MORINGA OLEIFERA LEAF EXTRACT AND ITS PROMISING SYNERGISTIC ANTIMICROBIAL EFFECT WITH TYPHOID FEVER VACCINE IN IMMUNIZED MICE

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EKSTRAKT LIŠĆA MORINGA OLEIFERA I NJEGOV OBEĆAVAJUĆI SINERGISTIČKI ANTIMIKROBNI EFEKAT SA VAKCINOM PROTIV TIFUSNE GROZNICE KOD IMUNIZOVANIH MIŠEVA

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Received/Primljen: 09.09.2018. Accepted/Prihvaćen: 12.12.2018.

ABSTRACT

Typhoid fever, a systemic infection caused by Salmonella typhi has maintained a high morbidity and mortality profile around the globe especially in developing countries. Though currently licensed vaccines are efficacious in prevention of the infection, their potency is ephemeral; hence, they require a boost by employing adjuvants that are safe and instrumental in achieving a better prolonged protective immune defense outfit. In this work, Moringa oleifera ethyl acetate leaf extract was evaluated for its possible adjuvant property to a heat-killed ST vaccine. Mice were vaccinated with typhoid vaccine and subsequently, daily weight of mice was measured. Also, post-vaccination microbial colony counts were enumerated after challenging the mice with Salmonella typhi cells. From the blood culture results, MO extract demonstrated an excellent synergistic antimicrobial effect as the mice group administered our formulated vaccine-MO extract combination had the lowest microbial load (12.25 ± 4.86) colony forming units following microbial challenge, when compared to the mice groups administered the vaccine alone (37.25 ± 4.5) and the MO extract alone (31.25 ± 9.43). Furthermore, assessment of the mice body weight of treated groups showed a growth pattern that did not deviate significantly from those of the control group. In conclusion, MO extract demonstrated a promising synergistic antimicrobial effect on coadministration with the typhoid fever vaccine against S. typhi and did not lead to adverse side effects in mice.

Keywords: *Typhoid fever, Moringa oleifera, Salmonella typhi, adjuvant, colony forming units, body weight.*

SAŽETAK

Tifusna groznica, sistemska infekcija izazvana Salmonelom tiphi održavala je visok profil morbiditeta i smrtnosti širom sveta, posebno u zemljama u razvoju. Iako su trenutno licencirane vakcine efikasne u sprečavanju infekcije, njihova moć je efemerna; stoga im je potreban podsticaj upotrebom adjuvansa koji su sigurni i instrumentalni za postizanje bolje produžene zaštite imune odbrane. U ovom radu, ocenjen je ekstrakt lišća Moringa oleifera etil acetata zbog mogućeg pomoćnog svojstva. Miševi su vakcinisani vakcinom protiv tifusa i posle toga je izmerena težina miševa. Takođe, navedeno je brojanje mikrobioloških kolonija posle vakcinacije miševa sa ćelijama Salmonella tiphi. Iz rezultata kulture krvi, MO ekstrakt je pokazao odličan sinergistički antimikrobni efekat, jer je grupa miševa koja je primenjivala našu formulisanu kombinaciju vakcina-MO imala najmanje opterećenje mikrobnog opterećenja (12,25 ± 4,86) kolonija posle izazivanja mikroba, u poređenju sa grupama miševa koji su primili samo vakcinu (37,25 ± 4,5) i sam ekstrakt MO (31,25 ± 9,43). Dalje, procena telesne težine miševa tretiranih grupa pokazala je obrazac rasta koji nije značajno odstupio od kontrolne grupe. Zaključno, ekstrakt MO pokazao je obećavajući sinergistički antimikrobni efekat na zajedničku primenu sa vakcinom protiv tifusa i nije doveo do štetnih nuspojava kod miševa.

Ključne reči: *Tifusna groznica, Moringa oleifera, Salmonella tiphi, adjuvans, jedinice koje formiraju koloniju, telesna težina.*

sciendo
UDK: 615.322 :582.683.4
616.937-085:615.322
Ser J Exp Clin Res 2020; 21 (4): 317-323
DOI: 10.2478/sjecr-2018-0092

