The Chemical and Biological Investigation of Plants that Target the Human Estrogen Receptor: *Trigonella foenum-graecum* (fenugreek)

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ABSTRACT

The occurrence of chemicals of natural origin that possess the capacity to mimic the hormonal properties of estrogen has been previously reported in the biomedical literature. From these reports, it is apparent that the structural nature of these estrogen mimics is very diverse. This estrogen mimic can be found in plants used in the treatment of health conditions as alternative medicine, such as the treatment of symptoms associated with menopause. Many of these plants have been investigated, but there still exist many instances in which estrogenic activity is unsuspected and exposure unknown.

This research focuses on *Trigonella foenum-graceum* (Fabaceae), commonly known as Fenugreek, which has been used since antiquity as a galactogogue to increase milk production in nursing women. It has been suggested that Fenugreek contains phytoestrogens responsible for this galactogogic activity. Although previous reports support Fenugreek's estrogenic activity and many of its complex chemical constituents have been elucidated, no specific chemical responsible for the purported estrogenic activity has been identified with certainty. The objective of this investigation is to study the chemistry of Fenugreek for estrogenic activity and using a bioassay-guided approach to isolate and elucidate principle(s) associated with the putative hormonal activity.

For the chemical and estrogenic study of Fenugreek, seeds were ground up and extracted with organic solvents of various polarities. Further fractionation of extracts was performed with the use of Sephadex LH-20 column chromatography and preparative Thin Layer Chromatography (TLC). The chemistry in these extracts has been explored using TLC and various diagnostic spray reagents for composition profiles. These crude extracts were used to test for estrogenic activity and further chemical elucidation. The estrogenic activity was assayed using the strain YGY13 of *Saccharomyces cerevisiae* transformed with the plasmid pGEV-HIS3, which contains the human estrogen receptor and a β-galactosidase reporter gene. Initial investigation of Fenugreek revealed the presence of the steroidal saponin Diosgenin in the active chloroform/methanol extract as shown by the use of the Liebermann-Burchard reagent and an authentic sample, as well as, the use of mass spectrometry. Sequential bioassay guided fractionation of this extract revealed the presence of additional non-steroidal estrogenic constituents resulting in the detection by mass spectrometry of a family of isoflavones. This is the first time that the presence of these isoflavones in Fenugreek have been directly associated with its estrogenic activity.

The Acetylcholinesterase inhibition activity of Fenugreek was explored using an in vitro bioautographic method on TLC, and the antibiotic properties were assayed against yeast as a test organism. Extracts of fenugreek were found to possess both pharmacologic activities.

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Chapter 1

Estrogenic Activity

I. Introduction

Hormones play diverse regulatory roles, effecting blood pressure, electrolyte balance, embryogenesis, sexual differentiation, development and reproduction, just to name a few. Hormones have a diverse chemical nature such as peptides, catecholamines, eicosanoids, steroids, and retinoids. Thus hormones have diverse functions, as well as diverse structures. A particular class, steroidal hormones, also known as adrenocortical and sex hormones, reach their target cells by binding to circulating plasma proteins traveling through the bloodstream and act through nuclear receptors in the cell to change the level of expression of a certain gene (Ackerman & Carr, 2002). The relatively small and lipophilic nature of steroids allows them to easily pass through the cellular plasma membrane to bind to intracellular receptors. The steroid hormones, including progesterone, cortisol, aldosterone, testosterone and estradiol, are synthesized from cholesterol in many of the endocrine tissues (Nelson and Cox, 2000).

One of the most widely discussed types of steroidal hormones are estrogens. The principal estrogens are estrone, estradiol, estriol and catechol metabolites, which are shown in Figure 1. The effects of estrogen are mediated by two estrogen receptor subtypes, ERα and ERβ, which are differentially sensitive to various estrogens. Some estrogenic molecules can be full agonists for one of the receptors while being full antagonists for the other receptor (Ketzenellenbogen et al. 2000). This further illustrates the complexity of estrogen and its physiological effects. Estrogens affect the female reproductive system, regulation of skeletal homeostasis, lipid and carbohydrate metabolism, electrolyte balance, skin physiology, the cardiovascular system and the central nervous system (Hall, 2011; Nilsson & Gustafsson, 2011; Vrtačnik et al. 2014). Consequently,

estrogen affects both males and females in several different manners. Not only is the presence of estrogen necessary for proper physiological function, but the long-term deficiency of estrogen can cause osteoporosis, cardiovascular disease, and possibly Alzheimer's disease (Harlow & Signorello, 2000). As a result, there might be instances in which there could be reasons why one would want to take estrogen or estrogen-like compounds for medicative or preventative purposes.

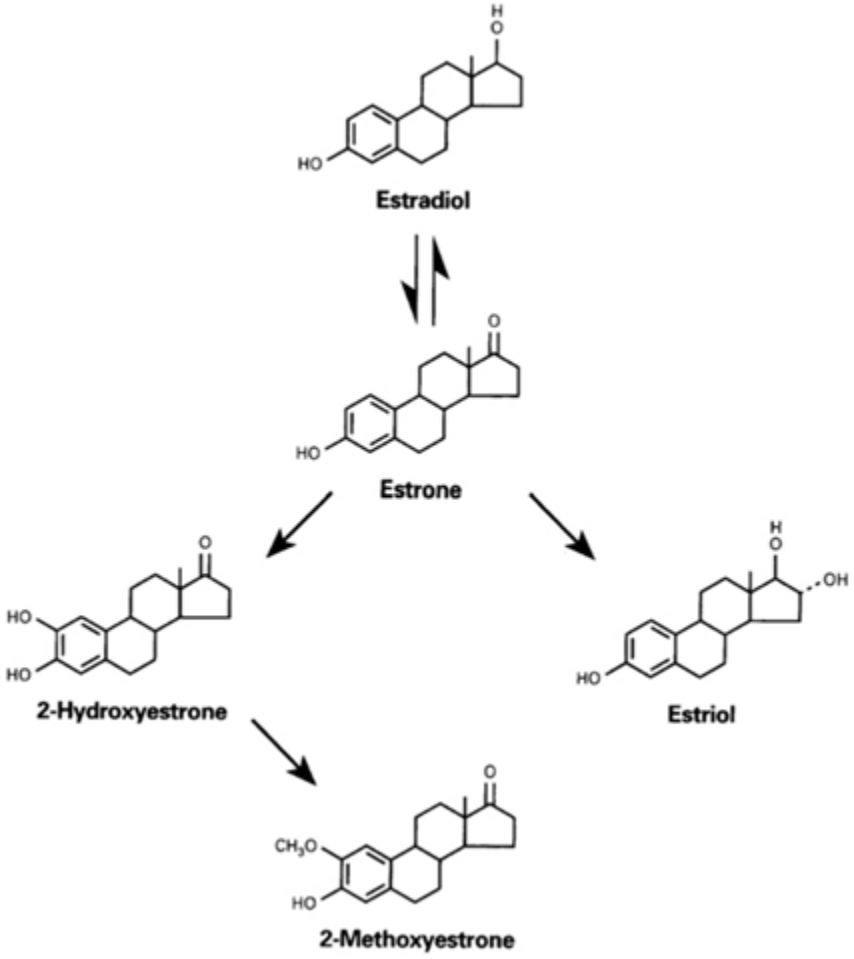


Figure 1. The main estrogens and metabolites in women (Ackerman & Carr, 2002)

For instance, many women turn to herbs and plant extracts as an alternative to hormone replacement therapy (HRT) and estrogen replacement therapy (ERT) because of their undesirable side effects, such as increased risk of breast and endometrial cancer and irregular bleeding (Sreeja

et al. 2010). Such an alternative is the case of Black Cohosh (*Actaea racemosa*, Ranunculaceae) used in the treatment of symptoms associated with menopause or the soy derived products that were recently found to contain estrogenic isoflavones (Newton et al. 2006). Although Black Cohosh was a very popular herbal medicine for women's reproductive health in the 19th century, several studies have concluded that Black Cohosh lacks an estrogenic mechanism of action and its efficacy in treating menopausal symptoms is doubtful (Peng et al. 2014; Ross, 2014). This raises the concern of the validity of the use of herbal medicines with purported estrogenic activity and issue as to whether or not some ethnobotanical claims need to be scientifically tested.

On the other hand, there is much more certainty with soy. Soy contains the isoflavones genistein, daidzein and glycitein, which are non-steroidal compounds that exhibit estrogen-like properties, shown in Figure 2 (Messina & Wood, 2008). These isoflavones preferentially bind to ERβ, which classifies them as selective estrogen receptor modulators (SERM) and they exhibit weak estrogen-like effects under certain experimental conditions (Ye et al. 2009). Recent evidence suggests that soy isoflavones reduce the risk of breast cancer, which could be because the binding of isoflavones to estrogen receptors blocks the action of the stronger endogenous estrogen binding on these receptors (Blei et al. 2015; *Breast Cancer and Environmental Risk Factors*, 2002). Despite controversy over isoflavones and their relationship to breast cancer, it is accepted that soy contains natural chemicals that mimic the hormonal properties of estrogen. These type of molecules are referred to as phytoestrogens.

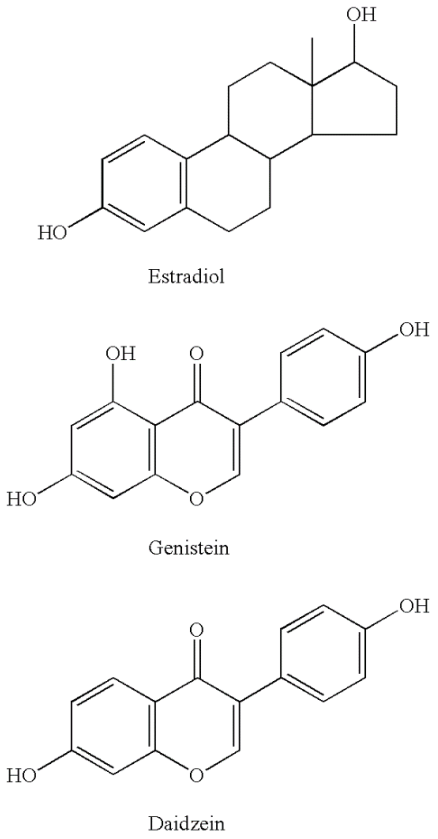


Figure 2. A structural comparison of the phytoestrogens genistein and daidzein found in soy to estradiol. The 15-carbon structures of these phytoestrogens is similar to the 17-carbon structure of estradiol, as shown above. (Notelivitz et al. 2003)

Phytoestrogens are molecules present in certain plants that are structurally and functionally analogous to endogenous human estrogen (Jargin et al. 2014). They have the potential to act as estrogen agonists or antagonists, which could interfere with the proper functioning of the endocrine system (Mortel & Mehta, 2013). When seeking out phytoestrogens, plants used as galactagogues are a promising source. Galactogogues are substances that assist the initiation, continuation, or augmentation of breast milk production that may act via the estrogen receptor (ER) to stimulate the female reproductive system (Academy of Breastfeeding Medicine Protocol Committee, 2011). The galactogogue effect associated with estrogen is believed to be caused by its stimulating effect on the ductal epithelial cells or the potentiation of prolactin production (Mortel & Mehta, 2013).

Such is the case for *Asparagus racemosus*, or shatavari, a plant used as a galactagogue in India. Studies have demonstrated the plant's estrogenic effects in the mammary glands and genital organs of rats, which are thought to be caused by the presence of phytoestrogens in the form of saponins and steroidal saponins in shatavari roots (Gupta & Shaw, 2011).

The use of galactagogues like shatavari is widespread, with many clinicians recommending the use of herbs to improve milk output. An estimated 15% of breastfeeding women use herbal galactogogues in the United States and 43% in Norway, however there is limited evidence on the mechanism of action behind these herbs and their galactogogue properties (Mortel & Mehta, 2013; The National Children's Study, 2003; Nordeng & Havnen, 2004). While some of the reported galactogogue effects may be linked to phytoestrogens, it is likely that there are many different mechanisms of action involved in the efficacy of these plants. Fenugreek, or *Trigonella foenum-graceum* (Fabaceae), is the most commonly used over the counter (OTC) herbal galactagogue in published literature, yet the mechanism of action and exact compound(s) behind its reported lactation enhancing activity is unclear (Mortel & Mehta, 2013). However, since Fenugreek has not only been used as a galactogogue, but also for its hypocholesterolemic, aphrodisiac and hypoglycemic activities among many others, compounds exhibiting hormonal activity like phytoestrogens may be present (Parthasarathy et al. 2008). Estrogenic activity of Fenugreek seeds was confirmed by Sreeja et al. in a study in which MCF-7 cells were treated with a chloroform extract of the seeds. This resulted in the discovery that molecules in the extract bind to the ER and act as an agonist for ER mediated transcription (Sreeja et al. 2010).

Despite studies that have confirmed Fenugreek's estrogenic activity, there is still conflicting evidence on its efficacy (Mortel & Mehta, 2013). Currently, the most supported hypothesis for Fenugreek's reported estrogenic activity is the presence of diosgenin in Fenugreek

seeds, which is the main natural steroidal sapogenin used in the commercial manufacture of steroidal drugs like cortisone and sexual hormones (Mortel & Mehta, 2013; Brenac & Sauvaire, 1996; Kang et al. 2013, Chan & O'Malley, 1976). However, there are many other steroidal saponins present in Fenugreek that have the potential to be structurally similar to endogenous estrogen (Kang et al. 2013, Chan & O'Malley, 1976). Furthermore, Fenugreek has diverse chemistry that goes beyond steroidal saponins like flavonoids, coumarin compounds and alkaloids which could contribute to its estrogenic activity (Chan & O'Malley, 1976). Reported flavonoids present in Fenugreek include vitexin, tricin, naringenin, quercetin, kaempferol, and luteolin, shown in Figure 3 (Parthasarathy et al. 2008). One study even reports the presence of the isoflavones daidzein and genistein in Fenugreek, however this finding is not well documented in the scientific literature and needs to be confirmed (Kaur, 1998).

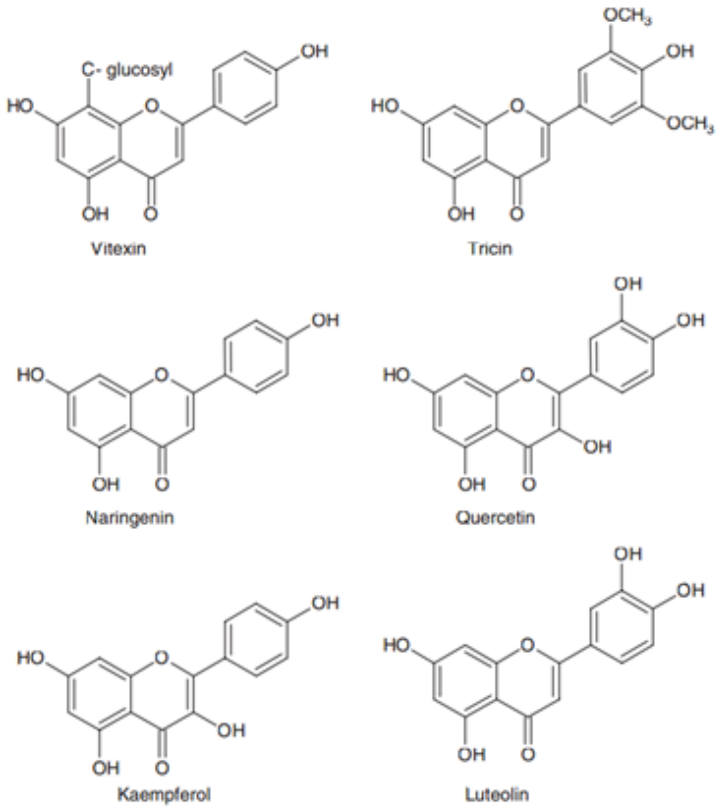


Figure 3. Flavonoids found in Fenugreek (Parthasarathy et al. 2008)

Overall, Fenugreek is a good candidate for a plant possessing phytoestrogens. Its widespread uses may imply that a hormonal modulator is at play and its diverse chemistry gives a robust collection of compounds with the potential to exhibit estrogenic activity, which has been confirmed various times. Nonetheless, the exact chemistry behind this reported estrogenic activity is ambiguous and may be due to the synergistic effect of several different phytoestrogens that include saponins, isoflavones, or other chemical compounds. The elucidation of phytoestrogens in Fenugreek bears significance in both the pharmaceutical and toxicological realms of science since estrogens exhibit such a broad range of effects in humans. Although this study does not focus on the toxicology of phytoestrogens, as with any therapeutic use of a plant, the toxicology of a plant must be taken into consideration. This study can be used to alert the potential adverse effects of Fenugreek in individuals sensitive to estrogen exposure, like certain Breast Cancer patients/survivors, and raise awareness for safer medicinal uses of Fenugreek based on this particular endocrinopharmacology (Patiaul & Jefferson, 2013).

Nevertheless, the aim of this investigation is to isolate the chemical compound(s) responsible for Fenugreek's purported estrogenic activity utilizing an estrogen activity reporting biological system to guide the fractionation of active crude extracts.

