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Adjuvant effect of *Vernonia amygdalina* leaf extract on host immune response to hepatitis B virus subunit vaccine

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Aim of the study: Adjuvants can increase the efficiency and reduce the number of required doses for hepatitis B vaccination. Thus the study was designed to investigate whether *V. amygdalina* leaf extract may be used as an adjuvant to the conventional hepatitis B surface antigen-based vaccine through humoral response analyses. *Methodology:* The toxicity/safety margin of *V. amygdalina* was determined using Lorke's method. Immunization was carried out in mice in two phases, phase 1 employed a 3-times vaccination schedule while phase 2 tested 2-times vaccination schedule. The humoral immune response was determined using ELISA test. The total white blood count, different white blood count, aspartate aminotransferase level, alanine aminotransferase level were determined and the body weight of the mice periodically monitored. *Results:* Our data show that *V. amygdalina* was not toxic up to the dose of 5000 mg/kg bodyweight (bw). At a concentration of 250 mg/kg bw as an adjuvant in a three times vaccination schedule, it increased IgM, IgG1 and IgA antibody responses. In a 2-times vaccination schedule, 1000 mg/kg of *V. amygdalina* as an adjuvant to hepatitis B vaccine was able to elicit effective antibody production (0.174±0.002) significantly (P<0.05) higher than the conventional hepatitis B vaccine group (0.109±0.002) which received 3-times vaccine dose. It equally enhanced innate cell-mediated immune response by increasing total white blood cell, neutrophil and lymphocyte counts. The adjuvant-vaccine combination did not produce side effects as the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were within the normal ranges. The liver excised from the sacrificed mice at the end of the vaccination series showed no sign of congestion, inflammation or colour change. Periodic mice body weight monitoring showed similar growth pattern between the treatment and control groups. *Conclusion:* Results obtained suggest that *V. amygdalina* may serve as an effective adjuvant to hepatitis B virus vaccine.

1. Introduction

Hepatitis B virus infection is a life-threatening, common infectious disease of the liver affecting millions of people all over the world (Lok 2002). It is caused by the hepatitis B virus (HBV) which is an enveloped virus containing a partially double-stranded, circular DNA genome classified within the family hepadnavirus. This infection is prevalent in sub-Saharan Africa where about 5–10 % of the adult population is chronically infected (WHO 2016). This infection is a major public health problem; because there are no specific treatments for acute viral hepatitis B and the efficacy of interferon, lamivudine and nucleoside analogues which have been widely used to treat chronic hepatitis B virus infection is limited (D'Souza and Foster 2004). Vaccination using commercial recombinant hepatitis B surface antigen (HBsAg) is an effective approach to prevent hepatitis B virus infection. Primary vaccination consists of three doses of hepatitis B vaccine administered intramuscularly at 0, 1, and 6 months (alternative vaccination schedules use 0, 1, and 4 months or 0, 2, and 4 months) which produces protective antibody response in more than 90 % of infants, children and young adults (Andre 1989). The requirement of hepatitis B vaccine for multiple injections presents compliance issues and significant logistic challenges. Adjuvants can allow immunization with fewer doses of a vaccine, it can also enable reductions in the quantity of antigen contained in an individual vaccine dose and also increase the response to a vaccine (Banzhoff et al. 2009; Onah et al. 2017). The combined effect of dose reduction and antigen sparing can have important implications for improving global vaccine supply and global hepatitis B vaccination compliance.

Vernonia amygdalina is a small shrub of 2-5 m height that grows in tropical Africa with petiolate leaf of about 6 mm diameter and elliptic shape, the leaves are green with a characteristic odour and a bitter taste, it has a grey or brown coloured bark, which has a rough texture and is flaked. *V. amygdalina* has antioxidant (Iwalewa et al. 2005), antidiabetic (Nwaoguikpe 2010) and anticancer activity (Izevbigie et al. 2004), in addition it has been shown to strengthen the immune system through cytokine regulation (Sweeney et al. 2005). The organic fraction extracts of the plant has been shown to possess cytotoxic effects towards human carcinoma cells of the nasopharynx (Kupchen et al. 1969).

2. Investigations and results

2.1. Vernonia amygdalina is not toxic

The result of the acute toxicity test of *V. amygdalina* in mice showed no mortality after 24 h of treatment and the mean lethal dose (LD_{50}) was greater than 5000 mg/kg body weight (Table). Furthermore, no death was recorded in all the groups throughout the 14 days monitoring period, it was equally observed that the *V. amygdalina* induced a dose-dependent percent body weight change of 47.12 % (10 mg/kg bw), 29.37 % (100 mg/kg bw), 14.83 % (1000 mg/kg bw), 5.43 % (1600 mg/kg bw), 0.62 % (2900 mg/kg bw) and 0.18 % (5000 mg/kg bw) as shown in Fig. 1. The vital organs were not affected by the extract as revealed by the percent organ-body weight ratio except for slight increase or decrease observed in some groups (Fig. 2) which were not significant (P<0.05).

