

SUPPRESSIVE EFFECT OF *GARCINIA KOLA* ON HUMORAL IMMUNE RESPONSE OF mice to HEPATITIS B VIRUS SUBUNIT VACCINE

ABSTRACT

Aim: Due to the therapeutic effects of most medicinal plants, humans have resorted to their frequent and sometimes daily consumption, thus there is a need therefore to investigate the effect of medicinal plants consumption while on vaccination.

Study design: This study was designed to investigate the interference of *Garcinia kola* seed extract and Hepatitis B 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 *Garcinia kola* seeds were obtained, identified, dried, pulverised and stored in an air-tight container until extraction. Cold maceration technique was used for extraction. Methanol was the solvent used. Lorke's method of acute toxicity testing was used to ascertain the toxicity of the extract. Afterwards the experimental animal were grouped and vaccinated accordingly. After vaccination, sera collected from the animal were used for immunogenicity studies while the whole blood was used for total white blood cell count. During the study period the experimental animal were monitored frequently and weighed.

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

Conclusion: The result of the study suggests that 250mg/kg of *G. kola* seed extract have a suppressive effect on hepatitis B surface antigen. Thus *G. kola* should not been consumed while on vaccination so that the antigen in the vaccine can induce the most robust antibody response

Keywords: Vaccine, Hepatitis B virus, *Garcinia kola*, antibody responses, immunogenicity.

40 1 INTRODUCTION

41 Microorganism surrounding our environment causes numerous diseases, most of which have
 42 no cure, making preventive measures the only way out of such diseases. Vaccination exercise
 43 is one of the best known and effective means of preventing infectious diseases as it has
 44 reduced the rate of chronic infection in many countries [1]. Our study evaluated the effect of
 45 *Garcinia kola* seed consumption while on vaccination. *Garcinia kola* is highly valued in
 46 Africa thus highly consumed; it is used socially as refreshment and medicinally for the
 47 treatment of abdominal pain, cough, laryngitis, liver disease, infection and erectile problems
 48 [2]. The seeds are also considered a poison anti-dote. The seeds have been proven to possess
 49 numerous physiological and pharmacological effects which include hepatoprotective,
 50 antiinflammatory, antioxidant, antifertility, haematological and anticancer effects [3]. The
 51 seeds are mostly eaten by the elderly because of their belief that it could prolong life.
 52 Chinedu *et al.* [4] in his report encourages the daily consumption of *Garcinia kola* as it
 53 induces hypoglycaemic effect. Phytochemical screening of the seed revealed the presence of
 54 flavonoids, biflavonoids, xanthenes, triterpenes, cycoartenols, benzophenones, phenol,
 55 alkaloids, saponin and tannin[5]. *Garcinia kola*, locally called bitter kola and known
 56 variously as “Orogbo”(Yoruba), “Namijin gworo”(Hausa) and “Aki ilu” (Igbo) is an
 57 angiosperm, belonging to the family of *Guttiferae*. it is found in moist forest and is widely
 58 distributed throughout west and central Africa.

59 2 MATERIALS AND METHODS

60 2.1 Collection, identification and extraction of plant material

61 *Garcinia kola* seeds were purchased from Ogige market in Nsukka area of Enugu State,
 62 Nigeria and authenticated by a botanist from the Department of Botany, University of
 63 Nigeria, Nsukka having the voucher number UNH NO 55 (University of Nigeria Herbarium
 64 number 55). These seeds were air-dried under shade (for 21 days) to avoid sunlight thereby
 65 conserving the volatile constituent in the plant materials, then pulverized and the powder
 66 obtained were stored in an air tight container until extraction. Methanolic extract of the
 67 pulverized seeds was obtained using cold maceration method [6]. Exactly 300g of the
 68 pulverized *G.kola* was macerated with 2.5 liters of methanol (Sigma-Aldrich) using a glass
 69 jar. The preparation was sealed properly to avoid evaporation. This was shaken vigorously
 70 after 24 hours. At 48 hours, the extract was filtered using a muslin cloth and Whatman No. 1
 71 filter paper. The filtrate obtained was evaporated to dryness at room temperature for 1 week.

