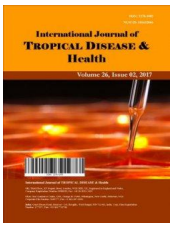

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Suppressive Effect of *Garcinia kola* on the Humoral Immune Response of Mice to Hepatitis B Virus Subunit Vaccine

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Authors' contributions

This work was carried out in collaboration between all authors. Author DCO conceptualized the study. All authors contributed in the design of the study. Authors DCO and IAO wrote the protocol, performed the experiments, statistical analysis and wrote the manuscript. All authors managed the analyses of the study, literature searches, read and approved the final manuscript.

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ABSTRACT

Aim: Due to the health benefits of most medicinal plants, humans have resorted to their frequent and sometimes daily consumption, thus there is need to investigate the effect of medicinal plants consumption while on vaccination.
Study Design: This study was designed to investigate the biological interaction between *Garcinia kola* (GK) seed extract and Hepatitis B virus Surface Antigen.
Place and Duration of Study: The research was carried out in the Department of Pharmaceutical Microbiology and Biotechnology, University of Nigeria Nsukka, within six (6) months.
Methodology: Fresh GK seeds were obtained, identified, dried, pulverized and stored in an air-tight container until extraction. Cold maceration technique was used for extraction. Methanol was the solvent used. Locke's method of acute toxicity testing was used to ascertain the toxicity of the extract. Afterwards, the experimental animals were grouped and vaccinated accordingly. After vaccination, sera collected from the animals were used for immunogenicity studies while the whole

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blood was used for total white blood cell count. During the study period, the experimental animals were monitored frequently and weighed.

Results: The percentage yield after extraction was 16.7%. The extract was non-toxic up to 5000 mg/kg. The vaccination induced antibody responses (IgM, IgG1 and IgA) in all the groups but the response in the hepatitis B vaccine group was significantly higher than that of the hepatitis B vaccine/GK extract combination group (P<0.05), suggesting an inhibitory/ suppressive effect of *G. kola* on immune response to the hepatitis B surface antigen. The total white blood cell count equally revealed a suppressive effect of the extract on the hepatitis B virus surface antigen. The periodic weight monitoring reveals similar growth pattern across all other groups except the hepatitis B vaccine/GK combination group that seems not to be growing rapidly.

Conclusion: The outcome of this present study shows that at ≥250 mg/kg body weight GK seed extract demonstrates a suppressive effect on the immunogenic responses to hepatitis B surface antigen. Therefore, cautious consumption or total abstinence from *GK* is adviced in subjects receiving hepatitis B vaccination.

Keywords: Vaccine; hepatitis B virus; Garcinia kola; antibody responses; immunogenicity.

1. INTRODUCTION

Microorganism surrounding our environment causes numerous diseases, most of which have no cure, making preventive measures the effective means of managing such diseases. Vaccination exercise is one of the best known and effective means of preventing infectious diseases having been shown to reduce the rate of chronic infection in many countries [1]. Hepatitis-B virus (HBV) infection is the major cause of the development of liver cirrhosis and hepatocellular carcinoma [2]. Approximately 2 billion people worldwide are infected with the virus [3] with the highest infection in sub-Saharan Africa and East Asia [4]. Majority of individuals who get infected in highly endemic areas acquire the infection either perinatally or in early childhood, whereas in low prevalence areas the infection is acquired primarily in adulthood [5]. Hepatitis B virus belongs to the *Hepadnaviridae* family, possess a 3.2 kb partially double-stranded DNA, is an enveloped virus, and infects hepatocytes by interacting with sodium-taurocholate co-transporting polypeptide present on the surface of these cells [6,7]. Treatment of hepatitis B virus infection could be either palliative or preventive. Currently, the preventive approach involves vaccination with the recombinant hepatitis B virus vaccine based on the recombinant Hepatitis-B surface antigen (rHBsAg) [8,9].

Garcinia kola (GK) is highly valued in Africa thus highly consumed; it is used socially as refreshment and medicinally for the treatment of abdominal pain, cough, laryngitis, liver disease, infection and erectile problems [10]. The seeds are also considered a poison anti-dote. The seeds have been proven to possess numerous physiological and pharmacological effects which include hepatoprotective, antiinflammatory, antioxidant, antifertility, haematological and anticancer effects [11]. The seeds are mostly eaten by the elderly because of their belief that it could prolong life. Chinedu et al. [12] in his report encourages the daily consumption of GK as it induces hypoglycaemic effect. Phytochemical screening of the seed revealed the presence of flavonoids, bioflavonoids, xanthones, triterpenes, cycloartenol, benzophenones, phenol, alkaloids, saponin and tannin [13]. GK, locally called bitter kola and known variously as “Orogbo” (Yoruba), “Namijin gworo” (Hausa) and “Aki ilu” (Igbo) is an angiosperm, belonging to the family of *Guttiferae*. It is found in moist forest and is widely distributed throughout west and central Africa.

