text the Kama Sutra, a guide on desire, pleasure and related erotica. The leaves and roots of the plant are used in oil for treating impotence and infertility, and it is considered an aphrodisiac in females (Romm 2010). In Ayurveda, the species is classified as a rasayana and vajikarana which are said to further love and surrender while helping with the weaknesses of the female genital organs as well as overall sexual weakness, and infertility (Rege, Thatte et al. 1999, Dalal, Tripathi et al. 2013). Shatavari is well known as the main Ayurvedic tonic for females, and is highly effective in problems of the female reproductive system; however, similarly to TCM, Ayurvedic principles are not easily translated into a western model of medicine. The purported traditional use has not been studied in a female animal model though a review mentions a study in male rats conducted in 2009 (Thakur, Bhargava et al. 2009). Unlike some of the other traditional used aphrodisiacs, Shatavari is a standout in terms of its recognition as such in Ayurvedic medicine, and specifically for females which is uncommon in traditional medicine. For that reason alone, it is worth looking into further.

2.3.27 Stinging nettle (*Urtica dioica* L.)

Stinging nettle (Urticaceae) is an herbaceous perennial forb/ herb native to Eurasia that is in the listed as an aphrodisiac from Dioscorides' De Materia Medica, and afterward appears in the Assyrian Book of Botany (Romm 2010, Kew 2019). Dioscorides states that "a decoction of the seed taken as a drink with

raisin wine is an aphrodisiac and opens the womb" (Dioscorides 1518). In Avicenna's Canon of Medicine, the aphrodisiac potential of Nettle is also highlighted where the powdered seeds are used as a liniment to stimulate sexual desire (Gruner 1930). On the Iberian Peninsula Nettles are used to improved circulation, lower blood pressure, as well as to clean or purify the blood (Calvo, Akerreta et al. 2011). In Turkey the circulatory use is also applied as well as a depurative, and nerve tonic, while Southern Turkey has ethnobotanical use as an aphrodisiac that isn't expanded upon (Ozdemir and Alpinar 2015). In a polyherbal formulation, the seeds are utilized with Myrrh to act as an emmenagogue and open the mouth of the uterus. (Rätsch 2013). This uterine affinity has also led to traditional use in a multitude of gynecological conditions (Ilhan, Ali et al. 2019). Limited information exists on the extent of Nettles being utilized as female aphrodisiac, though it may be due to the circulatory effects and uterine affinity which it has been traditionally employed for. The literature shows complementary medicine has made attempts to progress in evidence for Nettles in women's health in terms of menstruation, androgenic activity, and effects of luteinizing hormone, as well as testosterone; however, in FSD specifically no literature is present (Hamedi, Afifi et al. 2017, Kargozar, Salari et al. 2019).

2.3.28 Tribulus (*Tribulus terrestris* L.)

Tribulus terrestris (Zygophyllaceae) also known as puncture vine, is an annual forb/herb that is widely distributed around the world (Kew 2019). In the herbal context, it is seen as an aphrodisiac and hormone regulating (WHO 2009, Romm 2010). One of the first records of this species is in Avicenna's Canon of Medicine claiming that it stimulates sexual desire (Rätsch 2013). Its use also ranges from India where all parts of the species are employed as aphrodisiacs, as well as Africa and Indonesia where the fruits are smoked or drank, and in TCM and Ayurveda practices. *T. terrestris* has been evaluated in multiple studies, though the difference in herbal preparation, dosing, country of study, as well as endpoints make it challenging to draw a conclusion that isn't general.

The first qualitative-quantitative study in Brazil was conducted based on hospital records of 144 female patients presenting with sexual dysfunction and treated with 250 mg of *T. terrestris* extract TID for a period of 90 days. Results showed a significant improvement in total FSFI scores, increased levels of DHEA, and decreased free testosterone, while serum testosterone levels decreased (Gama, Lasmar et al.

2014). Although the investigators provided exclusion criteria, the medical history provided brings up concerns for those included especially regarding the menstrual conditions, history of depression, and previous contraceptive use. The study population demographics are also widely spread regarding relationship which is known to play a factor in sexual dysfunction and resulting treatment. The article is weak due to an assortment of these confounding variables skewing any interpretation of the findings. Even with quantitative information deduced from the study, the method of qualitative data analysis is plagued by research bias.

A randomized, double-blind, placebo-controlled clinical trial took place in Iran where patients were treated with 5 mL of syrup containing 3.5 g of ethanolic extract of *T. terrestris*. The dried leaves of Tribulus were obtained by a licensed distributer and approved and registered by the herbarium but no other analytical testing was done. Participants were recruited through a singular question "Do you suffer from loss of libido?", followed by a phone interview with inclusion criteria, and finally an easement based on the DSM codes for HSDD. Whether or not these patients were diagnosed is not discussed. The study preparation was 7.5 mL of syrup BID for four weeks, and patients were evaluated using the FSFI. At the termination of the study results showed a significant improvement in all domains of the FSFI as well as in the total FSFI score (Akhtari, Raisi et al. 2014).

Another randomized, double-blinded, placebo-controlled study was conducted in Brazil evaluating the efficacy of *T. terrestris* for the treatment of HSDD in postmenopausal women. The treatment group of 45 women received 750 mg/day of Tribulus referenced to be from an Herbarium in Brazil. No information on phytoconstituents or any other validation methods are present. Outcomes were measured using the FSFI, validated for the Portuguese language and the QS-F questionnaire originally developed for Brazilian women. Only 36 women completed the study due to personal reasons (*n*=3), and side effects of nausea (*n*=6, three from treatment group, three from placebo). A secondary outcome of serum testosterone was also assessed. Termination of the study after 120 days (17 weeks) resulted in total FSFI scores increasing significantly for treatment and placebo group when assessed independently. However, like other species studied before, when compared between the two groups no significance was determined. The lubrication domain specifically showed significant improvement in the treatment group that was not demonstrated by placebo. The QS-F questionnaire also showed significant improvements across all domains (desire,

arousal/lubrication, pain, anorgasmia) solely for the treatment group. Free and bioavailable testosterone levels also significantly increased in the treatment group (de Souza, Vale et al. 2016).

Another randomized, double-blinded, placebo-controlled study used *T. terrestris* for the treatment of premenopausal women with HSDD also evaluated the effect on serum levels of testosterone. The study design mimicked the one conducted in 2016. Similarly, the results showed independent significance in baseline and post treatment scores of the FSFI for the treatment group; however, the placebo group also showed significance in all but the "lubrication" and "pain" domain. The QS-F questionnaire again showed significant improvement across all domains, while the placebo did not. Although the free and bioavailable testosterone levels pre and post treatment significantly increased for the treatment group, total testosterone levels did not vary significantly (Vale, Zanolla Dias de Souza et al. 2018).

A third prospective, randomized, double-blind, placebo controlled clinical trial was conducted in 60 postmenopausal women with sexual dysfunction. The treatment group received blister packs containing *T. terrestris* and were instructed to take one tablet (250 mg) orally TID for 90 days. Although a batch number is listed there is no further information on the medicinal product. Endpoints were measured utilizing the Sexual Quotient-Female version (SQ-F), in addition to the Female Intervention Efficacy Index (FIEI). After three months of treatment there was a significant difference between the treatment and control group in multiple SQ-F domains including "desire and sexual interest" and "arousal in women and harmonious interaction with the partner". The FIEI questionnaire demonstrated significant improvement across all domains including lubrication, sensation, and ability to orgasm. Adverse incidents were reported within both groups, though not determined to be significant (Postigo, Lima et al. 2016).

Overall the studies suggest that potentially *T. terrestris* exerts its' effects due to a modulation of testosterone. The mechanism in which this occurs, or the constituent responsible has not identified though it may be due to the steroidal saponins present in the species. The characteristically high placebo response confounds any positive response to Tribulus; however, the lack of a response from placebo in specific domains such as lubrication and pain indicate that Tribulus may be more suitable for another type of FSD outside of libido desire disorders.

Please refer to the above section on Ginkgo (4.11) for information on a polyherbal formula containing Tribulus.

2.3.29 Valerian (*Valeriana officinalis* L.)

Valeriana officinalis (Valerianaceae) is an herbaceous perennial native to Europe utilized traditionally for its sleep-promoting and relaxing properties (Kew 2019). It is listed in the PDR with an unproven use in menstrual states of agitation, menopause, and uterine spasticity, and in the American Dispensatory, it is listed as an aphrodisiac (AHP 1999, PDR 2007). The sedating and relaxing properties may aid pre-orgasmic women in being receptive to stimuli as the basis for inclusion in formulas; however, no specific literature on this species use in FSD exists.

2.4 DISCUSSION

This review set out to evaluate the medicinal plants listed on the ingredient label of the female aphrodisiac products on the market in the United States.

Out of 53 species gathered from AP ingredient labels, the following lacked any clinical evidence but did have traditional use: *T. cacao, V. agnus-castus*, *E. grandiflorum*, *A. sativa*, *M. pruriens*, *R. glutinosa*, *S. repens*, *A. racemosus.* Many of the herbs listed do have ethnobotanical use as an aphrodisiac throughout history but are lacking in specific research done to determine whether they have any measurable effect in FSD. Those without clinical or traditional evidence in females included: *A. officinalis, T. catuaba, C.monnieri, Crataegus spp., C. zedoaria, D. villosa, Epimedium spp (exception of E. grandiflorum),E. longifolia,* Tongkat ali, Flower pollen, *C. johimbe, P. vulgare, P. montana var. lobata, S. rosea, R. idaeus, Schisandra spp., Smilax spp., U. dioica, X. scabrosa.* We believe that it may be useful in the future to study some of the species with traditional evidence in females, as well as the species that may have no sex assigned aphrodisiac role. However, for the field to progress, focusing on those herbs which have clinical evidence seems the most appropriate.

Five herbs had clinical evaluations in a polyherbal formula: *T. diffusa, G. biloba, P. ginseng, T. foenum-graecum, T. terrestris.* In polyherbal formulations, even with a statistically significant finding, there is no way to determine which herb is responsible for the biological activity. In some cases, it may also be that a synergism exists between herbs in a formula so that one potentiates the action of another. However, due to the infancy of the clinical research of herbs being assessed for their aphrodisiac potential or

application in FSD, we must evaluate these species alone to determine not only their efficacy but safety and toxicity profiles as well which has been done in this case. Adding to the list of species with any amount of clinical evidence is *W. somnifera*, *A. sinensis*, *T. foenum-graecum*, *G. biloba*, *L. meyenii*, and *Passiflora incarnata.* We suggest continuing work on these species as priority over any other.

From the literature of plants being used female sexual dysfunction, there is currently no substantial amount of evidence to fully support those claimed on the label of aphrodisiac products on the market. although almost all of the herbal products have traditional and anecdotal use as an aphrodisiac, the clinical evidence of such is lacking. This can be due to a variety of reasons, perhaps most importantly that HSDD and FSD, in general, are heterogeneous amongst the female population, making it difficult to establish a consensus for controlled measures across studies. For those plants with clinical evidence behind them, the studies are sometimes too dissimilar to conclude; for instance, in preparation of the botanical (aqueous/alcoholic extract), dosing, and even carrier for administration (pill, tea, syrup).

We suggest that due to the evident neurobiological underpinnings of FSD libido desire disorders, that an *in vitro* model, or multiple *in vitro* models should be used as screening tools to evaluate the aphrodisiac potential in herbs. While there is no current model with specificity for aphrodisiac potential in females, we suggest looking into models reporting on dopamine, serotonin, and melanocortin's affinities as well as any of the other neurotransmitters discussed in the inhibitory/excitatory circuits of sexual response. We believe that aphrodisiacs as a group may function by several different mechanisms, so assessment of estrogenic or androgenic potential of pure extracts is feasible and warranted. It is after this screening that researchers can then move forward with an *in vivo* animal model, although it is our understanding that an agreed upon FSD model does not exist. Therefore, it may serve the research in this area to first focus on evaluating behavioral conditions in specific subsets of the population, such as HSDD in menopause.

CHAPTER 3 IN VITRO CYTOTOXICITY ASSESSMENT AND MELANOCORTIN RECEPTOR ACTIVATION SCREENING OF BOTANICAL APHRODISIACS AND THEIR SPECIALIZED METABOLITES

3.1 OVERVIEW

One of the foremost concerns of the dietary supplement industry in general is the adulteration of herbal products whether through intentional spiking of major constituents, addition of foreign plant material whether a similar species as in the case of cinnamon or a very distinct species such as *Eleutherococcus senticosus* and *Periplopum sepium.* Therefore, authentication of botanical material is vital for experimentation so researchers can say with certainty this is the genus and species it is purported to be. We proceeded with our target botanicals based on their availability in the NCNPR repository with the requirement that it must be a voucher specimen. This designation in the repository meant that it had previously been through authentication testing by a team of botanists and analytical chemists, often using a combination of micro/macroscopic methods along with molecular ones including an array of chromatographic and spectroscopic techniques.

Three of our species were discussed in Chapter 2 and their ethnobotanical and clinical evidence for use as an aphrodisiac product in particular covered, *T. terrestris* (2.3.38), *A. racemosus* (2.3.26), *T. foenum-graecum* (2.3.10). The other three species which were not covered will be detailed below, *L. pumila*, *C. johimbe*, and *E. longifolia*.

L. pumila is a small flowering forest floor plant with a native range from Indo-China to W. Malesia. It is highly regarded in Malaysia as the “queen of female herbs”. Traditionally the species commonly called “Kacip Fatimah”, is employed to induce and expedite labor, as well as used in post-partum recovery (Burkhill 1935). Safety and efficacy pilot studies have been conducted on the aqueous extract of *L. pumila* previously. A randomized double-placebo controlled study investigated the extracts on sexual health, lipid profile, and inflammatory markers in pre and postmenopausal women (Annie 2014). Although the investigators determined no significant differences in FSFI scores after twelve weeks, the

population excluded women with a sexual dysfunction; therefore, comparison between placebo and treatment cannot say whether this would improve women with a score indicative of FSD. They also determined no significant changes in serum-estradiol levels which conflicts previous data suggesting presence of estrogenic like compounds, as well as studies in which *L. pumila* aqueous extract exhibited high estrogenic activity *in vitro* (Poh 2012). Other studies however have not found estrogenic activity believing a necessary metabolic transformation must take place for activity (Jamal 1998;2011). In general demographics of the study such as smoking, and alcohol consumption may have confounded variables tested. Another randomized, double-blind, placebo-controlled study was conducted which found the extract to be safe and effective for improving QOL, as well as vasomotor and menstrual symptoms though no changes in gonadotrophin hormones or estradiol were seen. (Norhayati 2014). The conflicting data as well as popularity of this species warrants further exploration into mechanistic studies of its purported activity especially due to a favorable safety profile tested in humans.

C. johimbe is an evergreen tree species with a native range from Nigeria to W. Central Tropical Africa growing upwards of 30 m with a reddish hued bark commonly employed in traditional medicine (Kew 2019). Primary interest of the sexual effects of this species belong to the isolated and well characterized indole-alkaloid yohimbine, an alpha-2-adrenoceptor antagonist. The general consensus is that it is efficacious over placebo for erectile dysfunction; however, it lacks patented commercialization potential which is thought to be why rigorous human clinical trials have not been conducted. (Ernst, Pittler 1998; Tam 2001). There has been an evaluation of yohimbine in combination with l-ariginine glutamate on sexual effects in post-menopausal women with arousal disorders, finding an increase in vaginal pulse amplitutde with treatment, though no significant change in subjective arousal effects (Mestone, Worcel 2002). The vasodilatory erectogenic and CNS effects of yohimbine suggest that it may have similar effects on clitoral tissue. The availability of this compound commercially as well as the history of whole species use as an aphrodisiac certainly necessitates futher experimentation.

Eurycoma longifolia Jack (Simaroubaceae) grows as an evergreen shrub native Indo-China to W. Malesia (Kew 2019). It is extremely popular as a male enhancing medicine where it has been shown to increase testosterone levels. Many of the studies conducted on the species have been limited to male animal models; however, the presence and sexual function impact of testosterone is established in both

sexes (Bolour, Braunstein 2005). Furthermore, the sample to be experimented has already undergone standardization which should make further attempts to isolate the bioactive component feasible. (Bhat, Karim 2010; Rehman et al 2016)

Although some of the botanical species we are evaluating here have been primarily used as male aphrodisiacs with limited reports or experiments regarding female specific use, the melanocortin system is present in both sexes functioning to produce not only erectogenic effects of tissue, but stimulating arousal and desire as well.

3.2 TARGET BOTANICALS

C. johimbe (NCNPR1110) bark was collected by Nkuinkeu Rober from the Yoyo Locality, Littoral province, Cameroon 80 m; *L. pumila* (NCNPR 5004) leaf was collected by Tropical Botanics in Perak Malaysia; *A. racemosus* (NCNPR 7472) tuber was collected by Mr. N.K Pandey from Gwalior, Madhya pradedh; *T. foenum-graecum* (NCNPR 8105) whole seed was provided by the Missouri Botanical Garden*; T. terrestris* (NCNPR 9753) whole fruit was collected in the Shanxi province Xi'an county, and was provided by Harvard Medical School; *E. longifolia* was supplied as a dietary supplement in pill form (NCNPR854PR); all voucher material was verified by Vijayasankar Raman and stored in The Natural Products Research Repository housed within The University of Mississippi. Samples were ground to a uniform powder utilizing a Retsch pm 400 machine prior to performing sequential extractions. For all species aside from *E. longifolia*, twenty-five grams of ground material were packed according to the manufacturer's instructions within medium sized stainless-steel cylinders and extracted sequentially with three cycles each of hexanes, ethyl acetate, methanol, and water using a Dionex ASE (Dionex Corporation, Sunnyvale, CA, USA) at 40 °C and 1500 PSI followed by a rinse volume of 100%. Fractions were then concentrated and dried before being prepared in a 20 mg/mL stock solution in DMSO to make a working solution for *in vitro* experiments.

3.3 EXTRACTION AND ASSESSMENT OF CRUDE BOTANICAL APHRODISIACS IN VITRO

3.3.1 METHODS

CELL CULTURING: Commercially obtained HEK293T cells from the American Type Culture Collection (ATCC, Manassa, VA, USA) were thawed in a 37 °C water bath and agitated rapidly. Cells were washed, re-suspended, plated in flat bottomed T75 culturing vessels, and maintained in High Glucose Dulbecco's Modified Eagle's Medium (Gibco, 11995073) containing 1% penicillin-streptomycin solution (Gibco, 15140122) as well as 10X FBS (Gibco 10437028), and incubated at 37 °C with 5% CO_2. Prior to treatment, cells were sub cultured into 96-well tissue culture treated plates at a density of 37K cells/well for cytotoxicity, and according to the manufacturer's instructions for the assay.

IN VITRO CYTOTOXICITY: After 2 passages, cells were split with serum free DMEM and pipetted into 96-well plates. Plated cells were incubated for overnight to reach ≥ 80% confluence prior to treatment with botanical fractions. Following 24 hrs. of incubation, well media was aspirated and replaced with fresh serum free media before being treated in triplicates with the botanical extracts at 7 concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.5625 µg/mL, DMSO at 10% diluted in media was used as a vehicle control). Cells were incubated overnight before addition of 2 µL MTS reagent and then incubated further for three hrs. prior to reading cells with the MTS procedure at 490 nm on a Biotek Synergy HTX Multi-Mode plate reader (BioTek, Winooski, VT, USA) in order to determine an MTC.

MC4R ASSAY: A Melanocortin-4 Receptor Reporter Assay Kit was purchased (Cayman Chemical, 600190). Wells were seeded at a density of 50,000-100,000 cells/well according to the manufacturer's instructions and incubated overnight. Media was aspirated and 100 µL of fresh serum free media added. Extracts were prepared as a 20 mg/mL stock solution in DMSO at 2x the final concentration in serum free media. Untreated control wells received 100 µL of serum free media, positive control wells were treated with dilutions of alpha-MSH for a concentration curve. Cells were stimulated for 19 hrs. before performing the SEAP Assay according to the manufacturer's instructions. Plates were read on CLARIOStar Plus (BMG Labtech Inc., Cary, NC, USA) microplate reader capable of detecting chemiluminescence.

STATISTICS: We analyzed our cytotoxicity data in Graph-Pad Prism with a Two-way RM ANOVA, factoring in concentration and solvent as our independent variables and their effect on cell viability in relation to control cells at 10% DMSO. Significance is demonstrated by a p value ≤0.05. The two way-ANOVA will break down the source of variation into four categories (concentration x solvent, concentration, solvent, plant sample). We followed this up with Dunnett's multiple comparisons test (alpha=0.05) to determine

significance levels between treatment and control wells. The MC4R data was conversely measured by an Ordinary one-way ANOVA followed by Tukey's multiple comparisons test.