72 2.2 Animal

73 Young female Swiss albino mice (7 - 9 weeks old) purchased from Faculty of Veterinary
 74 Medicine, University of Nigeria Nsukka and kept under standard pathogen-free conditions in
 75 an animal facility of the Department of Pharmacology and Toxicology of the University of
 76 Nigeria, Nsukka were used. These animals were fed with standard feed and water *ad libitum*
 77 throughout the study period. A total of 39 animals were used.

78 2.3 Vaccine

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

81

82 2.4 Acute toxicity Study

83 Lorke's method for acute toxicity study, as modified by Bulus *et al.* [7] was employed for the
84 acute toxicity study. The study was carried out in two stages:

85 Stage 1: Twelve (12) mice was used in this stage, these were divided into four groups (A, B,
86 C and D) of 3 mice each. Group A, B, C were given 10, 100 and 1000 mg/kg bw of the
87 methanolic *G. kola* seed extract respectively, Group D (control group) received distilled
88 water (10ml/kg bw). All these were given in a single dose via parenteral route after 4 days of
89 acclimatization.

90 Stage 2: This stage was carried out based on the result of the first stage. Here further specific
91 doses of 1600, 2900 and 5000 mg/kg bw of the extract were administered to 3 mice (one mice
92 per dose) to further determine the correct LD₅₀ value.

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

98 2.5 Vaccination

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

105 2.6 Immunogenicity studies

106 The sera were harvested by intraocular eye puncture and analyzed for IgG1, IgA and IgM
107 rHBsAg specific antibody levels using conventional ELISA method as described by Ternette
108 *et al.* [8]. ELISA plate (Brandplates (immunograde), Wertheim, Germany) were coated with
109 hepatitis B vaccine at a dose of 2 µg/ml (0.2 µg / 100 µl / well) overnight at 4°C in coating
110 carbonate-bicarbonate buffer (pH 9.5). The plates were blocked with phosphate Buffered
111 Saline (PBS) containing 0.05% Tween 80 (PBS-T) and 5% fat free milk for 1 hour at room
112 temperature. The plate was washed three times with PBS-T. Mice sera diluted in PBS-T with
113 2 % fat free milk were added to each well and incubated for 1 hour at room temperature.
114 After washing three times with PBS-T. The IgG1, IgA and IgM subclass specific antibodies
115 conjugated with horseradish peroxidase were added to the different wells and incubated for 1
116 hour at room temperature. The plates were washed as done previously and 3,3',5,5'-
117 Tetramethylbenzidine (TMB) substrate solution (Sigma Aldrich, Taufkirchen, Germany) was
118 added. The reaction was stopped with 2M H₂SO₄ and absorbance at 450nm was taken using
119 an ELISA reader (GM 2000).

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

123 2.7 Determination of total White Blood Count (WBC)

124 Using the method outlined by Verma [9], 0.38ml (380µl) of diluting fluid (10% glacial
125 Acetic acid and 1 drop of Gentian violet) was measured and dispensed into a small tube and
126 0.02ml (20µl) 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:

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

2.8 Periodic body weight assessment

The mice used for the study were weighed periodically using a digital sensitive weighing balance and their weight recorded accordingly.

2.9 Statistical analysis

The data obtained was expressed as mean $\pm$ standard error of mean (Mean $\pm$ 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.

3.0 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 [10]. 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	<i>Garcinia kola</i> seed extract
Weight extracted	300g
Weight of extract	50.1g
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 [11] and it revealed a mean lethal dose (LD₅₀) 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. [12] reported that substances with LD₅₀ values higher than 5000 mg/kg are regarded as being safe or practically non-toxic, thus the extract is practically non-toxic. The LD₅₀ gotten is in line with the findings of Komolafe et al. [13]. At the end of the 14 days monitoring period the percent body weight change and mean organ-body weight ratio were determined. Increase in body weight was noticed in all the groups. The percent body weight change for *G. kola* 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 $17.04 \pm 0.93\%$ (Figure 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 Figure 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 [14]. The organ weight were 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 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 serve as a sensitive indication of the general health status of the experimental animals [15]. The percent body weight change (Figures 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.