II. Methods and Materials

i) Plant Processing

Commercial seeds of Fenugreek (*Trigonella foenum-graecum*, Fabaceae) were obtained from Greenstar Coop. Natural Foods Market in Ithaca, NY. The Fenugreek seeds used for a comparative analysis to chemical composition between cultivars were obtained from Wegmans in Ithaca, NY. The seeds (250g) were finely ground in a Waring blender and transferred into a flask with 1.5L of Hexane. The flask was placed on a magnetic stirring plate for two days for extraction. After two days the Hexane solution was filtered by gravity into an Erlenmeyer flask using Whatman No. 1 filter paper. The filtrate was collected as the hexane extract and the filter papers with the solid residue were allowed to dry in a fume hood. The filtrate was evaporated to dryness in a Buchi RII Rotary Evaporator, the yield of the dried hexane extract calculated, and the residue re-suspended in Hexane. A hexane fenugreek extract (HFG) at a concentration of 235.4 g/L resulted from this extraction. To approximate the molar concentrations of the extracts, the average molecular weight of the compounds was assumed to be 250g/mol. Consistent with Lipinski's rule, it has been proved from a drug-like natural product library of 126,140 compounds that the average molecular weight of natural products, like the secondary metabolites being isolated in fenugreek, have an average molecular weight of 250g/mol (Quinn et al. 2008). Once the solid seed residue was dried, the same extraction procedure was followed in subsequent extractions using a semi-polar solvent mixture of 1:1 Chloroform/Methanol and then a polar solvent of 70% aqueous ethanol. This resulted in a chloroform/methanol fenugreek extract (CMFG, 129.6 g/L) and an aqueous ethanol fenugreek extract

(AEFG, 312 g/L). Further fractionation of the semi-polar extraction was performed by partitioning the extract into a methanol soluble portion and a chloroform soluble portion. This was done by evaporating the CMFG extract to dryness and mixing the dried residue with methanol in the rotary evaporator for 30min over in a 30°C water bath. This solution was then filtered and evaporated to dryness. The same procedure was performed again, but with chloroform as the solvent. The resulting fractions were 262.5g/L and 76g/L for the methanol soluble portion (mFG) and chloroform soluble portion (cFG) respectively.

ii) Thin Layer Chromatography Analysis

Silica gel Thin Layer Chromatography (TLC) was used to profile the chemical composition in various extracts and diagnose for the presence of specific compounds using various spray reagents. TLC Silica gel 60 F_{254} 5X10cm glass plates (Merck, Germany) were used to perform the chromatography. The main solvent system used was ethyl acetate/methanol/water (10:2.7:2). Various visualization and diagnostic spray reagents were used on the TLCs (i.e., UV light short/long wavelength, vanillin, $FeCl_3$, iodine). The Liebermann-Burchard reagent (5mL acetic anhydride, 2.5mL sulphuric acid, 2.5mL ethanol) was used to detect the presence of steroidal compounds.

iii) Ferric Chloride Diagnostic Test

The ferric chloride spray reagent was used as a diagnostic test for phenols. Ferric Chlordie (0.25g) and 95% aqueous ethanol 925mL) were mixed to create the spray reagent. The reagent was sprayed onto developed TLC plates to test for the presence of phenols. Since ferric chloride is an oxidizing and chelating agent, it causes a color change with the presence of phenolic compounds by the production of a coordination

complex formed between the iron(III) and three phenolate ions (Mohrig et al. 2003). Not all phenols produce colored complexes under test conditions, but if a color change is apparent, a phenol is present. The only exception to this observation is the development of a blue dark color caused by certain non-phenolic compounds. Apart from this exception, catechols produce a green color with the ferric chloride spray reagent, phenols with H-bonded OH groups produce a purple color and highly acidic phenolic OH groups give a brown color (Krishnaswamy, 1999).

iv) Column Chromatography Fractionation of Crude Extracts

A Sephadex LH-20 Lipophilic size-exclusion column chromatography was used to fractionate the mFG extract using methanol as the eluent. Eight fractions resulted from this fractionation according to their molecular size. The different bands were distinguished by color under visible light and their fluorescence, if any, under a portable UV lamp. The fractions resulting from this column have been assayed for estrogenic activity.

v) Preparative Thin Layer Chromatography

Preparative Thin Layer Chromatography (TLC) was used to separate the distinct bands present on the TLC for mFGcons. A Silica gel 60 F_{254} 20x20cm glass plate (Merck, Germany) was used to separate the compounds in mFGcons using an ethyl acetate: methanol: water (10:7:2) solvent system. After the TLC was developed in a glass chamber, a pencil was used to indicate the bands that had been separated and the silica gel was etched off of the glass plate using a chisel for each band. Each of the sets of chiseled silica gel were ground to powder using a mortar and pestle, dissolved in methanol, and filtered through a Whatman No. 1 filter paper.

vi) Yeast Transformation to Construct a Strain that Expresses the Human Estrogen Receptor

The yeast strain, *Saccharomyces cerevisiae*, YGY13 (*MAT***a** *ade1 leu2-3, 2-112::UASGAL10-lacZ- LEU2 his3*Δ *gal4*Δ *ura3-52*) was transformed with plasmid pGEV-HIS3 (Gao, 2000) using the "lazy bones" plasmid transformation of yeast colonies protocol described in Burke *et al.* (2000). Briefly, a single yeast colony was incubated with 1 µg plasmid pGEV-HIS3 and 100 µg herring sperm DNA in PLATE solution (40% polyethylene glycol (MW 3350), 0.1 M lithium acetate, 10 mM Tris-HCl pH 7.5, and 1 mM EDTA) containing dimethyl sulfoxide at 30 °C for 15 min and heat shocked at 42 °C for 15 min. Cells were subsequently pelleted, re-suspended in water, and plated on chemically defined growth medium lacking histidine. Transformants were isolated after incubation at 30 °C for 3 days and used to inoculate experimental cultures.

vii) Quantification of Estrogenic Activity using a β-galactosidase Reporting Assay

The estrogenic activity is measured as β-Galactosidase activity using the commercial β-Galactosidase Assay Kit (Thermo Scientific). To run the assay, transformed yeast cells were grown overnight in test tubes with an I^+-His medium in a shaker incubator at 30^0C. Then, the OD of the cultures was measured at 600nm in a UV-vis spectrophotometer and cultures were diluted back to an OD of around 0.4. Theses test tubes were then placed back in the incubator until the cells reached an OD between 1.0 and 1.5, or the exponential phase of yeast cell growth. Once the cells reached this point, they were ready to be used in the β-galactosidase Assay. For each assay performed, an estradiol (100nM) treatment was used as a positive control and a

water treatment was used as the control. Other materials used in this assay include a water bath at 37^0C, Eppendorf tubes, pipettes, vortex mixer, centrifuge and the Y-PER Yeast Protein Extraction Reagent, 2X β-Galactosidase Assay Buffer, and β-Galactosidase Assay Stop Solution. Final absorbance of the samples at 420 nm was measured and β-Galactosidase expressed as Miller Units. β-Galactosidase measurements are interpreted as estrogenic activity measurements. The following equation is used to calculate estrogenic activity in Miller units (Instructions: β-Galactosidase Assay Kit, 2012).

$$\beta galactosidase\ activity = \frac{1{,}000 * A_{420}}{t * V * OD_{660}}$$

$$t = time\ of\ incubation = 15min$$

$$V = volume\ of\ cells\ used\ in\ the\ assay = 2.5mL$$

$$A_{420} = absorbance\ of\ treatments\ at\ 420nm$$

$$OD_{660} = opitcal\ density\ of\ cells\ before\ treatment$$

viii) Electrospray Ionization Tandem Mass Spectrometry (ESIMS/MS) Analysis

Mass spec work was done at Cornell University at the Proteomics and Mass Spectrometry Facility at the Institute of Biotechnology. Samples were prepared for ESIMS/MS at appropriate concentrations and cleaned from particulates with the aid of a syringe filter (PTFE or Nylon respectively; 0.2 mm, Alltech). The samples were analyzed in the negative ion mode using Electrospray Ionization Tandem Mass Spectrometry (ESIMS/MS) in a 4000 Q Ion Trap LC/MS (Applied Biosystems) spectrometer, at an infusion rate of 10ml/hr and a capillary voltage of ±3500V. Mass spectra were acquired and selected parent ions were subjected to MS/MS analysis.

ix) Statistics

The statistical software Matlab R2014a, The MathWorks, Inc. (Natick, Massachusetts, United States) was used to perform statistical analysis on the data. A two sample t-test was used to compare estrogenic activity between samples, using the function “ttest2”. Results were considered statistically significant if $p<0.05$ or otherwise noted. Microsoft Excel 2013 (Redmond, Washington) was used to calculate the standard error of the estrogenic activity of samples.

III. Results and Discussion

To establish the putative estrogenic activity of fenugreek seeds, crude extracts were prepared using organic solvents with increasing polarity. Three extracts were prepared, a non-polar extract using hexane (HFG), an intermediate polarity extract with a mixture of chloroform/methanol (1:1) (CMFG), and a polar extract prepared with 70% aqueous ethanol (AEFG).

The outline of the investigation is detailed in Figure 4. The overarching guiding principle of the research process in this project was to use the biological activity of the extracts to lead to the chemistry associated with the estrogenic activity and ultimately elucidate the phytoestrogens present in Fenugreek. In this case, the estrogenic activity of extracts tested using the β-galactosidase reporting assay was used to decide which fenugreek fractions would be pursued for elucidation of the chemistry responsible for the reported estrogenic activity.

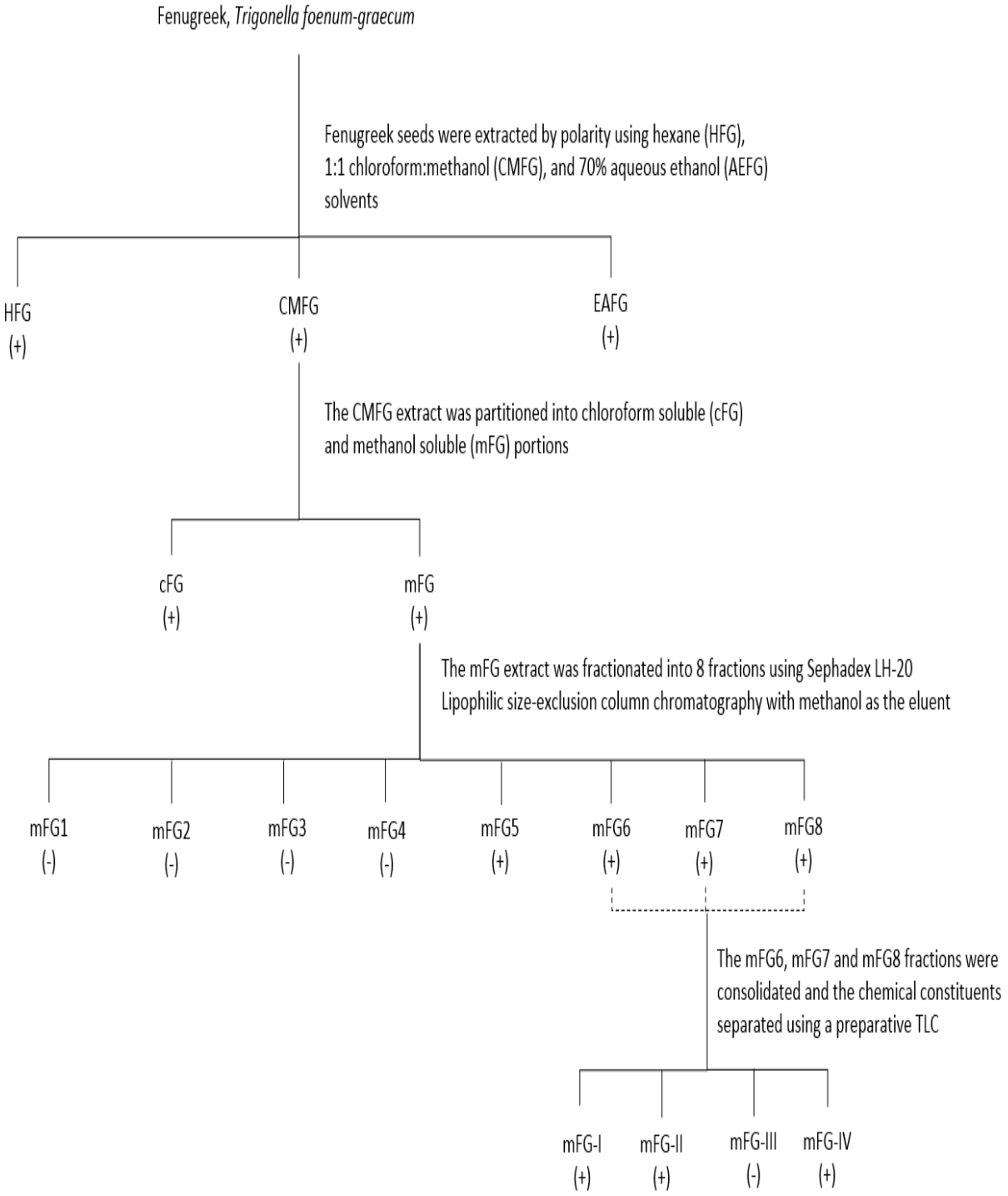


Figure 4. Flow chart of the bioassay guided fractionation of fenugreek seeds to isolate chemistry with estrogenic activity. Estrogenic activity: (+) present, (-) absent

Two commercial samples of fenugreek seed were purchased locally and used for the project. The chemical composition profiles of the hexane, chloroform/methanol and aqueous alcohol extracts were thoroughly compared for consistency between cultivars. The chemical composition of both fenugreek seeds was shown to be basically identical. To come to this conclusion, TLC was used exhaustively using various solvent systems and visualization techniques including several spray reagents. Figure 5 shows the similarity between fenugreek seeds obtained from two different venders. Additionally, the chemical stability of fenugreek seed constituents from the same vendor extracted one year apart was verified by examining old and new extracts to validate using samples from the previous year's extraction in the research. Figure 6 shows the chemical stability of fenugreek seed constituents over this time span. Although the TLC's of the new extracts were applied to the TLC plate in higher concentrations than the old extracts (evident by the bolder spots), the same chemical compounds appear to be present in each of the samples. This concludes that the chemistry present in the fenugreek seed extractions is relatively stable over time.

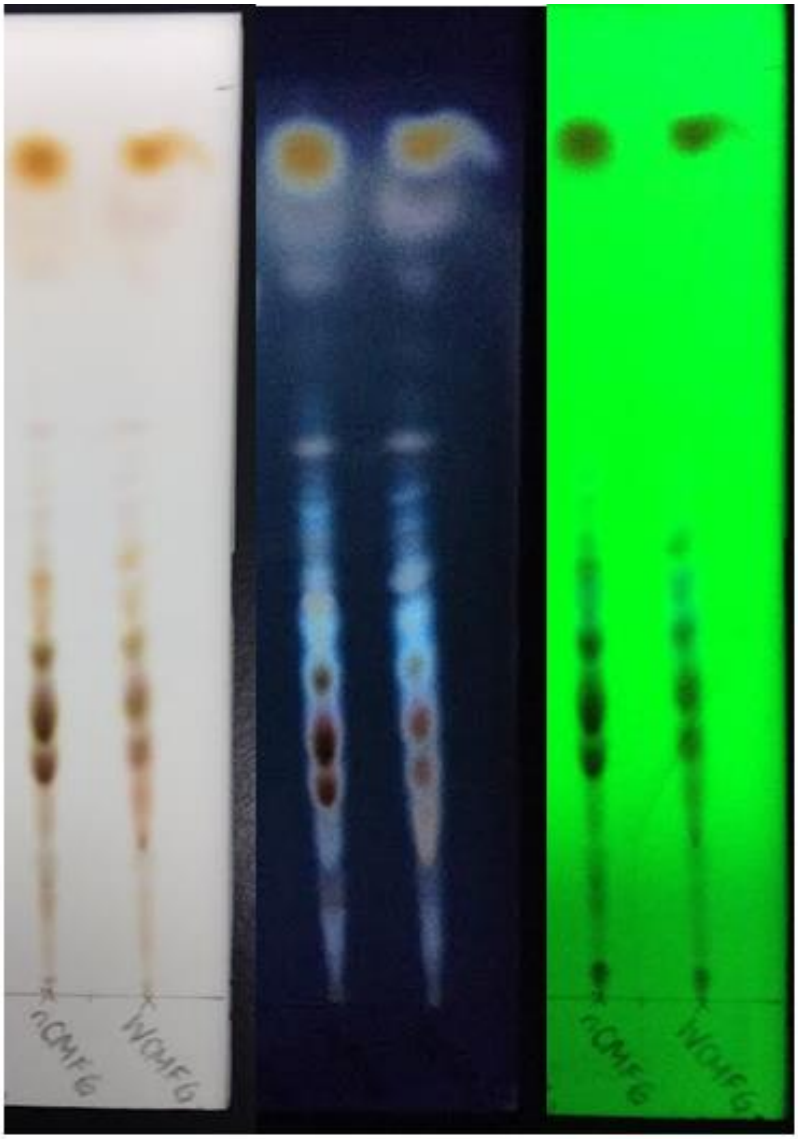


Figure 5. The CMFG extract purchased from Greenstar Coop. Natural Foods Market in Ithaca, NY (nCMFG) compared to fenugreek seeds purchased from Wegmans in Ithaca, NY (WCMFG). An ethyl acetate:methanol:water (10:2.7:2) solvent system with Liebermann-Burchard reagent applied. Plate viewed under normal lighting, long wavelength and short wavelength UV (left to right).

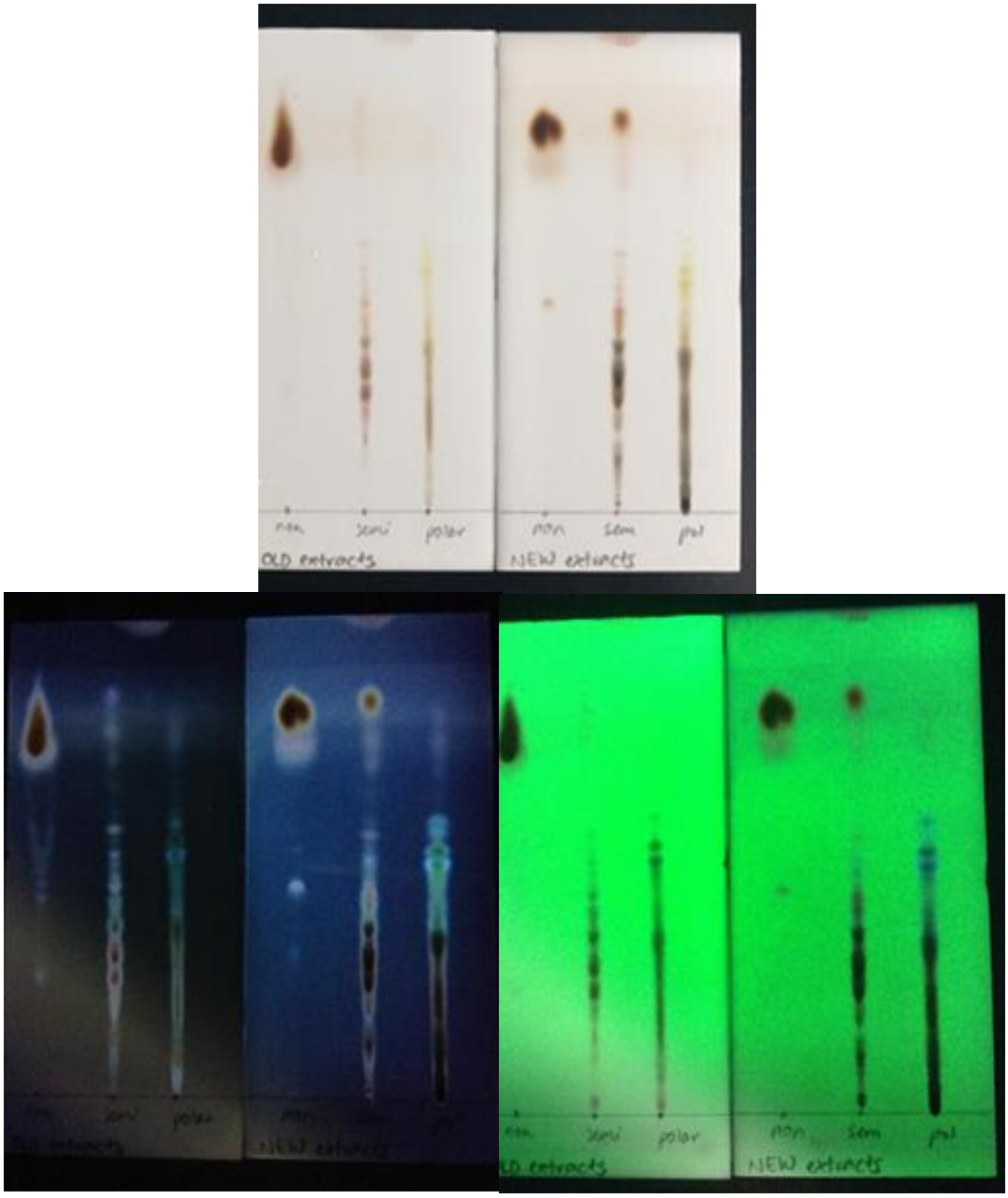


Figure 6. A comparison of two distinct Fenugreek seed extractions separated using an ethyl acetate:methanol:water (10:2.7:2) solvent system with Liebermann-Burchard reagent applied. Three extractions, HFG, CMFG, AEFG (from left to right), are shown on each plate. The plates are presented under normal lighting (top), long wavelength UV light and short wavelength UV light (from left to right).