Booster doses of the HBV vaccine are required to achieve a lasting immune response against the virus [9,14]. This trend seems to suggest the inherent relative weakness of the vaccine immunogen or the possibility that factors that tend to suppress the immune response to HBV vaccine exist. Is it possible that frequent consumption of Garcinia kola (as evaluated in our study) negatively impact the HBV vaccine response? Our current findings reported in this paper attempt to answer this question. Therefore, our study evaluated the effect of *Garcinia kola* seed extract on HBV vaccine response.

2. MATERIALS AND METHODS

2.1 Collection, Identification and Extraction of Plant Material

GK seeds were purchased from Ogige market in Nsukka area of Enugu State, Nigeria and

authenticated by a botanist from the Department of Botany, University of Nigeria, Nsukka having the voucher number UNH NO 55 (University of Nigeria Herbarium number 55). These seeds were air-dried under shade (for 21 days) to avoid sunlight thereby conserving the volatile constituent in the plant materials, then pulverized and the powder obtained was stored in an air tight container until extraction. Methanolic extract of the pulverized seeds was obtained using cold maceration method [15]. Exactly 300 g of the pulverized *G. kola* was macerated with 2.5 litres of methanol (Sigma-Aldrich) using a glass jar. The preparation was sealed properly to avoid evaporation. This was shaken vigorously after 24 hours. At 48 hours, the extract was filtered using a muslin cloth and Whatman No. 1 filter paper. The filtrate obtained was evaporated to dryness at room temperature for 1 week [16].

2.2 Test Animal

Young female Swiss albino mice (7 - 9 weeks old) purchased from 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 was used. These animals were fed with standard feed and water *ad libitum* throughout the study period. A total of 39 animals were used. The use and care of laboratory animals in the study were in accordance with ethical guidelines of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka.

2.3 Test Vaccine

Hepatitis B vaccine from Berna Biotech Korea Corporation known as Hepavax-Gene® (Hepatitis B virus vaccine, recombinant) was used.

2.4 Acute Toxicity Study

Lorke's method for acute toxicity study, as modified by Bulus et al. [17] was employed for the acute toxicity study. The study was carried out in two stages:

Stage 1: Twelve (12) mice were used in this stage, these were divided into four groups (A, B, C and D) of 3 mice each. Group A, B, C were given 10, 100 and 1000 mg/kg BW of the methanolic GK seed extract respectively, Group D (control group) received distilled water (DW) (10 ml/kg BW). All these were given in a single dose via parenteral route after 4 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 mouse 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 recorded.

2.5 Vaccination

Twenty-four (24) mice randomly divided into four groups (groups A - D) of six mice each was used. These were immunized on days 0, 28 and 56 as follows; Group A received 0.1 ml of distilled water only (control); Group B received 0.1 ml (2 µg) of Hepatitis B vaccine only; Group C received 0.1ml *GK* (250 mg/kg BW) only; Group D received 0.1 ml (2 µg) Hepatitis B vaccine + 0.1 ml *GK* (250 mg/kg BW). The vaccine was administered intramuscularly while the *GK* was given intraperitoneally.

2.6 Immunogenicity Studies

The sera were harvested by eye puncture at the retro-orbital plexus and analyzed for IgG1, IgA and IgM rHBsAg specific antibody levels using conventional ELISA method as described by Ternette et al. [18]. ELISA plate (Brandplates (immunograde), Wertheim, Germany) were coated with hepatitis B vaccine antigen 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 hour 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 hour 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 hour 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 2 M H_2SO_4 and absorbance at 450 nm was measured using an ELISA reader (GM 2000).

At the end of the 3-times vaccination schedule, the mice were sacrificed, livers excised, examined, weighed and the liver homogenate was equally screened for the presence of these antibodies.

2.7 Determination of Total White Blood Count (WBC)

Using the method outlined by Verma [19] 0.38 ml (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 (Sigma-Aldrich, Germany) 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:

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

2.8 Periodic Body Weight Assessment

The mice used for the study where weighed periodically using a digital sensitive weighing balance (Sartorius, Germany) and their weight recorded accordingly.