3.3.2 RESULTS

Corynanthe johimbe: The hexanes and methanol fractions did not demonstrate any significant change in MTS absorbance compared to control at all concentrations tested. The ethyl acetate fraction demonstrated a significant increase in MTS absorbance over control for the following concentrations: 25 µg/mL($p \leq 0.05$), 50 µg/mL ($p \leq 0.05$), 100 µg/mL($p \leq 0.05$). The water fraction demonstrated a significant increase in MTS absorbance over control for the following concentrations 6 µg/mL($p \leq 0.05$), 12 µg/mL($p \leq 0.01$), 50 µg/mL($p \leq 0.05$), 100 µg/mL($p \leq 0.05$). As no significant decreases in MTS absorbance were demonstrated, we proceeded to the MC4R assay with 100 µg/mL for every fraction of *C. johimbe*.

C. johimbe demonstrated a significant decrease in MC4R activation over control from the hexanes ($p \leq 0.01$), ethyl acetate ($p \leq 0.0001$), and methanol fractions ($p \leq 0.001$). While we did observe an increase in MC4R activation from the water fraction, it was not enough to be significant ($p = 0.0790$).

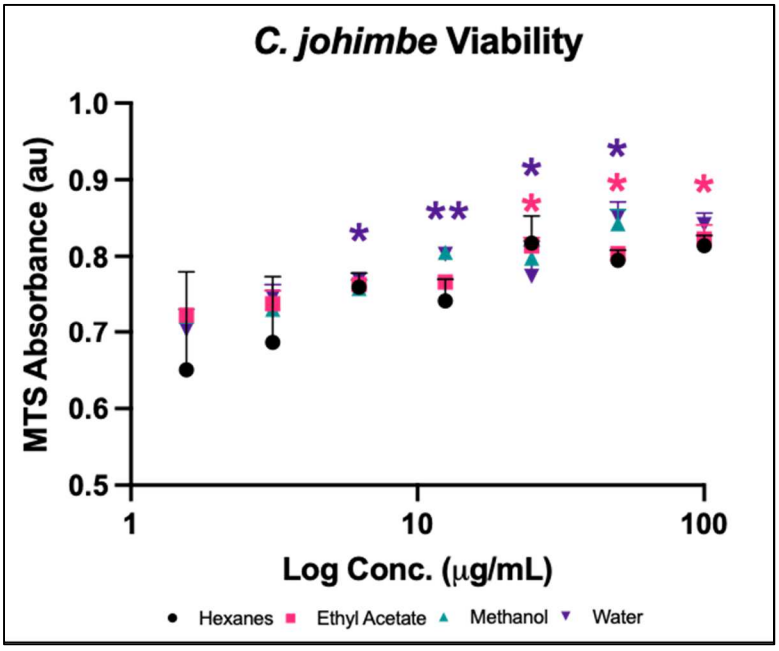

Figure 3a: Cytotoxicity of crude *C. johimbe* fractions in HEK293 Cells
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

C. johimbe demonstrated a significant increase in MTS absorbance for the H_2O fraction at 6,12,50,100 µg/mL, as well as the EtOAc fraction at 25, 50, and 100 µg/mL (n=3).

Table 3A: *Corynanthe johimbe* Cytotoxicity ANOVA					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Concentration x Solvent	0.02733	21	0.001302	F (21, 56) = 1.476	$p = 0.1245$
Concentration	0.2319	7	0.03313	F (1.613, 12.90) = 37.57	$p < 0.0001$
Solvent	0.02215	3	0.007385	F (3, 8) = 1.517	$p = 0.2830$
***C. johimbe* Fraction**	0.03895	8	0.004869	F (8, 56) = 5.522	$p < 0.0001$
Residual	0.04938	56	0.0008817		

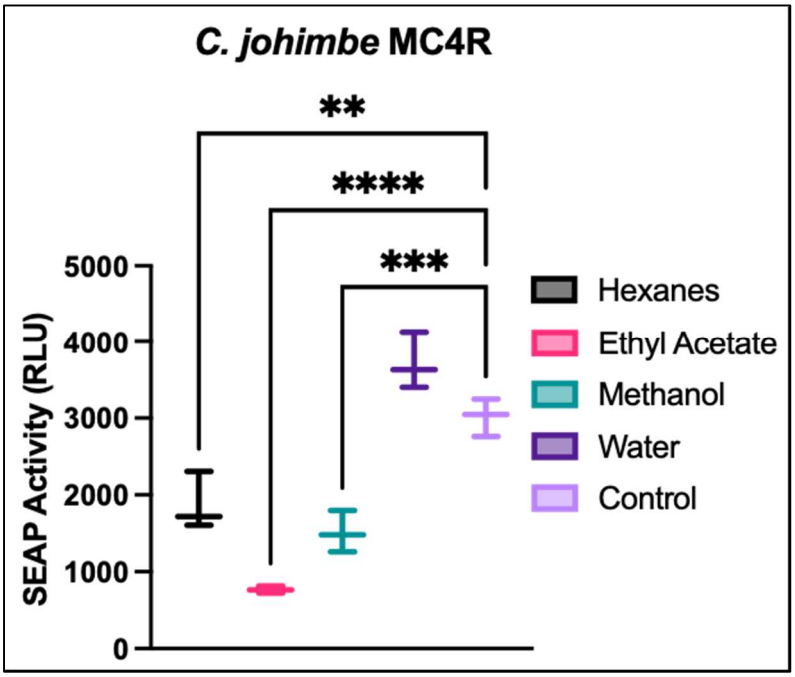

Figure 3b: Activation of MC4R *in vitro* by *Corynanthe johimbe*
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

C. johimbe displays an increase in activation of MC4R over control by the water fraction at 100 µg/mL (p= 0.08), and a significant decrease in activation by the hexanes, EtOAc, and MeOH fractions all at 100 µg/mL (*n*=3).

Table 3B: *Corynanthe johimbe* MC4R ANOVA					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	16851279	4	4212820	F (4, 10) = 50.65	$p < 0.0001$
Residual (within columns)	831722	10	83172		
Total	17683001	14			

Asparagus racemosus: The hexanes fraction demonstrated a significant increase in MTS absorbance at 50 µg/mL and 100 µg/mL. The ethyl acetate fraction demonstrated a concentration dependent decrease in MTS absorbance as the concentration increased from 25 µg/mL ($p \leq 0.005$). The methanol fraction demonstrated a significant decrease in MTS absorbance at 100 µg/mL ($p \leq 0.005$). The water fraction did not demonstrate a significant increase or decrease in MTS absorbance at any concentration. Therefore, we proceeded to the MC4R assay with 100 µg/mL for the hexanes fraction, 12.5 µg/mL for the ethyl acetate fraction, 50µg/mL for the methanol fraction, and 100 µg/mL for the water fraction. *A. racemosus* demonstrated a significant decrease in MC4R activation compared to control in all fractions tested (hexanes, water at 100 µg/mL, EtOAc at 12.5 µg/mL, MeOH at 50 µg/mL) (fig4b).

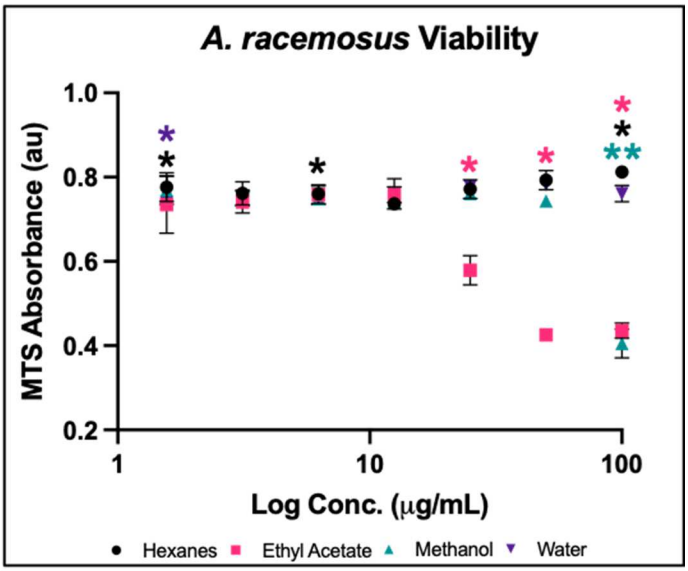

Figure 4a: Cytotoxicity of crude *A. racemosus* fractions in HEK293 Cells
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

A.racemosus demonstrated a significant increase in MTS absorbance for the hexanes fraction at 1,6, 100 µg/mL, as well as the H_2O fraction at 1 µg/mL; however, it demonstrated a significant decrease in MTS absorbance from the EtOAc fraction at 25,50,100 µg/mL, as well as the MeOH fraction at 100 µg/mL

Table 4A: *Asparagus racemosus* Cytotoxicity ANOVA

	SS	DF	MS	F (DFn, DFd)	P value
Concentration x Solvent	0.5890	21	0.02805	F (21, 56) = 39.94	$p < 0.0001$
Concentration	0.2288	7	0.03268	F (2.623, 20.98) = 46.54	$p < 0.0001$
Solvent	0.2018	3	0.06727	F (3, 8) = 230.0	$p < 0.0001$
***A. racemosus* Fraction**	0.002340	8	0.0002925	F (8, 56) = 0.4165	$p = 0.9063$
Residual	0.03932	56	0.0007022		

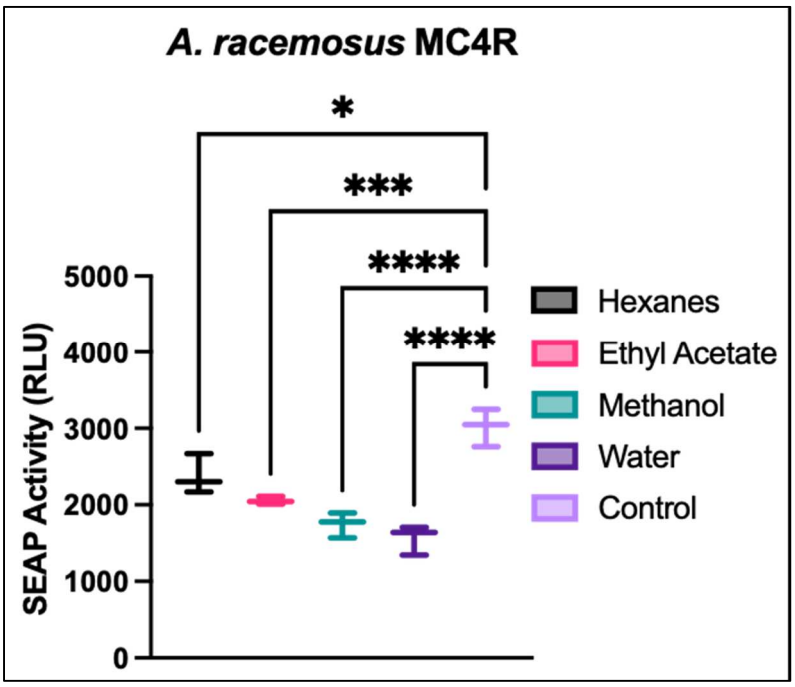

Figure 4b: Activation of MC4R *in vitro* by *A. racemosus*
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

A. racemosus displays a significant decrease in activation for all fractions including hexanes, and water at 100 µg/mL, EtOAc at 12.5 µg/mL, and MeOH at 50 µg/mL (*n*=3).

Table 4B: *Asparagus racemosus* MC4R ANOVA

	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	3969154	4	992288	F (4, 10) = 25.60	*p* < 0.0001
Residual (within columns)	387576	10	38758		
Total	4356730	14			

Tribulus terrestris: The hexanes and water fractions did not demonstrate any significant increase or decrease of MTS absorbance against control at all concentrations tested. The EtOAc fraction demonstrated a significant increase in MTS absorbance over control at the following concentrations: 3 µg/mL($p \leq 0.05$), 6 µg/mL ($p \leq 0.01$), 12 µg/mL ($p \leq 0.05$), 50 µg/mL ($p \leq 0.001$), 100 µg/mL ($p \leq 0.05$). The methanol fraction demonstrated a significant increase in MTS absorbance over control at the following concentrations: 6 µg/mL ($p \leq 0.05$), 25 µg/mL ($p \leq 0.01$), 50 µg/mL ($p \leq 0.001$), 100 µg/mL ($p \leq 0.01$). As no significant decreases in MTS absorbance were observed in respect to control levels, we proceeded with testing 100 µg/mL for each fraction in the MC4R assay.

T. terrestris demonstrated a significant decrease in MC4R activation from control ($p \leq 0.05$).

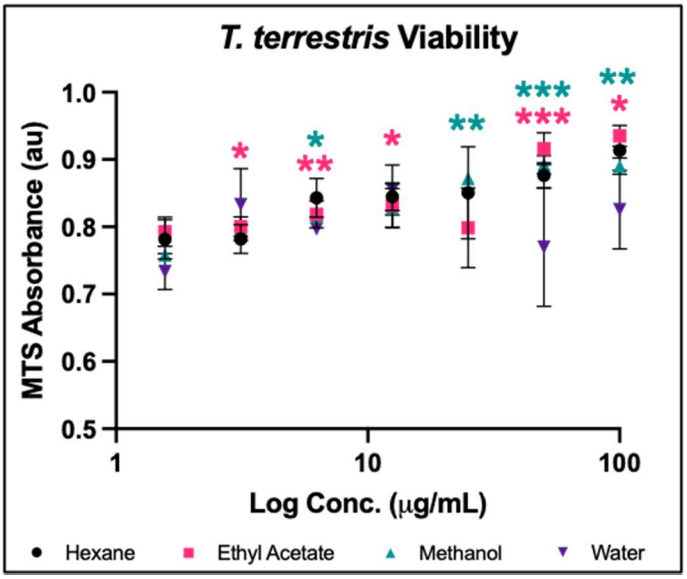

Figure 5a: Cytotoxicity of crude *T. terrestris* fractions in HEK293 Cells
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

T. terrestris demonstrated a significant increase in MTS absorbance for the EtOAc fraction at 3,6,12,50, and 100 µg/mL, as well as the MeOH fraction at 6,25, 50, and 100 µg/mL (n=3).

Table 5A: *Tribulus terrestris* Cytotoxicity ANOVA

	SS	DF	MS	F (DFn, DFd)	P value
Concentration x Solvent	0.2517	21	0.01199	F (21, 56) = 1.229	$p = 0.2649$
Concentration	0.1202	7	0.01718	F (1.163, 9.302) = 1.760	$p = 0.2196$
Solvent	0.005492	3	0.001831	F (3, 8) = 0.1038	$p = 0.9555$
***T. terrestris* Fraction**	0.1411	8	0.01764	F (8, 56) = 1.807	$p = 0.0949$
Residual	0.5464	56	0.009758		

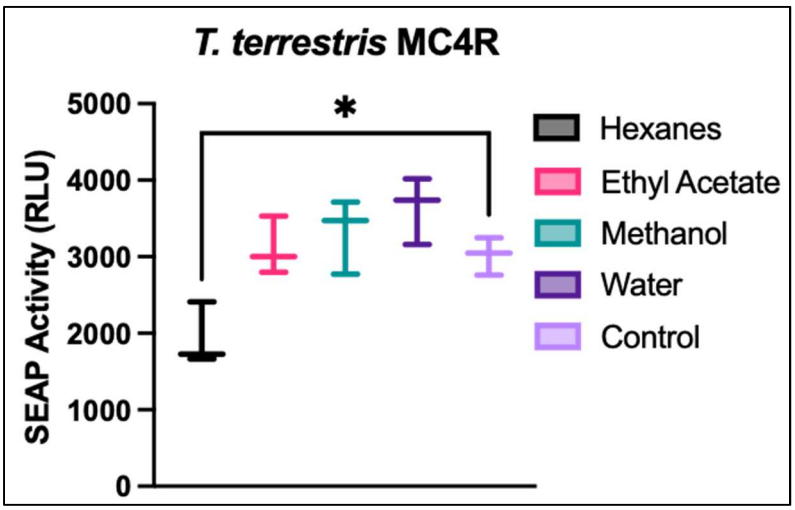

Figure 5b: Activation of MC4R *in vitro* by *T. terrestris*
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

T. terrestris demonstrated a significant decrease in activation for the hexanes fraction at 100 µg/mL (*n*=3)

Table 5B: *Tribulus terrestris* MC4R ANOVA					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	4978400	4	1244600	F (4, 10) = 7.717	*p* = 0.0042
Residual (within columns)	1612745	10	161274		
Total	6591145	14			

Trigonella foenum-graecum: The hexanes fraction did not demonstrate any significant increase or decrease in MTS absorbance compared to control. The ethyl acetate fraction demonstrated a significant increase in MTS absorbance over control for the following concentrations: 6 µg/mL ($p \leq 0.05$), 25 µg/mL ($p \leq 0.05$), 100 µg/mL ($p \leq 0.05$). The methanol fraction demonstrated a significant increase in MTS absorbance over control at 100 µg/mL ($p \leq 0.05$). The water fraction demonstrated a significant increase in MTS absorbance over control at 100 µg/mL ($p \leq 0.01$). Due to no observation of any significant decrease of MTS absorbance for each of our fractions we proceeded to the MC4R assay with 100 µg/mL for every fraction of *T. foenum-graecum*.

T. foenum-graecum demonstrated a significant increase in MC4R activation for the hexanes ($p \leq 0.001$) and ethyl acetate fraction ($p \leq 0.01$).

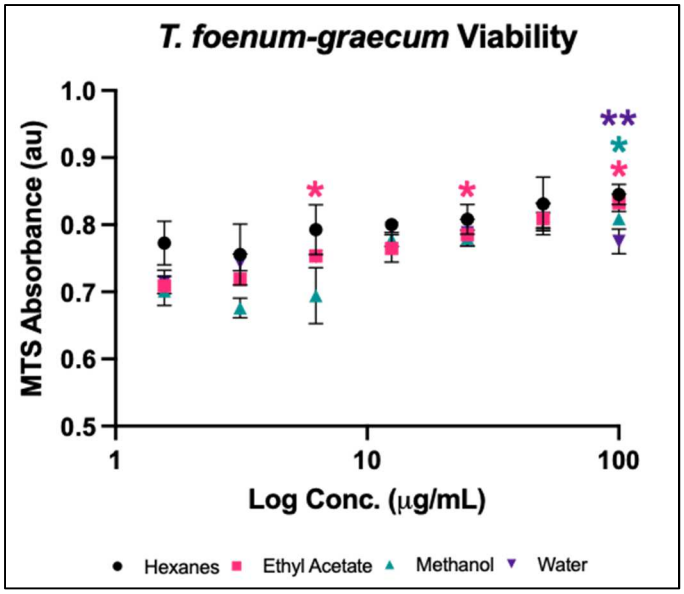

Figure 6a: Cytotoxicity of crude *T. foenum-graecum* fractions in HEK293 Cells
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

T. foenum-graecum demonstrated an increase in MTS absorbance from the EtOAc fraction at 6,25,100 µg/mL, as well as the MeOH and H_2O fractions at 100 µg/mL each

Table 6A: *Trigonella foenum-graecum* Cytotoxicity ANOVA

	SS	DF	MS	F (DFn, DFd)	P value
Concentration x Solvent	0.06811	21	0.003243	F (21, 56) = 0.9095	$p = 0.5806$
Concentration	0.1172	7	0.01674	F (1.152, 9.218) = 4.695	$p = 0.0540$
Solvent	0.06342	3	0.02114	F (3, 8) = 2.407	$p = 0.1426$
***T. foenum graecum* Fraction**	0.07025	8	0.008781	F (8, 56) = 2.462	$p = 0.0232$
Residual	0.1997	56	0.003566		

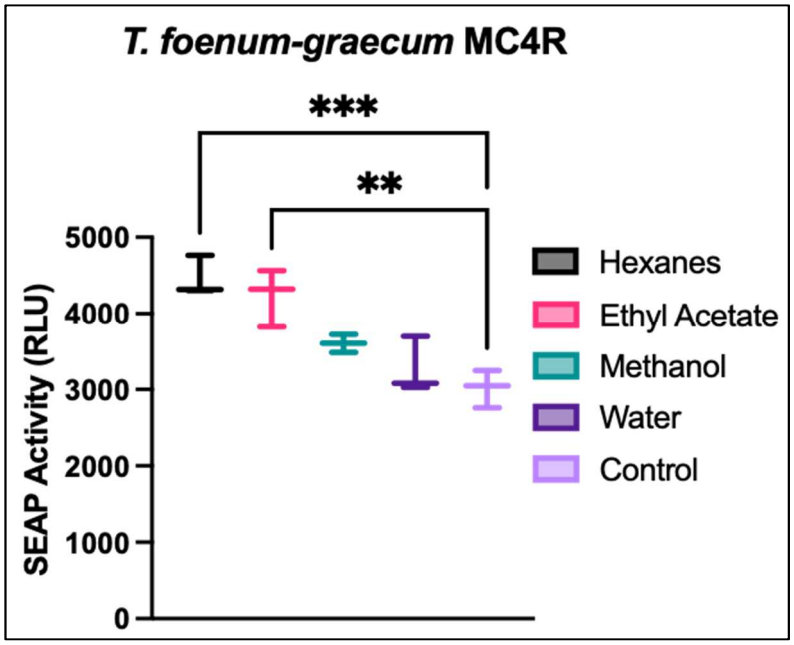

Figure 6b: Activation of MC4R *in vitro* by *T. foenum-graecum*
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

T. foenum-graecum demonstrated a significant increase in activation for the hexanes and EtOAc fraction at 100 µg/mL (*n*=3).

