EVALUATION OF THE EFFICACY OF SAPONIN EXTRACT FROM *GLYCINE MAX (L.) MERR*. AS AN ADJUVANT ON THE HEPATITIS B VACCINE AND HEPATITIS B SURFACE ANTIGEN IN BALB/C MICE

FAITH NJOKI NDUNG'U

MASTER OF SCIENCE

(Molecular Biology and Bioinformatics)

JOMO KENYATTA UNIVERSITY

OF

AGRICULTURE AND TECHNOLOGY

2024

Evaluation of the Efficacy of Saponin Extract from *Glycine Max (L.) Merr*. as an Adjuvant on the Hepatitis B Vaccine and Hepatitis B Surface Antigen in Balb/C Mice

Faith Njoki Ndung'u

A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Molecular Biology and Bioinformatics of the Jomo Kenyatta University of Agriculture and Technology

2024

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature ……. Date……

Faith Njoki Ndung'u

This thesis has been submitted for examination with our approval as University Supervisors

Signature ……. Date……

Dr. Steven Nyanjom, PhD

JKUAT, Kenya

Signature ……. Date……

Dr. James H. Kimotho, PhD

KEMRI, Kenya

DEDICATION

This work has been dedicated to my parents, Mr. Eutycus Ndung'u and Mrs. Hannah Ndung'u for their support, financially, morally and in prayers.

ACKNOWLEDGEMENT

Special appreciation to my supervisor: Dr. James H. Kimotho, working on this project under your supervision has been a delightful learning experience. Your insights and encouragement ensured the successful completion of this work. Dr. Steven Nyanjom, your sacrifice, unwavering support, criticism and encouragement throughout the MSc training period have been very instrumental in completing this research. You have both been great mentors throughout this journey, offering guidance and advice through all the stages of the project. The completion of this study would not have been possible without your expertise. God bless you.

I would also like to give a special thanks to my university, Jomo Kenyatta University of Agriculture and Technology, and the Kenya Medical Research Institute for giving me an opportunity and for facilitating this project, to its completion.

KEMRI-ITTD staff: To Samuel Omari and Eunice Wainaina, thank you for your immense help throughout the research period.

KEMRI- Animal house staff: Mr. Steve and Ms. Lucy, thank you for your help and guidance when handling the mice.

I wish to sincerely thank my parents, Mr. and Mrs. Ndung'u, for cheering me on. Your love, encouragement and prayers can't go unmentioned. God bless you.

Finally, I deeply thank God for the strength to do this work. Through all the difficult situations, I have experienced His grace day by day.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

APCs	Antigen Presenting Cells
Arg	Arginine
cccDNA	Covalently Closed Circular DNA
CD4+	Cluster of Differentiation 4
CFA	Complete Freund Adjuvant
COVID-19	Coronavirus Disease 2019
DAMPs	Damage-Associated Molecular Patterns
DC	Dendritic Cells
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FCT	Fold Change in Transcription
FC	Fold Change
FTIR	Fourier Transform Infrared Analysis
HBcAb	Hepatitis B Core Antibody
HBeAg	Hepatitis B e Antigen
HBsAg	Hepatitis B Surface Antigen
Hb	Hemoglobin
HCT	Hematocrit

HBV	Hepatitis B Virus
HRP	Horseradish Peroxidase
HSPG	Heparan Sulfate Proteoglycan
IFN	Interferon
Ig G	Immunoglobulin G
Ig M	Immunoglobulin M
IL	Interleukin
ISCOMs	Immunostimulating Complex
ITTD	Innovation and Technology Transfer Division
KEMRI	Kenya Medical Research Institute
Lys	Lysine
MeOH	Methanol
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MPL	Monophosphoryl Lipid
NETs	Neutrophil Extracellular Traps
NODs	Nucleotide-Binding Oligomerization Domain
NTCP	Sodium Taurocholate Co-Transporting Polypeptide

OD	Optical Density
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
Pro	Proline
PRRs	Pattern Recognition Receptors
QS-21	*Quillaja Saponaria-21*
RBC	Red Blood Cells
Rcdna	Relaxed-Circular DNA
RIG-I	Retinoic Acid-Inducible Gene I
RNA	Ribonucleic Acid
SBA	Saponin-Based Adjuvants
SBM	Soybean Meal
SPC	Soybean Protein Concentrate
STING	Stimulator of Interferon Genes
TBP	TATA-Box Binding Protein
TGF	Transforming Growth Factor
Th1	T-Helper Cells 1
Th2	T-Helper Cells 2

Thr	Threonine
TLRs	Toll-Like Receptors
Tm	Melting Temperatures
TNFα	Tumor Necrosis Factor Alpha
WBC	White Blood Cells

ABSTRACT

The most prevalent and dangerous type of liver infection worldwide is hepatitis B. Hepatitis B virus infects and damages the liver. A chronic hepatitis B infection affects roughly 300 million people, whereas two billion people, or one in three, have already contracted the virus. Hepatitis B is preventable and treatable, but it nevertheless claims the lives of up to a million people annually. Hepatitis B vaccination is the primary means of preventing infections and complications caused by the Hepatitis B Virus (HBV). Subunit vaccines such as the recombinant hepatitis B vaccines have been shown not to activate many facets of the immune response, as compared to whole-organism-based vaccines. Immuno-stimulatory adjuvants that improve immune responses caused by low immunogenic antigens are critical for improving immunogenicity in subunit vaccines. Saponin-based adjuvants extracted from *Quillaja saponaria* and *Glycine max (L.) Merr.* can stimulate cell-mediated and enhance antibody production. These saponins are now being applied in human vaccines, with several clinical trials proving safety and efficacy. Using mice models (N=51), this study assessed the adjuvant activities of saponins extracted from *Glycine max (L.) Merr.* on BALB/c mice vaccinated with either Revac B^{TM} vaccine, a commercial hepatitis B vaccine or purified hepatitis B surface antigen. Saponins were extracted from soybean meal and characterized using the Fourier Transform Infrared Analysis and Ultra-violet/Visible Spectrophotometry. Eight-weeks-old female BALB/c mice were divided into 17 groups and vaccinated in triplicate, with 50µl of either Revac B^{TM} vaccine, or Hepatitis B surface Antigen (HBsAg) supplemented with 100%, 50% or 25% concentrations of the saponin extract. A booster shot was administered two weeks after the first vaccination. A negative control involved the administration of phosphate buffer saline. Enzyme-linked Immunosorbent Assay was used to compare the humoral immune response using serum collected on day 14 and day 30. Spleen tissues were harvested on day 30 and used to analyze the cellular immune response by determining the mRNA expression levels of the Interleukin-6 (IL-6) and Tumor Necrosis Factor-alpha (TNF-α) genes. Hematological analysis was performed on the whole blood collected from the mice through a cardiac puncture. The findings from this study demonstrated that soybean saponin extracts did not have a statistically significant effect on the immune response of BALB/c mice. The immune response was slightly higher for Revac-B™ vaccine (0.758 ± 0.012), and HBsAg (0.799 ± 0.013) than vaccination with the Revac-B™ vaccine without the saponin extract. A novel finding was that the HBV vaccine suppressed the mRNA expression on IL-6 gene (Fold change in transcription (FCT) = 0.603, while promoting the expression of the TNF-α gene (FCT = 28.84). This study demonstrated that the *Glycine max (L). Merr.* saponin extract did not have any effect on most of the hematological parameters. However, the neutrophil and platelet counts were reduced (*p value*= 0.027 and 0.592 respectively). *Glycine max (L). Merr.* saponin extract showed minimal adjuvant activity for HBV vaccine, unique expression profile of IL-6 and TNF-α genes, and high safety profile in BALB/c mice. The findings of this study demonstrated that the saponin extract from *Glycine max (L). Merr.* did not elicit significant immune response in BALB/c mice and hence cannot be used in combination with either the Revac-B^{TM} or the HBsAg.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Hepatitis B virus (HBV) is a DNA virus responsible for acute and chronic liver disease. Infection with HBV continues to be the main cause of liver cancer and is a major source of morbidity and mortality globally, making hepatitis B immunization very important (Pattyn et al., 2021). The World Health Organization (WHO) reported that in 2024, the mortality rate due to viral hepatitis is increasing, with 1.3 million deaths reported globally. In 2022, it was estimated that 254 million people live with hepatitis B virus (W.H.O, 2024). In Africa, HBV in endemic, with an estimated 64.8 million people chronically infected, and 300,000 deaths reported as of 2022 (Razavi-Shearer et al., 2023). According to reports, the general population in Kenya has a 6-12% prevalence of chronic HBV infection (Mercy Jelagat et al., 2020).

The primary means to avert infections and difficulties brought on by the HBV is to get vaccinated against hepatitis B (Guimarães et al., 2015; Schillie et al., 2018). The WHO advised in 1991 that the HBV vaccination be a part of every country's national immunization program (Kane, 1996). In order to eradicate HBV infection as a worldwide health hazard by 2030, the World Health Organization unveiled a plan in 2016. The plan called for a 90% reduction in HBV incidence and a 65% reduction in mortality (W.H.O., 2016). More than 95% of healthy newborns, kids, and young people who received the HBV vaccine showed signs of sero-protection (Van Damme P et al., 2018).

Hepatitis B surface antigen (HBsAg) was isolated from the plasma of asymptomatic HBsAg carriers to create the first hepatitis B vaccine that was approved for use. The development of a recombinant hepatitis B vaccine was then made possible via recombinant DNA technology. More than 30 years of long-term protection can be obtained from a vaccination that requires three doses. Hepatitis B immunoglobulin and hepatitis B vaccination used concurrently have significantly decreased the amount of HBV transmitted from mother to child; for example, children of carriers

who have negative hepatitis B e antigen (HBeAg) have almost no infection, while children of mothers who have positive HBeAg have 5–10% infection (Zhao et al., 2020a).

Current HBV vaccines are produced using the recombinant technology by expressing the S gene in *Saccharomyces cerevisiae* (Zhao et al., 2020b). A dose of the vaccine consists of 5-40 μg of recombinant HBsAg adsorbed on aluminum hydroxide or aluminum phosphate adjuvant, and is administered on a three-dose schedule (Zhao et al., 2020b). The administration of a hepatitis B virus vaccine triggers the T cell-mediated immunity, resulting in the generation of anti-HBs (Honorati & Facchini, 1998). After one month of finishing the three-dose vaccination schedule, more than 90% of the immunized persons achieve protective anti-HBs levels. Despite a considerable decline in antibody titres over the first few years following vaccination, memory immunity alone is adequate to prevent infection regardless of the antibody levels (Di Lello et al., 2022a). Revac-B^{TM} vaccine is a hepatitis B vaccine manufactured using the recombinant DNA technology and contains purified, non-infectious major surface antigen of the hepatitis B virus. This vaccine is manufactured by Bharat Biotech and contains aluminum hydroxide as an adjuvant.

Adjuvants can be used in a variety of ways to increase the immune response to vaccination antigens, including increasing the immunogenicity of weak antigens, enhancing the speed and duration of the immune response, modulating antibody avidity, specificity, isotype distribution, stimulation of cell-mediated immunity and enhance the immune response in immunologically immature individuals. They can reduce the dose of antigen and reduce vaccine costs, or they can help overcome antigen competition in combination vaccines (Singh & O'Hagan, 2003). Many diverse classes of compounds have been assessed including mineral salts, microbial products, emulsions, saponins, cytokines, polymers, microparticles, and liposomes (Guy, 2007)**.**

Soya bean (*Glycine max),* is a major leguminous crop of the Fabaceae family. Regular consumption of soya beans has been associated with reduced incidences of diseases such as osteoporosis, cancer, and cardiovascular disease. Many health

benefits of soya bean are derived from its secondary metabolites such as isoflavones, phytosterols, lecithins, and saponins. Isoflavones improve digestive tract function, prevent breast, prostate, and colon cancer, and promote bone health. Lecithins improve lipid metabolism and also improve memory and learning abilities. Saponin on the other hand regulates lipid metabolism and also acts as an antioxidant (Dixit et al., 2011).

Saponin-based adjuvants can stimulate the cell-mediated immune system, as well as enhance antibody production. These adjuvants have the advantage that only a low dose is required for adjuvant activity. Saponins induce a strong adjuvant effect on T-dependent and T-independent antigens (Rajput et al., 2007a). Additionally, saponins also induce strong cytotoxic CD8+lymphocyte responses and potentiate the response to mucosal antigens. Studies have reported that saponins induce the production of cytokines such as interleukins and interferons that might mediate their immuno-stimulant effects (Jie et al., 1984).

1.2 Statement of the Problem

Vaccines require optimal adjuvants as immune-potentiators and delivery systems to offer long-term protection from infectious diseases in animals and human beings (Rajput et al., 2007b). Around 5–10% of people are not protected against HBV by the most popular second-generation vaccines now in use (Di Lello et al., 2022b). The currently licensed Hepatitis B vaccines, e.g. Engerix-B vaccines have limitations including hypo-responsiveness in older adults, poor compliance, and the extended time for most people to develop sero-protection, e.g. more than 6 months. The aluminum-hydroxide-adjuvanted Hepatitis B vaccines require three doses over 6 months to achieve high rates of protection in adults (Halperin et al., 2012; Heyward et al., 2013).

Modern vaccines use subunit or recombinant antigens, improving the safety of the vaccines, albeit at the cost of reduced immunogenicity (Poolman, 2014). Adjuvant discovery has lagged behind other vaccine areas such as antigen discovery. Consequently, only a limited number of adjuvants based on aluminum salts, monophosphoryl lipids and oil emulsions are currently approved for human use, with

alum being the dominant adjuvant in use (Petrovsky, 2015). According to Petrovsky *et al.* (Petrovsky, 2015), alum is able to induce a good antibody (Th2) response. However, it has little capacity to stimulate cellular (Th1) immune response. Additionally, alum has the potential to cause severe local and systemic side-effects. Oil-based adjuvants can induce immunoglobulin E antibody response, but have been associated with allergic reactions in human subjects. Mineral oil-based adjuvants have also been reported to cause local reactions and granulomas at the injection site. There is also a public concern about the risk of mineral oil contamination by carcinogenic polycyclic aromatic hydrocarbons (Spickler, 2003).

1.3 Justification

The use of highly pure antigens to improve vaccine safety has led to reduced vaccine immunogenicity and efficacy, rendering the necessity to use adjuvants. Adjuvants help antigens to elicit an early, high, and long-lasting immune response with less antigen, thus saving on vaccine production costs. With the development of new-generation vaccines like recombinant subunit and mucosal vaccines, that are less immunogenic, the search for more potent vaccine adjuvants has intensified. The ideal adjuvant should maximize vaccine immunogenicity without compromising on safety and tolerability (Petrovsky, 2015).

Saponin-based adjuvants can stimulate the cell-mediated immune system as well as enhance antibody production and have the advantage that only a low dose is required for adjuvant activity (Rajput *et al.,* 2006). *Glycine max (L.) Merr.* seeds contain 184 mg/gm of saponins, which is a rather a high level of concentration (Kunatsa and Katerere, 2021). Studies have established that female rainbow trout fed (orally) with *Glycine max (L.) Merr.* saponins augmented immune response to Furogen vaccine following intraperitoneal vaccination (Penn, 2005). The increased specific antibody levels at four weeks post-vaccination in fish from the SBM-SAP diet suggests that orally administered *Glycine max (L.) Merr* saponins can exert a systemic immunomodulatory effect, resulting in an increased humoral immune response following vaccination with *A. salmonicida bacterin* (Penn, 2005). Currently, the saponin QS-21 from the bark of *Quillaja saponaria* has proven to induce humoral

and T-cell responses (Wu *et al.,* 1992). The use of saponin-based adjuvant on HBV vaccine could potentially stimulate both the humoral and cellular immune response, and reduce the number of doses required to stimulate a long-lasting immunity.

1.4 Research Questions

1. What is the characteristic profile of *Glycine max (L. Merr.* Saponin extract?
2. Are saponin extracts from *Glycine max (L.) Merr*. efficacious in BALB/c mice when vaccinated with either Revac-B^{TM} vaccine or HBsAg?
3. How does the supplementation of *Glycine max (L.) Merr*. saponin extract affect the expression levels of Interleukin-6 (IL-6) and Tumor Necrosis Factor-alpha (TNF-α) genes in BALB/c mice vaccinated with either Revac-B^{TM} vaccine or HBsAg?
4. Does the supplementation of either Revac-B^{TM} vaccine or HBsAg with *Glycine max (L.) Merr.* saponin extract affect the hematological profile of BALB/c mice?

1.5 Null hypotheses

Glycine max (L). Merr. does not contain saponins with adjuvant efficacy when vaccinated with either Revac B^{TM} vaccine or HBsAg in BALB/c mice.

1.6 Objectives

1.6.1 General Objectives

To assess the adjuvant efficacy of saponin extract from *Glycine max (L.) Merr.* on Revac-B^{TM} vaccine and HBsAg in BALB/c mice.

1.6.2 Specific Objectives

1 To profile the saponins extracted from *Glycine max (L.) Merr.*

2 To assess the humoral immune response of BALB/c mice vaccinated with either the Revac-B^{TM} vaccine or HBsAg, both supplemented with *Glycine max (L.) Merr*. saponin extract.

3 To determine the expression levels of the (IL-6) and TNF-α genes in BALB/c mice vaccinated with either Revac-B^{TM} vaccine or HBsAg, both supplemented with *Glycine max (L.) Merr.* saponin extract.

4 To determine the effect of Revac-B^{TM} vaccine or HBsAg supplemented with *Glycine max (L.) Merr.* saponin extract on the hematological profile of BALB/c mice.

CHAPTER TWO

LITERATURE REVIEW

2.1 Hepatitis B virus Disease

Hepatitis B virus is a hepadnavirus that has the ability to cause systemic infection and hepatocellular damage in humans (Honorati & Facchini, 1998). The small, DNA-encased hepatitis B virus exclusively infects hepatocytes. The viral capsid is carried to the nucleus after entry and uncoating of the HBV, where the double-stranded relaxed-circular DNA (rcDNA) genome is released and altered into covalently closed circular DNA (cccDNA) by host enzymes. (Köck et al., 2010; Y. Qi et al., 2016). This cccDNA persists as a minichromosome and serves as a template for transcription of four viral RNAs through the cellular transcription machinery. A number of liver-enriched transcription factors and nuclear receptors have been shown to bind HBV promoter or enhancer elements and to be critical in activating and regulating HBV transcription (Block et al., 2007). The (Figure 2.1) shows a simplified figure of the hepatitis B virus particle and surface antigens as illustrated by (Pattyn et al., 2021).

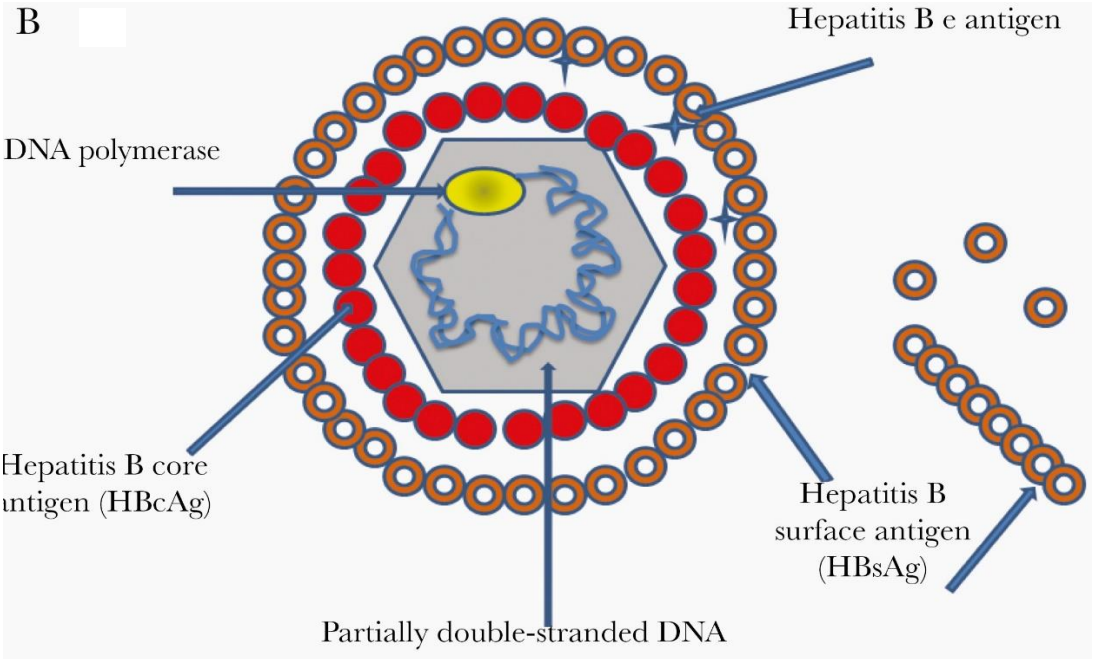


Figure 2.1: Hepatitis B Virus Particle

Globally, approximately 350 million people are chronic carriers of the hepatitis B surface antigen, vertical transmission of the virus from mother to neonate being the main route of infection (Lok & McMahon, 2009). According to the World Health Organization (WHO), in 2019, an estimate of 296 million people were living with chronic hepatitis B infection, and an estimated 820,000 deaths reported. These deaths were mainly from cirrhosis and hepatocellular carcinoma (WHO, 2022). With over 60 million infections, the developing region of Africa ranks second only to Asia in terms of the burden of chronic HBV carrier rates. Seventy percent of new HBV infections globally occur in countries in this region, including Kenya, where infection rates are over five percent. Unfortunately, due to inadequate record keeping and underreporting, it has been challenging to accurately estimate the burden of HBV in Africa. Moreover, due to inadequate financing and infrastructure, relatively few research has been conducted and published to clarify the occurrence of infections. However, the scant figures that are currently available indicate that this region has a 60% lifetime probability of contracting HBV infection (Sonderup & Spearman, 2022).