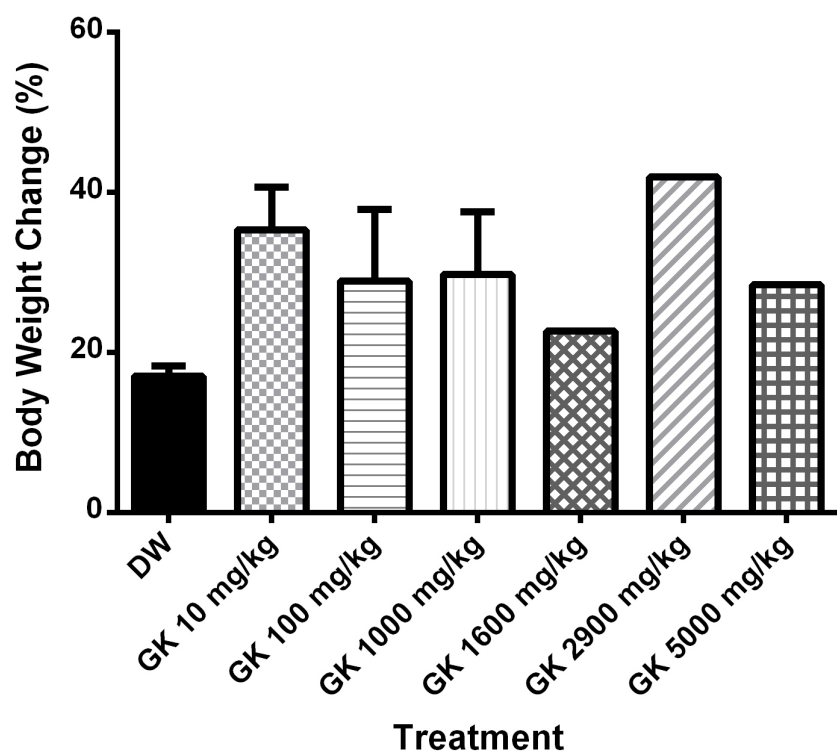
Table 2: Acute toxicity effect of methanolic extract of *Garcinia kola* seed administered intraperitoneally to Swiss albino mice

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

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)	10mg/kg bw	100mg/kg bw	1000mg/kg bw	1600mg/kg bw	2900mg/kg bw	5000mg/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

187



188

189 **Figure 1: Percentage body weight change in mice treated with *Garcinia kola* during**
 190 **acute toxicity test**
 191 **Mice (n=3 for DW, 10, 100, 1000 mg/kg and n=1 for 1600, 2900 and 5000 mg/kg dose)**
 192 **were weighed at the end of the acute toxicity study and the body weight change**
 193 **illustrated.**
 194 **DW = Distilled water, GK = *Garcinia kola*.**