Table 6B: *Trigonella foenum-graecum* MC4R ANOVA					
	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	4584410	4	1146103	F (4, 10) = 13.52	*p* = 0.0005
Residual (within columns)	847416	10	84742		
Total	5431826	14			

Labisia pumila: The hexanes and ethyl acetate fractions did not demonstrate any significant increase or decrease in MTS absorbance over control for any of the concentrations tested. The methanol fraction demonstrated a significant decrease in MTS absorbance at the following concentrations: 25 µg/mL ($p \leq 0.05$), 50 µg/mL ($p \leq 0.05$), 100 µg/mL ($p \leq 0.05$). The water fraction demonstrated a significant decrease in MTS absorbance in comparison to control at 100 µg/mL ($p \leq 0.01$). Therefore, we proceeded at 100 µg/mL for the hexanes and ethyl acetate fractions, while dropping to 50 µg/mL for the water fraction and 12.5 µg/mL for the methanol fraction.

L. pumila demonstrated a significant increase in MC4R activation from the water fraction ($p \leq 0.05$), and a significant decrease in MC4R activation from the ethyl acetate ($p \leq 0.05$) and methanol fractions ($p \leq 0.05$).

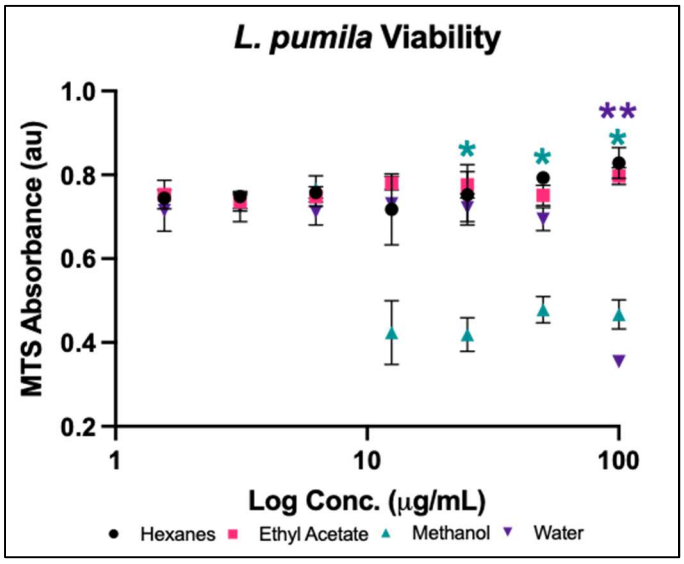

Figure 7a: Cytotoxicity of crude *L. pumila* fractions in HEK293 Cells
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

L. pumila demonstrated a significant decrease in MTS absorbance for the MeOH fraction at 25, 50, and 100 µg/mL, as well as a significant decrease in MTS absorbance for the H_2O fraction at 100 µg/mL (n=3).

Table 7A *Labisia pumila* Cytotoxicity ANOVA					
	SS	DF	MS	F (DFn, DFd)	P value
Concentration x Solvent	0.7154	21	0.03407	F (21, 56) = 30.94	$p < 0.0001$
Concentration	0.1975	7	0.02821	F (2.720, 21.76) = 25.63	$p < 0.0001$
Solvent	0.4669	3	0.1556	F (3, 8) = 57.59	$p < 0.0001$
***L. pumila* Fraction**	0.02162	8	0.002702	F (8, 56) = 2.455	$p = 0.0236$
Residual	0.06165	56	0.001101		

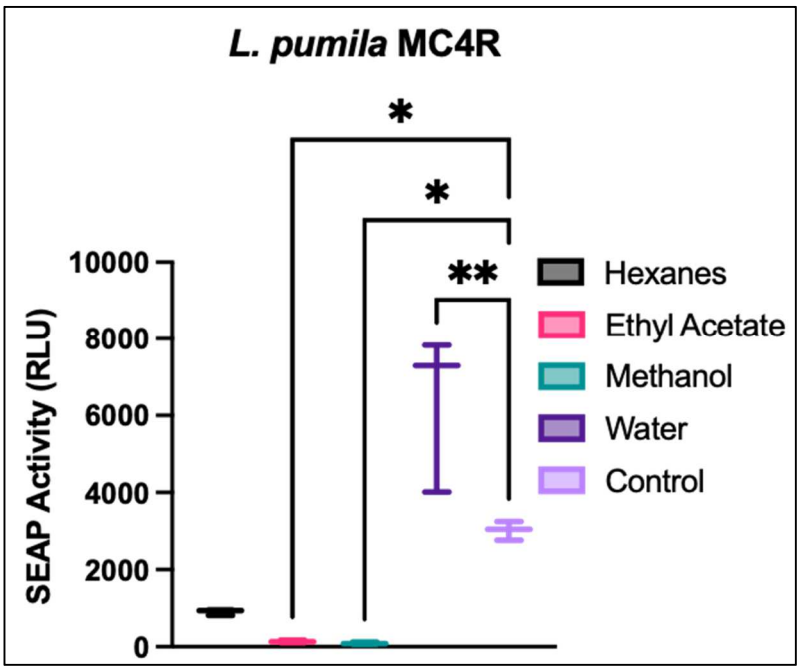

Figure 7b: Activation of MC4R *in vitro* by *L. pumila*
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

L. pumila demonstrated a significant increase in MC4R activation for the water fraction at 50 µg/mL, and a significant decrease in EtOAc (12.5 µg/mL) and MeOH (50 µg/mL) fractions (*n*=3).

Table 7B *Labisia pumila* MC4R ANOVA

	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	85565691	4	21391423	F (4, 10) = 24.66	$p < 0.0001$
Residual (within columns)	8673665	10	867366		
Total	94239356	14			

The significant activation of the *L. pumila* water fraction led us to further fractionate to narrow the scope of potential compounds causing the activity. The water fraction was further fractionated utilizing both a 1 g C-18 column, and 1 g Sephadex G-10 column in order to reduce compound column incompatibility. The C-18 column was eluted with 3 mL each of each system H_2O: acetonitrile (100%, 80%, 60%, 20%, 100%) for a total of 5 sub-fractions, due to yield two sub-fractions were selected W1, W3, numbered in order of their elution. The G10 column was eluted with 2 mL of H_2O per fraction for a total of five sub fractions, and again due to low yield, sub-fractions G1 and G3 were chosen to evaluate. We also wanted to reassess the initial water fraction to confirm previous findings of MC4R activation. The W1 ($p \leq 0.05$), W3 ($p \leq 0.0001$) and G3 ($p \leq 0.05$) sub-fractions demonstrated a significant decrease in MC4R activation. As demonstrated previously the crude water fraction demonstrated a significant increase in MC4R activation over control ($p \leq 0.0001$)

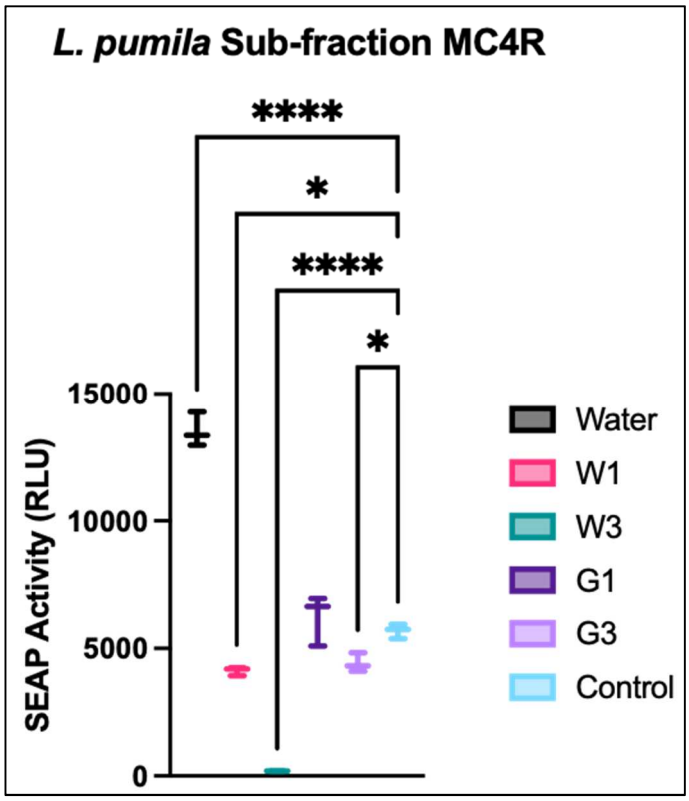

Figure 8: Activation of MC4R *in vitro* by *L. pumila* crude H_2O fraction and sub-fractions
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

L. pumila H_2O fraction demonstrated a significant increase in MC4R activation over control while fractions W1, W3, and G3 all demonstrated a significant decrease in activation.

Table 8*: L. pumila* Fraction and Sub-fraction MC4R ANOVA

	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	288716906	5	57743381	F (5, 12) = 203.6	$p < 0.0001$
Residual (within columns)	3402945	12	283579		
Total	292119852	17			

3.4 VALIDATION OF THE EXTRACTION AND ASSESSMENT OF CRUDE BOTANICAL APHRODISIACS IN VITRO

3.4.1 OVERVIEW: A challenge of botanical experimentation is the variability of plant material often obtained at different times. In attempt to reproduce and validate our findings in Chapter 3, we replicated the experiment from extraction to MC4R activation with vouchered specimens and included only the samples which had shown significant activity, as well as *C. johimbe* as justified previously. To this, we also added a fourth botanical in the form of a highly popular dietary supplement containing an encapsulated aqueous extract of *E. longifolia* roots, a species commonly known as Tongkat Ali and purported to have aphrodisiac ethnobotanical activity. The supplement had been standardized to 40% glycol-saponins, 28%eurypeptides, 30% polysaccharides, and eurycomanone.

3.4.2 METHODS:

TARGET BOTANICAL EXTRACTION: Voucher specimens of the three species of interest were extracted identically to the protocol outlined in section 3.3.

EURYCOMA LONGIFOLIA EXTRACTION**:** Ten pills of the dietary supplement, "NCNPR854PR", were taken apart weighing approximately 3.154 g, sample was then extracted three times with 50 mL HPLC grade H2O using a Velp Scientifica SER158 Solvent Auto extractor utilizing the same plant material at 100 °C. The fractions were then combined, frozen, and lyophilized prior to solubilizing for the experiment.

CELL CULTURING**:** HEK293 cells were cultured the same as in section 3.3

CELL CYTOTOXICITY: Following HEK 293 cell treatment with five concentrations of each respective extract, cell viability/cytotoxicity was quantified using the LIVE/DEAD® (Thermo Fisher Scientific) commercial kit assay. After treatment, cell media was aspirated followed by a gentle wash of 1X PBS prior to adding Calcein-AM (1:1000), and ethidium homodimer-1 (1:500) diluted in PBS. Cells were incubated in the dark at room temperature for thirty minutes prior to imaging on the Nikon Ti2-E microscope with automated image acquisition. Total cell counts and subsequent percent toxicity were calculated by NIS-Elements acquisition and analysis software (fig 9)

MC4R ASSAY: A Melanocortin-4 Receptor Reporter Assay Kit was purchase from Cayman Chemicals (Cayman Chem, Ann Arbor, MI, USA). Wells were seeded at a density of 50,000-100,000 cells/well

according to the manufacturer's instructions and incubated overnight. Media was aspirated and 100 µL of fresh serum free media added. Extracts were prepared as 5 mg/mL stock solutions in their respective solvents (*C. johimbe* and *L. pumila* in water, *T. foenum-graecum* in a 1:1:8 water; EtOH; kolliphor) and prepared at 2x the final concentrations to be assessed in serum free media. Untreated control wells received 100 µL of serum free media, positive control wells were treated with a 1:500 dilution of alpha-MS. Plates were read on a GloMax (Promega) plate reader with the specific luminescence program. Plates were briefly defrosted at room temperature prior to performing the SEAP Assay according to the manufacturer's instructions. As we assessed three timepoints, the instructional step where substrate is added to the plate was performed on each plate separately, then read, to ensure plates were incubated with substrate evenly for 2 minutes prior to reading on the luminescence program of a Glomax (Promega) plate reader.

MC4R INITIAL TIME CURVE: Cells were stimulated for a total of 48 hrs. with 15 µL aliquots taken out, snap frozen over dry ice, and stored at -80 °C prior at the following timepoints 6, 12, 24, 36, and 48 hrs. prior to completing the assay.

MC4R SUBSEQUENT FRACTION CONCENTRATION CURVE**:** Cells were stimulated for a total of 40 hrs. with 15 µL aliquots removed, snap frozen, and stored at -80 °C at 16, 24, and 40 hrs. prior to completing the SEAP portion of the assay.

STATISTICS: The MC4R fraction curve data was measured by a two-way ANOVA followed by Tukey's multiple comparisons test. For our initial time curve experiments; we calculated a 4 parameter least squares fit of treatment vs response.

3.4.3 RESULTS: Alpha-MSH: The positive control diluted at 1:500 of alpha MSH exhibited an exponential increase of SEAP activity over an initial period of 24 hrs. before plateauing the final two timepoints T_{50}=14.86 hrs. (fig 10). Vehicle control remained close to baseline throughout the course of the experiment slightly increasing from the 12 hr mark to 48 hrs.

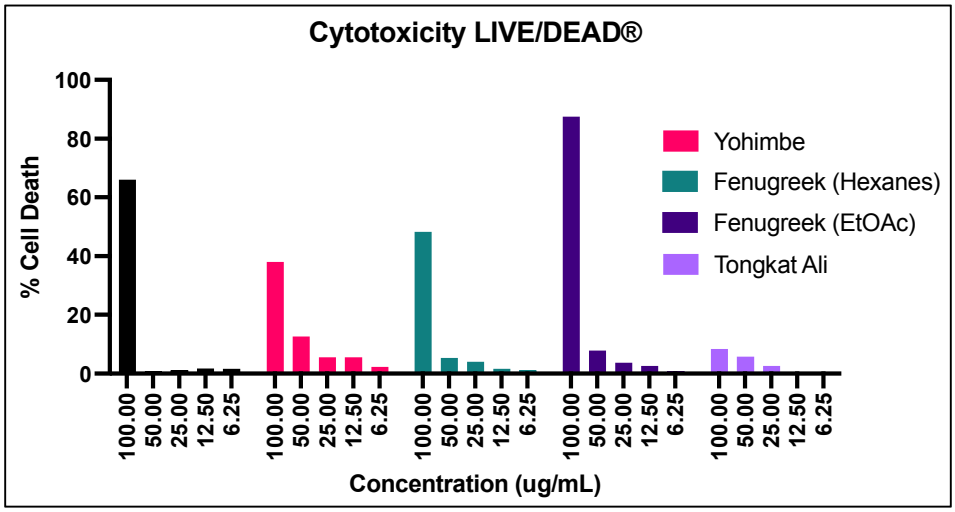

Figure 9. Cytotoxicity of Resolubilized Extracts in HEK293 Cells
Labisia (*L. pumila*) displays over 60% toxicity at 100 µg/mL with no significant effects on cell death at lower concentrations.
Yohimbe (*C. johimbe*) No cytotoxicity observed over 50% for any concentration tested.
Fenugreek hexanes (*T. foenum-graecum*)- No cytotoxicity observed over 50% for any concentration tested.
Fenugreek EtOAc (*T. foenum-graecum*)- Cytotoxicity over 50%. observed at 100 µg/mL
Tongkat Ali (*E. longifolia*) No cytotoxicity observed over 50% for any concentration tested.

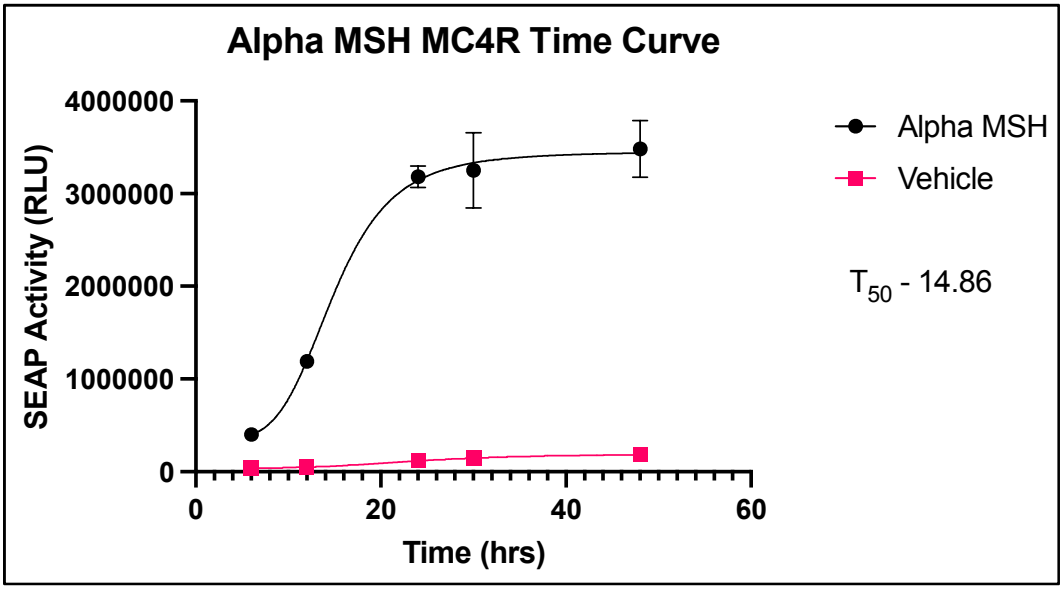

Figure 10. Time Curve Alpha-MSH over 48 hrs.
Alpha-MSH demonstrates clear SEAP activation over a 48 hr period, steadily increasing over the first 24 hrs. before plateauing in activation

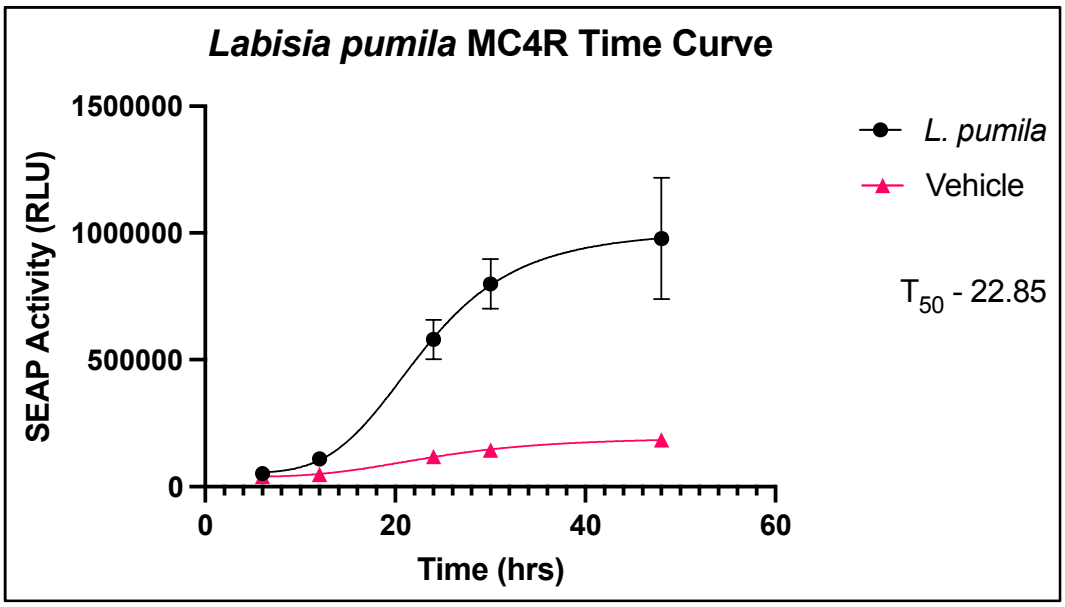

Figure 11. *L. pumila* MC4R Time Curve
L. pumila increases SEAP activity over 48 hrs.