2.9 Statistical Analysis

The data obtained were expressed as a mean ± standard error of the mean (Mean ± SEM) being consistent with the Tukey's test. One way analysis of variance (ANOVA) followed by pair wise multiple comparisons according to Tukey's post hoc test was used to test for significance. $P<0.05$ was considered significant. Graphpad Prism (version 6.0) was used for the analysis.

3. RESULTS AND DISCUSSION

3.1 Physical Properties of the *Garcinia kola* Seed Extract

Polar solvents have been shown to be effective in extracting organic and inorganic materials from plants [20]. Methanol, the polar solvent used gave a percentage yield of 16.7 as shown in Table 1 below.

Table 1. Some physical properties of the methanolic extract of *Garcinia kola* seed

Physical properties	*Garcinia kola* seed extract
Weight extracted	300 g
Weight of extract	50.1 g
Percentage Yield	16.7%
Description	A brown sticky extract that contains oil

3.2 Acute Toxicity

The acute toxicity test was carried out on the extract to determine possible toxicity/ safety margin [21] and it revealed a mean lethal dose (LD_{50}) greater than 5000 mg/kg as there was no mortality recorded after 24 hours of treatment (Table 2). Furthermore, no death was recorded in all the groups throughout the 14 days monitoring period (Table 2). Kennedy et al. [22] reported that substances with LD_{50} values higher than 5000 mg/kg are regarded as being safe or practically non-toxic, thus the extract is practically non-toxic. The LD_{50} gotten is in line with the findings of Komolafe et al. [23]. At the end of the 14 days monitoring period the percent body weight change and mean organ-body weight ratio was determined. Increase in body weight was noticed in all the groups. The percent body weight change for *GK* doses of 10, 100, 1000, 1600, 2900 and 5000 mg/kg were 35.36 ± 3.07, 28.96 ± 5.18, 29.80 ± 4.51, 22.70, 41.92 and 28.49 % respectively and the distilled water (DW) control group had a body weight change of 17.04 ± 0.93% (Fig. 1). Macroscopic examination of the internal organs showed no sign of congestion, inflammation or change of colour. The mean organ-body weight ratio revealed there was no significant difference in the weight of the various organs when compared with the control groups as indicated in Fig. 2 suggesting the extracts have no effect on normal growth. The usefulness of weighing organs in toxicity studies includes their sensitivity to predict

toxicity, enzymes induction, physiologic perturbation and acute injury. It correlates well with histopathological changes [24]. The organ weight was expressed as a percentage of the body weight (organ-body weight ratio) rather than as absolute weight so as to take into consideration differences in the organ weight that may solely be attributable to differences in the body weight of the respective mice. The result of the study revealed that the heart, spleen, kidneys, liver and lungs did not show clinical sign of toxicity throughout the treatment since there was no significant (P<0.05) reduction or increase in the organ-body weight ratio of the treated animals. The body weight change monitored to serve as a sensitive indication of the general health status of the experimental animals [25]. The percentage body weight change (Fig. 1) revealed all animal demonstrated an increase in body weight during the period. This increase in weight might be as a result of the extract having an appetite-stimulating effect, which resulted to increase in food intake and consequently increase in weight. The increase in body weight might also be as a result of natural growth since the mice were young animals.

Table 2. Acute toxicity effect of methanolic extract of *Garcinia kola* seed administered intraperitoneally 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%
	100	-	-	100%
	1000	-	-	100%
Control	10 ml/kg	-	-	100%
Stage 2	1600	-	-	100%
	2900	-	-	100%
	5000	-	-	100%

