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Novel Natural Compounds Derived From TCM In The Treatment of Food Induced Anaphylaxis

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Contents

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List of Abbreviations

APS – Ammonium Persulfate

ASHMI - Antiasthma Simplified Herbal Medicine Intervention

B cells – B lymphocytes

BAL – Broncho Alveolar Lavage

BCR - B cell receptor

BSA – Bovine Serum Albumin

CD23 - Fc epsilon RII, or FcεRII, is the "low-affinity" receptor for IgE

CSR - Class switch recombination

DMSO - Dimethyl sulfoxide

ELISA – Enzyme Linked Immunosorbent Assay

FcεRI – High Affinity IgE receptor on Mast cells

FcεRII - Low Affinity IgE receptor on B cells

FBS - Fetal Bovine Serum

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GATA-3 – Transcription factor

H1-NMR - Proton nuclear magnetic resonance

HPLC - High-performance liquid chromatography

IFN γ – Interferon gamma

IgA Immunoglobulin A

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IgG – Immunoglobulin G

IgG1 – Immunoglobulin G type 1

IgM – immunoglobulin M

IL-10 – Interleukin-10

IL-12 - Interleukin-12

IL-13 - Interleukin-13

IL-2 - Interleukin-2

IL-4 - Interleukin-4

IL-6 - Interleukin-6

IL-8 - Interleukin-8

ITAM - Immunoreceptor tyrosine-based activation motif

NF- κB - Nuclear Factor kappa-light-chain-enhancer of activated B cells

OVA – Ovalbumin

PBS – Phosphate Buffered Saline

p-IκBα Phosphorylated Inhibitor of NF- κB

PPAR - Peroxisome proliferator-activated receptor

pSTAT6 - Signal transducer and activator of transcription 6

RIPA buffer - Radioimmunoprecipitation assay buffer

RPM - Revolutions per minute

RPMI- 1640 - Roswell Park Memorial Institute 1640 Medium

SDS - Sodium Dodecyl Sulfate

T bet – Transcription factor

TBST - Mixture of tris-buffered saline (TBS) and Polysorbate 20 (also known as Tween 20)

TCM- Traditional Chinese Medicine

TH2 – Type 2 Helper T cell

TH1 – Type 1 Helper T cell

TNF-α – Tumor Necrosis Factor - alpha

U266 cell line– Myeloma Cell line

UV – Ultraviolet light

β-ME - 2-mercaptoethanol

Abstract

Food allergy is a highly prevalent disease affecting about 30 million people in the U.S. It is managed primarily by food avoidance due to lack of promising treatment options. Immunoglobulin E, IgE plays an important pathologic role in most, if not all, allergic conditions. ASHMI (anti-asthma herbal intervention) which consists of three components, *Sophorae flavescentis, Ganoderma lucidum, Glycyrrhiza uralensis* has been shown to inhibit allergic lung inflammation in antigen sensitized and challenged mice. It decreased airway hyperactivity, eosinophilic inflammation and downregulates IgE and Th2 cytokines IL-4, IL-13, in murine models of asthma. The active compounds in *Sophorae flavescentis* are yet to be identified. In this study we isolate and identify the active compound in *Sophorae flavescentis*, characterized the mechanism of IgE inhibitory effect, bio markers and potential to prevent food anaphylaxis.

To separate and identify the compounds we used column chromatography, high-performance liquid chromatography, mass spectrometry and proton nuclear magnetic resonance. We screened the potential for IgE inhibition in IgE producing myeloma cell line U266 cells. IgE production was measured with ELISA. Gene expression of the targets regulated was determined with qRT-PCR. The biological targets regulated by the identified compound (formononetin) in food allergy, mast cells, IgE diseases were obtained from Mala Card, Gene Card, and Swiss Target databases. We characterized the molecular pathways, cellular processes and biological functions regulated by formononetin that are relevant to food allergy by mapping the obtained targets into the gene ontology pathway, KEGG pathway, protein-protein interaction network. The targets that were highly relevant to food allergy were validated using qRT-PCR. Finally, to determine if formononetin

prevents food anaphylaxis. We used an established peanut mice model. The mice were sensitized to peanut protein by intraperitoneal administration of crude peanut extract and alum, followed by oral food challenge after the sensitization period. Mice were subsequently given formononetin in a dose escalation manner or given vehicle as sham treatment. Unsensitized /untreated mice were naïve controls. After completing therapy, mice underwent oral peanut challenges. Anaphylaxis was evaluated by visual assessment of symptom scores and measurement of body temperature. ELISA was used to measure serum antibodies peanut specific IgE, IgG1, IgG2a, plasma histamine and Th2, Th1 cytokines IL-4, IL-13 and IFN ɣ.

The IgE inhibitory compound isolated from *Sophorae flavescentis* was identified as formononetin. Formononetin decreased IgE production in a dose dependent fashion without cytotoxicity. Its inhibitory mechanism is specific to IgE production as it did not inhibit IgG production in an IgG producing myeloma ARH-77 cell line. Formononetin potentially regulates 25 targets in food allergy, 51 in IgE diseases and 19 in mast cell disease. The targets regulated by formononetin were mapped into KEGG and gene ontology pathways to uncover potential disease, biological and cellular processes regulated such as Th17 pathway, AGE-RAGE pathway, primary immunodeficiency, Th1, Th2, B cell activation, differentiation, and proliferation. This uncovers the mechanism of formononetin in food allergy. We validated the highly relevant targets identified from network analysis using qRT-PCR. Formononetin significantly reduced the expression of the BTK, TYK2, CASP8 genes and significantly increased the expression of the P53, BCL2, and NFKBIA genes. Peanut allergic mice treated with formononetin but not vehicle was significantly protected from anaphylaxis to oral peanut challenges as indicated by lower symptom scores, better

retention of body temperature and reduced plasma histamine. Importantly, formononetin treatment did not cause anaphylactic reactions, or any abnormality during the treatment. Treatment was associated with a significant decrease in peanut specific IgE, increase peanut specific IgG2a. There was an increase in IFNɣ and a decrease in IL-4, IL-13 indicating a shift in response from Th2 to Th1. There is a strong correlation between peanut specific IgE and symptom score and plasma histamine levels, and body temperature.

Taken together, we have shown that formononetin is the major component of *Sophorae flavescentis* that inhibited IgE production. We identified the potential biological targets, molecular pathways regulated by formononetin using computational technology and showed that it can prevent food anaphylaxis in a peanut allergic mouse. This study has shown that formononetin might be a potential treatment and preventive option for food anaphylaxis reaction seen in food allergy and a clinical trial is needed as next steps.

1. INTRODUCTION

A. Overview of the biology, functions, pathophysiology of B cell, Mast cell

B lymphocytes develop in the bone marrow from hematopoietic precursor cells (Pieper *et al.,* 2013). B cells and their antibodies perform central functions in the humoral immunity and participate in adaptive immune system (Ahangarani *et al.,* 2009). Naïve B cells differentiate upon receiving appropriate signals from T cells into memory B cells and long-lived plasma cells (Rajewsky 1996). Memory B cells recirculate in the blood, their main reservoir is in the bone marrow (Paramithiotis and Cooper 1997). Plasma cells are from antigen activated B cells in spleen and lymph nodes. After formation they will migrate to the bone marrow where they can remain for a long period of time – months to years (Northrup and Allman 2008).

Mast cells were identified as granular cells in the mesentery of frog in 1863 by Dr von Recklinghausen and it was they were named “Mastzellen” by Dr Paul Ehrlich in 1878 (Moon *et al.,* 2010). Mast cells can be found near blood or lymphatic vessels and are common at mucosal surfaces (Selye 1966). Mast cells can detect and initiate immune response to microbes. Products from microbes activate mast cells through the toll like receptors, complement receptors and Fc receptors (Marshall *et al.,* 2019). Mast cells have diverse physiologic functions such as in wound healing, hair follicle recycling, bone growth and remodeling (Moon *et al.,* 2010). Mast cell are discriminately activated by microbial products to release preformed mediators which recruit effector cells such as neutrophils (Marshall *et al.,* 2019). Mast cells are involved in the pathophysiology of IgE-mediated allergic diseases such as asthma, allergic rhinitis, atopic dermatitis and food allergy (Bischoff 2007).

B. Food Allergic Reaction, Food Anaphylaxis

To trigger an allergic reaction, the ingested or inhaled allergen will bind to IgE which is bound to the FcεRI high affinity IgE receptors present on the surface of mast cells or basophils. This causes the crosslinking of the receptors and downstream degranulation to release the pharmacologic mediators responsible for clinical symptoms seen in allergic reactions.

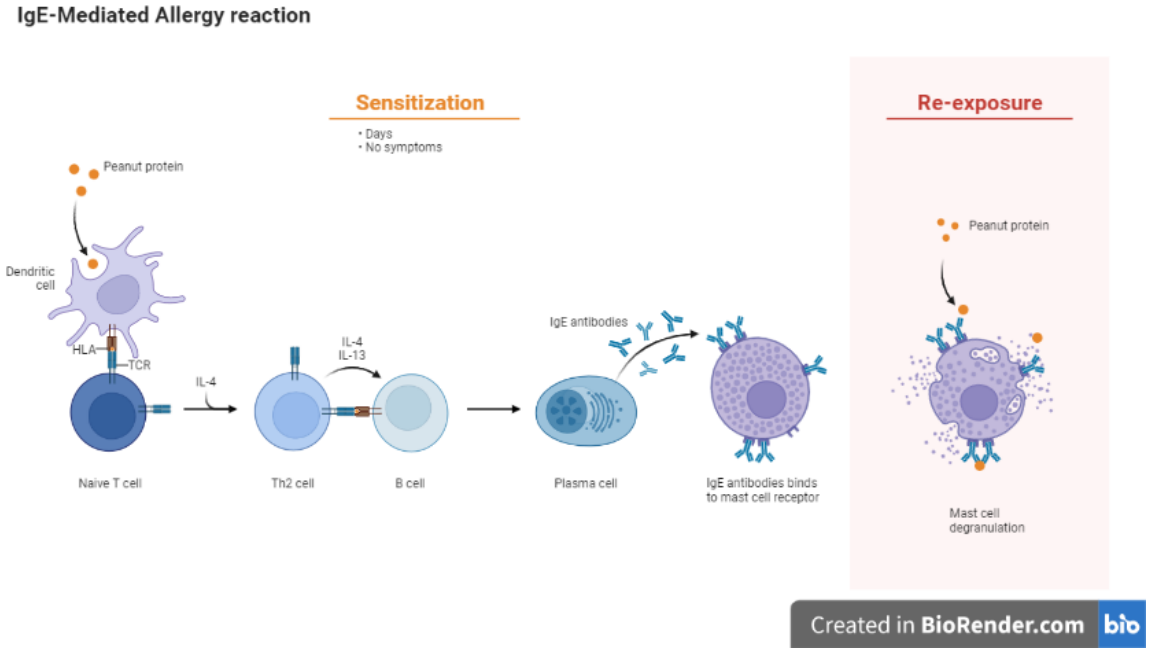


Figure 1: Mechanism of IgE mediated allergy reaction. IgE is produced from plasma B cells upon activation by Th2 cytokines IL-4, IL-13. Secreted IgE binds to Mast cells and upon second exposure to allergen, bound IgE triggers mast cell degranulation [original image].

C. IgE biology & function, High Affinity IgE Receptor-FcεRI

I. IgE Biology, Function, Half life

Immunoglobulin has a short life and is the Isotype with the lowest abundance of all the immunoglobulin isotypes. It has a serum half-life of 2 days in humans (Wu 2014) and a serum concentration of about 50-100ηg/mL of blood (Wu 2014). IgE mediates type I hypersensitivity reactions – systemic and localized anaphylaxis (Gould 2015). It is the isotype that mediates the first line of defense against helminths. It has also been implicated in the pathogenesis of allergic diseases, with several studies implicating it as the central molecule in diseases such as asthma, allergic rhinitis, and atopic dermatitis.

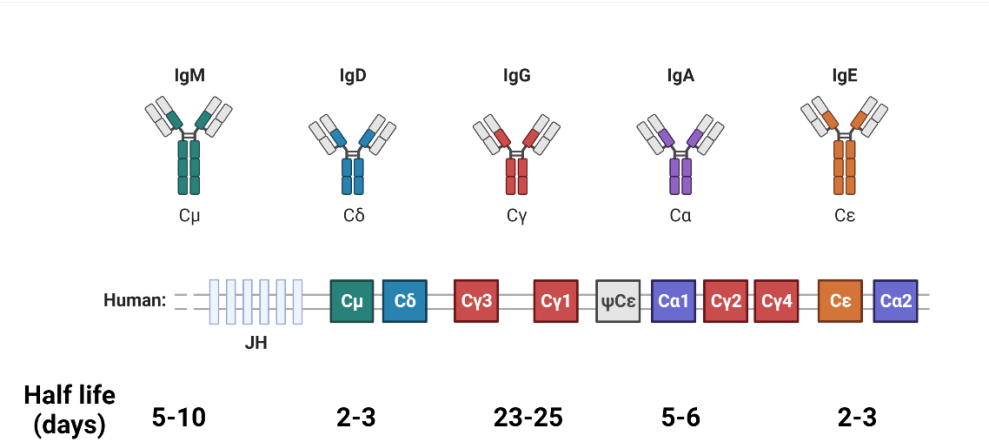


Figure 2: The structure and half-life of the five immunoglobulin classes (isotypes) of antibody molecules found in serum: IgG, IgM, IgA, IgD, IgE [original image].

II. Forms of IgE, IgE Receptors, IgE mediated Early and Late Responses

There are two forms of IgE – the membrane B cell receptor form which is expressed on the surface of the B cells and the secreted form (Wu 2014). The secreted form of IgE is responsible for binding to mast cells after first exposure to an antigen and triggers mast cell degranulation upon second exposure to the antigen (Wu 2014). The membrane IgE, on the other hand, is responsible for B cell antigen uptake and presentation, B cell proliferation and differentiation (Wu 2014). IgE receptors are present on immune cells such as basophils and mast cells. There are two types of IgE receptors, the high affinity (FcεRI) and the low affinity (FcεRII) also known as CD23 (Wu 2014)). FcεRI is present on mast cells, basophils, dendritic cells and macrophages while FcεRII is expressed on B cells and other immune cell types (Wu 2014).

There are two types of IgE responses, the early response and the late response (Wu 2014). The early response are mediated by low affinity short lived IgE plasma cells arising from extrafollicular sources (Wu 2014). The late response is mediated by germinal center B cells that are activated by T follicular helper T cells (Wu 2014).

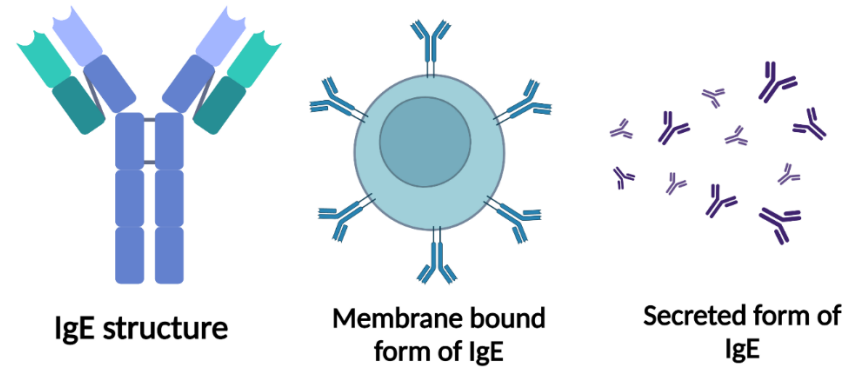


Figure 3: IgE structure, membrane bound form of IgE, secreted form of IgE [original image].

J. Class switch recombination

The human ε-germline gene promoter region consists of several promoters which includes STAT6 promoter region and NF-κB region. NF-κB binds to two sites in the ε-germline gene promoter and cooperates with STAT6 in synergistic action of transcription (Goenka 2011). The activity of STAT6 is highly influenced by NF-κB (Goenka 2011). Signal transducer and activator of transcription (STAT) proteins are mediators of cytokine signaling (Nutt *et al.*, 2011). STAT6 is one of the types of STAT proteins, is principally activated by IL-4 and IL-13, and once activated its conserved tyrosine residue becomes phosphorylated (Nutt, Taubenheim *et al.*, 2011). Phosphorylated STAT6 forms homodimers, which translocate into the nucleus where it has the ability to bind directly to DNA (Nutt *et al.*, 2011). In B cells STAT6 promotes class switching to IgE and IgG1. IgE class switching in B cells requires the action of multiple enhancer binding proteins which

are coordinated to regulate the gene expression (Nutt *et al.,* 2011) . In human B cells activation of transcription factors of the NF-κB family and then the IκB kinase leads to the degradation of IκBα and IκBβ. This leads to the eventual release of NFκB – p50 and NFκB – p65 which translocate to the nucleus to induce transcription of target genes (Karin 2000) (Rolling *et al.,* 1996). Decreased levels of phosphorylated IKBα is an indicator of NFκB inhibition**.** Upon activation mature B cells undergo immunoglobulin class switch recombination and differentiate into antibody-secreting plasma cells (Shaffer *et al.,* 2004). Xbp1 is a positively acting transcription factor in the CREB/ATF family that is expressed at a high level in plasma cells (Shaffer *et al.,* 2004). Xbp1 induces physical expansion of the ER, in addition to increasing cell size, organelle biosynthesis, total protein synthesis, therefore Xbp1 plays a critical role in the secretion of immunoglobulins (Bae *et al.,* 2011). Xbp1 is uniformly expressed in all multiple myeloma cell lines (Bae *et al.,* 2011). Activation of ε germline gene is an essential step towards IgE expression (Nutt *et al.,* 2011), which leads to generation of transcripts ε mRNA transcripts after class switch recombination. The presence of IgEH transcripts in the B cell is evidence that class switch recombination has occurred in the germline region.

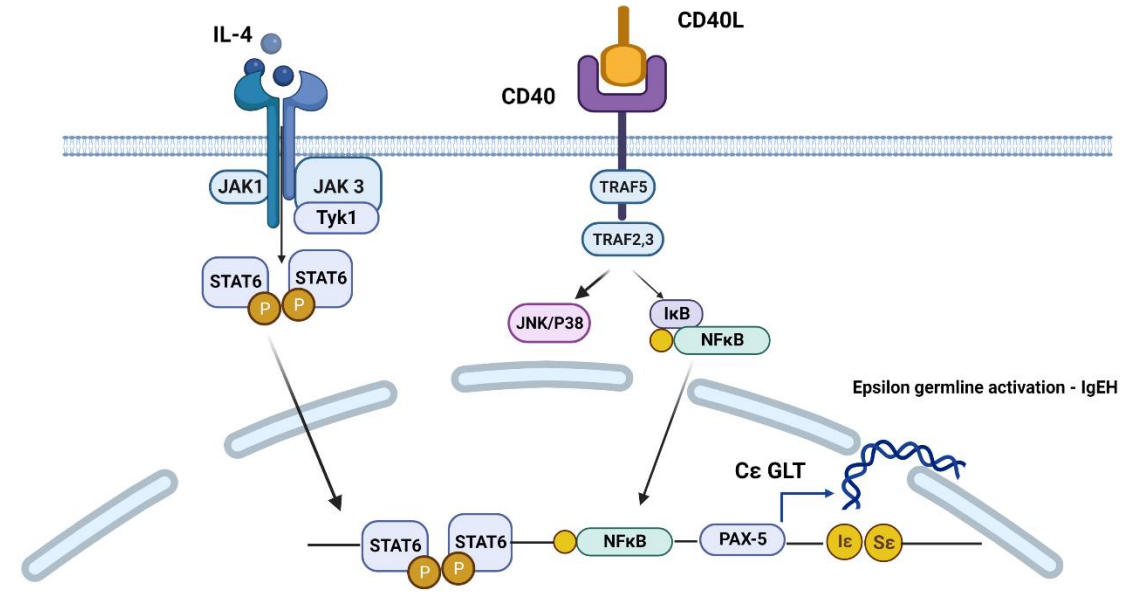


Figure 4: Mechanism of IgE class switch recombination in plasma B cells. Activation of epsilon germline leads to transcription of IgE heavy chain transcripts [original image].

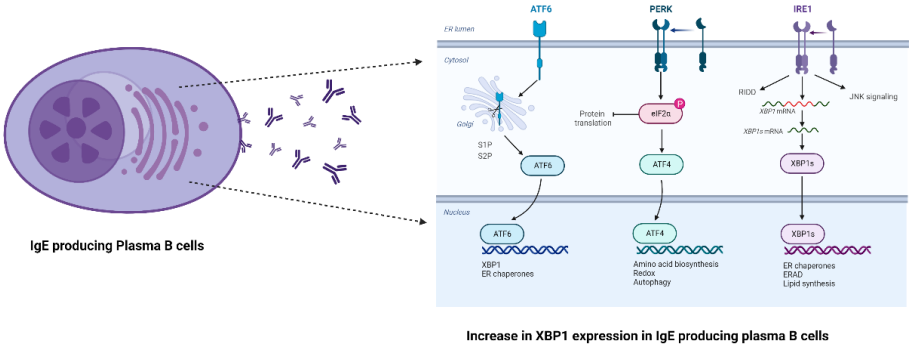


Figure 5: Increase in XBP-1 gene expression is important for IgE production in plasma B cells [original image].

K. Current Treatments of Allergic diseases

Therapies that have been in clinical trial or are currently used to decrease serum IgE are classified into categories such as – agents that block the transcription factors for IgE production, agents that induce transcription factors that suppress IgE synthesis, agents that block the synthesis of IgE promoting cytokines of the T_H2 lymphocyte profile – IL-4 and IL-13, and monoclonal antibodies such as omalizumab (Stokes 2004). T_H1 cells produce IL12 and IFN γ. These factors suppress IgE production while T_H2 cells produced IL-4, IL-13 which cause increase in synthesis of IgE (Finotto *et al.,* 2002). There are two transcription factors – T bet and GATA-3 that are important for T_H1 and T_H2 differentiation (Finotto *et al.,* 2002). T bet suppresses T_H2 phenotype causing the release of IFN γ and IL-12, decreasing IgE production (Finotto *et al.,* 2002). GATA-3 regulates IL-4, IL-4 producing a T_H2 phenotype and repressing T_H1 development. PPAR have been used to inhibit GATA3 to decrease both IL-13 production and IgE (Finotto *et al.,* 2002).

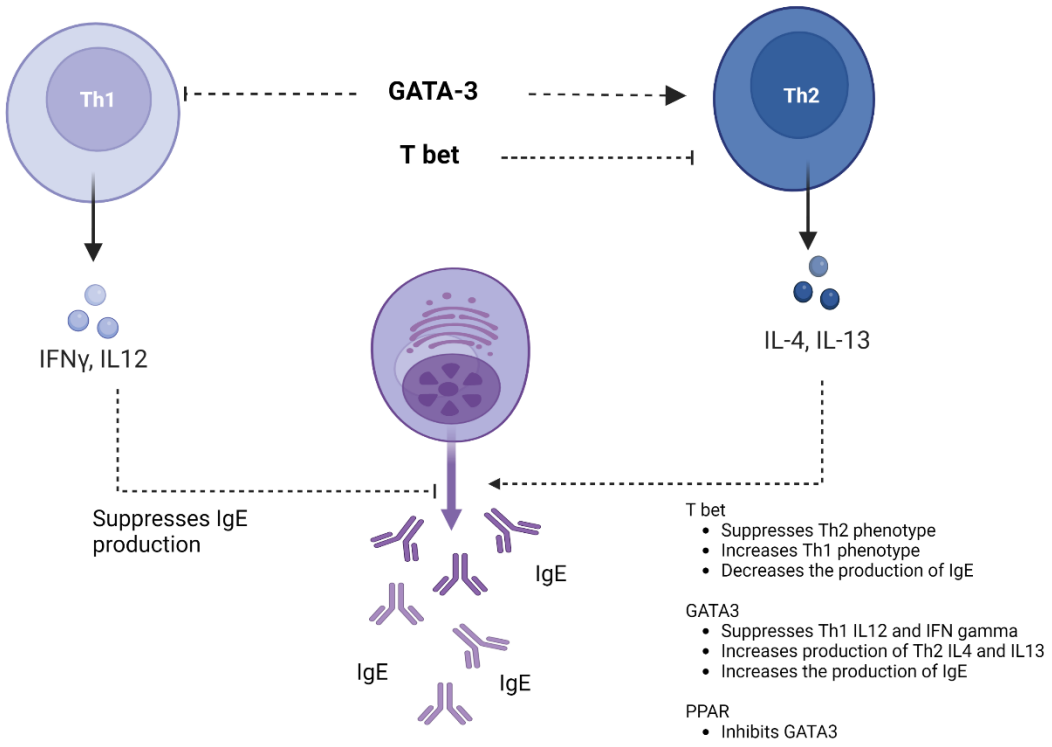


Figure 6: Transcription factors T bet and GATA3 regulating Th1 and Th2 cytokines that increases or suppresses B cell IgE production [original image].

Suplatast tosilate is a selective inhibitor of synthesis of IL-4, IL-5., It has been shown to inhibit synthesis of IgE (Tamaoki *et al.*, 2000, Yoshida *et al.*, 2002, Sano *et al.*, 2003). Consistent with the inhibition of IL-4 and IL-5 production, suplatast decreased levels of both IgE and eosinophil cationic protein (Tamaoki *et al.*, 2000, Yoshida *et al.*, 2002, Sano *et al.*, 2003). Clinical trials have shown that suplatast is effective in reducing cough variant asthma symptoms and the presence of eosinophils and eosinophil cationic protein in sputum of mild asthmatic patients (Tamaoki *et al.*, 2000, Yoshida *et al.*, 2002, Sano *et al.*, 2003). Thus, by its inhibitory effects on both IL-4 and IL-5, suplatast might prove more useful than therapies aimed at a single cytokine, or a single pathway thought to be

important in allergic airway diseases (Tamaoki *et al.,* 2000, Yoshida *et al.,* 2002, Sano *et al.,* 2003).

IL-12, IFN γ promote T_H1 response and inhibit T_H2 response. Intravenous infusion of recombinant human IL-12 cause a reduction in both blood and sputum eosinophil levels following airway allergen challenges (Yamaguchi *et al.,* 1991). There have been significant adverse effects associated with recombinant IL-12 including flu like symptoms, cardiac arrhythmias and when given in consecutive days, it causes an increase in mortality (Yamaguchi *et al.,* 1991). An anti-IL-4 monoclonal antibody trial showed a negative effect regarding clinical end points. Strategies aimed at inhibiting effects of IL-13 have been successful in animal models, but human clinical trials are yet to be performed. Strategies that block cytokines have not been proven to be effective in modulating allergic inflammatory responses in humans (Yamaguchi *et al.,* 1991).