Labisia pumila: The *L. pumila* water fraction demonstrated 66% cell death at 100 µg/mL compared to control; therefore, we proceeded with 50 µg/mL as was done in the previous experiment assessing melanocortin activation (fig. 9). The fraction exhibited a consistent increase in SEAP activity over the five timepoints seeming to plateau off in response after the 48 hr. timepoint T_{50}=22.85 hrs. Vehicle control increased marginally throughout the experiment appearing to plateau around 40 hrs. (fig 11). The concentration curve demonstrated significant decreases in SEAP activity compared to control for concentrations less than 50 µg/mL at 16 hrs., and less than 25 µg/mL at 24 hrs. and 40 hrs. Though demonstrating an increase over control in SEAP activity by the L. pumila fraction at 50 µg/mL at 24 hrs., the difference does not become significant until the 40 hr timepoint (fig 12).

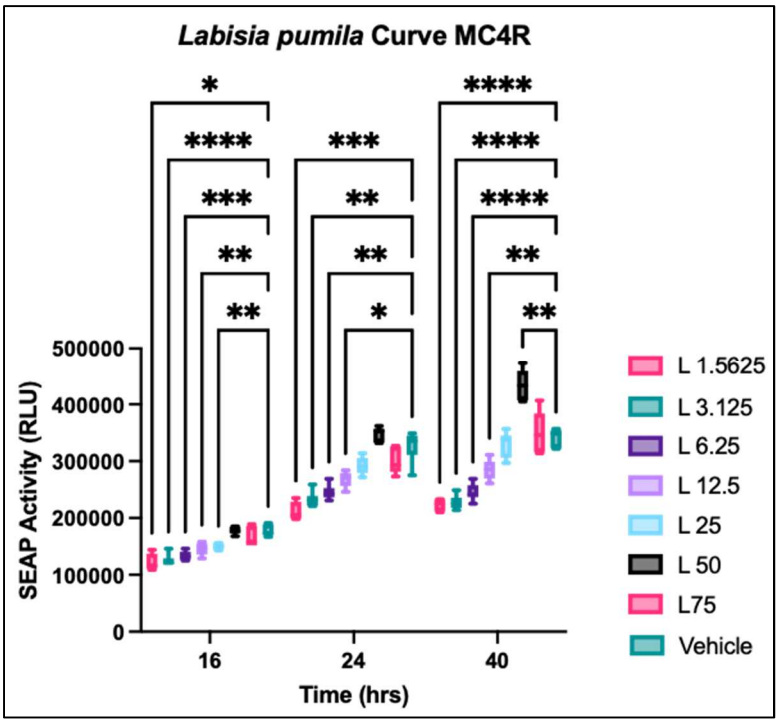

Figure 12. L. *pumila* MC4R Concentration Curve * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001

L. pumila demonstrates a concentration and time dependent increase of SEAP Activity. A significant increase is seen at 40 hrs. by the 50 µg/mL sample

Table 9: *Labisia pumila* Concentration Curve MC4R ANOVA					
	SS	DF	MS	F (DFn, DFd)	P value
Time x Concentration	58292640097	14	4163760007	F (14, 76) = 24.21	*p* < 0.0001
Time	622207513072	2	311103756536	F (1.939, 73.69) = 1809	*p* < 0.0001
Concentration	256944439614	7	36706348516	F (7, 38) = 65.77	*p* < 0.0001
Subject	21208111111	38	558108187	F (38, 76) = 3.245	*p* < 0.0001
Residual	13072722222	76	172009503		

Corynanthe johimbe: The *C. johimbe* water fraction demonstrated 38% cell death at 100 µg/mL in comparison to control; however, in order to mitigate any confounding data due to cell death, we proceeded with 50 µg/mL for this experiment (fig. 9). The fraction exhibited an exponential increase in SEAP activity over the five timepoints and appeared to reach a linear zone following the last timepoint with a T_{50}=28.03 hrs. Vehicle control increased marginally throughout the experiment appearing to plateau around 40 hrs. (fig 13). The concentration curve demonstrated a significant decrease in SEAP activity from 75 µg/mL, as well as less than 25 µg/mL at both 16 and 24 hrs. A significant increase in SEAP activity was seen from 50 µg/mL at the 40 hr timepoint (fig 14).

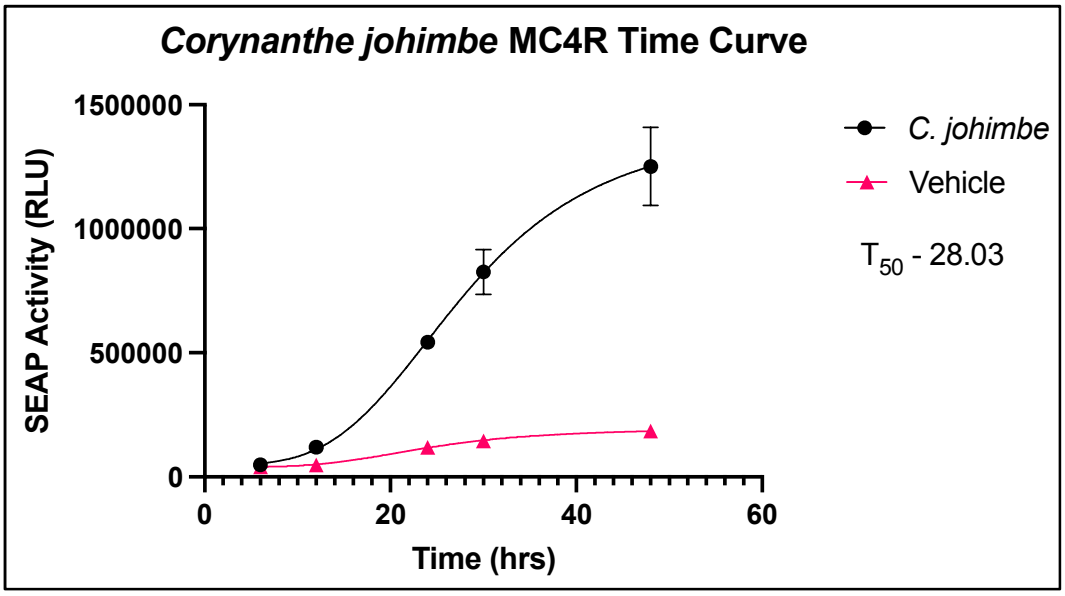

Figure 13. *C. johimbe* MC4R Time Curve
C. johimbe increases SEAP activity over a 48 hr period

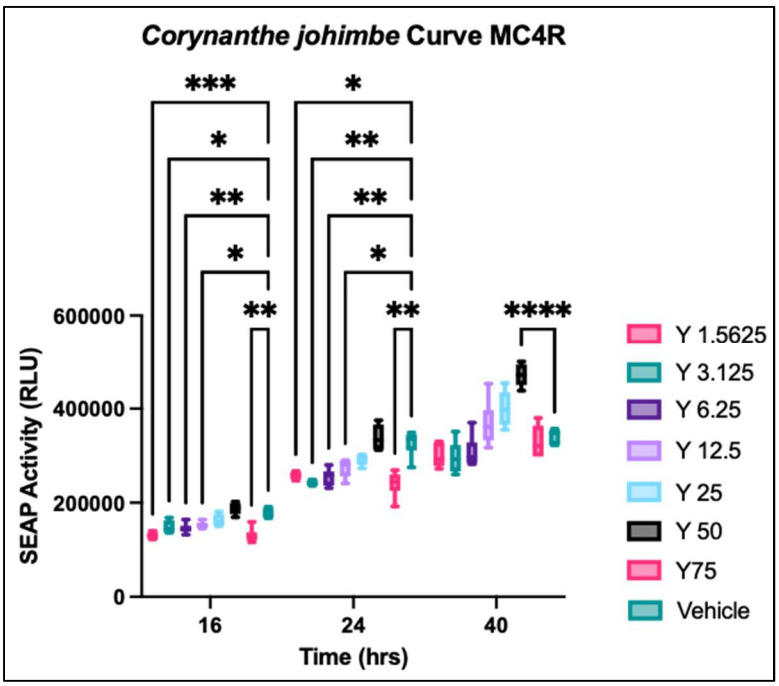

Figure 14. *C. johimbe* MC4R Concentration Curve
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

C. johimbe demonstrates a concentration and time dependent. Increase of SEAP activity with the exception of 75 µg/mL. Significant increase over SEAP activity is seen at 40 hrs. by the 50 µg/mL sample.

Table 10: *Corynanthe johimbe* Concentration Curve MC4R ANOVA

	SS	DF	MS	F (DFn, DFd)	P value
Time x Concentration	55876100236	14	3991150017	F (14, 78) = 9.159	$p < 0.0001$
Time	936487279675	2	468243639837	F (1.318, 51.40) = 1075	$p < 0.0001$
Concentration	169120757210	7	24160108173	F (7, 39) = 33.48	$p < 0.0001$
Subject	28146788889	39	721712536	F (39, 78) = 1.656	$p = 0.0297$
Residual	33990311111	78	435773219		

Trigonella foenum-graecum: The *T. foenum-graecum* hexane fraction demonstrated 48.25% cell death at 100 µg/mL; however, the significant difference in cytotoxicity between this concentration and 50 µg/mL enabled us to move forward testing the latter concentration (fig. 9). The *T. foenum-graecum* hexanes fraction started to demonstrate an exponential response in SEAP activity starting at 20 hrs., and then plateauing after 30 hrs. T_{50}= 23.24 hrs. On this smaller scale, the vehicle control could be seen increasing SEAP activity over time slightly faster than treatment and plateauing after the last measurement we observed (fig 15)

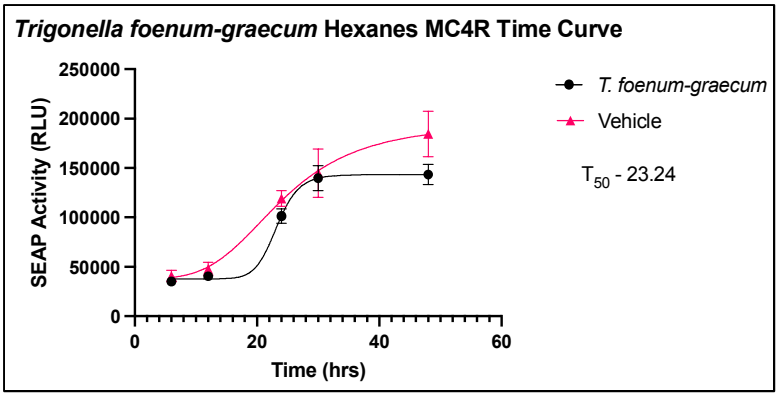

Figure 15. *T. foenum-graecum* hexanes MC4R Time Curve
T. foenum-graecum hexanes fraction has lower SEAP activation compared to vehicle.

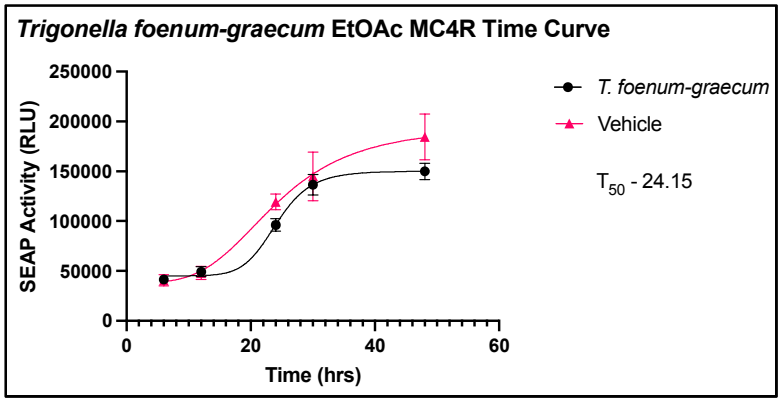

Figure 16. *T. foenum-graecum* EtOAc MC4R Time Curve
T. foenum-graecum EtOAc has lower SEAP activity than control

The *T. foenum-graecum* EtOAc fraction demonstrated 87.5% cell death at 100 µg/mL and similarly to the hexanes fraction had a significant decrease in cell death at 50 µg/mL; therefore, we proceeded with this

concentration (fig. 9). The EtOAc fraction as well as the vehicle control followed a similar pattern of SEAP activity increase over time T_{50}= 24.15 hrs., while the treatment plateaued in activity after 40 hrs., the vehicle marginally increased (fig 16)

Eurycoma longifolia: The *E. longifolia* fraction did not demonstrate a high percentage of cell death at 100 µg/mL (8.4%); however, we moved forward testing every extract at 50 µg/mL to retain consistent concentrations (fig. 9). The fraction demonstrated a consistent increase in SEAP activity over time with a half maximal activity calculated beyond the limits of our experiment T_{50}= 62.36. The vehicle control demonstrated a marginal increase in SEAP activity trending closer to baseline (fig 17). In the curve experiment the concentrations < 50 µg/mL demonstrated significant decreases in SEAP activity for all three timepoints with no increases in SEAP activity shown (fig 18)

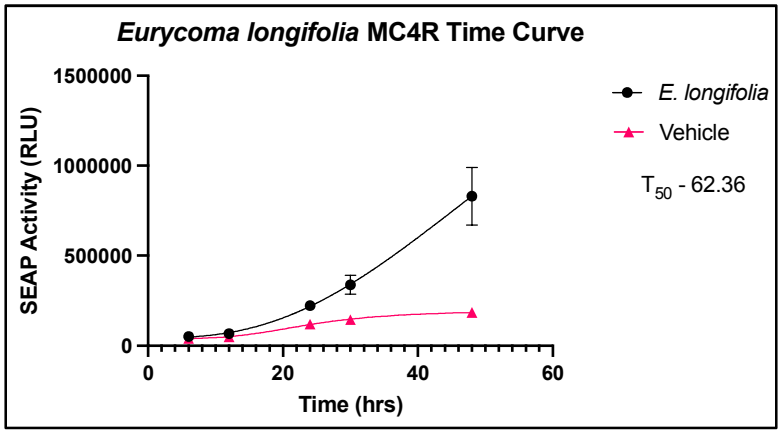

Figure 17. *E. longifolia* MC4R Time Curve
E. longifolia demonstrates increase in SEAP activity over 48 hrs.; however, not enough time was assessed to reach a half maximal effect.

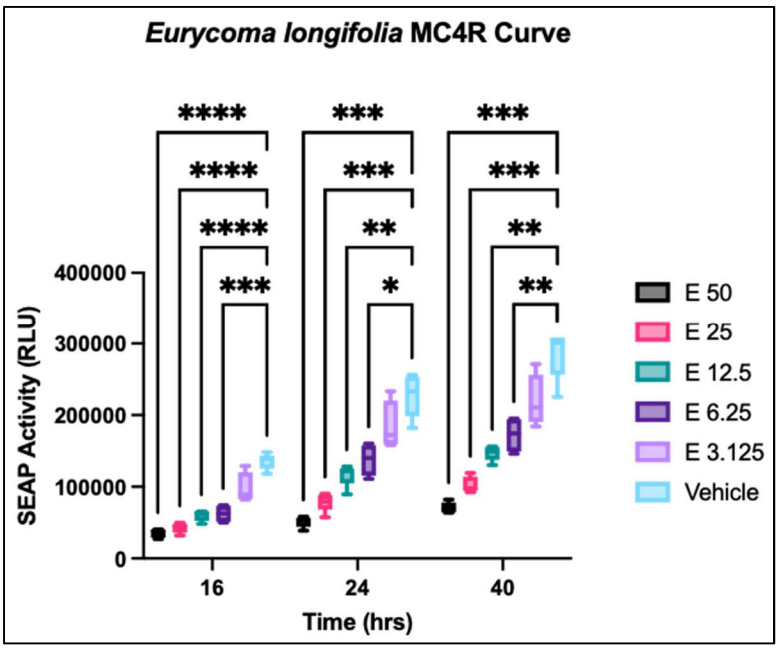

Figure 18. *E. longifolia* MC4R Concentration Curve
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

E. longifolia demonstrates a time dependent increase in SEAP activity. No significant activation over control is seen.

Table 11: *Eurycoma longifolia* MC4R Concentration Curve ANOVA					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Time x Concentration	23046150571	10	2304615057	F (10, 44) = 33.33	*p* < 0.0001
Time	126057288474	2	63028644237	F (1.593, 35.05) = 911.5	*p* < 0.0001
Concentration	266425883214	5	53285176643	F (5, 22) = 52.86	*p* < 0.0001
Subject	22175382500	22	1007971932	F (22, 44) = 14.58	*p* < 0.0001
Residual	3042383000	44	69145068		

3.5 IN VITRO CYTOTOXICITY OF SPECIALIZED METABOLITES AND THEIR SUBSEQUENT ABILITY TO ACTIVATE MC4R IN HEK293 CELLS

3.5.1 OVERVIEW:

Botanical species pose a unique challenge of being complex mixtures of specialized metabolites in which it is often difficult to determine a compound responsible for a particular activity observed. While bio-assay or bio-activity guided fractionation can lessen fraction complexity, it also can lead to a scarcity of sample to proceed with structural elucidation (Weller 2012). Furthermore, assays which have not been adapted to be high-throughput screenings are not feasible to proceed with in this manner. One of our target botanicals, *L. pumila* had previously undergone isolation efforts by the NCNPR yielding several triterpene saponins, and alkenated-phenolics (Ali, Khan 2011). A follow-up experiment quantitatively determined the concentration of these compounds in multiple *L. pumila* samples both commercial and voucher specimens, of which, our particular sample was included. Therefore, we proceeded to evaluate seven previously isolated compounds, Ardisicrenoside B (L1), Ardisiacrispin A (L2), Ardismamilloside H (L3), Irisresorcinol (L4), Belamcandol B (L5), Demethylbelamcandaquinone B (L6), and 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-αL-arabinopynanosyl cyclamiretin A (L7) (Ali 2011, Avula et al 2011). For our other target species, we decided to evaluate major compounds that were already well characterized. We tested three of the major alkaloids present in *C. johimbe* bark, yohimbine (Sigma, Y3125), rauwolscine (Cayman Chemical,15596), and ajmalicine (Cayman Chemical,31213) (Betz 2010; Raman et al. 2012). For *T. foenum-graecum* we evaluated a major alkaloid, trigonelline(Cayman Chemical, 11904), a major steroidal saponin, diosgenin which was previously isolated, and added the ubiquitous flavonoid quercetin (Sigma, Q4951) which is known to be a pan assay interference compound (Ali et al 2013, Bisson et al 2015). By evaluating major compounds present in our target botanicals we obtained commercially available pure samples, while future isolation efforts should eliminate the re-isolation of these known and potentially inactive compounds.

3.5.2 METHODS

SPECIALIZED METABOLITE ANALYSIS: Untargeted mass spectrometry analysis was carried out according to the methods detailed (Adaikpoh et al 2020). We submitted our crude extracts to LC-MS/MS

analysis which was performed on an Orbitrap Fusion Instrument (Thermo Scientific, San Jose, CA, United States) controlled with Xcalibur version 2.0.7 and coupled to a Dionex Ultimate 3000 nano UHPLC system. Samples were loaded onto a PepMap 100 C18 column (0.3 mm × 150 mm, 2 µm, Thermo Fisher Scientific). Separation of the samples was performed using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at a rate of 6 µL/min. The samples were eluted with a gradient consisting of 5–60% solvent B over 15 min, ramped to 95% B over 2 min, held for 3 min, and then returned to 5% B over 3 min and held for 8 min. All data were acquired in positive ion mode. Collision-induced dissociation (CID) was used to fragment molecules, with an isolation width of 3 *m/z* units. The spray voltage was set to 3600 V, and the temperature of the heated capillary was set to 300 °C. In CID mode, full MS scans were acquired from *m/z* 150 to 1200. The orbitrap resolution for both the MS1 and MS2 scans was 120,000. The expected mass accuracy was < 3 ppm. Generated data were converted to .mzMXL files using MS-Convert and mass data representative of our pure compounds were confirmed with MZmine3.0.21 software (Pluskal et al. 2010).

CELL CULTURING: Cells were cultivated as described previously in section 3.3

CYTOTOXICITY: Treatments were assessed for cytotoxicity in cells as described in section 3.3.1 while percent toxicity was assessed visually after staining with concentrations showing over 50% cell death representing the MTC. *T. foenum-graecum* and *C. johimbe* compounds were tested at 10uM, and 1uM, while *L. pumila* isolated compounds were tested in a serial dilution from 100 µg/mL to 0.78126 µg/mL.

