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Evaluation of *Vigna subterranea* seed Extract as Possible Adjuvant for Typhoid Fever Vaccine

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Authors' contributions

This work was carried out in collaboration between all authors. Author DCO designed the study and wrote the protocol. Authors DCO and CO wrote the protocol and performed the experiments. Authors DCO and IAO managed the analyses of the study, statistics, literature searches, and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Typhoid fever is an essential communicable disease that is endemic in developing countries characterised by poor water supply, sanitary conditions and increasing population. Vaccination with the *Salmonella typhi* (ST) vaccine is an effective control measure. The two currently licensed ST vaccines are saddled with a lot of drawbacks which can be overcomed by the addition of adjuvant. *Vigna substerranea* (VS) ethanolic extract was evaluated as a possible adjuvant to ST vaccine. Mice were vaccinated with typhoid vaccine followed by daily weight measurement, and enumeration of microbial colony counts post-vaccination ST challenge. The blood culture results showed that mice group that received our formulated vaccine had the lowest microbial load (82.2±29.72) colony forming units (CFU) following microbial challenge, the mice body weight assessment results also emphasize this as the mice in this treatment group had an uninterrupted healthy growth. Further, from our results VS extract demonstrated a relative inability to function as effective adjuvant to the ST vaccine since the microbial load in this group (vaccine + VS extract) was high (152.3±47.67) hence the ineffectiveness of combining both agents.

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1. INTRODUCTION

Typhoid fever, a systemic infection caused by *Salmonella enterica* serotype *Typhi* (*S. typhi*) remains a significant global public health problem as it is a major cause of death in the developing countries [1]. In 2000, it was estimated that over 2.16 million episodes of typhoid occurred worldwide, resulting in 216 000 deaths [1]. This infection is highly prevalent in Nigeria [2,3,4]. Typhoid fever which has been reported in all age groups and classes in Nigeria [5] is commonly acquired from water or food contaminated by the faeces of an infected person. This infection occurs all year round in Nigeria but higher in April and July which coincides with the height of the hot, dry season and the onset of the rainy season, respectively [6]. In south east Nigeria, the highest number of cases of typhoid fever are recorded during the rainy season [7]. The onset of the illness is insidious, characterised with symptoms such as the gradual onset of sustained fever, chills and abdominal pain. In some cases, patients experience rash, nausea, anorexia, diarrhoea or constipation, headache, relative bradycardia and reduced level of consciousness [8]. Patients with untreated typhoid fever are reported to have case-fatality rates >10% [8] while those with early and appropriate antibiotic treatment is typically <1% [9]. Although improved water quality and sanitation constitute ultimate solutions to this problem, vaccination in high-risk areas is a potential control strategy recommended by WHO for the short-to-intermediate term [10]. This importance of vaccination is heightened by the increasing resistance of the aetiological agent to antimicrobial agents, including fluoroquinolones, in many parts of the world [11]. In 2003, the World Health Organization recommended strategies to reduce typhoid fever which are routine immunisation of school-aged children, immunisation of children beginning at 2 years of age in sites where typhoid fever occurs in younger children [10] and immunisation of travellers going to endemic areas. Two safe and efficacious typhoid vaccines, the injectable Vi capsular polysaccharide vaccine (Typhim Vi, manufactured by Sanofi Pasteur) and the oral live attenuated vaccine (Vivotif, manufactured from the Ty21a strain of Salmonella serotype Typhi by PaxVax), have been licensed; and new, improved candidate vaccines are currently being tested [12-14]. The Vi polysaccharide vaccine is administered as a single injection and is approved for adults and children aged ≥2 years and is given at least 2 weeks before potential exposure. The oral Ty21a vaccine is administered in 4 doses on alternating days over 1 week and is approved for adults and children aged ≥6 years; this is usually administered one week before potential exposure. Repeat doses of typhoid vaccine are needed to maintain immunity when repeated exposure to the *Salmonella typhi* is expected. An optimal revaccination schedule for the Vi polysaccharide vaccine has not been established; however, the manufacturer recommends a repeat dose every 2 years after the primary dose if continued or renewed exposure is expected [15]. The manufacturer of Ty21a recommends revaccination with the entire 4-dose series every 5 years if continued or renewed exposure to *Salmonella* serotype *Typhi* is expected [16]. An effective adjuvant can eliminate the need for revaccination or atleast ensure revaccination at a much longer time interval. Additionally, a suitable adjuvant can equally allow immunization with fewer doses of this oral vaccine, enabling reductions in the quantity of antigen contained in an individual vaccine dose, and can be used to increase the response to this vaccine especially in the under-aged population [16-18].