The interaction of IgE with CD23 receptor causes an increase in IgE synthesis. However cross linking of the membrane bound CD23 downregulates IgE synthesis. Crosslinking of membrane bound CD23 can be achieved with the use of anti – CD23 monoclonal antibodies[48-54]. In a mouse model of allergic airway disease, anti CD23 treatment reduced allergen induced bronchoconstriction, reduced IgE and IgG1, abolished eosinophilia and normalized airway hyperresponsiveness (Yamaguchi *et al.,* 1991).

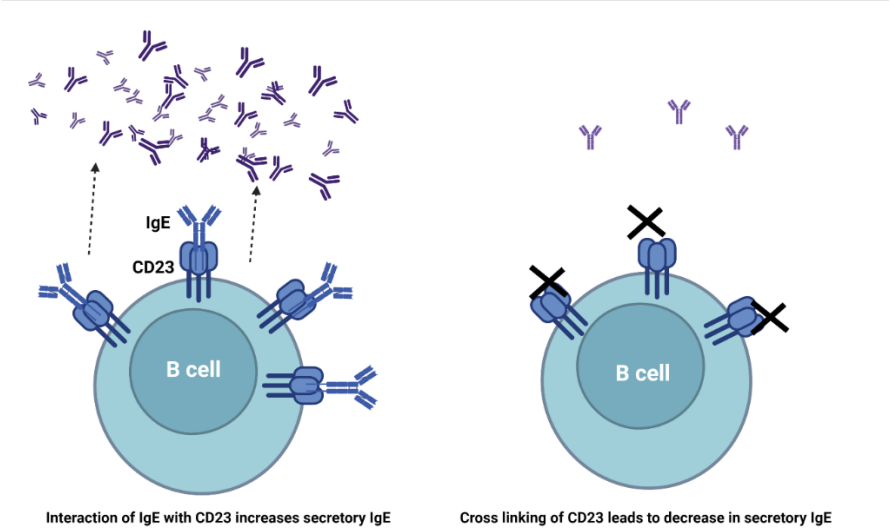


Figure 7: Crosslinking of CD23 leads to a decrease in IgE production [original image].

Recombinant monoclonal anti-IgE antibody therapy – omalizumab, was developed for treatment of allergic disease. It rapidly reduces IgE and expression of high affinity IgE receptors on critical effector cells. It's used in asthma, perennial allergic rhinitis (MacGinnitie *et al.*, 2017) (Chan *et al.*, 2013). Omalizumab is used to reduce rhinitis symptoms and improved quality of life in adults and children with either seasonal or perennial allergic rhinitis (MacGinnitie *et al.*, 2017) (Chan *et al.*, 2013) (Pillai *et al.*, 2016). Treatment with omalizumab and immune therapy was more effective in reducing symptom scores than immunotherapy alone in pediatric patients with seasonal allergic rhinitis (MacGinnitie *et al.*, 2017) (Chan *et al.*, 2013) (Pillai *et al.*, 2016). Peanut allergic patients tolerated a 15-fold increase in peanut flour during oral food challenge with high-dose anti-IgE antibody therapy (450 mg of TNX-901). Other IgE-mediated diseases, such as atopic dermatitis, venom allergy, and chronic urticaria with autoantibodies, represent theoretical uses of anti-IgE antibody therapy (MacGinnitie *et al.*, 2017) (Chan *et al.*, 2013) (Pillai *et*

al., 2016). Omalizumab is the first biologic response modifier approved by the US Food and Drug Administration for patients 12 years and older with moderate-to-severe perennial allergic asthma for the treatment of allergic asthma (MacGinnitie *et al.*, 2017) (Chan *et al.*, 2013) (Pillai *et al.*, 2016).

L. Traditional Chinese Medicine: ASHMI and Sophorae flavescentis

Traditional Chinese Medicine (TCM) is a system of medicine that has been practiced over 2000 years. The use of TCM for food allergy rose from 11% in 2002 to 18% in 2006. In 2016, the American Academy of Allergy, Asthma, and Immunology reported that food allergy has been one of the most common indications for TCM use (53.6%). Preclinical studies of TCM herbal formulas suggest an exciting potential for food allergy.

ASHMI is composed of three herbs; Ling-Zhi (Ganoderma lucidum), Ku-Shen (Sophora flavescens), and Gan-Cao (Glycyrrhiza uralensis) and is derived from a Traditional Chinese Medicine 14-herb formula MSSM-002 (Li *et al.*, 2000). It suppresses allergic airway hyperactivity and modulates T_h1 and T_h2 response.

The roots of Sophorae Radix known as “Kosam” in Korean and “Kushen” in Chinese. It is sometimes referred to as *Kushen*or *Ku Shen*, and it is known to have 'cooling' and antiphlogistic (anti-inflammatory) properties (Li *et al.*, 2020). The traditional Chinese medicine kushen is the dried roots of *Sophora flavescens* Aiton (Leguminosae). It was first described in the Chinese book *Shen Nong Ben Cao Jing* in 200 A.D. as a treatment for solid tumors, inflammation, and other diseases. It has been used as a functional food ingredient and as a traditional herbal medicine as an antipyretic, diuretic, and anthelmintic. It has been used for the treatments of diarrhea, gastrointestinal hemorrhage, and eczema (Yang *et al.*,

2013). It has also been used for anti-tumor, viral hepatitis, enteritis, viral myocarditis, arrhythmia, and skin diseases (He *et al.*, 2015).

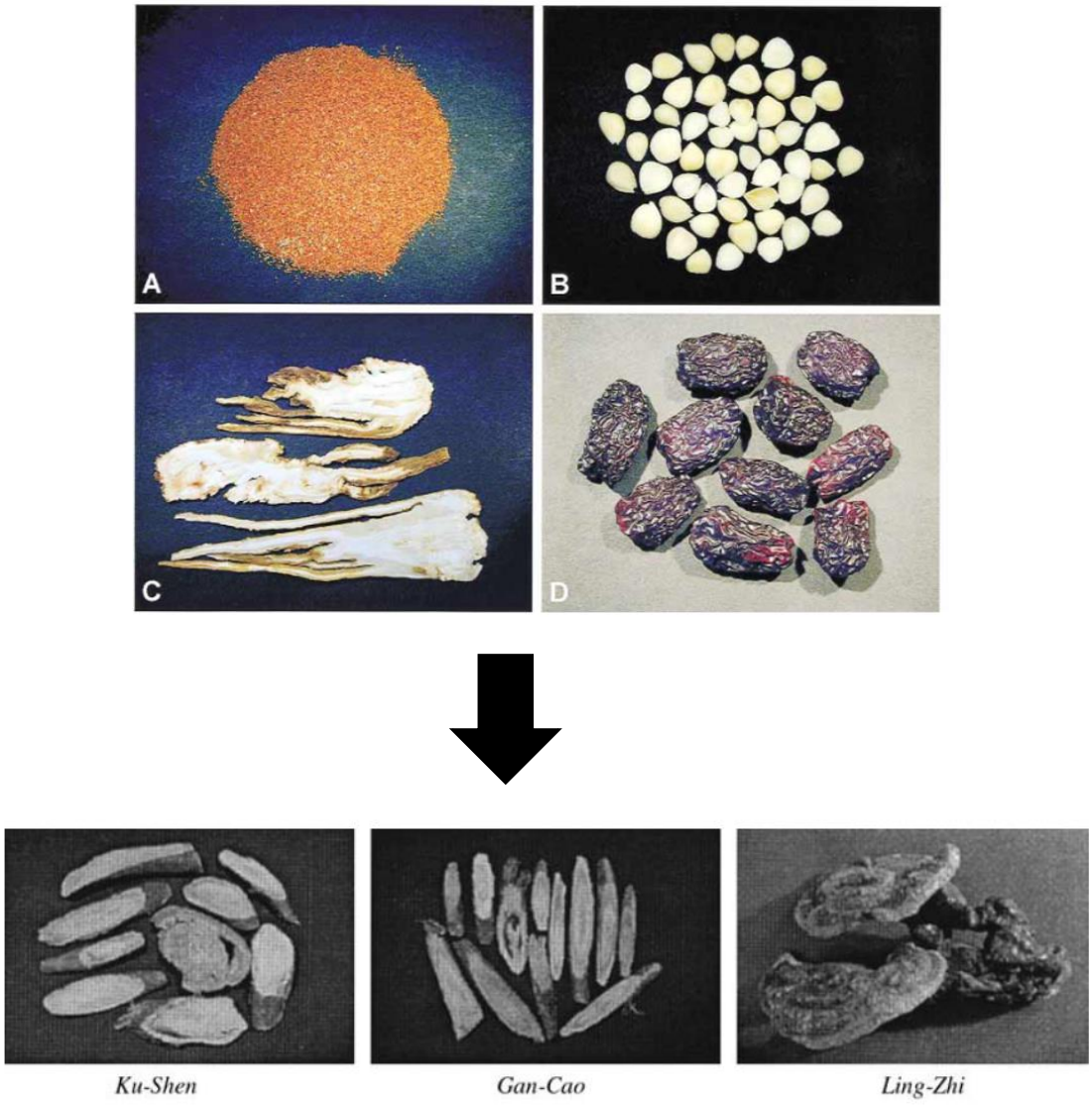


Figure 8: ASHMI – Antiasthma Simplified Herbal Medical Intervention derived from 14 herb formula MSSM-002 contains three components of ASHMI; *Ku-Shen, Gan-Cao, Ling Zhi*. Figure and legend taken from (Li *et al.*, 2000, Kelly-Pieper *et al.*, 2009).

M. Formononetin

I. Structure, Source and Beneficial Effects

Formononetin (7-hydroxy, 4'-methoxy isoflavone) is an O-methylated isoflavone that can be found in legumes, clovers such as red clovers *Trifolium pratense L., Sophorae flavescentis, Astragalus membraneus* (Heinonen *et al.,* 2004). Formononetin belongs to a class of phytoestrogens, it has a chemical structure partially similar to the estrogen structure (Kulling *et al.,* 2002). It belongs to a large family of secondary plant metabolites that have health benefits such as antioxidant, apoptotic, anti-inflammatory, antiproliferative (Burkard *et al.,* 2017). Clinical trials have demonstrated its beneficial effects in preventing bone loss, relieving menopausal pain (Nestel *et al.,* 1999). It has also been shown to be beneficial in the treatment of carcinomas (Wang *et al.,* 2018). This is due to its partial agonist activity on estrogen receptors namely the estrogen receptors α and β (Chaturantabut *et al.,* 2019).

The different sources of formononetin.		
#	**Plant species**	**Part of plant isolated**
1	*Actaea racemosa* L. (black cohosh)	Roots, rhizomes
2	*Amorpha fruticosa* L.	Roots, leaves
3	*Andira inermis* (Wright) DC.	Stems, leaves
4	*Astragalus membranaceus* (Fisch). Bunge	Roots
5	*Astragalus mongholicus* Bunge	Roots
6	*Baptisia australis* (L). R. Br.	Stem, leaves, flower
7	*Bolusanthus speciosus* (Bolus) Harms	Root wood
8	*Cicer arietinum* L. (Chickpea)	Germinated seeds, stem
9	*Dalbergia cearensis* Ducke	Wood
10	*Dalbergia ecastophyllum* (L). Taub	Wood
11	*Dalbergia stevensonii* Standl.	Bark, heartwood
12	*Euphorbia portlandica* L.	air-dried whole plant
13	*Glycyrrhiza glabra* L.	Roots
14	*Glycyrrhiza pallidiflora* Maxim.	Roots
15	*Glycyrrhiza uralensis* Fisch.	Roots
16	*Medicago sativa* L. (Alfalfa)	Root exudate, aerial parts
17	*Ononis spinosa* L.	Roots
18	*Pterocarpus indicus* Willd.	Heartwood
19	*Pueraria lobata* (Willd). Ohwi	Roots, vines
20	*Sophora flavescens* Aiton	Roots
21	*Trifolium pratense* L.	Aerial parts of plant
22	*Trifolium subterraneum* L.	Leaves
23	*Virola caducifolia* W. A. Rodrigues	Trunk wood
24	*Virola multinervia* Ducke	Wood

Table 1: The different isolation sources of formononetin. Table and legend taken from (Tay *et al.*, 2019).

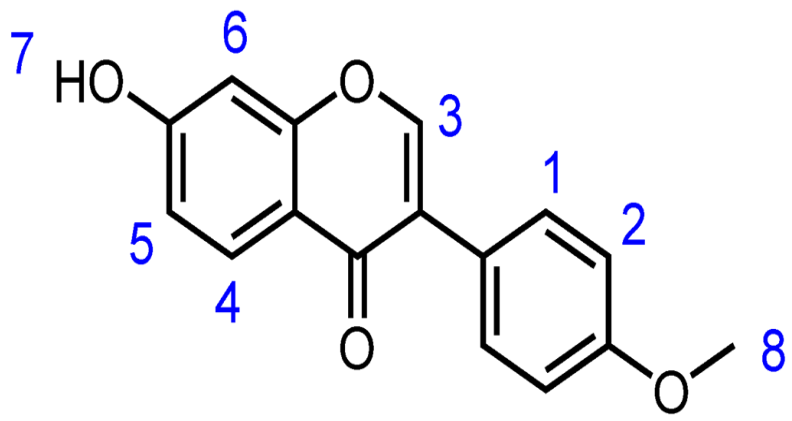


Figure 9: Structure of formononetin. Figure and legend taken from (Yang *et al.*, 2022)

II. Metabolism, Bioavailability and Pharmacokinetics

Formononetin is lipophilic therefore it is rapidly absorbed into the gut, it has a half-life of 2-3 h after oral administration. Upon oral administration of 20 mg/kg, formononetin has a bioavailability of about 22% in rats (Singh *et al.*, 2011). The main absorption site of formononetin is the small intestine (Luo, Fan *et al.*, 2018). The in vivo efficacy of formononetin is higher than predicted, this is probably due to biotransformation (Tay *et al.*, 2019). Biotransformation is a metabolic process that takes place in the liver, it is a series of reactions that alter the chemical structure of substance causing them to become inactive, active, or even toxic (Almazroo *et al.*, 2017).

N. Study Outcomes

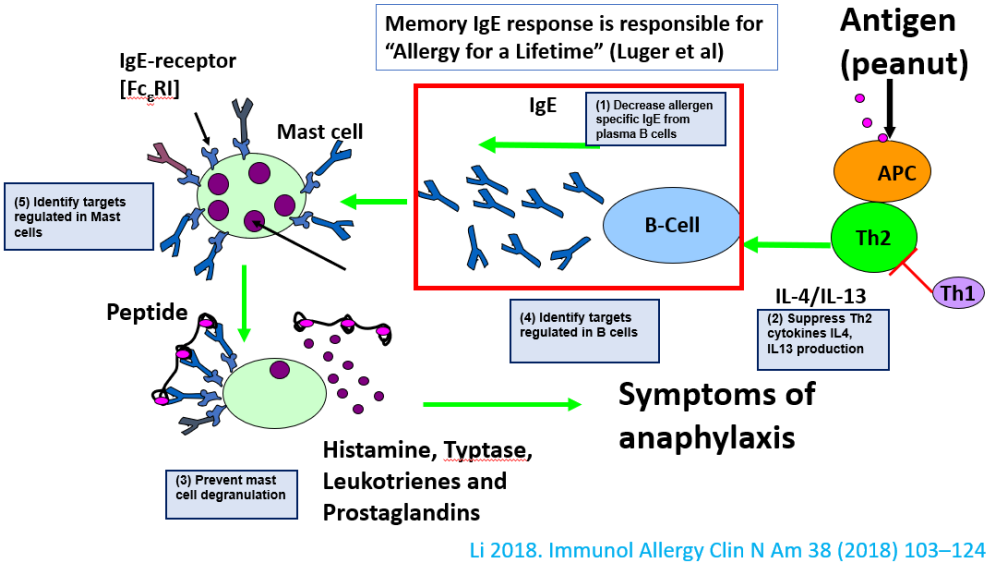


Figure 10: Study outcome. Image taken from (Li 2011).

O. Hypothesis

The overall goal of this study is to identify the bioactive components of ASHMI formula and determine if it prevents food induced anaphylaxis. We hypothesize that the bioactive component isolated from *Sophorae flavescentis* – a component of ASHMI (anti-asthma simplified herbal medicine intervention) prevents food anaphylaxis by regulating key mediators in T cells, B cells and Mast cells. To answer this question, we divided the study into three major aims: 1. Identify the IgE inhibitory compound(s) in ASHMI and the mechanism by which it mediates its effect in B cells, 2. Computational analysis to identify therapeutic targets, biological process regulated, and pharmacological mechanism of formononetin relevant to food allergy, 3. Investigate if formononetin prevents food anaphylaxis in vivo using a peanut allergic mice model.

P. Specific Aims

Aim 1: Identify the IgE inhibitory compound(s) in ASHMI and the mechanism by which it mediates its effect in B cells.

1.1. Determine IgE inhibitory potential of the components of ASHMI.

1.2. Investigate bioactive compound in the component with the most potent IgE inhibitory effect.

1.3. Determine effect on IgEH and XBP-1 in B cells critical for IgE production.

Aim 2: Computational analysis to identify therapeutic targets, biological regulated and pharmacological mechanism of formononetin relevant to food allergy.

2.1. Determine the therapeutic and biological targets of formononetin in food allergy via targets mining from databases.

2.2. Determine the biological functions, pharmacological mechanism, of formononetin relevant to food allergy via pathway analysis – KEGG pathway, Gene ontology, CTPD network and protein-protein interaction.

2.3. Invitro validation of therapeutic targets in U266 cell lines.

Aim 3: Investigate if formononetin prevents food anaphylaxis in vivo using a peanut allergic mouse model.

3.1. Intraperitoneal sensitization of mice model to peanut allergy, oral food challenge.

3.2. Administer treatment and determine if formononetin prevents peanut induced anaphylaxis.

3.3 Determine effect of formononetin on serum antibodies and cytokines relevant to food allergy.

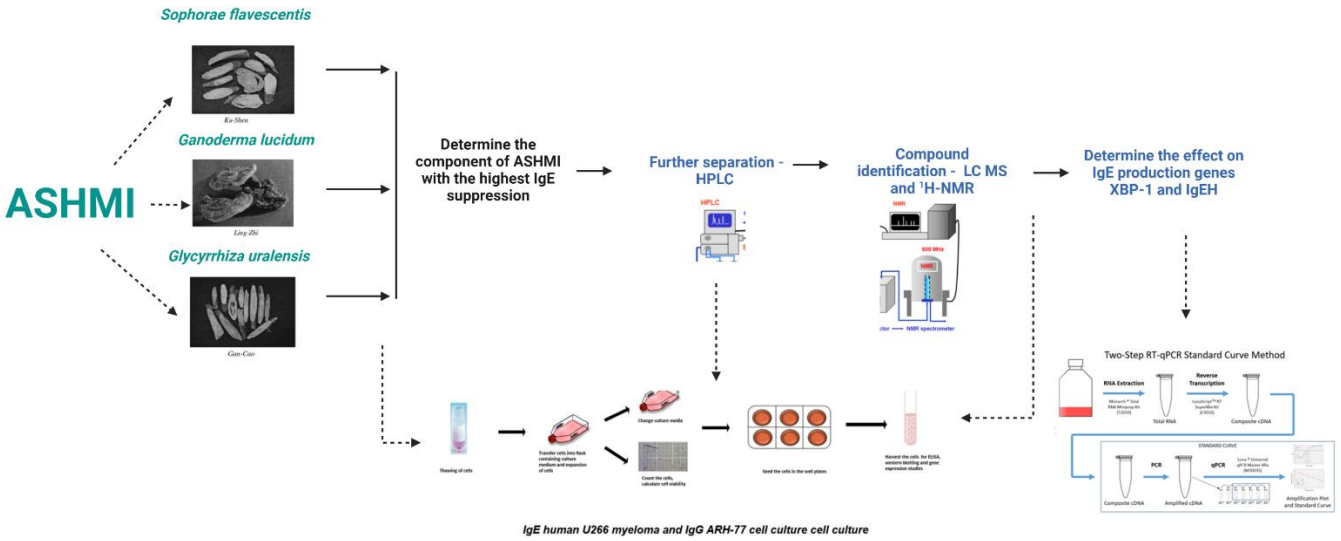
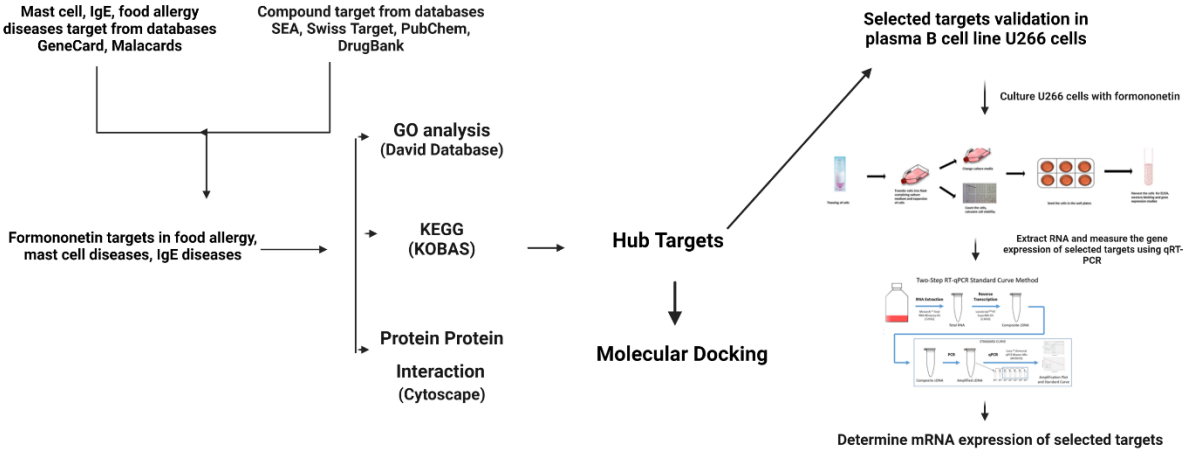


Figure 11: Methods and workflow of Aim 1 [original image].



Work flow: Computational analysis and In vitro Validation of effect of Formononetin in B cells IgE regulation and Mast cells activation relevant to food allergy

Figure 12: Methods and workflow of Aim 2 [original image].

2. MATERIALS AND METHODS

A. Cell culture reagents

"The reagents used to prepare the cell culture media used in this study were obtained as follows. We purchased 2-Mercaptoethanol (2-ME), sodium pyruvate, (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) and dimethyl sulfoxide (DMSO) from Sigma Aldrich (St Louis, MO). We purchased formic acid, ethyl acetate; methanol, dichloromethane and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). RPMI 1640 and penicillin-streptomycin were purchased from Mediates Inc (Manassas, VA) ". Adapted from (Yang *et al.*, 2022)

B. ASHMI, Sophorae flavescentis, Ganoderma lucidum and Glycyrrhiza uralensis

"ASHMI product is a dried aqueous extract obtained from MSSM-002, is composed of *Sophorae flavescens* , *Ganoderma uralensis* and *Gandoderma lucidum*, in capsule form. (Wen *et al.*, 2005) ASHMI and the water extract of all three herbal medicines were manufactured in the Sino-Lion Pharmaceutical Company in Weifang, China which is a GMP-certified facility (Wen *et al.*, 2005). This ensured that the product was manufactured according to good manufacturing guidelines, to ensure high product safety and product quality standard. The raw materials used for the manufacture of the herbs are all from China. These raw herbs have a high quality rating which is in accordance with the quality criteria as defined by the Pharmacopoeia of People's Republic of China.(China 2005). The raw herbs were chopped into pieces, these pieces were then soaked in water for 60 minutes. The soaked pieces were then boiled for 2 hours. The procedure was repeated twice, and the

decoctions were collected and concentrated under vacuum (Kelly-Pieper *et al.,* 2009)". Adapted from (Yang *et al.,* 2022)

C. Identification of active compounds and fractionation of Radix Sophora flavescens

*"*To identify the active compounds in *Sophorae flavescens* we used HPLC to separate the extract into fractions. Dichloromethane extraction of *Sophorae flavescens* was done utilizing liquid-liquid extraction method. To do this, we measured 50g of *Sophorae flavescens* obtained from the manufacturer Sino-lion Pharmaceutical Company. The weighed *Sophorae flavescens* was then dissolved into 4L water. After dissolving it in water, we then added 3L of CH_2Cl_2 in liquid-liquid extractor. This was left for 24 hours, after which we collected the non-polar fraction and using a rotary evaporator, we dried the fraction at low pressure. The dried residues were then dissolved again in methanol (300mL). After dissolving in methanol, the mixture was then centrifuged at 2500rpm for 30 minutes. The Auto Purification system coupled with UV/Vis Detector (Waters, MA) was used for further separation into fractions." Adapted from (Yang *et al.*, 2022)

D. NMR and LC-MS to analyze licorice flavonoids.

"To characterize the compounds from the fraction with the highest IgE inhibitory potential. we used a Waters 2695 HPLC system (Waters Corporation, Milford, MA) which is a high-performance liquid chromatography system with a quarternary, low-pressure mixing pump and inline vacuum degassing. The HPLC system was coupled to a Waters Micromass LCT Premier mass spectrometer that delivers high sensitivity, resolution and exact mass measurements for LC/MS applications. Samples were separated on an analytical Column:

Zorbax® SB C18 (5.0 µm, 4.6 x 150 mm) (Agilent Technologies, Santa Clara, CA) with 0.1% formic acid as mobile phase A and acetonitrile with 0.1% formic acid as mobile phase B. A gradient program was used as follows: 0-75min, linear change from A-B (98:2, v/v) to A-B (52:48, v/v). A 15 min equilibration time was used before each HPLC run. The flow rate was set at 1 mL/min. The UV spectra were recorded between 200 and 460nm. The detection wavelength was set at 254nm, as previously described (Yang *et al.,* 2013). Molecular weight was identified using a Time-of-Flight mass spectrometer (LC-MS/TOF, Micromass LCT Premier, Waters Corporation) in both electrospray (ESI) positive and negative mode. 10 µL of sample was analyzed under the same gradient condition described above. The parameters were set as: Capillary Voltage: 3200 v; Cone Voltage: 15 v; Aperture I: 25 v; Desolvation Temperature: 300 ˚C; Source Temperature: 110 ˚C; Desolvation gas: 500 L/h; Nebulizing gas: 40 L/h; Ionization mode: Electrospray; Positive Ion Acquisition Range: m/z 50-1000. The results were collected and analyzed by Empower and Masslynx software (Yang *et al.,* 2013). ^{1}H NMR spectra were obtained on a JOEL instrument at 300 MHz using DMSO-d_6 as the solvent (Yang *et al.,* 2013)". Adapted from (Yang *et al.,* 2022)

E. Cell culture method of IgE producing myeloma cell line

"To determine the IgE inhibitory extracts, fractions and compounds, we used the U266 myeloma cell line. This U266 cell line is an IgE producing myeloma cell that has been used widely in allergy research to screen the potential of IgE inhibitory compounds to prevent IgE production in B cells (Yang *et al.,* 2014). We purchased the U266 cell line from ATCC (American Type Culture Collection; Rockville, MD). The U266 Cells were cultured at

37°C under 5% CO_2 in complete media containing RPMI 1640 medium supplemented, 10% FBS, 1 mM sodium pyruvate, 1×10^{-5} M β-ME and 0.5% penicillin-streptomycin. The cells were cultured to a concentration of 2×10^5 cells/mL. At day 0 the U266 cells were cultured the ASHMI extracts, the three herbs, *Sophorae flavescens, Ganoderma lucidum, Glycyrrhiza uralensis,* and the four fractions of *Sophorae flavescens,* the compounds isolated and purified at different concentrations. The culture was incubated for 6 days. After the incubation period, the cells were harvested and the supernatant was separated by centrifuging at 3000rpm for 10 minutes. The Immunoglobulin E concentration was determined using ELISA Kit (Mabtech Inc, OH) according to the manufacturer's instructions". Adapted from (Yang *et al.,* 2022)

F. Cell culture method of IgG producing human ARH-77 cell line

"To test the specificity of the compound in inhibiting B cell IgE production only, we used the ARH-777 cell line. This cell line has been used to screen the effects of compounds on inhibiting Immunoglobulin G production in B cells. We purchased the ARH-77 cell line from ATCC (American Type Culture Collection; Rockville, MD) (Drewinko *et al.,* 1984). We cultured the ARH-77 cells in the growth medium which contains containing RPMI 1640 medium supplemented, 10% FBS, 1 mM sodium pyruvate, 1×10^{-5} M β-ME and 0.5% penicillin-streptomycin, incubated at 37°C under 5% CO_2. We cultured the cell line to an initial concentration of 2×10^5 cells/mL. At day 0 we added the test compound at the concentrations of 20,10,5, 2.5 (μg/mL). After adding the test compounds, we incubated for 3 days and harvested the cells at the end of the incubation period. Harvested cell culture

was centrifuged at 3000rpm for 10 minutes, and the supernatant was collected. The IgG concentration in the supernatant was then determined using ELISA Kit (Mabtech Inc, OH) ". Adapted from (Yang *et al.*, 2022)

G. Human U266 and ARH-77 cell viability using trypan blue exclusion

"To evaluate the cell viability of the cells we used the trypan blue exclusion assay (Yang, Patil *et al.*, 2013). We pipette 10µL of cell suspension from the cell culture into an Eppendorf tube. Then we add 10 µL of trypan blue dye into the cell suspension in the Eppendorf tube. The mixture of the cell suspension and the trypan blue dye was then loaded onto a hemocytometer and we counted the cells under a microscope. We calculated the cell viability using the formula: Viable cells (%) = (total number of viable cells) / (total number of cells) × 100". Adapted from (Yang *et al.*, 2022)

H. Cell cytotoxicity measured by CCK-8 assay

"We used another assay known as CCK-8 assay to evaluate cell cytotoxic effect of formononetin in U266 cells. This assay has been used in studies by (Kagawa, Nakano *et al.*, 2012, Wang, Li *et al.*, 2021). To evaluate the cytotoxicity we cultured formononetin over a dose dependently across the concentration 2.5–20 (µg/mL) and for the control, we used DMSO in a 96 well plate. We incubated the culture with the treatments for 3 days. After the culture time point we added 10µL of CCK8 to each well containing the culture with the treatments and incubated at 37°C for 10 minutes. After the incubation period we measured the optical density using a microplate reader at 450nm" Adapted from (Yang *et al.*, 2022).