MC4R ASSAY: The MC4R assay was carried out as described in section 3.3.1 with the following changes to this specific experiment. *T. foenum-graecum* compounds were solubilized in EtOH, while *C. johimbe* compounds were solubilized in DMSO at a 10mM stock solution before being prepared at 2X the final concentration to be used as treatments in wells. *L. pumila* isolated compounds were solubilized in DMSO and prepared in a 5mg/mL stock solution prior to being prepared at 2X the final treatment concentration.

STATISTICS: The MC4R pure and isolated compound significance was measured by a two-way ANOVA followed by Tukey's multiple comparisons test.

3.5.3 RESULTS:

Table 12: Cytotoxicity of Botanical Isolates in HEK293 Cells		
Sample	**Concentration tested**	**Toxicity (>50% cell death) Observed above**
L0- Labisia water crude	0.78125- 100 µg/mL	50 µg/mL
L1- Ardisicrenoside B	0.78125- 100 µg/mL	25 µg/mL
L2- Ardisiacrispin A	0.78125- 100 µg/mL	0.78125 µg/mL
L3- Ardisimamilloside H	0.78125- 100 µg/mL	12.5 µg/mL
L4- Irisresorcinol	0.78125- 100 µg/mL	12.5 µg/mL
L5- Belamcandol B	0.78125- 100 µg/mL	50 µg/mL
L6- Demethylbelamcandaquinone B	0.78125- 100 µg/mL	50 µg/mL
L7- 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-αL-arabinopynanosyl cyclamiretin A	0.78125- 100 µg/mL	All concentrations tested were cytotoxic
F0- Fenugreek hexanes crude	0.78125- 100 µg/mL	No toxicity observed
F1- Fenugreek EtOAc crude	0.78125- 100 µg/mL	50 µg/mL
F2- Diosgenin	1mM, 10mM	No toxicity observed
F3- Quercetin	1mM, 10mM	No toxicity observed
F4- Trigonelline	1mM, 10mM	No toxicity observed
Y0- Yohimbe water crude	0.78125- 100 µg/mL	No toxicity observed
Y1- Yohimbine	1mM, 10mM	No toxicity observed
Y2- Rauwolscine	1mM, 10mM	No toxicity observed
Y3- Ajmalicine	1mM, 10mM	No toxicity observed

Corynanthe johimbe: No toxicity was observed for yohimbine, rauwolscine, ajmalicine or crude water fraction (Table 12). None of the pure major alkaloids, or the crude water fraction demonstrated an increase in SEAP activity over control at any of the timepoints. We did observe a significant decrease in SEAP activity compared to control from both rauwolscine and the crude fraction tested at 75 µg/mL at 24 hrs. (fig 19).

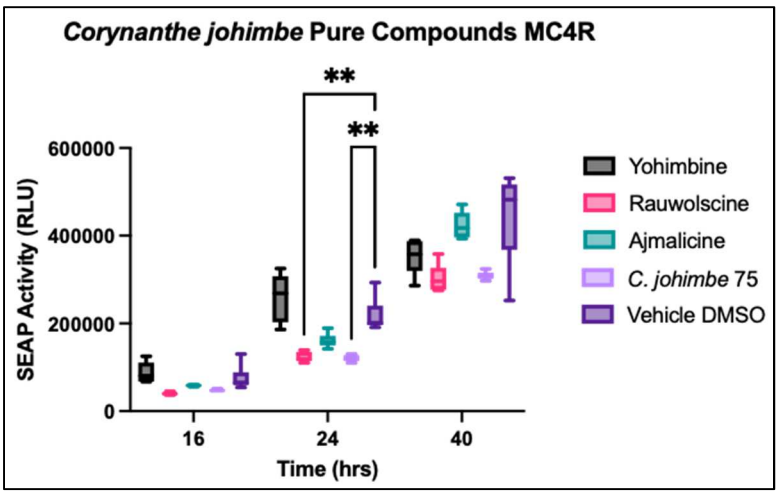

Figure 19. *C. johimbe* Pure Compounds MC4R * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001
Time dependent increase in SEAP Activity shown. Decrease in activation at 24 hrs. from rauwolscine and 75 µg/mL crude fraction.

Table 13: *C.johimbe* Pure Compounds MC4R ANOVA					
	SS	DF	MS	F (DFn, DFd)	P value
Time x Compound	73180813333	8	9147601667	F (8, 50) = 5.514	$p < 0.0001$
Time	1418188022222	2	709094011111	F (1.116, 27.90) = 427.4	$p < 0.0001$
Compound	127276206667	4	31819051667	F (4, 25) = 35.00	$p < 0.0001$
Subject	22730402222	25	909216089	F (25, 50) = 0.5480	$p = 0.9474$
Residual	82955471111	50	1659109422		

Labisia pumila: Previously isolated compounds from L. pumila were tested for their cytotoxicity. ardisicrenoside B demonstrated toxicity above 50 µg/mL, ardisiacrispin A above .78125 µg/mL, ardismamilloside H 12.5 µg/mL, irisresorcinol above 12.5 µg/mL, belamcandol B above 50 µg/mL, demethylbelamcandaquinone above 50 µg/mL, and the sugar compound was toxic at all concentrations assessed. We did not proceed to evaluate MC4R activation with compounds ardisiacrispin A or 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-αL-arabinopynanosyl cyclamiretin A due to toxicity concerns. Neither isolated compounds or the crude water fraction retested at 75 µg/mL demonstrated any significant increase in SEAP activity over control levels; however, we did witness a decrease in SEAP activity from compounds L3, and L5 at 24 hrs., and L3 again at the 40-hour timepoint.

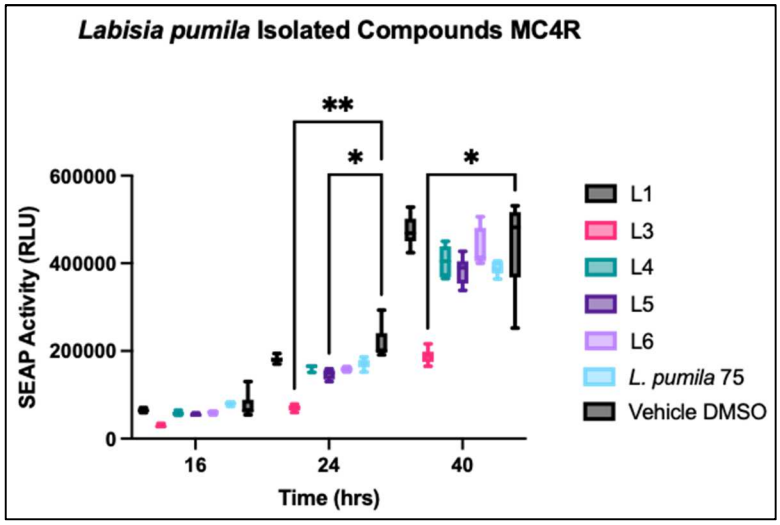

Figure 20. *L. pumila* Isolated Compounds MC4R
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**
Time dependent increases in SEAP activity. Decrease in activation at 24 and 40 hrs. by L4, and 24 hrs. by L6.

Table 14: *L. pumila* Isolated Compounds MC4R ANOVA					
	SS	DF	MS	F (DFn, DFd)	P value
Time x Compound	128926054444	12	10743837870	F (12, 70) = 9.915	$p < 0.0001$
Time	2383989107778	2	1191994553889	F (1.028, 35.97) = 1100	$p < 0.0001$
Compound	271246353175	6	45207725529	F (6, 35) = 74.84	$p < 0.0001$
Subject	21143233889	35	604092397	F (35, 70) = 0.5575	$p = 0.9700$
Residual	75853584444	70	1083622635		

Trigonella foenum-graecum: Pure compounds trigonelline, quercetin, and diosgenin were assessed for cytotoxicity in HEK293 cells at both 10 mM and, 1 mM with no toxicity observed at either concentration. No significant increases in SEAP activity were demonstrated in the MC4R assay; however, a significant decrease in SEAP activity was demonstrated by quercetin in respect to both vehicle control in DMSO and vehicle control in EtOH.

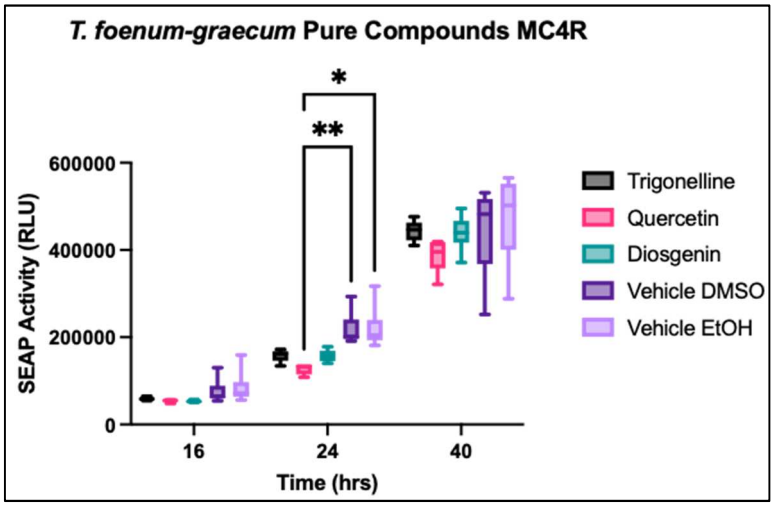

Figure 21. *T. foenum graecum* Pure Compounds MC4R
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

Time dependent increases in SEAP Activity by pure compounds. Decrease in activation at 24 hrs. by quercetin.

Table 15: *T. foenum-graecum* Pure Compounds MC4R ANOVA					
	SS	DF	MS	F (DFn, DFd)	P value
Time x Compound	15665259111	8	1958157389	F (8, 50) = 0.7368	$p = 0.6586$
Time	2194168768667	2	1097084384333	F (1.021, 25.52) = 412.8	$p < 0.0001$
Compound	52886176222	4	13221544056	F (4, 25) = 12.95	$p < 0.0001$
Subject	25517599444	25	1020703978	F (25, 50) = 0.3841	$p = 0.9943$
Residual	132873565556	50	2657471311		

3.6 CRUDE EXTRACTS ABILITY TO ACTIVATE MC3R

3.6.1 OVERVIEW: Our extracts have now demonstrated the ability to activate the MC4R in vitro. Known agonists of the MC4R such as alpha-MSH, and Bremelanotide which affect sexual functioning, also have activity to a lesser extent at the MC3R. Of the melanocortin family, the MC3R and MC4R share the highest homology and are both present in the CNS; therefore, in this experiment we aimed to determine whether our fractions could similarly activate the MC3R *in vitro*.

3.6.2 METHODS:

CELL CULTURING: Cells were cultivated as described previously in section 3.3

CYTOTOXICITY: Toxicity was assessed previously in HEK293 cells as detailed in section 3.3.1

MC3R ASSAY: A Melanocortin-3 Receptor Reporter Assay Kit was purchased (Cayman Chemicals, 600180). The assay kit was carried out identically to the MC4R kit experimented with previously.

STATISTICS: The MC3R curve significance was measured by a two-way ANOVA followed by Tukey's multiple comparisons test.

3.6.3 RESULTS:

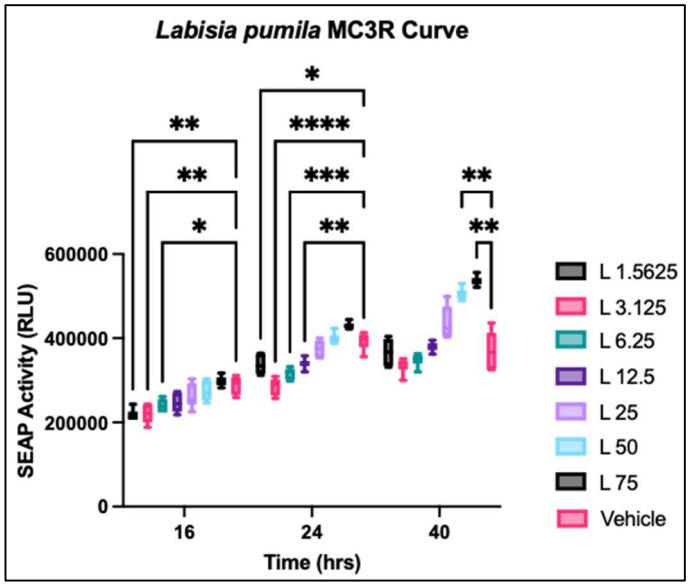

Figure 22. *L. pumila* MC3R Concentration Curve
*** = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001**

Concentration and time dependent increases of SEAP activity. Decrease of activation at 16 hrs. lower than 6.25 µg/mL, 24 hrs. lower than 12.5 µg/mL. Increase of activation at both 50 and. 75 µg/mL at 40 hrs.

Labisia pumila: At 16 hrs., concentrations of *L. pumila* including and below 6.25 µg/mL demonstrated a significant decrease in SEAP activity over vehicle. This trend continued to 24 hrs. and also included 12.5 µg/mL. At 40 hrs. both the 50 µg/mL and 75 µg/mL concentrations demonstrated a significant increase over vehicle in SEAP activity.

Table 16: *Labisia pumila* MC3R Concentration Curve ANOVA

	SS	DF	MS	F (DFn, DFd)	P value
Time x Concentration	79212500000	14	5658035714	F (14, 80) = 19.28	$p < 0.0001$
Time	596533388889	2	298266694444	F (1.337, 53.46) = 1016	$p < 0.0001$
Concentration	293724437500	7	41960633929	F (7, 40) = 50.07	$p < 0.0001$
Subject	33519055556	40	837976389	F (40, 80) = 2.855	$p < 0.0001$
Residual	23478111111	80	293476389		

Corynanthe johimbe: C. johimbe at 1.5625 µg/mL significant decreased SEAP activity in comparison to vehicle at both 16 and 24 hrs. At the 24 hr timepoint we see a significant increase in SEAP activity from the concentration 50 µg/mL which continues and shows a higher significance at the 40 hr mark where we also see the concentration 25 µg/mL demonstrate significant SEAP activity over vehicle.

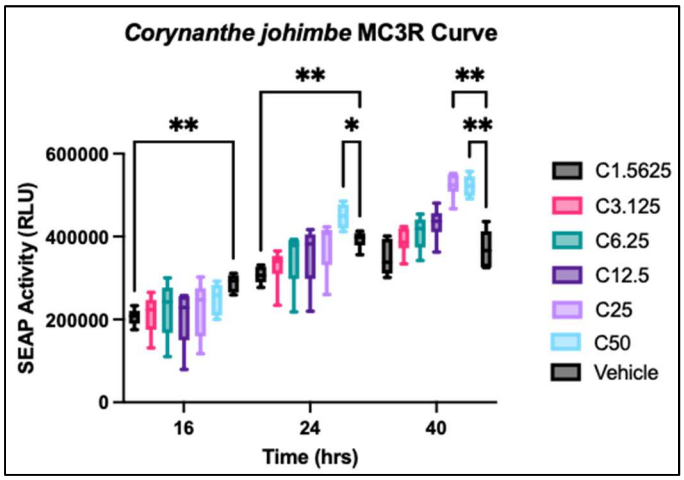

Figure 23. *C. johimbe* MC3R Concentration Curve
Time and concentration dependent increase of SEAP Activity. Decrease of SEAP activity at 1.56 µg/mL for. 16, 24 hrs. Increase of SEAP Activity at 25 and 50 µg/mL

Table 17: *Corynanthe johimbe* MC3R Concentration Curve ANOVA

	SS	DF	MS	F (DFn, DFd)	P value
Time x Concentration	108586806098	12	9048900508	F (12, 68) = 23.00	$p < 0.0001$
Time	834908176080	2	417454088040	F (1.558, 52.96) = 1061	$p < 0.0001$
Concentration	167837668496	6	27972944749	F (6, 34) = 4.729	$p = 0.0013$
Subject	201104058333	34	5914825245	F (34, 68) = 15.03	$p < 0.0001$
Residual	26755550000	68	393463971		

Eurycoma longifolia: *E. longifolia* concentrations all demonstrated a significant decrease in SEAP activity compared to control at the 16 hr timepoint which continues through the 24 hr. timepoint as well as the 40 hr. timepoint with the exception of 6.25 µg/mL.

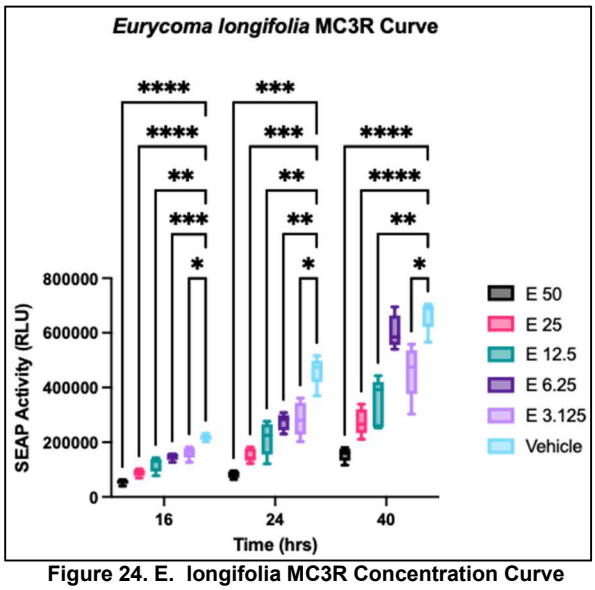

Figure 24. E. longifolia MC3R Concentration Curve

Time and concentration dependent increase of SEAP Activity. Decrease of activation at 16and 24 hrs. by all concentrations, which continues to 40 hrs. with the exception of 6.25 µg/mL

Table 18: *Eurycoma longifolia* MC3R Concentration Curve ANOVA

	SS	DF	MS	F (DFn, DFd)	P value
Time x Concentration	268736740444	10	26873674044	F (10, 48) = 34.60	$p < 0.0001$
Time	1183924246222	2	591962123111	F (1.270, 30.49) = 762.1	$p < 0.0001$
Concentration	1123330532889	5	224666106578	F (5, 24) = 47.91	$p < 0.0001$
Subject	112533280000	24	4688886667	F (24, 48) = 6.036	$p < 0.0001$
Residual	37284860000	48	776767917		

3.7 DISCUSSION CHAPTER 3:

Female Sexual Dysfunctions, namely libido desire disorders, are highly prevalent, underreported, and are a significant detriment to the quality of life of females affected (Clayton Kingsberg et al 2018) Currently, there are two FDA-approved drugs on the market for the most common form of FSD, HSDD, a now outdated diagnosis replaced by FSIAD (AmericanPsychiatricAssociation 2013). However, neither of these medications have been able to revolutionize female sexual functioning in the way that Viagra changed the landscape for male sexual dysfunctions. A clear mechanism through which females can increase arousal and desire has not been fully elucidated due to the biopsychosocial complexities of this diagnosis which

has largely been an excuse for the lack of progression. We believe that the identification of a mechanism of action for traditional botanical aphrodisiacs will progress the understanding of the machinery behind arousal and desire while eventually facilitating the development of novel therapies that may improve the gap in treatment availability for women.

In our first study, we assessed twenty crude botanical fractions for activation of the MC4R receptor to identify those whose reported ethnobotanical aphrodisiac activity could be potentially explained through this mechanism of action. The MC4R has been implicated in sexual functioning specifically desire by proceptivity behavioral responses of female mice studies, the approval of Bremelanotide which was previously discussed in chapter 1.2.5. No previous studies have assessed aphrodisiac botanicals for their activity at this receptor. Of the twenty crude fractions assessed for activation, three demonstrated significant activation over control including the *T. foenum-graecum* hexanes and ethyl acetate fractions, as well as the *L. pumila* water fraction. As the *L. pumila* water fraction returned a more robust SEAP activity response, we proceeded to sub-fractionate in order to narrow the range of potential specialized metabolites responsible for the activity. However, as is common in chasing activity in botanical extracts, we ended up losing the activity we previously saw.

As a preliminary screening tool, the sample size of our initial screening was insufficient to decrease the likelihood of misleading statistics of which we based our conclusions. While the finding of our sub-fractionation experiment may implicate a hypothetical additive, or synergistic effect amongst metabolites, to pharmacologically reproduce such effects in crude extracts with numerous metabolites at their individual concentrations is not possible. Though we could recombine our sub-fractionated material to test this hypothesis, again, it would be increasingly difficult to solubilize them in a way that would be representative of the initial extract. In regards to the original evaluation of the *L. pumila* hexanes and ethyl acetate fractions at MC4R, the significant decrease in MC4R activation was due to cell toxicity that had not been previously accounted for in the MTS assay screening step. Furthermore, when we proceeded to sub-fraction the *L. pumila* water fraction, we did not retest individual sub-fractions for toxicity which resulted very clearly in toxicity issues of the W3 sub-fraction, but could've potentially affected the others as well.