Table: Acute toxicity effect of aqueous extract of *Vernonia amygdalina* leaves administered orally to swiss albino mice

Experiment	Dose (mg/kg bw)	No. of mortality after 24 hours	No. of mortality after 14 days	Survival rate
Stage 1	10 100 1000	0/3 0/3 0/3	0/3 0/3 0/3	100% 100% 100%
Control	10 ml/kg	0/3	0/3	100%
Stage 2	1600 2900 5000	0/1 0/1 0/1	0/1 0/1 0/1	100% 100% 100%

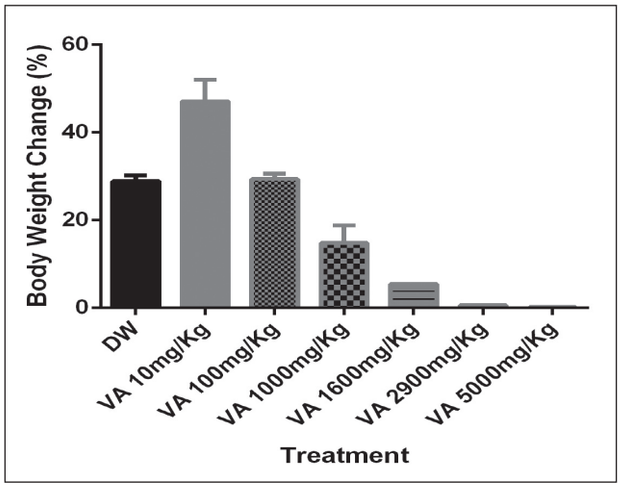

Fig. 1: Percentage body weight change in mice treated with *Vernonia amygdalina* during acute toxicity test. DW = Distilled water, VA = *Vernonia amygdalina*.

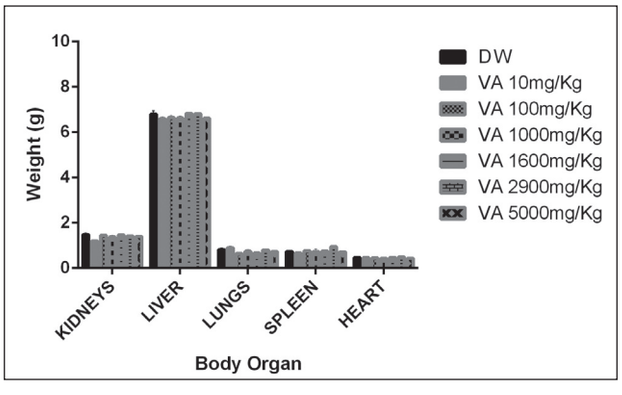

Fig. 2: Percent organ-body weight ratio of mice after *Vernonia amygdalina* acute toxicity study. DW = Distilled water, VA= *Vernonia amygdalina.*

2.2. V. amygdalina as an adjuvant increases humoral immune response

Immunogenicity studies revealed that the level of antibody response in mice immunized with hepatitis B vaccine (HV) + *V. amygdalina* (VA) was significantly (P<0.05) increased at the end of the vaccination compared to mice immunized with hepatitis B vaccine alone, *V. amygdalina* alone or distilled water (Figs. 3, 4, 5, and 6).

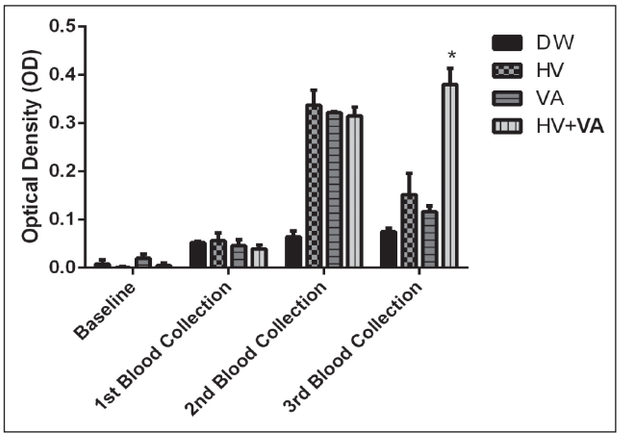

Fig. 3: Total HBsAg – Specific serum antibody response. DW = Distilled water, HV=Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at P<0.05.

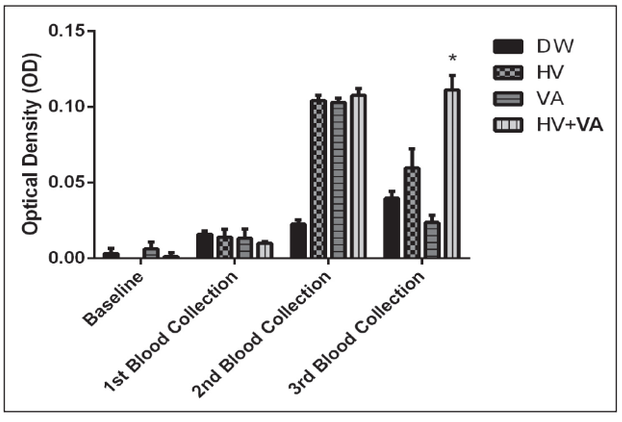

Fig. 4: HBsAg – Specific serum IgM response. DW = Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at P<0.05.

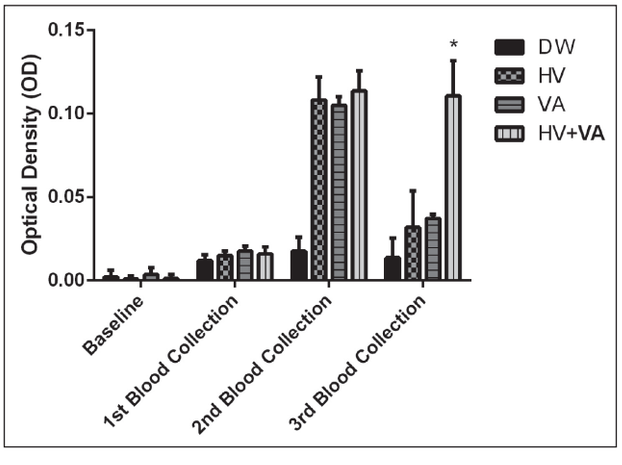

Fig. 5: HBsAg – Specific serum IgG1 response. DW = Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at P<0.05.