HBVs are highly infectious and are transmitted through contact with infected blood or other body fluids through the mucous membranes or broken skin (Van Damme P et al., 2018). The primary determinant of clinical manifestation of acute disease and the development of chronic infection is the age of HBV infection acquisition (Edmunds et al., 1993). The virus can survive for at least seven days outside of the body. If during this time the virus enters the body of an individual who is not immunized, it might still cause an infection. HBV incubation period ranges from 30 to 180 days. During the 30-60 days of infection, the virus can survive and cause chronic hepatitis B, especially if it is spread during infancy or childhood (WHO, 2023). Compared to 30% of infections in adults, less than 10% of children under the age of five show clinical signs or symptoms of the acute hepatitis B disease. Age has an inverse relationship with the risk of developing chronic HBV infection; 80%-90% of infants infected in the first year of life go on to acquire chronic HBV infection, compared to 30%-50% of children infected before the age of 6, and 1%-5% of adults. Viral persistence in infants infected at birth appears to be significantly influenced by neonatal immune tolerance to viral antigens (Bauer et al., 2011; Milich et al., 1990). The immunization of new-born and infants is currently a crucial strategy for the prevention of HBV infections because HBV infections that arise during pregnancy, infancy or the early years of childhood are more likely to become chronic (Pattyn et al., 2021).

Acute hepatitis b is the discrete onset of symptoms; headache, anorexia, nausea, vomiting, abdominal pain and diarrhea, the presence of jaundice, or elevated levels of serum alanine transaminase. Chronic hepatitis B on the other hand is the persistence of hepatitis B surface antigen for more than six months. Chronic hepatitis B increases the risk of hepatocellular carcinoma and cirrhosis. A definitive diagnosis of HBV requires serological testing since the clinical manifestations are indistinguishable from other causes of viral hepatitis (Hoofnagle et al., 1981). This testing determines if a person has acute or chronic HBV infection, is immune to HBV due to prior infection or vaccination, or is susceptible to infection by using a combination of serologic markers to identify distinct phases of HBV infection (Van Damme P et al., 2018). Table 2.1 shows an interpretation of HBV serologic test results (Pattyn et al., 2021).

Table 2.1: Interpretation of Serologic Test Results for Hepatitis B

	Acute HBV	Chronic HBV	Cleared HBV	Vaccination
HBcAb IgM	+	-	-	-
HBcAb IgG	+	+	+	-
HBsAg	+	+	-	-
Anti-HBs	-	-	+	+
HBeAg	+	+/-	-	-
Anti-HBe	-	+/-	+/-	-
HBV DNA	High/low	Low/high	-	-

Abbreviations; HbcAb IgG- hepatitis B core antibody immunoglobulin G, HBcAb IgM- hepatitis B core antibody immunoglobulin M, HBeAg- hepatitis B e antigen, HBsAg- hepatitis B surface antigen.

Treatment of hepatitis B is individualized, based on the clinical and the laboratory characteristics, and the risk of developing cirrhosis and hepatocellular carcinoma. Drug therapies that suppress HBV DNA replication and improve liver inflammation and fibrosis help to attain immunologic cure, which is the loss of the HBsAg with sustained HBV DNA suppression. The recommended treatment options for chronic hepatitis B are pegylated IFNα-2a, antecavir, and tenofovir (Wilkins et al., 2019). Although they are not curative, the current treatments for chronic hepatitis B can stop or slow the advancement of cirrhosis, lower the incidence of liver cancer, and enhance long-term survival and quality of life. Hence, the majority of people who begin hepatitis B treatment must continue for life.

2.2 Pathogenesis of Hepatitis B Virus Infection

HBV clearance is mainly dependent on the antiviral effect of the immune system (Zhong et al., 2021). The rapid activation of IFNα/β by the infected cell, which occurs frequently as a result of virus replication, signals the onset of an innate immune response (Samuel, 1991). The innate immune system acts as the first line of defense against HBV, reacting promptly and subsequently triggering the adaptive immune response by either killing virus-infected hepatocytes directly or exerting

non-cytolytic mechanisms mediated by soluble cytokines (Kapoor & Kottilil, 2014). Adaptive immune response largely mediates viral clearance and disease pathogenesis in HBV infection (Guidotti & Chisari, 2006). The antibody response to the Hepatitis B surface antigen (HBsAg) is a T-dependent process (Tsui et al., 1995a). Adaptive immune cells are crucial during chronic liver infection, and are involved in the pathogenesis of hepatic inflammation. The activity of T lymphocytes largely depends on the differentiation of naïve T cells into T-helper 1, Th2, Th9, Th17, and Th22 effector T-cells via the release of cytokines and other cofactors (He et al., 2013). The anti-envelop antibodies play a critical role in the clearance of the virus by complexing with any free viral particle and removing them from circulation or by preventing their attachment and uptake by hepatocytes. Neutralizing antibodies are unlikely to contribute to the early phase of viral clearance in acute infections since they occur relatively late after infection with HBV (Baumert et al., 2007). In patients with acute hepatitis, the peripheral blood CD4 T cell response is vigorous and multi-specific, thus ultimately clearing the infection (Ferrari et al., 1990). CD8 T cells are fundamental in pathogenesis of liver disease and viral clearance. A weak and narrowly focused T cell response is observed in chronically infected patients, whereas a vigorous polyclonal CD8 T cell response is readily detectable in acute hepatitis patients, who ultimately clear the infection (Tsui et al., 1995b). In low dose infections, failure to induce trigger early CD4+ T cell responses results to functionally impaired CD8+ T cell responses, resulting in a persistent infection (Baumert et al., 2007).

2.3 Hepatitis B Vaccines

Vaccines are administered as a preventative measure in advance of pathogen exposure, taking advantage of the immune system's ability to respond quickly to microorganisms following a second exposure (Cohen & Marshall, 2001). The principle of vaccination is the generation of a long-term immunization against antigens specific to a pathogen or a cancer cell through the development of antibodies and cytotoxic T cells (Korenkov et al., 2018). There are a variety of vaccine types that are currently in use, including, live, attenuated vaccines that contain weakened versions of the original pathogenic agent. With one or two doses

of the vaccine, these vaccines typically produce long-term immunity, and a strong cellular and antibody response. An example of such a vaccine is the measles vaccine. Inactivated vaccines, on the other hand, are not live, and cannot replicate. They are less affected by circulating antibodies, and so they may be given when an antibody is present in the blood. Although the inactivation of the microorganism gives the vaccine more stability, these vaccines produce a weaker immune response, thus requiring additional booster shots to maintain immunity. Inactivated vaccines include whole-cell inactivated vaccines, subunit vaccines, toxoids, conjugate vaccines and recombinant vaccines (Clem, 2011; Wodi & Morelli, 2016)

Vaccination with the Hepatitis B surface antigen (HBsAg) has proven to be the effective mode of protection against HBV infection. HBs antibody titers in serum are the basis for the evaluation of the efficacy of the HBV vaccine. Hepatitis B vaccine, as per the Advisory Committee on Immunization Practices is recommended for all medically stable infants weighing 2kgs or more within 24 hours of birth, unvaccinated infants and children, and unvaccinated adults requesting protection against HBV, or those at risk of infection (Schillie et al., 2018a). The vaccines are available in monovalent formulations, administered within 24 hours after delivery, and is recommended to reduce mother-to-child transmission, as well as combination vaccines, e.g. infant vaccines including diphtheria-tetanus-pertussis, *Haemophilus influenzae* type b, and inactivated polio vaccine, administered in three doses, 4 weeks apart, and aims to minimize horizontal transmission. According to the individual's age, the recommended immunization schedule for the adult population includes three vaccine doses. Adults with impaired immune systems or patients receiving dialysis therapy need higher or additional doses of the HBV vaccine (Di Lello et al., 2022a).

Studies have shown that persons over 40 are less likely to achieve a sero-protective response to the hepatitis B vaccine, and this likelihood lowers to 60%-70% in adults over 60 years of age (Van Den Ende et al., 2017). Lower response rates may also be as a result of obesity, smoking, HIV infection, hereditary factors, and chronic illnesses. The recombinant hepatitis B vaccines are highly immunogenic. Due to their manufacture in yeast, recombinant HBsAg particles differ from natural viral particles in that they lack both the preS domain of HBsAg and glycosylation. In addition to

the main HBsAg protein, mammalian cell-derived vaccines also include glycosylated pre-S1 and pre-S2 proteins (Shouval et al., 2015). Table 2.2 lists the vaccines currently available for Hepatitis B as compiled by (Zhao et al., 2020b).

Table 2.2: Chart of Internationally or Locally Available Recombinant Hepatitis B Vaccines

Vaccine name	**Company, Country**	**HBsAg (μg)/volume (mL)**	**Applicable subject**
Engerix-B	GlaxoSmithKline, USA	10/0.5	From birth to age 19 y
		20/1.0	Adults (≥20 y)
		40/2.0	Immunocompromised individuals
Recombivax HB	Merck & Co, USA	5/0.5	From birth to age 19 y
		10/1.0	Adults (≥20 y)
		40/1.0	Immunocompromised individuals
Hepavax-Gene	Berna, Korea	10/0.5	Infants and children
		20/1.0	Adults
Euvax-B	Lucky Goldstar Chemical, Korea	10/0.5	Infants and children
		20/1.0	Adults
Revac-B	Bharat Biotech, India	10/0.5	≤10 y old children
		20/1.0	>10 y old children and adults
Heberbiovac-HB	Heber Biotec, Cuba	10/0.5	≤10 y old children
		10/1.0	>10 y old children and adults
HBvaxPro	MSD Pharma, Singapore	5/0.5	Pediatric/adolescent
		10/1.0	Adults
		40/1.0	Predialysis/dialysis patients
Hanxin	Hiss Bio, China	10/0.5	All ages
		20/1.0	All ages
Tiantan	Tiantan, China	10/0.5	<16 y
		20/1.0	≥16 y
Kangtai	Kangtai, China	10/0.5	<16 y
		20/1.0	≥16 y

2.4 Immune Response against Hepatitis B Vaccine

The most commonly used immune marker is the measurement of the humoral immune response as it correlates with protection against HBV infection (Plotkin, 2010). Titers above 10IU/ml are associated with protection and considered protective. However, an estimated 4-10% healthy individuals vaccinated for HBV fail to achieve the 10IU/ml titer, and are considered non-responders. Moreover, 13-60% of healthy vaccinated persons eventually lose their protection as their anti-HBs levels tend to decrease over time, a phenomenon described as "waning immunity" (Sjogren, 2005). The activation of the CD4+ T helper lymphocytes that in turn elicit B cell proliferation and their differentiation into Ab-secreting plasma cells is required for the production of HBV-specific antibodies by B lymphocytes. The adjuvants included in HBV vaccines, such as alum, stimulate the migration of dendritic cells (DCs) towards the site of injection and, after antigen capture, their homing into the lymph nodes, where antigens will be processed and presented to CD4+ T cells. These CD4+ T cells will trigger antibody production by secreting cytokines, thus establishing a long-lasting immune memory (Lambrecht et al., 2009; Michel & Tiollais, 2010).

The titers attained at the end of the vaccination regimen are directly correlated with the duration of antibody levels (Mendy et al., 2013). Protective antibodies levels tend to decrease over time, especially during the first years of vaccination. Several studies have shown that vaccination in adolescence produces greater and long-lasting titers than infant vaccination, with the age at vaccination being an independent variable linked with an anti-HBs titer below 10mIU/ml. Nevertheless, vaccination against HBV throughout childhood, when combined with other vaccinations, ensures a better coverage rate (Coppola et al., 2015; Pileggi et al., 2017; Stefanati et al., 2019). Different variables, including the host's genetic makeup, age, body weight, smoking status, and coexisting illnesses, have been proven to influence the vaccines effectiveness (Yang et al., 2016). Notably, while the HBV vaccine induces a protective immunity against infection, it would not be sterilizing. As a result, even vaccinated persons are susceptible to infection, but most cases are asymptomatic and self-limited (Werner et al., 2013).

2.5 Control of Hepatitis B by Cytokines

To control a viral infection, inhibition of virus entry is the best approach, and targeting either of the two host factors; Heparan sulfate proteoglycan (HSPG) or Sodium taurocholate co-transporting polypeptide (NTCP) is crucial. Cytokines and cytokine-induced mediators target these host factors, thus exploiting HBV entry (Xia & Protzer, 2017). The cytokines IL-6 and IL-1 β inhibit viral entry by regulating the expression of NTCP. A recent study by Bouezzedine et al. revealed that cells pretreated with IL-6 inhibited HBV entry by up to 90%, resulting in strong reduction of cccDNA HBsAg secretion (Bouezzedine et al., 2015). The inhibition of HBV entry by IL-6 through the downregulation of viral entry receptor is possibly through the inhibition of hepatocyte nuclear factor 4α transcription (Hösel et al., 2009). Studies have also shown that both IFN-γ and TNF-α interfered with cccDNA integrity and stability in HBV-infected primary human hepatocytes (Xia et al., 2016). Transforming growth factor (TGF)-β, via the activation-induced cytidine deaminase (AID), is able to induce the deamination and degradation in hepatocytes (Qiao et al., 2016). Interleukin (IL)-4 has shown to have direct antiviral effect on HBV, by inducing differentiation of naïve helper T cells to T helper cells

2.6 Saponins

Saponins are a class of bioorganic compounds found in particular abundance in the plant kingdom. They are phytochemicals consisting of polycyclic aglycones covalently attached to one or more sugar side chains mainly found but not exclusively in plants. The aglycone part (sapogenin), is either a steroid (C27) or a triterpene (C30) (Majinda, 2012). Some plants are rich in saponins that are usually used as natural detergents. There are 11 main classes of saponins: dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes, and steroids (Figure 2.2) (Madland, 2013). The oleanane skeleton is the most common, present in most orders of the plant kingdom (Szakiel et al., 2005). They contain a steroidal or triterpenoid aglycone to which one or more sugar chains are attached (Oda et al., 2003). Triterpenoids are predominantly found in cultivated plants such as soybeans, peas, tea, and spinach, while steroid saponins

are common in plants used as herbs, such as oats, capsicum, and tomato seeds, and asparagus (Rajput *et al.*, 2007).

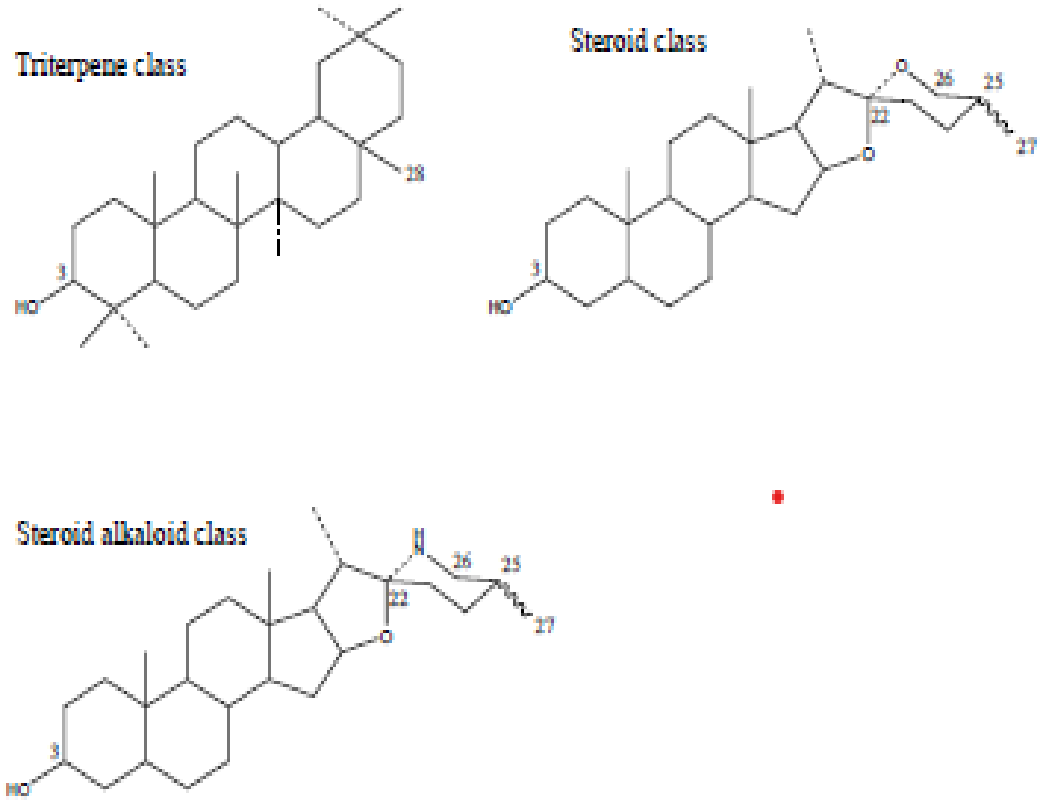


Figure 2.2: Skeletal Types of Sapogenins

The structural characteristics of saponins and their amphiphilic nature, which results from the presence of a hydrophilic sugar moiety and a hydrophobic genin, are thought to be responsible for surface activity responsible for foaming properties as well as some other biological function, including hemolytic activities (Podolak et al., 2010). Saponins possess a variety of pharmaceutical properties including the ability to increase immune response, antibacterial properties, anticancer, antidiabetic and anti-obesity properties, cholesterol-lowering activity and also as food additives (Guclu-Ustundag & Mazza, 2007).

2.7 Vaccine Adjuvants and their Roles

An adjuvant is a substance that is added to a vaccine to stimulate and enhance the magnitude, durability, and breath of the immune response. They are non-immunogenic substances that can improve or modulate antigen-specific immune responses toward their co-administered antigens (Pulendran et al., 2021; Wang, 2021). Vaccine adjuvants have been broadly divided into delivery systems and immune-stimulatory adjuvants, based on their principal mechanisms of action (Singh & O'Hagan, 2003). Modern adjuvant development is based on enhancing and shaping vaccine-induced responses without compromising safety. Adjuvant selection and formulation are based on several factors including, the physical and chemical natures of the vaccine, the type of immune response desired, the age of the target population, and the route of vaccine administration. Adjuvants with specific properties are necessitated by the desired qualities of each particular vaccine (Reed et al., 2013).

Adjuvants approved for human vaccines are aluminum, MF59, in some viral vaccines, MPL, AS04, AS01A, and AS02A against viral and parasitic infections, virosomes for hepatitis B virus (HBV), human papillomavirus (HPV), hepatitis A virus (HAV), and cholera toxin for cholera. Oil-based adjuvants include incomplete Freund adjuvant, which contains water in oil emulsion, complete Freund adjuvant (CFA), similar to IFA, but it also contains inactivated and dried mycobacteria in addition to oil in water emulsion. CFA is usually used for primary vaccination, while IF is used for boosting (Guimarães et al., 2015)

2.7.1 Aluminum hydroxide

This is the oldest and the most commonly used adjuvant (Pulendran et al., 2021). It induces antibody responses and CD4+ T helper cell responses in humans. It is postulated that alum mediates its adjuvant effects through the slow release of antigens from the site of vaccination (Moyer et al., 2020). In addition, alum is known to exert several effects on the immune system. Alum can also enhance adaptive immunity by causing tissue damage that induces uric acid-mediated activation of inflammatory DC. Injection of alum rapidly recruited various cells, including

neutrophils, which released neutrophil extracellular traps (NETs) composed of chromatin. The DNA released in NETs partially mediated the adjuvant activity of alum (Marichal et al., 2011).

2.7.2 MF59

This is a squalene-based oil-in-water emulsion that has been found to induce fast priming of influenza antigen-specific CD4+ T-cell responses, induce strong and long-lasting memory T- and B-cell responses, and broaden the immune response beyond influenza strains included in the vaccine (Kommareddy et al., 2017). It is composed of squalene (4.3%) oil/dispersed phase, Tween 80 (0.5%) and Span 85 (0.5%) both as Non-ionic surfactant/emulsifier, and sodium citrate buffer (10mM) (Figure 2.3) (Kommareddy et al., 2017).