The HFG, CMFG and AEFG extractions were tested for estrogenic activity in the β-galactosidase reporting assay. The plasmid containing the human estrogen receptor used in the transformation of the yeast cells is shown in Figure 7 and the protocol for this assay is shown in Figure 8. The manner in which the assay functions is that if estrogen, or an estrogen mimic, is present in the medium the cells are growing in, it will bind to the human β-estradiol α-receptor (hER) that is present in the promoter region of the pGEV-HIS3 plasmid. The promotor region includes a vector encoding the β-estradiol inducible activator, GAL4.ER.VP16 (GEV), which is analogous to the steroid inducible Gal4p-directed expression systems in mammalian cells. Once an estrogenic compound binds to hER, the expression of GAL4 is induced. GAL4 is a protein that is a positive regulator for the expression of galactose-induced genes such as GAL1 and GAL10. The expression of these galactose-induced genes produces β-galactosidase, an enzyme generally used to convert galactose to glucose. In this assay, the β-galactosidase activity is measured by its hydrolyzing action on the substrate *o*-nitrophenyl-β-galactopyranoside (ONPG) that releases *o*-nitrophenol (ONP), which is measured at 420nm. This produces a system in which the expression of β-galactosidase, and as a result, formation of ONP, is dose dependent on the amount of estrogen or estrogen like compounds the cells are exposed to (Gao & Pinkham, 2000).

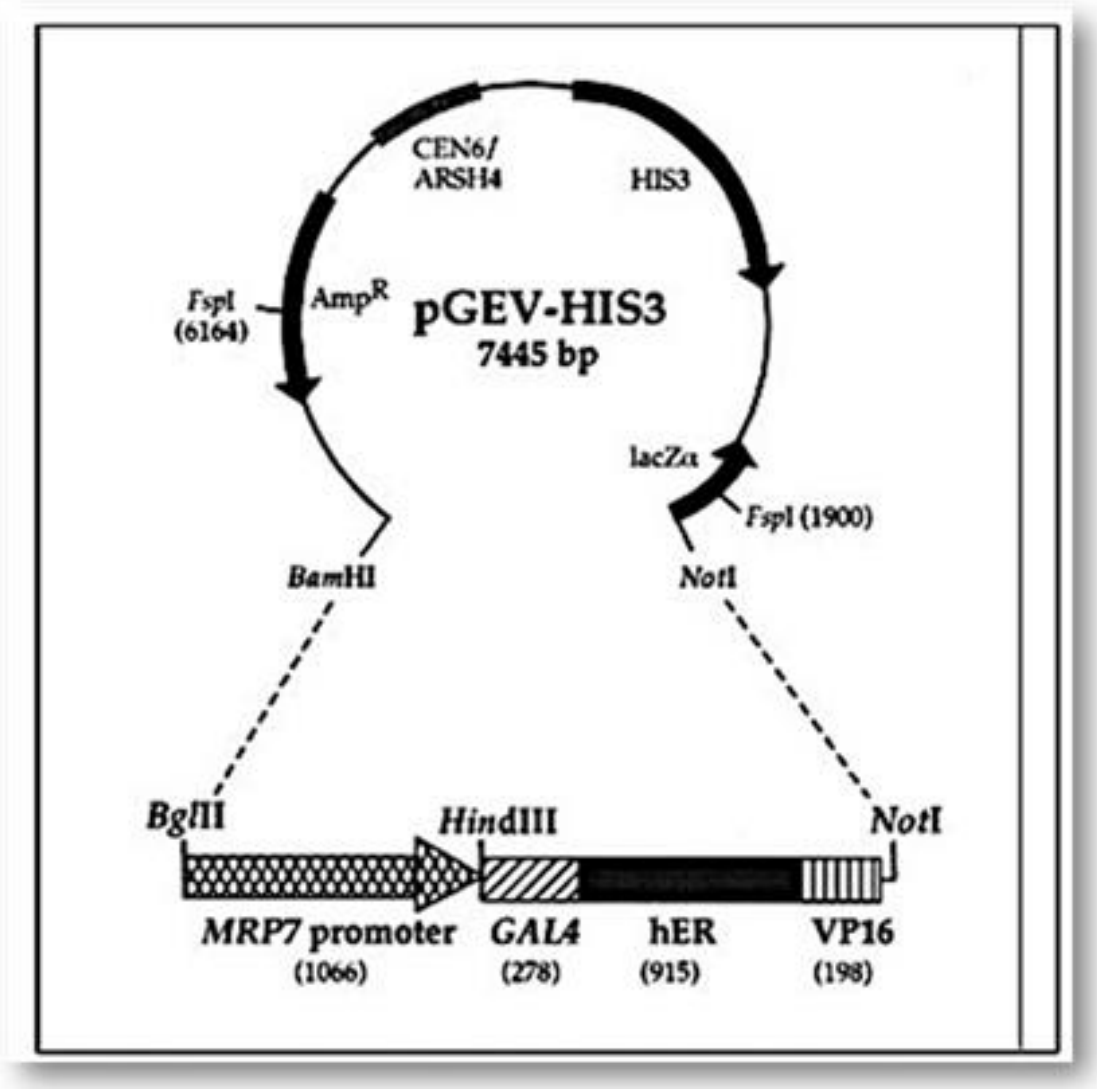


Figure 7. The pGEV-HIS3 plasmid used in the transformation of yeast cells with the human estrogen receptor. The GEV chimera contains the following domains: the N-terminal 93 amino acids (aa) of Gal4p, aa 282-576 of the human β-estradiol α-receptor, the C-terminal 424-490 aa of VP16. The numbers in parenthesis indicate the positions of *FspI* sites in pGEV-HIS3, the sizes of the DNA fragments encoding the MRP7 promoter, and the domains of the chimeric activator in base pairs (Gao & Pinkham, 2000).

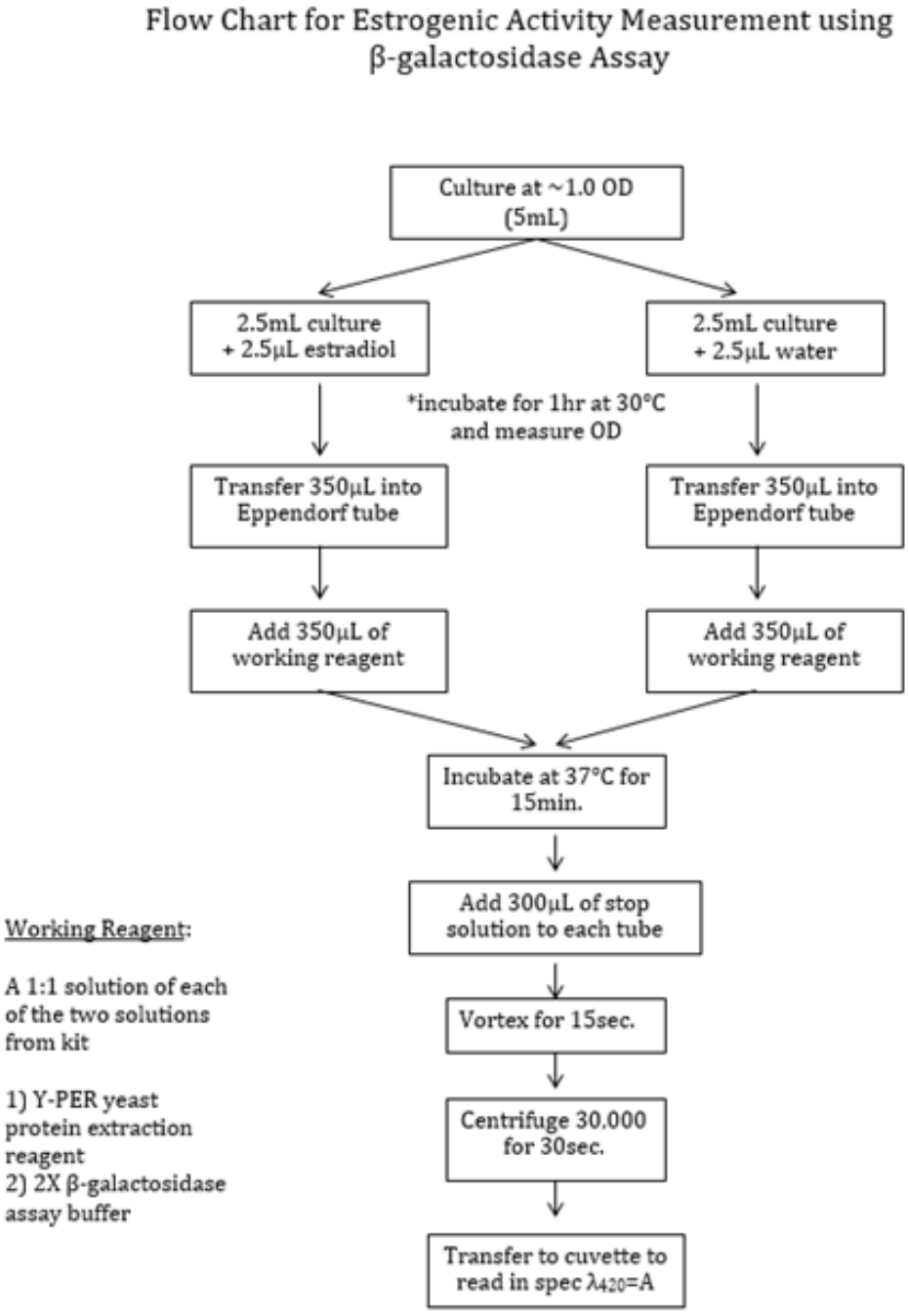


Figure 8. A simplified outline of the protocol used to measure estrogenic activity using the β-galactosidase assay.

In the first step of the bioassay guided fractionation of fenugreek seeds, the comparative assessment of the estrogenic activity of the various extracts prepared from fenugreek seeds (Figure 9) showed higher estrogenic activity for the CMFG sample than the AEFG and HFG. The extracts were tested in duplicate for this assay and the estrogenic activities of HFG, CMFG and AEFG were found to be 13.3 Miller units/M, 18.2 Miller units/M and 7.10 Miller units/M respectively. The estrogenic activity of the HFG was statistically significantly different ($p<0.05$) from the CMFG with $p=0.0168$ as well as the AEFG, with $p=0.00810$. The estrogenic activity of CMFG compared to AEFG was not statistically significant, with a p-value of 0.0780. However, since the difference between the estrogenic activity of CMFG and AEFG was significant to the level of significance $p<0.10$, this was convincing enough to pursue the CMFG extract for further study.

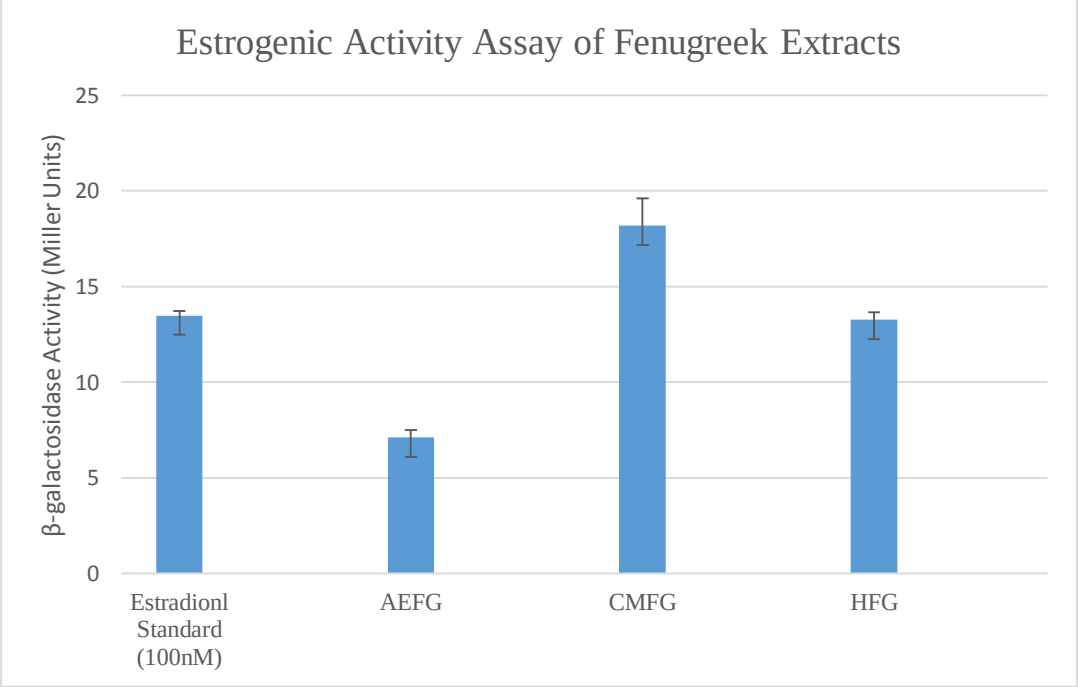


Figure 9. Estrogenic activity of nonpolar (HFG), semi-polar (CMFG) and polar (AEFG) fenugreek extracts with the standard error shown (SE=0.257, 0.391, 1.42, 0.398 from left to right). The estradiol standard is at a concentration of 100nM while the extracts are at concentrations of ~1M.

The presence of diosgenin in fenugreek that is reported in the literature (Mortel & Mehta, 2013; Brenac & Sauvaire, 1996; Kang et al. 2013, Chan & O'Malley, 1976) was confirmed using TLC analysis, diagnostic reagents for steroidal saponins (ie. Liedermann Burchard), and mass spectrometry. A commercial diosgenin standard (93%, Sigma Chemical Company) was used to compare to the HFG, CMFG and AEFG extractions. Use of the Liebermann-Buchard reagent guided the identification of diosgenin (Figure 10).

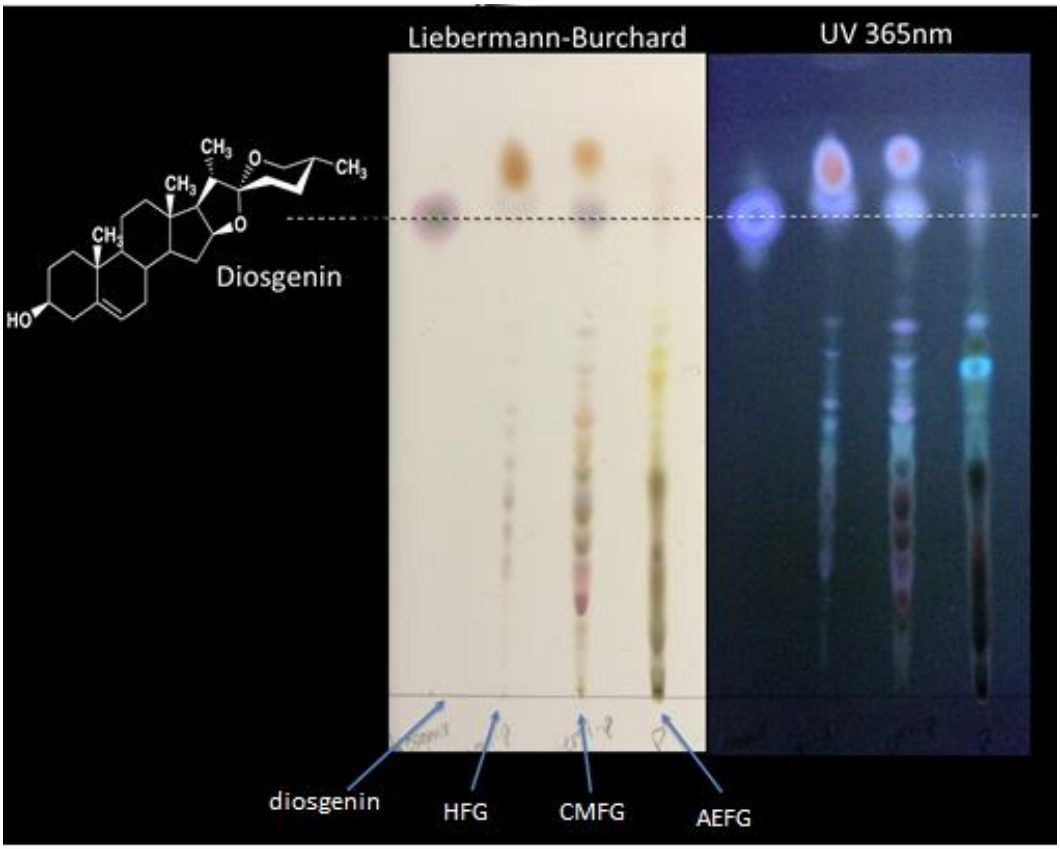


Figure 10. A TLC of diosgenin, HFG, CMFG and AEFG extracts run in an ethyl acetate:methanol:water (10:2.7:2) solvent system and sprayed with the Liebermann-Buchard reagent. The TLC plate is shown under normal light and long wavelength UV light (left to right).

Further analysis of the CMFG extract using mass spectrometry confirmed the presence of diosgenin (Figure 11). The estrogenic activity of the diosgenin standard was verified (Figure 12), which confirms that if the CMFG extract contains diosgenin, part of its estrogenic activity is due to the presence of diosgenin.