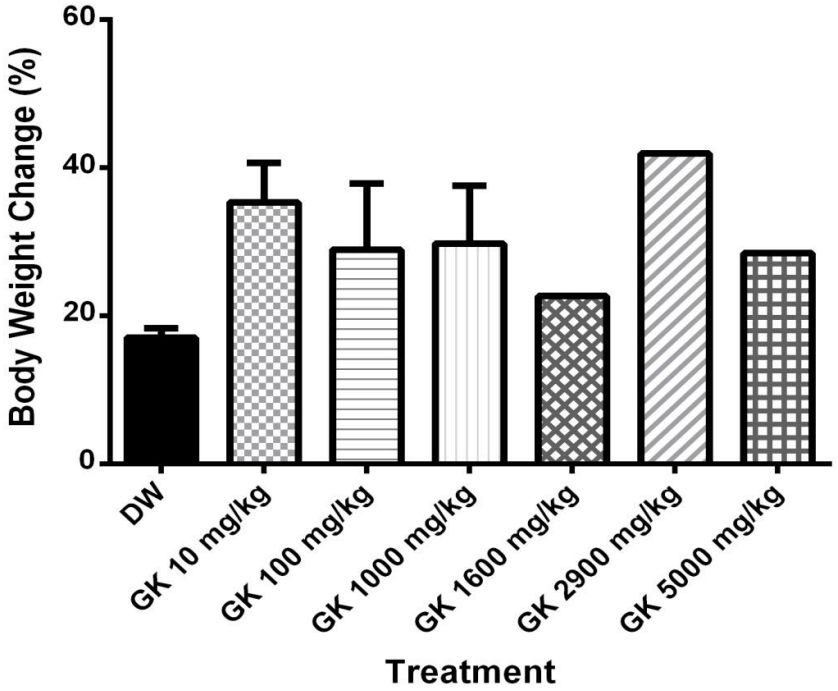

Fig. 1. Percentage body weight change in mice treated with *Garcinia kola* during acute toxicity test

Mice (n=3 for DW, 10, 100, 1000 mg/kg and n=1 for 1600, 2900 and 5000 mg/kg dose) were weighed at the end of the acute toxicity study and the body weight change illustrated.
DW = Distilled water, GK = Garcinia kola

Table 3. Effect of the intraperitoneal administration of methanolic extract of *Garcinia kola* seed on percent organ-body weight ratios of mice following acute toxicity study

Organs	Control (DW)	10 mg/kg bw	100 mg/kg bw	1000 mg/kg bw	1600 mg/kg bw	2900 mg/kg bw	5000 mg/kg bw
Kidneys	1.378 ± 0.054	1.175 ± 0.016	1.304 ± 0.025	1.253 ± 0.020	1.240	1.168	1.316
Liver	6.426 ± 0.063	6.418 ± 0.148	6.370 ± 0.111	6.640 ± 0.061	6.284	6.502	6.117
Lungs	0.829 ± 0.055	0.810 ± 0.095	0.681 ± 0.011	0.731 ± 0.006	0.656	0.703	0.703
Spleen	0.778 ± 0.052	0.684 ± 0.064	0.708 ± 0.083	0.675 ± 0.035	0.646	0.703	0.699
Heart	0.464 ± 0.034	0.407 ± 0.003	0.405 ± 0.009	0.463 ± 0.016	0.477	0.494	0.410

3.3 Effect of the Co-administration of Vaccine and GK Extract on the Humoral Immune Response

The immunogenicity studies revealed HBsAg – specific serum antibody response. Antibodies revealed were the IgG1, IgA and IgM. Following vaccination, the antibody response (total immunoglobulin i.e IgA, IgG1 and IgM) increased from first vaccination through second vaccination and declined after the third vaccination for all the groups (Fig. 3) except the DW groups which did not decline after the third vaccination. The antibody titre (OD) trend as observed were 0.052 ± 0.001 to 0.064 ± 0.007, then 0.075 ± 0.004 for the distilled water (DW) treated group; 0.137 ± 0.009 to 0.337 ± 0.018, then 0.182 ± 0.011 for hepatitis B vaccine (HV) group; 0.104 ± 0.003 to 0.281 ± 0.010, then 0.175 ± 0.007 for *GK* (GK) treated group; 0.113 ± 0.009 to 0.267 ± 0.012, then 0.148 ± 0.010 for the hepatitis B vaccine and the *GK* (HV + GK) combination. The antibody response seen in the HV + GK group was less than that seen in the HV positive control group in all the three vaccinations with the decrease observed after second and third vaccination in the HV + GK group been significant (P<0.05), this decrease was more evident in the IgA specific responses (Fig. 6).

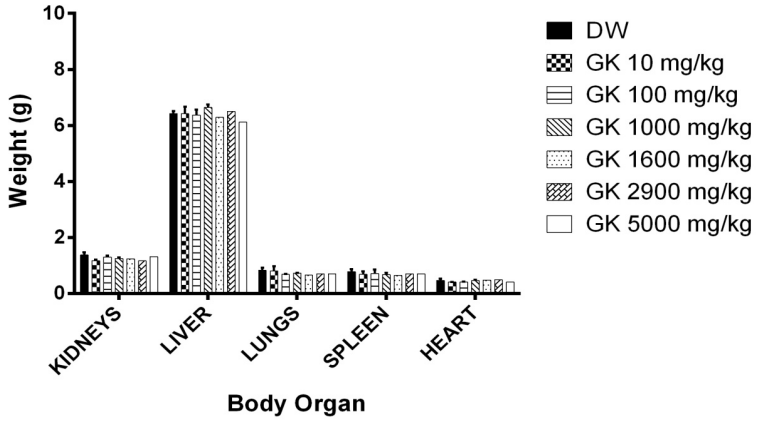

Fig. 2. Percent organ-body weight ratio of mice after *Garcinia kola* acute toxicity study