Vigna subterranea (L.) *Verdc* also was known as Bambara groundnut, Bambara bean, "Okpa", earth pea, Congo goober, hog-peanut or ground bean to natives is evaluated as an adjuvant to typhoid fever vaccine. This legume, whose origin has been traced back to Africa, is the third important legume after groundnut (*Arachis hypogea*) and cowpea (*Vigna unguiculata*) [19] however, it is one of the underutilized crops [20]. It is highly nutritious, and has been termed a complete food. Its seed consists of 50–65% carbohydrate, 15–20% protein, 4–9% fat, 3–5% fiber, 3.2–4.4% ash and 2% mineral [21]. Aside nutrition, there exist another broad category of health benefit exhibited by this plant which is its use in traditional medicine therapies. The Seeds are used by the Igbo tribe in Nigeria to treat venereal diseases, and, according to the Luo tribe in Kenya, seeds are also used to overcome diarrhea [21]. The consumption of roasted seeds is recommended in the treatment of polymenorrhea. Among the beans family, *Vigna subterranea* seeds have the highest concentration of soluble fiber, these are a non-nutrient believed to reduce the incidence of heart disease and to help prevent colon cancer. The

chewing and swallowing of immature fresh seeds is believed to arrest nausea and vomiting; this remedy is often used to treat morning sickness in pregnant women [22]. The seeds have also been used to treat some malignancies and inflammatory disorders in Africa [21]. The antioxidant and antimicrobial properties of *Vigna subterranea* has scientifically been reported [21-25], and thus might be a useful immunological adjuvant.

2. MATERIALS AND METHODS

2.1 Plant Collection, Identification and Extraction

VS 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. This was pulverized and the powder obtained was stored in an air tight container until extraction. Ethanolic extract of the fine powder was obtained using cold maceration technique [26] in which 250g of powder was macerated with one (1) liters of absolute ethanol in a glass jar. The preparation was sealed properly to avoid evaporation. After 24 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.

2.2 Test Animals

Twenty-five (25) young female Swiss albino mice (8 - 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 chicks grower mesh (vital feed) and water *ad libitum* throughout the study period.

The use and care of the laboratory animals in this present study was done in accordance with the "NIH guidelines for laboratory animal care and use" [27] and the University of Nigeria regulations for laboratory animal use.

2.3 Typhoid Fever Vaccine

The vaccine used was prepared locally by heat denaturation method following the steps outlined below.

2.3.1 Collection and isolation of *Salmonella typhi* (ST)

ST was obtained from the University of Nigeria Teaching Hospital, Ituku-Ozalla Enugu. Pure culture of the isolate was prepared by subculturing into freshly prepared Salmonella-shigella agar by streak method and then incubated at 37˚C for 24 hours. After incubation, distinct colonies were again transferred/subcultured into freshly prepared and sterilized nutrient broth and incubated for another 24 hours.

2.3.2 Determination of bioload

Exactly 1 ml of the broth culture of the microorganism ST was collected and transferred aseptically into a test tube containing 9ml of sterile water and this was tagged 10^{-1} (10-fold serial dilution); this was continued to the ninth test tube (10^{-9}) and the last 1ml (i.e. from this test tube) transferred into a beaker to be discarded appropriately. Nine well-labeled nutrient agar plates corresponding to the nine test tubes (10^{-1} to 10^{-9}) were each divided into 8 sections and from each test tube/dilution (e.g. 10^{-2}), one drop each on the 8 sections were made (a total of 8 drops of that same dilution) on the corresponding nutrient agar plate. The plates were then incubated at 37˚C for 24 hours, after which the viable cell count (using an appropriate dilution, i.e. one that is clear enough to be counted) was done to determine the bioload or concentration of the microorganism.

2.3.3 Determination of death time of the microorganism

A loopful was transferred from the selected dilution test tube at time 0, into a recovery test tube containing nutrient broth. The test tube with its content was then heated (at a temperature of about 55-60˚C) and at various time intervals (i.e. 10, 20, 30, 40, 50, 60, 70, 80, 90minutes), subculturing into the respective recovery test tubes (labeled according to the time). The recovery test tubes were then incubated for 48 hours at 37˚C, after which the test tubes were examined for microbial growth (indicated by turbidity) so as to determine the death time.