2.3. V. amygdalina as an adjuvant can reduce the number of doses required for a complete hepatitis B vaccination

To further determine whether *V. amygdalina* as an adjuvant can reduce the number of doses required for a complete hepatitis B vaccination, a two-times vaccination schedule was designed in which various *V. amygdalina* concentrations were co-administered with hepatitis B vaccine as against a three-times vaccination schedule. The IgM response was determined exactly one week after the first vaccination

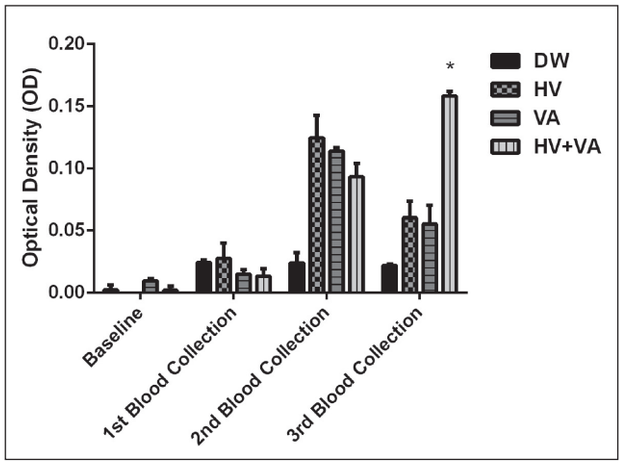

Fig. 6: HBsAg – Specific serum IgA response. DW = Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at $P<0.05$.

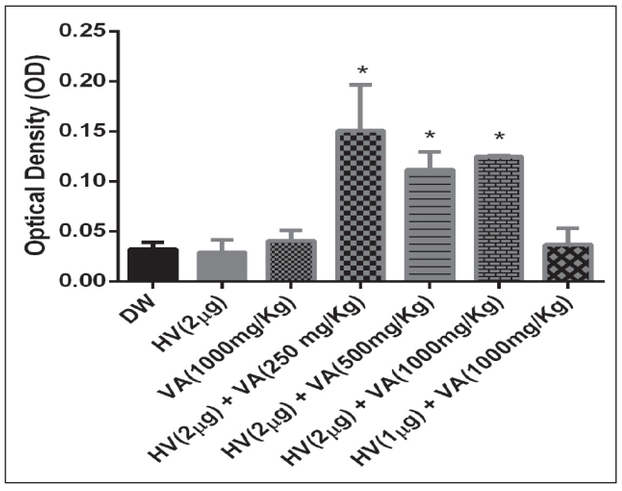

Fig. 7: HBsAg – Specific serum IgM response. DW= Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at $P<0.05$.

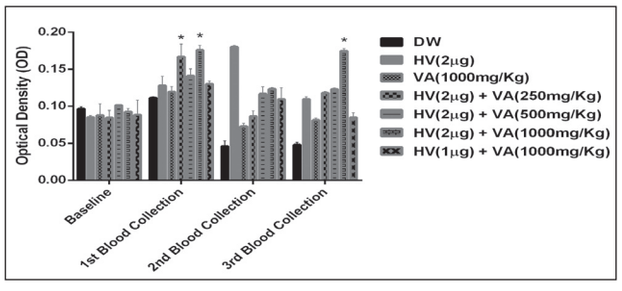

Fig. 8: Total HBsAg – Specific serum antibody response. DW= Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at $P<0.05$.

and the responses in HV (2 µg)+VA (250 mg/kg bw), HV (2 µg)+VA(500 mg/kg bw) and HV (2 µg)+VA (1000 mg/kg bw) groups were significant ($P<0.05$) (Fig. 7). IgG1 and IgA antibody responses were determined periodically. At the end of the vaccination series, significant ($P<0.05$) IgG1 and IgA antibody titres were elicited by mice vaccinated with HV (2 µg) + VA (1000 mg/kg bw) compared to the conventional HV (2 µg) group (Figs. 8, 9, and 10).

2.4. V. amygdalina as an adjuvant enhances antibody retention in the liver, the site for hepatitis B virus infestation

At the end of the vaccination, the animals were sacrificed and the livers excised. IgG1 titre observed in the liver homogenate of HV

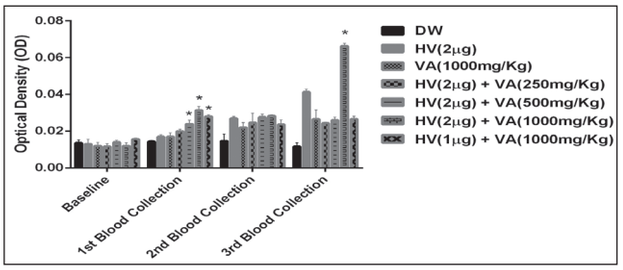

Fig. 9: HBsAg – Specific serum IgG1 response. DW= Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at $P<0.05$.

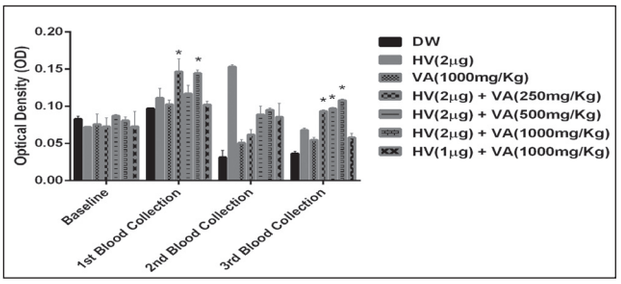

Fig. 10: HBsAg – Specific serum IgA response. DW= Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at $P<0.05$.