I. Quantitative real time polymerase chain reaction (qRT-PCR)

"To measure the relative gene expression of the targets XBP-1, IgEH, BCL-2, NFKBIA, BTK, TYK2, CASP8, Rag1, TP53 we used quantitative real time polymerase chain reaction (qRT-PCR). We cultured U266 cells with formononetin at the concentration of 10(µg/mL) and for the control/untreated culture group, we used DMSO. We incubated the cultured cells with the treatments for 3 days at 37°C under 5% CO_2. After the culture time point, we harvested the cells. After harvesting the cells, the supernatant was collected by centrifuging at 3000rpm for 10 minutes. Then the cells were washed by adding 5mL of PBS and centrifuging at 3000 rpm for 10 minutes. After washing the cells, the total RNA was isolated according to manufacturer's instructions using Trizol regent (Gibco BRL, Rockville, MD). The RNA concentration was measured using triplicate optical density (OD) readings (Bio-Rad SmartSpect 3000; Bio-Rad, Hercules, CA). Using reverse transcription, we obtained cDNA from the isolated RNA. To do this we used the RevertAid RT Reverse Transcription Kit (ThermoFisher, Waltham, MA) and followed the manufacturer's instructions. The thin- walled sterile tubes were chilled on ice, the nuclease free water were chilled on ice. We added RNA in the thin walled tube, and then added the cDNA primer in nuclease free water into the tube. Then we placed the tube into a 70 ˚C for 5 minutes and then after this we placed the tube back on ice to chill it down. We spinned down the tube and returned the spined tube back on ice to prepare the reverse transcription mix. We added the following according to the manufacturers to make the reverse transcription mix recombinant Ribonuclease Inhibitor, reverse transcriptase, dNTP, $MgCl_2$, 5X reaction buffer. Then we added nuclease free water to get a final volume of 15 µl. The mixture of the reverse transcription mix was then added to the mixture of RNA and primer.

Finally we used PCR to complete the reverse transcription. After the completion of reverse transcription we placed the tube on ice and prepared the master mix for RT-PCR. To prepare the mix for RT-PCR, we used maxima SYBR Green qPCR Master Mix (2X) kit (ThermoFisher, Waltham, MA). The master mix prepared was added to the cDNA, then we added the primers for the target genes and GAPDH. The primers that we used were purchased from Sigma-Aldrich Corporation (St. Louis, MO). The primer list can be seen in Table 2. Adapted from (Yang *et al.*, 2022)

J. Protein expression determined using western blotting

"To measure the effect of formononetin on the protein expression of XBP-1. We used wester blotting analysis. To do this we cultured U266 cells to 3.0 x 10^6 cells/mL. Then we added formononetin at a concentration of 10 (µg/mL) and for the untreated control we added DMSO. We incubated the cell culture with the treatments for 3 days at 37°C under 5% CO_2. After the culture time point we harvested the cells and collected the supernatant. To collect the supernatant we centrifuged the cell suspension at 3000rpm for 10minutes. Then we washed the cells by adding 5mL of PBS and centrifuging at 3000rpm for 10minutes. To extract the protein from the washed cells we used the RIPA lysis buffer. We added 100-200 µL of RIPA lysis buffer to the cells and kept it on ice for about an hour followed by intermittent vortexing every 10 minutes. Then we isolated the protein by centrifuging at 14,000rpm for 20 minutes at 4°C and the protein concentration was quantified using Nanodrop Spectrophotometer. We run an electrophoresis gel to separate the proteins at 90V for 15 minutes and then at 100-110V for the rest of the run. After running the gel electrophoresis, we used the semi dry transfer to transfer the gel onto a

PVDF membrane. We blocked the membrane and then incubated overnight with primary antibodies of XBP-1, β-actin. After the overnight incubation, we washed the membrane and incubated the membrane with the secondary antibodies for 2 hours. Then the membrane was washed four times and then using the chemiluminscence imaging we obtained the image of the bands. We obtained the primary and secondary antibodies from the Cell Signaling (Danvers, MA, USA). The primary antibodies-XBP1 and β-actin were used at a 1:1000 dilution while the secondary antibody was used at a 1:10,000 dilution". Adapted from (Yang *et al.,* 2022).

K. Biological and Therapeutic Target analysis

From literature (Liu *et al.*, , Chen *et al.,* 2012) and databases such as, Hit Pick Swiss Target Prediction (Daina *et al.*, 2019), Similarity Ensemble Approach (Keise *et al.,* 2007), PubChem (Kim *et al.,* 2019) and Drug Bank (Wishart *et al.*, 2008) we mined the biological targets of formononetin. The formononetin targets in food allergy and mast cell diseases were identified from the Therapeutic Target Database (Wang *et al.*, 2020), Gene Association Database (Piñero *et al.*, 2020), Gene Cards (Safran *et al.,* 2002), Open Targets Platform (Carvalho-Silva *et al.*, 2019), and Comparative Toxicogenomic Database (Davis *et al.*, 2019). The top 200 genes mined from each of the databases were mapped into the UniProt Database (2019) for normalization. Finally, the common targets of formononetin in food allergy, IgE and mast cell diseases were identified as potential regulated targets which were then used for gene ontology, KEGG pathway and protein-protein interaction analysis.

L. Biological process and function analysis

Target enrichment gene ontology, pathway, and protein-protein interaction (PPI) analyses provides a molecular-level mechanistic insight into biological function. GO was introduced by mapping potential targets to the DAVID database (Huang *et al.,* 2008). The GO biological process terms with a false discovery rate of (FDR) <0.01 were selected. Pathways were obtained by mapping targets to KOBAS 3.0 (Xie *et al.,* 2011) and the significant pathways with FDR <0.01 were selected. Potential targets were mapped to String database, obtaining their interaction. The protein interactions were further used to construct the PPI network using Cytoscape (v3.2.1).

M. Compound-target-pathway-disease (C-T-P-D) network construction and analysis

With obtained targets and significant pathways, C-T-P-D biological networks were constructed using Cytoscape (v3.2.1). The C-T-P-D network, containing formononetin, its related targets for food allergy, IgE and mast cells related diseases, and significant principal pathways provides general information at the molecular level of how formononetin regulates targets in food allergy, IgE and mast cell diseases. The Cytoscape plugin; Network Analyzer (Assenov *et al.*, 2007), was used to validate the results obtained from the CTPD network.

N. Binding mode and interaction analysis

Molecular docking analysis via Auto Dock Vina (Trott and Olson 2010) was used to predict the mode of binding between formononetin and the identified biological targets. Protein crystal structures of TYK2, BTK, P53, BCL2, NFKBIA, CASP8 were obtained from RCSB protein data bank (5-30-2021) (Berman *et al.*, 2000) while the structure of formononetin was obtained from PubChem without further optimization. PyMOL system (DeLano 2002) (5-30-2021) and Discovery Studio (DassaultSystèmesBIOVIAD 2020) were used for the 3D molecular visualization.

O. β-Hexosaminidase enzymatic release

To determine the effect of formononetin in preventing mast cell degranulation we used the β-hexosaminidase enzymatic release assay. To do this we cultured the rat basophilic leukemic cell line. We added the cell culture to at a concentration 2.5 X 10^5 cells/well in a 24 well plate. Then we added the treatments of formononetin at the desired concentration. After adding the treatments, we incubated the plate overnight at 37°C under 5% CO_2. On the second day we added the anti-DNP IgE in all the wells, treatment wells, positive control wells and negative control wells. Then we incubated the plate overnight at 37°C under 5% CO_2. On the third day, we washed the plates using Tyrode buffer three times and then added DNP-BSA 150ηg/mL to the treatment and negative control well, while we added Triton X-100 to the positive control well. Then we incubated the plate for 60min at 37°C under 5% CO_2. After the period of incubation, we transferred 50µL of the supernatant into a 96 well plate and added 5050µL of p-nitrophenyl-N-acetyl-β-D-glucosamide (PNAG) substrate solution (1.3mg/mL in 0.1M citric acid solution, pH 4.5). The plates were then incubated at

37°C under 5% CO_2 for another 60minutes. At the end of the incubation period we used glycine solution in water (0.2M, pH 10.7) to stop the reaction and measured the optical density using a plate reader. We used 3-(4,5 dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay to measure the cell viability according to methods as previously published in (Li *et al.,* 2008).

P. Mice

To conduct the in vivo study, we used six-week-old female C3H/HeJ mice. These mice were purchased from Jackson Laboratory (Bar Harbor, ME). Upon arrival in the animal facility, the mice were placed on a peanut free chow and in accordance with the standard for care and animals. We grouped the mice into three and labelled it as sham group, the naïve group, and the treatment group.

Q. Establishing peanut allergic mice model via intraperitoneal sensitization, and intragastric challenge

To sensitize the mice to peanut allergy, we administered intraperitoneally crude peanut extract at 500μg and alum 2mg of alum in a 500μL of phosphate buffered saline (PBS). This administration was done weekly for four weeks. Two weeks after the sensitization period we challenged the mice by administering 200mg of freshly ground peanut (White Rose brand, NJ) in 200μL PBS intragastrically. The intragastric administration was done twice in 1hour and the rectal temperature and anaphylactic symptoms were measured.

R. Measure of anaphylactic symptoms and rectal temperature

Upon challenging the mice via intragastric administration of freshly ground peanut we measured the anaphylactic symptoms displayed by the mice. We scored these symptoms using the rating criteria: 0, no signs of anaphylaxis; 1, scratching and rubbing around the mouth and head of the mice; 2, redness and puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration and cyanosis around the mouth and tail; 4, no activity after prodding or tremor and convulsions and 5, death. We measure the rectal temperature using a temperature probe purchased from (Harvard Apparatus, USA). The temperature was measured every 10 minutes over a period of 1 hour.

S. Determine mast cell degranulation in mice via measure of plasma histamine

To determine if the formononetin prevents anaphylaxis by inhibiting mast cell degranulation we measured the level of histamine after the oral food challenge. To do this we drew blood 30 minutes after the challenge from the submandibular region. The blood was collected in a heparinized tube and plasma was obtained. Using an enzyme immunoassay kit purchased from (Immunotech, France and Moredun Scientific, UK for histamine) and we measured histamine level according to the manufacturer's instruction.

T. Determination of the level of serum peanut specific antibodies IgE, IgG1 and IgG2a

To determine if the peanut specific serum antibodies were affected by the treatment of formononetin. To do this we drew blood from the submandibular region of the mice. The serum was isolated from the blood drawn and used for the measure of the serum peanut

specific antibodies via ELISA as previously described (Srivastava, Siefert *et al.,* 2016). To do this we coated the ELISA plates with crude peanut extract in coating buffer for the sample wells, DNP-HSA diluted in coating buffer for peanut specific IgG1 and IgG2a, and rat anti-mouse IgE for peanut specific IgE for the reference wells in the first day. After coating the plate, we incubated the plate overnight at 4°C. On the second day we washed the plate three times and blocked the plate with 2% BSA in PBS or 10% FBS in PBS for 2 hours in room temperature. After blocking the plates, we added serum samples diluted at 1:20 for peanut specific IgE measurement and 1:1000 for peanut specific IgG2a measurement and 1:10,000 for peanut specific IgG1 to the peanut coated wells. Then we added the mouse anti DNP-IgG1 (Accurate Antibodies, Westbury, NY) to the DNP coated reference wells for the peanut specific IgG1; mouse anti DNP-IgG2a (Accurate Antibodies, Westbury, NY) to the DNP coated reference wells for the peanut specific IgG2a and purified mouse IgE for the peanut specific IgE(BD Biosciences). After we incubated the plate overnight at 4°C. On the third day we washed the plates three times. After washing the plates six times we added; biotinylated rat anti-mouse IgE detector antibody diluted in 2% BSA in PBS or 10% FBS in PBS for the peanut specific IgE, biotinylated rat anti-mouse IgG1 detector antibody diluted in 2% BSA in PBS or 10% FBS in PBS for the peanut specific IgG1, biotinylated rat anti-mouse IgG2a detector antibody diluted in 2% BSA in PBS or 10% FBS in PBS for the peanut specific IgG2a. We incubated the plate at room temperature for 45 minutes to 1 hour after adding the biotinylated antibody. After incubating the plates at room temperature, we washed the plates six time and added avidin-peroxidase (AVP) diluted in 2% BSA in PBS or 10% FBS in PBS at 1:4000. After adding AVP we incubated for 45 minutes to 1 hour at room temperature. After the incubation

period we washed the plate nine times. After washing the plates, we added the ABTS substrate purchased from (KPL, St Paul, Minn). We allowed the color to develop for 30 minutes to 1 hour and measured the optical density using a microplate reader at 405nm.

U. Determination of the level of Th1, Th2t cytokines from mesenteric lymph nodes and splenocyte culture

To determine if formononetin treatments influences the Th1 and Th2 cytokines. We collected splenocytes and mesenteric lymph nodes (MLN) from the mice groups. The spleens collected from the mice were gently crushed and collected in a 6 well plate and resuspended in growth media containing RPMI 1640, penicillin streptomycin and fetal bovine serum. The cells were then cultured in a 24 well plate (4×10^6 /well/mL) in the presence or absence of concanavalin A (2.5μg/mL) or crude peanut extract (2005μg/mL). The culture was incubated for 72 hours at 37°C under 5% CO_2. At the end of the culture time point the cells were harvested and the supernatant were collected by centrifuging at 3000rpm for 10 minutes. The cytokines were determined from the supernatant using ELISA according to the manufacturer's instructions. The ELISA kit for cytokine measurements were obtained from R&D Systems, Minneapolis, Minnesota for IL-13 cytokine only and the remainder cytokine kits were all obtained from BD Pharmingen, San Jose, California.

V. Data analysis

"The data obtained from this project was analyzed using the Sigma Stat 3.5 application purchased from Systat Software Inc., Chicago, Illinois and using GraphPad Prism purchased from GraphPad Software, Inc, San Diego, California. The figures were generated

using GraphPad Prism. Data analysis was done using One way analysis of variance (ANOVA) followed by Bonferroni correction for pairwise comparisons or by Donne's method for skewed data. *p* value of ≤ 0.05 is statistically significant, ≤ 0.01 is very significant, ≤ 0.001 is extremely significant". Adapted from (Yang *et al.,* 2022)

Primer	**Sequence (5'-3')**
GAPDH	F- 5'-GAGGCAGGGATGATGT TCTG-3' R- 5' CAGCCTCAAGATCATCAGCA-3'
BCL2	F - 5' GTTGATGGGATCGTTGCCTTA 3' R - 5' CCTTGGCATGAGATGCAGGA 3'
NFKBIA	F - 5'-AAG TGG TCC GCC AAG TGA AG-3′ R 5′-CGATTTCTGGCTGGTTAGTGATC-3′
BTK	F 5'-ACAGATTCCGAGGAGAGGTGAGG-3' R 5'-GGTCCTTCATCATATACAACCTGGAATGG-3'
TYK2	F 5'-GCCGTCTAGACTTCAAGGACTGCATCCCG-3' R 5'-GCCGATCGATAGCAGGGGTCCGTGGATC-3'
CASP8	F, 5'-CTGCTGGGGATGGCCACTGTG- 3' R, 5' –TCGCCTCGAGGACATCGCTCTC- 3'
Rag1	F 5′-CTCATTGCCAGAGTTTTCCG-3′ R 5′-TGCTGACCCTAGCCTGAGTT-3′
P53	F 5'-TGCGTGTGGAGTATTTGGATG-3' R 5'-TGGTACAGTCAGAGCCAACCTC-3'
XBP1	F - 5'- TCACCCCTCCAGAACATCTC -3' R - 5'- AAAGGGAGGCTGGTAAGGAA -3
IgEH	F - 5'- ACCCTGGTCACCGTCTCCTCAG -3' R - 5'- CAGAGTCACGGAGGTGGCATT -3'

Table 2. List of primer sequences

3. Results

A. Aim 1: Identify the IgE inhibitory compound(s) in ASHMI and the mechanism by which it mediates its effect in B cells.

I. Anti-Asthma Simplified Herbal Medicine Intervention (ASHMI), *Sophorae flavescens, Ganoderma lucidum, Glycyrrhiza uralensis* inhibited IgE production dose dependently

"We determined the effect of formula anti-asthma simplified herbal medicine intervention, and its constituents *Sophorae flavescens, Ganoderma lucidum, Glycyrrhiza uralensis* on IgE production in IgE producing human myeloma cell line known as U266 cells. To do this we cultured ASHMI over a concentration range from 31 – 500 µg/mL and used DMSO for the untreated or control group. The culture was incubated for 6 days (Yang, Zhao *et al.*, 2014). After the incubation period of 6 days, we harvested the cells, collected the supernatant and measured Immunoglobulin E levels using ELISA. We saw that ASHMI inhibited IgE production significantly in a dose dependent manner (Figure 13A, $p<0.05$; $p<0.001$), the IC_{50} value for this inhibition was 49.76 µg/mL (Figure 13B). Then we measured cytotoxic effect of ASHMI by using trypan blue exclusion to determine the cell viability in both the treated and untreated or control group. Our results showed that ASHMI did not have any significant cytotoxic effect on IgE producing human myeloma cells (Figure 13C).

Upon seeing the effect of ASHMI on IgE production, next we wanted to determine the effect of each of its three constituents individually on IgE production, additionally we wanted to determine which of the three constituents has the most potent IgE inhibitory effect. We believe the three components inhibit IgE, however at a different potency level.

Our goal is to identify the most potent of the three components of ASHMI. Once the component with the highest IgE potency has been identified we will further isolate and identify the IgE bioactive component. To do this we cultured each of the three constituents of ASHMI: *Sophora flavescens*, *Glycyrrhiza uralensis*, and *Ganoderma lucidum* with IgE producing human myeloma cells. Each of the component was cultured at a concentration of 250 µg/mL. After the culture we harvested the cells and collected the supernatant. We measured IgE production using ELISA. Our results showed that each of the three components inhibited IgE production. However, *Sophora flavescens* was determined to have the most potent IgE inhibitory effect. Our results showed that Sophora flavescens had an IgE inhibition percentage of 96.54 % ± 3.01 %; *Glycyrrhiza uralensis* had an IgE inhibition percentage of 33.90 % ± 5.63 % and *Ganoderma lucidum* had a percentage of inhibition of 33.35 % ± 8.20 % (Figure 14, $p<0.001$). With the data showing that *Sophora flavescens* had the most potent IgE inhibitory effect, we determined the IgE inhibitory over a concentration range of 13.2 - 211.3 µg/mL. Our results showed that *Sophora flavescens* significantly inhibited IgE production dose dependently (Figure 15A, $p<0.01$) with an IC_{50} value of 43.13 µg/mL (Figure 15B). Cell viability using trypan blue exclusion also showed that there was no significant cytotoxicity (Figure 15C)". (Yang *et al.,* 2022).

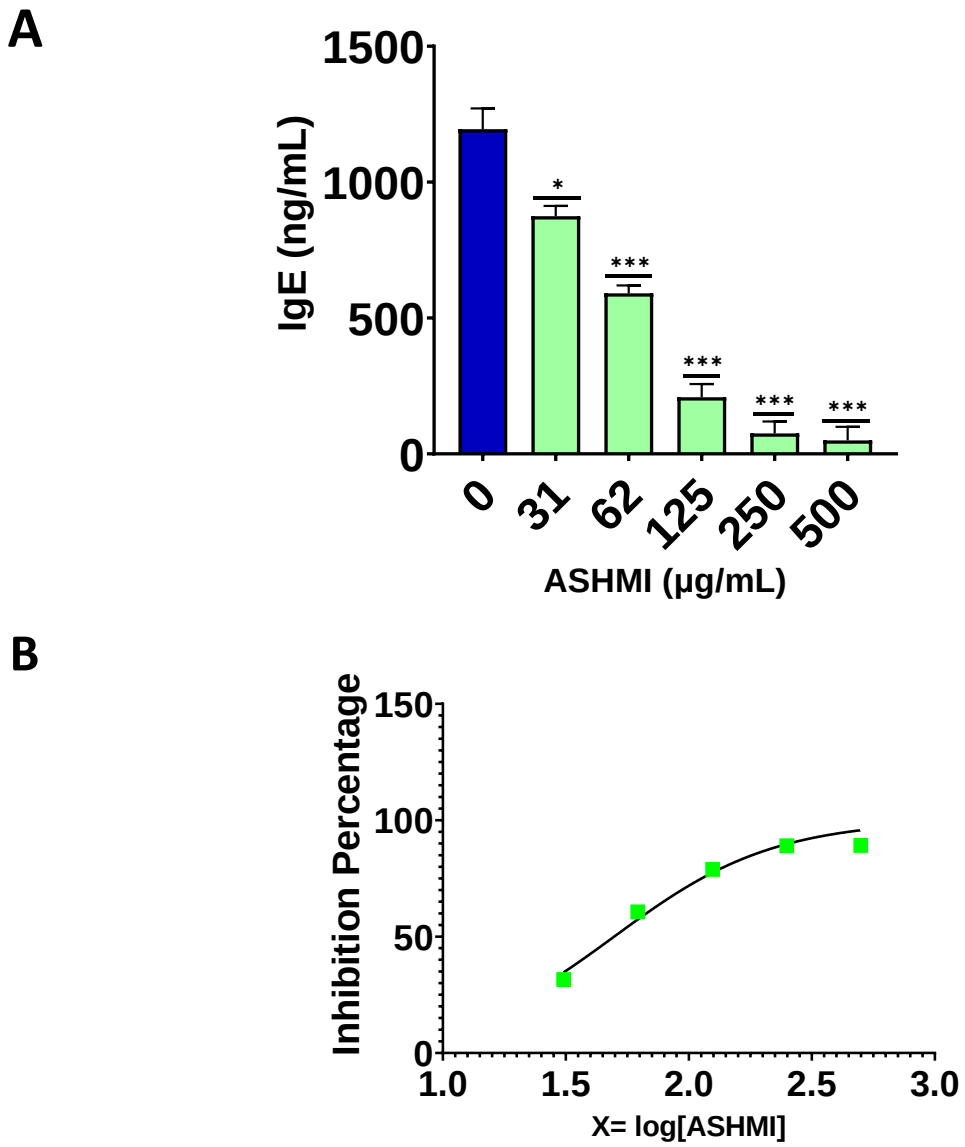


Figure 13. "Effect of Anti-Asthma Simplified Herbal Medicine Intervention (ASHMI) on IgE production by IgE producing human myeloma cells. IgE producing human myeloma cells at (2×10^5 cells/mL) were cultured with ASHMI across a concentration of 31, 62, 125, 250 and 500µg/mL for 6 days. After the culture time point, the cells were harvested and IgE concentration was determined by ELISA from the supernatant. A. Effect of ASHMI on IgE production by IgE producing human myeloma cells. B. IgE inhibition percentage vs. log [ASHMI concentration] curve. N=3, data represents triplicate experiments. *$p<0.05$; ** $p<0.01$; *** $p<0.001$. Results expressed as mean± S.D. Figure and legend taken from (Yang *et al.,* 2022) Panel A, B was provided by Dr. Yang.