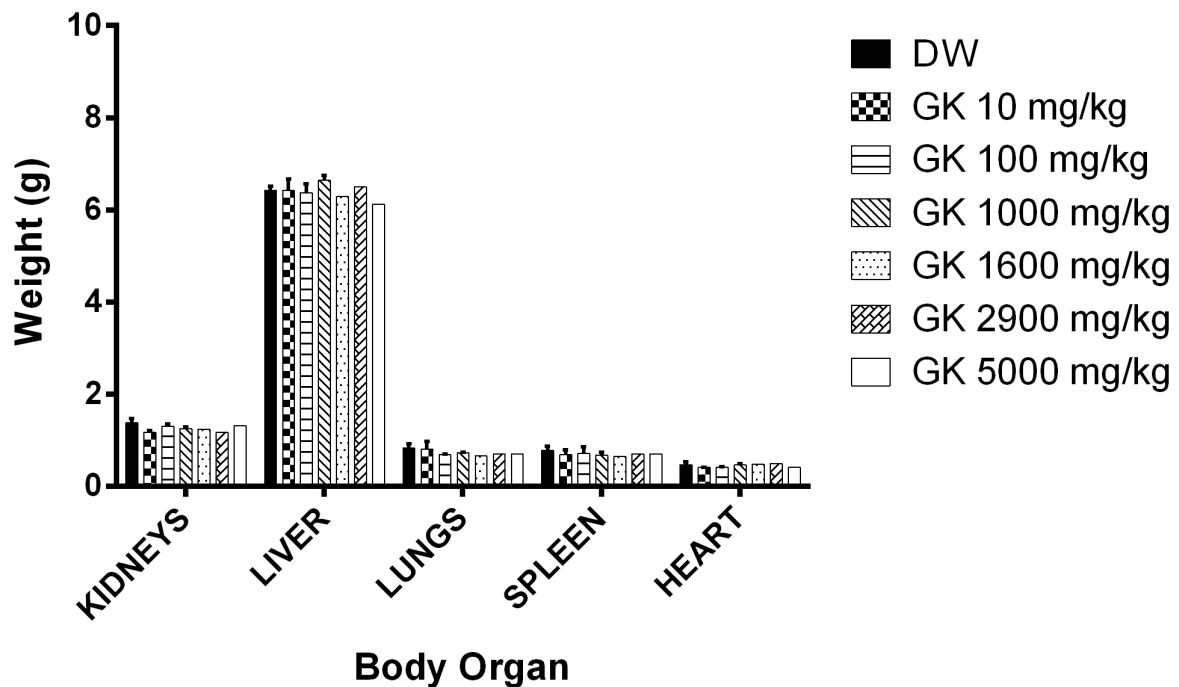


Figure 2: Percent organ-body weight ratio of mice after *Garcinia kola* acute toxicity study

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 $P<0.05$.

3.3 Effect of the co-administration of vaccine and *G. kola* extract on 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 (Figure 3) except the DW groups which did not decline after third vaccination. The antibody titre (OD) trend as observed are 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 *G. kola* (GK) treated group; 0.113 ± 0.009 to 0.267 ± 0.012 , then 0.148 ± 0.010 for the hepatitis B vaccine and the *G. kola* (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 is more evident in the IgA specific responses (Figure 6).