Mice (n=3 for DW, 10, 100, 1000 mg/kg and n=1 for 1600, 2900 and 5000 mg/kg dose) were sacrificed and vital organs isolated and weighed. The mean organ-body weight ratio was calculated and illustrated.
*DW = Distilled water, GK = Garcinia kola, * Significant at P<0.05.*

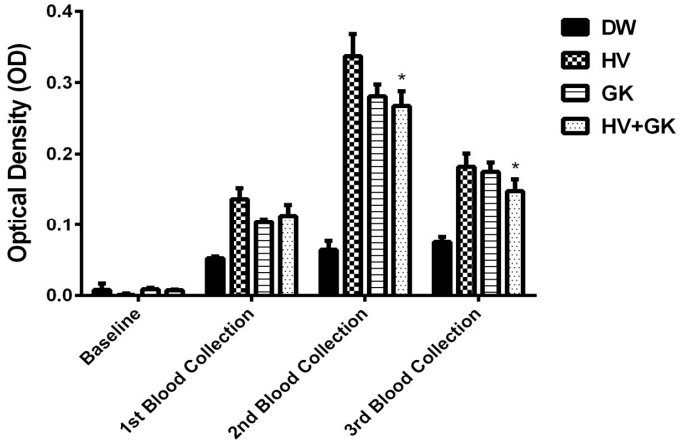

Fig. 3. Total HBsAg – specific serum antibody response

Mice (n=6) were vaccinated 3 times, sera were collected after each vaccination and examined for the presence of HBsAg specific antibodies, this was summed up and presented as total HBsAg – Specific serum antibody
*DW = Distilled water, HV= Hepatitis B vaccine, GK= Garcinia kola, * Significant at P<0.05.*

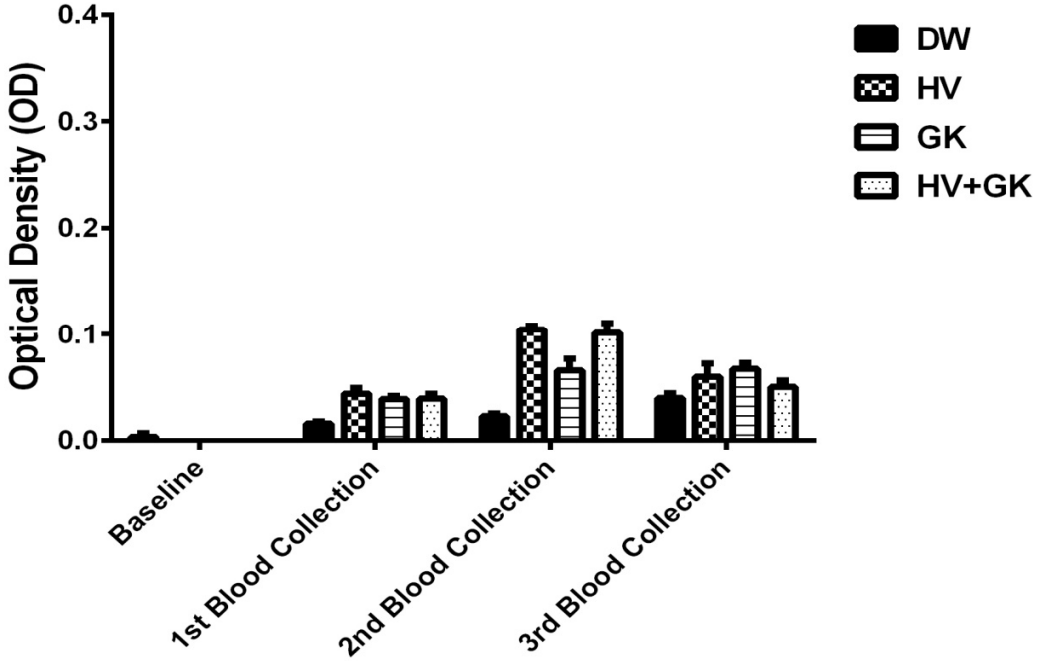

Fig. 4. HBsAg – specific serum IgM response

Mice (n=6) were vaccinated 3 times, sera were collected after each vaccination and examined for the presence of HBsAg specific IgM.