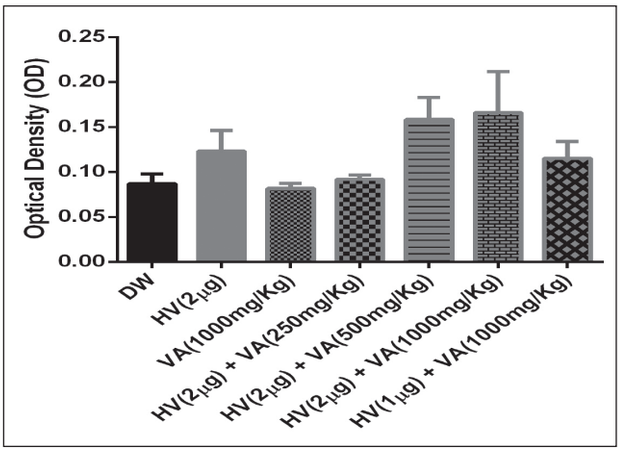

Fig. 11: HBsAg – Specific IgG1 titre in liver homogenate. DW= Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at $P<0.05$.

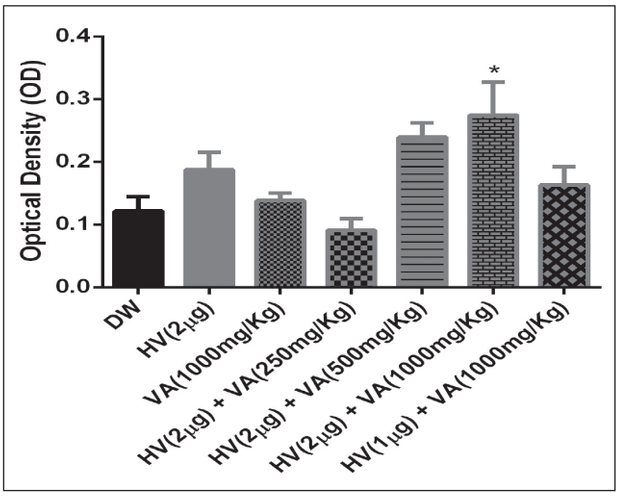

Fig. 12: HBsAg – Specific IgA titre in liver homogenate. DW= Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at $P<0.05$.

(2 µg) + VA (500 mg/kg bw) and HV (2 µg) + VA (1000 mg/kg bw) groups were higher than HV (2 µg) group (Fig. 11). Also the IgA response observed in these groups were higher than what was seen in HV (2 µg) group with that of HV (2 µg) + VA (1000 mg/kg bw) being significant (P<0.05) (Fig. 12).

2.5. V. amygdalina as an adjuvant enhances innate cellular immune response

To investigate further whether the adjuvant influences innate cellular immune response, periodic total white blood cell counts and differential white blood cell counts were evaluated. The result of the total white blood cell (WBC) count showed that only the increase elicited by the HV (2 µg) + VA (1000 mg/kg bw) group was significant (P<0.05) by the end of the vaccination series (Fig. 13). Differential WBC count revealed an increase in absolute neutrophil count at the end of the vaccination in all the groups, of which HV (2 µg) + VA (1000 mg/kg bw) group was significant (P<0.05) (Fig. 14). The absolute lymphocyte count after vaccinations equally increased (Fig. 15), with the HV (2 µg) + VA (1000 mg/kg bw) group having an absolute lymphocyte count that was significant (P<0.05) at the end of the vaccination series. The absolute monocyte count was not significant in any of the groups, likewise the absolute eosinophil count.

2.6. V. amygdalina as an adjuvant did not cause an increase in liver enzymes

ALT and AST are sensitive indicators used to monitor the liver function under drug treatment; they are determinants for hepatotoxicity and liver disease. Result from biochemical analysis showed the AST and ALT level fell within the normal range reported by University of Pennsylvania (University of Pennsylvania 2002). An AST level of 18.67±1.45, 16.00±2.89, 22.67±0.88, 24.00±0.58, 21.00±4.62, 29.33±0.33 and 26.33±6.57 were observed for the DW, HV (2 µg), VA (1000 mg/kg bw), HV (2 µg) + VA (250 mg/kg bw), HV (2 µg) + VA (500 mg/kg bw), HV (2 µg) + VA (1000 mg/kg bw) and HV (1 µg) + VA (1000 mg/kg bw) groups respectively (Fig. 16). An ALT level of 30.67±0.88, 33.67±2.03, 21.67±0.88, 23.67±2.03, 30.00±0.58, 23.00±1.16 and 30.00±3.00 were recorded in the DW, HV (2 µg), VA (1000 mg/kg bw), HV (2 µg) + VA (250 mg/kg bw), HV (2 µg) + VA (500 mg/kg bw), HV (2 µg) + VA (1000 mg/kg bw) and HV (1 µg) + VA (1000 mg/kg bw) groups respectively (Fig. 17).

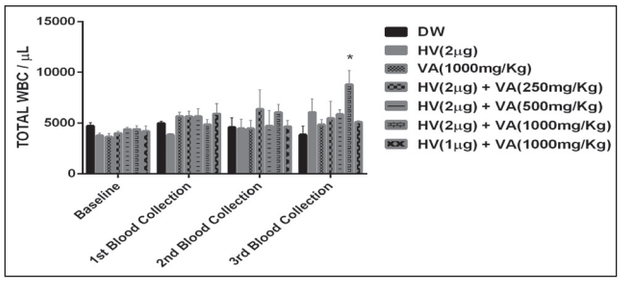

Fig. 13: Total WBC count after vaccination. DW= Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at P<0.05.