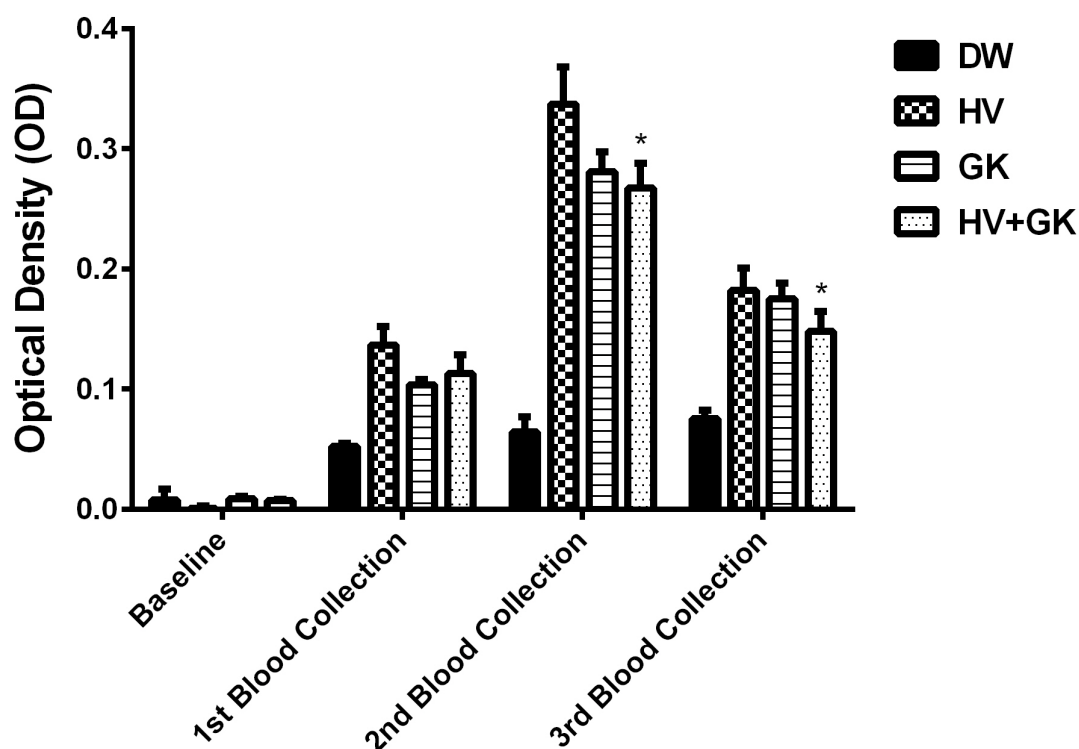


Figure 3: Total HBsAg – Specific serum antibody response

Mice (n=6) were vaccinated 3 times, sera was 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 $P<0.05$.