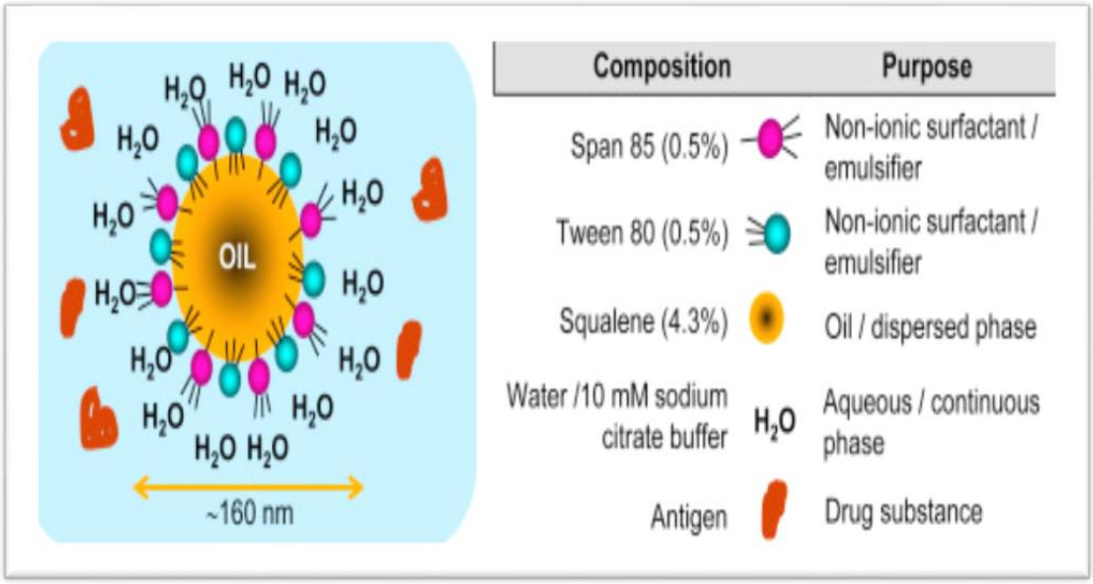


Figure 2.3: Diagrammatic Representation of MF59

MF59 acts by rapid induction of chemokines, inflammatory cytokines, recruiting multiple immune cells, uric acid, and benign apoptosis of some innate immune cells (Ko & Kang, 2018).

2.7.3 Virosomes

Virosomes are adjuvants that contain a membrane-bound hemagglutinin and neuraminidase obtained from the influenza virus. These two components facilitate the uptake into antigen-presenting cells (APC) and mimic the natural immune response (Glück, 1999).

2.7.4 TLR Related Adjuvants

Leucocyte membranes have membrane-bound pattern recognition receptors (PRR) called toll-like receptors (TLRs), which are responsible for detecting most antigen-mediated infections. Their activation leads to an adaptive immune response. It is for this reason that many adjuvants being used today are directed to PRRs. These adjuvants are called Toll-like receptors related adjuvants (Beutler, 2004).

2.7.5 Tufstin

Tufstin is an auto adjuvant, which is a natural self-immunostimulating tetra-peptide (Thr-Lys-Pro-Arg). The tetra-peptide is a fraction of the IgG heavy chain molecule produced by enzymatic cleavage in the spleen (Siemion 1999). Tufstin functions include binding to receptors on neutrophils and macrophages to stimulate their phagocytic activity, increasing tumor necrosis factor-alpha (TNF-α) release from human Kupffer cells enhancing the secretion of IL1 by activating macrophages, and enhancement of murine natural cell-mediated cytotoxicity in vitro. Immunological boosting effects of adjuvants can be attributed to five immune functional activities: 1) translocation of antigens to the lymph nodes where they can be recognized by T cells, 2) antigen protection, enabling longer exposure, 3) enhanced local reaction at the injection site, 4) induction of the release of inflammatory cytokines, 5) interaction with PRRs, specifically TLRs (Schijns 2000).

2.7.6 Saponin-Based Adjuvants

The adjuvant activity of saponins was believed to be related to branched sugar chains or aldehyde groups, or an acyl residue bearing the aglycone. However, soyasaponins and lablabosides were found to show strong adjuvant activity despite lacking acyl

residues and possessing only unbranched sugar chains (Bomford, 1992; Oda, 2000). Saponins have been patented as vaccine adjuvants in form of an aqueous solution comprising a saponin in the amount of about 0.7 mg/dose to about 1.3 mg/dose, wherein the saponin consisted of an anion-exchange column purified extract of *Quillaja saponaria Molina* bark (Detraz & Riguat, 2017).

Triterpene saponin adjuvants such as QS-21 have the ability to influence T cells and dendritic cells either individually or in combination through both receptor-mediated and non-receptor-mediated processes. A costimulatory signal is sent to the T cell via the aldehyde group on QS-21 forming an imine with an e-amino group from a T cell surface receptor, most likely CD2. This signal replaces the one that results from contacts between the DC's CD80 (B7-1 ligand) and the T cell's CD28 receptor. At the level of mitogen-activated protein (MAP) kinase (ERK2) tyrosyl phosphorylation, this signal converges with T cell receptor (TCR)-mediated signaling. This, along with modifications in the cell's K+ and Na+ transport, stimulates T cell activation biased toward Th1 immunity, leading to the secretion of Th1 cytokines (Marciani, 2018).

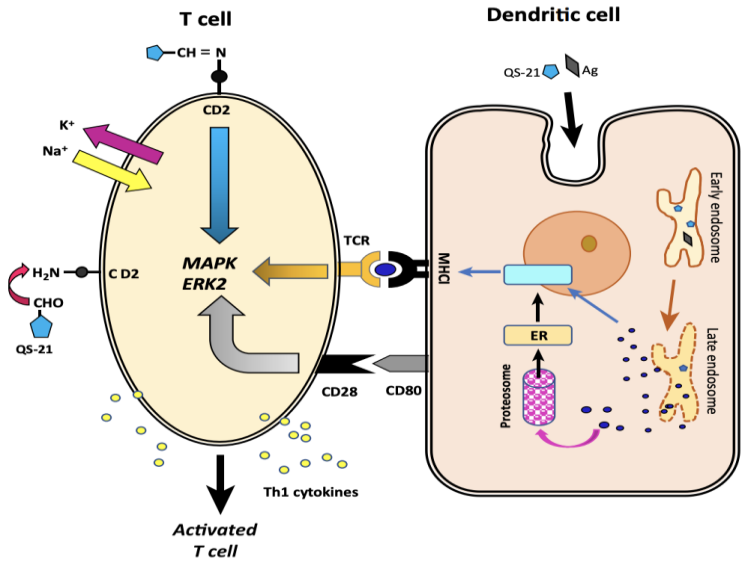


Figure 2.4: Proposed Mechanism of Action for QS-21 and Related Adjuvants Derived from Saponin

Saponin purified from *Quillaja saponaria* alone or introduced as part of the immune-stimulating complexes (ISCOMs), has proved to be a powerful adjuvant in human cytomegalovirus vaccines, influenza vaccines, and polysaccharide vaccines (Sharma *et al.,* 2020). Some of the known plants that are rich in saponins include Licorice (*Glycyrrhiza glabbra*) root (22.2–32.3%), Quillaja bark (*Quillaja saponaria*) (9-10%), sugar beet *(Beta vulgaris)* leaves (5.8%), Chinese ginseng (*Panax ginseng*) (2-3%), Soybean (*Glycine max*) (0.22–0.49%) and Green pea (*Pisum sativum*) (0.18–4.2%) (Kregiel *et al.,* 2017). Triterpenoid saponins have been detected in *Quillaja saponaria* and many legumes such as soybeans, beans, peas, lucerne, etc., and also in alliums, tea, spinach, sugar beet, quinoa, licorice, sunflower, horse chestnut, and ginseng. Purified Quillaja saponins have been shown to boost antibody production without producing any reaginic antibodies (Rajput *et al* 2007). BALB/c mice vaccinated with experimental vaccine formulations containing the saponin adjuvant QS-21 produced significantly higher titers of antibodies than mice vaccinated with only the alum-adsorbed HIV-1 160D (Wu *et al.,* 1992).

CHAPTER THREE

METHODOLOGY

3.1 Materials and Methods

This study required the following materials; BALB/c mice, cages, wood shavings, feeds, syringes and needles, dissection kit, cotton wool, chloroform, partitioning solvents, soybean meal, commercial HBV vaccine (Revac-B^{TM}), HBsAg, RNA extraction kit, cDNA synthesis kit, PCR kit, ELISA kit, olive oil, spectroscopic grade potassium bromide, and PBS.

3.2 Study Site

This laboratory-based experimental study was conducted at the Innovation and Technology Transfer Division (ITTD) of Kenya Medical Research Institute.

3.3 Study Animal

Female BALB/c mice (20 ± 2 g, 8 weeks old) were used in this study. They were procured from the Institute of Primate Research (IPR) Kenya and acclimatized for 7 days at the temperature of 21 ± 3 °C, a humidity of 40–70%, 12-hour light/dark cycle, and *ad-lib* access to appropriate mice chow and water. All efforts were made to ensure minimal harm and suffering to the mice by treating them according to the obligations enlisted in the 3Rs; replacement, reduction, and refinement (Hubrecht & Carter, 2019)

3.4 Sample Size Determination

The number of mice to be used in this study was determined using the resource equation approach (Arifin & Zahiruddin, 2017)

$$DF = N - k = kn - k = k\,(n - 1),$$

Where N = total number of subjects, k = number of groups, and n = number of subjects per group.

By rearranging the formula, n is given as $n = DF/k + 1$.

Minimum $n = 10/k + 1$, maximum $n = 20/k + 1$.

In total, the minimum and maximum numbers of animals required were:

Minimum N = Minimum n x k

Maximum N = Maximum n x k

In this case, the number of groups (k) is 19.

Minimum $n = 10/17 + 1 = 1.7$, and maximum $n = 20/17 + 1 = 2.7$

Thus, maximum N= 3x17= 51

3.5 Preparation of *Glycine Max (L.) Merr*. Extract

3.5.1 Extraction Process of Saponins from Plants

The organic extraction of saponins was done by soaking three portions of 500 gm of soybean meal in one litre of 80% methanol (Sigma-Aldrich, Switzerland) for one hour and then filtered with Whatman filter paper (Merck, Darmstadt, Germany). The final volume of the combined filtrate was transferred into a round-bottomed flask and concentrated using a rotary evaporator (Goel Scientific Glass Works, India) at 65 ºC. The solvent was partitioned sequentially using diethyl ether, distilled water, n-butanol, and acetone as described by Kim & Park (Kim & Park, 2001). Briefly, 150 mL of diethyl ether (Merck, Darmstadt, Germany) and 50 mL of distilled water were mixed with the extract, and the resulting slurry was transferred into a separatory funnel (Duran®, New Jersey, USA). Diethyl ether was discarded after the water layer was washed. This process was repeated twice. The water layer was combined with 100 mL of n-butanol (Merck, Darmstadt, Germany) in the separatory funnel (Duran®, New Jersey, USA), and the mixture was vigorously shaken After setting

still, the resulting n-butanol was collected. The process was repeated once more and the combined n-butanol fraction was concentrated at 82 ºC. Acetone (Merck, Darmstadt, Germany) was added to the n-butanol fraction and the resulting slurry was shaken vigorously and centrifuged (Beckman Coulter, USA) at 1,250 rpm for 15 minutes, at room temperature. The resulting supernatant was concentrated *in vacuo* at 56 ºC.

3.6 Characterization of the Extracted Saponins

3.6.1 Test for Saponins

To confirm the presence of saponins in the soybean meal (SBM), a standard foam test was carried out on the ground SBM, as described by Harbone (1998). 3g of the dry plant material was weighed and extracted using 300ml of hot distilled water in a beaker (Duran®, New Jersey, USA). After filtration, 5ml of the filtrate was placed in a test tube, and diluted with 5ml of distilled water. This mixture was shaken vigorously for two minutes and observed for the appearance of a persistent foam. 3 drops of olive oil were added to the mixture and shaken to examine the formation of an emulsion.

3.6.2 Ultraviolet Spectroscopy

For the purpose of qualitative analysis, the absorption spectrum of the extract was measured as described by Harbone (1998). 100 µl of the extract was diluted using 2900µl distilled water, and the absorbance was measured at a wavelength of 203nm against a distilled water blank, using an automatic recording spectrophotometer.

3.6.3 Fourier Transform Infrared (FTIR) Analysis

Infrared analysis was performed as described by (Wangia et al., 2018) to determine the characteristic profile of the saponin extract. Briefly, 2mg of the extract was mixed with 300mg of spectroscopic grade Potassium Bromide (KBr) in a crucible and compressed into a disk. The disk was subjected to FTIR spectroscopy. The infrared spectrum was recorded using an FTIR spectrophotometer (Shimadzu, Japan) in the absorption range between 4500cm^{-1} to 500 cm^{-1}.

3.7 Assessment of the Immune Response of BALB/c Mice Vaccinated with Hepatitis B Vaccine Supplemented with *Glycine Max (L.) Merr.* Saponin Extract

3.7.1 Vaccination Protocol

Treatments (in triplicate) for the various experimental groups was administered as shown in (Table 3.1).

Table 3.1: Sample Distribution

			Proportion of *Glycine max (L.) Merr.* saponin extract and number of samples			
			0%	**25%**	**50%**	**100%**
Types of treatment, their proportion and the number of samples	Revac-B^{TM}	50%	0	3	3	3
		100%	3	3	3	3
	HbsAg	25%	-	3	3	3
		50%	-	3	3	3
		100%	3	3	3	3
	PBS (Control)		3			

N =51

Immunogen concentrations; 100% - 0.0067ug/ml, 50% - 0.00335, 25% - 0.001675. Saponin concentrations; 100% - 0.2mg/ml, 50% - 0.1mg/ml, 25% 0.05mg/ml

Revac-B^{TM} was employed as a vaccine candidate. The mice were given intramuscular injections with either 50 µL of vaccine alone, 50µL of vaccine + 50µL of saponin extract, 50µL HBsAg alone, 50µL HBsAg + saponin extract or 50µL of the saline buffer. The doses were administered on day one and day 15. On day 14, 50 µL of blood samples was collected through the tail vein before administration of the booster shot. The blood was centrifuged at 5000rpm for 10 minutes to obtain clear sera, which was stored at -20 ºC, awaiting the ELISA test.

On day 30, the mice were euthanized by the method of cervical dislocation, by a trained researcher, ensuring a painless animal sacrifice. Spleens were harvested and placed in sterile 1.5 ml centrifuge tubes and stored at -80 ºC before they were used

for total RNA extraction. Through a cardiac puncture, 500 µl of whole blood for hematological analysis was collected using a 1 ml syringe (BD Luer-Lok™, Canada) and a 22 gauge needle and collected in 1.5 ml EDTA-containing blood collection tubes (BD Vacutainer®, Canada) and stored at 8 ºC awaiting analysis.

3.8 ELISA of Total Antibodies

The total HBsAg antibodies (HBsAb) were evaluated using an adapted ELISA method as described by (Wolters et al., 1979). Briefly, 100 µL of HBsAg (a working standard donated by a KEMRI Scientist, Dr James Kimotho) was diluted 2-fold serially with 100 µL murine generated HBsAb (also donated by Dr James Kimotho). The experimental samples were tested with the HBsAg ELISA test kit (Creative Biolabs, NY. US) to develop a calibration graph. From the serum obtained, 50 µL were mixed with 50 µL of the working standard HBsAg and tested using the ELISA kit following the manufacturer's instructions. Briefly, 50 µL of the mixture was added into the plate wells, followed by 50 µL of HRP-conjugate, and mixed by gently tapping the plate. The plate was covered and incubated at 37 ºC for 1 hour. After incubation, the plate was washed 5 times with the diluted washing buffer. Chromogen A and Chromogen B solutions, 50 µL each were added to each well, and the plate was incubated at 37 ºC for 15 minutes. After incubation, 50 µL of the stop solution was added to each well. Absorbance was then read at 450_{nm} and the optical densities noted.

3.9 Assessment of the Expression Profile of the IL-6 and TNF-α Genes in BALB/c Mice Vaccinated with HBV Vaccine and HBsAg with and without *Glycine Max (L.) Merr*. Saponin Extract

3.9.1 RNA Extraction

The total RNA from the murine splenocytes was extracted using the Direct-zol™ RNA Miniprep (Zymo Research, CA USA) according to the manufacturer's instructions. Briefly, 1 µL of proteinase K was added to 100 µL of the sample and incubated at room temperature for 15 minutes. Then, 300 µL of the TRI Reagent was added to the mixture and mixed thoroughly. To the lysed sample, 400 µL of

molecular grade ethanol (Merck, Darmstadt, Germany) was added and mixed thoroughly. The mixture was then transferred into a Zymo-spin column in a collection tube and centrifuged at 16000 x g for 1 minute. The column was transferred to a new collection tube. To the column, 400 µL of RNA wash buffer was added and centrifuged at 16000 x g for 1 minute. A mixture of 5 µl DNases and 75 µL DNA digestion buffer was added to the column matrix and incubated at room temperature for 15 minutes. This was followed by the addition 400 µL of Direct-zol RNA pre-wash to the column and centrifuging at 16000 x g for 1 minute. This step was carried out twice. RNA wash buffer, 700 µL, was added to the column and centrifuged at 16000 x g for 1 minute. This step was also carried out twice to ensure the complete removal of the wash buffer. The column was transferred into an RNase-free tube and 50 µL of DNase/RNase-free water was added directly to the column matrix for elution. The tube was centrifuged at 16000 x g for 1 minute. The RNA purity and concentration were assessed using the NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at absorbance 260/280. The samples were stored at -80 °C awaiting analysis.

3.9.2 cDNA Synthesis and Amplification

The RNA from 8 representative groups was pooled and the cDNA for each of the samples was synthesized and subsequently amplified in a single tube using the Accuris™ One Step RT-PCR Kit, with the components for the qPCR reaction set-up mixed as shown in Table 3.2.

Table 3.2: cDNA Synthesis and qPCR Reaction Components

Components	Volume (µL)
Accuris 2X One-Step Mix	10
Forward primer (10µM)	0.8
Reverse primer (10µM)	0.8
20X RTase Blend	1
Template RNA	3
PCR grade water	4.4
Total reaction volume	20

The reaction was performed using the Applied Biosystems Quant Studio 5 qPCR machine (PE Applied Biosystems, USA). The experiment used the thermocycling conditions as shown in Table 3.3.

Table 3.3: Quantitative Real-Time Thermal Profile

Step	Temperature (°C)	Time	Cycles
Reverse transcription	55	10 minutes	1
Pre-denaturation	95	2 minutes	1
Denaturation	95	5 seconds	40
Annealing	60	30 seconds	

Relative quantification analysis to determine the expression of IL-6 and TNF-α mRNA levels was performed with QuantStudio™ 5 Real-Time PCR system (Applied Biosystems) using cDNA synthesized from the RNA from the spleen. All levels of expression quantities were expressed in fold-changes, comparing the animal groups using the 2-ΔΔCt method, formula ΔCt=Ct (gene of interest) - Ct (housekeeping gene) (Livak & Schmittgen, 2001). The TATA Binding Protein (TBP) gene was used as the housekeeping for normalization of the genes of interest. The sequences of the primers used in the experiments are shown in Table 3.4.

Table 3.4: Sequences for the IL-6, TNF-α and TBP Primers

Primer	Sequence	Reference
IL-6	5′-GAGGATACCACTCCCAACAGACC-3'	(Atmaca, 2019)
	5′-AAGTGCATCATCGTTGTTCATACA-3′	
TNF-α	5’-GATCTCAAAAGACAACCAACATGTG-3’	(Sudo et al., 2008)
	5’-CTCCAGCTGGAAGACTCCTCCCAG-3'	
TBP	5’- GGTCGCGTCATTTTCTCC-3’	(Medrano et al., 2017)
	5’- GGGTTATCTTCACACACCATGA-3’	

3.10 Assessment of Hematological Profiles

Whole blood count analysis for six representative groups was performed using the HumaCount 30^{TS} (Human Diagnostics Worldwide, Wiesbaden, Germany) hematology analyzer machine following the manufacturer's instructions. Briefly, 100 µL of the anti-coagulated whole blood sample was aliquoted into sterile micro-centrifuge tube. Then, 25 µL of the blood was automatically aspirated by the machine for analysis. The profiles determined were white blood cells (WBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, red blood cells (RBC), hemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and Platelets.

3.11 Data Analysis

The optical densities from the ELISA analysis were expressed as the mean of experiments using Microsoft excel (2016). The *p-value* was calculated using Pearson correlation coefficient (r) for immune response values. Relative quantification of gene expression was calculated using the delta-delta threshold cycle (ΔΔCt) formula (Pfaffl, 2007). Cytokine expression levels were expressed in fold-changes, and a fold change <0.5 or >2 was considered as differentially expressed. Microsoft excel (2016) was used to create the bar charts to display the fold change in expression levels from the experimental groups. The data obtained for the hematological parameters were expressed as mean values using Microsoft excel (2016) and displayed in bar charts. A p-value of <0.05 was taken as significant for all statistical analyses.

3.12 Ethical Considerations

Approval to carry out this study and ethical clearance were obtained from the KEMRI Scientific Ethics Review Unit (SERU), protocol No. KEMRI/CBRD/SERU/ 005/500/4641. All animal work was carried out in accordance to relevant national and international standards as approved by KEMRI-Animal Care and Use Committee. The commercial HBV vaccine, Revac-B^{TM} (Bharat Biotech

International, India) was used in this study solely for research and not for modification or commercialization.