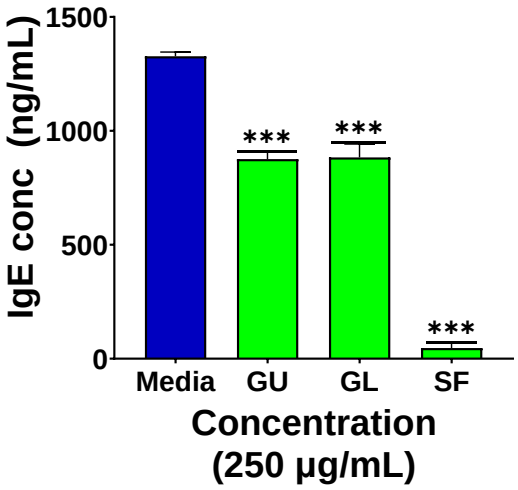
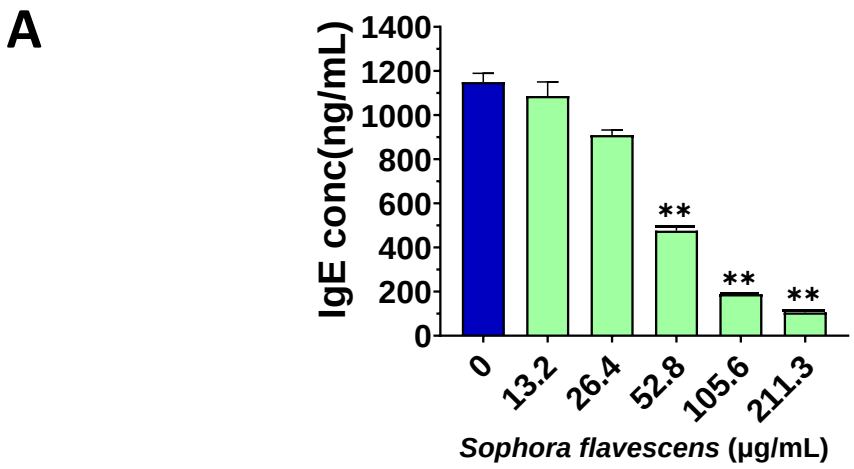


Figure 14. "Effect of the constituents of ASHMI- *Sophora flavescens*, *Glycyrrhiza uralensis*, and *Ganoderma lucidum* on IgE production. IgE producing human myeloma cells at (2×10^5 cells/mL) were cultured with the constituents at a concentration of 250µg/mL for 6 days. After the culture time point, the cells were harvested, and ELISA was used to determine IgE concentration from the supernatant. N=3, data represents triplicate experiments. *p<0.05; ** p<0.01; *** p<0.001. Results expressed as mean± S.D. Figure and legend taken from (Yang *et al.,* 2022) data provided by Dr. Yang.



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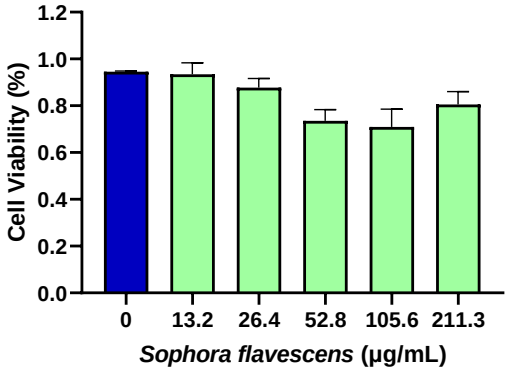

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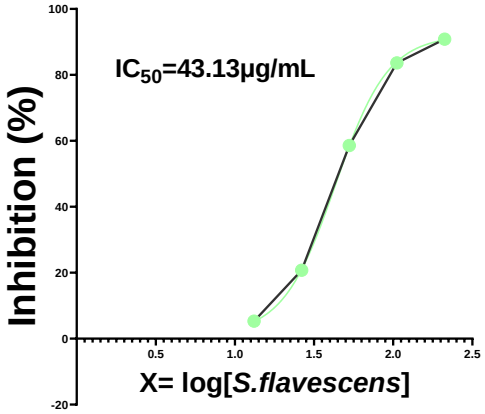


Figure 15. "Sophorae flavescens inhibits IgE inhibit in a dose dependent manner without cytotoxicity. A. *Sophorae flavescens* inhibited IgE production dose dependently. B. Effect of *Sophorae flavescens* on cell viability. C. IgE inhibition percentage vs. log [Sophora flavescens concentration] curve. IgE producing human myeloma cells at (2×10^5 cells/mL) were cultured with *Sophorae flavescens* across a concentration of 13.2, 26.4, 52.8, 105.6, 211.3µg/mL for 6 days. After the culture time point, the cells were harvested, and ELISA was used to determine IgE concentration from the supernatant. N=3, data represents triplicate experiments. *p<0.05; ** p<0.01; *** p<0.001. Results expressed as mean± S.D. Figure and legend taken from (Yang *et al.*, 2022) Panel A, B was provided by Dr. Yang.

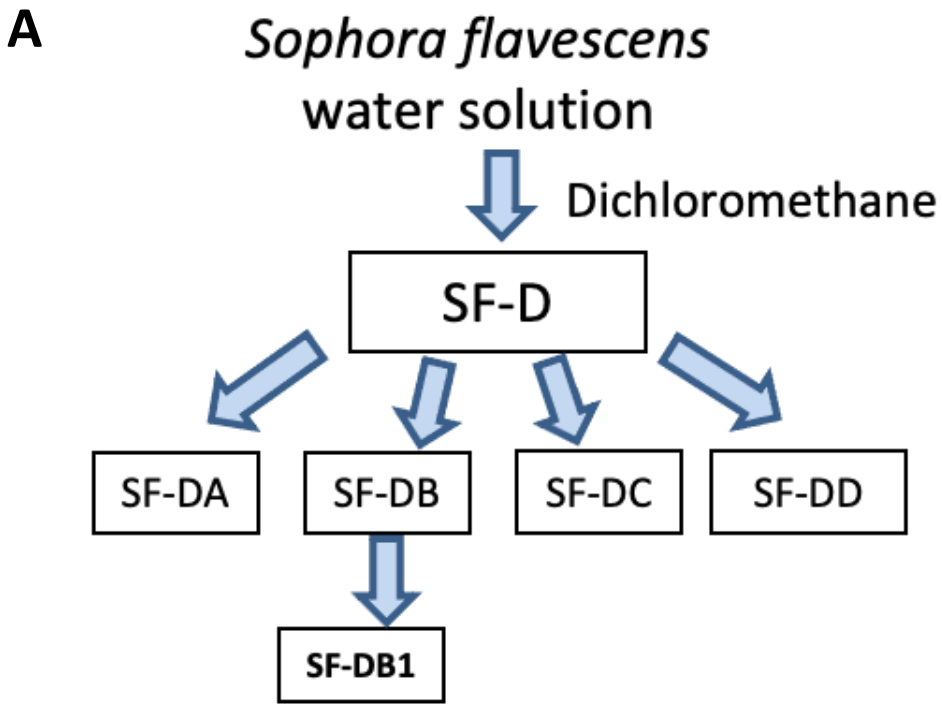
II. Inhibition of Immunoglobulin E production in IgE producing human myeloma cells by the dichloromethane extract of *Sophora flavescens* and its sub fractions

"Our previous data showed that *Sophora flavescens* has the highest potency of the three components of ASHMI: *Sophora flavescens*, *Glycyrrhiza uralensis*, and *Ganoderma lucidum.* Therefore, we wanted to identify and isolate the IgE bioactive component. The flow chart show in (Figure 16A) shows the steps used in extraction of the bioactive components of *Sophora flavescens.* The extract from *Sophora flavescens* was done using the organic solvent dichloromethane. The extract named SF-D was further separated according to polarity using Auto Purification system coupled with UV/Vis Detector into four subfractions know as SF-DA, SF-DB, SF-DC, SF-DD (Figure 16B). Bioactivity fractionation process was used to further isolate the active compounds in the subfraction. We determined the effect of the dichloromethane extract of *Sophora flavescens* on IgE production in IgE producing human myeloma cells. Our results showed that the dichloromethane extract of *Sophora flavescens* inhibits immunoglobulin E production across the concentration tested and dose dependently (Figure 17A $p<0.001$). The IC_{50} value of inhibition of the dichloromethane extract of *Sophora flavescens* is 1.612 µg/mL (Figure 17C). The cytotoxicity determined by measure of cell viability showed that the inhibition occurred with our significant cytotoxicity (Figure 17B).

Upon seeing the IgE inhibitory effect of the dichloromethane extract of *Sophora flavescens*, we next determined the IgE inhibitory effect of each of the subfractions. We cultured subfractions; SF-DA, SF-DB, SF-DC, SF-DD with IgE producing human myeloma cells. We measured IgE production and determined the percentage of inhibition. Our results showed a significant inhibition of IgE production by all four subfractions. The inhibition

percentage of the subfractions is as follows; SF-DA -10.93 % ± 3.17 %; SF-DB - 97.37 % ± 0.03 %; SF-DC - 97.30 % ± 0.25 %; and SF-DD - 94.37 % ± 0.1 % (Figure 18A $p<0.01$; $p<0.001$).

Upon seeing these results, we decided to isolate the active compounds from the fraction with the most potent IgE inhibition percentage. SF-DB and SF-DC had the highest IgE inhibition percentage. SF-DB had the highest isolation efficiency and two major peaks from the HPLC chromatogram. SF-DC on the other hand two small peaks but with a relatively low yield which made it difficult to isolate the pure compound. While SF-DD which was collected as the washout contained many small peaks and would pose difficulty in isolating the pure compounds in it. Therefore, we focused on SF-DB to isolate the pure compounds in it. Before isolating and purifying the compounds in SF-DB, we cultured SF-DB across a concentration of 1.6-25µg/mL with IgE producing human myeloma cells and determined the levels of IgE using ELISA. Our results showed that SF-DB significantly inhibited IgE production in a dose dependent manner. We measured its cytotoxic effect via and our results showed that this inhibition occurred in a non-cytotoxic manner. (Figure 18B, $p<0.05$; $p<0.01$, Figure 18C). Upon seeing this we further isolated the compound and determined the purity of the isolated compound to be >96% using analytical HPLC". Adapted from (Yang, Musa *et al.*, 2022)



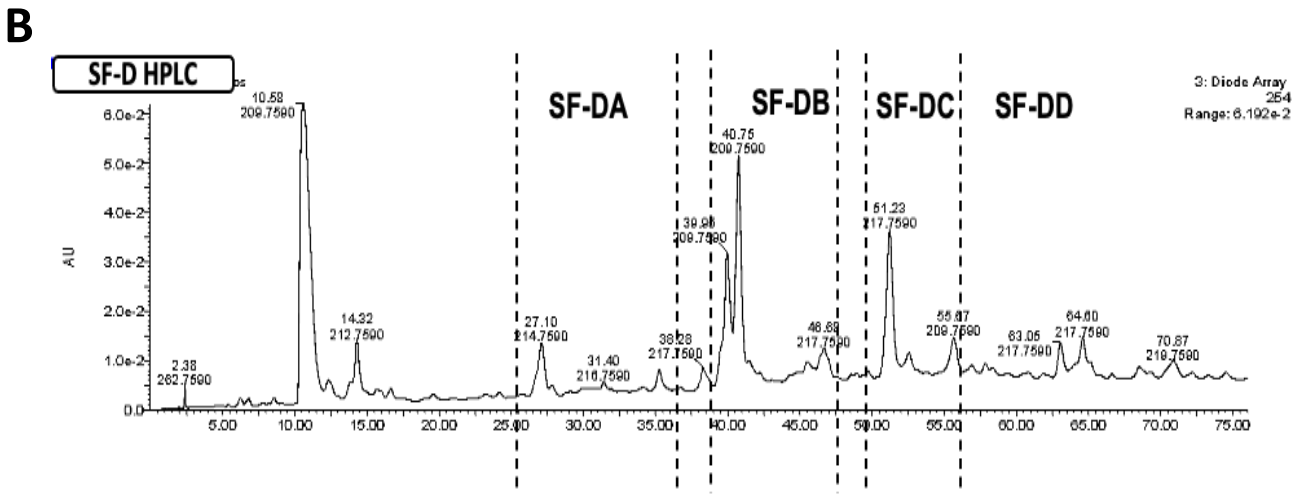


Figure 16. "The process of isolating the compound in Sophorae flavescens. A. Process of isolating and purifying the compounds shown in a flow chart. B. HPLC chromatogram obtained from the Auto-Purification system". Figure and legend from (Yang *et al.*, 2022). Panel A, B was data provided by Dr. Yang.

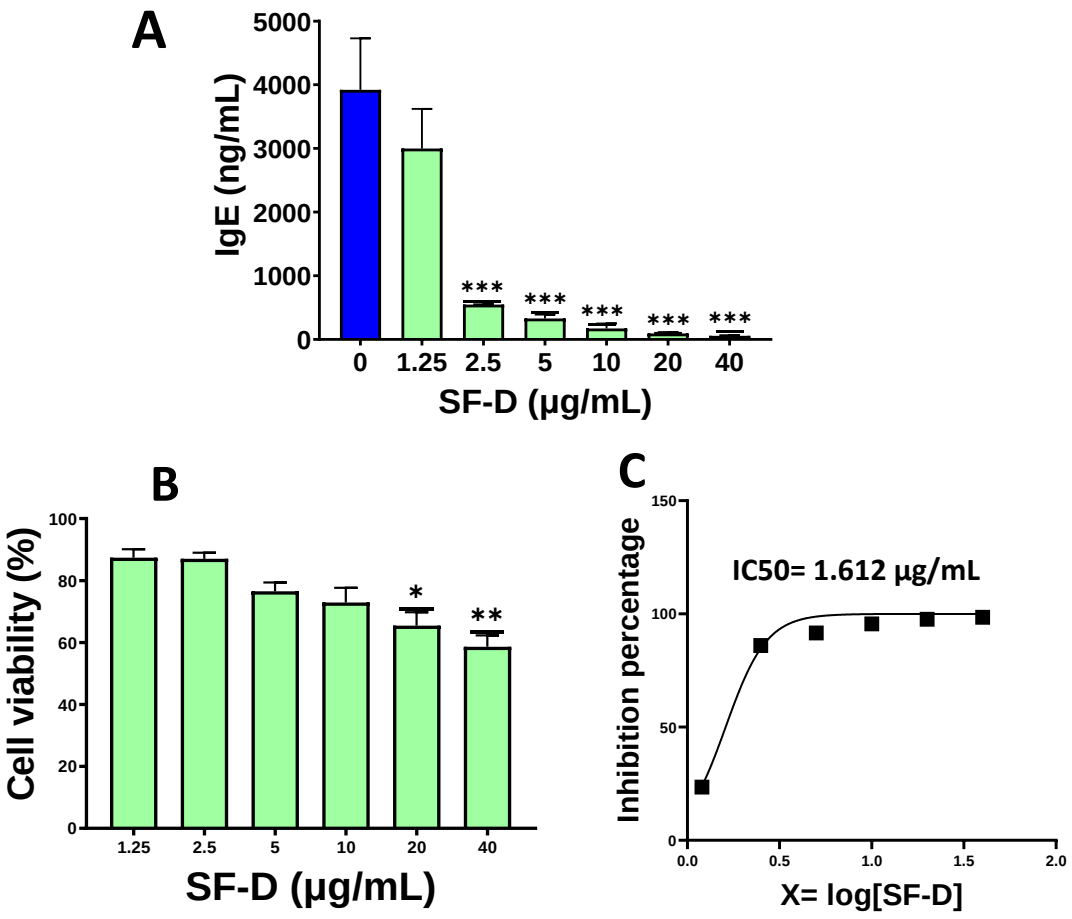


Figure 17. "The dichloromethane extract of Sophora flavescens (SF-D) inhibits IgE without cytotoxicity A. The dichloromethane extract of Sophora flavescens (SF-D) inhibits IgE production in IgE producing human myeloma cells. B. Cell viability of dichloromethane extract of Sophora flavescens (SF-D) cultured with IgE producing human myeloma cells. C. IgE inhibition percentage vs. log [SF-D concentration] curve. IgE producing human myeloma cells at (2×10^5 cells/mL) were cultured with dichloromethane extract of *Sophorae flavescens* across a concentration of 1.25, 2.5, 5, 10, 20µg/mL for 6 days. After the culture time point, the cells were harvested, and ELISA was used to determine IgE concentration from the supernatant. N=3, data represents triplicate experiments. *$p<0.05$; ** $p<0.01$; *** $p<0.001$. Results expressed as mean± S.D. Figure and legend taken from (Yang *et al.*, 2022) Panel A, B,C was provided by Dr. Yang

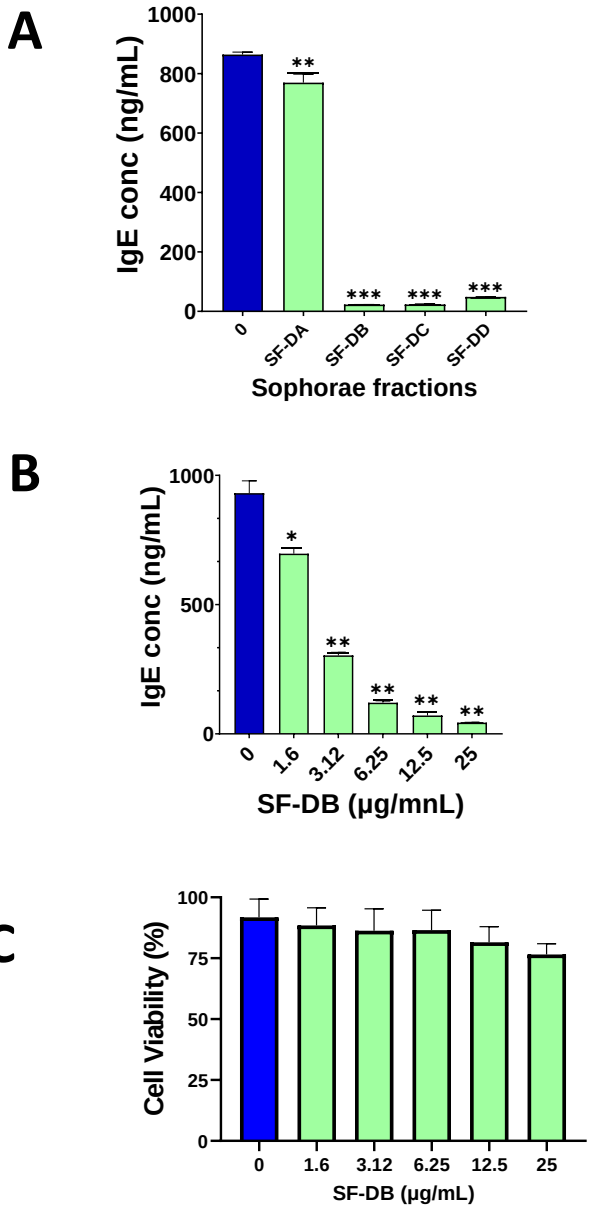


Figure 18. "SF-D-sub-fractions inhibited IgE production without cytotoxicity. A. The SF-D-sub-fractions inhibited IgE production in IgE producing human myeloma cells. B. SF-DB inhibited IgE production dose dependently across across a concentration of 1.6, 3.12, 6.25, 12.5, 25µg/mL. C. Cell viability of SF-DB cultured with IgE producing human myeloma cells. IgE producing human myeloma cells at (2×10^5 cells/mL) were cultured with the four subfractions of the dichloromethane extract of *Sophorae flavescens* across a concentration of 1.6, 3.12, 6.25, 12.5, 25µg/mL for 6 days. After the culture time point, the cells were harvested, and ELISA was used to determine IgE concentration from the supernatant. N=3, data represents triplicate experiments. *$p<0.05$; ** $p<0.01$; *** $p<0.001$. Results expressed as mean± S.D. Figure and legend taken from (Yang *et al.*, 2022) Panel A, B,C was provided by Dr. Yang

III. Formononetin is identified as the IgE bioactive compound in the sub-fraction of Sophora flavescens

"The sub fraction of *Sophorae flavescens* D, SF-DB which showed very strong IgE inhibition. Therefore, we wanted to identify the compound. To do this we utilized mass spectroscopy and nuclear magnetic resonance to conduct further analysis and identify the compounds in the subfraction. Using LC-MS we identified the molecular weight of the major compound that we isolated from the subfraction SF-DB in both the positive and negative mode. (Figure 19A). The results from the mass spectra data showed that there was a significant $[M+H]^+$ ion peak at m/z 269 and $M-H]^-$ value of 267. Using the nuclear magnetic resonance data, we determined the molecular of the compound to be 268 g/mol. In addition, the ^{1}H-NMR spectrum showed eight proton signals (Figure 19B). Based on the ^{1}H-NMR, we identified the compound to be formononetin. Our result (Figure 19B) was also consistent with the data that was shown by (Zheng *et al.,* 2015) for formononetin. Therefore using data from both liquid chromatography – mass spectrometry and nuclear magnetic resonance, we identified the IgE bioactive compound in the SF-DB sub fraction of *Sophorae flavescens* to be formononetin (Figure 20A). A second compound was also isolated and identified in the SF-DB sub fraction, this compound is maackiain (Figure 20B). We determined if this compound has potential IgE inhibitory effect by culturing with IgE producing human myeloma cells. This compound showed strong inhibition of IgE. The isolation efficiency of this compound was however very low using HPLC system. Due to the low efficiency of isolation, we focused on formononetin for further studies to determine the mechanisms by which it inhibits IgE production". Adapted from (Yang *et al.,* 2022).

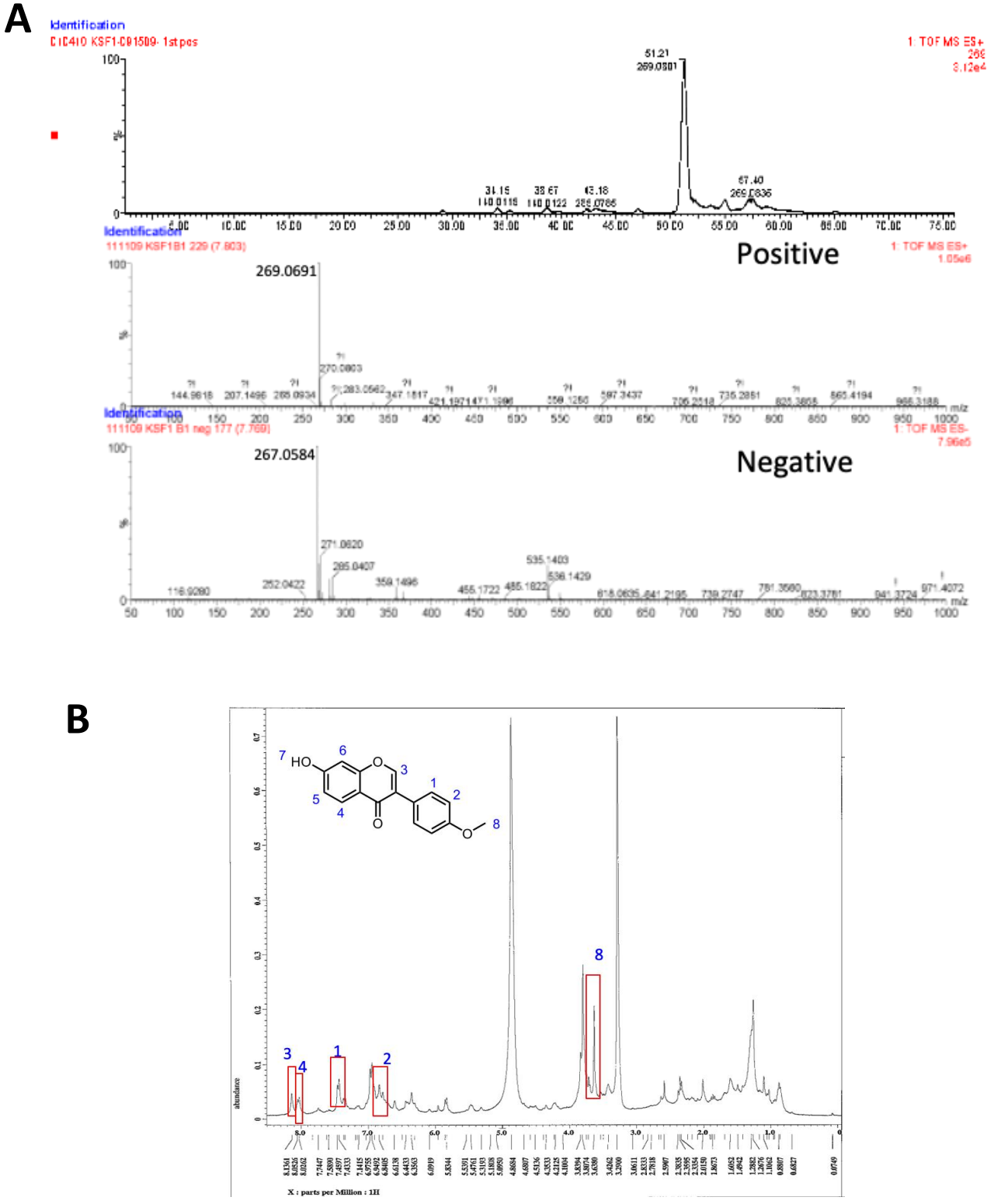


Figure 19. "Characterization of the compound isolated from the sub fraction SF-DB. A. Mass spectra data of SF-DB1. B. The ^{1}H NMR spectrum of the compound isolated from sub fraction SF-DB1". Figure and legend taken from (Yang *et al.*, 2022) Panel A, B was provided by Dr. Yang

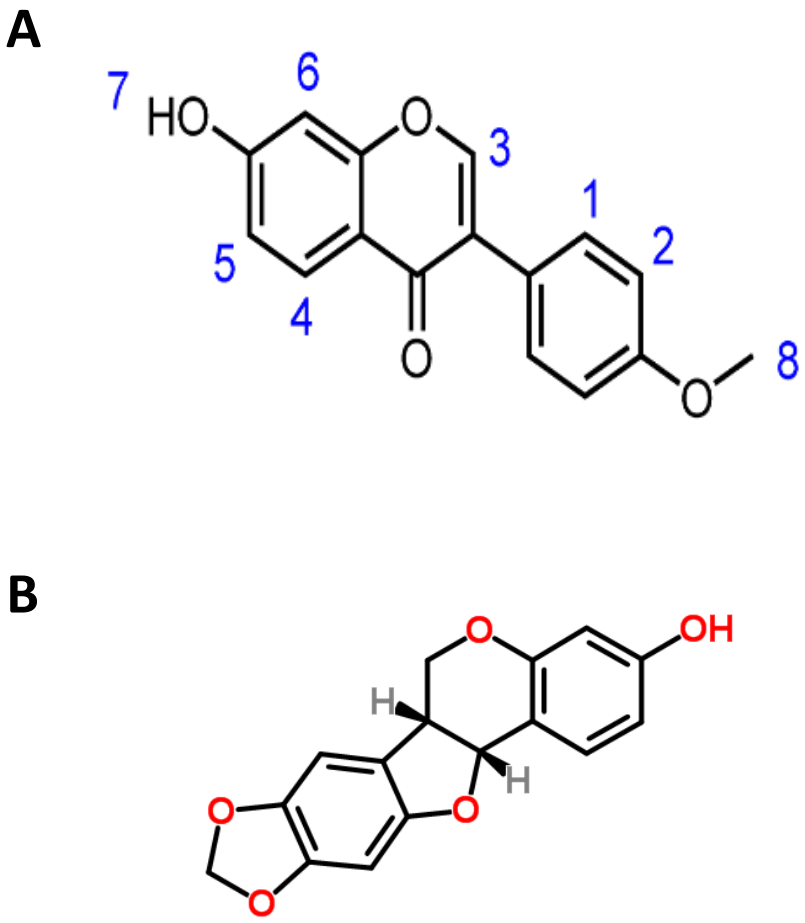


Figure 20. “Characterization of the compound isolated from the sub fraction SF-DB. A. The chemical structure of the main isolated compound SF-DB1, identified as formononetin**.** B. The chemical structure of the second isolated compound SF-DB1, identified as maackiain”. Figure and legend taken from (Yang *et al.*, 2022) Panel A, B was provided by Dr. Yang

IV. The Compound isolated from SF-DB sub fraction of *Sophorae flavescens* – formononetin, inhibited immunoglobulin E production in U266 cells and immunoglobulin G in ARH-77 cells

"Upon isolating and identifying that the IgE inhibitory compound to be formononetin, we wanted to further investigate its bioactivity. To do this we cultured formononetin across a concentration of 0.15 - 20 µg/mL with IgE producing human myeloma cells. At the end of the culture, we harvested the cells and collected the supernatant. We used ELISA to measure the concentration of IgE. Our results showed that formononetin significantly inhibited IgE production dose dependently in IgE producing human myeloma cells. (Figure 21A $p<0.05$; $p<0.001$). Our data showed that at the low concentration of 0.15µg/mL there was a significant inhibition of IgE. Our cell viability data also showed that formononetin did not cause any significant cytotoxicity (Figure 21B). The calculated IC_{50} value of formononetin inhibition of IgE is 0.16 µg/mL (Figure 21C). In seeing the strong IgE inhibitory results of formononetin, we obtained formononetin commercially from Sigma Aldrich, (St. Louis, MO). We cultured this version of formononetin across a concentration of 2.5 – 20 µg/mL with IgE producing human myeloma cells. We harvested the cells and collected the supernatant after the culture time point. Using ELISA, we determined the IgE concentration. Our results showed a dose dependent significant inhibition of IgE by formononetin in IgE producing human myeloma cells (Figure 22A $p<0.001$). The cell viability data also showed that there was no significant cytotoxicity measured by trypan blue exclusion assay (Figure 22B) as well as by CCK-8 assay.