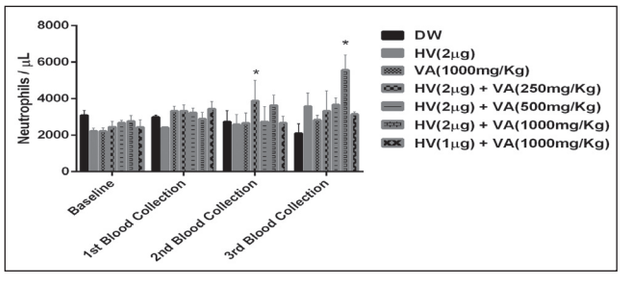

Fig.. 14: Absolute neutrophil count after vaccination. DW= Distilled water, HV=Hepatitis B vaccine, VA=*Vernonia amygdalina,* * Significant at P<0.05.

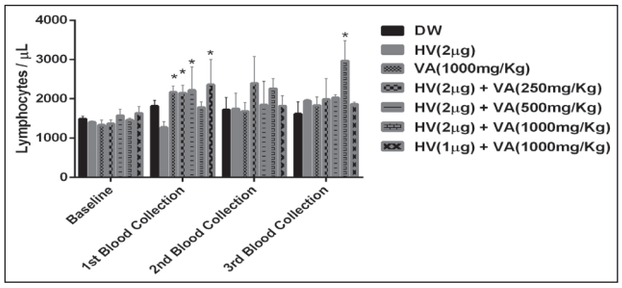

Fig. 15: Absolute lymphocyte count after vaccination. DW= Distilled water, HV=Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at P<0.05.

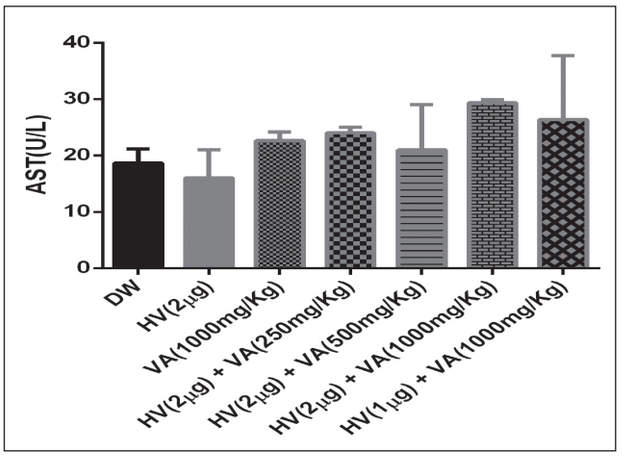

Fig. 16: AST level in the blood following vaccination. AST= Aspartate aminotransferase, DW= Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at P<0.05.

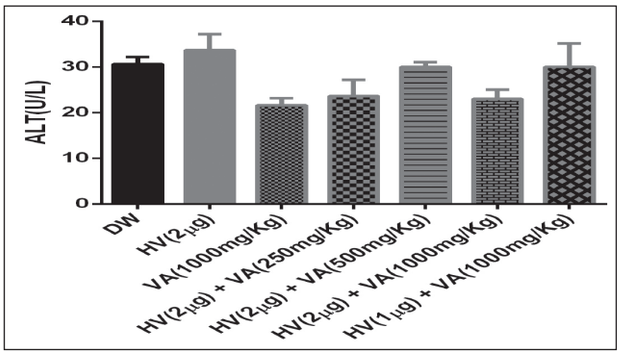

Fig. 17: ALT level in the blood following vaccination. ALT= Alanine aminotransferase, DW= Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at P<0.05.

2.7. V. amygdalina as an adjuvant did not cause an inflammation of the liver

Liver inflammation and dysfunction, acute exacerbation of autoimmune hepatitis and acute hepatitis B infection after vaccination have been reported (Ballinger and Clark 1994). Thus liver excised from the sacrificed mice were examined, it showed no colour changes or sign of congestion or inflammation of the livers. The liver weight recorded were 1.341±0.061, 0.999±0.082, 1.184±0.019, 0.991±0.109, 0.933±0.087, 1.060±0.029 and 0.998±0.032 for the DW, HV (2 µg), VA (1000 mg/kg bw), HV (2 µg) + VA (250 mg/kg bw), HV (2 µg) + VA (500 mg/kg bw), HV (2 µg) + VA (1000 mg/kg bw) and HV (1 µg) + VA (1000 mg/kg bw) groups, respectively (Fig. 18). There was no significant (P<0.05) change across the groups.

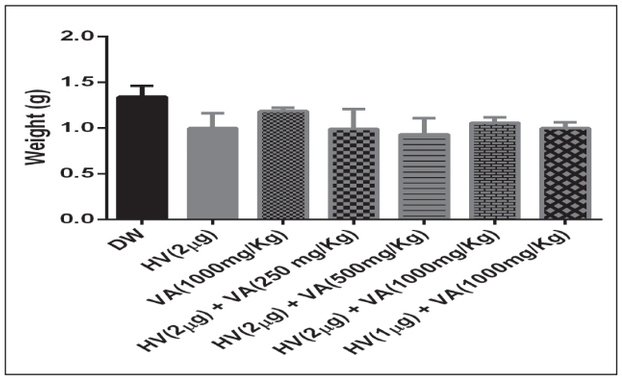

Fig. 18: Liver weight following vaccination. DW= Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina,* * Significant at $P<0.05$.