For future experiments we have considered the additional testing of the water fraction of *C. johimbe* as it did increase SEAP activity (p=.0790). By increasing the sample size for the three fractions which

produced significant activity, as well as adding the *C. johimbe* fraction, we will increase our power and precision and be able to draw much sounder conclusions from our data. For our cytotoxicity data, the MTS assay in particular cannot directly give information on cytotoxicity, as the reduction of the reagent tetrazolium to generate a colored formazan dye can be due to cell proliferation as well as increased metabolism. While the majority of our extracts did produce clear concentration responses for those that reduced dye formation compared to the control wells, we did observe toxicity past the initial screen. In order to directly assess cell death future experiments, we utilized the commercially available LIVE/DEAD assay kit.

In our second study we increased the sample size assessed, and redid toxicity data with the more specific and reliable assay suggested. In general, botanical experiments in the literature are varied by sample preparation differences. We decided to replicate our initial extraction procedure of our active botanical species prior to retesting them for activity at the MC4R to ensure no false positives were seen. Whereas our first experiment focused on a single concentration of the fractions and one timepoint, we assessed a time curve of our MTC over 48 hrs. in order to determine three timepoints that would be most representative of MC4R activation over time according to the positive control alpha-MSH. We then proceeded with a concentration curve of the four fractions which produced significant activation over vehicle control, as well as a new species *E. longifolia.*

We observed that previously active *T. foenum-graecum* fractions did not replicate prior results over the first-time curve experiment; therefore, we did not proceed with a concentration curve of these fractions. The discrepancy in results may have been due to the fact that fractions were initially solubilized in DMSO, and subsequently solubilized in a 1:1:8 ratio of water: EtOH: Kolliphor. This indicates a need for complete solubility of individual fractions as well as a more robust sample size to be assessed in preliminary screenings of botanical extracts to account for variability and increased power in analysis of results. The hexanes fraction should've pulled out primarily non-polar metabolites, while the ethyl acetate would've extracted moderately polar metabolites, fortunately DMSO has the unique ability of generally being able to dissolve both polarities; however, predicting which compounds will be soluble is something that even computer modeling has had difficulty with (Balakin 2006). We believe it would be worthwhile to pursue

spectroscopic analysis of the two soluble fractions in order to determine metabolite discrepancies which could illuminate why previous experiments demonstrated activation aside from inadequate sample size.

We confirmed our preliminary findings that *L. pumila* activated the MC4R in a concentration and time dependent manner with optimal significant activation occurring from 50 µg/mL at 40 hrs. The inclusion of *C. johimbe* to reassess proved to be successful with a concentration and time dependent activation of MC4R occurring optimally at 50 µg/mL at 40 hrs. The time curve of *C. johimbe* suggested that if we had assessed MC4R activation further optimal activation may occur later than the 40hr timepoint. The addition of *E. longifolia* similarly demonstrated a concentration and time dependent activation of MC4R; however, we did not observe any significance over vehicle control. From the time course experiment we can clearly see that activation of MC4R by this fraction did not even reach its half maximal effective concentration which would've occurred past hour 60.

The time curve experiment for each fraction determined the amount of time we measured SEAP-activity *in vitro* which was capped at 40 hrs. due to full saturation by the positive control*;* furthermore, we were limited by our assay which did not have parameters for increasing the amount of time to reflect delays in activation such as seen in *E. longifolia.* Future studies should aim to include timepoints which overlap with the EC_{50} of target botanical species which should give a more accurate representation of the activation.

As a follow-up study we proceeded with assessing the major specialized metabolites of the respective hits for their potential to activate the MC4R receptor in order to determine whether an individual compound could be held responsible for the activity seen. As stated previously, a major limitation in proceeding with botanicals in the framework of developing a therapeutic is the complexity of the mixture. Currently, a structure does exist for developing what is called a Botanical Drug Substance (BDS), and to date two products exist and have been approved for marketing as prescription drugs, while others are included in over the counter drug reviews (Wu et al. 2020). The individual compounds through which these botanical species exert their effects of activating the MC4R remain unclear. Identification of the specific bioactive metabolites which activate MC4R will facilitate the advancement of therapeutic molecular scaffolds which can target this receptor while explaining how these botanicals work mechanistically.

We continued with an approach focusing on previously isolated and characterized major specialized metabolites of our hits which we had confirmed presence of via LC-MS/MS, see Appendix for

chromatogram data, as well as thorough previous work by Avula et al. utilizing LC-UV/ELSD with confirmation by LC-ESI-TOF. As in prior experiments, we evaluated these compounds for their cytotoxicity in HEK293 cells before assessing them at the MTC for activation of the MC4R in our commercial kit. We did not identify any isolated or pure compounds that were able to demonstrate a significant activation of the MC4R over vehicle control, though we did observe across all compounds a noticeable time dependent activation of the receptor in which the SEAP activity trended to increase over time. It is possible that the concentrations of these compounds were not sufficient enough to cause activation of the receptor.

Our results demonstrated that none of the major compounds from these three species are activating MC4R through the cAMP pathway as assessed by our assay while it is clear that there is/are a bioactive metabolite (s) which does work through this pathway. We cannot rule out that these compounds may activate MC4R through another mechanism as previous studies have indicated the endogenous agonist activates three signal pathways, cAMP which our kit directly assesses, as well as calcium and mitogen-activated protein kinase pathways (Shinyama et al 2003). Hydrophilic specialized metabolites are notoriously difficult to isolate and it may be a minor compound or a combination of minor compounds acting to produce this effect. There is also concern if the metabolite is in small quantities in the extract of being able to procure enough plant material in order to carry out a full isolation and structure elucidation.

Our final study aimed to assess whether our active fractions could similarly activate the MC3R, the only other melanocortin receptor which is predominately expressed in the brain, and to a lesser extent shown to be involved in sexual functioning through response to internal or external environmental challenges less than internal homeostasis. It has been shown that agonists which activate the MC4R also activate the MC3R including the endogenous alpha-MSH, as well as developed synthetic compounds like Bremelanotide.

For this study we again utilized a commercial MC3R kit and transfected HEK cells and measured SEAP activity which correlates to receptor activation *in vitro*. Similar to previous observations of interactions with this receptor, we observed a time dependent increase in MC3R activation. The *L. pumila* which activated the MC4R at 50 µg/mL was likewise able to significantly activate the MC3R at this concentration as well as 75 µg/mL at the 40hr timepoint; *C. johimbe* which also activated the MC4R at 50 µg/mL was able to significantly activate the MC3R at the same concentration and timepoint. *E. longifolia* on the other hand

did not demonstrate a significant activation in either receptor. We believe that activation of either receptor by *E. longifolia* may potentially take longer due to T_{50} being at a much further timepoint than was measured in our experiment.

Overall, we were able to reproduce much of our preliminary data with an increased sample size which also allowed us to exclude confounded fractions in secondary experiments. As the first project to assess botanical aphrodisiacs for activity at both the MC4R and MC3R we have provided preliminary evidence for a potential mechanism behind their reported ethnobotanical use.

In regard to sustainability of these species we are assessing, great concern exists for deforestation of growth habitats and improper harvesting of material in the wild (Tchoundieu et al. 2004). *C. johimbe* has also been plagued by the commercialization of the alkaloid yohimbine which has led to a decreased interest of exploring new phytochemical entities outside of the alkaloidal fractions. Efforts to increase commercial cultivation of *L. pumila* has been extensively undertaken in Malaysia where the plant is native (Ariff, Hamzah 2014). Although individual compounds of *E. longifolia* were not assessed in this section, this species as well is at risk with conservation efforts undertaken since the 1990's (Hussein 2012, Lee 2004). We would caution against obtaining bulk commercial material and advocate eco-consumerism of these delicate species. Traditional grind and find techniques would devastate these plant populations, therefore, moving forward with targeted isolation and dereplication steps at a small scale first is imperative.

CHAPTER 4 ABILITY OF MC4R ACTIVATING APHRODISIAC EXTRACTS TO STIMULATE THE RELEASE OF GNRH IN AN IN VITRO GT1-7 MOUSE HYPOTHALAMUC NEURONAL CELL LINE

4.1 OVERVIEW

GT1-7 cells are a mature hypothalamic neuronal cell line which secrete gonadotrophin releasing hormone (GnRH) upon depolarization in a pulsatile manner while also exhibiting high levels of GnRH1 mRNA (Wetsel 1991). These cells exhibit circadian rhythms which are controlled by particular clock genes that follow a twenty-four-hour cycle. These daily changes of clock gene expression have been shown to affect the coordination of cells to produce GnRH pulsatility. However, the rhythm of these genes could be reset with a serum shock of 50% FBS prior to treatment. (Chappell et al 2003). *In vivo*, this corresponds to neuronal signaling from the suprachiasmatic nucleus to neurosecretory cells in the brain responsible for daily hormone secretion (Zhao and Kriesfeld 2009). Interestingly these cells encode the gene for the MC4R (Mayer et al. 2009), and it has been demonstrated that a functional MC4R receptor is present on the homologous GT1-1 cell line. This cell line when exposed to alpha-MSH concentrations from 1nM-1uM agonize the MC4R and couple to GnRH release *in vitro* (Khong et al. 2001). Therefore, we hypothesized that botanical fractions which activate the MC4R will also be able to then stimulate the release of GnRH through the functional melanocortin receptor present in the GT1-7 cell line.

4.2 METHODS

GT1-7 CELL CULTIVATION: Frozen cells were rapidly thawed via incubation in a 37 °C water bath and expanded in DMEM containing 4.5 g/L glucose, 110 mg/L pyruvate, and 548 mg/L L-glutamine (Gibco, 11995040) with 10% FBS (Gemini Bio Products, 900-108), and 1% penicillin-streptomycin solution (Gibco, 15140122). They were then plated in 60 mm tissue treated culture disks and incubated at 37 °C in a humidified incubator with 5% CO_2 with media being replaced every 2-3 days until 90% confluency reached. Cells were passaged by rinsing with room temp 1X PBS (Gibco, 20012027), followed by treatment with

trypsin-EDTA (Gibco, 25200072) for 1-2 minutes until cells lifted off plate, then subcultured 1:3 in 60 mm dishes and incubated to reach ≥80% confluency before being sub cultured further into 96 well plates at a density of 12K cells/well. Cell growth time for each of the individual experiments were as follows (Experiments 1,2a/b,6- 1 day; Experiments 3,4,5- 3 days). Cell media was aspirated, then cells were serum shocked with 50% FBS and incubated for 1 hr prior to treatment, serum shocking media was then aspirated and replaced with complete media.

CELL STIMULATION

Exp. 1 - 1mM alpha-MSH was solubilized in DMSO. Cells were incubated with treatment and vehicle for one hour at 37 °C, aliquots were removed then snap frozen over dry ice before storage at -80 °C prior to the ELISA assay (*n*=4). Incubation time in the ELISA was 1.5 hrs.

Exp. 2a- Treatments of *L. pumila* at 50 and 100 µg/mL were solubilized and applied to cells for final in well concentrations of 25 and 50 µg/mL respectively. Treatments of *C. johimbe* were identically prepared and applied. One 96-well plate of cells was incubated for 24 hrs., the second for 48 hrs.; at the end of the timepoint, an aliquot was removed, snap frozen and stored at -80 °C prior to assaying (*n*=6). Incubation time in the ELISA was 1.5 hrs. Cells were assessed at each timepoint for cytotoxicity utilizing the LIVE/DEAD kit as outlined in prior experiments.

Exp 2b Cells were treated exactly as detailed in experiment 2a.

Exp. 3- Treatments of *L. pumila* at 50 and 100 µg/mL were solubilized and applied to cells for final in well concentrations of 25 and 50 µg/mL respectively. Treatments of *C. johimbe* were identically prepared and applied for 24 hrs. Aliquots were removed then snap frozen over dry ice before storage at -80ºC prior to the ELISA assay (*n*=6). Incubation time in the ELISA was 2 hrs.

Exp 4. L. *pumila* aqueous extract was prepared in a concentration curve serially diluted at the following concentrations; 50, 25, 12.5, 6.25, and 3.125 µg/mL. Cells were incubated with treatment or vehicle over a 24-hr. period before 10µL aliquots were removed, snap frozen over dry ice and then stored at -80ºC prior to the ELISA assay (*n*=10).

Incubation time in the ELISA was 2 hrs.

Exp 5. Treatments of *L. pumila* and *C. johimbe* at 100 µg/mL were solubilized and applied to cells for final in well concentrations 50 µg/mL respectively. Cells were treated for 24 hrs. and ran in duplicate with 464

nM of MCL 0020 (Tocris, 3438) a potent and selective MC4R antagonist. Aliquots were removed, snap frozen over dry ice and then stored at -80 °C prior to the ELISA assay (*n*=12). Incubation time in the ELISA was 2 hrs.

Exp 6. Treatments of *L. pumila* at 100 µg/mL was solubilized and applied to cells for final in well concentration of 50 µg/mL. Cells were incubated for one hour prior to starting a clock where aliquots of treatment and vehicle were taken in five-minute intervals for a total of six timepoints over 30 minutes. (*n*=4) Incubation time in the ELISA was 2 hrs.

CELL VIABILITY/CYTOTOXICITY: Following cell treatment, cell viability/cytotoxicity was quantified using the LIVE/DEAD® (Invitrogen, L3224) commercial kit assay. After treatment, cell media was aspirated followed by a gentle wash of 1X PBS prior to adding Calcein-AM (1:1000), and ethidium homodimer-1 (1:500) diluted in PBS. Cells were incubated in the dark at room temperature for thirty minutes prior to imaging on the Nikon Ti2-E microscope with automated image acquisition. Total cell counts and subsequent percent toxicity were calculated by NIS-Elements acquisition and analysis software.

ELISA: A commercial ELISA kit with the ability to detect native mouse GnRH1 from cell media was purchased (MyBioSource, MBS264939) and ran according to the manufacturer's instructions. Incubation times are noted for each experiment in the cell stimulation part of the methods.

4.3 RESULTS

CYTOTOXICITY: We did not observe any toxicity greater than 50% by our treatment concentrations within the 48 hr testing period; therefore, we proceeded to assess both concentrations in our experiments. We did however, observe an increase in cell death over the two-day period for our treatments as well as media control.

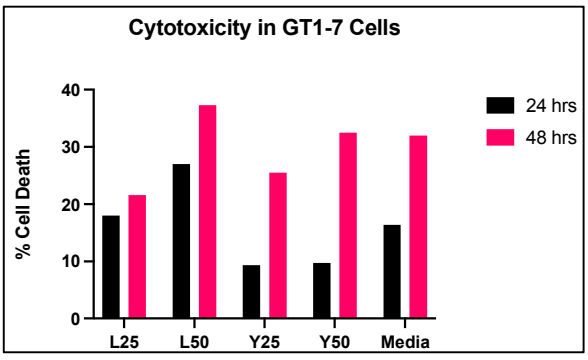

Figure 25. Cytotoxicity of Botanical Extracts in GT1-7 Cells Over 48 hrs.

Exp 1: Alpha-MSH demonstrates an average GnRH secretion of 3pg/mL while vehicle control secrets 73 pg/mL.

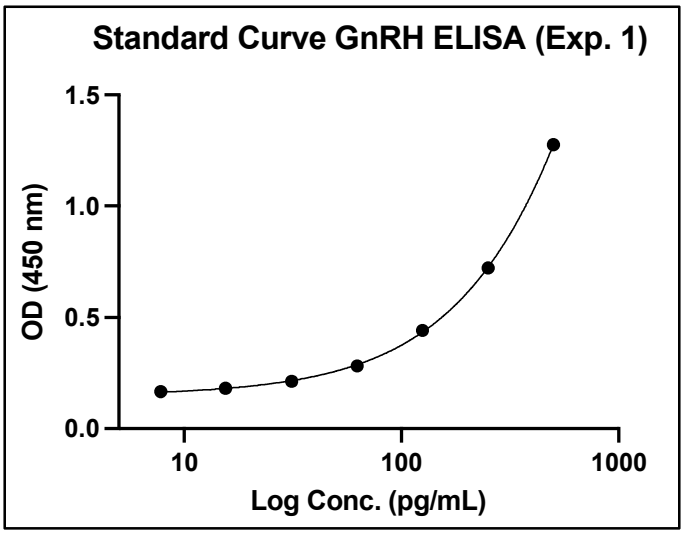

Figure 26A. Standard Curve Exp. 1

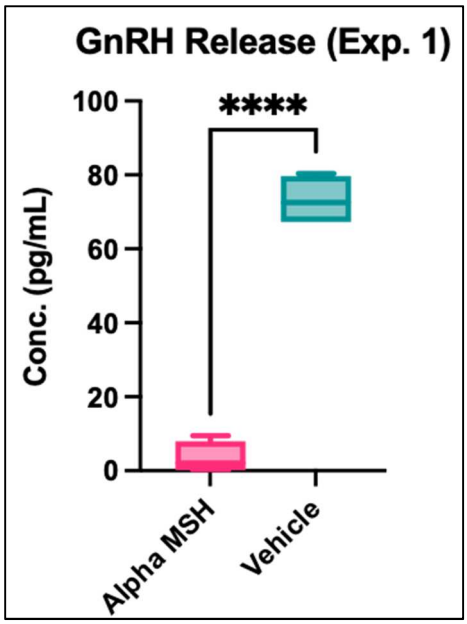

Figure 26B. GnRH Release Exp. 1

Table 19: 4PL Standard Curve GnRH ELISA (Exp. 1)	
A	0.133917
B	0.950161
C	71518046
D	89511.5
R2	0.99878

Exp 2a: (*n*=6) *L. pumila* at 25 µg/mL shows an average GnRH secretion of 87pg/mL while at. 50 µg/mL increases average GnRH secretion to 143 pg/mL. C. yohimbe demonstrates an average secretion of 39 pg/mL at 25 µg/mL and slightly increases to 54 pg/mL at 50 µg/mL. Media demonstrates an average secretion of 7.5 µg/mL.

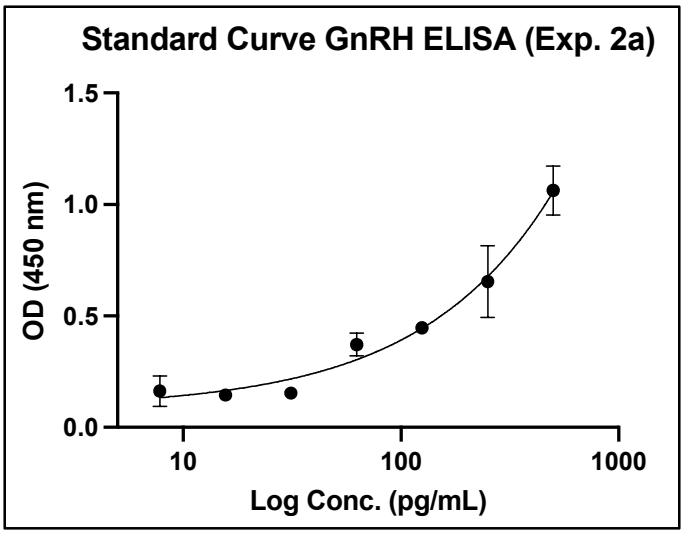

Figure 27A. Standard Curve Exp. 2A

Table 20: 4PL Standard Curve GnRH ELISA (Exp. 2a)	
A	0.106218
B	0.760841
C	1.98E+08
D	16874.73
R2	0.954505

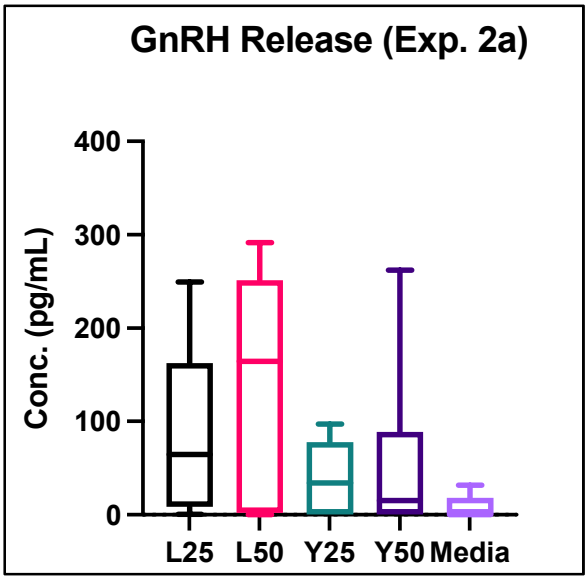

Figure 27B. GnRH Release Exp. 2A

Exp 2b: At 25 µg/mL *L. pumila* demonstrates an average. GnRH secretion of 27 pg/mL and at 50 µg/mL only 25 pg/mL. *C. johimbe* demonstrates an average of 7 pg/mL at the lower concentration, and .5 pg/mL for the highest concentration. Media control had an average of 3 pg/mL.