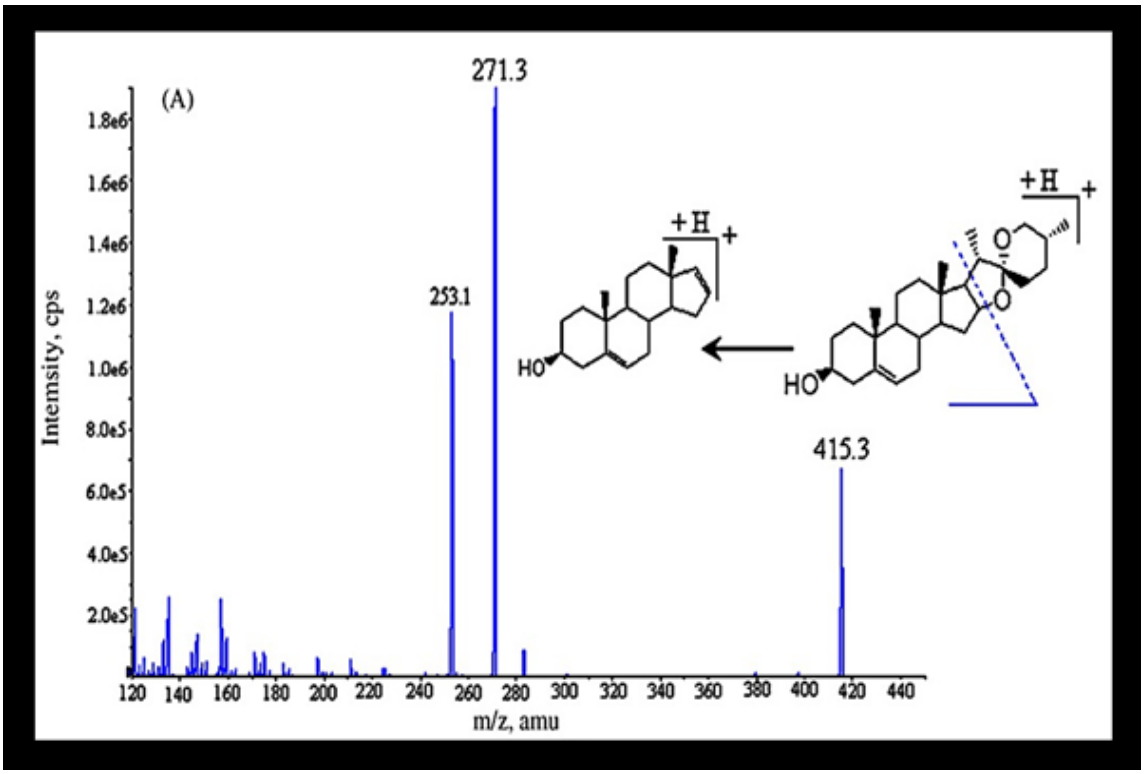


Figure 11. The product of ion mass spectra of diosgenin.

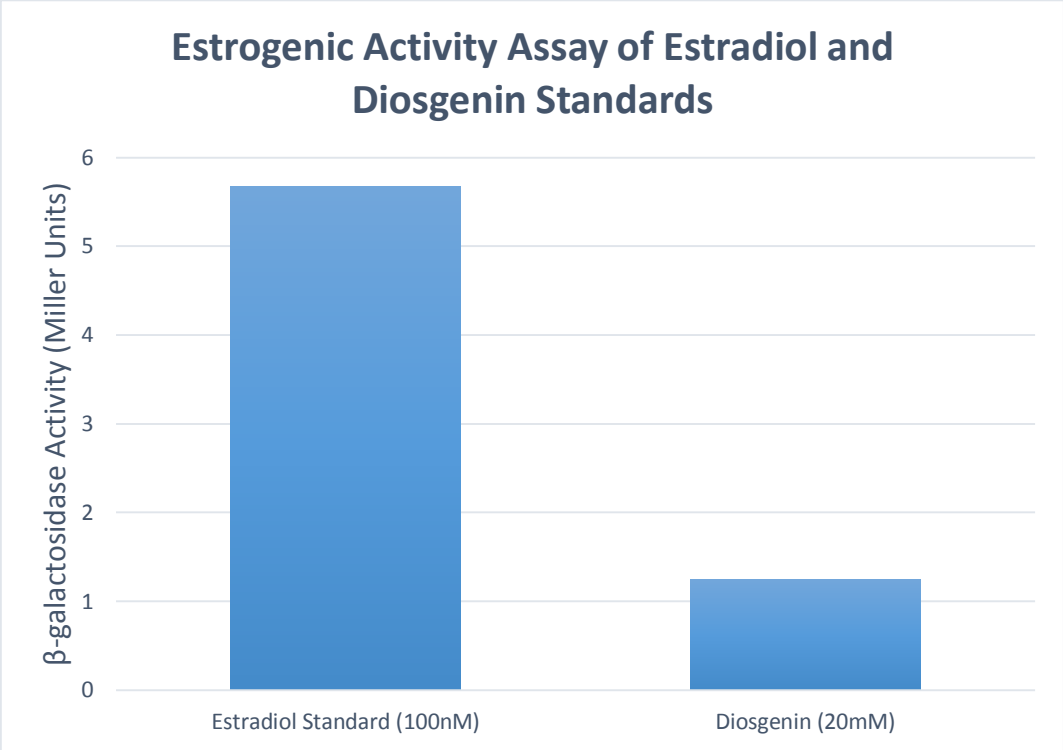


Figure 12. The measured estrogenic activity of the estradiol (100nM) and diosgenin standards (20mM) reported using the β-Galactosidase Estrogenic Activity Assay.

The CMFG fraction was further investigated by bioassay guided fractionation to assess the presence of any other additional estrogenic components. To initiate the fractionation of the CMFG extract, it was partitioned into its methanol soluble fraction (mFG) and chloroform soluble fraction (cFG) (see methods section). The composition of the CMFG extract with its resulting fractions is shown in the TLCs in Figure 13. However, the CMFG and cFG separated into two phases, a bottom phase (bp) and a supernatant phase (sp). Since the diosgenin is not present on the TLC in Figure 13, it is hypothesized that the diosgenin is present in the bottom phase of the cFG, which was not applied to the TLC. Figure 13 shows the supernatant cFG (cFGsp) and mFG compared to the supernatant CMFG (CMFGsp).

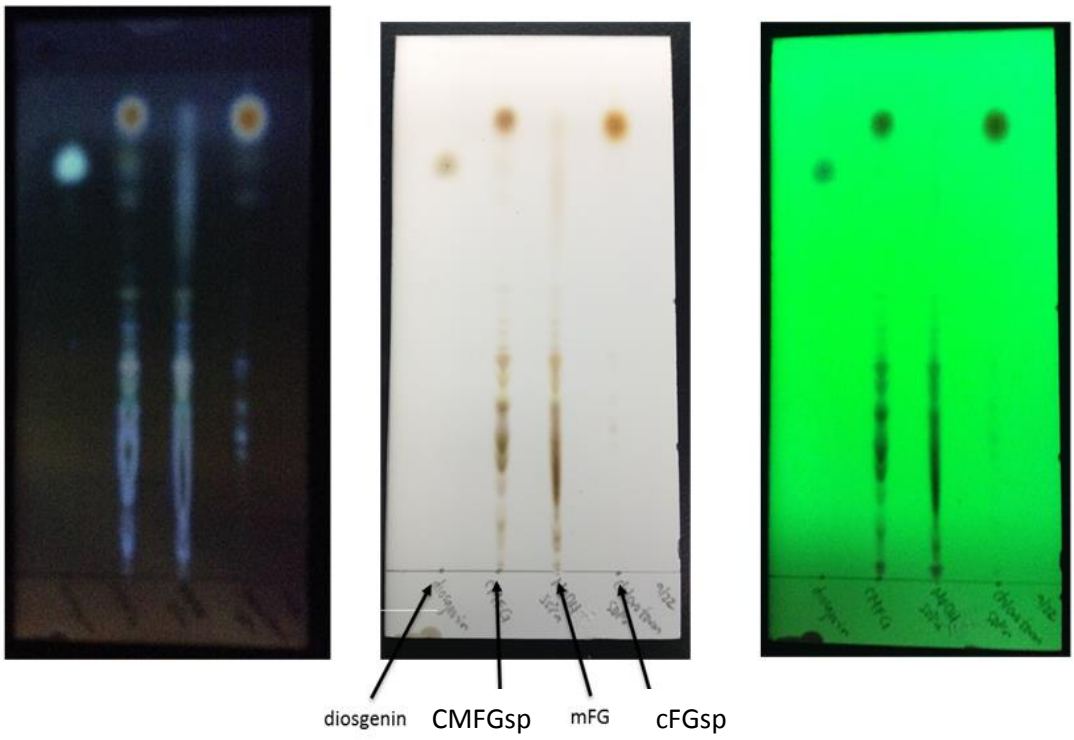


Figure 13. An ethyl acetate:methanol:water (10:2.7:2) solvent system was used with the Liebermann-Burchard reagent applied to visualize the cFGsp and mFG portions of the CMFG. Plate viewed with long wavelength UV, normal lighting, and short wavelength UV (left to right).

The estrogenic activity of the cFG and mFG samples was measured using the β-galactosidase assay. Estrogenic activities were 1.00 Miller unit/M, 1.28 Miller units/M and 0.35 Miller units/M for CMFGsp, mFG and cFGsp respectively. Results are shown below in Figure 14. To qualify these results, they suggest that the estrogenic activity of CFGsp is notably reduced compared to CMFGsp and mFG. Since the methanol portion (mFG) reported more estrogenic activity than the chloroform portion (cFGsp), the estrogenically active chemistry from the CMFG extract was preferentially soluble to methanol and this fraction was further pursued to isolate the estrogenic compounds. Although there weren't replicates produced in order to perform statistical

analyses, the main purpose of these assays is to test for the presence or absence of estrogenic activity in order to reach the isolation of compounds that are estrogenically active. Since all of these extracts possess estrogenic activity, if any of them were pursued, the isolation of estrogenically active compounds would be likely. However, under the time constraints, the fraction with the highest amount of estrogenic activity was pursued in each subsequent round of estrogenic activity assays.

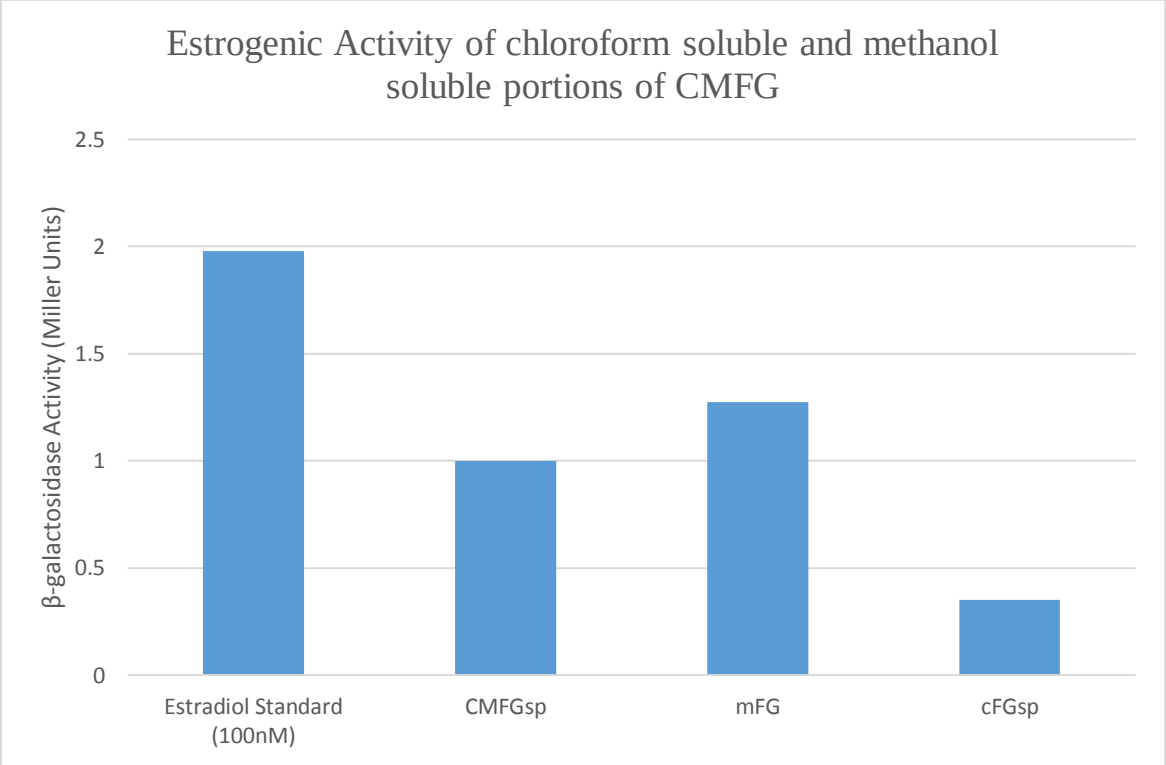


Figure 14. Estrogenic activity of supernatant of CMFG (CMFGsp), the methanol soluble portion of CMFG (mFG) and the supernatant of the chloroform soluble portion of CMFG (cFGsp) fenugreek extracts. The estradiol standard is at a concentration of 100nM while the extracts are at concentrations of ~1M.

The mFG extract was fractionated using size exclusion Sephadex LH-20 column chromatography, shown in Figure 15. Based on the different bands distinguished by color under visible light and their fluorescence, if any, under a portable UV lamp, eight fractions resulted from the fractionation.

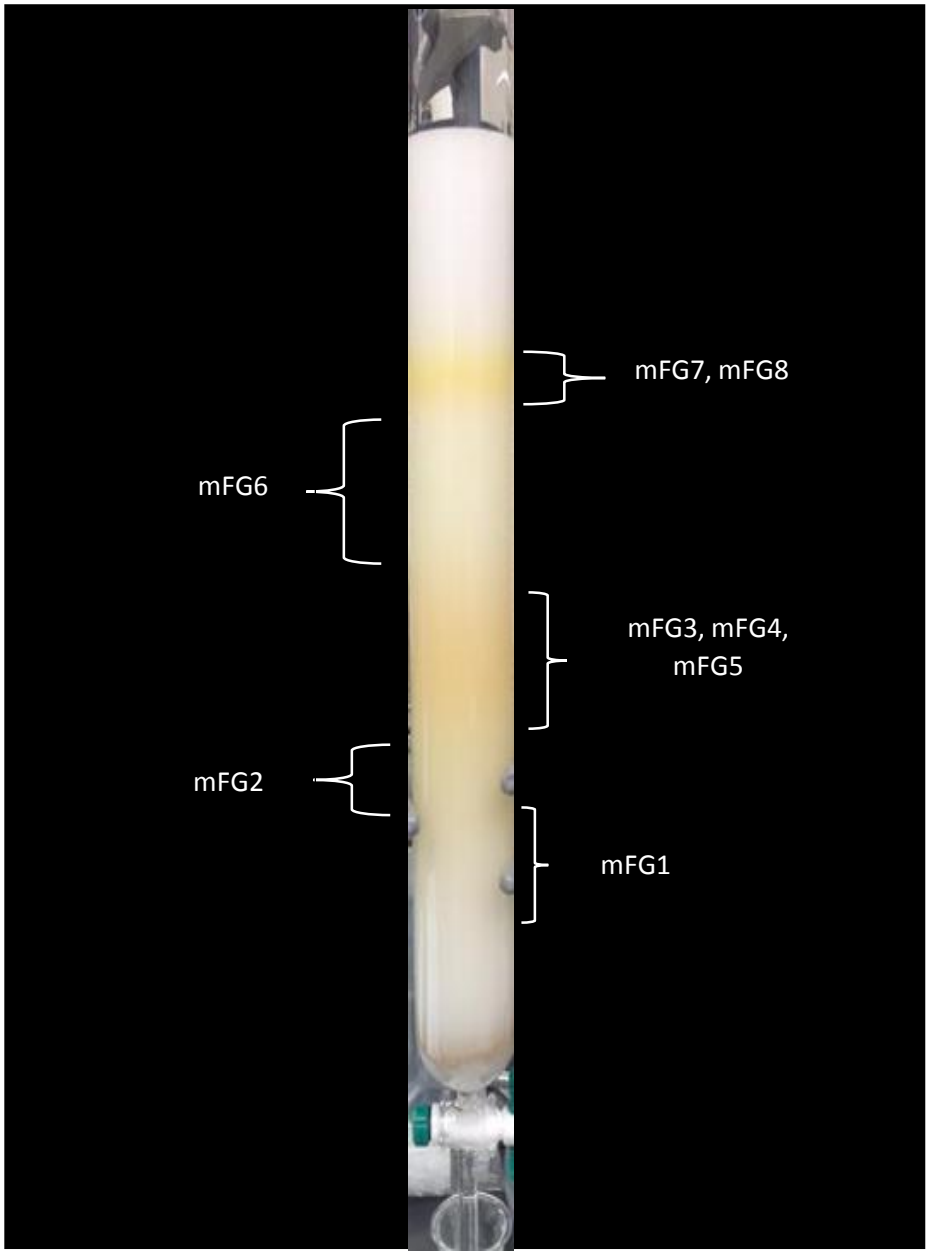


Figure 15. Fractionation of mFG using Sephadex column chromatography with methanol as the solvent system. Fractions were labeled mFG1-mFG8 as they exited the column.

Subsequently, the estrogenic activity of the methanol fractions, mFG1-mFG8, was determined using the estrogenic activity β-galactosidase assay. The concentration of each fraction was standardized to a concentration of 0.1g/mL. However, it must be taken into consideration that the minute amounts of methanol used to re-suspend some of the fractions and the limitations of the mass balance may have caused some error in the standardization of concentrations. The results for the estrogenic activity were as follows: mFG (9.78 Miller units/M), mFG1 (0 Miller units/fraction), mFG2 (0.17 Miller units/fraction), mFG3 (0 Miller units/fraction), mFG4 (0 Miller units/fraction), mFG5 (5.06 Miller units/fraction), mFG6 (9.03 Miller units/fraction), mFG7 (6.53 Miller units/fraction), mFG8 (4.54 Miller units/fraction). Results are shown in Figure 16.

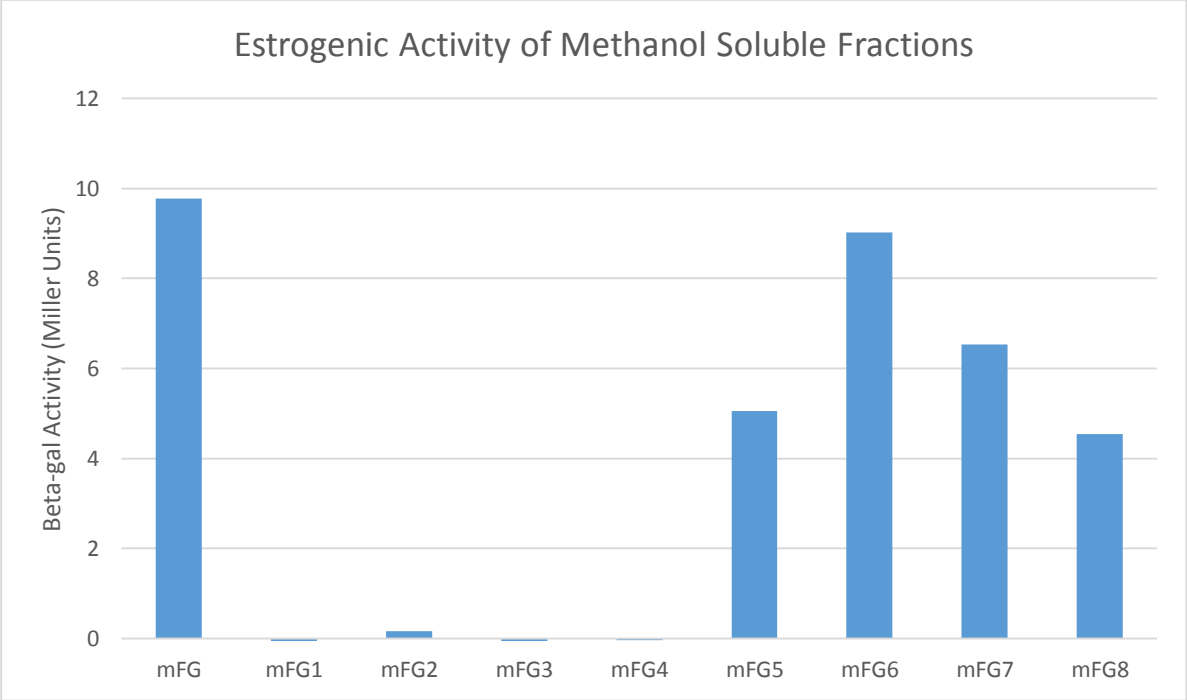


Figure 16. The estrogenic activity reported in Miller units of the mFG1-mFG8 fractions separated from the mFG extract using size exclusion Sephadex LH-20 column chromatography. Concentrations among the fractions were not standardized.