2.3.4 Formulation of ST vaccine

The selected broth culture was centrifuged at 3000 revolutions per minute for 5 minutes. The supernatant was then removed with the aid of the

micropipette and the cells washed twice with normal saline by centrifuging and removing the supernatant in each case. The cells were then re-suspended in a specified volume of normal saline (5 ml) and heated (at the same temperature) for a period of time equivalent to the predetermined death time. The vaccine, thus formulated, was then aseptically transferred into bijou bottles and made up with normal saline, labeled appropriately and stored in the refrigerator.

2.4 Vaccination

The experimental animal were divided into five groups named A, B, C, D and E of five mice each and vaccinated intraperitoneally as follows; Group A received absolute ethanol (0.4 ml) only (solvent used in extraction), Group B received 500mg of the VS extract /kg body weight only, Group C received normal saline only (solvent used in vaccine constitution), Group D received 0.4ml of the vaccine only (which contains 10^8 cells). Group E received 0.4 ml of the vaccine and 500 mg of VS extract/kg body weight of the mice. The vaccination was repeated once after two weeks. Blood samples were collected from the mice by intraocular eye puncture using the method described by [19] at 1 week and 2 weeks post first vaccination and 1 week post second vaccination.

2.5 Weight Monitoring of the Experimental Animals

From day 1 post second vaccination, each of the experimental animals was weighed on a daily basis using a digital sensitive weighing balance and the weight recorded accordingly. This continued till the animals were sacrificed.

2.6 Challenge of Animals with Live ST

After the 1 week post second vaccination blood sample collection, the experimental animals were challenged on the 3rd day with 10^7 live ST organisms contained in 0.04ml preparation through the intraperitoneal route.

2.7 Blood Culture

Blood culture is the most definitive method of diagnosing typhoid fever, especially in the 1st week of infection [28] thus four days after the challenge, blood samples were collected from each of the mice and 2 fold serial dilutions of the blood samples were done by diluting 25µL of the blood sample with 25 µL of normal saline. 10µL of each of the diluted blood samples was then aseptically placed on each of the respective portions of the properly labeled nutrient agar plates. This was carried out for all the mice. The plates with its contents were incubated for 24 hours at 37°C and then examined for growth of microorganisms via colony count.

2.8 Statistical Analysis

The data obtained was expressed as mean ± standard error of mean (Mean ± SEM). 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. RESULTS

3.1 Mice Body Weight

From the periodic mice body weight monitoring, the mice were seen to grow progressively in all the groups except the normal saline treated group which had a decline from the 10th day (Figs. 1-6).

3.2 Blood Culture

From the blood culture, distinct colony forming units (cfu) were seen which were counted and recorded as shown in Fig. 7.

4. DISCUSSION

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 [29]. ST infection is known to be associated with significant weight loss [1] hence the need for weight monitoring of the mice in the different treatment groups. The weight assessment results showed a pronounced decrease in weight after challenge for the normal saline treated group C (Fig. 4) compared to other groups this is attributed to the fact that normal saline has no antimicrobial, prophylactic and therapeutic effects thus serving as control, it equally shows that the organism administered during challenge are live and viable ST. The ST vaccine group D (Fig. 5) had an uninterrupted growth despite the challenge and this goes a

long way to buttress the observation that our laboratory-prepared vaccine is efficacious, thus offering immunity to the infection. The (vaccine + adjuvant) group E (Fig. 6) also had a progressive growth after the challenge possibly because of the vaccine present in the formulation. The ethanol-treated group A (Fig. 2) although slightly interrupted at day 12 equally had a progressive growth. This progressive growth despite the microbial challenge can be attributed to the inherent effect of ethanol which could have provided a sterilizing effect (antimicrobial activity) which may have inhibited the growth of the ST administered during the challenge. The VS treated group B (Fig. 3) also had a slightly disturbed progressive growth. The antimicrobial properties of this plant could provide an explanation of the limitation posed to ST invasiveness in the group of mice hence the positive growth curve observed for the mice.