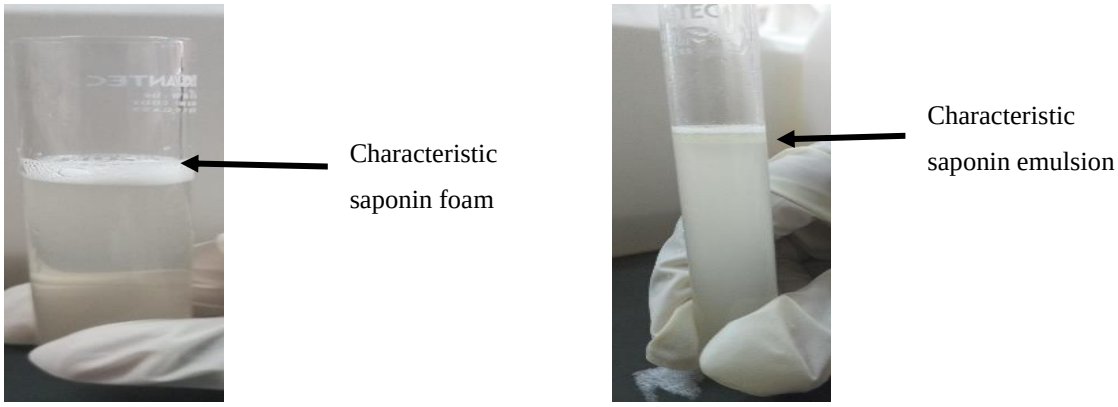
CHAPTER FOUR

RESULTS

4.1 Profiled Saponins Isolated from *Glycine Max (L.) Merr.*

4.1.1 Phytochemical Screening

On shaking the aqueous solution of the plant extract, frothing that is associated with saponins was observed (Figure 4.1(A) A). The addition of 3 drops of olive oil to the aqueous solution *Glycine max (*L.) *Merr*. extract and shaking resulted in the formation of characteristic saponin emulsion (Figure 4.1(A) B).



(A) (B)

Figure 4.1(A): The Formation of a Characteristic Saponin Foam

Figure 4.1(B): Emulsion Formation upon Shaking

4.1.2 Qualitative Analysis of Saponins – UV/Vis Spectra

The UV/Visible spectroscopy of the crude saponins showed the characteristic absorbance spectra of saponins, with a maximum absorption peak at 300nm, (Figure 4.2).

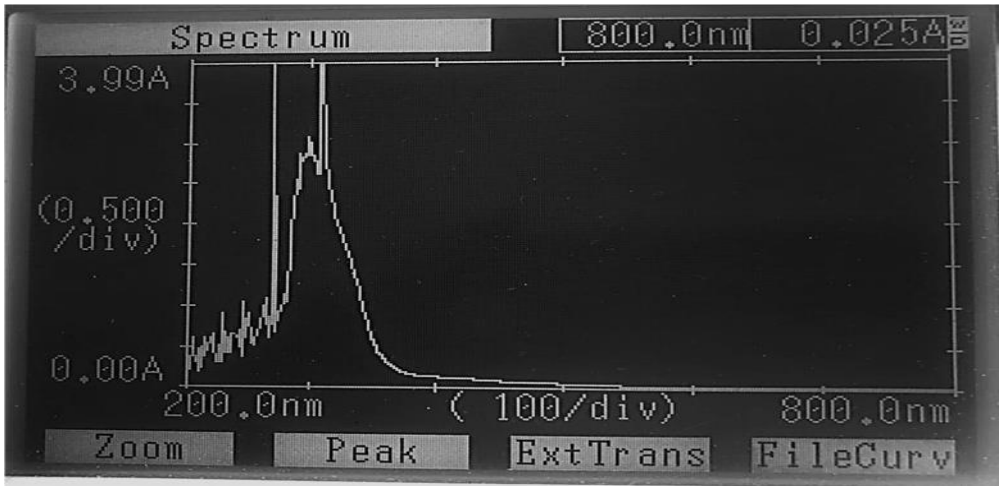


Figure 4.2: UV/Vis Spectra for *Glycine Max (L.) Merr*. Saponin Extract

4.1.3 Qualitative Analysis of Saponins – FTIR Spectra

The Fourier-Transform Infrared Spectroscopy (FTIR) of the crude saponins showed the characteristic infrared absorbance of saponins; the hydroxyl group (-OH) at 3382cm^{-1}, C-H bond at 2928cm^{-1}, C=C absorbance at 1635cm^{-1}, and C-O-C bond at 1055cm^{-1}, (Figure 4.3).

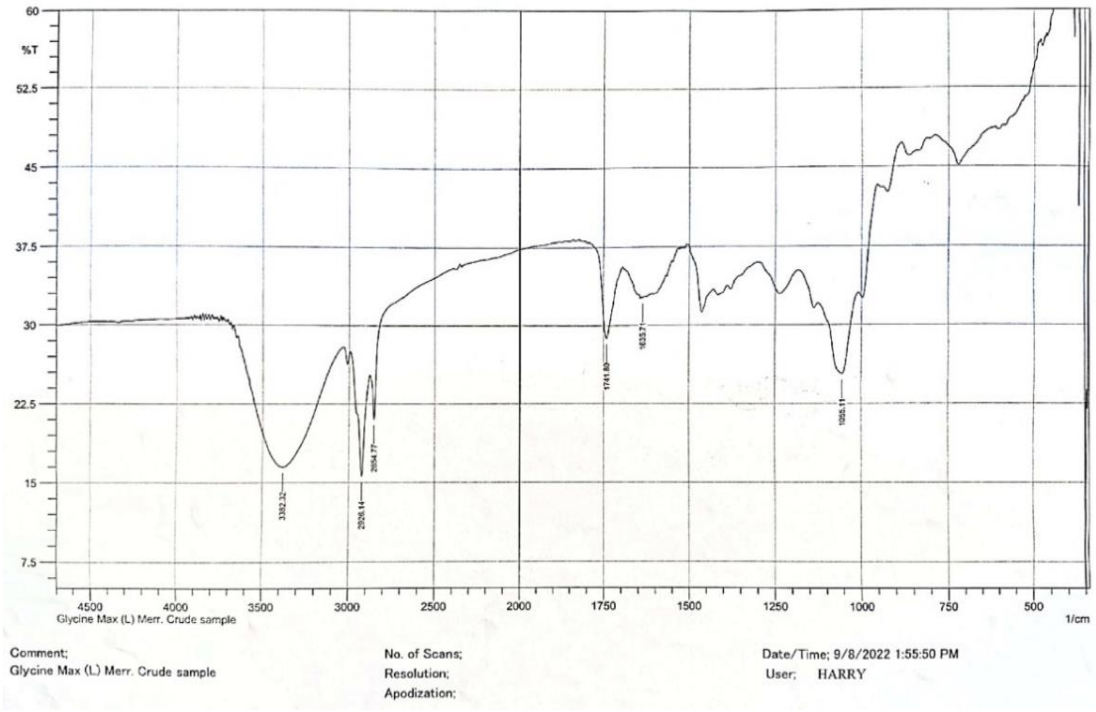


Figure 4.3: FTIR Spectra for *Glycine Max (L.) Merr.* Saponin Extract

4.2 Assessment of the Immune Response of BALB/c Mice Vaccinated with either Revac-B^{TM} Vaccine or HBsAg Supplemented with *Glycine Max (L.) Merr.* Saponin Extract

4.2.1 Vaccination of BALB/c Mice with either Revac-B^{TM} Vaccine or HBsAg Supplemented with *Glycine Max (L.) Merr.* Saponin Extract

When BALB/c mice were vaccinated with Hepatitis B vaccine, HBsAg, supplemented with *Glycine max (L.) Merr.* saponin extract, and PBS buffer (negative control), there was no reactogenicity at the injection sites.

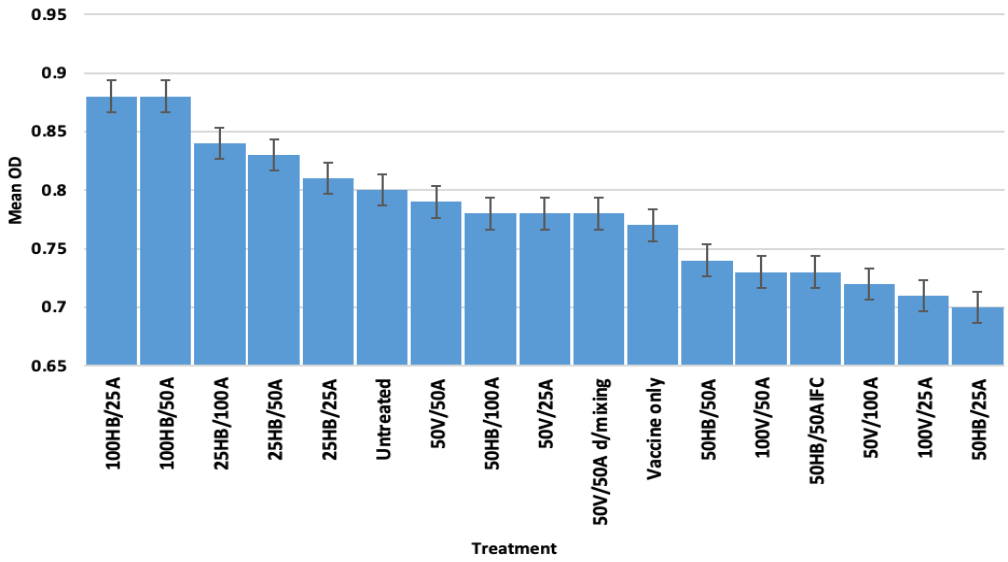
4.2.2 Humoral Response of BALB/c Mice Vaccinated with either Revac-B^{TM} Vaccine or HBsAg Supplemented with *Glycine Max (L.) Merr.* Saponin Extract

4.2.2.1 General Trend

The optical density readings from serum samples of BALB/c mice vaccinated with Revac B^{TM} vaccine, HBsAg supplemented with *Glycine max (L.) Merr*. saponin

extract were obtained. Since the Inhibition ELISA method was used, the higher the optical density that was observed, the lower the antibody titer.

The least inhibition or the lowest antibody titer (OD=0.88) was obtained with samples 100HB/50A and 100HB/25A while the highest inhibition (OD=0.70) was obtained from sample 50HB/25A. Vaccination with 50HB/25A produced the highest immune response followed by 100HB/25A (Figure 4.4).



Key: OD = Optical density, V = vaccine, A = adjuvant, HB = HBV antigen, and IFA=Incomplete Freund's adjuvant

Figure 4.4: General Trend of the Immune Response of BALB/c Mice 14 Days Post-Vaccination.

4.2.2.2 Comparison of the Immune Response between BALB/c Mice Treated with either Revac B^{TM} Vaccine and HBsAg

Generally BALB/c mice treated with HBsAg showed higher immune response (Mean OD = 1.263) than those treated with commercial vaccine, (V) (mean OD = 1.338) (Figure 4.5).

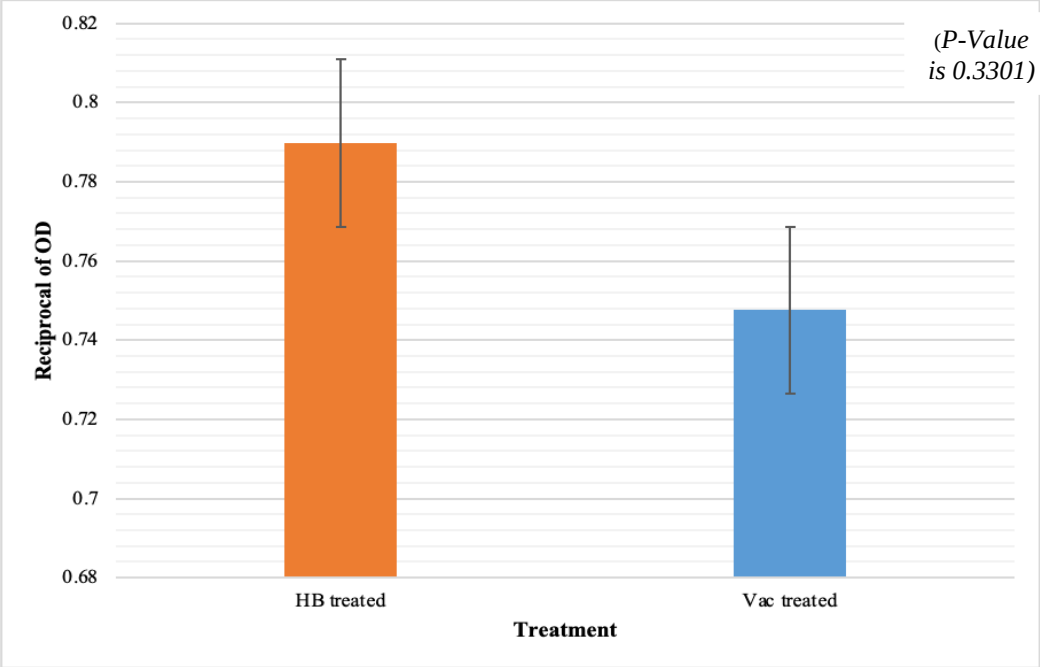


Figure 4.5: Comparison of Response of HBsAg Treated and Revac B^{TM} Vaccine (Comm Vac) Treated Mice

Lowest immune response (OD= 0.88) was observed in mixtures containing 100% HBsAg with 25% and 50% saponin extract. In comparison to the 50% concentration of the Revac B^{TM} vaccine, 50% HBsAg had higher inhibition (OD= 0.74 and 0.70) when in combination with 50% and 25% saponin extract respectively. Supplementing the 50% Revac B^{TM} with 100% saponin extract resulted in a higher antibody titer than 50% HBsAg supplemented with 100% extract (Figure 4.6).

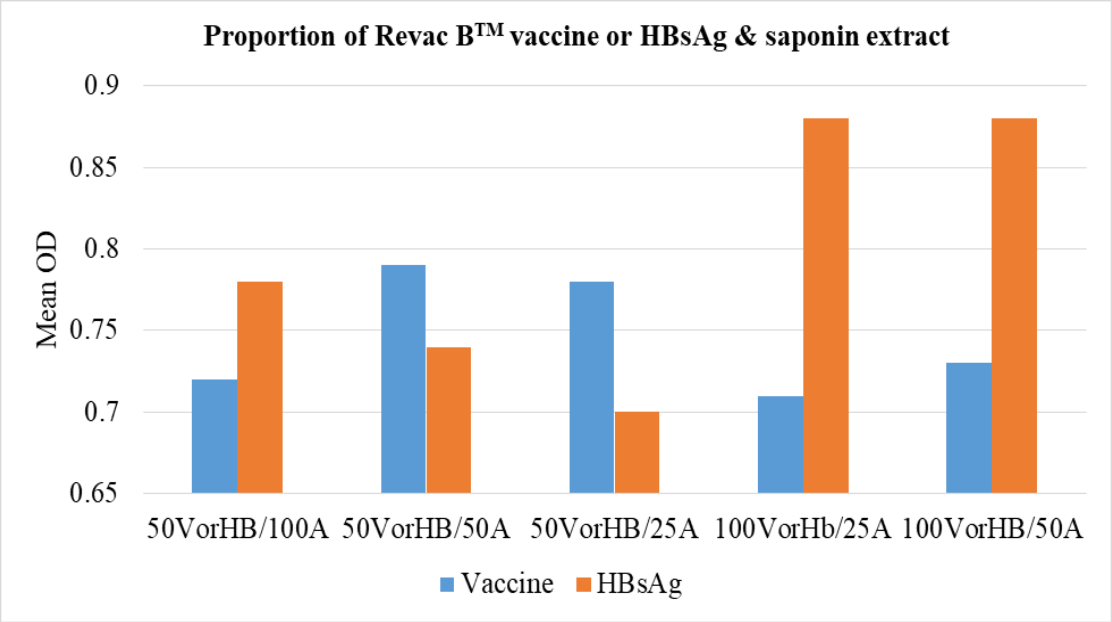


Figure 4.6: Comparison of Immune Response to the Similar Revac B™ Vaccine and HBsAg Concentrations with Different Saponin Extract Concentrations (100%, 50%, and 25%)

4.2.2.3 Comparison of the Immune Response among Different Saponin Extract Concentrations in BALB/c Mice Treated with Revac B™ Vaccine and HBsAg

No significant change was observed in the immune response with the use of 100%, 50%, and 25% concentrations of saponin extracts (two-tailed P value = 0.7507). When combined with HBsAg, the different saponin extract concentrations did not demonstrate significant difference in the immune response in the mice. However, 100% extract (100A) that was used with Revac B™ vaccine indicated some difference though not statistically significant (with p values of 0.7507 and 0.9431 in respect to 25A and 50A) (Figure 4.7). Unexpectedly mice treated with 50% HBsAg with various saponin extract concentrations produced significantly lower response than those treated with 25% and 100% HBsAg (two-tailed P = 0.0248 of 50% HBsAg in respect to 25% HBsAg treatment and P = 0.0184 in respect to 100% HBsAg treatment) respectively.

Statistical Comparison of 50% and 25% Saponin Concentrations

Unpaired t test results: P value and statistical significance: The two-tailed P value equals 0.0248

By conventional criteria, this difference is considered statistically significant. Confidence interval:

The mean of Group One minus Group Two equals 0.0867, 95% confidence interval of this difference: From 0.0180 to 0.1553. Intermediate values used in calculations: t = 3.5058, df = 4, standard error of difference = 0.025.

Statistical Comparison of 25% and 100% Saponin Concentrations

Unpaired t test results: P value and statistical significance: The two-tailed P value equals 0.0184

By conventional criteria, this difference is considered statistically significant. Confidence interval: The mean of Group One minus Group Two equals 0.0533, 95% confidence interval of this difference: From 0.0171 to 0.0896 Intermediate values used in calculations: t = 4.6843, df = 3, standard error of difference = 0.011

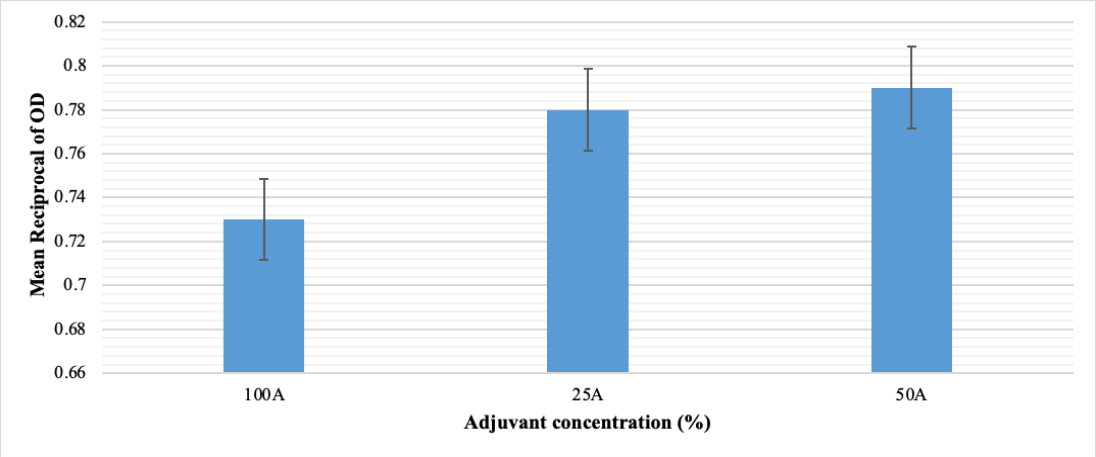


Figure 4.7: Comparison of Immune Response for Different Saponin Extract Concentrations (100%, 50% and 25%)

Effect of Mixing HBsAg and saponin Extract Prior to Mixing and Addition of Incomplete Freud's Adjuvant (IFA)

The vaccination of the mice with 50% HBsAg supplemented with 50% IFA resulted to a high immune response (OD= 0.73) in comparison to mixing 50% of either the Revac B^{TM} vaccine or HBsAg with 50% saponin extract (OD= 0.79 and 0.74 respectively) (Figure 4.8).