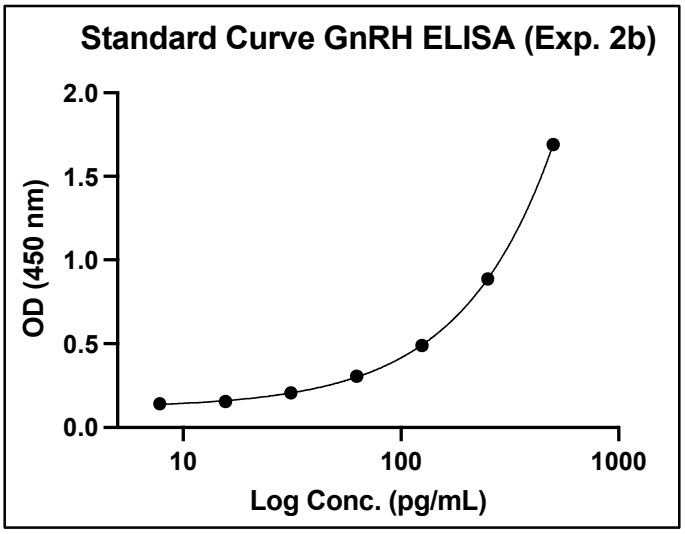

Figure 28A. Standard Curve Exp. 2B

Table 21: 4PL Standard Curve GnRH ELISA (Exp. 2b)	
A	0.115194
B	1.030883
C	48279784
D	216902.8
R2	0.999951

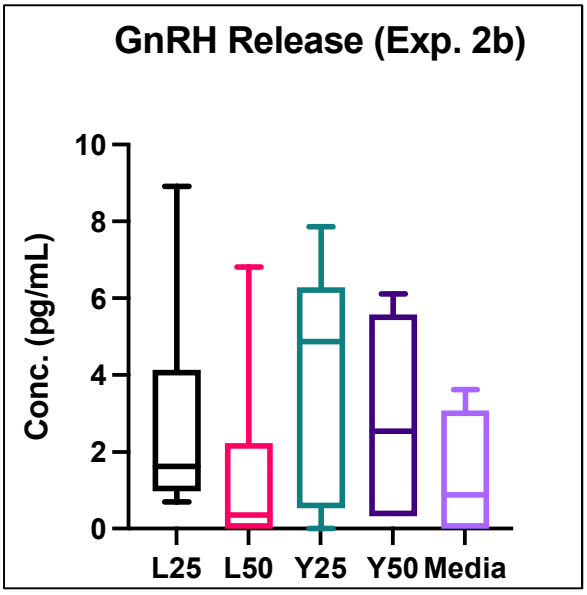

Figure 28B. GnRH Release Exp. 2B

Exp 3: For L. p*umila* at 25 µg/mL we observed an average section of 27 pg/mL and at 50 µg/mL an average secretion of 25 pg/mL. For C. johimbe we observed an average secretion of 7 pg/mL at the 25 µg/mL concentration, and .5 pg/mL at the 50 µg/mL concentration. Media had an average secretion of 3 pg/mL.

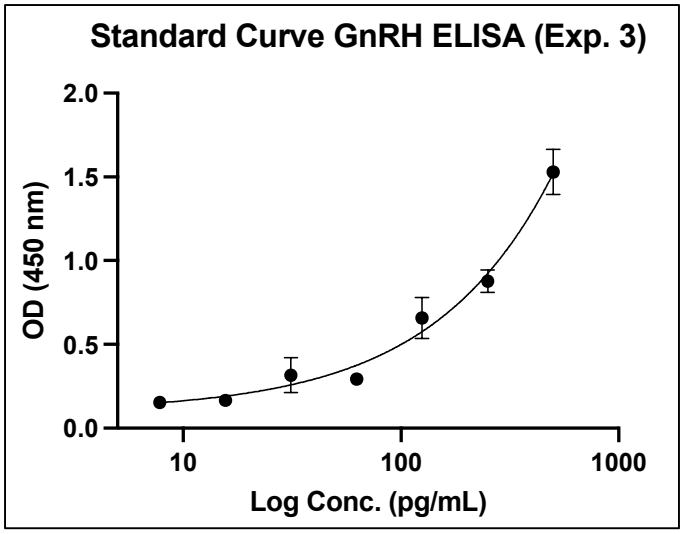

Figure 29A. Standard Curve Exp. 3

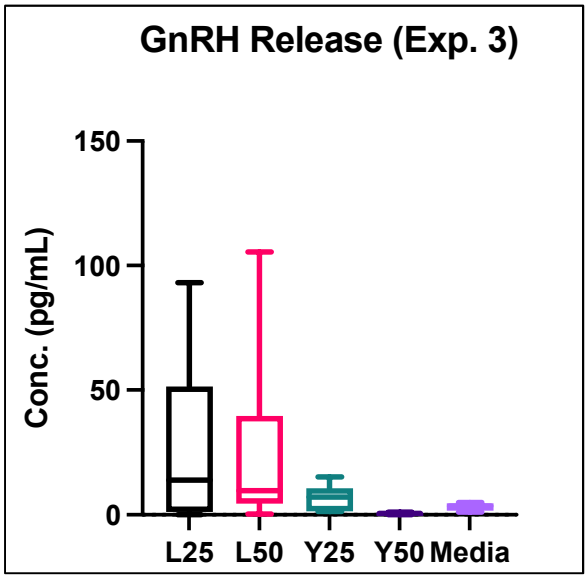

Figure 29B. GnRH Release Exp. 3

Table 22: 4PL Standard Curve GnRH ELISA (Exp. 3)	
A	0.097223
B	0.786033
C	2.07E+08
D	36976.32
R2	0.987126

Exp 4: At 3.125 µg/mL we observed secretion of 7 pg/mL, at 6.25 µg/mL there was an average of 9 pg/mL secretion, at 12.5 µg/mL secretion averaging 23 pg/mL, at 25 µg/mL secretion averaging 32 pg/mL, at 50 µg/mL secretion averaging 23 pg/mL and media showed 15pg/mL secretion.

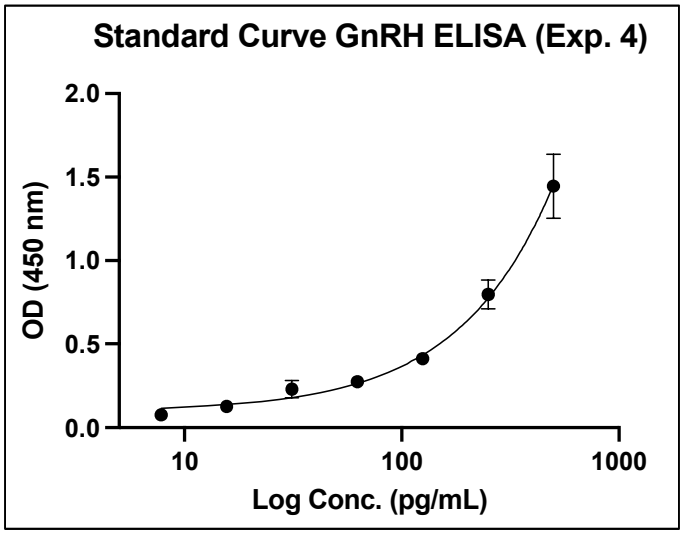

Figure 30A. Standard Curve Exp. 4

Table 23: 4PL Standard Curve GnRH ELISA (Exp. 4)	
A	0.077969
B	0.946469
C	80363134
D	115681.8
R2	0.996686

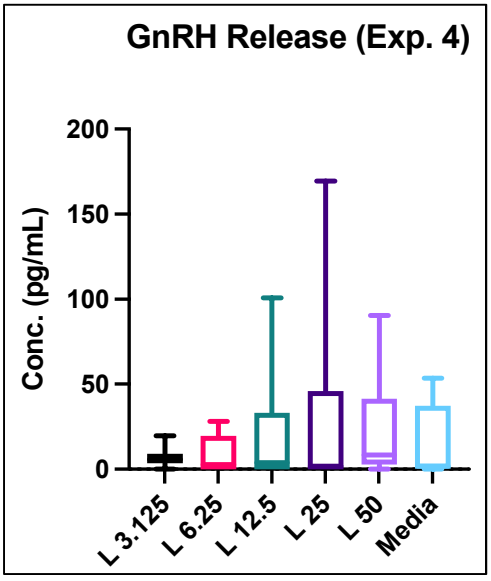

Figure 30B. GnRH Release Exp. 4

Exp. 5. 14/16 L50 samples were below the asymptote and unable to be quantified. L50 plus inhibitor had similar results where 15/16 samples were unable to be quantified. No samples from *C. johimbe* with or without the inhibitor were able to be quantified, and likewise no media values could be quantified.

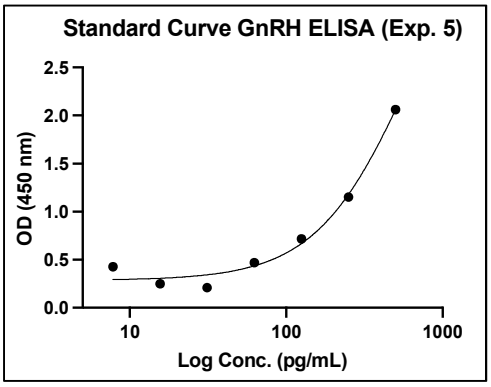

Figure 31. Standard Curve Exp. 5

Table 24: 4PL Standard Curve GnRH ELISA (Exp. 5)	
A	0.304501
B	2.496403
C	207.4428
D	2.259265
R2	0.987872

Exp 6: No data from the time course experiment was able to be quantified as values were below the limit of detection.

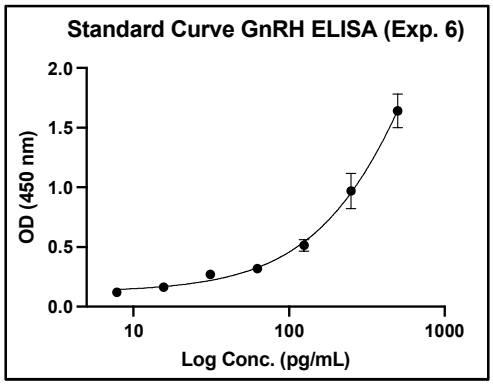

Figure 32. Standard Curve Exp. 6

Table 25: 4PL Standard Curve GnRH ELISA (Exp. 6)	
A	0.123539
B	1.10111
C	1226.536
D	5.727316
R2	0.997624

4.4 DISCUSSION

The GT1-7 cells are an immortalized hypothalamic pituitary cell capable of secreting GnRH upon depolarization. They are known to contain MC4R receptors which have been shown to couple to GnRH secretion. GnRH is a major signaling hormone which upon pulsatile secretion causes the pituitary gland to make and secret luteinizing and follicle-stimulating hormone which are responsible for regulating steroidogenesis and gametogenesis. We have botanical extracts which have been shown to activate the MC4R receptor *in vitro*. Although this receptor has been linked to sexual functioning, we wanted to evaluate whether our fractions were affecting the HPG axis in some way. If our extracts demonstrated the ability to secrete GnRH through the functional MC4R receptor present we would further understand the ethnobotanical relevance to use as an aphrodisiac.

We undertook several experiments in order to determine whether two botanical fractions which activated MC4R and MC3R, could cause secretion of GnRH in GT1-7 cells. Through sandwich ELISA's, we have identified that GnRH release is highly variable in our cells, and often secreted in amounts that are undetected by this assay type.

While we were able to observe a paucity of replicates that would suggest our extracts caused secretion of this hormone, the variability was far too high to conclude with certainty. Our ELISA parameters suggested it could read amounts as low as 15.6 pg/mL which clearly did not cover several of our measurements; although our individual standard curve calculations allowed us to quantify lower than this amount. We had initially increased our sample size in order to sufficiently account for the variability initially demonstrated between samples; however, the well-adapted method of perfusion cell culture coupled with radio-immunoassay seem to be ideal for detection of GnRH secretion when the amount is unknown or potentially thought to be low. The benefit of perfusion cell culture specifically is that GnRH is released in a pulsatile manner following depolarization and the ability to take multiple measurements at various timepoints without having to remove culture media is an advantage. The final step of any experiment with these cells should be to challenge the cells with 60 mM potassium chloride which will depolarize them and allow an assessment of viability and general secretory function. We do believe future studies should take this methodology into account to potentially demonstrate a more accurate depiction of whether these extracts can in fact increase GnRH secretion

CHAPTER 5 ABILITY OF MC4R ACTIVATING APHRODISIAC EXTRACTS TO EFFECT GENE EXPRESSION IN GT1-7 CELLS

5.1. OVERVIEW We proceeded with qRT-PCR experiments because our receptor activation data suggested there was something happening over a period of time to affect activation, perhaps on a transcriptional level. For this experiment we looked at three genes known to be expressed in GT1-7 cells, POMC, MC4R, AGRP, as well as a gene that is directly downstream of the MC4R, BDNF (Mayer 2009).

Expression changes of both MC4R and AGRP has been shown to change over the estrous cycle in an *in vivo* rat model. While humans typically have a 28-day menstrual cycle, rodents spend 4-5 days in estrous; however, both mammals undergo a significant spike in the hormones, estrogen, progesterone, LH, and FSH. In humans our LH and FSH occurs prior to and triggers ovulation, in rodents this occurs at the end of the proestrus phase. In the proestrous phase, MC4R mRNA is the highest suggesting it may be involved in preovulatory surge of GnRH which causes secretion of LH and FSH, conversely AGRP mRNA is at its lowest suggesting an inhibitory effect (Zandi et al 2014). Hormonal inputs also can affect POMC expression and neuronal activity, it is believed that POMC neurons relay metabolic inputs to GnRH populations and regulate their activity (Roa and Herbison 2012).

5.2 METHODS:

CELL CULTIVATION: Cells were cultivated as outlined above in Chapter 4 but instead sub cultured into 6-well plates and grown to 90% confluency ~ 4 days.

RNA- EXTRACTION/ QUANTIFICATION- QIAGEN RNEASY KIT (Qiagen, 74116)

Following cell culture and incubation with test compounds, cells were harvested by direct lysis on plate with 350 µL per well of Buffer RLT with 10 µL ß-mercaptoethanol, then scraped using the base of a sterile pipette tip. 350 µL of EtOH were then added. To the lysate and mixed by pipetting. All 700 µL of sample were transferred to a RNeasy Mini spin column placed in a 2 mL collection tube. Samples were centrifuged at

8000g for 15 sec with flow through discarded. Buffer RW1 (700 µL) were added to the column then centrifuged again at 8000g for 15 sec with the flow through discarded. Buffer RPE (500 µL) were then added to the column, centrifuged for 15 sec at 8000g, and flow through discarded. Finally, 500 µL of Buffer RPE was added to the column, centrifuged for 2 min at 8000g. Column was removed and placed into a new 2 mL collection tube, then centrifuged at full speed to dry the membrane. Column was again removed and placed into a new 1.5 mL collection tube, 50 µL of RNase-free water added directly to the membrane, then centrifuged for one minute at 8000 X g to elute the RNA prior to quantification.

RNA was quantified and using a NanoDrop One^{C} Microvolume UV-Vis Spectrophotometer (Thermo Fisher) which provided a concentration of RNA in ng/µL as well as the ratio of absorbance at 260 nm/280 nm to indicate RNA purity, as well as the ratio of absorbance at 260 nm/230 nm to indicate nucleic acid purity.

CDNA CONVERSION: RNA was immediately converted to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, 4387406). First, we calculated the volume of RNA needed to make 422 ng of cDNA, then the volume of nuclease-free water to bring to total to 9 µL per sample in PCR strip tubes. To this we added 10 µL of 2X RT Buffer Mix and 1 µL of RT Enzyme Mix for a total of 20.0 µL per reaction. Tubes were labeled, sealed, and centrifuged before being incubated in a thermocycler under the program High Cap RNA-cDNA which incubates the reaction for 60 min at 37 °C, then heats to 95 °C for 5min, and finally held at 4 °C before being frozen at -20 °C.

Q-PCR: Initially, samples (*n*=12) were defrosted on ice then diluted with 120 µL of RN-ase free water. Nine microliters of diluted cDNA were added to the optical reaction plate well along with 10 µL of TaqMan Master Mix (Applied Biosystems, 4305719), and 1µL of each individual TaqMan Primer (GnRH1-Mm01315604_m1, BDNF-Mm04230607_s1, POMC-Mm00435874_m1, AGRP-Mm00475829_g1, MC4R-Mm00457483_s1 , HPRT-FAM, GAPDH-VIC, B2M-FAM) for a total of 20 µL per reaction well.. Plates were covered with a MicroAMP Optical Adhesive film, and briefly centrifuged to spin down contents and eliminate air bubbles from the solutions. Plate was then inserted into the RT-qPCR system (Applied Biosystems 7200 real-time cycler with TaqMan detection chemistry (Applied Biosystems) with the following thermal cycling parameters 50 °C for 2 min, 95 °C for 10 min for polymerase activation, 95 °C 15 sec to denature, 60 °C for 1min to anneal/extend for a total of 50 cycles averaging 2 hrs. Samples were screened in duplicated

followed by $2^{-\Delta\Delta CT}$ method evaluation. Housekeeping genes assessed were HPRT-Fam, GAPDH-VIC, and B2M-FAM

STATISTICS Grubbs outlier analysis was performed with an alpha of 0.05 on each of the samples/gene *n*=12. Following any outlier removal, an Ordinary One-Way ANOVA was calculated with Tukey's multiple comparisons test followed afterwards.

5.3 RESULTS

No outliers were detected in the treatment data for the MC4R, BDNF, and AGRP Gene Expression samples. We did not see any significant increase or decrease in either the MC4R or BDNF gene expression from either of our treatment groups in respect to each other or in comparison to control; however, we did observe a significant increase in AGRP gene expression over control from the *L.pumila* treatment group, as well as a significant increase by the *C. johimbe* treatment group..

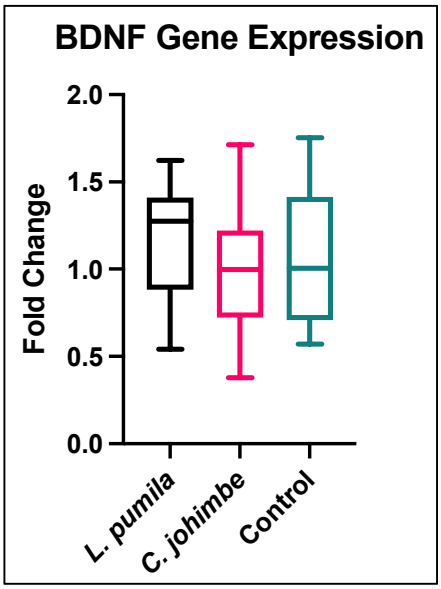

Figure 33A MC4R Gene Expression in GT1-7 Cells

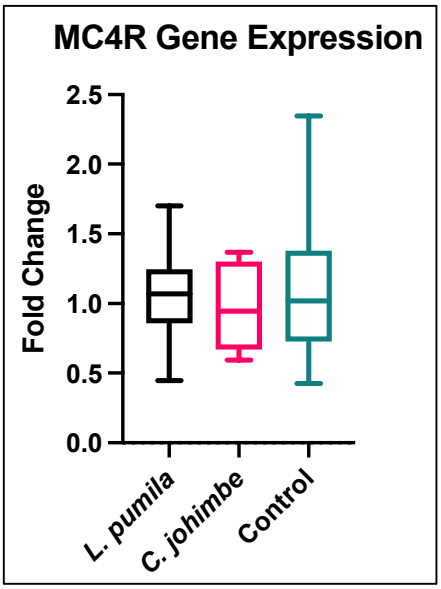

Figure 33B BDNF Gene Expression in GT1-7 Cells

An outlier was detected in the POMC control group which in order to be removed we needed to recalculate the $^{\Delta\Delta CT}$ to update the data. After removal and recalculation, we did not see any significant changes in gene expression from our treatment groups in comparison to control.

An outlier was also detected in the GnRH control group which upon removal caused a recalculation of $^{\Delta\Delta CT}$. Afterwards we were able to observe a significant decrease in gene expression as expressed by fold change by both of our treatment groups in respect to our control.