2.8. V. amygdalina as an adjuvant did not affect the growth of the mice

Animal weight monitoring is important as it provides information as regards the health status of experimental animals (Odimegwu et al. 2008; 2011). Thus periodic assessment of mice body weight was carried out and it showed a similar growth pattern for the various groups (Fig. 19).

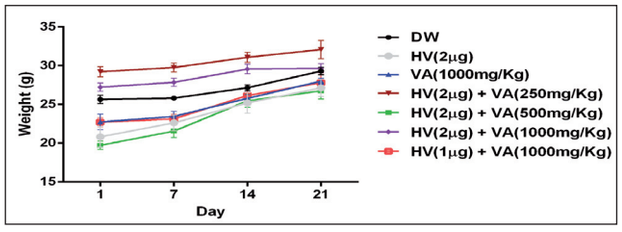

Fig. 19: Mice body weight curve. DW = Distilled water, HV= Hepatitis B vaccine, VA= *Vernonia amygdalina.*

3. Discussion

Hepatitis B vaccination is the most effective measure of preventing hepatitis B virus (HBV) infection and its consequences. Although the vaccine for this immunization is available, the fact that it has to be given in three doses present compliance issue and in several parts of the world logistic challenges (Teena et al. 2013). Adjuvants can help to reduce the number of doses required for a complete protection. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic and prophylactic benefits (Onah et al. 2017; Odimegwu et al. 2008, 2011; Farnsworth 1988). *V. amygdalina* has proven useful in folkloric medicine, where it is employed in the treatment of amoebic dysentery (Moundipa et al. 2000), gastrointestinal disorders (Akah and Ekekwe 1995), laryngitis, liver disorders, and bronchitis (Iwu 1982) and has antimicrobial and antiparasitic activities (Hladik et al. 2005). This plant was evaluated for a possible adjuvant effect to hepatitis B vaccine. Our study shows *V. amygdalina* is a possible adjuvant for the HBV subunit vaccine, which can improve both hepatitis B vaccine specific humoral and innate cellular immune responses. This plant is non toxic as the acute toxicity studies showed an LD_{50} greater than 5000 mg/kg bw and Kennedy et al. (1986) reported that substances with LD_{50} values higher than 5000 mg/kg are regarded as being safe or practically non toxic. $LD_{50} > 5000$ mg/kg obtained for *V. amygdalina* tallies with Yeap et al. (2013) and Adiukwu et al. (2012). At the end of the 14 days monitoring period the result of the percent body weight change and the mean organ body weight ratio further reaffirms the safety of the extract with the tested doses. Immunogenicity studies revealed 250 mg/kg bw of *V. amygdalina* potentiated the effect of hepatitis B vaccine as there was a significant IgM, IgG1 and IgA antibodies response in the group given HV+VA compared to the HV group. At a higher concentration of 1000 mg/kg bw it was shown to reduce the number of doses required to produce the same effect. The response in the HV (2 µg) + VA (1000 mg/kg bw) group tends to be progressive and sustained while that from the HV group seems to rapidly rises and fall (Figs. 8, 9 and 10). Long-term memory in humoral immunity is acquired by the survival and continued function of plasma B cells to generate antibody against pathogens. *V. amygdalina* as an adjuvant seems to extends the stimulation of the immune system and hence antibody production. Adjuvants increase the speed of initial response to a vaccine, which may be critical in a pandemic outbreak of infection (Huleatt et al. 2007). IgM being significantly produced at one week after vaccination suggest *V. amygdalina* could enhance early response to hepatitis B virus surface antigen which might be useful in the vaccination of non responder population such as the elderly and hemodialysis patient. The presence of IgM equally shows the vaccine-extract combination is immunogenic. IgG1 acts against bacteria and viruses by opsonizing and neutralizing them, vaccination with this combination could increase the rate of opsonisation of hepatitis B virus for recognition and phagocytosis by neutrophils and macrophages, also being the predominant antibody in secondary response, this combination might provide long term immunity. The significant IgA produced shows the extract–vaccine combination could prevent the spread of hepatitis B virus as the IgA will prevent the attachment of the virus to mucosal surfaces and invasion of host tissues.

Hepatitis B is an infection of the liver, during which it causes inflammation of the liver, liver cirrhosis and liver cancer. The presence of these antibodies specifically in the liver will help in the prevention and clearance of the virus. This was evaluated from the liver homogenate, which revealed HV (2 µg) +VA (1000 mg/kg bw) group had an IgA presence (Fig. 12) and an IgG1 titre (Fig. 11) that was significantly ($P<0.05$) higher than that seen from the conventional hepatitis B vaccine group.

The total white blood cell (WBC) count revealed a significant increase in this same group (Fig. 13). WBCs form the basis of an immune response to invading microbes and foreign substances, with some functioning in the innate system, whereas others are part of an adaptive immune response. It is thought that the primary mechanism of action of adjuvants is on the innate immune response. Neutrophils are indispensable first line of defense against microorganisms, as they can activate different cell types, such as dendritic cells, macrophages, B lymphocytes and natural killer cells (Laeger et al. 2012) and also secrete cytokines, such as IL-1β (Cho et al. 2012). Differential WBC count revealed that this same group had a significant absolute neutrophil and lymphocytes count. This significant increase in neutrophil count suggests that *V. amygdalina* potentiates the ability of hepatitis B vaccine in producing neutrophils which are phagocytes with excellent microbiocidal abilities. Lymphocytes on the other hand are the major cells of the specific immune system; they include T cells, B cells, and null cells (which include natural killer cells). Mature, activated B cells (plasma cells) secrete large quantities of antibodies which can directly neutralize toxins and viruses, and are important in stimulating an efficient phagocytic response. The high lymphocytes count seen in this group suggest 1000 mg/kg bw of *V. amygdalina* can potentate B and T cell inducing and/or producing ability of hepatitis B vaccine.