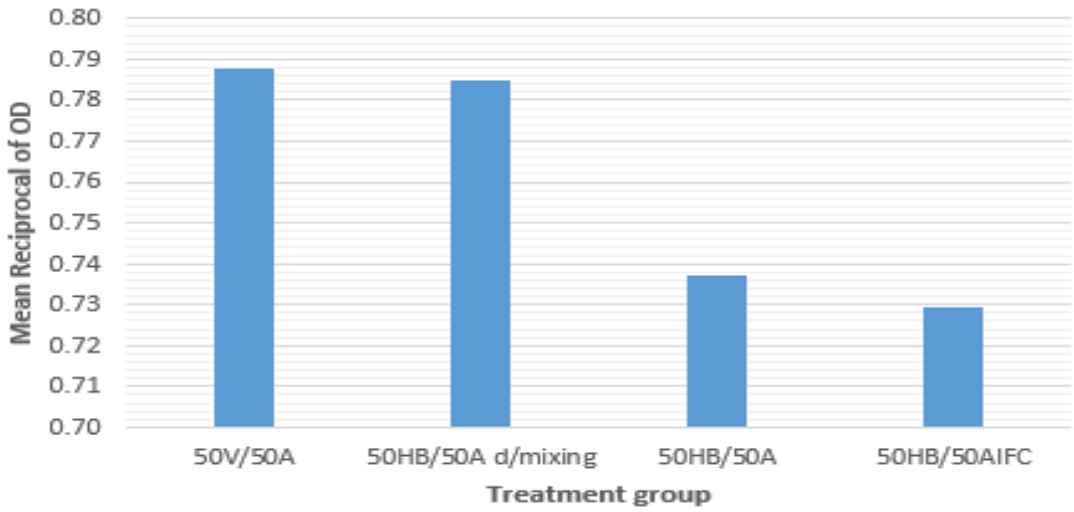


Figure 4.8: Comparison of Immune Response to Different Vaccination Procedures and Formulations: V; Vaccine, HB; Hepatitis B Surface Antigen, A; Saponin Extract, D/Mixing; Direct Mixing, IFC; Incomplete Freund's Adjuvant

4.2.3 Humoral Immune Response, 14 Days after Administration of Booster Shot

Indirect ELISA method was used to obtain the optical density readings from serum samples of BALB/c mice, 14 days after the administration of the booster shot that comprised of either the HBV vaccine or HBsAg supplemented with *Glycine max (L.) Merr.* saponin extract. From the readings, the mice vaccinated with 25Hb/100A had the highest immune response (OD=0.062), while the mice vaccinated with 100V/100A and 50V/25A had the least immune response (OD=0.051).

Generally, vaccination with 25HB/100A produced the highest response (OD= 0.062), while 100V/100A and 50V/25A produced the least response (OD=0.051) (Figure 4.9).

General Response Trend

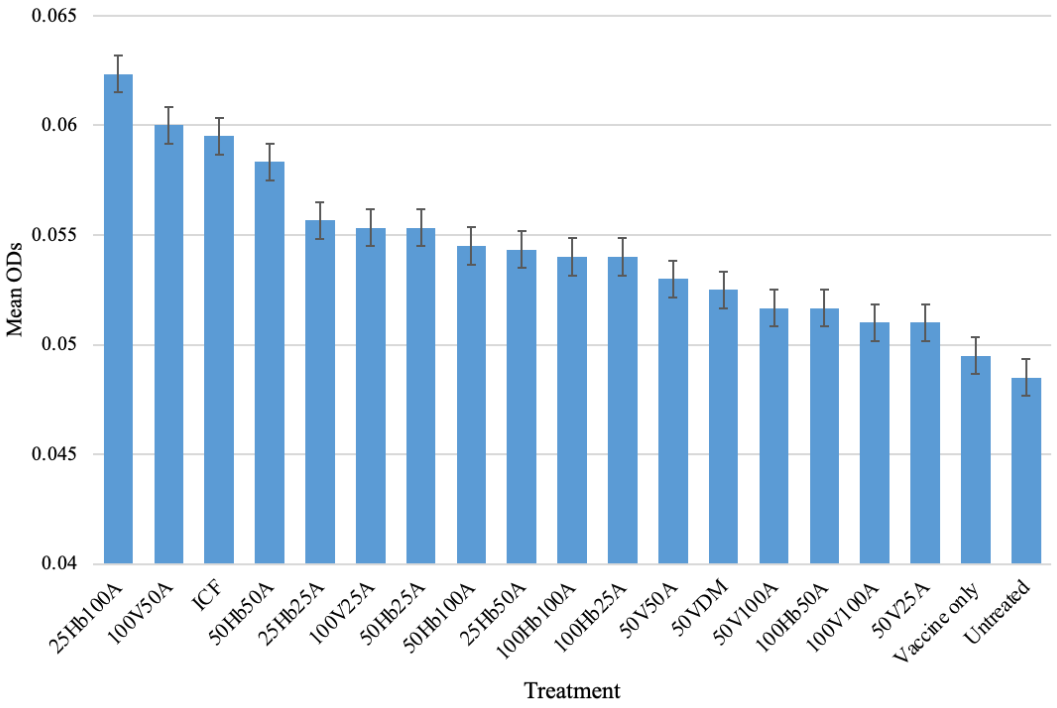


Figure 4.9: General Trend of the Immune Response of BALB/c Mice 14 days after Administration of the Booster Shot V; Vaccine, HB; Hepatitis B Surface Antigen, A; Saponin Extract, d/Mixing; Direct Mixing, IFC; Incomplete Freund's Adjuvant

4.2.3.1 Comparison of Immune Response among Animals Vaccinated with Revac B™ Vaccine and the Saponin Extract

In comparison to the other treatment groups, the animals treated with 100V50A had the highest response (OD=0.06), while 50V/25A (OD= 0.051) had the least immune response (Figure 4.10).

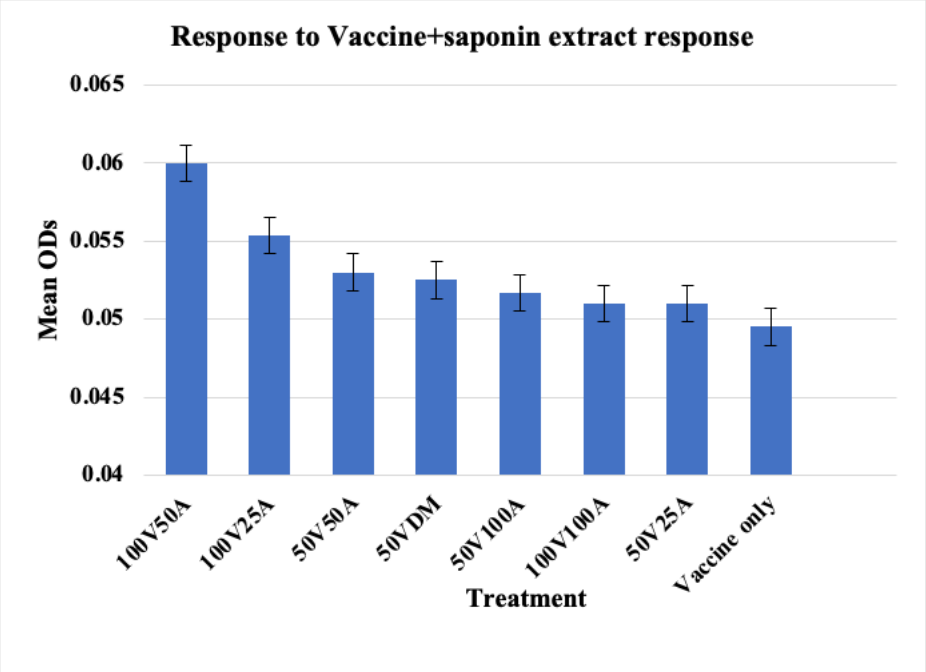


Figure 4.10: Comparison of Immune Response among Groups Treated with Revac B™ Vaccine Supplemented with *Glycine Max (L). Merr.* Saponin Extract

4.3 Expression of IL-6 and TNF-α Genes in BALB/c Mice Vaccinated with Revac-B™ Vaccine, HBsAg Supplemented with Saponins from *Glycine Max (L.) Merr.*

4.3.1 Comparison of the Cellular Immune Response, and Expression of IL-6, and TNF-α Genes

The fold change values for both IL-6 and TNF-α genes were tabulated and analyzed. The relationship between the expression of IL-6 gene and TNF-α gene was a moderately negative correlation ($R = -0.7444$ and $p=0.0343$, at $p<0.05$). Thus TNF-α expression demonstrated stronger relations with cellular immune response than IL-6 expression.

The (Table 4.3) illustrates the comparison in gene expression between the IL-6 gene and TNF- α genes.

Table 4.1: Gene Expression Levels of IL-6, and TNF-α Genes

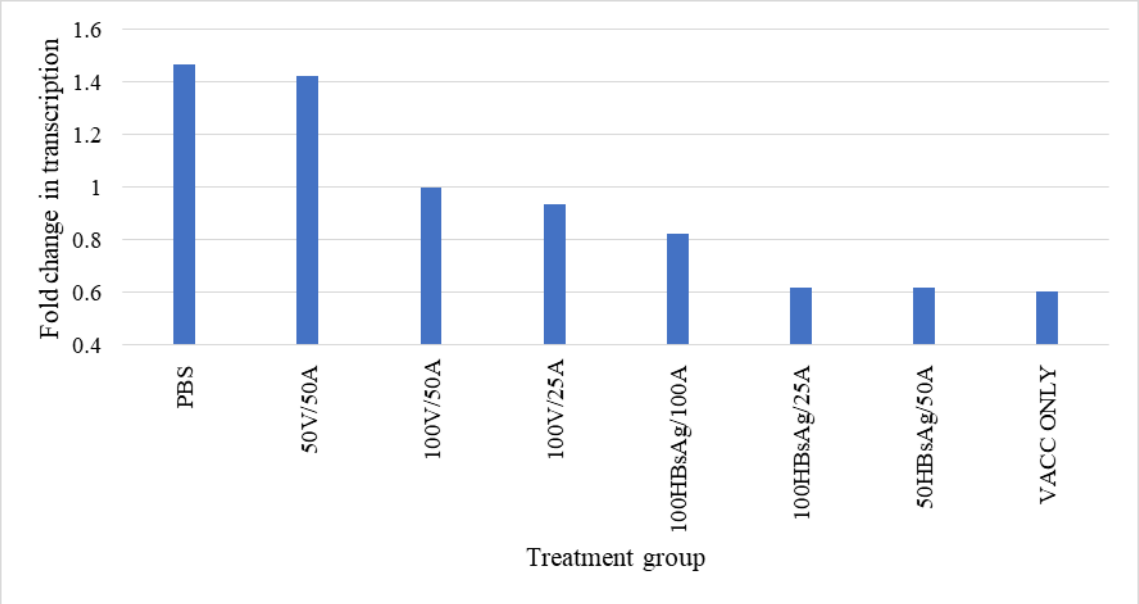
SAMPLE ID	IL-6 Fold change in transcription	TNF Fold change in transcription
Vaccine	0.603	28.836
100V/50A	1	2.112
100V/25A	0.935	4.436
50V/50A	1.423	0.370
100HBsAg/100A	0.822	5.904
100HBsAg/25A	0.618	9.654
50HBsAg/50A	0.618	16.227
PBS	1.469	1

V-Vaccine, HBsAg hepatitis B surface antigen, and A - *Glycine max (L.) Merr.* saponin extract

4.3.2 Expression of IL-6 Gene in BALB/c Mice Vaccinated with Revac-B™ Vaccine, HBsAg and *Glycine Max (L.) Merr.* Saponin Extract

From the assays performed with total RNA extracted from the spleen, the addition of the *Glycine max (L.) Merr.* saponin extract suppressed the expression of the IL-6 gene. The mice treated with Revac B^{TM} vaccine/extract (50%/50%) and those treated with Revac B^{TM} vaccine/extract (100%/50%) elicited slightly high levels of the IL-6 gene expression (Fold change (FC) = 1.42 and 1.0 respectively) compared to those treated with the Revac B^{TM} vaccine only (FC = 0.6) (Figure 4.11) With the *Glycine max (L.) Merr.* extract at 50% and increment of the Revac B^{TM} vaccine concentration from 50% (FC =1.42) to 100% (FC = 1) reduced the expression levels of this gene by 42%. The reduction of the *Glycine max (L.) Merr.* extract from 100% (FC =0.82) to 25% (FC =0.62) with the 100% concentration of the HBsAg suppressed the expression of the IL-6 gene by 20%. Increasing the concentrations of both the HBsAg and the *Glycine max (L.) Merr.* extract from 50% to 100% promoted the expression of the gene from FC= 0.62 to FC = 0.82, a 20% increment. Mice vaccinated with Revac-B™ vaccine only elicited expression of less quantities of IL-6

(FC = 0.6) in comparison to mice treated with PBS only (FC = 1.47). However, the results were not statistically significant (p = 0.620) (Figure 4.11).



Key: UNTRTD = Untreated, Vacc - Revac-B™ vaccine, and A - *Glycine max (L.) Merr* saponin extract

Figure 4.11: Relative Expression of IL-6 30-Days Post-Administration of Revac-B™ Vaccine, HBsAg Supplemented with *Glycine Max (L.) Merr.* Saponin Extract in BALB/c Mice.

The confirmatory test indicated that the vaccination of BALB/c mice with the Revac-B^{TM} vaccine suppressed the expression of the interleukin-6 cytokine gene. The mice treated with saline buffer had a higher expression level of the IL-6 gene, in comparison to the mice treated with the vaccine only (Figure 4.12).

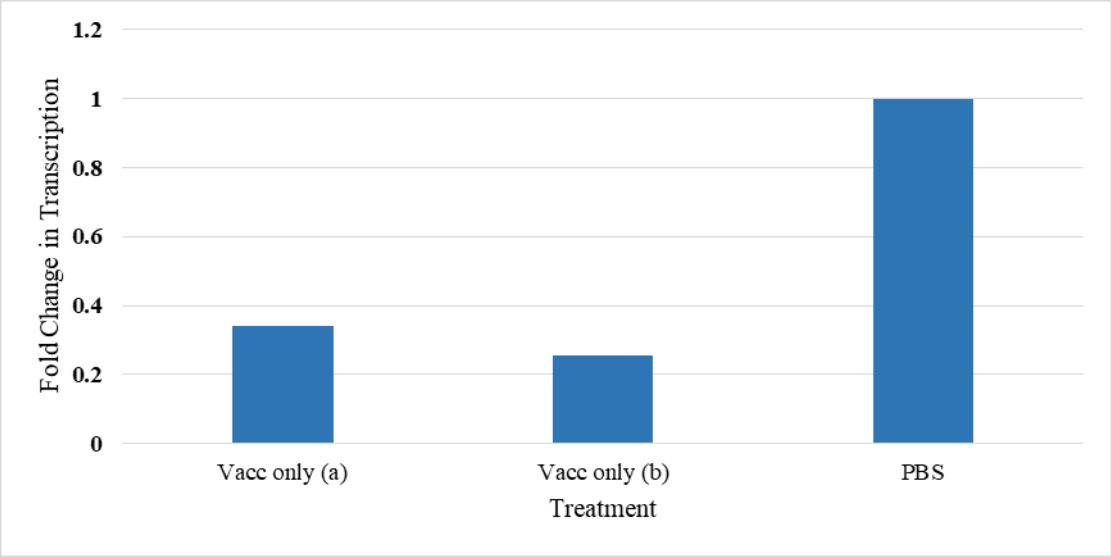


Figure 4.12: A confirmatory Test for the Relative Expression of IL-6 Gene

4.3.3 Expression of TNF-α Gene in BALB/c Mice Vaccinated with Revac-B™ Vaccine, HBsAg Supplemented with *Glycine Max (L.) Merr.* Saponin Extract

The group treated with the Revac-B^{TM} vaccine only showed the highest expression levels of the TNF-α gene than any other group (FC = 28.84). The animals treated with and HBsAg/extract at 50%/50% (FC =16.23) and 100%/25% (FC= 9.65), presented high expression levels of the TNF-α gene in comparison to the other treatment groups supplemented with the extract. The group treated with PBS only showed suppressed TNF-α gene levels (FC = 1) than the group treated with the commercial vaccine only. In both animal groups that were treated with HBsAg and vaccine the addition of the extract reduced the expression of TNF-α *(P = 0.063542)*. The highest suppression levels, (FC = 0.37) was observed in mice treated with the commercial vaccine and extract at 50%/50% concentrations (Figure 4.13).

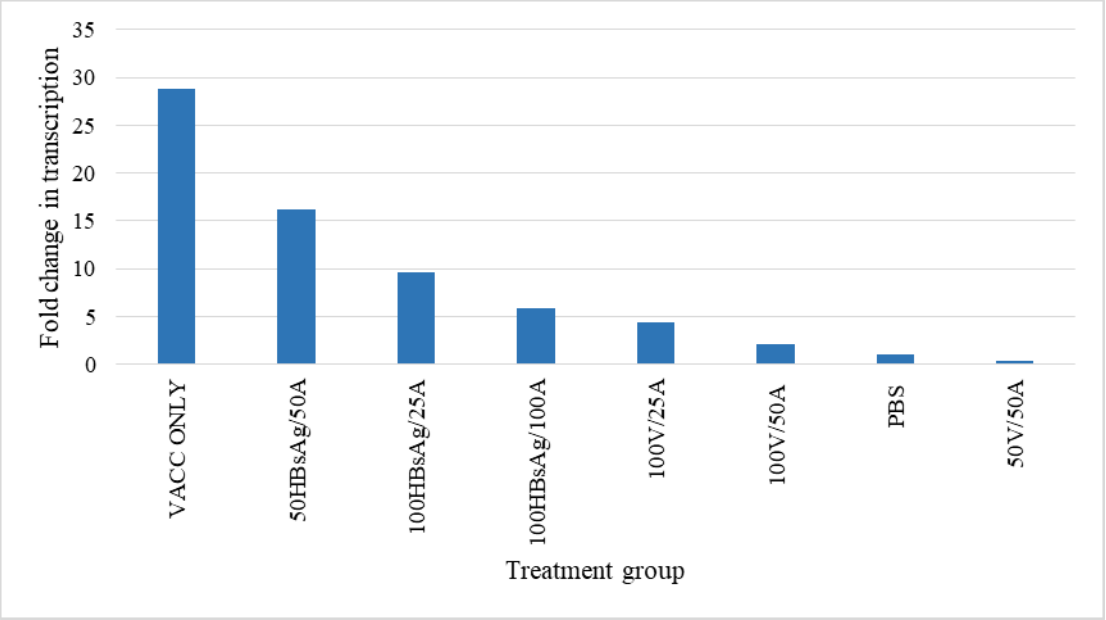


Figure 4.13: Relative Expression of TNF-α 30-Days Post-Vaccination

4.4 Effect of Administration of Revac-B™ Vaccine, HBsAg with *Glycine Max (L.) Merr.* Saponin Extract on Hematological Parameters in BALB/C Mice 30 Days Post-Administration

The administration of Revac-B™ vaccine, HBsAg and *Glycine max (L.) Merr.* saponin extract to BALB/c mice did not affect hematological parameters apart from white blood cells (WBC), neutrophil and platelet counts that were reduced by the extracts (Table 4.2).

Table 4.2: Results of Hematological Testing

Sample	Parameters					
	PBS	**Vacc only**	**Vacc/Adj DM**	**100Hb**	**50v/50A**	**100Hb/50a**
WBC *10*3/ul	3.6	4.8	7	3.15	1.05	0.95
Neutrophils %	12	16	13	4.5	4	5
Lymphocytes %	78.5	74	77	87	86	87.5
Monocytes %	8.5	9	9	7.5	9	6.5
Eosinophils %	1	1	1	1	1	1
Basophils %	0	0	0	0	0	0
RBC 10*6/ul	7.955	8.91	6.39	8.69	6.43	6.815
HB g/dl	14.15	15.2	13.6	14.65	11.8	12.55
HCT %	40.3	44.9	38.6	41.7	33.6	35.2
MCV fl	50.8	50.4	60.3	48.1	52.2	51.6
MCH Pg	17.8	17.1	21.2	16.95	18.55	18.4
MCHC g/dl	35.05	34	35.2	35.15	35.55	35.55
Platelets 10*3/ul	476	563	1030	1215	131.5	175.5

In comparison to the PBS-treated mice the WBC count increased by 33.3% in mice vaccinated with Revac-B™ vaccine alone and even increased (by 94.4%) in mice vaccinated with Revac-B™ vaccine that was mixed with *Glycine max (L.) Merr.* extract 50% immediately prior to injection. There was no significant change in WBC count in mice vaccinated with HBsAg. However, vaccination with Revac-B™ vaccine and HBsAg combined with *Glycine max (L.) Merr.* saponin extract 50% marked reduced WBC count by 72.2% (Figure 4.14) (*p-value = 0.333*).