The chemical compositions of the fractions were then examined using silica gel TLC and the vanillin spray reagent to analyze the differing chemical composition of the samples (Figure 17). As shown in the TLC's, fractions mFG6, mFG7 and mFG8 all have very similar chemical components, which are also fractions that possess estrogenic activity.

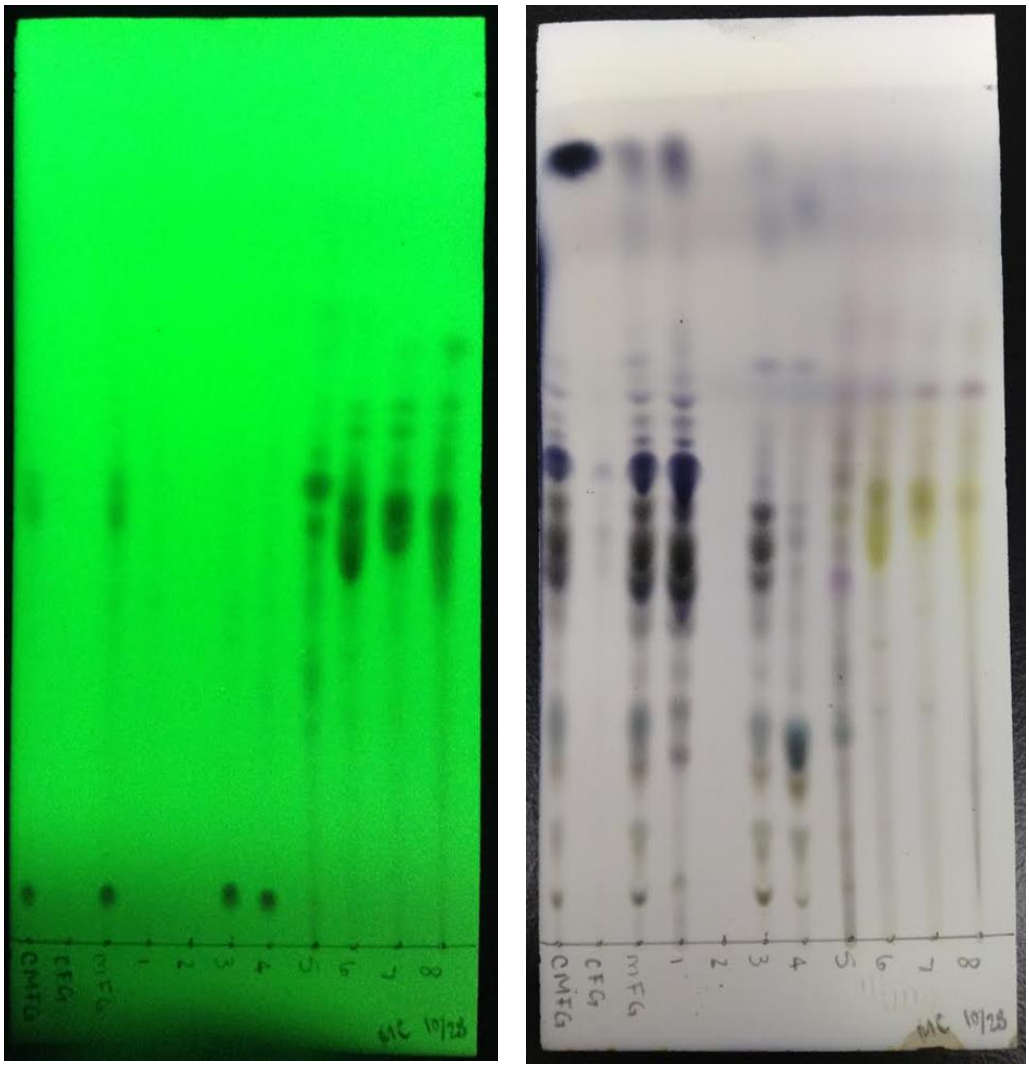


Figure 17. An ethyl acetate: methanol: water (10:2.7:2) solvent system used to separate components of the CMFG, cFG, mFG and the eight methanol fractions fractionated by the Sephadex LH-20 column (from left to right). The TLC plate on the left was examined under short wavelength UV light prior the application of the vanillin spray and the plate on the right was sprayed with the vanillin spray reagent.

For further chemical analysis, the estrogenic fractions mFG5, mFG6, mFG7 and mFG8 were examined for the presence of phenolic compounds using a ferric chloride spray reagent on a TLC plate (Figure 18). Since a brown-green color developed for spots in the lane of each of the fractions on the TLC, the test was positive for phenolic compounds in all four fractions (Krishnaswamy, 1999; Mohrig et al. 2003).

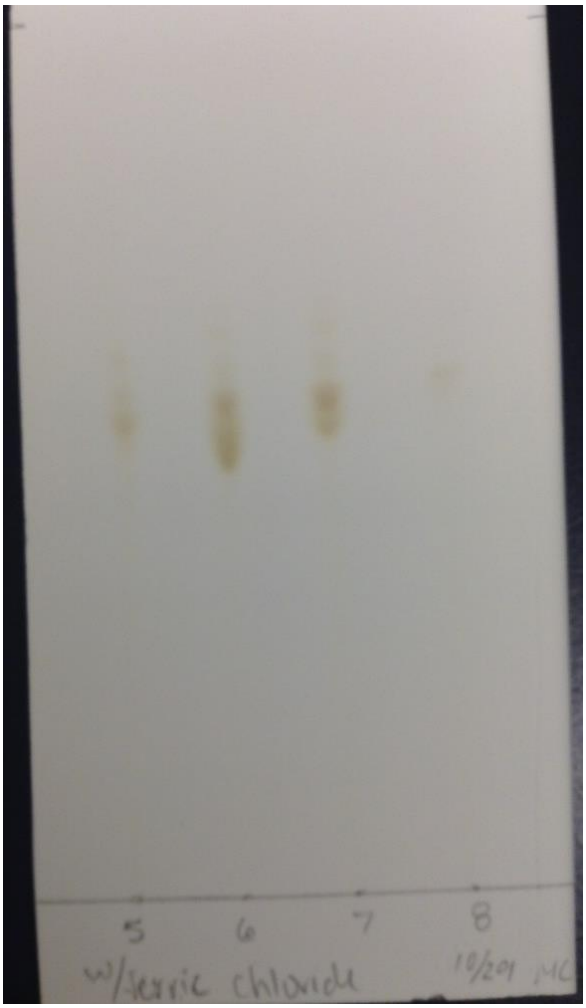


Figure 18. A TLC of mFG5, mFG6, mFG7 and mFG8 ran with a 10:2.7:2 ethyl acetate: methanol: water solvent system developed with the ferric chloride spray reagent to test for the presence of phenolics. The development of a green or brown color is a positive test for the presence phenolics.

A better solvent system was discovered to more clearly separate the compounds in fractions mFG5-mFG8, which was an ethyl acetate: methanol: water (10:7:2) solvent. Since the mFG7 fraction gave the highest yield in of extract in grams, it was examined again for the presence of phenolic compounds using the ferric chloride spray reagent to see if all of the components were phenolic in nature when spread out (Figure 19.

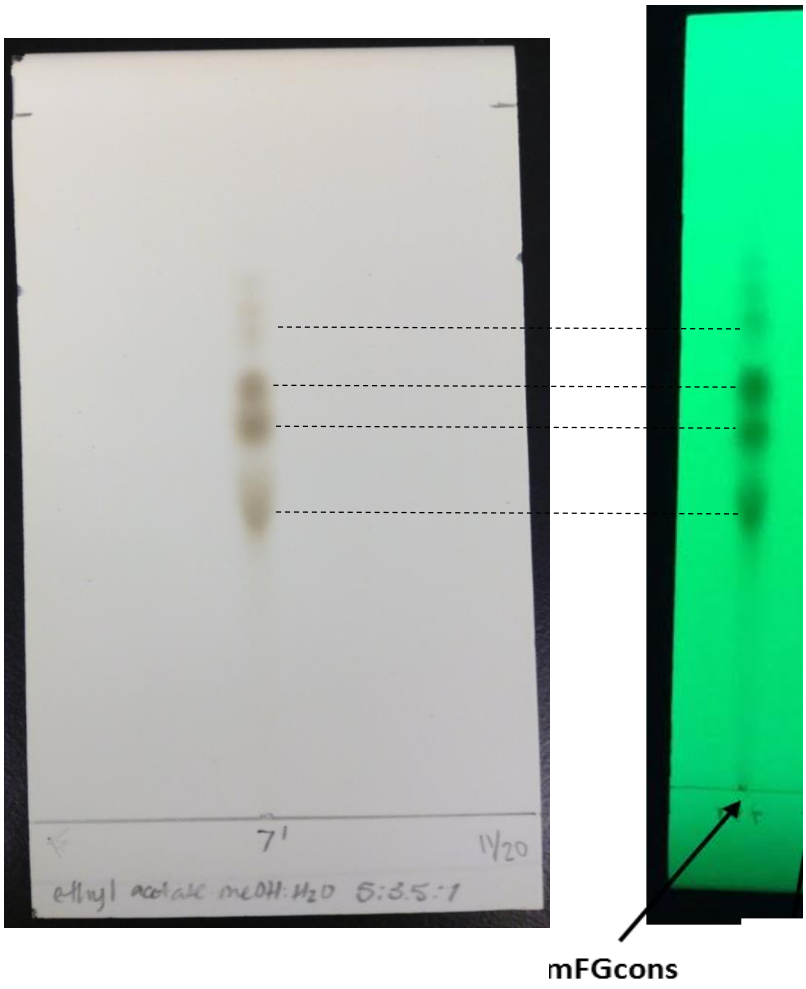


Figure 19. A diagnostic test for the presence of phenolics was performed by spraying a Ferric Chloride spray reagent on a TLC of mFG7 using an ethyl acetate: methanol: water (10:7:2) solvent system. The development of a greenish color denotes a compound phenolic in nature. The right TLC shows mFGcons under short wavelength UV. Dotted lines indicate the spots in mFG7 that correlate with the compounds present in mFGcons.

Since the majority of the spots on the TLC of mFG7 gave a positive on the ferric chloride test, the estrogenic activity is likely associated with compounds that are phenolic in nature. This result strongly suggests that one of those phenolics, or more, are linked with the estrogenic activity associated with mFGcons. Isoflavones are the known phenolic compounds that possess estrogenic activity (Messina & Wood, 2008). Therefore, one hypothesis is that the estrogenically active compounds in mFGcons are estrogenic isoflavones.

Subsequently, fractions mFG6, mFG7 and mFG8 were consolidated based on the similarity of their chemical composition, as shown in Figure 17, to produce a sample that contained the compound(s) exhibiting estrogenic activity. The consolidated fraction (mFGcons) was examined on a silica gel TLC with many different solvent systems. The solvent system that best separated the compound in mFGcons was found to be an ethyl acetate: methanol: water (10:7:2) solvent system. A preparative TLC was then used to separate the components in the mFGcons extract. The preparative TLC of mFGcons yielded four samples, mFG-I, mFG-II, mFG-III, mFG-IV. The TLC in Figure 20 shows the samples in relation to the mFGcons. As seen in the TLC, five distinct compounds can be visualized on the TLC plate (A,B,C,D and E).

The estrogenic activity of the four samples, mFG-I, mFG-II, mFG-III and mFG-IV, was then measured. The samples reported 0.66 Miller units/sample, 0.902 Miller units/sample, 0 Miller units/sample and 0.47 Miller units/sample of estrogenic activity respectively. The samples were standardized to similar concentrations before the assay. As was the case before, since such minute amounts of a sample are being dealt with, it is important to keep in mind that errors may arise from the limitations of the mass spec. Results are shown in Figure 21. Since mFG-III, which contains compounds C and D, did not report any estrogenic activity, yet mFG-II and mFG-IV did, it can be deduced that neither compound C nor D possess estrogenic activity while compounds A, B, and E

do report estrogenic activity. This leaves compounds A, B and E estrogenically active, and hence, to be phytoestrogens.

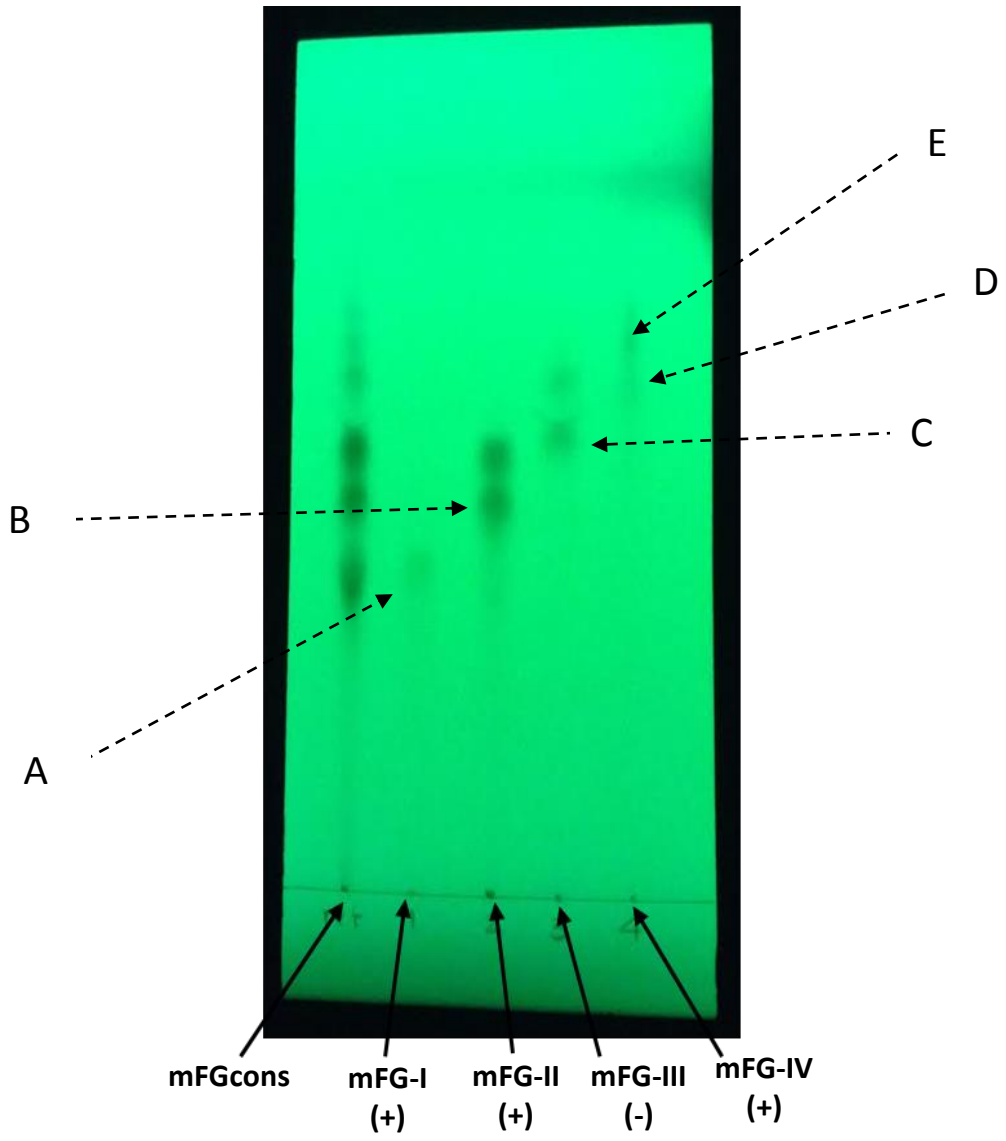


Figure 20. A silica gel TLC of mFGcons, mFG-I, mFG-II, mFG-III and mFG-IV separated using an ethyl acetate: methanol: water (10:7:2) solvent system shown under long wavelength UV light. Five distinct compounds can be identified in the separation of the fractions, which are labeled A-E. The fractions with (+) tested positive for estrogenic activity, while the fraction with (-) tested negative for estrogenic activity.

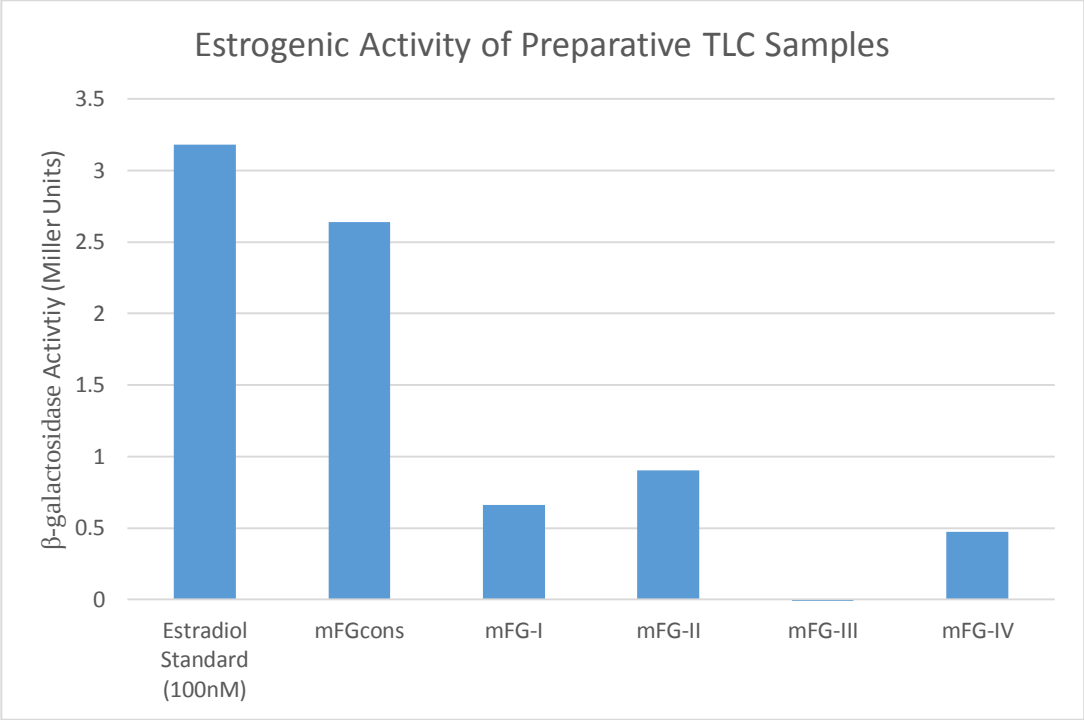


Figure 21. Estrogenic activity of mFG-I, mFG-II, mFG-III and mFG-IV compared to the estradiol standard and mFGcons. Activity is reported in Miller untis.