*DW = Distilled water, HV= Hepatitis B vaccine, GK= Garcinia kola, * Significant at P<0.05.*

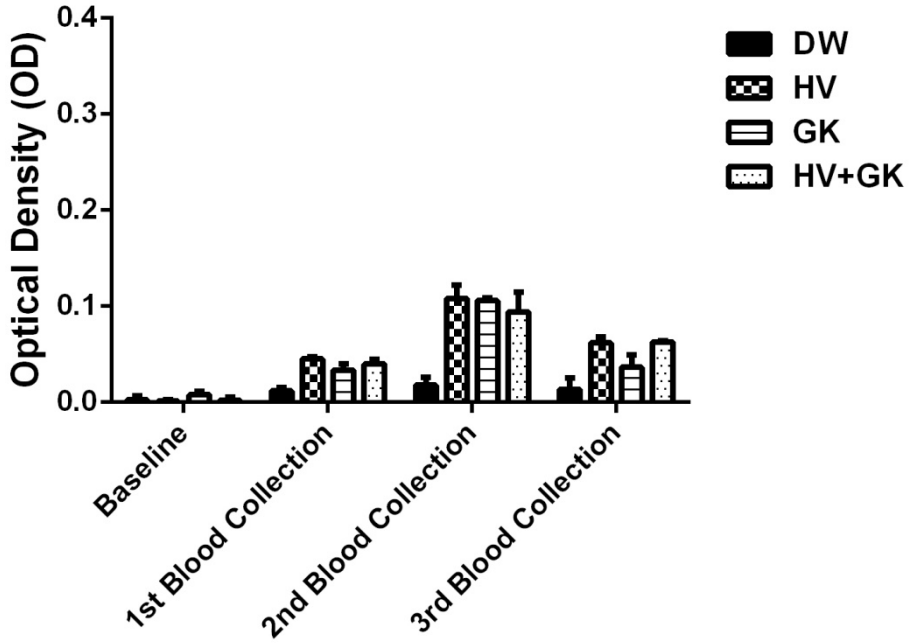

Fig. 5. HBsAg – specific serum IgG1 response

Mice (n=6) were vaccinated 3 times, sera were collected after each vaccination and examined for the presence of HBsAg specific IgG1.

*DW = Distilled water, HV= Hepatitis B vaccine, GK= Garcinia kola, * Significant at P<0.05*

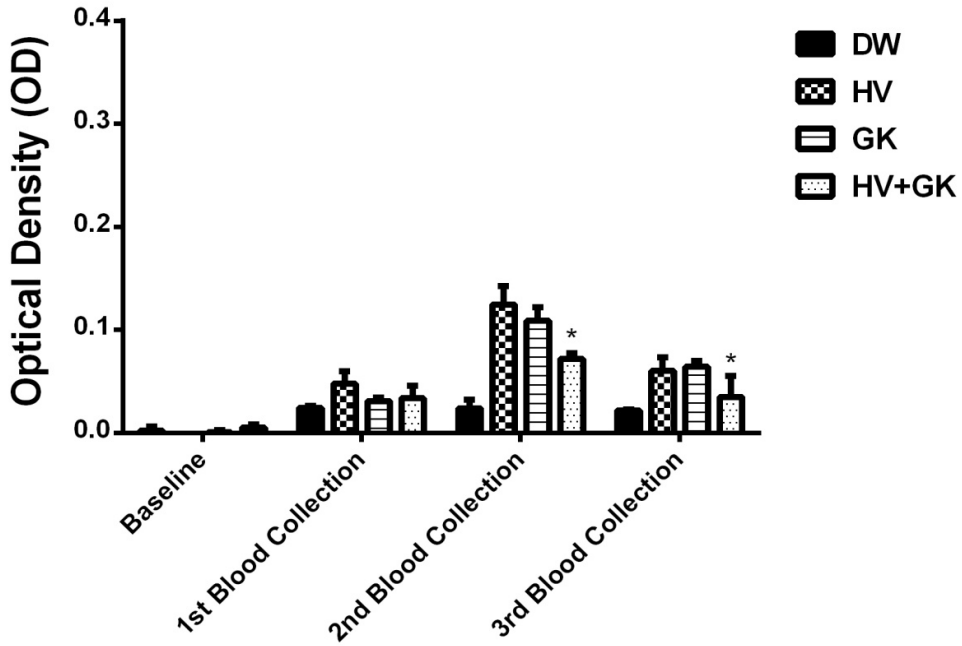

Fig. 6. HBsAg – specific serum IgA response

Mice (n=6) were vaccinated 3 times, sera were collected after each vaccination and examined for the presence of HBsAg specific IgA.