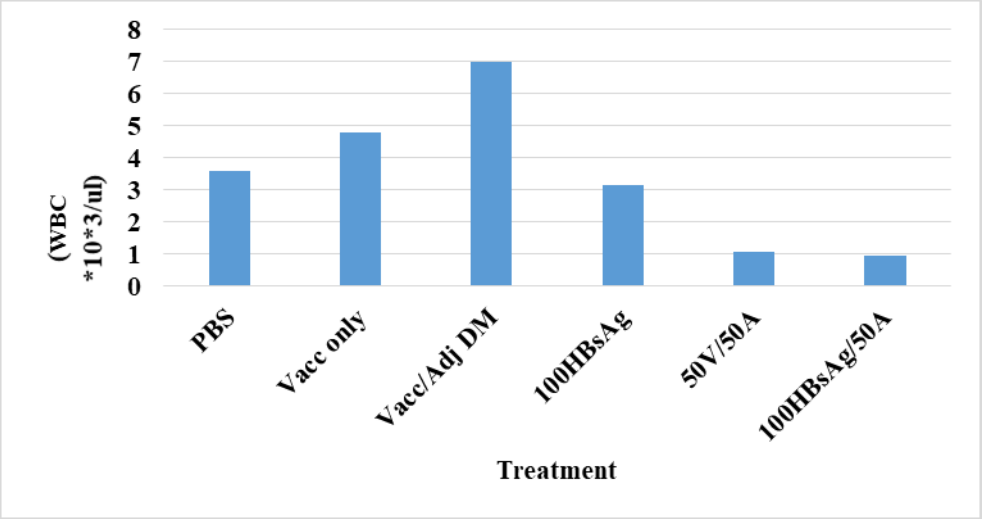
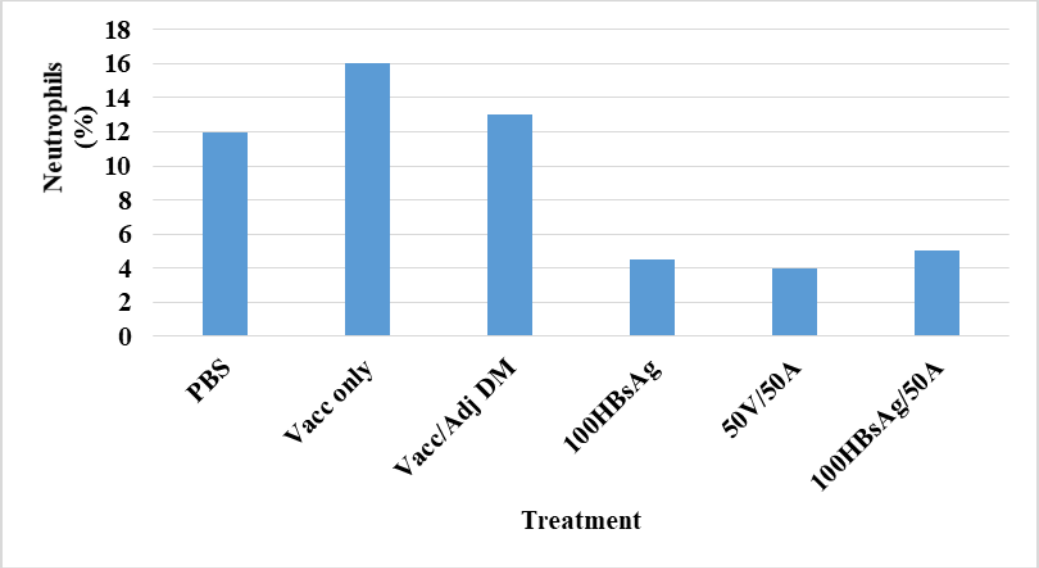


Figure 4.14: Variations of White Blood Cell (WBC) Count with 30-Days Post-Administration of Revac-B™ Vaccine, HBsAg and *Glycine Max (L.) Merr.* Saponin Extract in BALB/C Mice

The neutrophils count increased by 33.3% in mice vaccinated with Revac-B™ vaccine alone and increased by 11.00% in mice vaccinated with Revac-B™ vaccine that was mixed with *Glycine max (L.) Merr.* saponin extract 50% immediately prior to injection. There was no significant change in neutrophils count in mice vaccinated with HBsAg. However, vaccination with Revac-B™ vaccine and HBsAg combined with *Glycine max (L.) Merr.* saponin extract 50% marked reduced neutrophils count by 66.7% (Figure 4.15) *(p-value = 0.026, which is significant)*

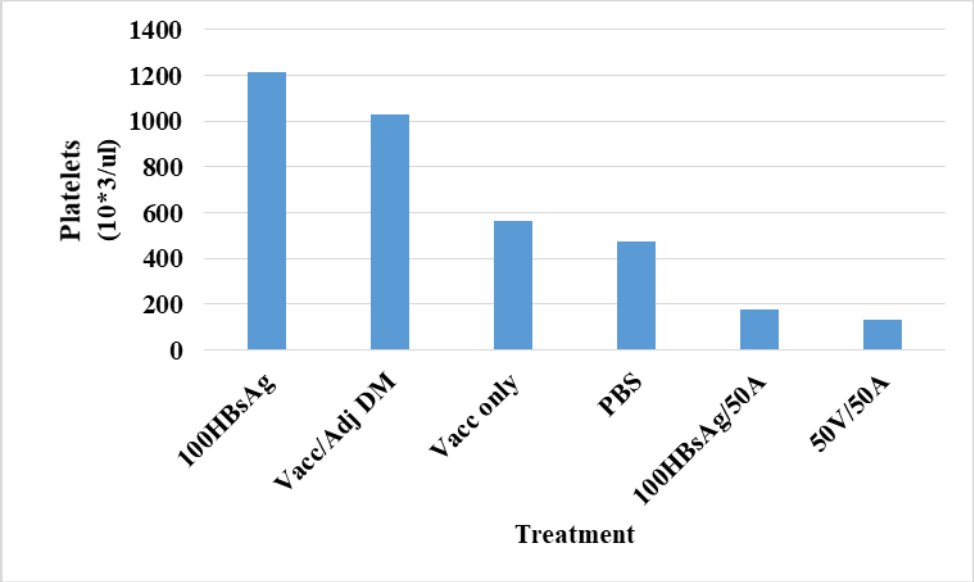
The Pearson's Correlation Coefficient between WBC and neutrophils counts was a moderate and significant correlation *(R = 0.724, P-Value = 0.027 at $p < 0.05$).*



Key: Vacc - Revac-B™ vaccine, Vacc/adj DM - Revac-B™ vaccine mixed with *Glycine max (L.) Merr.* saponin extract 50%, HB- HbsAg, and A - *Glycine max (L.) Merr.* saponin extract.

Figure 4.15: Variations of Neutrophil Count, 30-Days Post-Vaccination

The platelet count increased by 19.8% in mice vaccinated with Revac-B™ vaccine alone and increased by 216.0% in mice vaccinated with Revac-B™ vaccine that was mixed with *Glycine max (L.) Merr.* saponin extract 50% immediately prior to injection and 255.3% in mice vaccinated with HBsAg. However, vaccination with Revac-B™ vaccine and HBsAg combined with *Glycine max (L.) Merr.* saponin extract 50% marked reduced neutrophils count by 72.2% (Figure 4.16) (*p-value = 0.592, which is not significant).*



Key: WBC – White Blood Cells, Vacc - Revac-B™ vaccine, Vacc/adj DM - Revac-B™ vaccine mixed with *Glycine max (L.) Merr* saponin extract 50%, HB- HbsAg, and A - *Glycine max (L.) Merr* saponin extract.

Figure 4.16: Variations of Platelet Count, 30-Days Post-Vaccination

CHAPTER FIVE

DISCUSSION

5.1 Discussion

This study focused on the assessment of the efficacy of the *Glycine max (L.) Merr.* saponin extracts to enhance the humoral and cellular immune responses as adjuvants in BALB/c mice when vaccinated with either a commercial Hepatitis B vaccine (Revac-B^{TM}) or HBsAg.

5.1.1 Profiling of Saponins Extracted from *Glycine Max (L.) Merr.*

The testing of the aqueous solution of *Glycine max (L.) Merr.* saponin extract by shaking produced a characteristic frothing demonstrating the presence of saponin. The same evidence was obtained by using olive oil testing and the formation of an emulsion layer. The carbohydrate portion of the saponin molecule is water-soluble, while the sapogenin is fat soluble (Savage, 2003). When in contact with water, saponins lower the surface tension and are thus able to give a stable foam (Madland, 2013). The presence of both hydrophilic and hydrophobic groups in their molecular structure gives saponins their emulsifying properties (Dahlawi et al., 2020). The study also confirmed successful extraction of saponin extract by generation of characteristic UV/Vis spectra. These findings were in agreement with studies that had previously demonstrated the presence of saponin in *Glycine max (L). Merr.* (Sun et al., 2014) and tea (Yuan et al., 2018). UV is used for the detection of absorption peak of saponin compounds (L. W. Qi et al., 2006). The Fourier Transform Infrared Spectroscopy (FTIR) of the crude saponins also showed the characteristic infrared absorbance of saponins; the hydroxyl group (-OH) at 3382cm^{-1}, C-H bond at 2928cm^{-1}, C=C absorbance at 1635cm^{-1}, and C-O-C bond at 1055cm^{-1}, which is similar to studies carried out by (El-Keiy et al., 2019). This technique enables the identification and characterization of functional groups in the phytochemicals (Saxena & Saxena, 2012b)

5.1.2 Effects of administration of either Revac-B^{TM} Vaccine or HBsAg, both Supplemented with *Glycine Max (L.) Merr.* Saponin Extract on Humoral Immune Response in BALB/c Mice.

The results from this study indicate that saponins derived from *Glycine max (L). Merr*. did not have statistically significant adjuvant effect on the humoral immune responses when administered with either the Revac-B^{TM} vaccine or HBsAg. While mice vaccinated with either the vaccine or the HBsAg in combination with the extract had a higher immune response than those vaccinated with the Revac B^{TM} vaccine only, this difference was not statistically significant. Soybean extracts have been found to elicit substantial immune responses to ovalbumin in mice (Qiao et al., 2014). However, a study carried out by (Cossarini-Dunier, 1985) had conflicting results, where a saponin-based adjuvant did not have any significant effect on the humoral immune response of rainbow trout.

Probable reason why the saponin extracts in this study did not show substantial humoral immune response with the immunogens is that the extract was not innately effective with this vaccine, as adjuvants are not universal, and they tend to be specific with a vaccine or a group thereof (Singh & O'Hagan, 2003; Wilson-Welder et al., 2009). A Matrix M saponin-derived immunologic adjuvant (QS21) could not raise adequate immune response in HBV vaccine alone, but its adjuvant activity was markedly boosted upon the addition of 3-O-desacyl-4'-monophosphoryl lipid A (MPL) (Vandepapelière et al., 2008). Another plausible explanation is that the structural basis of the soysaponins affected the adjuvant activity of the saponin extract. (Kenji et al., 2000) suggests that the overall conformation of the functional groups affects the adjuvanticity of saponins. Additionally, the saponin extract was simultaneously used with an aluminum hydroxide-adjuvanted vaccine, which may have negatively affected the efficacy. This observation was also made in a study carried out by (Ayele et al., 2023) that reported significantly lower antibody levels in animals injected with a vaccine combined with aluminum hydroxide and a saponin.

5.1.3 Effects of Administration of either Revac-B™ Vaccine or HBsAg, both Supplemented with *Glycine Max (L.) Merr.* Saponin Extract on Cellular Immune Response in BALB/c Mice

Saponin-based adjuvants induce protective cellular immunity, hence are being used in animal and human vaccines (Den Brok et al., 2016). TNF-α plays two crucial roles in HBV infection, inducing liver inflammation and acting as a mediator of anti-HBV immunity (Zhong et al., 2021). To further explore the immune response elicited by this saponin extract as an adjuvant in mice, this study sought to determine the expression of the IL-6 and TNF-α cytokine genes.

Vaccination of mice with Revac-B™ vaccine, HBsAg, and various formulations of *Glycine max (L.) Merr*. saponin extract depressed the expression of IL-6 as untreated mice demonstrated the highest level of the cytokine. Mice vaccinated with 50V/50A did not demonstrate any marked depression of expression of the IL-6 gene in comparison to the untreated mice. Generally, vaccination of mice with Revac-B™ vaccine and different formulations of *Glycine max (L.) Merr*. saponin extracts oppressed expression of IL-6 gene less than mice vaccinated with HBsAg and various concentrations of the extract. The *Glycine max (L.) Merr*. saponin extracts appeared to prevent depression of IL-6 expression as the higher the ratio of the extract the lower the depression of IL-expression.

The decreased expression of IL-6 noted in this study was contrary to several studies (Cox et al., 2011; Valdez et al., 1999) that have found an increase of IL-6 expression levels upon vaccination. Increased IL-6 levels were associated with systemic reactogenicity following vaccination with HBsAg $AS01_B$/Alum vaccine in humans (Burny et al., 2019). The reduction of the expression of IL-6 after administration of vaccine was unexpected as it was supposed to increase rather than reduce. The confirmatory test carried out supported these results, where the animals treated with the Revac-B™ vaccine had suppressed expression of the IL-6 gene, in comparison to the PBS-administered mice. The increase was expected as IL-6 is known to have major effects on the adaptive and innate immune system promoting the development of pathogenic T-helper 17 T cells and the maturation of B lymphocytes (Choy &

Rose-John, 2017). This effect should be explored further as the IL- 6 inhibitors have been known to offer benefits to COVID-19 patients who are exposed to cytokine storm. IL-6 inhibitors include anti-IL-6 receptor monoclonal antibodies (mAbs) (e.g., sarilumab, tocilizumab) and anti-IL-6 mAbs (i.e., siltuximab) (Du et al., 2021).

Vaccination of mice with Revac-B™ vaccine alone demonstrated the highest level of expression of TNF-α compared to the untreated mice. The addition of *Glycine max (L.) Merr*. saponin extract appeared to reduce the expression of the same. The suppression of expression of TNF-α by various concentrations of *Glycine max (L.) Merr*. saponin extract was more in Revac-B™ vaccine/extract group than with the HBsAg treated group. The increase in the expression levels of TNF-α was as expected after vaccine administration as it is produced by the effector CD4 and CD8 T cells or innate cells hence a good marker of stimulation of the cell-mediated immunity (Jang et al., 2021).

According to Murphy *et al.* (Murphy et al., 2023), the cytokine profile induced by trained immunity differs depending on the microbial stimulus. For example, Bacillus Calmette-Guerin (BCG) vaccination increased TNF-α production while *Mycobacteria tuberculosis* stimulation decreased TNF-α production (D'Agostino et al., 2020; Kleinnijenhuis et al., 2012). Other studies have not observed changes with the expression of IL-6 and TNF-α (Xu et al., 2005). Increased expression of TNF-α could be exploited to develop a mucosal adjuvant consisting of a HBV vaccine leveraging on the observation by Kayamuro *et al.* (Kayamuro et al., 2009) that a mutant TNF-α has substantial mucosal adjuvant activity.

5.1.4 Effects of Revac-B™ vaccine or HBsAg supplemented with *Glycine max (L.) Merr.* saponin extract on the hematological profile of BALB/c mice

Hematological parameters are used in diagnosing infections, as indicators in diagnosis of organ or tissue injuries and other pathologies. RBC, MCV, MCH, and MCHC parameters enable the analysis of the correlation between the size of erythrocytes and the hemoglobin concentration in its interior (Silva-Santana et al., 2020). According to the findings of this study, the administration of Revac-B™ vaccine or HBsAg combined with *Glycine max (L.) Merr* saponin extract to BALB/c

did not affect most of the hematological parameters apart from WBC, neutrophil, and platelet counts. The neutrophils and platelet counts increased in mice vaccinated with Revac-B™ vaccine alone and in mice vaccinated with Revac-B™ vaccine that was mixed with *Glycine max (L.) Merr.* saponin extract 50%. However, vaccination with Revac-B™ vaccine and HBsAg combined with *Glycine max (L.) Merr.* saponin extract 50% reduced neutrophils count by 66.7% and platelets by 72.2%. Therefore, it can be concluded that the Revac B^{TM} vaccine stimulates production of neutrophils while *Glycine max (L.) Merr.* saponin extract suppress their production. In human subjects' neutropenia is associated with vaccination with various vaccines (Muturi-Kioi et al., 2016). However, this study recorded neutrophilia (high neutrophil counts) in vaccine-alone vaccinated mice. Possibly the situation could change in human subjects. This finding is contrary to the one of Lin *et al.* (Lin et al., 2021) who showed the increase in neutrophils and decrease in lymphocytes in female rats after treatment with quinoa saponins. Cases of vaccine-associated changes in platelet profiles are known such as COVID-19 mRNA induction of immunologic thrombocytopenia (low platelets count) that often causes spontaneous bleeding (Cines & Bussel, 2021). Thrombocytosis (increased levels of platelet counts) have been reported with some vaccines such as ChAdOx1 nCoV-19 adenoviral vector vaccine where it was found to cause venous thrombosis (Schultz et al., 2021). In this study the neutropenia and thrombocytopenia could be associated mainly to saponins from *Glycine max (L.) Merr*, and the literature search has not demonstrated any other study on these effects. Other studies have established that saponins, such as saponins from quinoa (Lin et al., 2021), *Amaranthus cruentus seeds* (Oleszek et al., 1999), and *Momordica dioica* (Jha et al., 2019), have limited acute toxicity effects. Injection of BALB/c mice with HBV vaccine (positive control), HBV, and PBS buffer (negative control) together with *Glycine max (L.) Merr.* saponin extract did not produce any injection or reactogenic reaction at the injection sites. These results concur with findings from a study carried out by (Sun et al., 2014) that evaluated various concentrations of soysaponins and observed no toxicities.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study aimed to assess the adjuvant activity and safety of *Glycine max (L) Merr.* saponin extract vaccinated together with either a hepatitis B virus vaccine or HBsAg in BALB/c mice. Following data collection and analysis, the following conclusions were made:

a) The study demonstrated that *Glycine max (L) Merr.* contains saponins with characteristic profile, using the Fourier Transform Infrared analysis, UV/Vis spectrophotometry, and the standard foam test methods.
b) Saponin extracts from the *Glycine max (L) Merr.* have an adjuvant effect on the humoral and cellular responses of the BALB/c mice when vaccinated with either the Revac B^{TM} vaccine or the HbsAg, however this effect is not statistically significant.
c) The addition of *Glycine max (L) Merr.* saponin extract suppressed the expression of TNF-α gene, while it promoted the expression of the IL-6 gene.
d) The supplementation of the various vaccine formulations with the *Glycine max (L) Merr.* saponin extract did not induce any effect on the hematological profile in the BALB/c mice.

6.2 Recommendations

From the observations of this study, the following recommendations may be made;

a) This study showed *Glycine max (L) Merr.* contains significant amounts of saponins. This study recommends the use of *Glycine max (L) Merr.* as potential source of saponin-based adjuvants.
b) Having demonstrated the efficacy of saponins from *Glycine max (L) Merr.* to enhance the humoral immune response in BALB/c mice, this study recommends investigation of the potential of *Glycine max (L) Merr.* saponins as adjuvants in vaccine formulations.

c) The findings from this study call for detailed investigations on the non-specific effects of hepatitis B vaccine on the expression levels of the IL-6 gene and other cytokines.
d) This study recommends the use of *Glycine max (L) Merr*. saponins as vaccine adjuvants, having demonstrated their safety.

6.3 Study Limitations

a) This study had limited funding hence the analysis of all the samples for gene expression and hematological profile was a challenge.

REFERENCES

Agmon-Levin, N., Paz, Z., Israeli, E., & Shoenfeld, Y. (2009). Vaccines and autoimmunity. *Nature Reviews Rheumatology*, *5*(11), 648-652.

Arifin, W. N., & Zahiruddin, W. M. (2017). Sample size calculation in animal studies using resource equation approach. *Malaysian Journal of Medical Sciences, 24*(5), 101–105. https://doi.org/10.21315/mjms 2017.24.5.11

Atmaca, H. T. (2019). Expression of serotonin 2A, 2C, 6 and 7 receptor and IL-6 mRNA in experimental toxoplasmic encephalitis in mice. *Heliyon*, *5*(11). https://doi.org/10.1016/J.HELIYON.2019.E02890

Ayele, G., Getachew, B., Bari, F. D., Bayissa, B., Muluneh, A., Abayneh, T., Gelaye, E., & Edao, B. M. (2023). Combined Adjuvant Formulations Enhanced an Immune Response of Trivalent Foot and Mouth Disease Vaccine in Cattle. *Veterinary Medicine: Research and Reports*, 47-62.

Bauer, T., Sprinzl, M., & Protzer, U. (2011). Immune control of hepatitis B virus. *Digestive Diseases (Basel, Switzerland)*, *29*(4), 423–433. https://doi.org/10.1159/000329809

Baumert, T. F., Thimme, R., & von Weizsäcker, F. (2007). Pathogenesis of hepatitis B virus infection. *World Journal of Gastroenterology*, *13*(1), 82–90. https://doi.org/10.3748/wjg.v13.i1.82

Block, T. M., Guo, H., & Guo, J. T. (2007). Molecular virology of hepatitis B virus for clinicians. *Clinics in Liver Disease*, *11*(4), 685–706. https://doi.org/10.1016/J.CLD.2007.08.002

Bouezzedine, F., Fardel, O., & Gripon, P. (2015). Interleukin 6 inhibits HBV entry through NTCP down regulation. *Virology*. *481*, 34-42. https://doi.org/10.1016/j.virol.2015.02.026

Burny, W., Marchant, A., Hervé, C., Callegaro, A., Caubet, M., Fissette, L., Gheyle, L., … & Yarzabal, J. (2019). Inflammatory parameters associated with systemic reactogenicity following vaccination with adjuvanted hepatitis B vaccines in humans. *Vaccine*, *37*(14), 2004–2015. https://doi.org/10.1016/J.VACCINE.2019.02.015

Choy, E., & Rose-John, S. (2017). *Interleukin-6 as a multifunctional regulator: inflammation, immune response, and fibrosis*. *Journal of Scleroderma and Related Disorders*, *2*(2_suppl), S1-S5.