There is strong evidence to suggest that these phytoestrogens are isoflavones. The compounds were confirmed to be phenolic in nature using the ferric chloride diagnostic test and fenugreek contains several flavonoids like vitexin, tricin, naringenin, quercetin, kaempferol and luteolin (Parthasarathy et al., 2008). Furthermore, analysis of samples using mass spectrometry indicated the presence of multiple isoflavones. The total ion chromatogram acquired in the negative mode for CMFG (Figure 22) shows peaks at *m/z* = 253.30, 285.50 and 297.40 amu, which suggests the presence of several isoflavones. The hypothesized isoflavones are shown in Figure 23. Note that all isoflavones have the capacity to lose a hydrogen and become negatively charged. Therefore, in the negative mode of the mass spectrometry, the molecular weight is 1g/mol less than its actual molecular weight. By a comparative process of elimination based on the total ion

chromatogram (TIC) for the four samples and their estrogenic activity, a group of molecular ion peaks are concluded to be the active principles. For example, mFG-II is the most active sample. Figure 24 shows the TIC of the sample and the molecular ion peaks of *m/z* 281.2 and 283.2 which have been tentatively identified as 5-hydroxy-6,7-methylenedioxyflavone and Biochanin A respectively. (Figure 25). Biochanin A has been previously isolated form Fenugreek, but never associated with estrogenic activity (Wang et al. 2010).

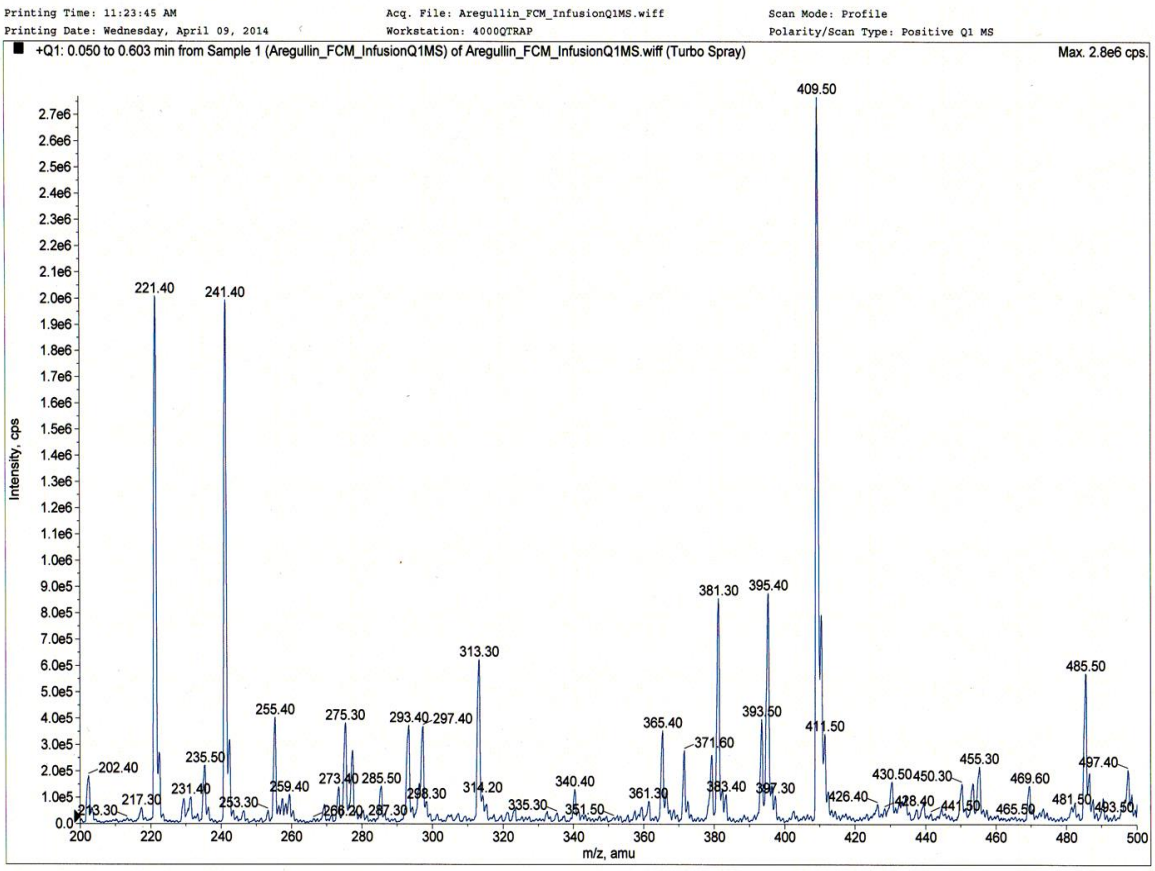
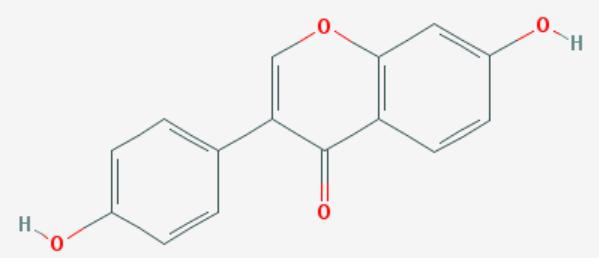
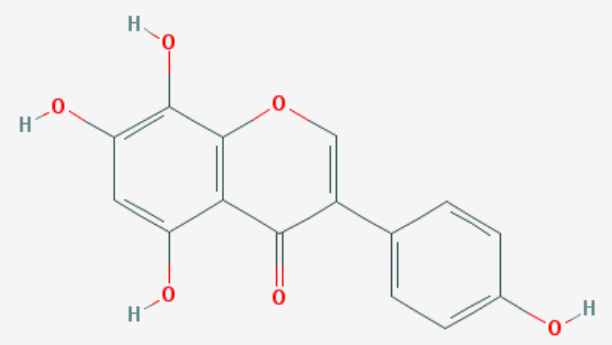


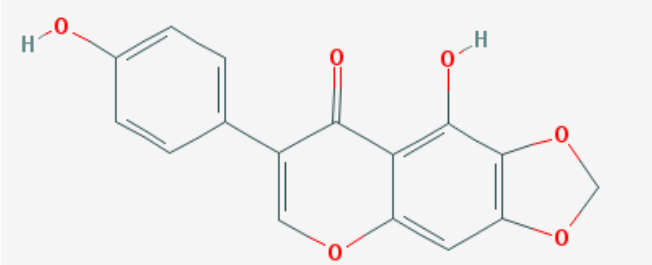
Figure 22. Total Ion Chromatogram TIC of CMFG extract.



Daidzein
Molecular weight: 254.2375 g/mol
Corresponds to 253.30 m/z peak



8-Hydroxygenistein
Molecular weight: 286.2363 g/mol
Corresponds to 285.50 m/z peak



Irilone
Molecular weight: 298.247 g/mol
Corresponds to 297.40 m/z peak

Figure 23. Isoflavones that may be present in fenugreek according to the m/z peaks of the total ion chromatogram of the CMFG extract

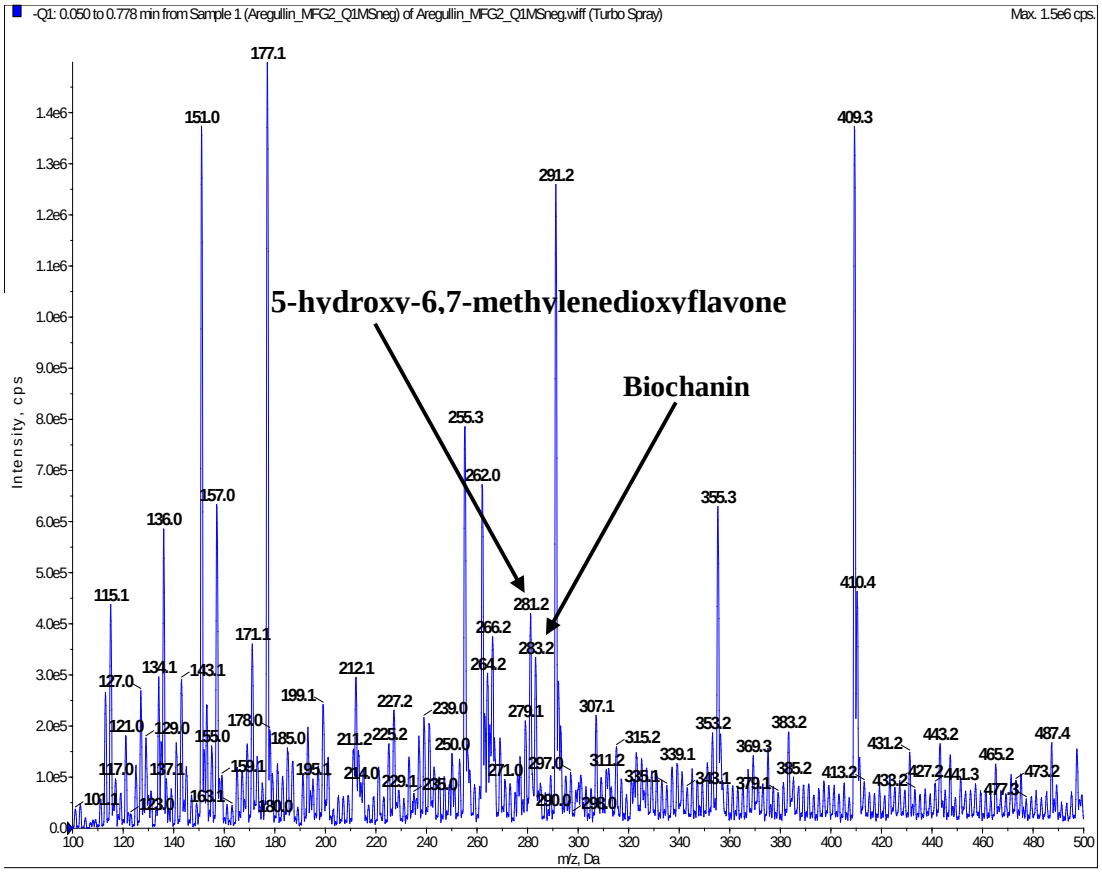
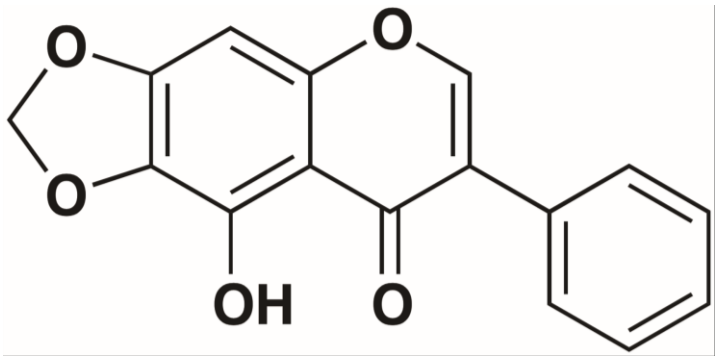
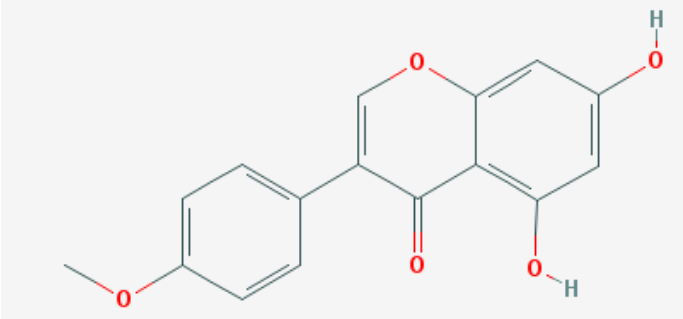


Figure 24. The Total Ion Chromatogram of mFG-II in the Negative Ion Mode Infusion MS Spectrum.



5-hydroxy-6,7-methylenedioxyflavone
Molecular weight: 282.05 g/mol
Corresponds to m/z peak 281.2



Biochanin A
Molecular weight: 284.26348 g/mol
Corresponds to 283.2 m/z peak

Figure 25. The structures of the tentatively identified isoflavones in mFG-II. Biochanin A corresponds to the *m/z* value of 283.2 from the mFG-II TIC.

Further evidence of the presence of isoflavones in fenugreek comes from the UV spectrum of mFGcons. As shown in Figure 26, the peaks of the compounds in mFGcons are within the range of typical peaks found in the UV spectrum of isoflavones. For example, the peaks for Biochanin A dissolved in methanol are 261 and 330nm (Mabry et al. 1970).

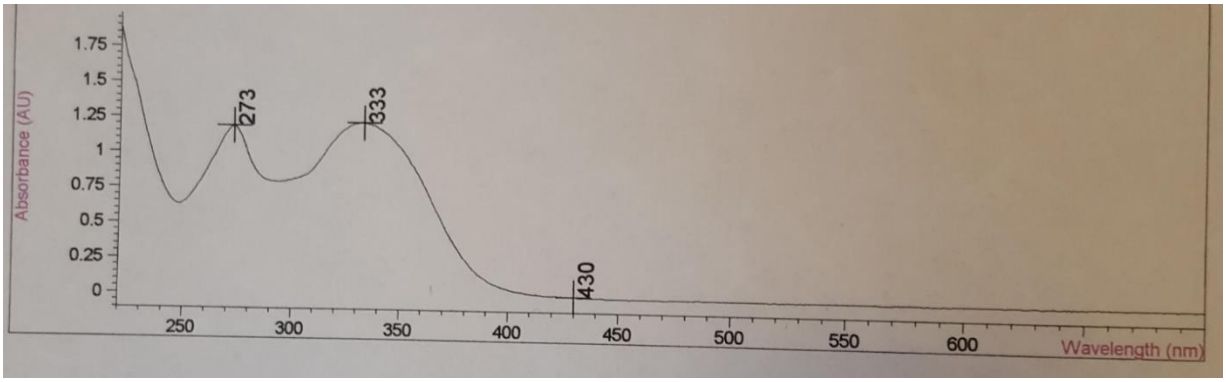


Figure 26. The UV spectra of mFGcons.

A model to explain the capacity of isoflavones to mimic the hormonal properties of estradiol has been proposed in the literature and shown in Figure 27 and 28. According to this

model, the structural similarity of isoflavones to estrogen is what enables isoflavones to exhibit estrogenic activity via the human estrogen receptor. Additionally, fenugreek belongs to the *Fabaceae* family, which synthesize isoflavones. Isoflavones are found exclusively in this family because the plants belonging to this family possess the enzyme responsible for transforming 2-phenylchroman to 3-phenylchroman, a necessary step in isoflavone production (Hanganu et al. 2010). Soy, which contains the estrogenically active isoflavones genistein, daidzein and glycitein, belongs to this family.

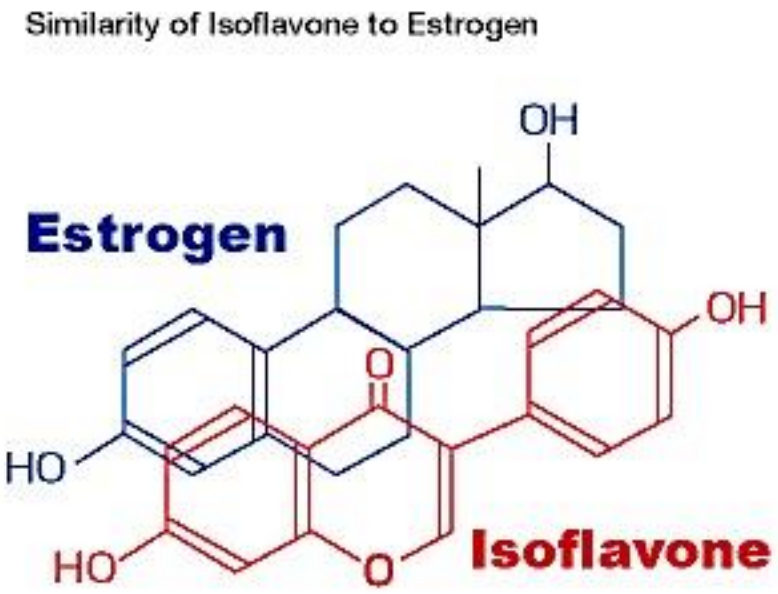


Figure 27. The structural similarity of isoflavones to estrogen.

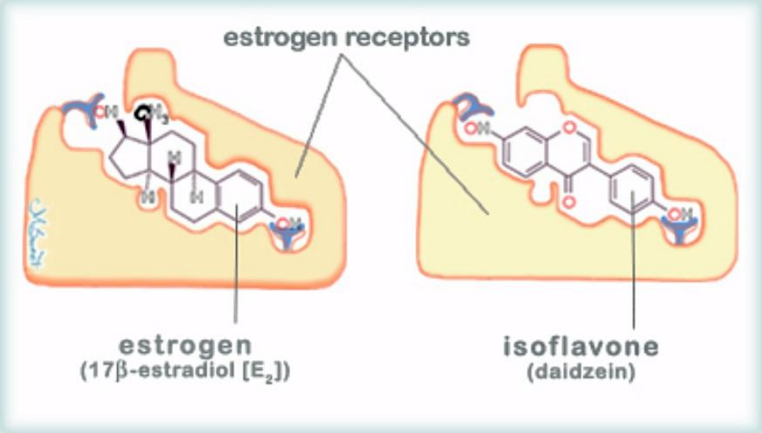


Figure 28. A pictorial depiction of the binding of estrogen compared with isoflavones to the estrogen receptor.

The results of this investigation suggest that in addition to diosgenin, non-steroidal estrogen mimics are present in fenugreek, which have never been previously reported. These additional estrogen mimics in fenugreek appear to be a family of isoflavones recurrent in the *Fabaceae* family. Although their relative concentrations appear to be low, since isoflavones act as hormones, trace amounts are sufficient to elicit a response.

Chapter 2

Acetylcholinesterase Inhibition

I. Introduction

One of the leading dementia pathologies is Alzheimer's disease (AD), which affects over 50 percent of dementia patients (Howes & Perry, 2011). Alzheimer's disease is a devastating disease that causes memory loss, especially the formation of new memories, and impairs cognition like language and social skills. Currently, an estimated 44 million people are diagnosed with AD, and this figure is expected to grow to 100 million people by 2050 (Thies & Bleiler, 2013). Although the number of people affected by AD is increasing rapidly, there is still no cure for the disease and the mechanism of action of the disease largely remains a mystery. Evidence has shown that genetics play a role in the development of AD. Of the two types of AD, early-onset and late-onset, early-onset AD is mostly inherited (Alzheimer's Disease Education and Referral (ADEAR) Center, 2011). However, early-onset AD represents less than 5 percent of all AD cases.