*DW = Distilled water, HV= Hepatitis B vaccine, GK= Garcinia kola, * Significant at P<0.05*

Vaccination is employed in the prevention or elimination of disease because it is possible to induce long-term protective immunity. This long-term protection is conferred by the maintenance of antigen-specific immune effectors and/or by the induction of immune memory cells that may be sufficiently efficient and rapidly reactivated into immune effectors in case of re-exposure to the pathogen. Vaccine-induced immune effectors are essentially antibodies. Antibodies are the main specific and robust defence against pathogens [26]. These antibodies were produced in HV and the HV + GK groups (Fig. 3) with the response as seen in the HV group been higher. It appears GK had a suppressive effect on HV immune response as the response seen in the HV + GK group was significantly (P<0.05) lower than that in the HV group (Fig. 3).

Specifically, IgM, IgG1 and IgA titres were maintained at low level in the HV + GK group (Figs. 5, 6 and 7) compared to the HV group. IgA titre, in particular, was significantly (P<0.05) low in the HV + GK group. The low antibody titre observed in this HV + GK group could be attributed to the presence of GK in the group as the group which received only the hepatitis B vaccine was high. IgM, which is the first antibody to appear in response to an initial exposure to an antigen was suppressed in the HV + GK group (Fig. 4), and this shows that the vaccine-extract combination is no longer immunogenic. Earlier research proved that HV is immunogenic [27,28]. Thus it is the presence of GK in our HV + GK group that made it less immunogenic, thus suggesting that *GK* could inhibit early immune responses to hepatitis B virus surface antigen.

IgG1, the naturally most abundant of all immunoglobulins was equally suppressed. The IgG1 class acts against bacteria and viruses by opsonizing and neutralizing them, thus vaccination with the combination of HV and GK could decrease the rate of opsonisation of hepatitis B virus for recognition and phagocytosis by neutrophils and macrophages.

IgA is principally found in saliva, respiratory, mucosal, intestinal and genital tract secretions [29] and hepatitis B virus is transmitted via this infected body fluid and blood [12,30]. Any vaccination that gives rise to abundant IgA in blood and body fluids will help prevent the transmission of the disease from one person to another. In the presence of GK, however, this may not be achieved.

Although, Chinedu et al. [31] reported that the daily consumption of GK guarantees the longevity of human life due to its hypoglycaemic effect. However, we found on vaccination, its consumption may not be advised as it has been shown to suppress the humoral immune response to hepatitis B vaccine, although Nworu et al. [16] had earlier reported the immune-modulatory action of *G. kola* extract.

3.3 Effect of the Co-administration of Hepatitis B Vaccine and *GK* Extract on Total White Blood Cell Count of Swiss Albino Mice

White blood cells form the basis for immune responses to invading microbes and foreign substances, some white blood cells function in the innate system, whereas others are part of an adaptive immune response. These white blood cells are important components in the surveillance and protection systems of host defence [32]. The total white blood cell count equally showed the HV + GK combination-treated group has low white blood cell count compared to the HV treated group.

3.4 Effect of the Co-administration of Hepatitis B Vaccine and *GK* Extract on Body Weight of Swiss Albino Mice

The result from the periodic assessment of mice body weight showed a progressive increase; from day 1 to day 29 and then decreased down to day 43, this was followed by a rapid increase in body weight up to day 57 (Fig. 8). The Distilled water group grew from 23.73 g ± 0.80 to 24.94 g ± 0.99 to 26.34 g ± 0.99 to 24.69 g ± 0.96 to 27.20 g ± 0.99 while the other groups grew as follows 23.31 g ± 0.73 to 24.09 g ± 0.76 to 24.87 g ± 0.45 to 23.69 g ± 0.61 to 25.12 g ± 0.65 (HV group); 23.65 g ± 0.86 to 25.49 g ± 0.96 to 26.42 g ± 1.01 to 26.10 g ± 1.22 to 27.96 g ± 0.56 (GK group); 25.05 g ± 1.19 to 25.63 g ± 1.03 to 26.57 g ± 1.21 to 26.50 g ± 1.16 to 27.20 g ± 1.15 (HV + GK group); on days 1, 15, 29, 43 and 57 respectively (Fig. 8).