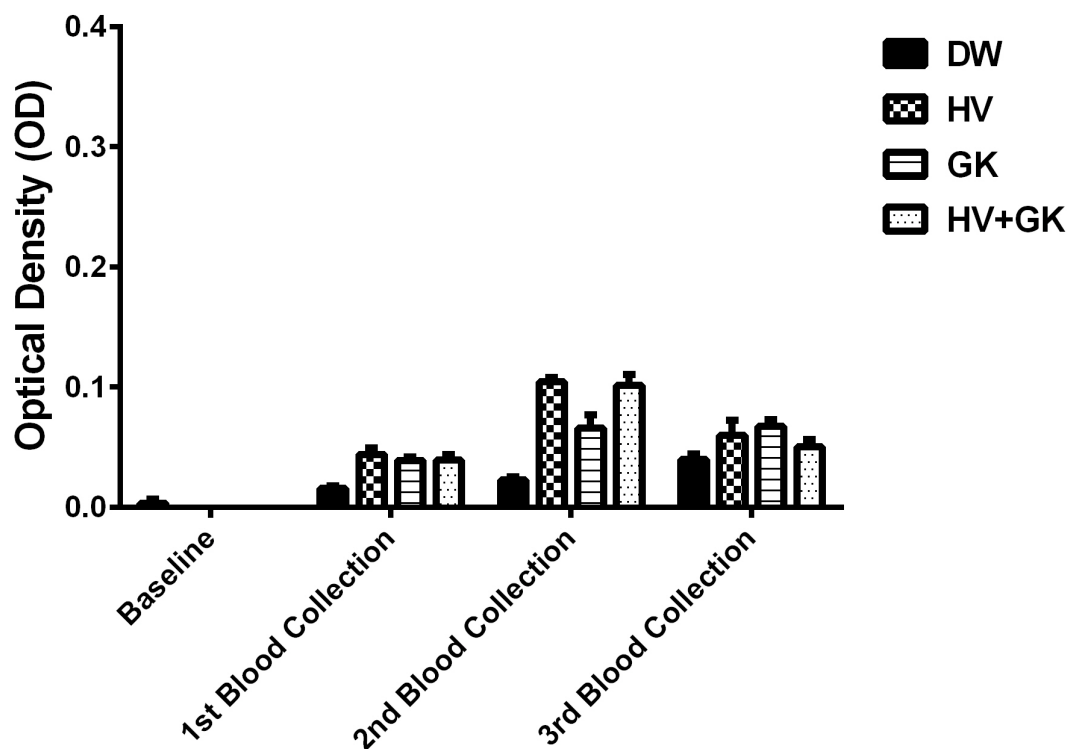


Figure 4: HBsAg – Specific serum IgM response

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

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

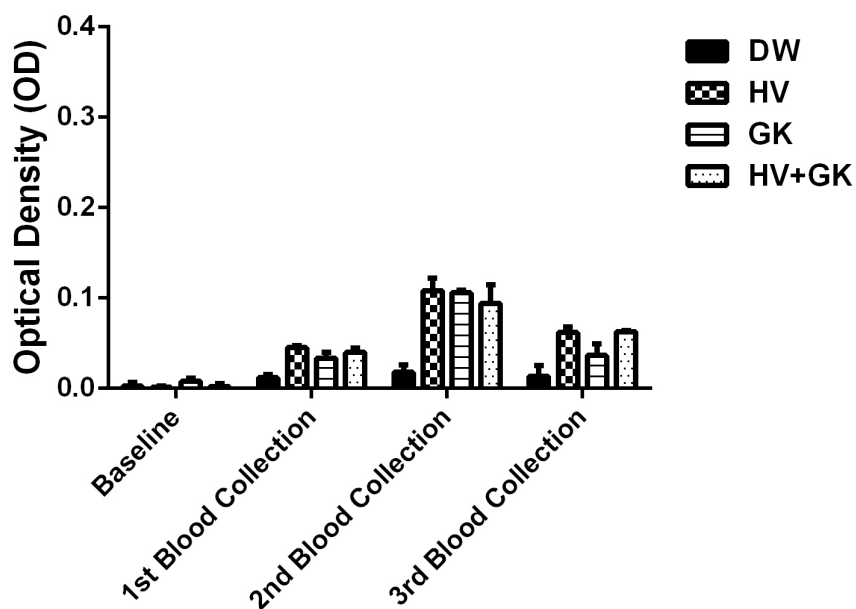


Figure 5: HBsAg – Specific serum IgG1 response

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

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

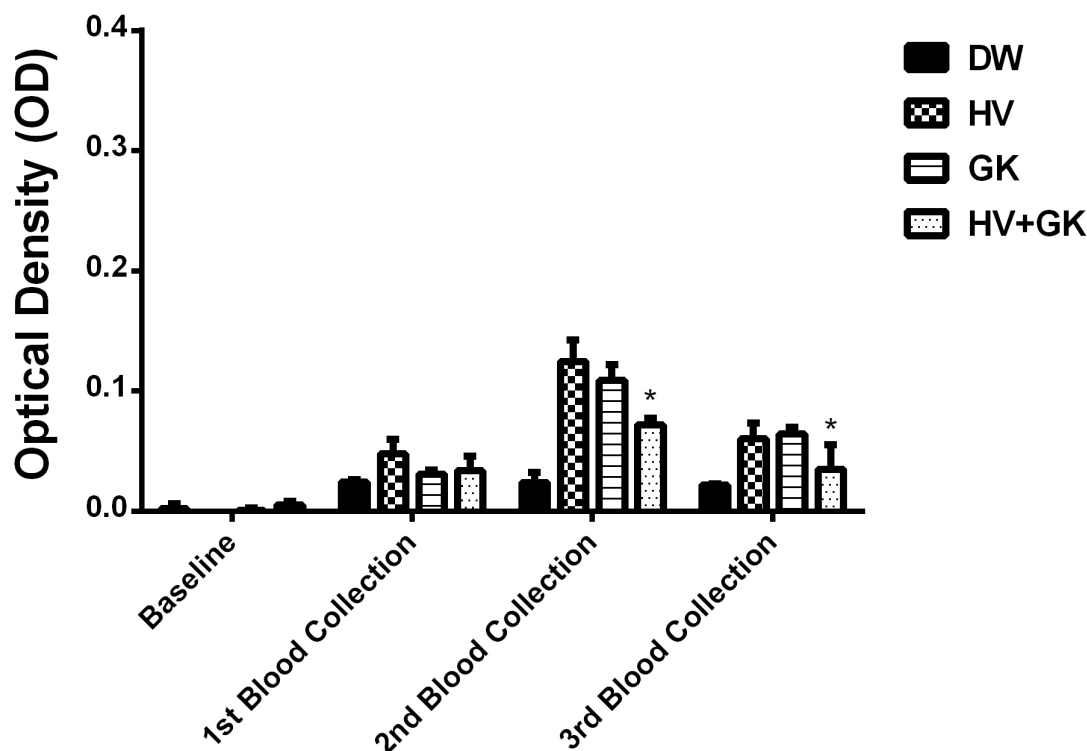


Figure 6: HBsAg – Specific serum IgA response

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

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

Vaccination is employed in the prevention or elimination of disease because it is able 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 pathogen exposure. Vaccine-induced immune effectors are essentially antibodies. Antibodies are the main specific and robust defence against pathogens [16]. These antibodies were produced in HV and the HV + GK groups (Figure 3) with the response 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 (Figure 3).

Specifically, IgM, IgG1 and IgA titres were maintained at low level in the HV + GK group (Figures 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 *G. kola* 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 (Figure 4), this shows that the vaccine-extract combination is no longer immunogenic. Earlier research proved that HV