One hypothesis on the pathological mechanism of Alzheimer's disease is cholinergic system degeneration (Howes & Perry, 2011). Decreases in biochemical indices of cholinergic function in the neocortex and hippocampus have been found to be correlated with the severity of dementia. Cholinesterases are a family of enzymes that catalyze the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetic acid. This reaction is necessary for cholinergic neurons to return to their resting state (Colovic et al. 2013). The cholinesterase most targeted in AD is Acetylcholinesterase. As shown in Figure 1, acetylcholinesterase breaks down

acetylcholine in the synaptic cleft. Therefore, inhibition of this enzyme increases the level and duration of acetylcholine's action as a neurotransmitter (Colovic et al. 2013).

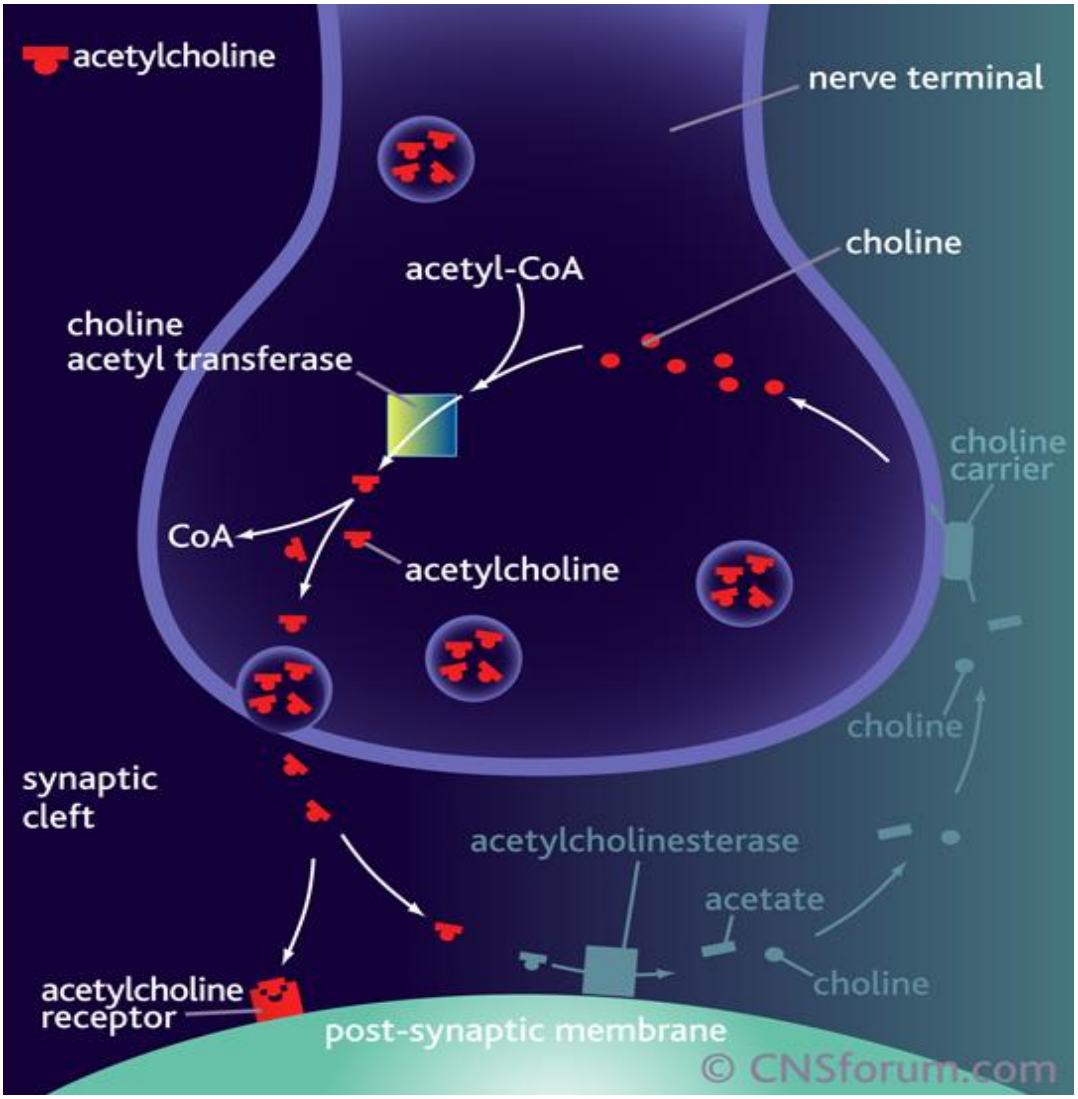


Figure 1. The mechanism of action of choline acetyl transferase. (The Lundbeck Institute, 2014)

Acetylcholinesterase (AChE) inhibitors have been proven to improve cognitive and neuropsychiatric symptoms over at least 6 months in patients with mild to moderate AD (Natarajan et al. ,2013). There are currently three different AChE inhibitors prescribed to AD patients, donepezil, rivastigmine and galantamine, all of which are reversible AChE inhibitors (Colovic et al. 2013). However, these drugs have limitations due to their short half-lives and undesirable side effects such as hepatotoxicity and gastrointestinal diseases. Although AChE inhibitors inhibit the same enzyme, they are structurally diverse (Brufani & Filocamo, 2000). Their structural diversity has led to an interest in finding better cholinesterase inhibitors, especially from natural products (Sung et al. 2002).

One popular natural product that has long been used as a cognitive enhancer in Chinese traditional medicine is Ginkgo biloba L. (Ginkgoaceae), which has the most mechanistic and clinical data relevant to dementia than any other plant species (Perry & Howes, 2011). Despite contradicting reviews on G. biloba's effect on cognition, G. biloba does show a dose dependent effect on AChE inhibition activity (Suganthy et al. 2013). G. biloba is an appealing natural product for treating dementia, like AD, because it has few indications of significant side effects (Perry & Howes, 2011). There are several different compounds that act as AChE inhibitors found in plants, like alkaloids, glycosides, and xanthones, and there is a lot of current interest in discovering new compounds with minimal side effects for therapeutic purposes (Suganthy et al. 2013).

Fenugreek, or *Trigonella foenum graecum* L. (Fabaceae) has shown potential for AChE inhibition, which is thought to be associated with the simple alkaloid Trigonelline (Suganthy et al. 2013). One study found AChE inhibition activity in a hydro alcoholic extract, ethyl acetate fraction, chloroform fraction and alkaloid fraction of Fenugreek using Ellman's method and TLC

bioassay detection (SatheeshKumar et al. 2010). In addition, there are other plants in the Fabaceae family that have promising AChE inhibitory activity. *Physostigma venenosum* L. (Fabaceae), commonly known as the calabar bean, showed AChE inhibitory activity from the compound physostigmine isolated from its seeds. Physostigmine is an indole alkaloid that has been approved by the FDA to treat mild to moderate AD, and is present in the formulation Synapton® (Suganthy et al. 2013).

However, despite multiple studies that report AChE inhibition activity in fenugreek, there is no conducive evidence of the chemistry associated with it. The aim of this study is to assess AChE inhibition activity in fenugreek extracts to see whether or not there are multiple compounds possessing AChE inhibitory activity in fenugreek, and to possibly establish the chemical identity of these compounds. The AChE inhibition activity was evaluated using a bioautographic method described in the literature.

II. Materials and Methods

i. Acetylcholinesterase Inhibition Activity Assay

1000 U of Acetylcholinesterase were dissolved in 149 mL of Buffer A solution (50 mM Tris-Hydrochloric acid buffer at pH 7.8) to have a solution of 6.7 U/mL of enzyme. The Fenugreek extracts were loaded onto the silica TLC plates and developed with a solvent solution of ethyl acetate:methanol:water (10:2.7:1) or appropriate mixture for optimal separation. After running the TLC, the plate was dried completely (with a blow dryer) before 2mL of the enzyme solution was sprayed onto the plate. The enzyme solution was at room temperature (~21°C) at the time of application. After completely spraying the enzyme solution onto the plate, the plate was dried again and then placed in a small, covered plastic container and then placed to incubate at 37°C for twenty minutes. The plastic container contained a small amount of water in it just enough to wet the bottom of the container so that the atmosphere within the chamber was humid. The container contained a layer of material to separate the water from the TLC plate.

After twenty minutes of incubation, the plate was removed from the incubator and dried again. To detect enzymatic activity, a 2mL solution of 13.4 mM naphthyl acetate and a 8mL solution of 7.4 mM FBS were combined and then sprayed onto the plate. After applying this mixture, the plate was then set aside to react in the dark for 12 hours. The plate turned red-purple while active fractions (fractions that inhibit Acetylcholinesterase) appeared as white spots (Adhami et al. 2011; Marston et al. 2001).

III. Results and Discussion

The Acetylcholinesterase Inhibition Activity Assay was performed on several of the Fenugreek extracts using a bioautographic assay that makes use of the AChE on a TLC plate (see Figure 1). Results of the assays are shown in the following Figures 2-4, where the white spots indicate compounds that inhibit the AChE.

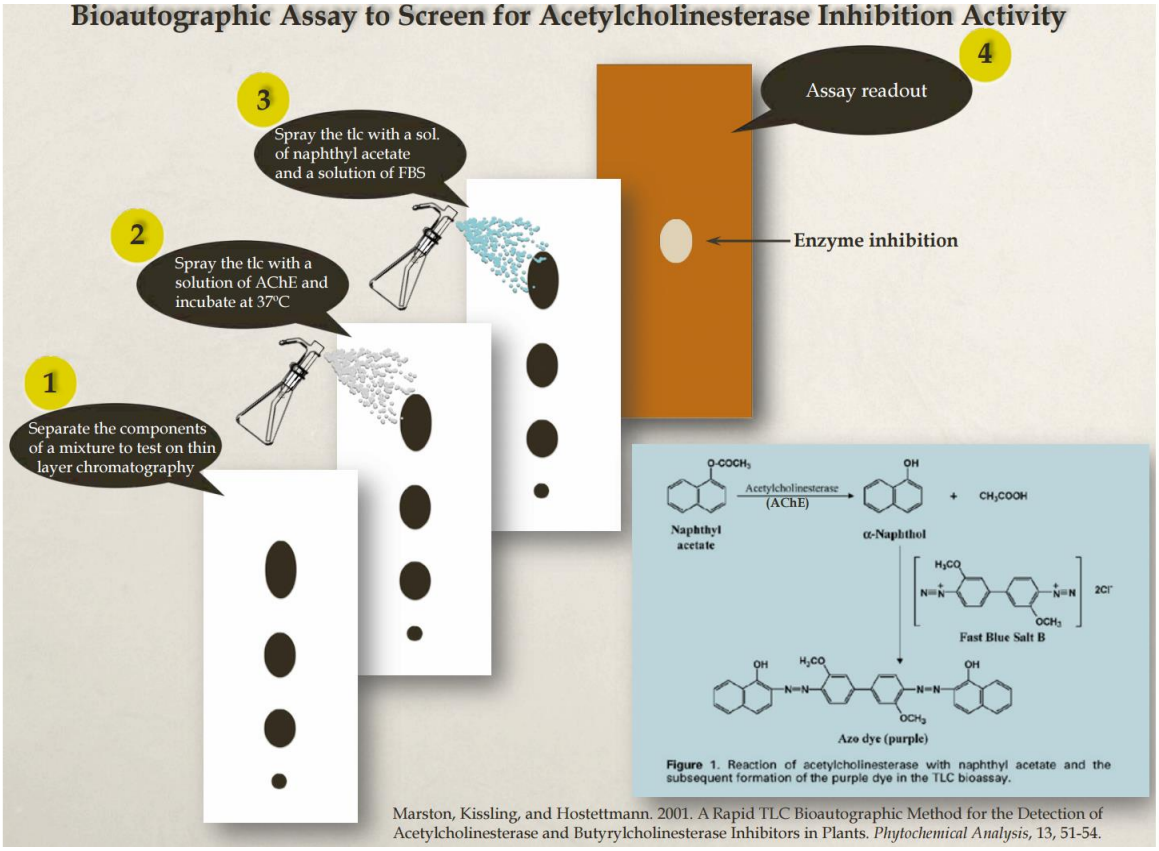


Figure 1. A schematic of the bioautographic assay used to screen the fenugreek extracts for acetylcholinesterase inhibition activity.

As shown in Figure 2, the HFG extract contains a compound at an Rf value of 0.87 that shows significant AChE activity by the area of discoloration, where there is lack in development of the purple-red background. These areas of discoloration indicate the presence of an AChE inhibitor, blocking the formation of 1-naphtol, which reacts with the fast blue salt to give a purple-red colored diazonium dye (Marston et al. 2002). The TLC from the bioautographic AChE inhibition assay is compared with a TLC of the HFG extract viewed with short UV light to show that the compound possessing AChE inhibition activity in the HFG extract is presumably the main compound present in this extract. Attempts to acquire the electrospray ionization mass spectrum (ESIMS) of this compound were unsuccessful as the solvent was too volatile to yield the molecular ion suitable for analysis, although the effort to identify the compound was made.

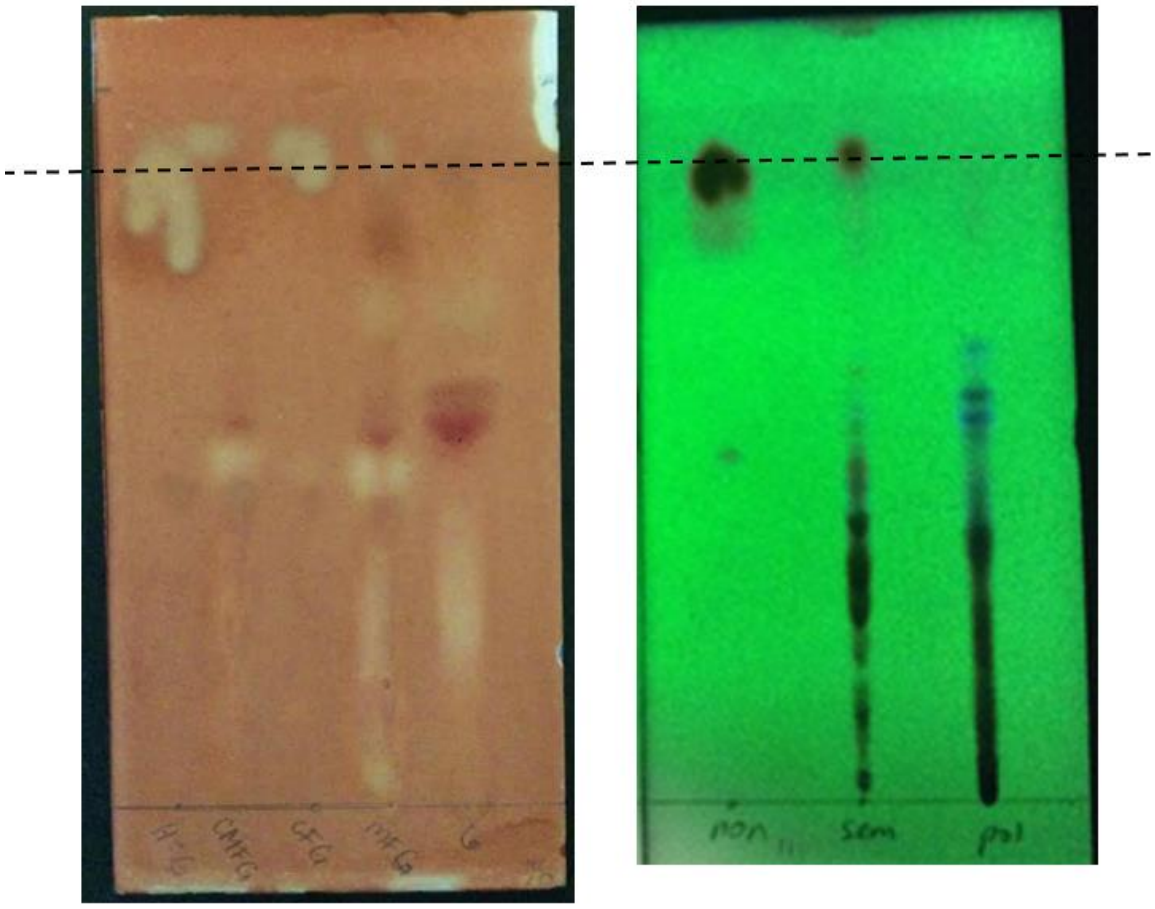


Figure 2. On the left, an Acetylcholinesterase Inhibition Activity Assay performed on a TLC with HFG, CMFG, cFG, mFG and mFG6 extracts applied (from left to right) using an ethyl acetate: methanol: water (10:2.7:2) solvent system. On the right, a TLC of HFG, CMFG and AEFG (from left to right) viewed under short wavelength UV light.

The CMFG extract showed two prominent zones of inhibition with Rf values of 0.5 and 0.91. The partition of CMFG into the chloroform and methanol soluble fractions resulted in the separation of these two AChE inhibitors. The compound with the Rf of 0.91 partitioned into the chloroform soluble fraction and the compound with an Rf of 0.5 partitioned into the methanol

soluble fraction. Additionally, the methanol soluble fraction showed a zone of inhibition between an Rf of 0.2 and 0.4 where more than one AChE inhibitor might be present.

Fractions 1-8 from the Sephadex LH-20 column chromatography of the methanol soluble fraction were assayed for AChE inhibition activity (Figure 3). The assay revealed the presence of some active components. The compounds with AChE inhibition activity in fractions mFG6 and mFG7 are of particular interest to the investigation of fenugreek because they appear to be present in the same fractions that are described to possess estrogenic activity in Chapter 1. For further investigation, the bioautographic assay for AChE inhibition was performed on the consolidated mFG6, mFG7 and mFG8 fractions (mFGcons, Figure 4).