Animal body weight monitoring is important in research studies, as it provides information as regards the health status of the experimental animal especially if infection or a disease state is involved, physiological changes due to administered immunogens could instigate changes in body weight [33]. Periodic body weight monitoring of the mice used in this study revealed a similar growth pattern for all groups (Fig. 8) except for the HV + GK group. The animals showed a progressive increase in body weight from day 1 up to day 29 for all the groups except the HV + GK groups which seems not to be growing, this was followed by a sharp decrease up to day 43, the reason for this sharp decrease is not known, it was however quickly followed by a rapid increase in body weight for all the groups (Fig. 8). The increase in body weight seen can be attributed to growth since the experimental animal was young mice between the ages of 7 and 9 week, thus are expected to grow progressively when properly fed.

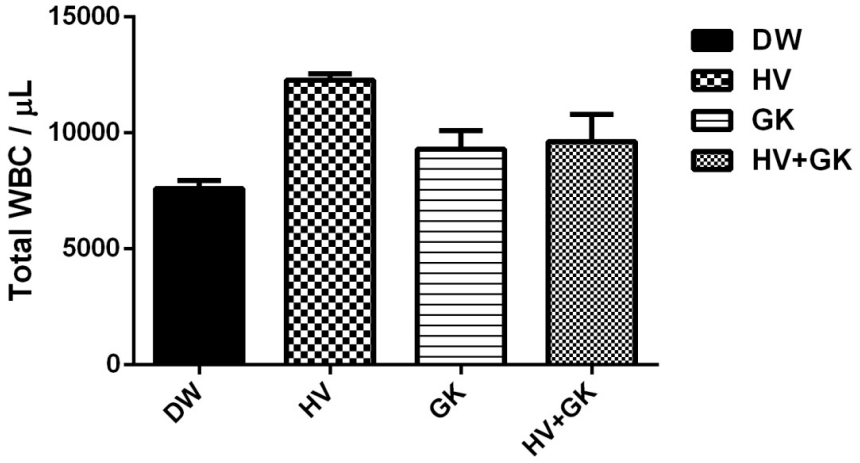

Fig. 7. Total WBC count after vaccination
*DW= Distilled water, HV= Hepatitis B Vaccine, GK= Garcinia kola, * Significant at P<0.05.*

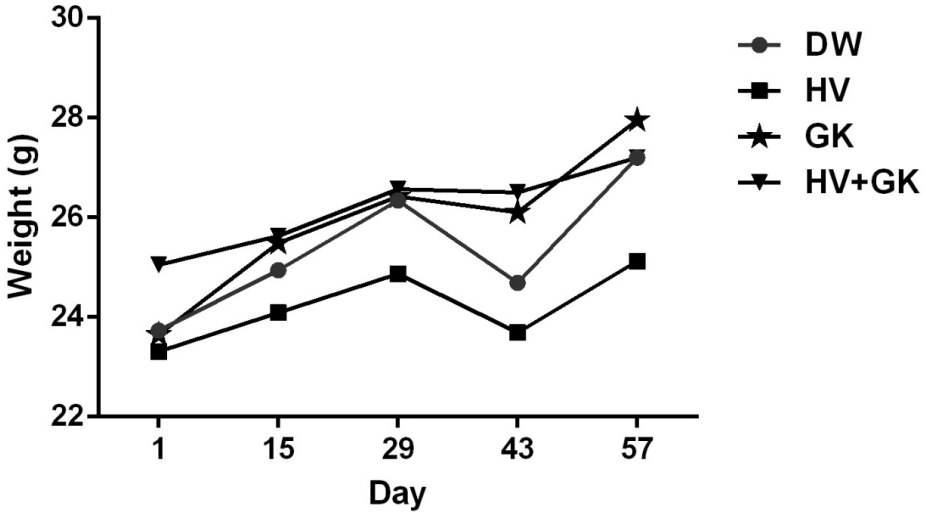

Fig. 8. Mice body weight curve

Mice used for this study were monitored periodically by means of body weight measurement to ascertain their health status.

DW = Distilled water, HV= Hepatitis B vaccine, GK= Garcinia kola.

4. CONCLUSION

The results of our study have shown that GK suppresses the humoral immune response to HBV vaccine. Although the mechanism of this immune suppression is not known, it nevertheless has far-reaching implications for the design and administration of vaccination schedules for HBV vaccines.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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