The blood culture results (Fig. 7) shows the relative performnace of the various treatment administered as prophylaxes against ST challenge. The outcome further supports the results obtained from the periodic mice body weight assessment. The very high microbial load (200 ± 0 CFU) seen in the normal saline treated group is expected since it is the negative control substance lacking antimicrobial, prophylactic and therapeutic potentials. The vaccine-treated group had the lowest microbial count (82.2 ± 29.72 CFU) because of the prophylactic effect of the formulated vaccine. The (vaccine + VS) group altough had a relatively high microbial load (152.3 ± 47.67 CFU), it is however less than that of the normal saline control group possibly because of the vaccine presence. However, the microbial load in this group is higher than what was seen in the group treated with vaccine only, suggesting that VS may not serve as an effective adjuvant to typhoid fever vaccine. Surprisingly, the VS-treated group B recorded a low microbial count of 85.5±38.52 CFU, and this could be attributed to the antimicrobial effect of the plant [15-18]. It is of course understandable that the ethanol solvent (although possessing antimicrobial properties) could not prevent the growth of the challenge ST organism in the mice since the amount administered (0.4 ml) is very small. Hence, a relatively high microbial load of 129.0±41.51 was recorded. It is not very clear why this was not reflected in the mice body weight. This disconnetion may be as a result of the robust immune status of the mice used in this group which prevented such weight loss outcome. Nevertheless, microbial enumeration in blood culture of challenged animals would serve as a more specific assessment of the effectiveness of the administered treatments viz-a-viz the microorganisms present in an animal host system.

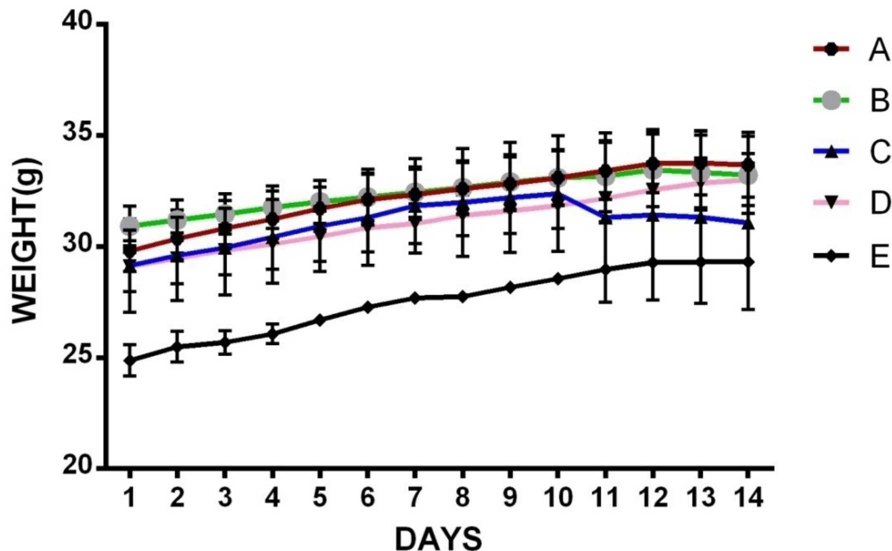

Fig. 1. Mice body weight curve

A= Ethanol; B= VS (500mg); C= Normal saline; D= ST vaccine (0.4ml = 10^8cells); E= ST vaccine (0.4ml =10^8cells) + VS (500mg)