Upon seeing IgE inhibitory potential of formononetin in both the forms isolated and the version obtained commercially, we decided to determine if this IgE inhibition is specific to IgE only. Therefore, we tested the effect of formononetin in inhibiting IgG production.

To do this we cultured formononetin with ARH-77 cells across a concentration of 2.5 – 20 µg/mL, a cell line that has been used to study the effect of compounds of IgG production. Our results showed that formononetin did not have any significant effect on IgG production of ARH-77 cells (Figure 23A-B)". Adapted from (Yang *et al.*, 2022).

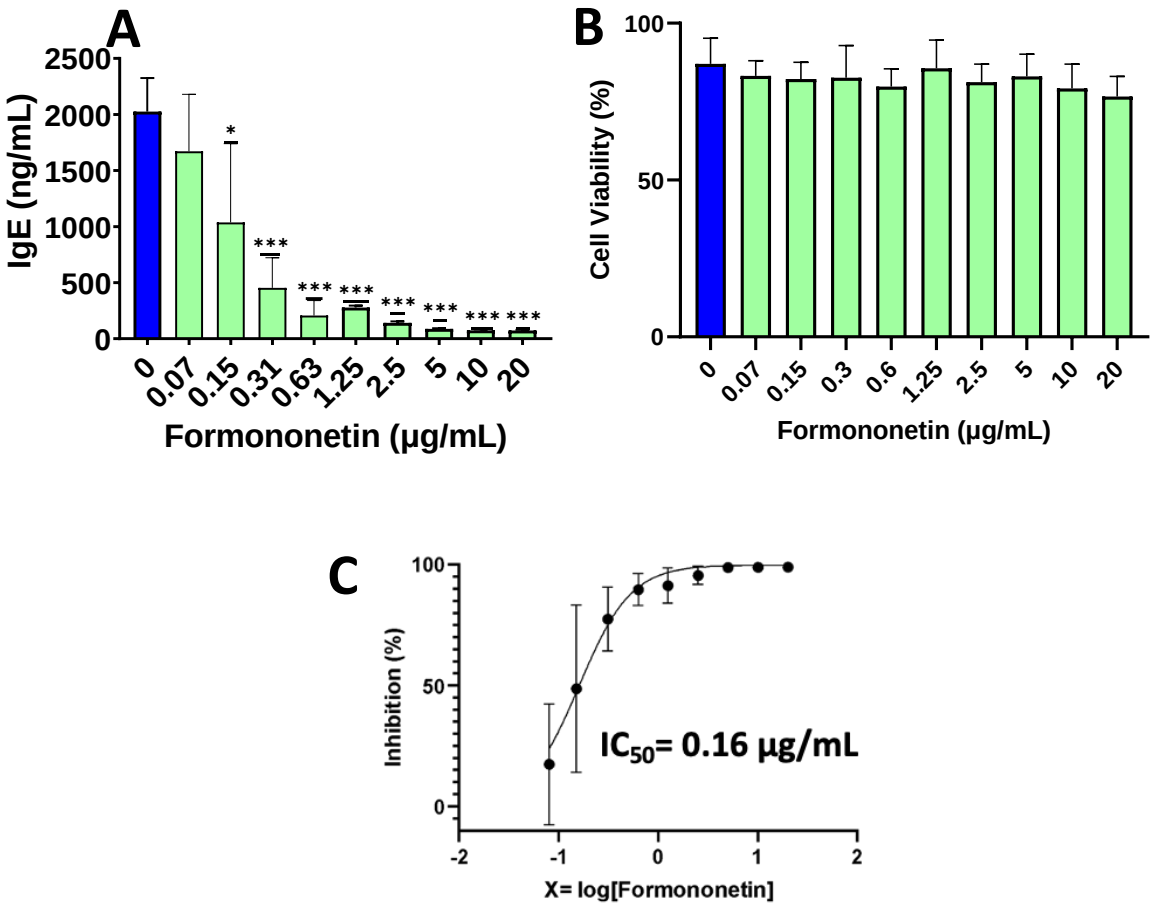


Figure 21. "Formononetin inhibits Immunoglobulin E production in IgE producing human myeloma cells. A. Dose dependent IgE inhibition by formononetin. B. Cell viability of formononetin cultured with IgE producing human myeloma cells. C. IgE inhibition percentage vs. log [formononetin concentration] curve. N=3, data represents triplicate experiments. *p<0.05; ** p<0.01; *** p<0.001. Results expressed as mean± S.D". Figure and legend taken from (Yang *et al.,* 2022)

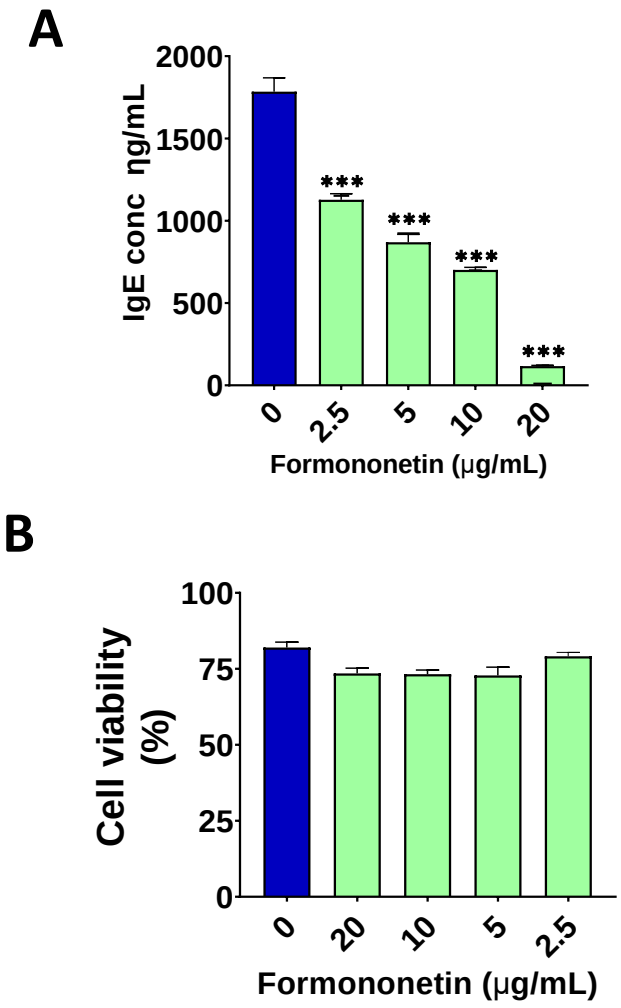


Figure 22. "Commercial formononetin inhibits immunoglobulin E production in IgE producing human myeloma cells. A. Dose dependent IgE inhibition by formononetin by IgE producing human myeloma cells. B. Cell viability of formononetin cultured with IgE producing human myeloma cells. N=3, data represents triplicate experiments. *$p<0.05$; ** $p<0.01$; *** $p<0.001$. Results expressed as mean± S.D O. D; optical density". Figure and legend taken from (Yang *et al.*, 2022).

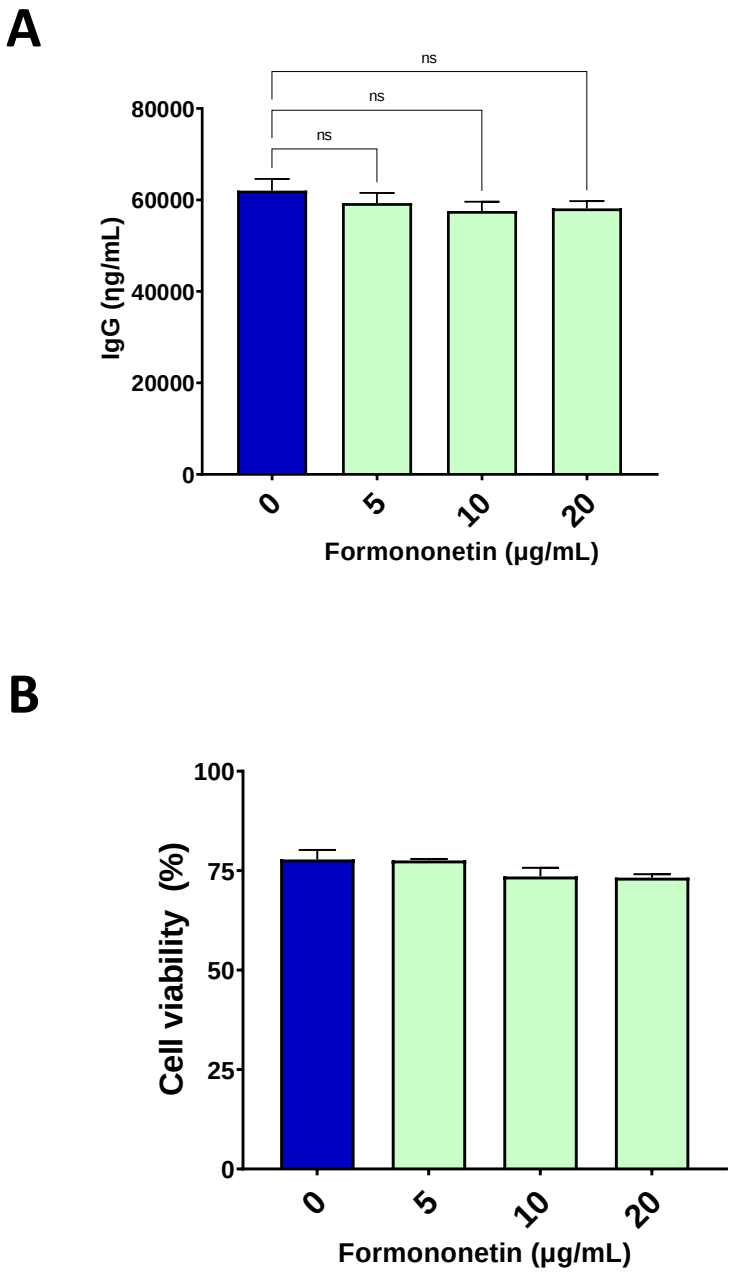


Figure 23. "Formononetin inhibits Immunoglobulin G production in IgG producing ARH-77 cell line. A. Effect of formononetin on IgG production by IgG producing ARH-77 cells. B. Cell viability of formononetin cultured with IgG producing ARH-77 cells. N=3, data represents triplicate experiments. ns – no significant difference". Figure and legend taken from (Yang *et al.,* 2022)

V. Formononetin inhibits the XBP1 and IgE heavy mRNA expression in U266 cells

"Immunoglobulin E production requires immunoglobulin E heavy chain heavy chain transcription. XBP-1 is critical for plasma cell function, the continuous production of immunoglobulin E requires the increased expression of X Box Protein -1 (XBP-1) to prevent the endoplasmic reticulum stress seen when plasma cells are seen in increased IgE production. To understand the mechanism by which formononetin inhibits IgE production we investigated the effect of formononetin on immunoglobulin E heavy chain and XBP-1. To do this we measured the mRNA expression of IgE heavy chain and XBP-1 in the IgE producing human myeloma cells using real time PCR. To do this we cultured formononetin at 10 µg/mL with IgE producing human myeloma cells. After the culture, we harvested the cells and isolated the RNA, converted the cDNA via reverse transcription. Then measured the relative gene expression of XBP-1 and IgE heavy chain using real time polymerase chain reaction. Our results showed that formononetin significantly inhibited the gene expression of XBP-1 and IgE heavy chain (Figure 24A,B, $p < 0.001$)". Adapted from (Yang *et al.*, 2022).

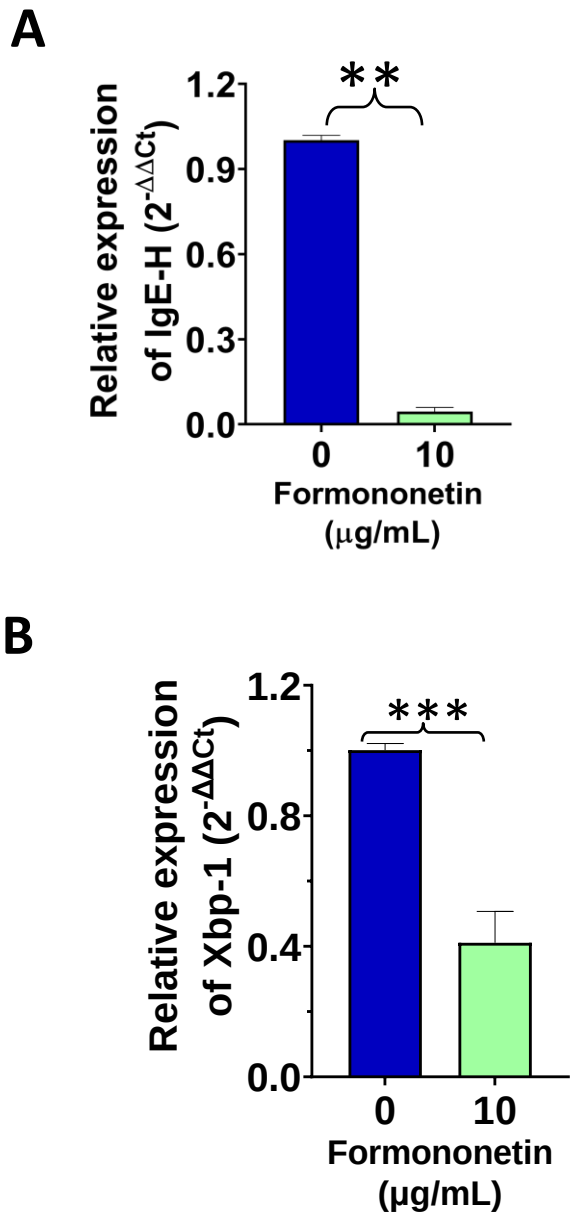


Figure 24. "Inhibition of Immunoglobulin E heavy chain (IgEH) and X Box Protein-1 (XBP-1) by Formononetin in IgE producing human myeloma cells.(A) Formononetin inhibits IgEH mRNA expression (B) Formononetin inhibits XBP-1 mRNA expression. N=3, data represents triplicate experiments. ** $p<0.01$; *** $p<0.001$. Results expressed as mean± SEM." Figure and legend taken from (Yang *et al.*, 2022)

VI. Inhibition of XBP-1 protein expression by formononetin in IgE producing human myeloma cells

"Our data showed that formononetin inhibited the mRNA expression of X Box Protein-1 (XBP-1). Therefore, we wanted to see if this effect is at the gene level or if it also inhibited the protein expression of XBP-1. To do this we cultured formononetin with IgE producing human myeloma cells. After the culture time point, we harvested the cells and isolated the protein and using western blotting determined the protein expression of XBP-1. Our results showed that formononetin inhibited the protein expression of XBP-1 protein significantly (Figure 25A, B, $p < 0.01$)". Adapted from (Yang *et al.*, 2022)

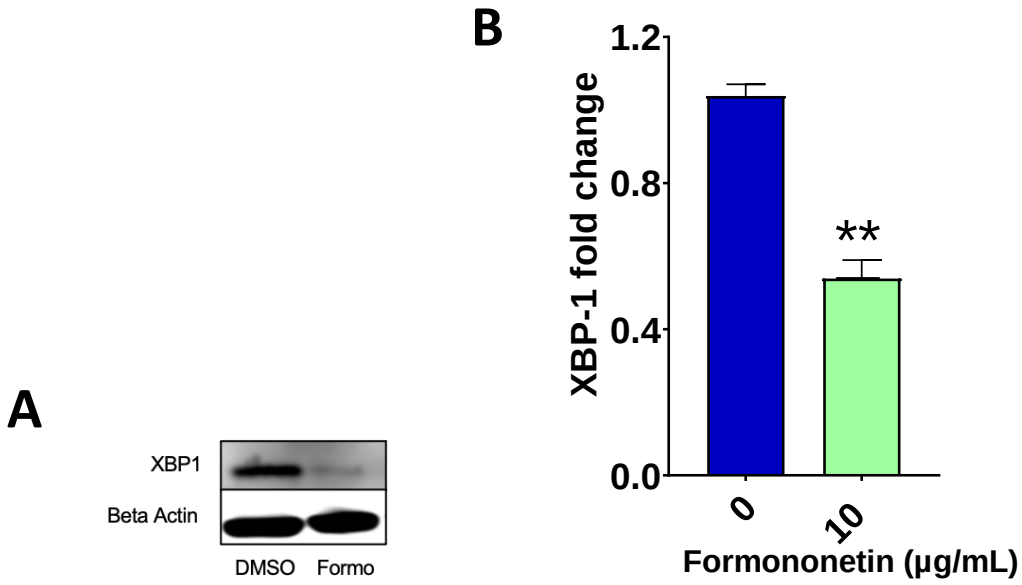


Figure 25. "Inhibition of XBP-1 protein expression in IgE producing human myeloma cells by formononetin. IgE producing human myeloma cells were cultured with formononetin for 72 hours and using western blotting to determine protein expression. (A). Western protein imaging (B). Relative protein expression of XBP-1. N=3, data represents triplicate experiments. *p<0.05; ** p<0.01; *** p<0.001. Results expressed as mean± S.D". Figure and legend taken from (Yang, Musa *et al.*, 2022)

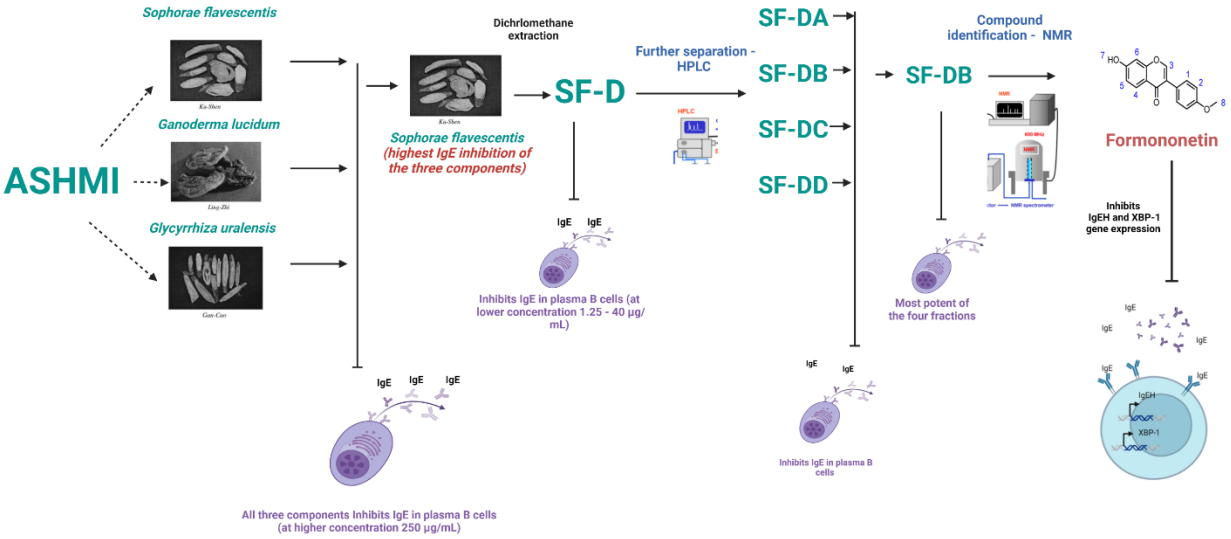


Figure 26: Illustration showing the summary of Aim 1 [original image].

B. Aim 2: Computational analysis to identify therapeutic targets, biological regulated and pharmacological mechanism of formononetin relevant to food allergy.

I. Biological targets of formononetin in Food allergy, IgE and Mast cell diseases identified by target mining

516 genes for food allergy, 308 genes for IgE and 332 genes for mast cell diseases were obtained from databases Opentarget platform, GeneCard and Malacard, Figure 1. 106 biological targets of formononetin were obtained from databases such as SEA, Swiss target prediction and PubChem and literatures, Figure 27. The biological targets of formononetin were then mined into the disease's targets, to obtain 25 targets of food allergy, 51 targets of IgE diseases and 19 targets of mast cell diseases Figure 28. These targets were further used for gene ontology, KEGG pathway analysis, CTPD network and protein-protein interaction.

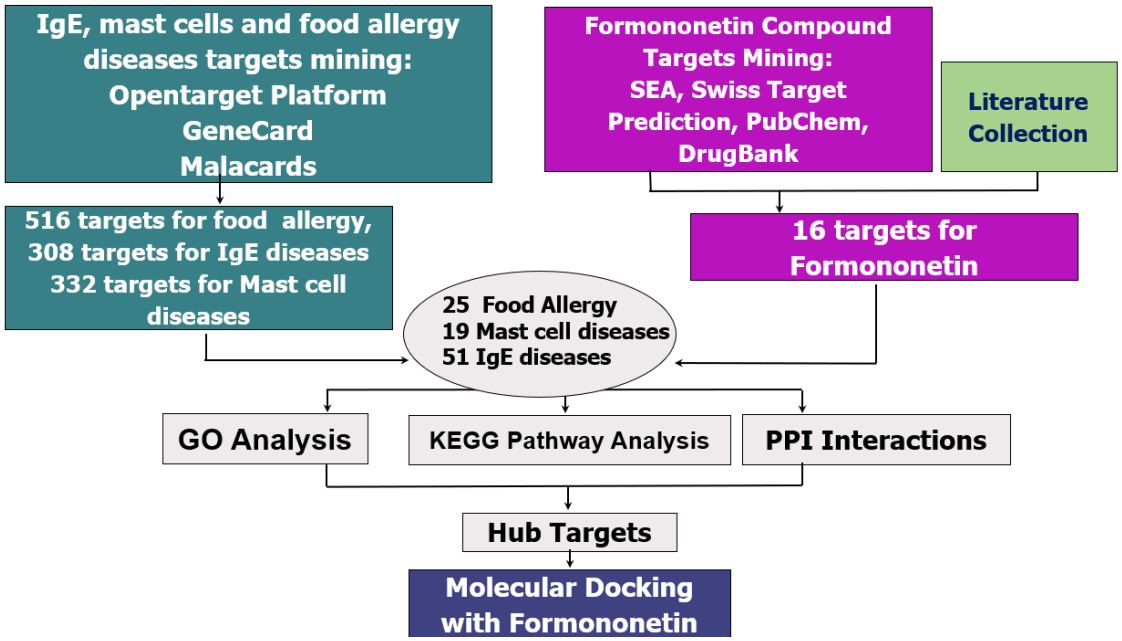


Figure 27. The workflow of computational modeling is used for analysis of Formononetin for treatment of food allergy, mast cell diseases and IgE diseases. First, total 516 genes for Food allergy, 308 genes for IgE diseases and 332 genes for Mast cell diseases were selected as their targets. 109 inflammatory and biological targets of formononetin were collected based on literatures and following published databases: Swiss target prediction, SEA, pub-chem and drug-bank. SEA, similarity ensemble approach; PPI, protein-protein interactions, GO, gene ontology, KEGG, Kyoto encyclopedia of genes and genomes [original data, unpublished].

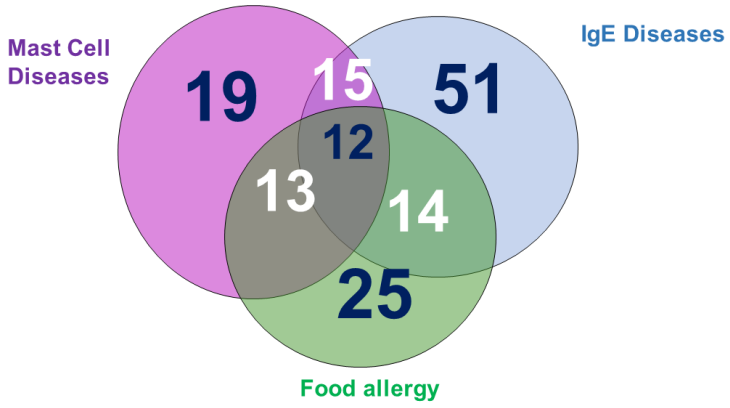


Figure 28. Predicted targets using computational modeling. Formononetin potentially regulate 25 targets for food allergy, 51 target for IgE diseases and 19 targets for mast cell diseases [original data, unpublished].