Cines, D. B., & Bussel, J. B. (2021). SARS-CoV-2 Vaccine–Induced Immune Thrombotic Thrombocytopenia. *The New England Journal of Medicine*, *384*(23), 2254–2256. https://doi.org/10.1056/NEJME2106315

Clem, A. S. (2011). Fundamentals of vaccine immunology. *Journal of Global Infectious Diseases*, *3*(1), 73–78. https://doi.org/10.4103/0974-777X.77299

Cohen, J., & Marshall, E. (2001). Vaccines for biodefense: A system in distress. In *Science*. 498-501. https://doi.org/10.1126/science.294.5542.498

Coppola, N., Corvino, A. R., De Pascalis, S., Signoriello, G., Di Fiore, E., Nienhaus, A., Sagnelli, E., & Lamberti, M. (2015). The long-term immunogenicity of recombinant hepatitis B virus (HBV) vaccine: contribution of universal HBV vaccination in Italy. *BMC Infectious Diseases*, *15*(1). https://doi.org/10.1186/S12879-015-0874-3

Cossarini-Dunier, M. (1985). Effect of different adjuvants on the humoral immune response of rainbow trout. *Developmental & Comparative Immunology*, *9*(1), 141–146. https://doi.org/10.1016/0145-305X(85)90067-9

Cox, S. J., Gubbins, S., & Barnett, P. V. (2011). IL-6 production following vaccination in pigs--an additional immune response parameter for assessing FMD vaccine efficacy? *Vaccine*, *29*(29–30), 4704–4708. https://doi.org/10.1016/J.VACCINE.2011.04.100

D'Agostino, M. R., Lai, R., Afkhami, S., Khera, A., Yao, Y., Vaseghi-Shanjani, M., Zganiacz, A., Jeyanathan, M., & Xing, Z. (2020). Airway Macrophages Mediate Mucosal Vaccine-Induced Trained Innate Immunity against Mycobacterium tuberculosis in Early Stages of Infection. *Journal of Immunology (Baltimore, Md. : 1950)*, *205*(10), 2750–2762. https://doi.org/10.4049/JIMMUNOL.2000532

Dahlawi, S. M., Nazir, W., Iqbal, R., Asghar, W., & Khalid, N. (2020). Formulation and characterization of oil-in-water nanoemulsions stabilized by crude saponins isolated from onion skin waste. *RSC Advances*, *10*(65), 39700–39707. https://doi.org/10.1039/d0ra07756a

Damme P., Ward J., Shouval., et al. (2018). Hepatitis B Vaccines. In *Plotkin's Vaccines*, 342–374.

Den Brok, M. H., Büll, C., Wassink, M., De Graaf, A. M., Wagenaars, J. A., Minderman, M., … & Adema, G. J. (2016). Saponin-based adjuvants induce cross-presentation in dendritic cells by intracellular lipid body formation. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms13324

Di Lello, F. A., Martínez, A. P., & Flichman, D. M. (2022a). Insights into induction of the immune response by the hepatitis B vaccine. *World Journal of Gastroenterology*, *28*(31), 4249–4262.

Di Lello, F. A., Martínez, A. P., & Flichman, D. M. (2022b). Insights into induction of the immune response by the hepatitis B vaccine. In *World Journal of Gastroenterology*, 28(31), 4249–4262.

Dixit, Ajay. K., Antony, J. I. X., Sharma, Navin. K., & Tiwari, Rakesh. K. (2011). Soybean constituents and their functional benefits. *Research Signpost*, *661*(2), 367–383.

Du, P., Geng, J., Wang, F., Chen, X., Huang, Z., & Wang, Y. (2021). Role of IL-6 inhibitor in treatment of COVID-19-related cytokine release syndrome.

International Journal of Medical Sciences, *2021*(6), 1356–1362. https://doi.org/10.7150/ijms.53564

Edmunds, W. J., Medley, G. F., Nokes, D. J., Hall, A. J., & Whittle, H. C. (1993). The influence of age on the development of the hepatitis B carrier state. *Proceedings. Biological Sciences*, *253*(1337), 197–201. https://doi.org/10.1098/RSPB.1993.0102

El-Keiy, M. M., Radwan, A. M., & Mohamed, T. M. (2019). Cytotoxic Effect of Soy Bean Saponin against Colon Cancer. *Journal of Biosciences and Medicines*, *07*(07), 70–86. https://doi.org/10.4236/jbm.2019.77006

Ferrari, C., Penna, A., Bertoletti, A., Valli, A., Antoni, A. D., Giuberti, T., Cavalli, A., Petit, M. A., & Fiaccadori, F. (1990). Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. *Journal of Immunology (Baltimore, Md. : 1950)*.

Guclu-Ustundag, Ö. & Mazza, G. (2007). Saponins: Properties, applications and processing. *Critical Reviews in Food Science and Nutrition*, *47*(3), 231–258. https://doi.org/10.1080/10408390600698197

Guidotti, L. G., & Chisari, F. V. (2006). Immunobiology and pathogenesis of viral hepatitis. In *Annual Review of Pathology*. *1*(1), 23-61.

Guimarães, L. E., Baker, B., Perricone, C., & Shoenfeld, Y. (2015). Vaccines, adjuvants and autoimmunity. In *Pharmacological Research*. *100*, 190-209.

Guy, B. (2007). The perfect mix: recent progress in adjuvant research. *Nature Reviews Microbiology*, *5*(7), 396–397. https://doi.org/10.1038/nrmicro1681

Halperin, S. A., Ward, B., Cooper, C., Predy, G., Diaz-Mitoma, F., Dionne, M., Embree, J., … & Martin, J. T. (2012). Comparison of safety and immunogenicity of two doses of investigational hepatitis B virus

surface antigen co-administered with an immunostimulatory phosphorothioate oligodeoxyribonucleotide and three doses of a licensed hepatitis B vaccine in healthy adul. *Vaccine*. *30*(15), 2556-2563.

He, D., Li, M., Guo, S., Zhu, P., Tan, Z., & Wang, Y. (2013). *Expression Pattern of Serum Cytokines in Hepatitis B Virus Infected Patients with Persistently Normal Alanine Aminotransferase Levels*. 1240–1249. https://doi.org/10.1007/s10875-013-9931-0

Heyward, W. L., Kyle, M., Blumenau, J., Davis, M., Reisinger, K., Kabongo, M. L., Bennett, S., Janssen, R. S., Namini, H., & Martin, J. T. (2013). Immunogenicity and safety of an investigational hepatitis B vaccine with a Toll-like receptor 9 agonist adjuvant (HBsAg-1018) compared to a licensed hepatitis B vaccine in healthy adults 40-70 years of age. *Vaccine*. *30*(15), 2556-2563.

Honorati, M. C., & Facchini, A. (1998). Immune response against HBsAg vaccine. *World Journal of Gastroenterology*, *4*(1–6), 464–466. https://doi.org/10.3748/wjg.v4.i6.464

Hoofnagle, J. H., Dusheiko, G. M., Seeff, L. B., Jones, E. A., Waggoner, J. G., & Bales, Z. B. (1981). Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. *Annals of Internal Medicine*, *94*(6), 744–748. https://doi.org/10.7326/0003-4819-94-6-744

Hösel, M., Quasdorff, M., Wiegmann, K., Webb, D., Zedler, U., Broxtermann, M., Tedjokusumo, R., … Protzer, U. (2009). Not interferon, but interleukin-6 controls early gene expression in hepatitis B virus infection. *Hepatology*, *50*(6), 1773–1782. https://doi.org/10.1002/hep.23226

Hubrecht, R. C., & Carter, E. (2019). The 3Rs and Humane Experimental Technique: Implementing Change. *Animals : An Open Access Journal from MDPI*, *9*(10). https://doi.org/10.3390/ANI9100754

Jang, D. I., Lee, A. H., Shin, H. Y., Song, H. R., Park, J. H., Kang, T. B., Lee, S. R., & Yang, S. H. (2021). The Role of Tumor Necrosis Factor Alpha (TNF-α) in Autoimmune Disease and Current TNF-α Inhibitors in Therapeutics. *International Journal of Molecular Sciences*, *22*(5), 1–16. https://doi.org/10.3390/IJMS22052719

Jha, D. K., Koneri, R., & Samaddar, S. (2019). Toxicity studies of a saponin isolated from the fruits of Momordica dioica in rats. *International Journal of Pharmaceutical Sciences and Research*, 4462–4476.

Jie, Y. H., Cammisuli, S., & Baggiolini, M. (1984). Immunomodulatory effects of Panax Ginseng C.A. Meyer in the mouse. *Agents and Actions*, *15*(3–4), 386–391. https://doi.org/10.1007/BF01972376

Kane, M. A. (1996). Global status of hepatitis B immunisation. *Lancet*, *348*(9029), 696. https://doi.org/10.1016/S0140-6736(05)65598-5

Kapoor, R., & Kottilil, S. (2014). Strategies to eliminate HBV infection. In *Future Virology*. *9*(6), 565-585.

Kayamuro, H., Abe, Y., Yoshioka, Y., Katayama, K., Nomura, T., Yoshida, T., Yamashita, K., … & Tsutsumi, Y. (2009). The use of a mutant TNF-alpha as a vaccine adjuvant for the induction of mucosal immune responses. *Biomaterials*, *30*(29), 5869–5876. https://doi.org/10.1016/J.BIOMATERIALS.2009.07.009

Kenji, O., Matsuda, H., Murakami, T., Katayama, S., Ohgitani, T., & Yoshikawa, M. (2000). Adjuvant and haemolytic activities of 47 saponins derived from medicinal and food plants. *Biological Chemistry*. *9*(6), 565-585.

Kim, N., & Park, I. S. (2001). Purification of saponin compounds in Bupleurum falcatum by solvent partitioning and preparative LC. *Bioscience, Biotechnology and Biochemistry*, *65*(7), 1648–1651. https://doi.org/10.1271/bbb.65.1648

Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L. A. B., Ifrim, D. C., Saeed, S., Jacobs, C., … & Netea, M. G. (2012). Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(43), 17537–17542. https://doi.org/10.1073/PNAS.1202870109

Ko, E. J., & Kang, S. M. (2018). Immunology and efficacy of MF59-adjuvanted vaccines. In *Human Vaccines and Immunotherapeutics*. *14*(12), 3041-3045.

Köck, J., Rösler, C., Zhang, J. J., Blum, H. E., Nassal, M., & Thoma, C. (2010). Generation of Covalently Closed Circular DNA of Hepatitis B Viruses via Intracellular Recycling Is Regulated in a Virus Specific Manner. *PLOS Pathogens*, *6*(9), e1001082.

Kommareddy, S., Singh, M., & O'Hagan, D. T. (2017). Chapter 13 – MF59: A Safe and Potent Adjuvant for Human Use. *Immunopotentiators in Modern Vaccines: Second Edition*, 249–263. https://doi.org/10.1016/B978-0-12-804019-5.00013-X

Korenkov, D., Isakova-Sivak, I., & Rudenko, L. (2018). Basics of CD8 T-cell immune responses after influenza infection and vaccination with inactivated or live attenuated influenza vaccine. *Expert Review of Vaccines*, *17*(11), 977–987. https://doi.org/10.1080/14760584. 2018. 1541407

Lambrecht, B. N., Kool, M., Willart, M. A., & Hammad, H. (2009). Mechanism of action of clinically approved adjuvants. In *Current Opinion in Immunology*. *21*(1), 23-29.

Lin, B., Qi, X., Fang, L., Zhao, L., Zhang, R., Jing, J., Zhang, S., Yang, X., Hou, Z., & Xue, P. (2021). In vivo acute toxicity and mutagenic analysis of

crude saponins from Chenopodium quinoa Willd husks. *RSC Advances*, *11*(8), 4829–4841. https://doi.org/10.1039/D0RA10170B

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *methods*, *25*(4), 402-408.

Lok, A. S. F., & McMahon, B. J. (2009). Chronic hepatitis B: Update 2009. *Hepatology*, *50*(3), 661–662. https://doi.org/10.1002/HEP.23190

Madland, E. (2013). Extraction, Isolation and Structure Elucidation of Saponins from Herniaria incana. *NTNU - Trondheim*, 1–67.

Majinda, R. R. T. (2012). Extraction and isolation of saponins. *Methods in Molecular Biology*, *864*(February 2012), 415–426. https://doi.org/10.1007/978-1-61779-624-1_16

Marciani, D. J. (2018). Elucidating the Mechanisms of Action of Saponin-Derived Adjuvants. In *Trends in Pharmacological Sciences*, 39(6), 573–585. Elsevier Ltd. https://doi.org/10.1016/j.tips.2018.03.005

Marichal, T., Ohata, K., Bedoret, D., Mesnil, C., Sabatel, C., Kobiyama, K., Lekeux, P., .. & Desmet, C. J. (2011). DNA released from dying host cells mediates aluminum adjuvant activity. *Nature Medicine*. *17*(8), 996-1002.

Medrano, G., Guan, P., Barlow-Anacker, A. J., & Gosain, A. (2017). Comprehensive selection of reference genes for quantitative RT-PCR analysis of murine extramedullary hematopoiesis during development. *PLoS One*, *12*(7), 1–15. https://doi.org/10.1371/journal.pone.0181881

Mendy, M., Peterson, I., Hossin, S., Peto, T., Jobarteh, M. L., Jeng-Barry, A., Sidibeh, M., … & Whittle, H. (2013). Observational study of vaccine efficacy 24 years after the start of hepatitis B vaccination in two

Gambian villages: no need for a booster dose. *PloS One*, *8*(3). https://doi.org/10.1371/JOURNAL.PONE.0058029

Mercy Jelagat, K., Fatuma Faraj, S., Hellen Irusa Lukhaka, D., Eric Wang'welo, D., Laban Kipkemei, M., & Adrian, G. (2020). Burden of Hepatitis B Infection among High Risk Populations in Western Kenya. *Journal of Infectious Diseases and Epidemiology*, *6*(3). https://doi.org/10.23937 /2474 -3658/1510132

Michel, M.-L., & Tiollais, P. (2010). Hepatitis B vaccines: Protective efficacy and therapeutic potential. *Pathologie Biologie*. *58*(4), 288-295.

Milich, D. R., Jones, J. E., Hughes, J. L., Price, J., Raney, A. K., & Mclachlan, A. (1990). Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? *Proceedings of the National Academy of Sciences of the United States of America*, *87*(17), 6599–6603. https://doi.org/10.1073/PNAS.87.17.6599

Moyer, T. J., Kato, Y., Abraham, W., Chang, J. Y. H., Kulp, D. W., Watson, N., Turner, H. L., … & Irvine, D. J. (2020). Engineered immunogen binding to alum adjuvant enhances humoral immunity. *Nature Medicine*, *26*(3). https://doi.org/10.1038/s41591-020-0753-3

Murphy, D. M., Cox, D. J., Connolly, S. A., Breen, E. P., Brugman, A. A. I., Phelan, J. J., Keane, J., & Basdeo, S. A. (2023). Trained immunity is induced in humans after immunization with an adenoviral vector COVID-19 vaccine. *The Journal of Clinical Investigation*, *133*(2). https://doi.org/10. 1172/JCI162581

Muturi-Kioi, V., Lewis, D., Launay, O., Leroux-Roels, G., Anemona, A., Loulergue, P., Bodinham, C. L., … & Podda, A. (2016). Neutropenia as an Adverse Event following Vaccination: Results from Randomized Clinical Trials in Healthy Adults and Systematic Review. *PLoS one*, *11*(8). https://doi.org/10.1371/JOURNAL.PONE.0157385

Oda, K., Matsuda, H., Murakami, T., Katayama, S., Ohgitani, T., & Yoshikawa, M. (2003). Relationship between adjuvant activity and amphipathic structure of soyasaponins. *Vaccine*, *21*(17–18). https://doi.org/10.1016/S0264-410X(02)00739-9

Oleszek, W., Junkuszew, M., & Stochmal, A. (1999). Determination and toxicity of saponins from Amaranthus cruentus seeds. *Journal of Agricultural and Food Chemistry*, *47*(9), 3685–3687. https://doi.org/10.1021/JF990182K

Pattyn, J., Hendrickx, G., Vorsters, A., & Van Damme, P. (2021). Hepatitis B Vaccines. *Journal of Infectious Diseases*, *224*(Suppl 4), S343–S351. https://doi.org/10.1093/infdis/jiaa668

Penn, M. H. (2005). *The effects of dietary soybean saponins on growth and performance, intestinal histology and immune response of first feeding rainbow trout Oncorhynchus mykiss*. Ohio: The Ohio State University.

Petrovsky, N. (2015). Comparative Safety of Vaccine Adjuvants: A Summary of Current Evidence and Future Needs. *Drug Safety*, *38*(11), 1059–1074. https://doi.org/10.1007/s40264-015-0350-4

Pfaffl, M. W. (2007). Relative quantification. *Real-Time PCR*, 64–82.

Pileggi, C., Papadopoli, R., Bianco, A., & Pavia, M. (2017). Hepatitis B vaccine and the need for a booster dose after primary vaccination. *Vaccine*, *35*(46), 6302–6307. https://doi.org/10.1016/J.VACCINE.2017.09.076

Plotkin, S. A. (2010). Correlates of protection induced by vaccination. In *Clinical and Vaccine Immunology*, *17*(7), 1055-1065.

Podolak, I., Galanty, A., & Sobolewska, D. (2010). Saponins as cytotoxic agents: A review. *Phytochemistry Reviews*, *9*(3), 425–474. https://doi.org/10.1007/s11101-010-9183-z

Poolman, J. T. (2014). Shortcomings of pertussis vaccines: Why we need a third generation vaccine. *Expert Review of Vaccines*, *13*(10), 1159–1162. https://doi.org/10.1586/14760584.2014.944902

Pulendran, B., S. Arunachalam, P., & O'Hagan, D. T. (2021). Emerging concepts in the science of vaccine adjuvants. *Nature Reviews Drug Discovery*, *20*(6), 454–475. https://doi.org/10.1038/s41573-021-00163-y

Qi, L. W., Yu, Q. T., Li, P., Li, S. L., Wang, Y. X., Sheng, L. H., & Yi, L. (2006). Quality evaluation of Radix Astragali through a simultaneous determination of six major active isoflavonoids and four main saponins by high-performance liquid chromatography coupled with diode array and evaporative light scattering detectors. *Journal of Chromatography A*, *1134*(1-2), 162-169.

Qi, Y., Gao, Z., Xu, G., Peng, B., Liu, C., Yan, H., Yao, Q., … & Li, W. (2016). DNA Polymerase κ Is a Key Cellular Factor for the Formation of Covalently Closed Circular DNA of Hepatitis B Virus. *PLoS Pathogens*, *12*(10). https://doi.org/10.1371/JOURNAL.PPAT.1005893

Qiao, N., Liu, Q., Meng, H., & Zhao, D. (2014). Haemolytic activity and adjuvant effect of soyasaponins and some of their derivatives on the immune responses to ovalbumin in mice. *International Immunopharmacology*, *18*(2), 333–339. https://doi.org/10.1016/J.INTIMP.2013.12.017

Qiao, Y., Han, X., Guan, G., Wu, N., Sun, J., Pak, V., & Liang, G. (2016). TGF-β triggers HBV cccDNA degradation through AID-dependent deamination. *FEBS Letters*, *590*(3), 419–427. https://doi.org/10.1002/1873-3468.12058

Rajput, Z. I., Hu, S. hua, Xiao, C. wen, & Arijo, A. G. (2007a). Adjuvant effects of saponins on animal immune responses. *Journal of Zhejiang University. Science. B.*, *8*(3), 153–161. https://doi.org/10.1631/jzus.2007.B0153

Rajput, Z. I., Hu, S. hua, Xiao, C. wen, & Arijo, A. G. (2007b). Adjuvant effects of saponins on animal immune responses. *Journal of zhejiang university science B*, *8*(3), 153-161.

Razavi-Shearer, D. M., Gamkrelidze, I., Pan, C. Q., Jia, J., Berg, T., Gray, R. T., Lim, Y. S., … & Razavi, H. A. (2023). Global prevalence, cascade of care, and prophylaxis coverage of hepatitis B in 2022: a modelling study. *The Lancet. Gastroenterology & Hepatology*, *8*(10), 879–907. https://doi.org/10.1016/S2468-1253(23)00197-8

Reed, S. G., Orr, M. T., & Fox, C. B. (2013). Key roles of adjuvants in modern vaccines. *Nature Medicine*, *19*(12), 1597–1608. https://doi.org/10.1038/nm.3409

Samuel, C. E. (1991). Antiviral actions of interferon interferon-regulated cellular proteins and their surprisingly selective antiviral activities. In *Virology*. *183*(1), 1-11. https://doi.org/10.1016/0042-6822(91)90112-O

Savage, G. P. (2003). Saponins. In B. Callabero (Ed.), *Encyclopedia of Food Sciences and Nutrition* (Second).

Saxena M., & Saxena J. (2012a). Evaluation of phytoconstituents of Acorus calamus by FTIR and UV-VIS spectroscopic analysis. *International Journal of Biological & Pharmaceutical Research*, 498–501.

Saxena M., & Saxena J. (2012b). Evaluation of phytoconstituents of Acorus calamus by FTIR and UV-VIS spectroscopic analysis. *International Journal of Biological & Pharmaceutical Research*, 498–501.