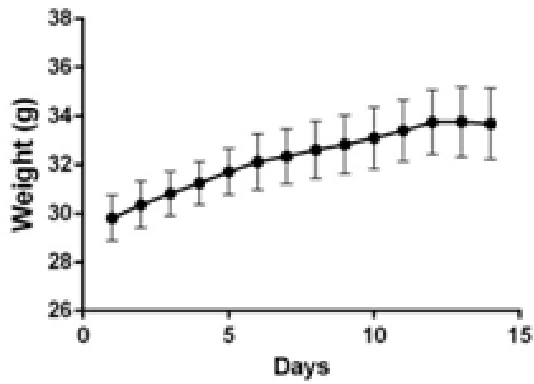

Fig. 2. Weight curve of ethanol treated group

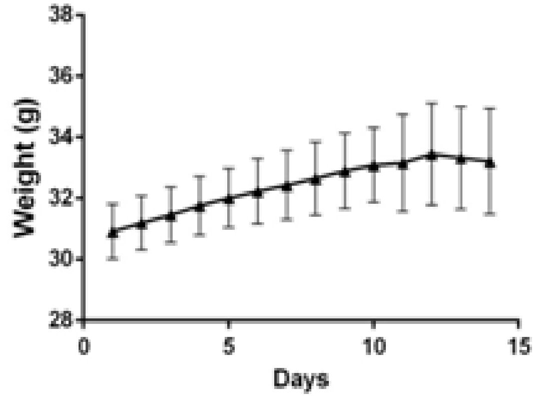

Fig. 3. Weight curve of *Vigna subterranea* (500mg) treated group

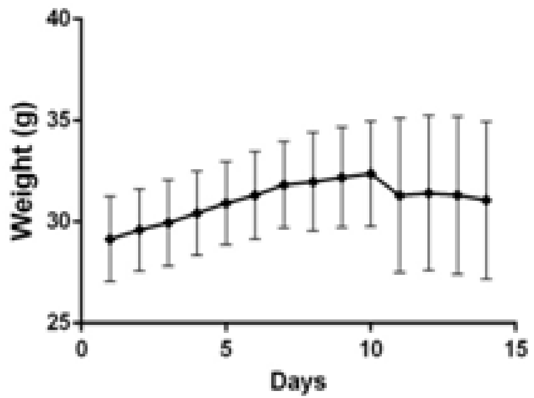

Fig. 4. Weight curve of normal saline treated group

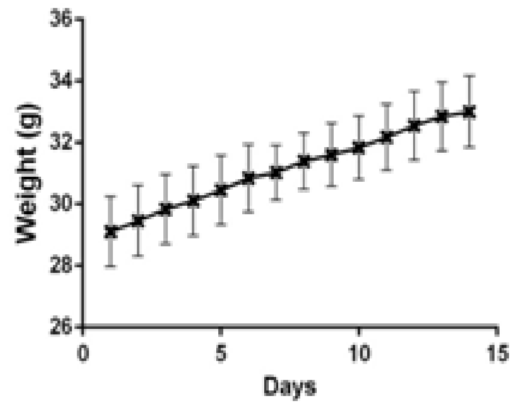

Fig. 5. Weight curve of *Salmonella typhi* vaccine treated group

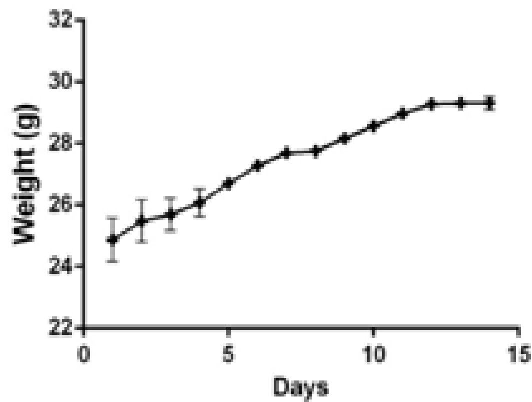

Fig. 6. Weight curve of *Salmonella typhi* vaccine + *Vigna subterranea* (500mg) treated group

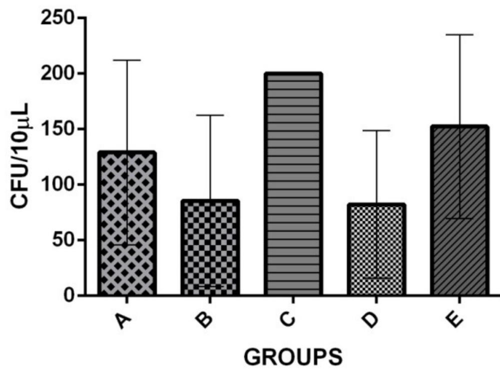

Fig. 7. A graphical representation of microbial count following blood culture

A= Ethanol; B= VS (500mg); C= Normal saline
D= ST vaccine (0.4ml =10^8 cells)
E= ST vaccine (0.4ml =10^8 cells) + VS (500mg)

5. CONCLUSION

Our work has evaluated the potential of VS as adjuvant for ST vaccine. Our findings showed that the combination of VS and ST vaccine demonstrated improved clinical outcome in comparison to the negative control (normal saline) only, but not to the ST vaccine-alone group. This result reflects the relative inefficacy of combining both agents. Therefore, the need for continuous search for adjuvants to ST vaccine to boost the immune response against ST infection is recommended.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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