II. KEGG pathway analysis of targets regulated by formononetin

The identified targets regulated were used for KEGG pathway analysis, Figure 29. The most relevant pathway in the top 15 pathway is the primary immunodeficiency pathway. Most common examples of primary immunodeficiencies are lymphomas and leukemias however allergic diseases have also been identified in patients with immune deficiency diseases (Raje 2015). T cell differentiation pathways - Th1, Th2 and Th17 ranked in the top pathway in KEGG analysis. Th17 cell differentiation has been reported to contribute to many allergic disorders with studies showing an increase in IL-17 mRNA in the lungs, sputum and Bronchoalveolar lavage (BAL) fluid of asthmatics (Wong *et al.*, 2001), the increase was also correlated with the degree of severity of airway hypersensitivity in asthmatic patients. IL-17 are also reported to be detectable in the nasal fluid of patients with allergic rhinitis (Klemens *et al.*, 2007), it was also shown to be increased in patients with psoriasis (van Beelen *et al.*, 2007). Due to the multiple roles that IL-17 play in allergic diseases; it has been considered as a marker for disease progression as a well a therapeutic candidate. The Th1 and Th2 cell differentiation pathway is important in the activation of B cells, an imbalance in Th1/Th2 can lead to disease states such as allergy and autoimmunity. Th1 cells stimulate cellular immunity against intracellular pathogens while Th2 cells stimulate IL-4, IL-5 and IL-13 that are necessary for inducing the humoral immunity as well as class switch recombination of IgG1 to IgE B cells. Improper balance between Th1 and Th2 cells as well incomplete development can lead to allergy (Amsen *et al.*, 2009).

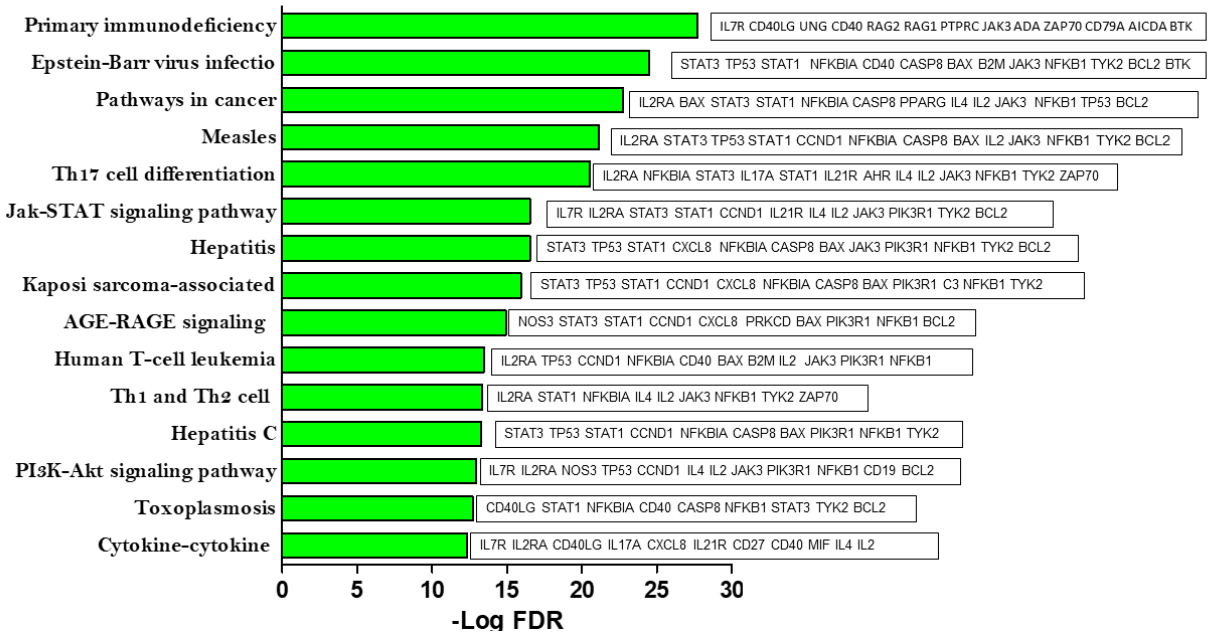


Figure 29. KEGG pathway analysis. Y-axis: Top enriched pathway with associated genes (right); X-axis: -log (false discovery rate) (FDR), level of significance [original data, unpublished].

III. Gene ontology (GO) reveals biological processes regulated by formononetin

The biological process from the top 15 obtained from gene ontology analysis were selected, see gene ontology terms in (Figure 30). The top biological processes regulated by formononetin are related to B cell development, differentiation, activation and activation. B cell activation is mediated by the delivery of specific cytokines from activated T cells (Katz *et al.,* 1973), (Chesnut 1981, Lanzavecchia 1985) and the binding of CD40L to CD40 receptor on B cells (Fanslow *et al.,* 1992). Activation of CD40 receptor by CD40L is sufficient to drive B cell proliferation (Armitage *et al.,* 1992), however, the presence of soluble cytokines increases the proliferation as well as initiate B class switch recombination in B cells (Cocks *et al.,* 1993). IL-2, IL-4 and IL-10 are cytokines that aid B cell proliferation and differentiation (Defrance *et al.,* 1992). IL-4 and CD-40 cross linking is particularly important for IgE production (Brière *et al.,* 1994) while IL-2 and IL-10 have been shown to induce IgM, IgG and IgA secretion (Callard *et al.,* 1993). Studies have shown that formononetin inhibits IL-4, IL-5, IL-13 by regulating Th2 cells (Yi *et al.,* 2020). The ability of formononetin to inhibit the cytokines that are critical in the activation of B cells and class switch recombination shows its therapeutic potential in preventing IgE production from B cells. Allergic diseases are mediated by the IgE production from B cells that are class switched. Therefore, understanding the mechanism of induction of IgE switching in B cells is important in developing therapeutics for preventing IgE mediated diseases. B cells class switch recombination requires at least IL-4 a class switch recombination factor and the engagement of CD40. There are several other regulatory factors that are involved in class switching, however studies have shown that CD27/CD70 interaction is very important in augmenting IgE secretion by promoting the differentiation

of memory B cells into plasma cells (Jabara *et al.*, 1990). IgE+ Memory B cells are short lived, 10-12 days, while IgE+ Plasma B cells are long lived, persisting for months to years. IgE+ plasma cells are responsible for the pathologic allergic responses seen mostly. CD27 was found to be a formononetin target that appeared in multiple GO pathways, therefore understanding the interaction between formononetin and CD27 will be important and a good area for future studies. Apoptosis has been shown to be an important GO ontology process that is regulated by formononetin. The anti-apoptotic Bcl-2 gene shown in the GO term - apoptosis is expressed at higher levels in plasma cells than in other B cells (Saunders *et al.*, 2019). Furthermore, a correlation was established between apoptosis and IgE expression, it was shown that the levels of IgE production increases in treatment with an apoptosis inhibitor (Saunders *et al.*, 2019). Studies have shown that formononetin has been shown to downregulate Bcl-2 gene expression thereby promoting apoptosis (Rabah 2002).

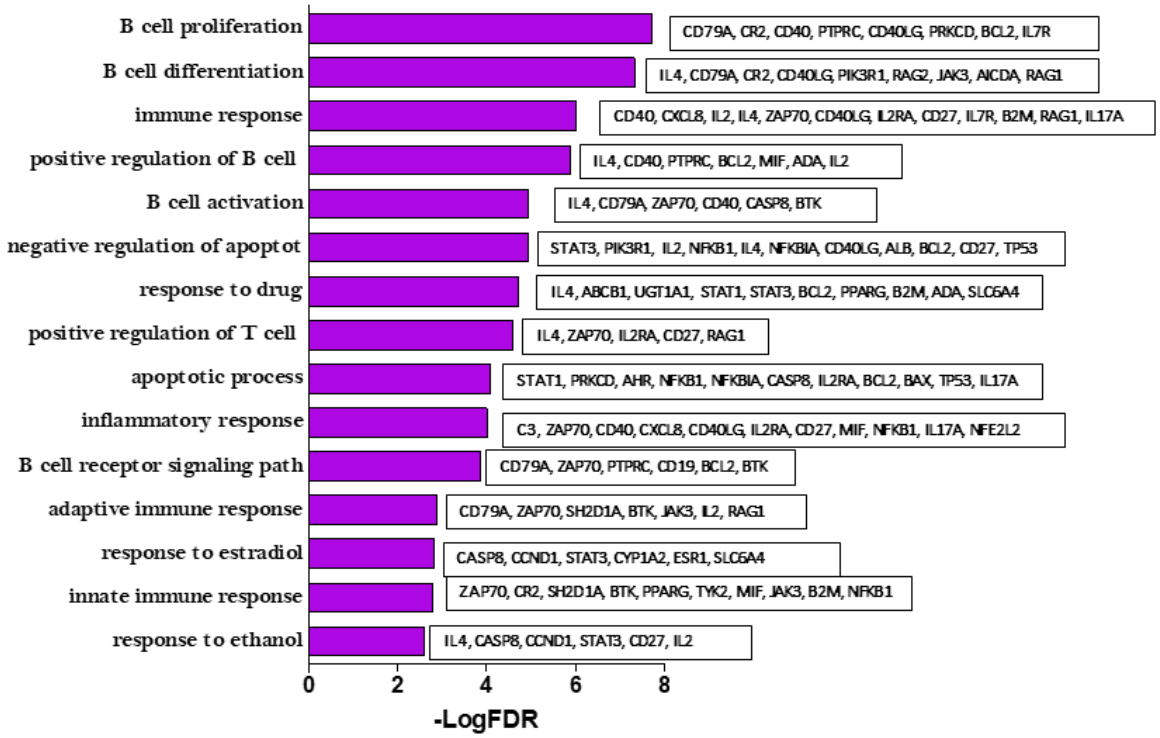


Figure 30. Gene Ontology: Top enriched pathway with associated genes (right); X-axis: -log (false discovery rate) (FDR), level of significance [original data, unpublished].

IV. Compound-target-pathway-disease (C-T-P-D) network construction of targets regulated by formononetin

C-T-P-D network containing formononetin, selected targets, top 15 pathways, in food allergy, IgE and mast cell diseases, provides general information about the potential pharmacological mechanisms of formononetin for prevention and treatment of food anaphylaxis at the molecular level Figure 31. The frequency of targets appearing in the top 15 pathways imply their influence and importance. Node sizes from large to small are proportional to degree value, displaying their importance from high to low in network. The encoded proteins of NFκBIA (IKBA) and NFκB1 (p50 and p105) identified in the network all play important roles in class switch recombination required for B Cell IgE production (Bishop 2004). Formononetin has been shown to inhibit NFκB activity by directly inhibiting phosphorylated IκBα. Formononetin has been shown to interact with PI3K pathway thereby decreasing apoptosis (Szklarczyk *et al.,* 2019). B cell receptor signaling, B cell activation genes identified in the top pathways of GO analysis are CD40, CD79A, PTPRC (CD45), CD27. CD40 is an important costimulatory molecule for B cells which was previously discussed to be important in IgE class switch recombination. CD79A is important for B cell development and function (Hu *et al.,* 2018) while the PTPRC gene encodes the CD45 protein required for antigen receptor signaling (Coughlin *et al.,* 2015). All these targets of formononetin are important for B cell receptor signaling and are potential targets for therapy.

Genes regulating mast cell activation and degranulation were also identified to be present in multiple top pathways, this includes the ADA, BTK, PI3K and PRKCD gene. BTK gene encodes the Bruton tyrosine kinase, BTK gene in B cell development is required for transmitting signals from the pre-B cell receptor that forms after successful

immunoglobulin heavy chain rearrangement (Middendorp *et al.*, 2003). It is also important in mast cell activation through the high-affinity IgE receptor (Hata *et al.,* 1998). In B cells, BTK has been shown to be important in the development of peripheral B cells, maturation and plasma cell differentiation (Maas 2001). The inhibition of PI3K pathway have been shown to inhibit antigen-mediated degranulation and cytokine production in human mast cells (Szklarczyk *et al.,* 2019). The PI3K pathway is also important in the regulation of B cell activation and survival. Formononetin has been shown to interact with PI3K pathway, it also regulates BTK gene which is upstream of PI3K as well as the NFκB which is downstream, the overall effect of formononetin in modulating this pathway as well as the P53 pathway is to initiate apoptosis (Wang *et al.,* 2018).

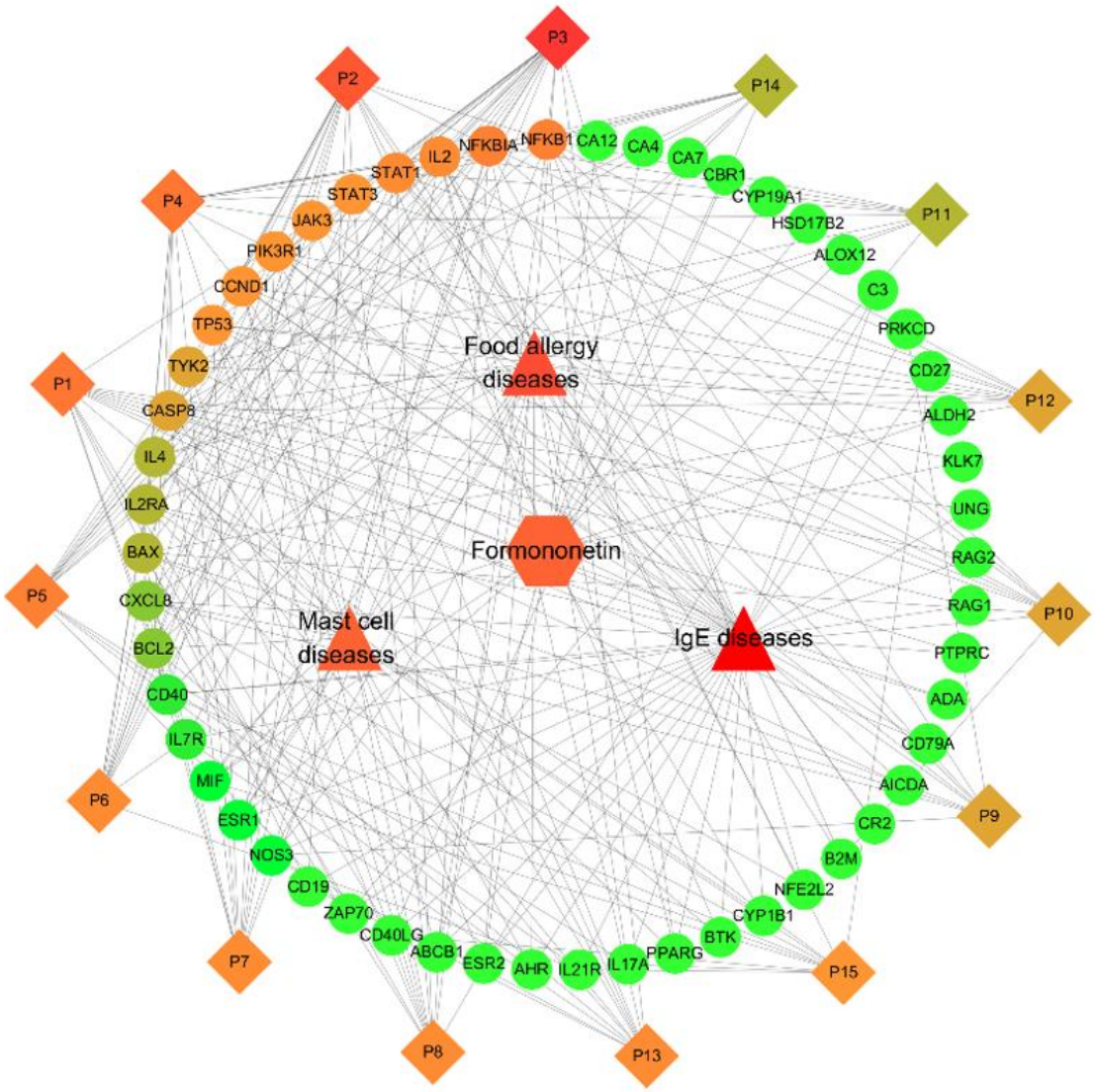


Figure 31. The targets of formononetin in food allergy, IgE and mast cell diseases. Diamonds, circles, squares, triangles represent formononetin, common targets, pathways, and diseases, respectively. The node size and color are proportional to the degree of relevance from green to red. The interaction between the nodes is represented by black lines [original data, unpublished].

V. Protein targets and functions regulated by formononetin.

The TP53 protein which showed a high degree of interaction regulates critical cell response to stress Figure 32. It has been shown that increased levels of P53 mRNA occurs during B cell differentiation, showing that it is an important target for B cell differentiation (Aloni-Grinstein *et al.,* 1993) P53 has also shown to negatively regulate IgE-mediated mast cell activation through interaction with the NF- κB pathways (Suzuki *et al.,* 2011). P53 is upregulated in mast cells activated by IgE crosslinking while P53 deficient mast cells were shown to have increased IgE mediated degranulation (Acciani *et al.,* 2016). Other proteins that have high degree of interaction include IL-2, IL-4, IL17A, PTPRC, CD40L, BTK, CD40, NFKB1, PIK3R1 have been shown to interact with formononetin in different studies.

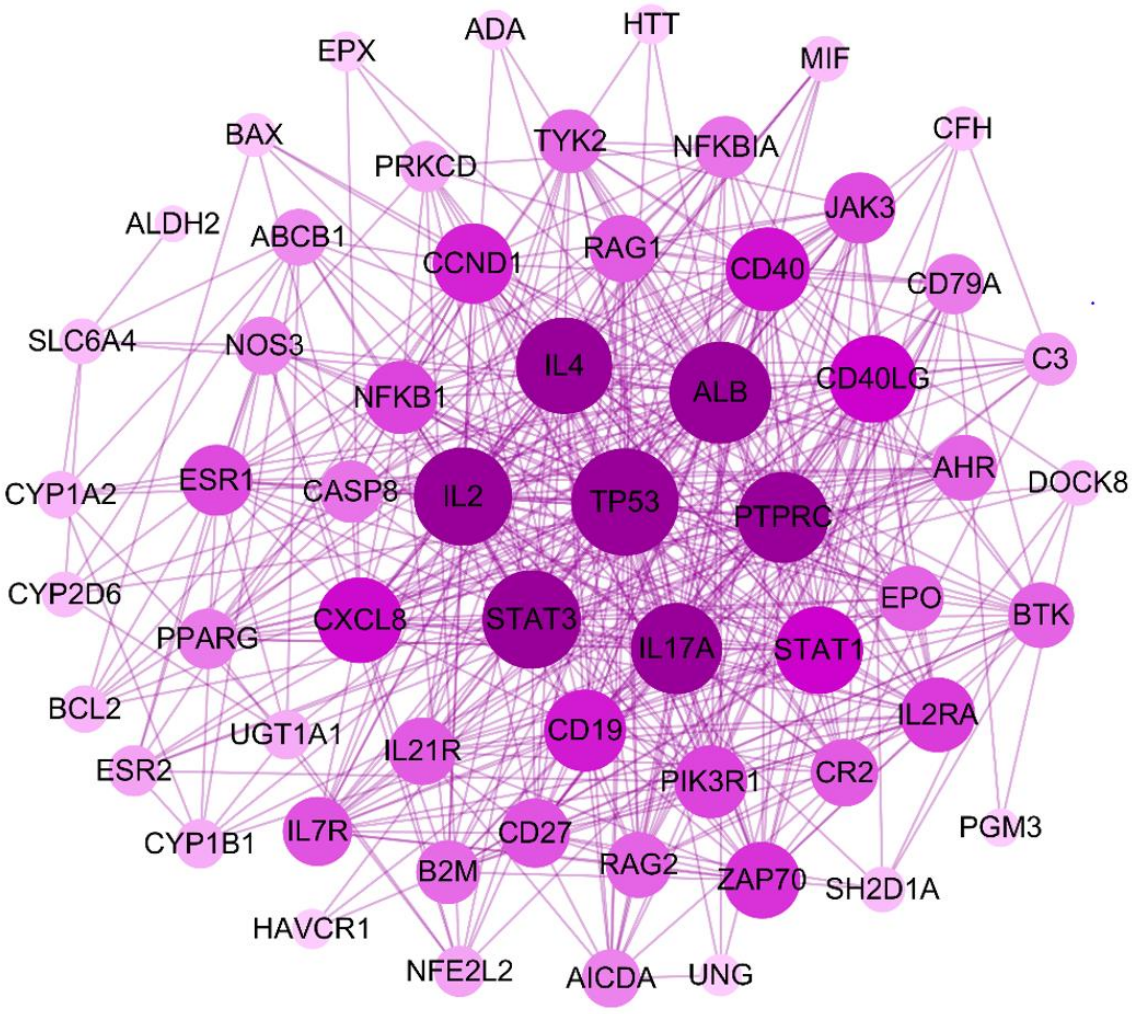


Figure 32. Protein-protein network generated by mapping potential targets in string database. The size of the node represents the degree of relevance; the small node-smaller degree; large node-higher relevance. The therapeutic targets are represented by circles while the interaction between the nodes are represented by purple lines [original data, unpublished].

VI. Prediction of the binding modes between formononetin and its crucial targets

Molecular docking analysis was used to predict the binding modes between formononetin and selected targets, the docking results (-5.9 to -8.7 kcal/mol) showed that formononetin is a potential regulator of the crucial targets, Table S1. The binding modes of formononetin with the targets TYK, BTK, TP53, BCL2, NFKBIA, CASP8 are shown in Figure 33. For complex formononetin-TYK, the hydrogen interactions were formed between formononetin and residues (ASP1041, ARG901). The π-π stacking, π-alkyl, and π-cation interactions with residues (LEU903, VAL911, LEU1030, MET978) further stabilize the complex, making the strongest binding of complex formononetin-TYK. Similar interactions between formononetin and residues (LEU542, THR474, PHE413, VAL416, ALA428, and LYS430) of BTK were constructed to form the complex formononetin-BTK. For complex formononetin-P53, hydrogen-binding with ARG273, the π-alkyl with PRO300, PRO318, the π-cation with LYS164, GLU326 facilitate the binding of formononetin with P53. Hydrophobic interactions of formononetin with residues (ALA100, ALA148, LEU201) of Bcl2 was generated to form complex formononetin-Bcl2.

The binding of formononetin with residues (GLU85, ALA88, LYS87, CYS360) of IKBA and residues (ARG260, ARG273, GLN358, ASN261, VAL450, CYS360, VAL406) of CASP8 supports the construction of complex formononetin-IKBA and formononetin-CASP8. The molecular docking indicated that formononetin might bind with these crucial proteins directly to regulate the following biological effects.

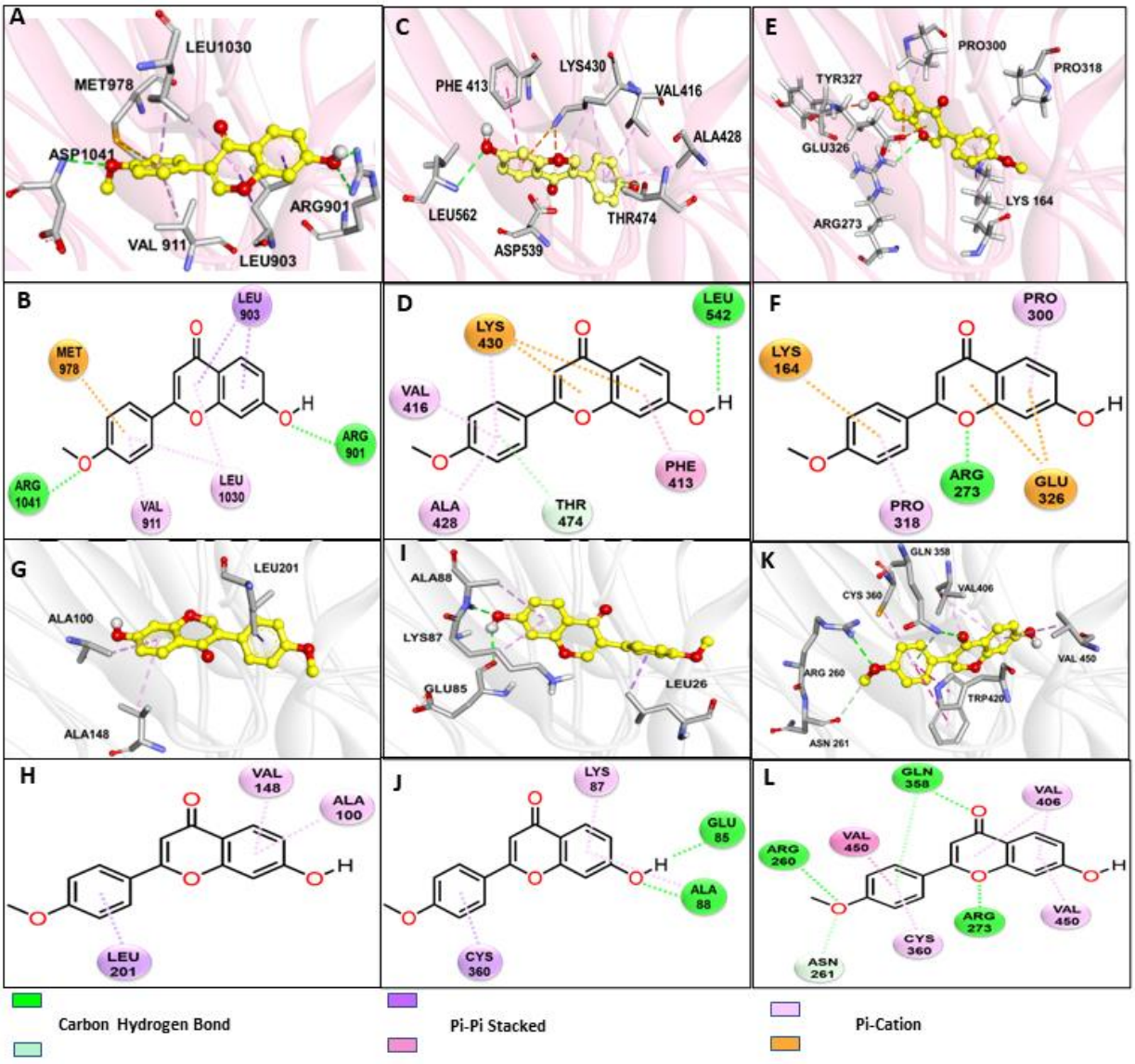


Figure 33. Binding explorations of complex between formononetin with targets TYK, BTK, P53, BCL2, IKBA, CASP8. Energy binding mode of formononetin in 3-dimensional figure with the following proteins: (A), (B) TYK; (C), (D) BTK; (E), (F) P53 ; (G), (H) Bcl2; (I), (J) NFKBIA; (K), (L) CASP8; the nitrogen, oxygen and carbon bonds are shown in blue, red and yellow [original data, unpublished].

Compound	Targets	Affinities (kcal/mol)
Formononetin	TYK	-8.7
	BTK	-8.5
	P53	-7.4
	Bcl2	-6.6
	NFKBIA	-6.1
	CASP8	-5.9

Table 3. Molecular docking between formononetin and highly relevant targets [original data, unpublished].