Schillie, S., Harris, A., Link-Gelles, R., Romero, J., Ward, J., & Nelson, N. (2018a). Recommendations of the Advisory Committee on Immunization Practices for Use of a Hepatitis B Vaccine with a Novel Adjuvant. *MMWR. Morbidity and Mortality Weekly Report*. https://doi.org/10.15585/mmwr.mm6715a5

Schillie, S., Harris, A., Link-Gelles, R., Romero, J., Ward, J., & Nelson, N. (2018b). Recommendations of the Advisory Committee on Immunization Practices for Use of a Hepatitis B Vaccine with a Novel Adjuvant. *MMWR. Morbidity and Mortality Weekly Report*. https://doi.org/10.15585/mmwr.mm6715a5

Schultz, N. H., Sørvoll, I. H., Michelsen, A. E., Munthe, L. A., Lund-Johansen, F., Ahlen, M. T., Wiedmann, M., … & Holme, P. A. (2021). Brief Report: Thrombosis and Thrombocytopenia after ChAdOx1 nCoV-19 Vaccination. *The New England Journal of Medicine*, *384*(22), 2124–2130. https://doi.org/10.1056/NEJMOA2104882

Shouval, D., Roggendorf, H., Roggendorf, M., Shouval, D., Roggendorf, H., & Roggendorf, M. (2015). Enhanced immune response to hepatitis B vaccination through immunization with a Pre-S1/Pre-S2/S Vaccine. *Med Microbiol Immunol*, *3*, 57–68. https://doi.org/10.1007/s00430-014-0374-x

Silva-Santana, G., Cunha Bax, J., Débora, |, Fernandes, C. S., Tendler, D., Bacellar, L., Hooper, … & Mattos-Guaraldi, L. (2020). *Clinical hematological and biochemical parameters in Swiss, BALB/c, C57BL/6 and B6D2F1 Mus musculus Funding information National Council for Scientific and Technological Development (CNPq); Coordination for the Improvement of Higher Level Personnel, Brazil (CAPES)*. https://doi.org/10.1002/ame2.12139

Singh, M., & O'Hagan, D. T. (2003). Recent advances in veterinary vaccine adjuvants. *International Journal for Parasitology*, *33*(5), 469–478. https://doi.org/https://doi.org/10.1016/S0020-7519(03)00053-5

Sonderup, M. W., & Spearman, C. W. (2022). Global Disparities in Hepatitis B Elimination A Focus on Africa. In *Viruses*, *14*(1). MDPI. https://doi.org/10.3390/v14010082

Stefanati, A., Bolognesi, N., Sandri, F., Dini, G., Massa, E., Montecucco, A., Lupi, S., & Gabutti, G. (2019). Long-term persistency of hepatitis B immunity: an observational cross-sectional study on medical students and resident doctors. *Journal of Preventive Medicine and Hygiene*, *60*(3), E184. https://doi.org/10.15167/2421-4248/JPMH2019.60.3.1315

Sudo, K., Yamada, Y., Saito, K., Shimizu, S., Ohashi, H., Kato, T., Moriwaki, H., Ito, H., & Seishima, M. (2008). TNF-α and IL-6 signals from the bone marrow derived cells are necessary for normal murine liver regeneration. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1782*(11), 671–679. https://doi.org/10.1016/J.BBADIS.2008.09.010

Sun, T., Yan, X., Guo, W., & Zhao, D. (2014). Evaluation of cytotoxicity and immune modulatory activities of soyasaponin Ab: An in vitro and in vivo study. *Phytomedicine*, *21*(13), 1759–1766. https://doi.org/10.1016/J.PHYMED.2014.09.002

Szakiel, A., Ruszkowski, D., & Janiszowska, W. (2005). Saponins in Calendula officinalis L. - Structure, biosynthesis, transport and biological activity. *Phytochemistry Reviews*, *4*(2–3). https://doi.org/10.1007/s11101-005-4053-9

Tsui, L. V., Guidotti, L. G., Ishikawa, T., & Chisari, F. V. (1995a). Posttranscriptional clearance of hepatitis B virus RNA by cytotoxic T lymphocyte-activated hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America*. *92*(26), 12398-12402.

Tsui, L. V., Guidotti, L. G., Ishikawa, T., & Chisari, F. V. (1995b). Posttranscriptional clearance of hepatitis B virus RNA by cytotoxic T lymphocyte-activated hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America*. *92*(26), 12398-12402.

Valdez, H., Purvis, S., Asaad, R., Valerio, I., Sha, B. E., Landay, A. L., & Lederman, M. M. (1999). Levels of Proinflammatory Cytokines in Plasma after Pneumoccoccal Immunization in Human Immunodeficiency Virus Type 1-Infected Patients. *Clinical and Diagnostic Laboratory Immunology*, *6*(3), 427. https://doi.org/10.1128/CDLI.6.3.427-428.1999

Van Damme P, Ward JW, Shouval D, & Zanetti A. (2018). Hepatitis B vaccines. In *Plotkin's Vaccines*, 342–374.

Van Den Ende, C., Marano, C., Van Ahee, A., Bunge, E. M., & De Moerlooze, L. (2017). The immunogenicity and safety of GSK's recombinant hepatitis B vaccine in adults: a systematic review of 30 years of experience. *Expert Review of Vaccines*, *16*(8), 811–832. https://doi.org/10. 1080/ 14760584. 2017.1338568

Vandepapelière, P., Horsmans, Y., Moris, P., Van Mechelen, M., Janssens, M., Koutsoukos, M., Van Belle, P., … & Leroux-Roels, G. (2008). Vaccine adjuvant systems containing monophosphoryl lipid A and QS21 induce strong and persistent humoral and T cell responses against hepatitis B surface antigen in healthy adult volunteers. *Vaccine*, *26*(10), 1375–1386. https://doi.org/10.1016/J.VACCINE.2007.12.038

Wang, P. (2021). Natural and synthetic saponins as vaccine adjuvants. *Vaccines*, *9*(3), 1–18. https://doi.org/10.3390/vaccines9030222

Wangia, C. O., Orwa, J. A., Muregi, F. W., Kareru, P. G., Cheruiyot, K., & Guantai, E. (2018). *Quantitative and Fourier Transform Infrared Analysis of Saponins from Three Kenyan,* Ruellia Species : Ruellia prostrata, Ruellia lineari-bracteolata and. June.

Werner, J. M., Abdalla, A., Gara, N., Ghany, M. G., & Rehermann, B. (2013). The Hepatitis B Vaccine Protects Re-Exposed Healthcare Workers, but Does Not Provide Sterilizing Immunity. *Gastroenterology*, *145*(5), 1026–1034. https://doi.org/10.1053/J.GASTRO.2013.07.044

W.H.O. (2016). *Global health sector strategy on viral hepatitis 2016-2021. Towards ending viral hepatitis*. Retrieved from https://apps.who.int/iris/handle/10665/246177

W.H.O. (2023). *Hepatitis B*. Retrieved from https://www.who.int/news-room/fact-sheets/detail/hepatitis-b#:~:text=The ongoing 2023 update of,die within months of diagnosis.

W.H.O. (2024). *WHO sounds alarm on viral hepatitis infections claiming 3500 lives each day*. Retrieved from https://www.who.int/news/item/09-04-2024-who-sounds-alarm-on-viral-hepatitis-infections-claiming-3500-lives-each-day

Wilkins, T., Sams, R., & Carpenter, M. (2019). *Hepatitis B : Screening , Prevention , Diagnosis , and Treatment*. *99*.

Wilson-Welder, J. H., Torres, M. P., Kipper, M. J., Mallapragada, S. K., Wannemuehler, M. J., & Narasimhan, B. (2009). Vaccine adjuvants: current challenges and future approaches. *Journal of Pharmaceutical Sciences*, *98*(4), 1278–1316. https://doi.org/10.1002/JPS.21523

Wodi, P. A., & Morelli, Valerie. (2016). Epidemiology and Prevention of Vaccine-Preventable Diseases. *CDC*, 263–278. https://doi.org/10.1002/9781119086512.ch28

Wolters, G., Kuijpers, L., & Schuurs, A. (1979). Detection of human antibodies to hepatitis B surface antigen (HBsAg) by an enzyme-immunoassay for HBsAg. *Journal of Clinical Pathology*, *32*(12), 1264–1271. https://doi.org/10.1136/JCP.32.12.1264

Xia, Y., & Protzer, U. (2017). Control of hepatitis B virus by cytokines. In *Viruses*. *9*(1), 18. https://doi.org/10.3390/v9010018

Xia, Y., Stadler, D., Lucifora, J., Reisinger, F., Webb, D., Hösel, M., Michler, T., … & Protzer, U. (2016). Interferon-γ and Tumor Necrosis Factor-α

Produced by T Cells Reduce the HBV Persistence Form, cccDNA, Without Cytolysis. *Gastroenterology*, *150*(1), 194–205. https://doi.org/10.1053/j.gastro.2015.09.026

Xu, D. Z., Huang, K. L., Zhao, K., Xu, L. F., Shi, N., Yuan, Z. H., & Wen, Y. M. (2005). Vaccination with recombinant HBsAg-HBIG complex in healthy adults. *Vaccine*, *23*(20), 2658–2664. https://doi.org/10.1016/J.VACCINE.2004.10.040

Yang, S., Tian, G., Cui, Y., Ding, C., Deng, M., Yu, C., Xu, K., … & Li, L. (2016). Factors influencing immunologic response to hepatitis B vaccine in adults. *Scientific Reports*, *6*. https://doi.org/10.1038/SREP27251

Yuan, C., Li, Y., Li, Q., Jin, R., & Ren, L. (2018). Purification of tea saponins and evaluation of its effect on alcohol dehydrogenase activity. *Open Life Sciences*, *13*(1), 56–63. https://doi.org/10.1515/biol-2018-0008

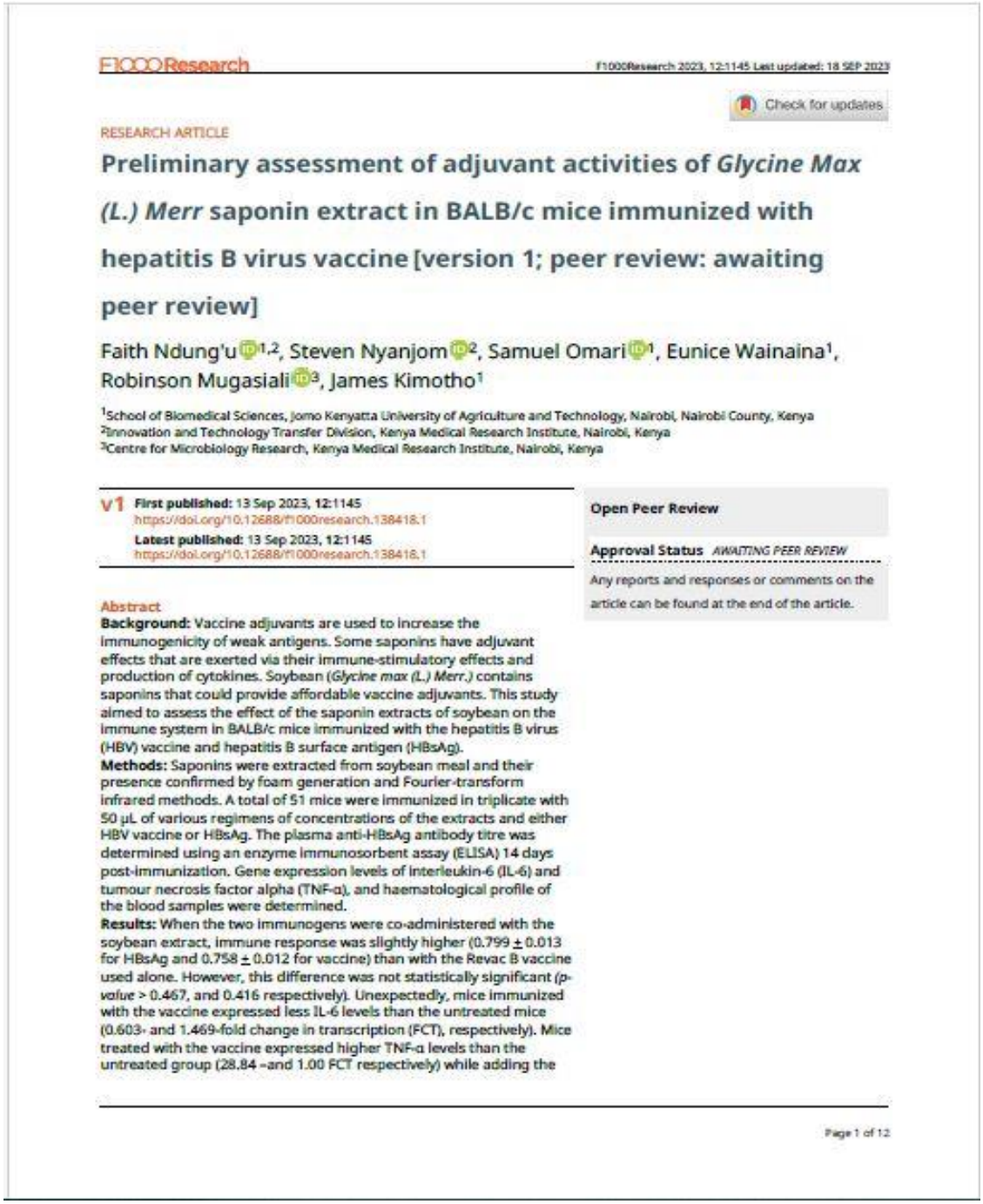
Zhao, H., Zhou, X., & Zhou, Y. H. (2020a). Hepatitis B vaccine development and implementation. *Human vaccines & immunotherapeutics*, *16*(7), 1533-1544.

Zhao, H., Zhou, X., & Zhou, Y. H. (2020b). Hepatitis B vaccine development and implementation. *Human vaccines & immunotherapeutics*, *16*(7), 1533-1544.

Zhong, S., Zhang, T., Tang, L., & Li, Y. (2021). *Cytokines and Chemokines in HBV Infection*. *8*(December), 1–15.

APPENDICES

Appendix I: Published Article in F1000Research journal- Impact Factor 0.939



F1000Research

F1000Research 2023, 12:1145 Last updated: 18 SEP 2023

Check for updates

RESEARCH ARTICLE

Preliminary assessment of adjuvant activities of *Glycine Max (L.) Merr* saponin extract in BALB/c mice immunized with hepatitis B virus vaccine [version 1; peer review: awaiting peer review]

Faith Ndung'u[1,2], Steven Nyanjom[2], Samuel Omari[1], Eunice Wainaina[1], Robinson Mugasiali[3], James Kimotho[1]

[1]School of Biomedical Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Nairobi County, Kenya
[2]Innovation and Technology Transfer Division, Kenya Medical Research Institute, Nairobi, Kenya
[3]Centre for Microbiology Research, Kenya Medical Research Institute, Nairobi, Kenya

v1 **First published:** 13 Sep 2023, **12**:1145
https://doi.org/10.12688/f1000research.138418.1
Latest published: 13 Sep 2023, **12**:1145
https://doi.org/10.12688/f1000research.138418.1

Abstract

Background: Vaccine adjuvants are used to increase the immunogenicity of weak antigens. Some saponins have adjuvant effects that are exerted via their immune-stimulatory effects and production of cytokines. Soybean (*Glycine max (L.) Merr.*) contains saponins that could provide affordable vaccine adjuvants. This study aimed to assess the effect of the saponin extracts of soybean on the immune system in BALB/c mice immunized with the hepatitis B virus (HBV) vaccine and hepatitis B surface antigen (HBsAg).

Methods: Saponins were extracted from soybean meal and their presence confirmed by foam generation and Fourier-transform infrared methods. A total of 51 mice were immunized in triplicate with 50 µL of various regimens of concentrations of the extracts and either HBV vaccine or HBsAg. The plasma anti-HBsAg antibody titre was determined using an enzyme immunosorbent assay (ELISA) 14 days post-immunization. Gene expression levels of interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α), and haematological profile of the blood samples were determined.

Results: When the two immunogens were co-administered with the soybean extract, immune response was slightly higher (0.799 ± 0.013 for HBsAg and 0.758 ± 0.012 for vaccine) than with the Revac B vaccine used alone. However, this difference was not statistically significant (*p-value* > 0.467, and 0.416 respectively). Unexpectedly, mice immunized with the vaccine expressed less IL-6 levels than the untreated mice (0.603- and 1.469-fold change in transcription (FCT), respectively). Mice treated with the vaccine expressed higher TNF-α levels than the untreated group (28.84 –and 1.00 FCT respectively) while adding the

Open Peer Review

Approval Status *AWAITING PEER REVIEW*

Any reports and responses or comments on the article can be found at the end of the article.

Appendix II: Ethical Clearance Approval Document.

In Search of Better Health

KENYA MEDICAL RESEARCH INSTITUTE

OFFICE OF THE DIRECTOR RESEARCH & DEVELOPMENT

Tell: +254 020 2722541, 2713349,
0722 205 901, 0733 400003

P.O. Box 54840-00200,
Nairobi Email:
ddrd@kemri.go.ke
Website: www.kemri.go.ke

KEMRI/RD/22

February 02, 2023

TO: **FAITH NJOKI NDUNG'U,**
PRINCIPAL INVESTIGATOR.

THROUGH: **THE DEPUTY DIRECTOR, CBRD,**
NAIROBI.

Dear Madam,

RE: **PROTOCOL NO. KEMRI/CBRD/SERU/005/500/4641 *(RESUBMISSION OF INITIAL SUBMISSION)*: ASSESSMENT OF ADJUVANT ACTIVITIES OF GLYCINE MAX (L.) MERR. EXTRACT ON BALB/C MICE INOCULATED WITH HEPATITIS B VACCINE.**

Reference is made to your email dated January 25, 2023. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledge receipt of the revised study documents on January 25, 2023.
This is to inform you that the Committee determined that the issues raised during the 331st Committee C meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **January 26, 2023** have been adequately addressed.

Consequently, the study is **granted approval** for implementation effective this day, **February 02, 2023** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **February 01, 2024.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **December 21, 2023.**

Please note that only approved documents including (informed consents, study instruments, Material Transfer Agreement) will be used. You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

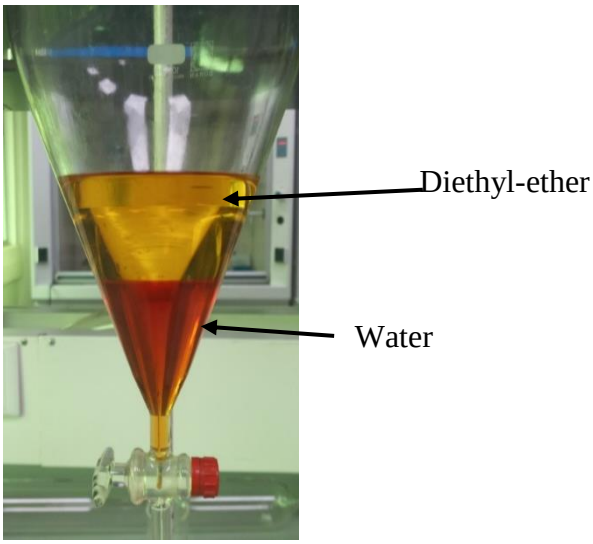
Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

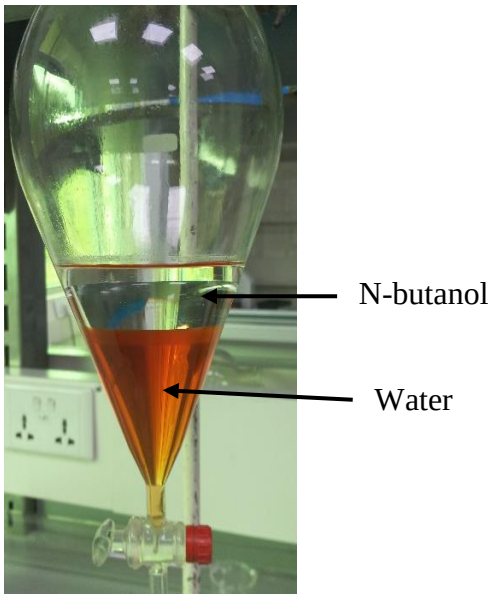
In Search of Better Health

Appendix III: Crude Methanol Extract from *Glycine Max (L). Merr*

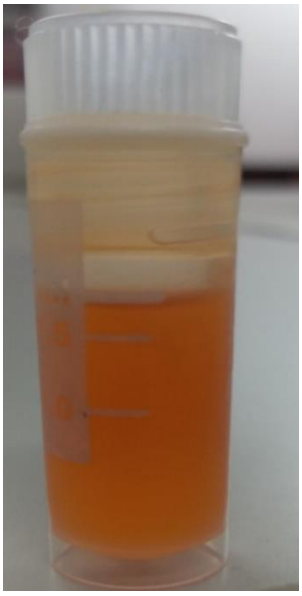
Appendix IV: Diethyl-Ether-Water Partition



Appendix V: N-Butanol-Water



Appendix VI: ***Glycine Max (L). Merr***



Appendix VII: Photo of the Animal Vaccination Activity