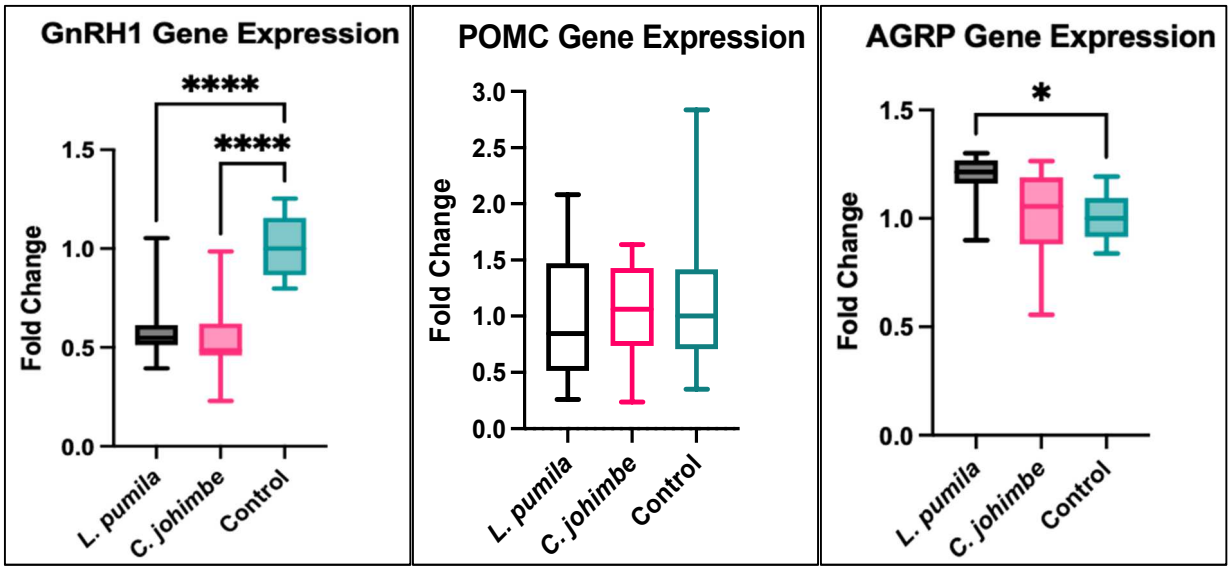

Figure 33C GnRH1 Gene Expression **Figure 33D POMC Gene Expression** **Figure 33E AGRP Gene Expression**

5.4 DISCUSSION

In this experiment we were looking to address whether any significant transcriptional changes took place in four relevant genes that may offer clues as to how our botanical fractions which activate the MC4R impact hypothalamic neuronal cells which *in vivo* are responsible for regulating metabolic inputs and reproductive function.

Our results showed a significant decrease of GnRH1 gene expression by both *L. pumila* and *C. johimbe* which would support our preliminary findings of Chapter 4, being that the majority of our samples were not able to reliably secrete GnRH. The lower GnRH gene expression exhibited by treatment with both *L. pumila* and *C. johimbe* may ultimately be responsible for the decrease in GnRH biosynthesis and release. Estrogens play a key role in the reproductive axis acting as a negative feedback mechanism, of which estradiol has been shown to downregulate GnRH mRNA levels in GT1-7 cells which contains both alpha and beta estrogen receptors (Ng et al. 2009). To validate the hypothesis that the phytoestrogenic activity of *L. pumila* downregulates GnRH1 mRNA in GT1-7 cells future experiments could co-incubate extracts with known estrogen antagonists such as tamoxifen, as well as prior to experimentation assess the estrogenic activity of the fraction. To date there appears to be no evidence for any estrogenic activity of *C.*

johimbe, but potentially due to its male potency effects, the extract may have androgenic activity which could explain the decrease in GnRH expression as 5-DHT has been shown to decrease GnRH mRNA through the androgen receptor of this cell line (Belsham 1998).

We also observed a significant increase of AGRP transcription by *L. pumila.* The product of this gene, agouti related protein, is coexpressed with neuropeptide Y in arcuate nucleus GABA neurons and is highly implicated in the regulation of the reproductive axis (Marshall et al 2020). Traditionally increases in AGRP mRNA would be seen in fasting states in animal models, but as cells were cultured in a complete media perhaps interactions with other metabolic mediators such as insulin caused this observation.

We believe that evaluating a neuronal population with genes that are highly regulated from several other cues in the body seems insufficient to put together a complete story of what our extracts are doing. Further experimentation should be done to delineate mechanisms by which transcriptional changes are taking place including downstream kinase pathways.

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APPENDIX

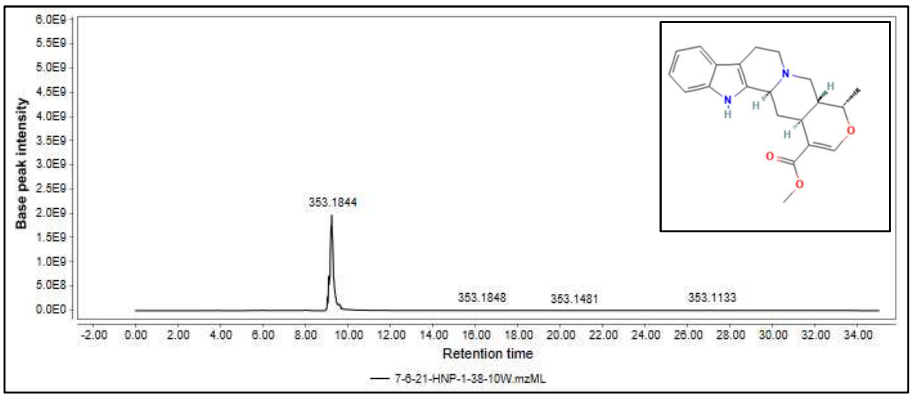

Fig 34. Ajmalicine in *C. johimbe* Water Fraction (MZmine Chromatogram)
Chemical Formula: $C_{21}H_{24}N_2O_3$
Exact Mass: 352.17869263
$[M+H]_{calc}$ = 353.18649263

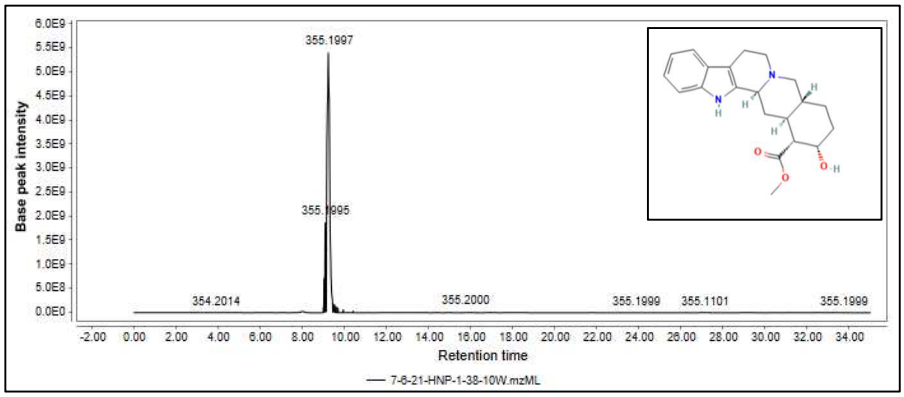

Fig A2. Yohimbine in *C. johimbe* Water Fraction (MZmine Chromatogram)
Chemical Formula: $C_{21}H_{26}N_2O_3$
Exact Mass: 354.19434270
$[M+H]_{calc}$ =. 355.2021427

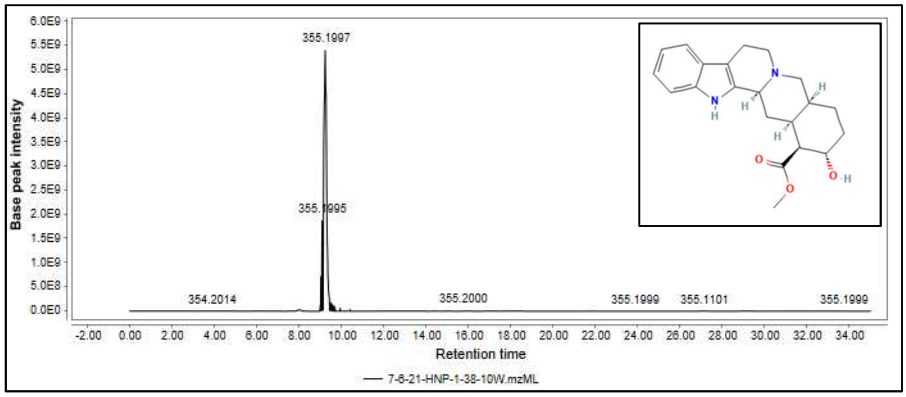

Fig A3. Rauwolscine in *C. johimbe* Water Fraction (MZmine Chromatogram)
Chemical Formula: $C_{21}H_{26}N_2O_3$
Exact Mass: 354.19434270
$[M+H]_{calc}$= 355.2021427

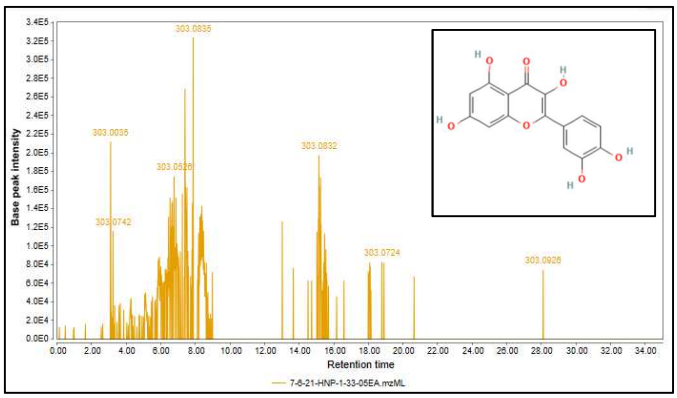

Fig A4. Quercetin in *T. foenum-graecum* EtOAc Fraction (MZmine Chromatogram)
Chemical Formula: $C_{15}H_{10}O_7$
Exact Mass: 302.04265265
$[M+H]_{calc}$= 303.05045265

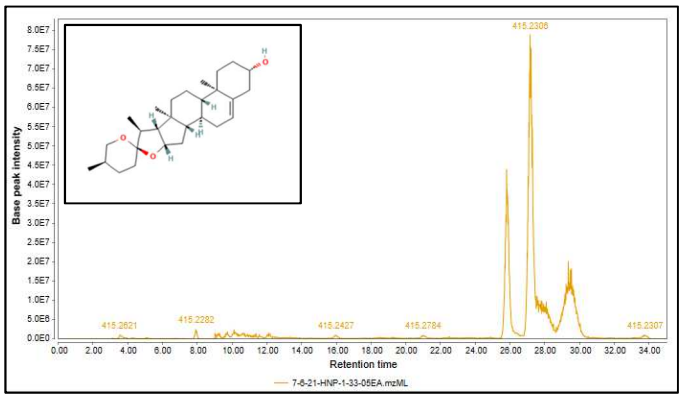

Fig A5. Diosgenin in *T. foenum-graecum* EtOAc Fraction (MZmine Chromatogram)
Chemical Formula: $C_{27}H_{42}O_3$
Exact Mass: 414.31339520
$[M+H]_{calc}$= 415.3211952

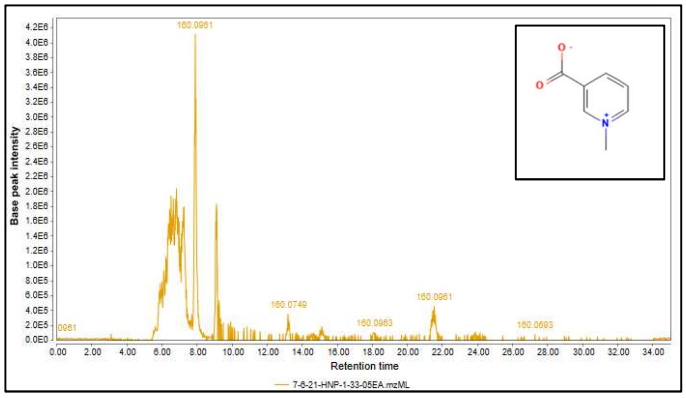

Fig A6. Trigonelline in *T. foenum-graecum* EtOAc Fraction (MZmine Chromatogram)
Chemical Formula: $C_7H_7NO_2$
Exact Mass: 137.047678466
$[M+Na]_{calc}$ = 160.037478466

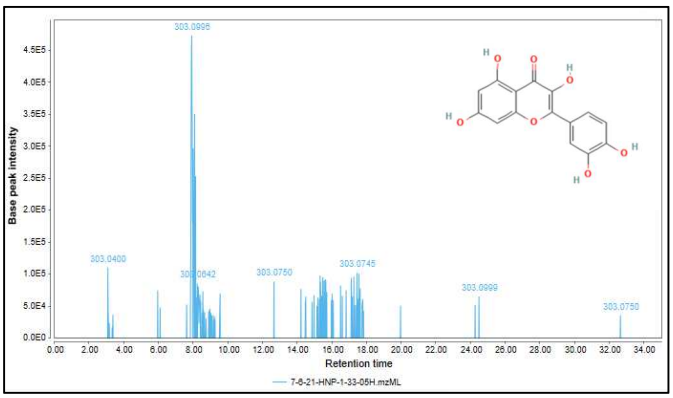

Fig A7. Quercetin in *T. foenum-graecum* hexanes Fraction (MZmine Chromatogram)
Chemical Formula: $C_{15}H_{10}O_7$
Exact Mass: 302.04265265
$[M+H]_{calc}$= 303.05045265

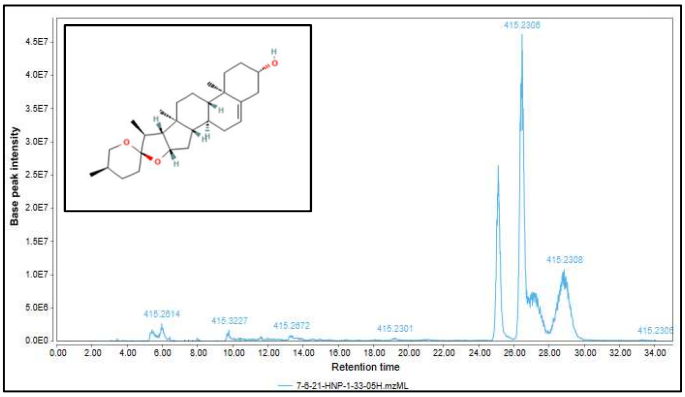

Fig A8. Diosgenin in *T. foenum-graecum* hexanes Fraction (MZmine Chromatogram)
Chemical Formula: $C_{27}H_{42}O_3$
Exact Mass: 414.31339520
$[M+H]_{calc}$= 415.3211952

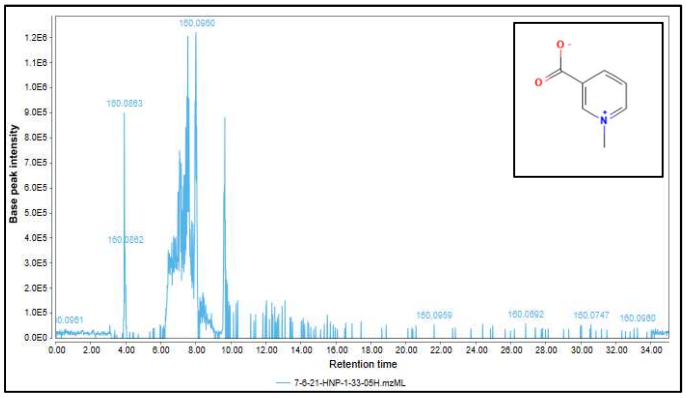

Fig A9. Trigonelline in *T. foenum-graecum* hexanes Fraction (MZmine Chromatogram)
Chemical Formula: $C_7H_7NO_2$
Exact Mass:137.047678466
$[M+Na]_{calc}$ =160.037478466

VITA

PERSONAL STATEMENT

hprescot@go.olemiss.edu

My unique undergraduate training encompassed all aspects of herbal medicine, from taxonomy and microscopic analysis, to QA/QC for the dietary supplement industry. I finished my program with an overall working knowledge comprising 120 plants that are commonly used globally. While studying, I joined an ongoing ethnobotanical research project in order to develop a standardized Botanical Drug Substance (BDS) for future clinical trial work. This gave me the experience of taking a whole plant extract through the FDA process of specific laboratory requirements and GMP's for developing a BDS. As a graduate student, I garnered expertise in natural product research namely, extraction, fractionation, and structural elucidation from botanical species. While gaining expertise in natural products research I focused on botanicals for women's health, specifically in female sexual dysfunctions. My work started with the assessment of dietary supplements currently marketed towards women experiencing a low libido, and I was able to succinctly review the plants for both traditional and clinical evidence. I also gained knowledge of cell cultivation and the application of botanicals in cell-based screening assays. I was able to successfully apply for The Anne S. Chatham Fellowship in Medicinal Botany, which afforded me the opportunity to expand my mechanistic research downstream from the receptor of interest. My unique perspective in natural products research allows for a wholly interdisciplinary and progressive approach and understanding to my work and future global impact. I believe my work will continue to push the innovation of discovering natural products drugs for development in the field of women's health.

EDUCATION

University of Mississippi, University, Mississippi — **2018-present**
Ph.D. in Pharmaceutical Sciences (Pharmacognosy Division)
Advisors: Dr. Ikhlas Khan, Dr. Nicole Ashpole

Bastyr University, Kenmore, Washington — **2014-2016**
B.Sc. Herbal Sciences
Mentor: Dr. Leanna Standish

Norwalk Community College, Norwalk, Connecticut — **2011-2014**
Associates (LAS-transfer)

EMPLOYMENT HISTORY

University of Mississippi, University, Mississippi
Graduate Research Assistant — **2018-present**
Graduate Teaching Assistant — **2019**

BioPure Healing Products, Redmond, Washington — **2016-2017**
Shipping Coordinator

Bastyr University, Kenmore, Washington — **2014-2017**
HPLC Lab Technician

GRADUATE/PHARMD LECTURES

- Foundations in BioMolecular Sciences II, PHCY 402 (guest lecture) 4/20, 4/21
- Pandemics and Society, BMS 474 (guest lecture on pathogen evolution and virulence) 9/21
- Drugs and Human Performance, PHCL 351 (guest lecture on Sexual Performance Enhancers) 10/21

SERVICE

- Department of Biomolecular Sciences Graduate Student Council Senate Representative 2020-2021
- BioMolecular Sciences Student Advocate 2019-2020
- STEM Panel Member: Graduate Orientation University of Mississippi 2019
- Habitat for Humanity- Local Oxford House Painting 2019

PUBLICATIONS

- Prescott, H., & Khan, I. (2019). Medicinal plants/herbal supplements as female aphrodisiacs: Does any evidence exist to support their inclusion or potential in the treatment of FSD? Journal of Ethnopharmacology, 251, 112464

FUNDING

Anne S. Chatham Fellowship in Medicinal Botany 4/2021
The Garden Club of America $4,500

AWARDS

- 2022; ICSB 2022 Attendance Grant, UM BDSRC
- 2021; Charles D. Hufford Award, Graduate Student in Pharmacognosy

CONFERENCES/PRACTICUMS

- 2022; International Conference on the Science of Botanicals: "*Botanical Aphrodisiacs for Women's Health"*
- 2021; NIH Office of Dietary Supplements 25th Anniversary Scientific Symposium
- 2021; NIH ODS Workshop on Bioactive Ingredients in Infant Formula
- 2021; NIH ODS Seminar Series
- 2021; Mary Frances Picciano Dietary Supplement Research Practicum
- 2021; UMSOP Responsible Conduct of Research Summer Section
- 2019; Natural Products Training Laboratory: Botanical Dietary Supplement Course
- 2019; International Conference on the Science of Botanicals
- 2017; Psychedelic Science "Standardization of *Banisteriopsis caapi* for a Phase I trial in Parkinson's"

TEACHING PORTFOLIO/ CERTIFICATIONS

- PHCL 611 Teaching in Pharmacology and Toxicology
- Center for Excellence in Teaching and Learning
  - Taking a Scientific Approach to Teaching Science
  - Essentials of Online Teaching and Content Delivery
  - Designing Online Instructional Media
  - Designing Online Learning Activities
  - Got Media? Creating and Captioning Course Video and Audio
  - 7 Steps to Create More Accessible Course Content
  - Online Teaching Endorsement Course

- Center for the Integration of Research, Teaching and Learning
  - The College Classroom
  - An Introduction to Evidence Based Undergraduate STEM Teaching
  - Bringing an Inclusive Mindset to Your Teaching
  - Writing an Effective Teaching Philosophy Statement