VII. Formononetin regulates selected targets in plasma cells and inhibits mast cell degranulation

We validated the selected targets P53, BTK, CASP8, NFKBIA, BCL2, TYK2, IgEH by culturing multiple myeloma cells with formononetin at a concentration of 20ug/mL and in the untreated culture we used 0.1% DMSO, for 72 hours We isolated the RNA and using qPCR we measured the gene expression. Formononetin significantly decreased the expression of BTK, TYK2, CASP8, IgEH while it significantly increased the expression of BCL2, NFKBIA, P53 (Figure 34 A-F). Using RBL-H3 cells we measured the effect of formononetin on inhibiting mast cell degranulation. We incubated formononetin with RBL-H3 cells and measured the beta hexosaminidase enzyme release, our results showed that formononetin decreased beta hexosaminidase release (Figure 35).

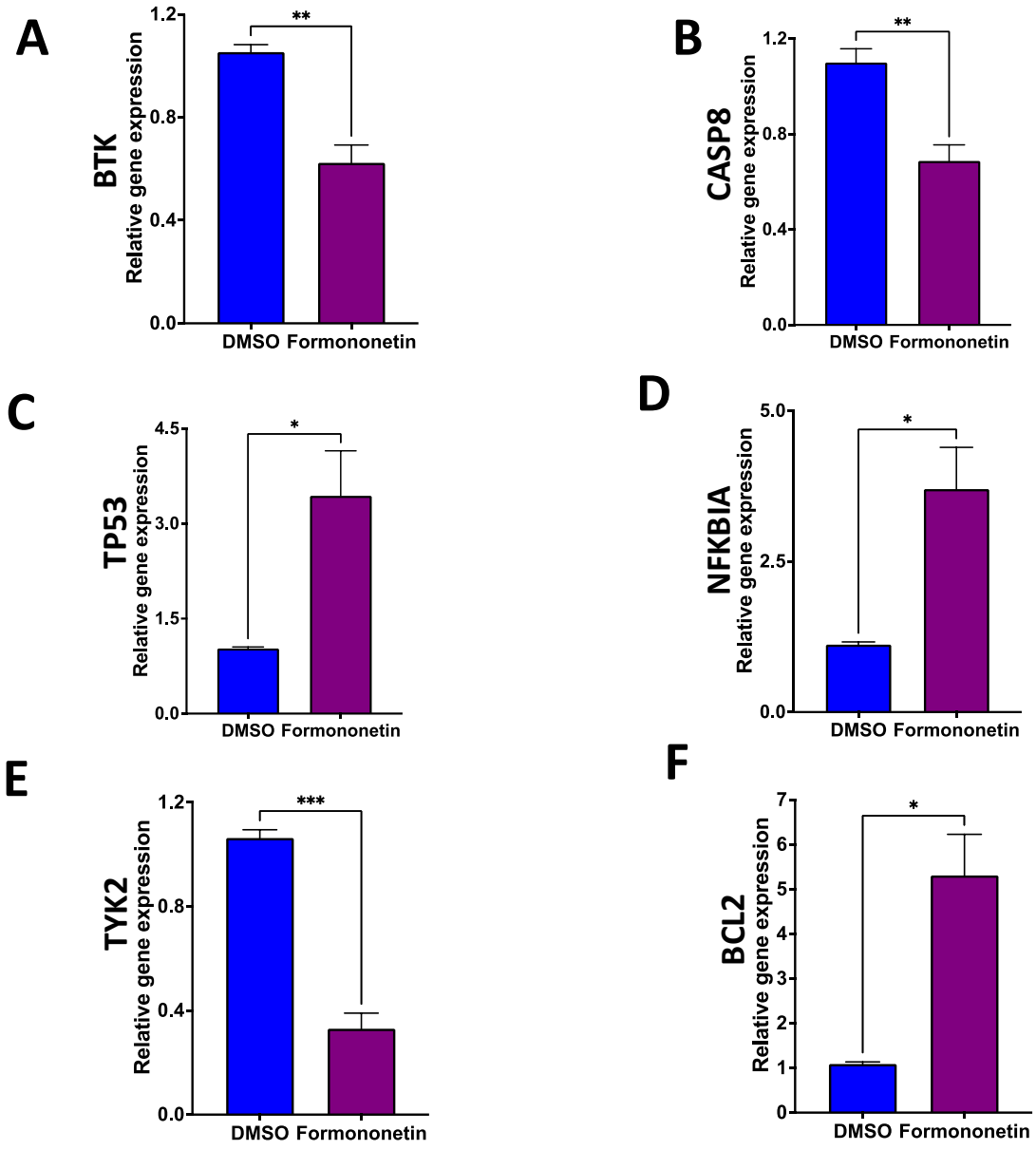


Figure 34. Formononetin regulates the mRNA expression of select targets. Formononetin was cultured with IgE producing human myeloma cells for 72 hours. (A). BTK (B). CASP 8 (C). TP53 (D). NFKBIA (E). TYK2 (F). BCL2. Data represents triplicate experiments, N=3, data represents triplicate experiments. *p<0.05; ** p<0.01; *** p<0.001 vs control (0.1%DMSO) [original data, unpublished].

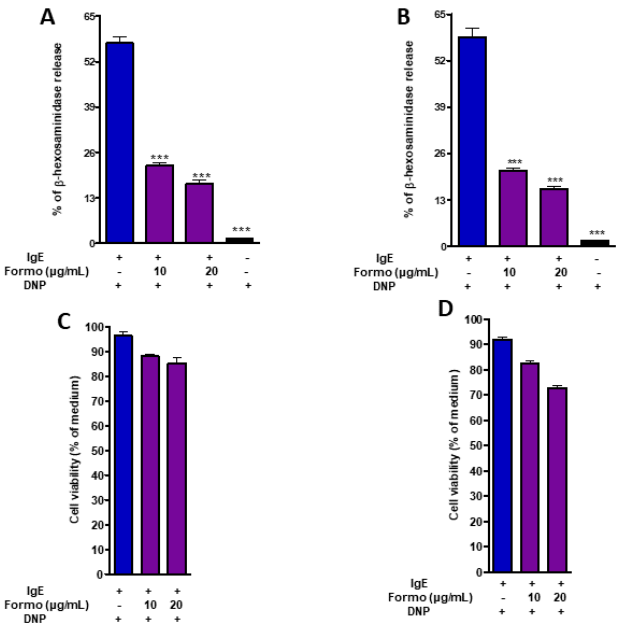


Figure 35. Formononetin decreases mast cell degranulation measured by Beta Hexosaminidase release assay in RBL-H3 cell. (A). After 24 hours incubation with formononetin (B). After 48 hours incubation with formononetin. Cell viability measured for toxic effects of formononetin on RBL-2H3 cells using CCK8 assay (C). After 24 hours incubation with formononetin (D). After 48 hours incubation with formononetin. Data represents triplicate experiments, n=3 and expressed as mean ±SD. *p<0.05; **p<0.01; *** p<0.001 vs control (0.1%DMSO) [original data, unpublished].

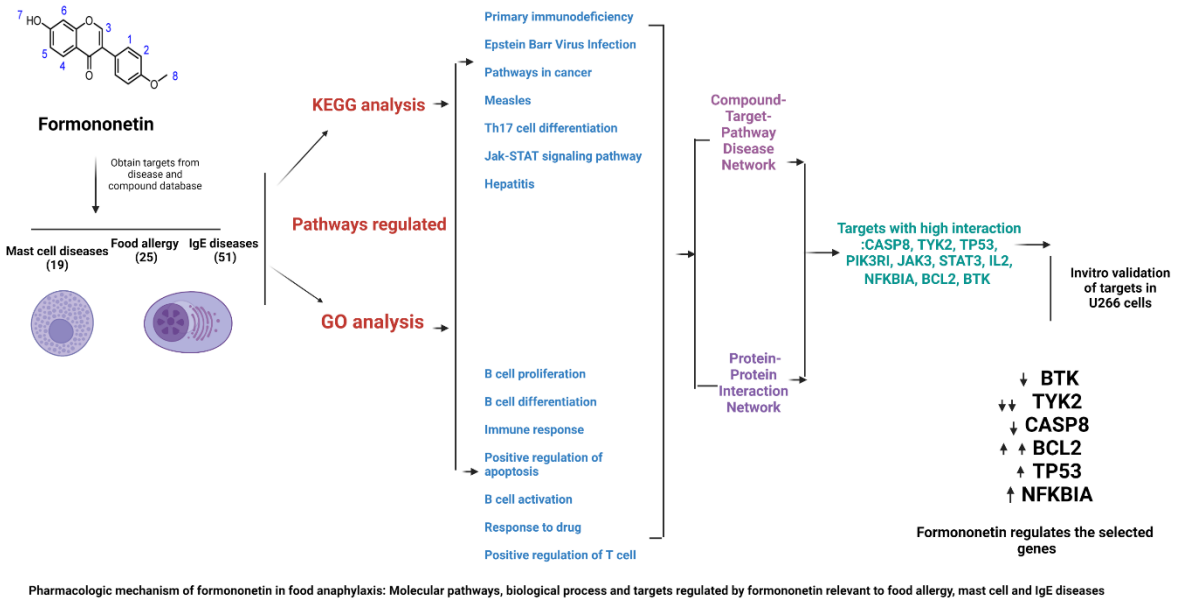


Figure 36: Summary of Aim 2 results.

C. Aim 3: Investigate if formononetin prevents food anaphylaxis in vivo using a peanut allergic mouse model.

I. Peanut-induced anaphylaxis in allergic mice model

To develop a peanut allergic mice model. 6 weeks old female mice were intraperitoneally sensitized with crude peanut extract 500 µg and 2 mg alum at weeks 0 through 3. Mice underwent oral peanut challenge at week 5, and rectal temperature was measured every 10 minutes, anaphylactic symptoms score was measured every 10 minutes. Blood was drawn and peanut specific Immunoglobulin E(IgE), Immunoglobulin G1(IgG1), Immunoglobulin G2a (IgG2a) was measured via ELISA. Group 1 and group 2 were sensitized while mice, group 3 mice were not sensitized (Figure 37). Mice in the sensitized group developed anaphylactic symptoms with symptoms scores between 1-3 compared to the group 3 naïve which showed 0 symptom scores (Figure 38A). Core body temperature measured correlates with the severity of the anaphylaxis, with temperature (Figure 38B). Peanut specific IgE, IgG1 and IgG2a were also increased compared to the unsensitized group ***$p<0.001$ (Figure 39 A-C).

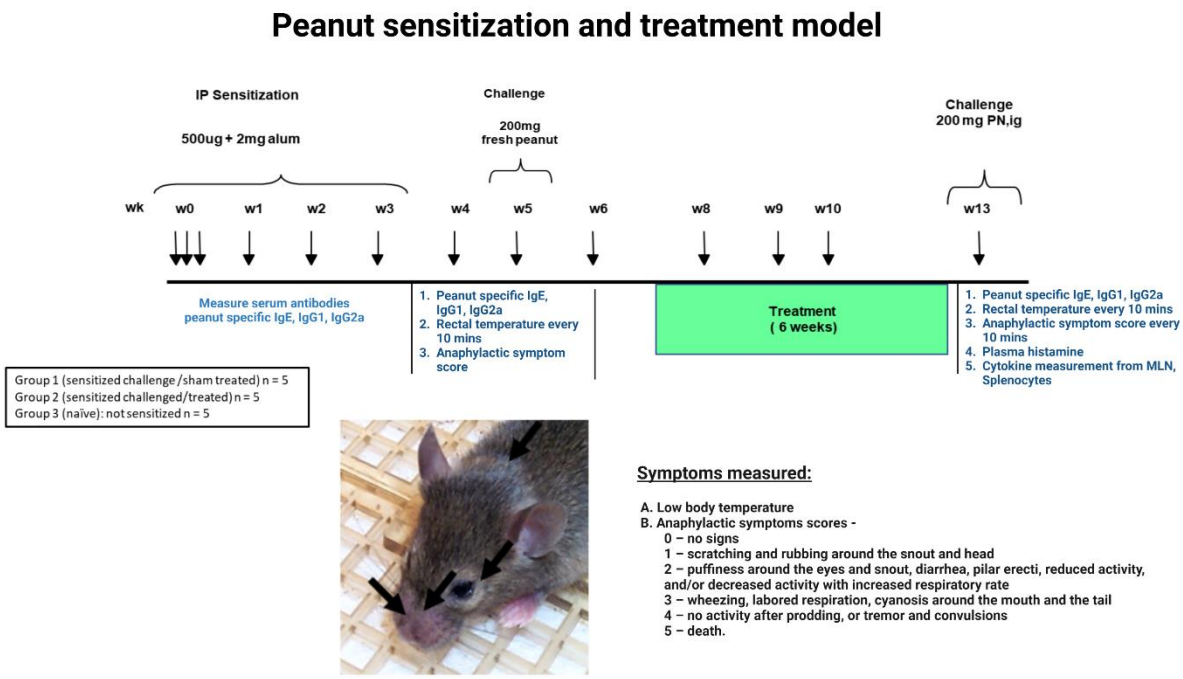


Figure 37. Peanut allergic mice model; sensitization, challenge, treatment: 6 weeks old C3H/HeJ mice received 500µg crude peanut extract and 2 mg alum intraperitoneally for 3 weeks. Followed by oral PN challenges with 200mg of freshly ground peanut in week 4. Each mouse received 600µL of formononetin daily. The treatment started in week 8 and continued every day for the next six weeks. At the end of the treatment, mice underwent oral peanut challenge (200 mg). N=4-5 mice/group [original image].

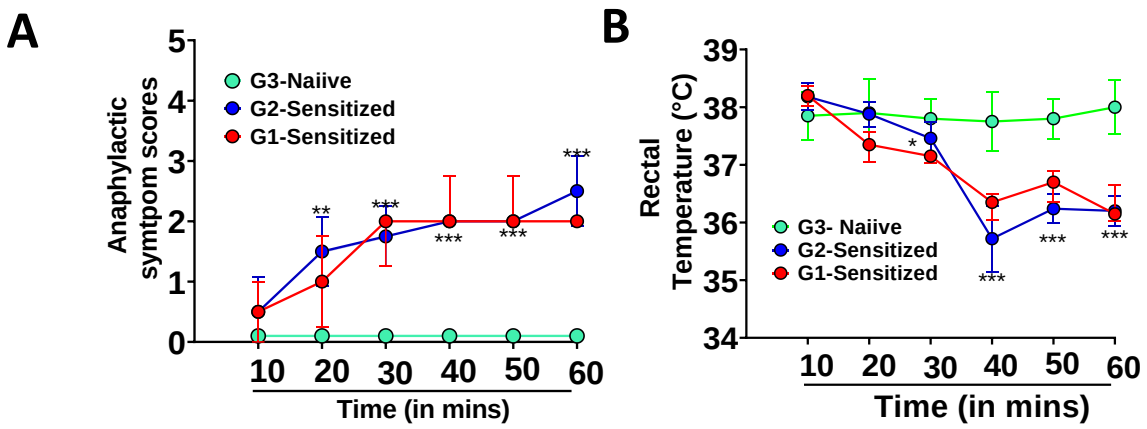


Figure 38. Peanut-induced anaphylaxis in allergic mice model: Mice underwent oral PN challenges 200 mg in week 5. A. Rectal temperature B. Anaphylactic symptoms score. N=4-5 mice/group. *, p<0.05, ***, p<0.001 [original data, unpublished].

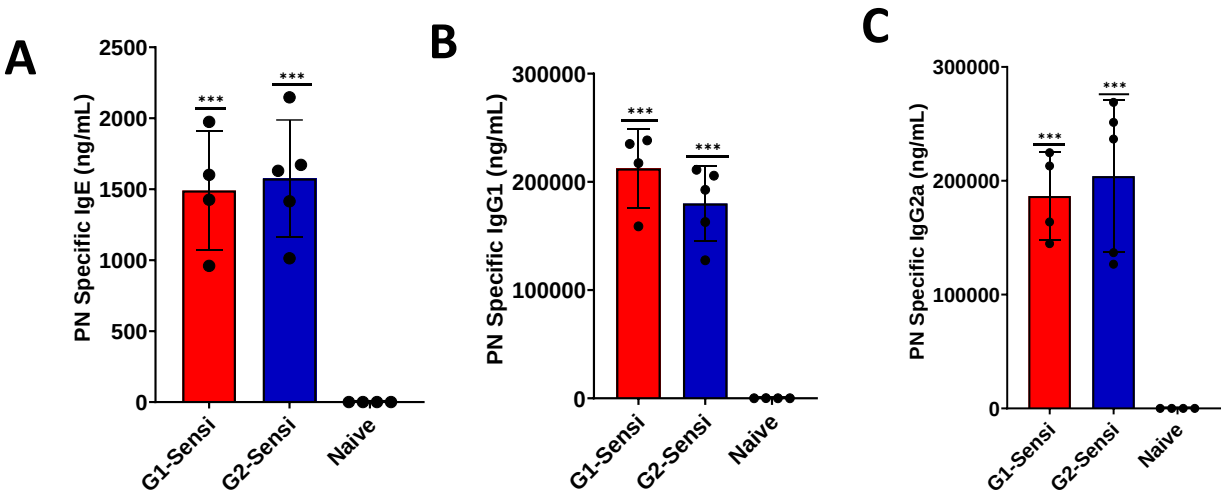


Figure 39. Peanut-induced anaphylaxis in allergic mice model: Serum antibodies measure after oral peanut challenge (200mg) in week 5. A. Peanut specific Immunoglobulin E B. Peanut specific Immunoglobulin G1 C. Peanut specific Immunoglobulin G2a. N=4-5 mice/group. *, p<0.05, ***, p<0.001 [original data, unpublished].

II. Formononetin decreased peanut specific IgE levels and increased IgG2a levels in peanut allergic mice

We monitored the levels of serum peanut specific IgE, IgG1, IgG2a to determine if formononetin has an effect on the levels of these antibodies. Before treatment, we measured serum peanut specific IgE, IgG1, IgG2a. Our results showed that the levels were high in all peanut allergic mice prior to the administration of formononetin (Figure 40A). We continued to measure the levels of serum antibodies. Our results showed that by the fourth week of treatment, mice receiving formononetin showed a significant decrease in peanut specific IgE levels compared to the sham treated group (Figure 41A, week 10, *p< .05). The decrease in peanut specific IgE in the formononetin treated mice continued into the 6^{th} week (week 12, *p<0.05). After 4 weeks (Figure 40B) we did not see a significant change in the levels of peanut specific IgG1 which continued until week 13 (Figure 41B). However peanut specific IgG2a were increased at week 4 (Figure 40C, (*p <0.05) of treatment and this increase was seen through week 13 (Figure 41C) (*p<0.05).

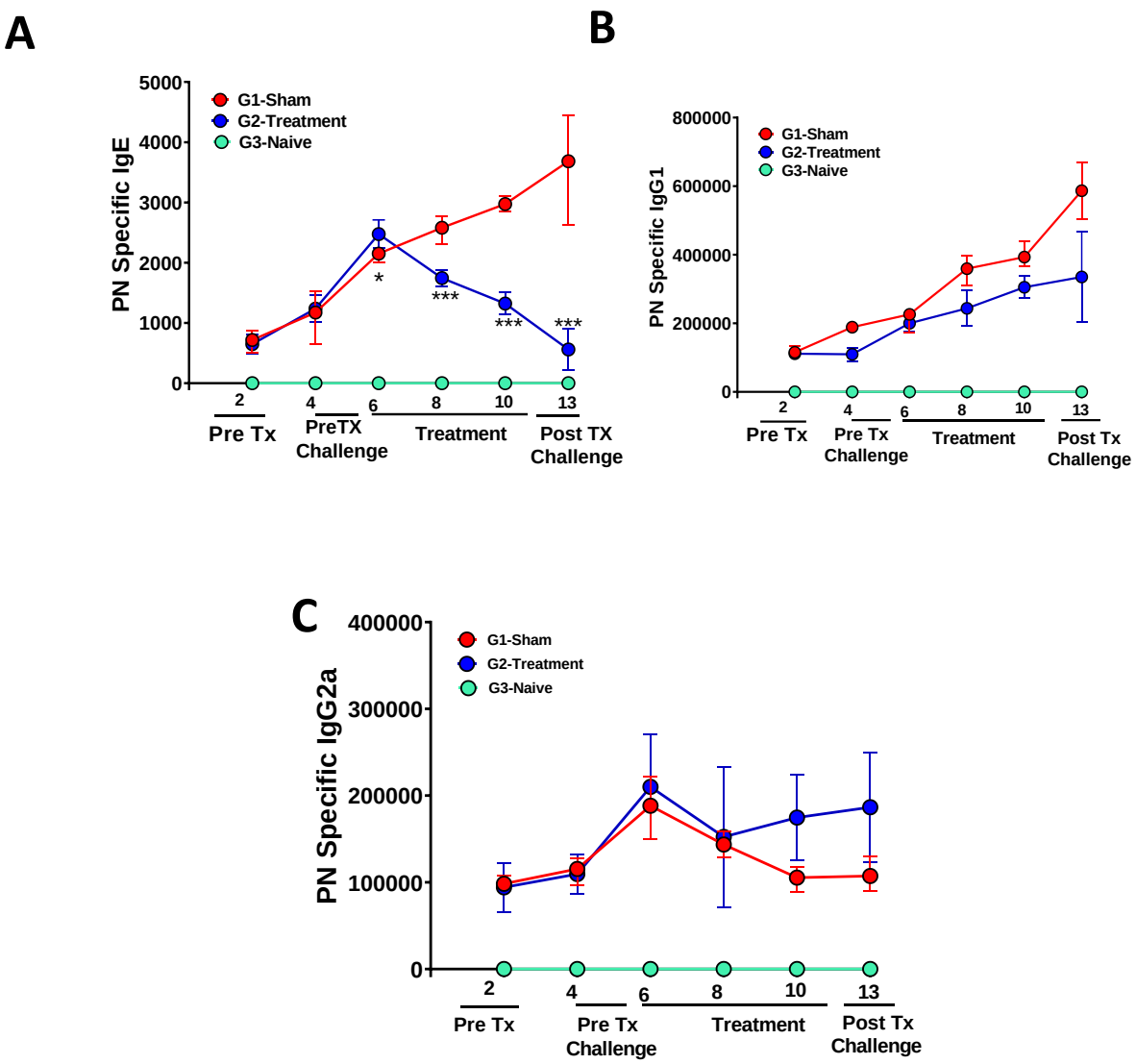


Figure 40. Formononetin decreased serum peanut specific IgE, IgG1 and decreased IgG2a. Serum was collected from venous blood drawn submandibularly from the mice. Using ELISA we determined the levels of serum peanut specific antibodies. A. IgE. B. IgG1. C. IgG2a. N=4-5 mice/group. *, $p<0.05$, ***, $p<0.001$ [original data, unpublished].

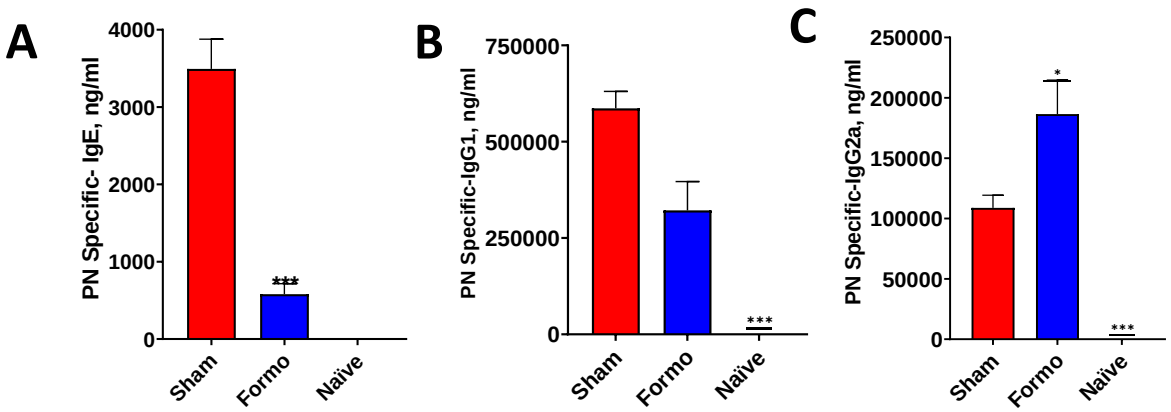


Figure 41. Formononetin decreased serum peanut specific IgE, IgG1 and increased IgG2a: Serum was collected from venous blood drawn submandibularly from the mice after oral peanut challenge. Using ELISA, we determined the levels of serum peanut specific antibodies. A. IgE. B. IgG1. C. IgG2a. N=4-5 mice/group. *, $p<0.05$, **$p < 0.01$; ***, $p<0.001$ [original data, unpublished].

III. Formononetin decreased peanut-induced anaphylaxis

All sham-treated mice showed symptoms of anaphylaxis after the oral peanut challenge at week 13 (Figure 42A). The formononetin treated mice showed decreased symptoms of anaphylaxis as indicated by lower anaphylactic symptom scores shown (median score 1; *P <0.05, Figure 42A, B).

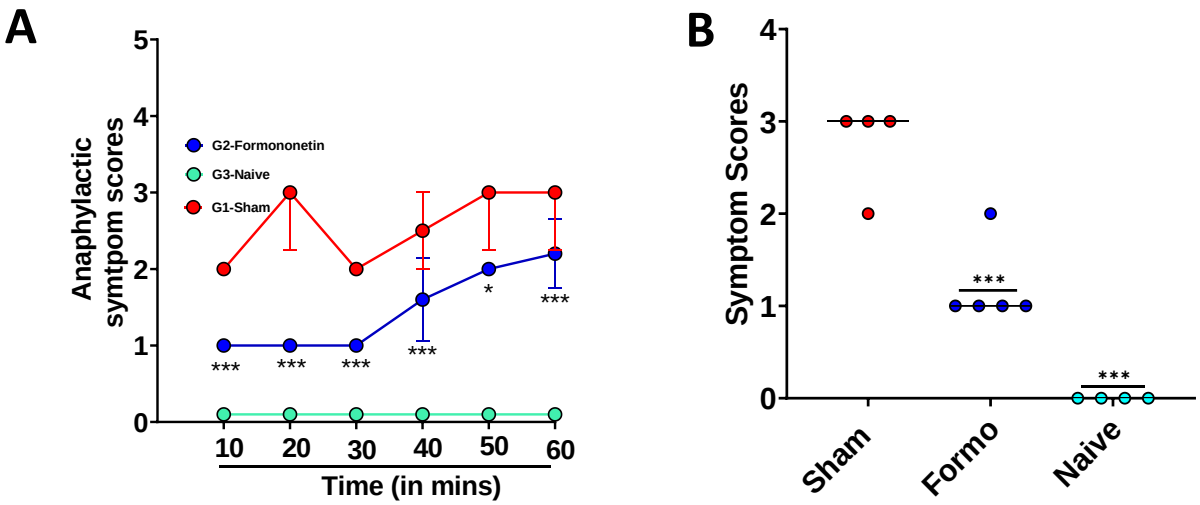


Figure 42. Formononetin decreased peanut-induced anaphylaxis. Anaphylactic symptom scores. A. Scores assessed every 10 minutes. B. Symptom score after 30 minutes. N=4-5 mice/group. ***, p<0.001 [original data, unpublished].