Adverse effects due to hepatitis B vaccine in adults and rarely in children have been reported since the 1980's after common use of hepatitis B vaccine (CDC 1996). Liver dysfunction, acute exacerbation of autoimmune hepatitis and acute hepatitis B infection after vaccination have equally been reported (Ballinger and Clark 1994). ALT and AST are used as the determinants for hepatotoxicity and liver disease. They are sensitive indicators used to monitor the liver function under drugs treatment. Their analysis showed the AST and ALT level were within the normal range for both the control and treated groups. This further supports the claim that the extract did not produce a toxic effect on mice and the extract-vaccine combination will possibly not have a side effect. Liver weights from the various concentration combination group animals showed no sign of inflammation as the weight observed was not significantly different from that of the control group. Peri-

odic weight monitoring of the mice revealed an increase in body weight which is a similar growth pattern for the different treatment groups. This increase in body weight can be attributed to growth since the experimental animals were young mice, thus are expected to grow progressively when properly fed. It could also be attributed to an appetite stimulating effect of *V. amygdalina* which led to an increase in food intake and subsequently increased body weight.

Following from the results seen in this study, one can deduce *V. amygdalina* has no toxic effect, can potentiate humoral immune response to hepatitis B vaccine and also reduce the number of doses required for a complete hepatitis B immunisation; though the mechanism by which this occurred is not known. Potentially, the *V. amygdalina* extracts potentiate the stimulation of the immune system through cytokine regulation as suggested by earlier research (Sweeney et al. 2005). Adjuvants enhance T and B cell responses by engaging components of the innate immune system, rather than by direct effects on the lymphocytes themselves (McCartney et al. 2009), this could also be suggested since the result from the study shows increased neutrophil and lymphocyte counts in the HV (2 µg) + VA (1000 mg/kg) group. It could also be that the phytocompounds, i.e. alkaloids, cardiac glycosides, steroids, saponins, tannins, flavonoids (Chinedu et al. 2015) present in *V. amygdalina* are responsible for the adjuvant effect in our study, as specific phytocompounds such as saponins (Igile et al. 1995), flavonoids (Sharma et al. 1996), tannins (Qari et al. 2014) and alkaloids (Omeje et al. 2011) have been known as immunostimulatory compounds.

With the results obtained from our study, it is plausible to conclude, that *V. amygdalina* is a potential and effective adjuvant to hepatitis B vaccine. Its incorporation into hepatitis B vaccine formulation might lead to a more potent, effective and safe candidate vaccine which when given in a two-dose schedule will lead to better clinical and demographic outcomes. However additional investigations are required to draw more definitive conclusions.

4. Experimental

4.1. Reagents and animals

Hepavax-Gene® (Hepatitis B vaccine, recombinant) from Berna Biotech Korea Corporation was used. *V. amygdalina* leaves were harvested from a local garden in Nsukka. The aqueous extract of the leaves used in this study was prepared freshly prior to every use using the method of Momoh et al. (2012). The plant was properly rinsed in distilled water then squeezed using the hands; the extracted solution was passed through muslin cloth and filtered paper before use. Young female Swiss albino mice (7 - 9 weeks old) purchased from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka, and kept under standard pathogen-free conditions in an animal facility of the Department of Pharmacology and Toxicology of the University of Nigeria, Nsukka, were used. These animals were fed with standard feed and water *ad libitum* throughout the study period.

4.2. Acute toxicity study

Lorke's method for acute toxicity study, as modified by Bulus et al. (2011) was employed for the acute toxicity study. The study was carried out in two stages:

Stage 1: Twelve mice were used in this stage, these were divided into four groups (A, B, C and D) of three mice each. Group A, B, C received 10, 100 and 1000 mg/kg bw of the aqueous *V. amygdalina* extract, respectively, Group D (control group) received distilled water (10 ml/kg bw). All these were given orally in a single dose after four days of acclimatization.

Stage 2: This stage was carried out based on the result of the first stage. Here further specific doses of 1600, 2900 and 5000 mg/kg bw of the extract were administered to 3 mice (one mice per dose) to further determine the correct LD_{50} value.

All animals were observed frequently on the day of treatment and surviving animals were monitored daily for 2 weeks for delayed signs of toxicity. At the end of 14 days, all surviving mice were sacrificed and the vital organs isolated. The weights of these organs were taken and the mean organ-body weight ratios calculated and compared with those of the control group. The body weight changes in the mice were also noted.

4.3. Immunization

In phase I, the usual three time vaccination schedule for hepatitis B vaccination was used to determine whether *V. amygdalina* can enhance humoral immune response. To further determine whether the extract (and at what concentration) as an adjuvant can reduce the number of dosing required for a complete hepatitis B vaccination, a two times vaccination schedule was designed for phase II.

Phase I: Twenty-four mice randomly divided into four groups (groups A - D) of six mice each were used. These were immunized on days 0, 28 and 56 as follows; Group A received 0.1 ml of distilled water only; Group B received 0.1 ml (2 µg) of hepatitis B vaccine only; Group C received 0.1 ml *V. amygdalina* (250 mg/kg bw) only; Group D received 0.1 ml (2 µg) hepatitis B vaccine + 0.1 ml *V. amygdalina* (250 mg/kg bw). The vaccine was administered intramuscularly while the *V. amygdalina* was given orally.