is immunogenic [17; 18]. Thus it is the presence of *G. kola* in our HV + GK group that made it non immunogenic, this suggest *G. kola* could inhibit early response 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 *G. kola* would decrease the rate of opsonisation of hepatitis B virus for recognition and phagocytosis by neutrophils and macrophages.

IgA is principally found in saliva, respiratory, intestinal and genital tract secretions [19] and hepatitis B virus is transmitted via this infected body fluid and blood [20; 4]. 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 *G. kola* however this will not be achieved.

Although, Chinedu *et al.* [21] reported that the daily consumption of *G. kola* guarantees the longevity of human life due to its hypoglycaemic effect. On vaccination however, its consumption will not be advised as it has been shown to suppress humoral immune response to hepatitis B vaccine. Nworu *et al.* [22] have earlier reported the immunomodulatory action of *G.kola* extract.

3.3 Effect of the co-administration of hepatitis B vaccine and *G. kola* 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 [23]. 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.

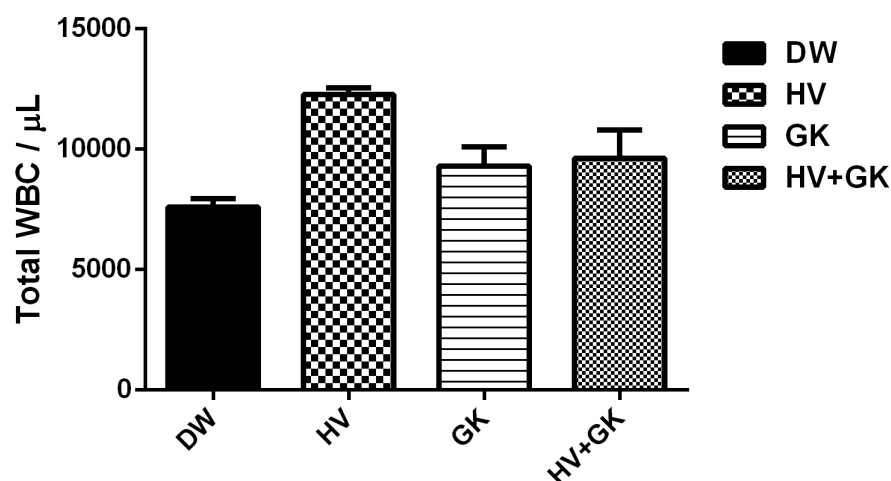


Figure 7: Total WBC count after vaccination. DW= Distilled water, HV= Hepatitis B Vaccine, GK= *G. kola*, * Significant at $P<0.05$.