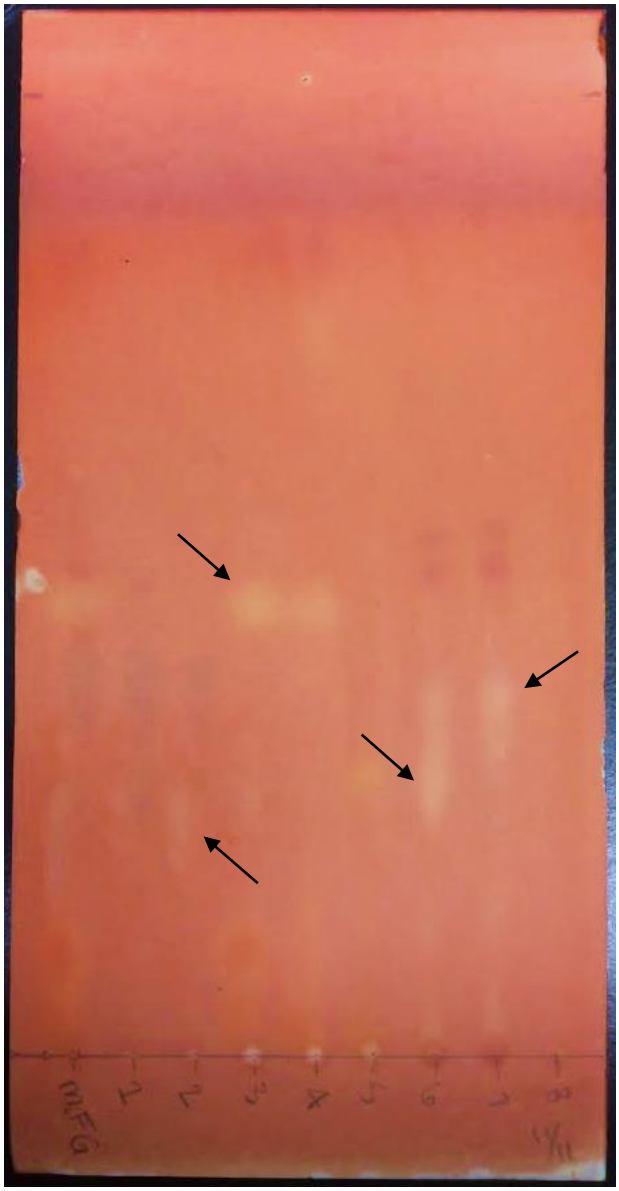


Figure 3. Acetylcholinesterase Inhibition Activity Assay performed on a TLC with mFG and mFG1- mFG8 extracts applied (from left to right) using an ethyl acetate: methanol: water (10:2.7:2) solvent system. Arrows show areas of enzyme inhibition.

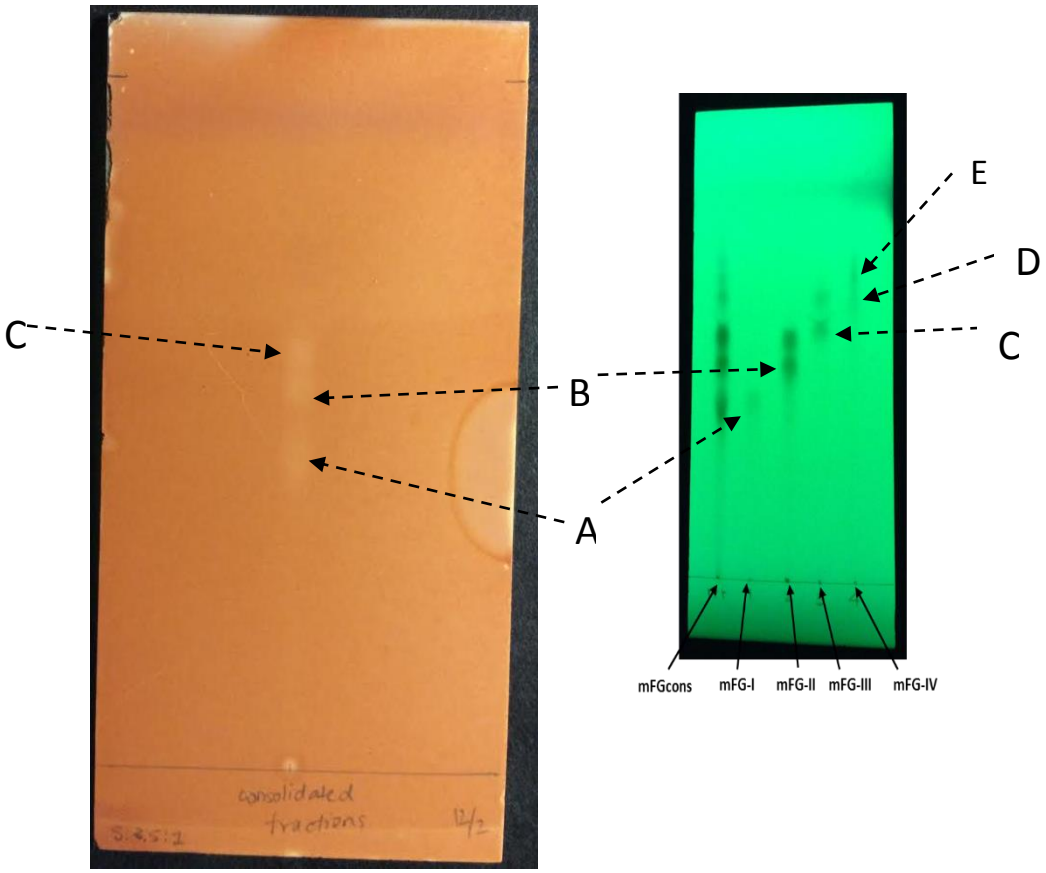


Figure 4. On the left, an acetylcholinesterase inhibition activity assay performed on a TLC of the mFGcons extract using an ethyl acetate: methanol: water (10:5:2) solvent system. On the right, a TLC of mFG, mFG-I, mFG-II, mFG-III and mFG-IV is shown (solvent system of ethyl acetate: methanol: water 10:7:2) under short wavelength UV. Arrows depict the identified compounds A-E from short wavelength TLC that exhibit AChE inhibition activity. Compounds A,B and C are presumably the same on each TLC because of their similar Rf values.

From the analysis of the TLC in Figure 4, there are three compounds in mFGcons with AChE inhibition activity that are primarily associated with the mFG-I and mFG-II samples from preparative TLC. Of the compounds, A, B and C, compounds A and B possess estrogenic activity.

These results suggest that possibly the same isoflavones are also responsible for the AChE inhibition activity.

This would not be the first plant to possess isoflavones with acetylcholinesterase inhibition activity. Venzke et al. identified four isoflavones (3′,4′-dihydroxy-6″,6″,6‴,6‴-tetramethylbis pyrano[2″,3″:5,6::2‴,3‴:7,8] isoflavone, 3′,4′-dimethoxy- 5-hydroxy-6 ″ , 6 ″ -dimethylpyrano[2 ″ , 3 ″ :7,6] isoflavone, 3′-methoxy-5,4′-dihydroxy-6″,6″-dimethylpyrano[2″,3″:7,6]isoflavone, 5,3′,4′-trihydroxy-6″,6″-dimethylpyrano[2″,3″:7,6] isoflavone) as AChE inhibitors from *Polygala molluginifolia* (2013). However, there is scarce literature on other isoflavones that have been explored for AChE inhibition activity.

Furthermore, there are no studies that have yielded compounds to be both estrogenic and AChE inhibitory. Nevertheless, several plants in the Fabaceae family report improving memory and dementia in ethnobotanical records (Adams et al. 2007). Such is the case for *Medicago sativa L.*, *Barbieria pinnata, Glycyrrhiza glabra L.* and several others. Therefore, it is not surprising that fenugreek possesses AChE inhibitory activity as well.

The finding that isoflavones in fenugreek contain both estrogenic and AChE inhibition activity requires further investigation. However, this study suggests that this is the case and opens the potential for medicinal uses of these isoflavones in the treatment of Alzheimer's, in addition to hormone replacement therapy. Despite estrogen's extensive involvement in the nervous system, no literature reports estrogen as an AChE inhibitor. Although this is all speculation, it may be worthwhile to investigate estrogen and its mimics for AChE inhibition activity.

The discovery of these phytoestrogens as AChE inhibitors also raises the question as to whether or not current AChE inhibitory drugs possess estrogenic activity. This could have profound implications for the side effects, which could be beneficial or negative, of AChE

inhibition drugs. This novel discovery in fenugreek could present an avenue for the synthetic production of drugs that are used to treat both Alzheimer's and have estrogenic activity, which elicits numerous physiological effects.

Bibliography

Academy of Breastfeeding Medicine Protocol Committee. ABM Clinical Protocol #9: Use of galactogogues in initiating or augmenting the rate of maternal milk secretion (first revision, January 2011). *Breastfeed Med.* 2011;6(1):41-49.

Ackerman GE, Carr BR. Estrogens. *Rev Endocr Metab Disord*. 2002;3:225-230.

Adams M, Gmunder F, Hamburger M. Plants traditionally used in age related brain disorders – A survey of ethnobotanical literature. *J Ethnopharmacol*. 2007. Vol. 113, 3: 363-381.

Adhami HR, Farsam H, Krenn L. Screening of medicinal plants from Iranian traditional medicine for acetylcholinesterase inhibition. *Phytother Res*. 2011; 25(8):1148-1152.

Alzheimer's Disease Education and Referral (ADEAR) Center. 2011. Alzheimer's Disease Genetics Fact Sheet. NIH Publicaiton NO. 11-6424.

Blei T, Soukup ST, Schmalbach K, Pudenz M, Moller FJ, Egert B, Wortz N, Kurrat A, Muller D, Vollmer G, Gerhauser C, Lehmann L, Kullin SE, Diel P. Dose-dependent effects of isoflavone exposrue during early lifetime on the rat mammary gland: Studies on estrogen sensitivity, isoflavone metabolism, and DNA methylation. *Mol Nutr Food Res*. 2015. 59:270-283.

Breast Cancer and Environmental Risk Factors: Estrogen and Breast Cancer Risk: What factors Might Affect a Woman's Exposure to Estrogen? *Cornell Unviersity Program on Breast Cancer and Environmental Risk Factors in New York Fact Sheet #10*. 2002.

Brenac P, Sauvaire Y. Chemotaxonomic Value of Sterols and Steroidal Sapogenins in the Genus *Trigonella*. *Biochem Sys Ecol.* 1996. Vol. 24, No. 2;157-164.

Brufani M, Filocamo L. Rational design of cholinesterase inhibitors. *Giacobini E, ed. Cholinesterase and Cholinesterase Inhibitors.* 2000. London, 27–46.

Burke, Dawon, and Stearns (2000). Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual. Cold Spring Harbor Laboratory Press.

Chan L, O'Malley BW. Mechanism of Action of the Sex Steroid Hormones. *New Engl J Med.* 1976; 294:1322-1328.

Colovic MB, Krstic DZ, Lazarevic-Pasti TD, Bondzic AM, Vasic VM. Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. 2013. 11(3): 315-335.

Gao and Pinkham (2000). Tightly regulated, beta-estradiol dose-dependent expression system for yeast. *Biotechniques.* **29**:1226-31

Gupta M, Shaw B. A double-blind randomized clinical trial for evaluation of galactogogue activity of Asparagus racemosus wild. Iranian J Pharm Res. 2011;10(1):167-172.

Hall JE. Guyton and Hall textbook of medical physiology. 12th ed. Philadelphia, PA: Saunders/Elsevier; 2011.

Hanganu D, Vlase L, Olah N. LC/MS Analysis of Isoflavones from Fabaceae Species Extracts. *Farmacia*. 2010. Vol. 58, 2.

Harlow BL, Signorello LB. Factors associated with early menopause. Maturitas 2000; 35 :3-9.

Howes, MJ, Perry, E. The Role of Phytochemicals in the Treatment and Prevention of Dementia. *Drugs Aging*. 2011. 28(6): 439-468.

Instructions: Yeast β-Galactosidase Assay Kit. *Thermo Fisher Scientific Inc*. 2012. Number 75768.

Jargin SV. Soy and phytoestrogens: possible side effects. *Ger Med Sci*. 2014; 12.

Kang J, Hick LA, Price WE. A fragmentation study of isoflavones in negative electrospray ionization by MS ion trap mass spectrometry and triple quadrupole mass spectrometry. *Rapid Commun. Mass Spectrom*. 2007. 21:857-868.

Kang L, Zhao Y, Pang X, Yu H, Xiong C, Zhang J, Gao Y, Yu K, Liu C, Ma B. Characterization and identification of steroidal saponins from the seeds of Trigonella foenum-graecum by ultra high-performance liquid chromatography and hybrid time-of-flight mass spectrometry. *J Pharm Biomed Anal*. 2013. 74;257-267.

Katzenellenbogen BS, Choi I, Delage-Mourroux R, Ediger TR, Martini PGV, Montano N, Sun J, Weis K, Katzenellenbogen JA. Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. *J Steroid Biochem Mol Biol*. 2000;Vol 74,5:279-285.

Kaur H. Estrogenic Activity of some Herbal Galactogogue Constituents. *Indian J Anim Nutr*. 1998. 15(3),232-234.

Krishnaswamy NR. Chemistry of Natural Products: A Unified Approach. *Universities Press*. 1999. Second Edition; 170.

Mabry TJ, Markham KR, Thomas MB. The systematic Identification of Flavanoids. *Springer-Verlag New York Inc*. 1970. 119.

Marston A, Kissling J, Hostettmann K. A rapid TLC bioautographic method for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants. *Phytochem Anal*. 2001; 13(1):51-54.

Messina MJ, Wood CE. Soy isoflavones, estrogen therapy, and breast cancer risk: analysis and commentary. *Nutr J*. 2008;7:17.

Mohrig JR, Hammond CN, Schatz PF, Morrill TC. Techniques in Organic Chemistry: Part 4, organic Qualitative Analysis. 2003. *W. H. Freeman and Company*. 34.

Mortel M, Mehta SD. Systematic Review of the Efficacy of Herbal Galactagogues. *J Hum Lact.* 2013;29:154.

Natarajan S, Shunmugiah KP, Kasi PD. Plants traditionally used in age-related brain disorders (dementia): an ethnopharmacological survey. *Pharm Bio*. 2013. 51(4): 492-523.

Nelson DL. Cox MM. *Lehninger Principles of Biochemistry*. Sixth Edition. New York (NY): W.H. Freeman and Company; 2000.

Newton KM, Reed SD, LaCroix AZ, Grothaus LC, Ehrlich K, Guiltinan J. Treatment of Vasomotor Symptoms of Menopause with Black Cohosh, Multibotanicals, Soy, Hormone Therapy, or Placebo. *Ann Intern Med*. 2006; Vol 145, No 12:869-881.

Nilsson S, Gustafsson J. Estrogen receptors: therapies targeted to receptor subtypes. Clin Pharmacol Ther. 2011;89:44–55. http://dx.doi.org/10.1038/clpt.2010.226. [PubMed]

Nordeng H, Havnen GC. Use of herbal drugs in pregnancy: a survey among 400 Norwegian women. *Pharmacoepidemiol Drug Saf.* 2004;13(6):371-380.

Notelivitz M, Clarkson TB, Pan Y, Anthony MS. (2003). Patent Identifier No. US6524616 B1. Wake Forest University Health Services. Web.

Parthasarathy V. A., Chempakam B, Zachariah TJ, ed. *Chemistry of Spices*. Oxfordshire: CAB International; 2008.

Patiaul HB, Jefferson W. The pros and cons of phytoestrogens. *Front Neuroendocrinol*. 2010. 31(4): 400-419. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3074428/. Accessed September 13, 2013.

Peng W, Adams J, David W. Frawley JE. Critical review of complementary and alternative medicine use in menopause: focus on prevalence, motivation, decision-making and communication. *Menopause*. 2014;21(5):536-548.

Perry E, Howes MR. Medicinal Plant s and Dementia Therapy: Herbal Hopes for Brain Aging? CNS Neuroscience & Therapeutics. 2011. 17:683-698.

Quinn RJ, Carroll AR, Pham NB, Palframan ME, Suraweera L, Pierens GK, Muresan S. Developing a Drug-like Natural Product Library. *J Nat Prod*. 2008. 71:464-468.

Rios JL. Recio MC. Medicinal plants and antimicrobial activity. *J Ethnopharmacol*. 2015. 100:80-84.

Ross SM. Menopause: A Standardized Isopropanolic Black Cohosh Extract (Remifemin) Is Found to Be Safe and Effective for Menopausal Symptoms. *Holistic Nursing Practice*. 2012;26(1):58-61.

SatheeshKumar N, Mukherjee PK, Bhadra S, Saha BP. Acetylcholinesterase enzyme inhibitory potential of standardized extract of Trigonella foenum graecum L and its constituents. Phytomed. 2010. 17(3-4)292-295.

Singh KP, Nair B, Jain PK, Naidu AK, Paroha S. Variability in the nutraceutical properties of fenugreek (*Trigonella foenum-graecum L.*) seeds. *Revista Colombiana de Ciencias Horticolas*. 2013. Vol. 7, No 2:228-239.

Sreeja S, Anju VS, Sreeja S. In vitro estrogenic activities of fenugreek Trigonella foenum graecum seeds. *Indian J Med Res*. 2010;131:814-819.

Srinivasan D, Nathan S, Suresh T, Perumalsamy PL. Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. *J Ethnopharmacol*. 2001. 74:217-220.

Sung SH, Kang SY, Lee KY, Park MJ, Kim JH, Park JH, Kim YC, Kim J, Kim YC. (+)-Alpha-viniferin, a stilbene trimer from Caragana chamlague, inhibits acetylcholinesterase. *Biol Pharm Bull*. 2002. 25, 125–127.

Suganthy N, Shunmugiah KP, Kasi PD. Plants traditionally used in age-related brain disorders (dementia): an ethnopharmacological survey. *Pharm Bio*. 2013. 51(4):492-523.

The Lundbeck Institute. The mechanism of action of choline acetyl transferase. *CNSforum*. 2014. https://www.cnsforum.com/educationalresources/imagebank/dementia_cholinergic/rcpt_sys_ach_acetransf. Accessed March 17, 2015.

The National Children's Study. Use of herbal products in pregnancy, breastfeeding and childhood workshop. December 2003.http://www.nationalchildrensstudy.gov/about/stayinginformed/listserv/studyassembly meetings/2003Dec/Pages/agenda 122003.aspx#day3. Accessed November 12, 2012.

Thies W, Bleiler L, Alzheimer's Association: 2013 Alzheimer's disease facts and figures. Alzheimers Dement 2013, 9:208–245.

Traore MS, Balde MA, Camara A, Balde ES, Diane S, Diallo MST, Ketia A, Cos P, Maes L, Pieters L, Balde AM. The malaria co-infection challenge: An investigation into the antimicrobial activity of selected Guinean medicinal plants. *J Ethnopharmacol*. 2015.

Venzke D, Carvalho FK, Ruani AP, Oliveira AS, Brighente IMC, Micke GA, Barison A, Pizzolatti MG. PAMPA Permeability, Acetylcholinesterase Inhibition and Antioxidant Activity of Pyranoisoflavones from *Polygala molluginifolia* (Polygalaceae). *J Braz Chem Soc*. 2013.Vol. 24, No. 12, 1991-1997.

Vrtačnik P, Ostanek B, Mencej-Bedrač S, Marc J. The many faces of estrogen signaling. *Biochem Med*. 2014; 24(3): 329-342.

Wang GR, Tang WZ, Yao QQ, Zhong H, Liu YJ. New flavonoids with 2BS cell proliferation promoting effect from the seeds of *Trigonella foenum-graceum* L. *J Nat Med*. 2010. 64:358-361.

WHO. Antimicrobial Resistance Fact Sheet Nº 194. *World Health Organization Media Centre*. 2014. Web. Accessed 30 March 2015. http://www.who.int/mediacentre/factsheets/fs194/en/

Ye L, Chan MY, Leung LK. The soy isoflavone genistein induces estrogen synthesis in an extragonadal pathway. *Mol Cell Endocrinol*. 2009; 302(1):73-80.