Phase II: Thirty-five mice randomly divided into seven groups (groups A - G) of five mice each were used. These were immunized on days 0 and 28 except for the hepatitis B vaccine and distilled water control groups that received treatments on days 0, 14 and 28. The groups were treated as follows; Group A received 0.1 ml of distilled water only; Group B received 0.1 ml (2 µg) of hepatitis B vaccine only; Group C received 0.1 ml of *V. amygdalina* (1000 mg/kg bw) only; Group D received 0.1 ml (2 µg) hepatitis B vaccine + 0.1 ml *V. amygdalina* (250 mg/kg bw); Group E received 0.1 ml (2 µg) hepatitis B vaccine + 0.1 ml *V. amygdalina* (500 mg/kg bw); Group F received 0.1 ml (2 µg) hepatitis B vaccine + 0.1 ml *V. amygdalina* (1000 mg/kg bw) and Group G received 0.1 ml (1 µg) hepatitis B vaccine + 0.1 ml *V. amygdalina* (1000 mg/kg bw). The vaccine was administered intramuscularly with the *V. amygdalina* given orally.

4.4. Immunogenicity studies

The sera were harvested by intraocular eye puncture and analyzed for IgG1, IgA and IgM rHBsAg specific antibody levels using conventional ELISA method as described by Ternette et al. (2007). ELISA plate (Brandplates (immunograde), Wertheim, Germany) were coated with hepatitis B vaccine at a dose of 2 µg/ml (0.2 µg / 100 µl / well) overnight at 4 °C in coating carbonate-bicarbonate buffer (pH 9.5). The plates were blocked with phosphate buffered saline (PBS) containing 0.05 % Tween 80 (PBS-T) and 5 % fat free milk for 1 h at room temperature. The plate was washed three times with PBS-T. Mice sera diluted in PBS-T with 2 % fat free milk were added to each well and incubated for 1 h at room temperature. After washing three times with PBS-T, the IgG1, IgA and IgM subclass specific antibodies conjugated with horseradish peroxidase were added to the different wells and incubated for 1 h at room temperature. The plates were washed as done previously and 3,3'5,5'-tetramethylbenzidine (TMB) substrate solution (Sigma Aldrich, Taufkirchen, Germany) was added. The reaction was stopped with 2M H_2SO_4 and absorbance at 450 nm was taken using an ELISA reader (GM 2000). At the end of the vaccination schedule the mice were sacrificed, livers excised and the liver homogenate was equally screened for the presence of these antibodies.

4.5. White blood count (WBC)

Using the method outlined by Verma (2000), 380 µl of diluting fluid (10 % glacial acetic acid and 1 drop of gentian violet) was measured and dispensed into a small tube and 0.02 ml (20 µl) volume of well-mixed EDTA anticoagulated blood was added. The Neubauer counting chamber was assembled. The diluted blood was re-mixed using a micropipette and one of the grids of the chamber was filled with the sample. The rulings of the chamber and white cells were focused carefully using the 10x objective until they appeared as small black dots. One area/chamber was counted and the total white blood cell is calculated using the formula below:

$$\frac{\textit{number of cells counted}}{1(\textit{the area counted}) \times 0.1(\textit{depth of the chamber})} \times 20(\textit{dilution factor})$$

4.6. Differential white blood cell count

The differential white blood cell count was determined also using the method outlined by Verma (2000). A drop of EDTA anticoagulated blood was placed on a microscope slide using another slide as a spreader a smear was made. The smear dried rapidly and was fixed with methyl alcohol for 2 min. Then Giemsa stain diluted 1:9 with buffer was poured over the smear for 8-10 min after which it was rinsed out with buffer and allowed to dry. The dried stained film was examined under oil immersion objective. The area where the morphology of the cells is clearly visible was used for counting, a total of 100 cells were counted in which every white cell seen was recorded under the following heading: neutrophil, eosinophil, basophil, lymphocyte, and monocyte thus representing the percentage of each type, this percentage was used to calculate the absolute differential white blood cell count.

4.7. Analysis of liver enzymes

The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined as described by Reitman and Frankel (1957) using Randox Diagnostic kit (Randox Laboratories Ltd, UK). Using a Reagent blank method, two clean test tubes, labeled A (for reagent blank) and B (for sample) was used, 0.5 ml of reagent 1 (from the kit) was added to the two test tubes, then 0.1 ml of the serum sample was added to test tube B while 0.1 ml of distilled water was added to test tube A, these were properly mixed and incubated for exactly 30 min at 37 °C in a water bath. Then 0.5 ml of reagent 2 (from the kit) was added to the two test tubes, mixed and allowed to stand for exactly 20 min at 25 °C, then 5 ml of 0.4 mol/l of NaOH was added to each test tube, mixed for 5 mins and the absorbance was read with a spectrophotometer at 546 nm wavelength.

4.8. Periodic body weight assessment

Mice used for the study where monitored periodically using a digital sensitive weighing balance and their weight recorded accordingly. At the end of the vaccination exercise the mice were sacrificed and the livers were isolated, examined and weighed.

4.9. Statistical analysis

The data obtained was expressed as mean ± standard error of mean (Mean ± SEM). Two way and One way analysis of variance (ANOVA) followed by Tukey's post hoc test were used to test for significance. $P<0.05$ was considered significant. Graph pad prism (version 6.0) was used for the analysis.

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