IV. Formononetin treatment prevented a decrease in body temperature compared to the sham treated mice

An important clinical symptom of anaphylaxis is a decrease in body temperature. To determine changes in body temperature, we measure the rectal temperature every 10 minutes after oral peanut challenge. Our results showed that the mice receiving formononetin treatment had higher body temperature compared to the naïve treated mice (***p<0.001, Figure 43). The mean temperatures in the group of mice receiving formononetin were also shown to be significantly higher compared to the naïve group (Figure 43A).

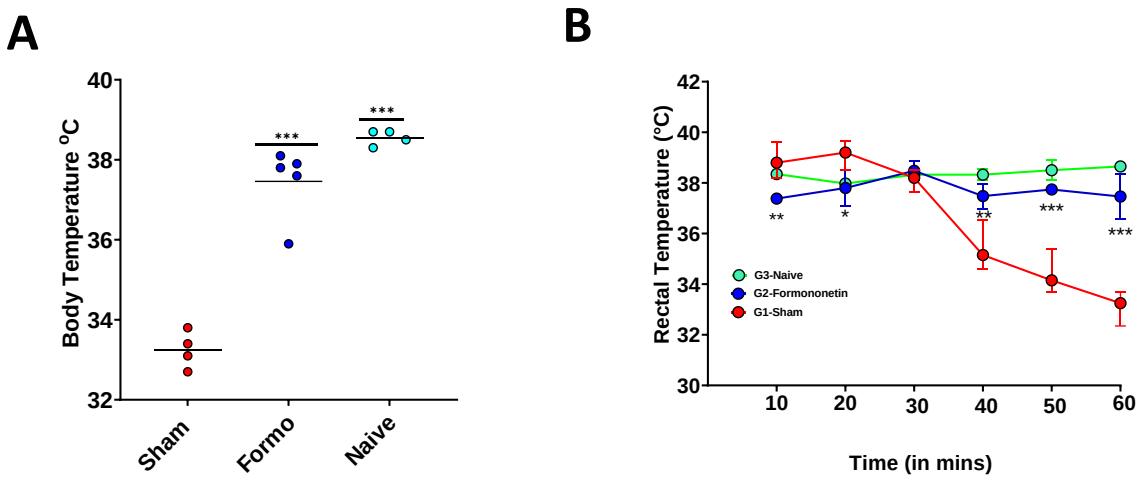


Figure 43. Decrease in body temperature, a symptom of anaphylaxis prevented by formononetin. Body temperature after oral peanut challenge. Post oral peanut challenge, rectal body temperature was measured every 10 minutes using a temperature probe. N=4-5 mice/group. ***, p<0.001 [original data, unpublished].

V. Histamine release was prevented in formononetin treatment mice after peanut challenge

Histamine release from mast cell degranulation is responsible for the symptoms of anaphylaxis. Therefore, it is important to measure the levels of plasma histamine to determine if formononetin prevents anaphylaxis by preventing plasma histamine release from mast cells. To do this we drew blood 30 minutes after oral peanut challenge and collected sera to measure plasma histamine levels. Our results showed that formononetin prevents histamine release compared to the naïve group (Figure 44, ***p<0.001).

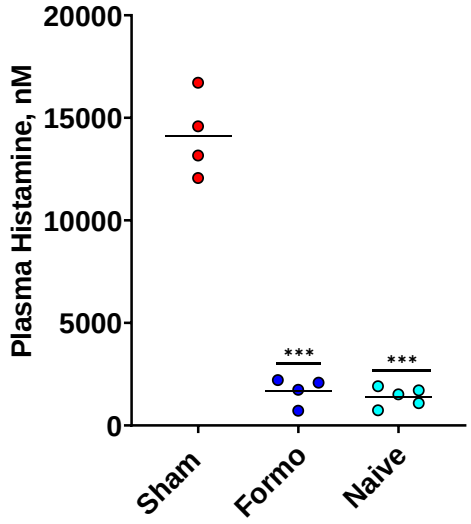


Figure 44. Formononetin treatment prevented histamine release. Blood was drawn after oral peanut challenge. Sera was collected and plasma histamine was measured using enzyme immunoassay. N=4-5 mice/group. ***, p<0.001 [original data, unpublished].

VI. Formononetin decreased Th2 cytokine IL-4, IL-13 and increased IFNγ

Post therapy we measured the Th2 cytokines IL-4, IL-13 in mesenteric lymph nodes and splenocytes. Our results showed that sham treated mice had higher levels of Th2 cytokines and lower levels of IFN γ (Figure 45A, B 46A, B) ***, p<0.001. While the formononetin treated mice showed a significant decrease in Th2 cytokines - IL-4 and IL-13 (Figure 45A, B 46A, B) ***,p<0.001 and a significant increase in IFN-gamma secretion in both cultured MLN and splenocytes (Figure 45C, 46C).

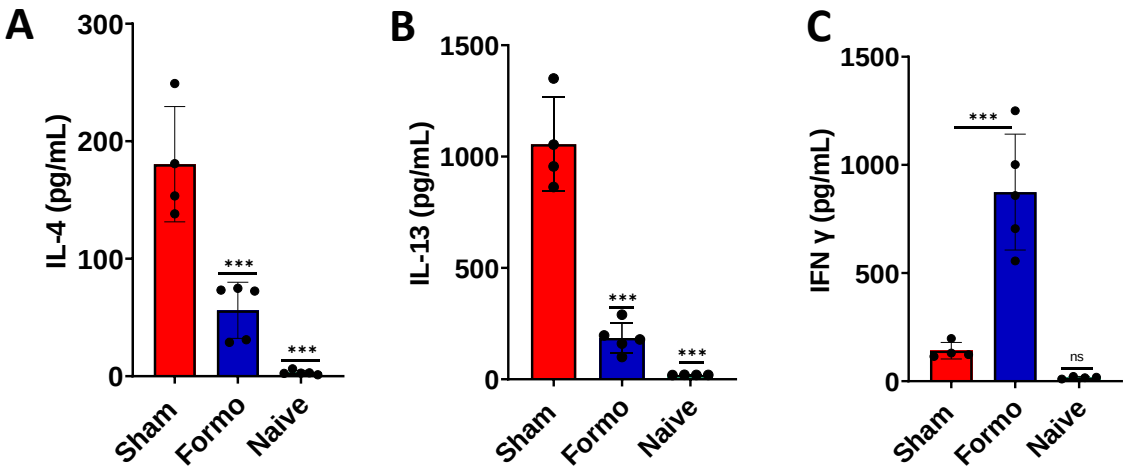


Figure 45. Formononetin decreased IL-4, IL-13 and increased IFN-gamma. After oral peanut challenge splenocytes were harvested from the mice and cultured for 72 hours. After the culture time point the supernatant was collected and using ELISA we measured cytokines (A). IL-4, (B). IL-13. (C). IFN-gamma. N=4-5 mice/group. ***, p<0.001 [original data, unpublished].

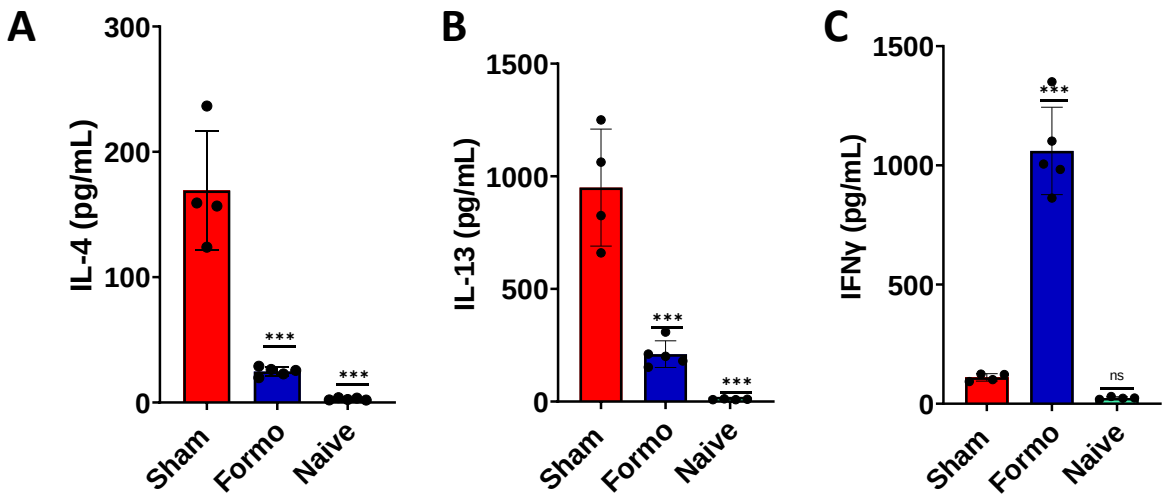


Figure 46. Formononetin decreased IL-4, IL-13 and increased IFN-gamma. After oral peanut challenge mesenteric lymph nodes were harvested from the mice and cultured for 72 hours. After the culture time point the supernatant was collected and using ELISA, we measured cytokines (A). IL-4, (B). IL-13. (C). IFN-gamma. N=4-5 mice/group. ***, $p<0.001$ [original data, unpublished].

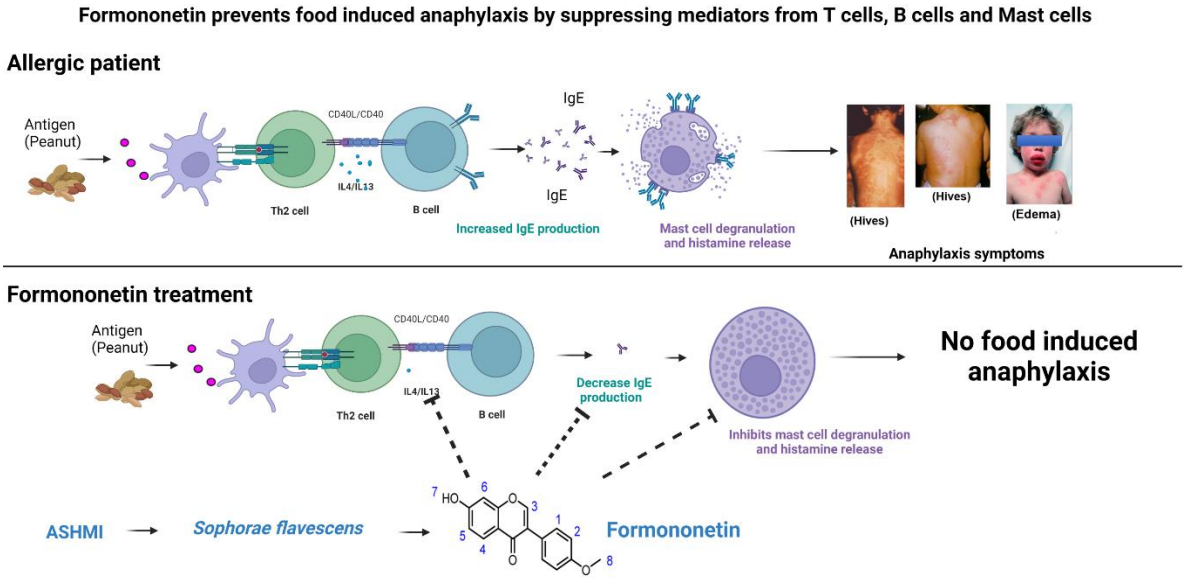


Figure 47: Summary of Aim 3

4. Discussion

"*Sophorae flavescens,* one of the herbal constituent of ASHMI, has been shown to reduce IgE It has been previously reported that *Sophorae flavescens* is used for the treatment of diseases such as skin diseases like psoriasis, eczema, gastrointestinal dysfunction, viral hepatitis, viral myocarditis and enteritis. *Sophorae flavescens* was previously determined to interfere with TNF-α cytokine and as such has anti-inflammatory properties and has been used in inflammatory diseases (Zhou *et al.,* 2009). It has been shown also that the main bioactive compound of *Sophorae flavescens* are major alkaloids that have antipyretic properties (The State Pharmacopoeia Commission of The People's Republic of China 2005). Alkaloids alone do not prevent inflammatory response that are type I hypersensitivity and allergy responses mediated by Immunoglobulin E. Therefore, it is important to determine the essential isoflavonoids that are responsible for IgE inhibition to identify the IgE inhibitory compounds. In this study we isolated and purified the IgE inhibitory compound in *Sophorae flavescens* using HPLC and H^1-NMR analysis, we identified the IgE inhibitory compound to be formononetin. The identified formononetin belongs to a class of naturally occurring active compounds knowns as flavonoids. Flavonoids belongs to a class of natural occurring products that have been shown to have favorable anti-carcinogenic, anti-inflammatory, biochemical, anti-mutagenic, antioxidant effect that are associated with diseases such as allergic, atherosclerosis, cancer and alzheimer's. There are different studies showing that flavonoids have the capability of decreasing IgE production. Therefore, it is important to determine the isoflavonoids that have the possibility of inhibiting IgE. Formononetin isolated from *Sophorae flavescens* belongs to the class of naturally occurring flavonoids. Therefore, we wanted to investigate

the mechanism by which it decreases immunoglobulin E production. Our data showed that formononetin significantly decreased the immunoglobulin E production from IgE producing human myeloma cells. The result is consistent with the result seen in whole herb extract, however we show that formononetin decreased immunoglobulin E at a relatively lower concentration compared to the herb extract. From our result formononetin is the main bioactive compound in *Sophorae flavescens* responsible for inhibition of immunoglobulin E. Our results also showed that the inhibition by formononetin occurred without any cytotoxicity as shown by the cell viability data". Adapted from (Yang *et al.,* 2022)

Formononetin regulates estrogen receptors which are present on Mast cells, B cells and T cells

Formononetin has a structure that is similar to endogenous estrogen (estradiol), it binds to the estrogen receptors α and β (Tay *et al.,* 2019). The anticancer properties of formononetin have been well researched. Studies have shown that formononetin inhibits STAT activation cascade, decreased DNA binding activities, reduced translocation of p-STAT3 and p-STAT5, inhibits upstream kinases of STAT3 activation (Kim *et al.*, 2018) in human myeloma cell. It also inhibits cell proliferation, induces cell cycle arrest, induces apoptosis, modulates MMP and ROS production, and regulates ERK1/2, P38 MAPK and PI3K/AKT signal transduction in human ovarian cancer (Park *et al.,* 2018). It has also been shown to have effects in human colon cancer (Auyeung *et al.,* 2012), human nasopharyngeal carcinoma cell, human breast cancer cell (Chen *et al.,* 2013), human prostate cancer (Li *et al.,* 2014), human osteosarcoma (Li *et al.,* 2014), human bladder cancer (Wu *et al.*, 2017), human cervical cancer (Jin *et al.,* 2014). Studies have shown that formononetin promotes angiogenesis and vascular recovery by regulating endothelial cell proliferation and migration at sites of vascular injury. Mast cells have estrogen receptors (Zhao *et al.,* 2001), expression

of estrogen and progesterone receptors by mast cells alone, but not lymphocytes, macrophages, or other immune cells in human upper airways. This shows that sex hormone may play a role in allergic diseases. This is also evidenced by the fact that 70% of all adult hospital admissions for asthma are women (Skobeloff *et al.,* 1992). (Grimaldi *et al.,* 2002) showed that estrogen receptors α and β are present on B cells and are functional. They activate molecular pathways that are important for survival and activation of naïve autoreactive B cells. The role of estrogen receptors α and β have been studied in effector T cells, as well as Th1, Th2, Th17, and T reg cells. It has been shown that estrogen receptor increases Th2 and Treg cells in mice via transcription factors such as GATA3 and Foxp3 (Lambert *et al.,* 2005). With formononetin activating estrogen receptors, it is important to investigate if formononetin regulates the activity of the immune cells, B cells, T cells and mast cells important in allergy via estrogen receptors. Understanding the pathways regulated downstream of estrogen receptor activation will be important to define the targets downstream of estrogen receptors activation regulated by formononetin relevant to food allergy.

Formononetin inhibits IgE production at a lower IC5O value.

Half maximal inhibitory concentration (IC50) is used for the measure of a drug's efficacy. It is a measure of the amount of drug that is needed to inhibit a biological process by half. The IC50 value measures the potency of an antagonist drug in pharmacological research (Aykul 2016). ASHMI - Antiasthma Simplified Herbal Medicine Intervention inhibits IgE production in IgE producing human myeloma cells dose dependently across a concentration of 31-500µg/mL, with an IC50 value of 49.76µg/mL. The components of ASHMI *Sophorae flavescentis, Glycyrrhiza uralensis* and *Ganoderma lucidum* were tested

individually for IgE inhibition. The three components of ASHMI inhibited IgE production significantly. *Sophorae flavescentis* had the strongest inhibition of the three components. The dichloromethane extract of *Sophorae flavescentis* was tested for its IgE inhibitory activity over a concentration of 1.25-40 μg/mL. Our results showed that the dichloromethane extract inhibited IgE production significantly with an IC50 value of 1.612 μg/mL. HPLC and LC-MS were used to identify the IgE inhibitory compound as formononetin. Formononetin was tested across a concentration of 0.07-20 μg/mL, our results showed that it inhibited IgE production significantly in a dose dependent manner with an IC50 value of 0.16 μg/mL. Taken together our data shows that formononetin is at least 300X more potent than ASHMI, and at least 30X more potent than the dichloromethane extract of *Sophorae flavescentis*. The results from the phase I clinical trial of ASHMI showed that ASHMI was safe in Twenty (20) nonsmoking, allergic subjects with asthma. Eight (8) subjects reported mild gastrointestinal symptoms. There were no grade 3 adverse effects observed and no abnormal immunologic symptoms were reported (Kelly-Pieper *et al.*, 2009). The results from the phase I clinical trial led to a larger phase II clinical trial. However, this was not terminated due to poor adherence and high daily dosage. Our results showing that formononetin is 300X more potent than ASHMI in inhibiting IgE shows that the ease of clinical application will be increased thereby increasing the chances of a successful clinical trial.

***Sophorae flavescentis* contains other fractions with IgE inhibitory compounds.**

"The dichloromethane extract of *Sophorae flavescentis* inhibited IgE production with an IC50 value of 1.612 μg/mL which is about 30X more potent than the IgE inhibitory potential of ASHMI. Further separation of the components was done using high-performance liquid chromatography (HPLC). Four fractions were collected based on the

major peaks presented in the analytic HPLC chromatogram data. The fraction from which formononetin was isolated was focused on because of the high isolation efficiency and the IgE inhibitory potency. However, the fractions SF-DC and SF-DD also contain potential IgE inhibitory compounds which may not be as potent as formononetin. The compounds in these fractions were not isolated because of low isolation efficiency. We believe further optimization and isolation of this compound should be considered. The potential IgE inhibitory compound in these fractions can be used as a combination therapy with other IgE inhibition compounds in the treatment and prevention of food anaphylaxis.

IgE production requires class switch recombination between large and highly repetitive switch (S) regions. The human ε-germline gene promoter region consists of several promoters which includes STAT6 promoter region and NF-κB region. NF-κB binds to two sites in the ε-germline gene promoter and cooperates with STAT6 in synergistic action of transcription. The activity of STAT6 is highly influenced by NF-κB.(Tinnell *et al.*, 1998, Goenka 2011) At the conclusion of Ig class switching, a B cell secretes one of the different classes of antibodies. (Geha *et al.*, 2003) Each of the three stages of class switching – germ line gene transcription, DNA recombination and B cell differentiation is very complex resulting in the activation of ε-germline gene transcription is essential. (Oettgen 2000)**.**

Upon activation mature B cells undergo immunoglobulin class switch recombination and differentiate into antibody-secreting plasma cells (Stavnezer *et al.*, 2008). Antagonistic influences from two groups of transcription factors control the differentiation of B cells into plasma cells (Stavnezer *et al.*, 2008). The group of transcription factors that maintain B cell includes Pax5, Bach 2, and Bcl6 while the group

of transcription factors that promote and facilitate plasma cell differentiation include Irf4, Blimp1 and XBP1(Stavnezer *et al.,* 2008). XBP1 is a positively acting transcription factor in the CREB/ATF family that is expressed at a high level in plasma cells. (Stavnezer *et al.,* 2008) XBP1 acts downstream of Blimp-1 gene to regulate a broad complement of genes encoding ER-associated proteins, many of which are involved in protein secretion (Shaffer *et al.,* 2004). XBP1 induces physical expansion of the ER, in addition to increasing cell size, organelle biosynthesis, total protein synthesis. This shows that XBP1 plays a critical role in the secretion of immunoglobulins (Shaffer *et al.,* 2004). XBP1 is uniformly expressed in all multiple myeloma cell lines (Shaffer *et al.,* 2004). Because of the role of XBP1 in secretion of immunoglobulin we decided to investigate whether it is inhibited in human B cell line U266 cultured with formononetin. U266 cells were cultured with 20 µg/mL of formononetin, and the cells were harvested after 6 days for RNA isolation and RT-PCR was done to analyze gene expression levels. Our result showed that formononetin decreased the XBP1 gene expression significantly when compared with untreated cell cultures. We believe that XBP1 inhibition is another mechanism that formononetin uses in decreasing IgE secretion. This is the only study to show that formononetin inhibits XBP1 in human B cell line U266 cells.

Activation of ε germline gene is an essential step towards IgE expression (Oettgen 2000, Geha *et al.,* 2003), which leads to generation of transcripts ε mRNA transcripts after class switch recombination. The presence of IgEH transcripts in the B cell provides evidence that class switch recombination has occurred in the germline region. Therefore, we used RT-PCR to compare the relative expression of IgEH transcripts in the U266 cultured with formononetin against untreated cultures. The result showed that there was a

significant decrease in IgEH mRNA transcripts in the treated culture compared to untreated cultures. This is an indication that the inhibition of IgE production by formononetin is also due to an alteration in the germline region which possibly affects class switch recombination. If indeed formononetin alters germ line transcription and possibly prevent class switch recombination, further study needs to be done to see if the cells are producing a different type of immunoglobulin because of class switching". Adapted from (Yang *et al.*, 2022).

Biological functions, pharmacological mechanisms of formononetin relevant to food allergy

Through data mining we found that formononetin potentially regulates 25 targets in food allergy, 51 in IgE diseases and 19 in mast cell diseases, we show the potential mechanisms that are regulated by formononetin in both B cells and Mast cells diseases. In B cells we showed that formononetin regulate genes involved in the receptor signaling, activation, proliferation, and class switch recombination. While in mast cells we show that formononetin target genes involved in mast cell activation and degranulation. ADA, BTK, PI3K and PRKCD genes appear in multiple GO terms indicating that these genes are very important in mast cell activation and degranulation, however more in vitro and in vivo studies should be done to further understand how formononetin interacts with these genes. CD40L and cytokines that are known to activate B cells and initiate class switching as well augment IgE secretion were also shown to be regulated by formononetin. IL-2, IL-4 and IL-10 are cytokines that aid B cell proliferation and differentiation (Defrance, Vanbervliet *et al.*, 1992). IL-4 and CD-40 cross-linking is particularly important for IgE secretion (Gascan *et al.*, 1991, Zhang *et al.*, 1991, Armitage *et al.*, 1993) while IL-2 and IL-10 have been shown to induce IgM, IgG and IgA secretion. Formononetin has been shown to inhibit IL-4,

IL-5, IL-13 and IgE by regulating Th2 cells. We also showed that formononetin interacts with CD27 to prevent class switch recombination.

The targets were analyzed in KEGG pathway, and the top 15 pathways were selected. In the top 15 pathways selected, primary immunodeficiency was identified as the top pathway. Examples of primary immunodeficiency disorders are atopic dermatitis, asthma, and malignancies such as lymphomas. Other pathways identified in the KEGG were Epstein Barr virus infection, measles virus infection, and hepatitis B and C virus infection all of which are infections that can cause allergy with several studies showing an elevation in serum IgE levels after an infection with these viruses, which leads us to suggest that allergy seen in later life might be due to an infection with these viruses at an early age.

Critical pathways involved in B cell differentiation – PI3K pathway and class switch recombination - NF-κB were also shown to be regulated by formononetin. The inhibition of the PI3K pathway, P53 gene, BCL-2 gene, and BTK gene by formononetin demonstrate its antiproliferative activity via inhibiting apoptosis, apoptosis plays a significant role in regulating B cells that are committed to IgE production, however the plasma IgE+ B cells responsible for the pathologic effects of IgE have been shown to have long life span months to years while the memory IgE B cells have a life span of about 10-12 days. We believe the longer life span seen IgE plasma B cells could be due to its regulation of this apoptotic genes.

Furthermore, we validated the computational results and our data showed that formononetin significantly decreased the expression BTK, TYK2, CASP8 while it significantly increased the expression of BCL2, NFKBIA, P53. We also show that formononetin decreased beta hexosaminidase release, which ultimately means that

formononetin can prevent mast cell degranulation. These findings of formononetin provide a comprehensive overview of how it can be a potential treatment candidate for food anaphylaxis in food allergy. By inhibiting activation, proliferation, differentiation of B cells as well as inhibiting mast cell activation and degranulation, formononetin can be used as a potential treatment and preventive option for food anaphylaxis.

Formononetin prevents food induced anaphylaxis via regulation of key mediators in T cells, B cells and Mast cells

Peanut mediated anaphylaxis which accounts for most of the fatal and near fatal anaphylactic reactions to foods is IgE mediated. Formononetin, which has previously shown to reduce IgE in vitro was tested to see if it can prevent peanut mediated anaphylaxis in an in vivo mouse model. Our results show that the mice treated with formononetin exhibited decreased anaphylactic symptoms which correlates with body temperature. Plasma histamine, which is also released during anaphylaxis, was also shown to be decreased. This might be due to the decrease in peanut specific IgE, which was shown to be decreased in the mice group that were treated with formononetin. Our results also showed that IgG2a levels were significantly increased in formononetin treated mice. IgG2a is a T_H1 driven antibody (Howard 1983) this indicates a shift from a T_H2 into a T_H1 response. This increase corresponds to the further suppression of T_H2 cytokines IL4, IL13 seen in splenocytes and mesenteric lymph nodes. In addition, we also saw an increase in T_H1 cytokines IFN-ɣ which further validates the shift from T_H2 to T_H1 response. IFN-ɣ is a potent negative controller of T_H2 responses. The role of T_H2 cytokines in the pathogenesis of food allergy has been well studied (Lorentz *et al.*, 1999).

Conclusion

Formononetin is the major component of Sophorae flavescentis that inhibited IgE production. We used computational technology to identify the biological targets and processes regulated by formononetin. Using our established peanut allergic mice model, we showed the formononetin prevents food anaphylaxis, by suppression of Th2 cytokines IL4, IL13 and increasing IFN gamma. This study has shown that formononetin might be a potential treatment and preventive option for food anaphylaxis reaction seen in food allergy.

Future directions

It is important to further investigate the long-term protective effect of formononetin. Further studies will also need to be conducted in vivo and ex vivo to determine if formononetin prevents food allergy to other allergens such as milk, fish, wheat etc. It is also important to study the drug and natural compounds interactions.

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