3.4 Effect of the co-administration of hepatitis B vaccine and *G. kola* extract on body weight of Swiss albino mice

Result from the periodic assessment of mice body weight showed 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 (Figure 8). The Distilled water group grew from $23.73 \text{ g} \pm 0.80$ to $24.94 \text{ g} \pm 0.99$ to $26.34 \text{ g} \pm 0.99$ to $24.69 \text{ g} \pm 0.96$ to $27.20 \text{ g} \pm 0.99$ while the other groups grew as follows $23.31 \text{ g} \pm 0.73$ to $24.09 \text{ g} \pm 0.76$ to $24.87 \text{ g} \pm 0.45$ to $23.69 \text{ g} \pm 0.61$ to $25.12 \text{ g} \pm 0.65$ (HV group); $23.65 \text{ g} \pm 0.86$ to $25.49 \text{ g} \pm 0.96$ to $26.42 \text{ g} \pm 1.01$ to $26.10 \text{ g} \pm 1.22$ to $27.96 \text{ g} \pm 0.56$ (GK group); $25.05 \text{ g} \pm 1.19$ to $25.63 \text{ g} \pm 1.03$ to $26.57 \text{ g} \pm 1.21$ to $26.50 \text{ g} \pm 1.16$ to $27.20 \text{ g} \pm 1.15$ (HV + GK group); on days 1, 15, 29, 43 and 57 respectively (Figure 8).

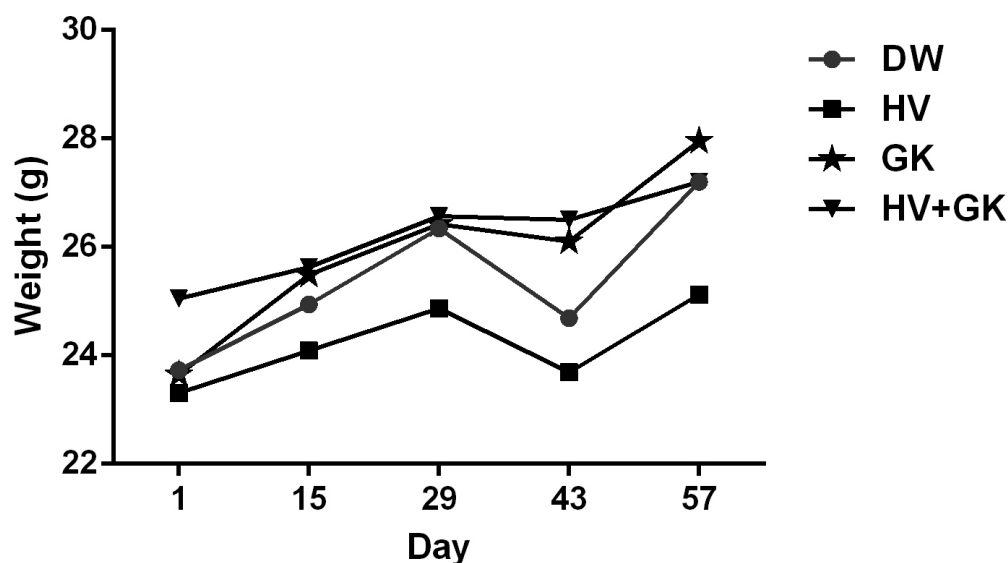


Figure 8: Mice Body Weight Curve

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*.

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 [24]. Periodic body weight monitoring of the mice used in this study revealed a similar growth pattern for all groups (Figures 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 (Figure 8). The

increase in body weight seen can be attributed to growth since the experimental animal were young mice between the ages of 7 and 9 week, thus are expected to grow progressively when properly fed.

4.0 CONCLUSION

From the results seen in this study, one can deduce *G. kola* although non- toxic, can suppress humoral immune response to hepatitis B vaccine; though the mechanism by which this occurred is not known, it could be that the *G. kola* extract inhibited the stimulation of the immune system through many cytokines inhibition. Suppression of the humoral immune response by *G. kola* extract has implication for the design of immunization schedule to protect the immunosuppressed and raises the possibility that *G. kola* use may be accompanied by more or various infection that complicates more conventional immunosuppression. More importantly, consideration should be given to harnessing the relatively specific effect of *G. kola* on antibody production to treat antibody-mediated diseases and in organ transplant.

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