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INTRODUCTION

Typhoid fever is a systemic disease associated with poor hygiene and socio-economic status with high morbidity and mortality profile in developing countries [1]. This disease is caused by the bacteria *Salmonella enterica* serotype Typhi (*S. typhi*), which is transmitted orally during the ingestion of food and water contaminated with faeces or urine of an infected individual [2]. Following ingestion, the bacteria spread from the intestine via the blood (where they multiply) to the intestinal lymph nodes, liver, and spleen [3]. The incubation period of the disease is usually 10-14 days and varies considerably from 8-15 days, but may be as short as 5 days and as long as 30 or 35 days depending on the inoculum size and the state of host defenses [4]. The onset of the disease is stealthy and characterized with clinical manifestations such as the gradual inception of persistent fever, chills and abdominal pain. In some cases, patients experience rash, nausea, anorexia, diarrhoea or constipation, headache, relative bradycardia and reduced level of consciousness [5]. *Salmonella typhi* is a member of the *salmonella* genus which belongs to the Gram-negative Enterobacteriaceae family of bacteria [6]. It is serologically positive for lipopolysaccharide antigens O9 and O12, polysaccharide capsular antigen Vi and protein flagellar antigen, Hd [2]. The polysaccharide capsule Vi is responsible for the defensive outfit posed by the bacterium against the bactericidal action of an infected individual's serum [7]. Differences in the structural patterns of the lipopolysaccharide create antigenic variations that influence the virulence of different strains [6]. The Widal test is the most common diagnostic method that aids the identification of the agglutinating antibodies against the *S. typhi* antigens, O (somatic) and H (flagellar), which appear about 7 to 10 days after disease onset. Nevertheless, the high numbers of false-positive and false-negative test results limit its clinical efficacy [8]. Before now, the preferred treatment for typhoid fever was Chloramphenicol, but owing to substantial relapse rates and the development of bacterial resistance during the 1970s and 1980s, this drug was replaced by co-trimoxazole and ampicillin [3]. More recently, increasing resistance to the latter antibiotics has prompted the use of quinolone derivatives and third-generation cephalosporins [8].

As Salmonella has become a major threat to the society due to the disease severity, recurrence of disease through carrier state and the emergence of multidrug resistance, an effective prophylactic measure is essential [4]. The development of a safe and effective vaccine remains a priority for controlling the spread of the disease especially if travelers to endemic areas are considered, as it will be of immense health benefit to them. Aside the two known licensed vaccines against typhoid fever – Parenteral Vi polysaccharide (ViCPS) and oral vaccine Ty21a (live-attenuated), other vaccines that have been recommended for use include; the conjugate vaccine (Vi-TT), where the Vi antigen is coupled to a carrier protein and multivalent combination vaccines (a combination of ViCPS and hepatitis A vaccines), and are administered parenterally [3]. The licensed vaccines have limitations such as poor seroconversion after first administration, hence requires repeated booster immunization schedules; sub-optimal antibody titre production, especially the Vi polysaccharide vaccine, and a short protective period. Currently, research focus is geared towards genetically attenuating strains of S. Typhi to achieve high immunogenicity while at the same time rendering the strain nonpathogenic and secondly, the development of new parenteral Vi polysaccharide protein conjugate vaccines, which are expected to produce higher antibody titres following initial and booster immunizations [3, 8]. In addition, a promising approach to circumvent the limitations is to employ vaccine adjuvants that are bio-compactible, biodegradable and non-toxic to subjects.

Adjuvants in immunology have been defined as substances added to vaccine formulations that enhance the immunogenicity of antigens and induce protection against infection [9]. Adjuvants can act like PAMPs (pathogen-associated molecular patterns), triggering the innate immune response through a variety of mechanisms, to identify the vaccine components as a "threat", with activation and maturation of APCs (antigen presenting cells) and initiation of downstream adaptive immune activities [10]. Benefits of adjuvants include the following; they decrease the dose of antigen needed to formulate a vaccine, decrease the number of vaccine booster doses required for immunization of subjects, enhance vaccine efficacy in infacts, the elderly and immunocompromised individuals, increase functional antibody titre, induce more rapid and long-lasting immune response, induce a robust cell-mediated immunity, provide broad protection (cross-reactivity), facilitate mucosal immunity and help to overcome antigen competition in combination vaccines [11]. Classes of adjuvants that have been explored include mineral compounds, bacterial products, oil-based emulsions, immune-stimulatory complexes (ISCOMs), virosomes, phytochemicals (saponin), liposomes and glycoproteins [12].

In this work, *Moringa oleifera* (MO), a member of the Moringaceae family also known as 'Horse radish' or 'Drumstick' was evaluated for a possible adjuvant property for typhoid fever vaccine [13]. It is a fast growing drought-resistant tree native widely cultivated throughout tropical countries including Nigeria. In folk medicine it is been reported to be used in the treatment of rheumatism, cardiac and circulatory disorders and also possesses antitumor, anti-inflammatory, antihypertensive, antidiabetic, hepatoprotective, cholesterol lowering, antioxidant, antibacterial and antifungal properties [14]. Moreover, some medicinal properties associated to different parts of Moringa have been acknowledged by both Unani and Ayurvedic systems of medicines. Studies have revealed that Moringa roots possess antispasmodic activity through calcium channel blockade which is the platform for its traditional use in treatment of diarrhea. Moringa leaves have been reported to be a rich source of β-carotene, protein, vitamin C, calcium, potassium and act as good source of natural antioxidants like ascorbic acid, flavonoids, phenolics and carotenoids that work mutually to strengthen immunity [15]. Hence, this work seeks to explore the plant leaves as a potential typhoid fever vaccine adjuvant.

MATERIALS AND METHODS

Plant Extraction

The leaves of *Moringaoleifera* were collected in Nsukka area of Enugu State, Nigeria and authenticated by a botanist from the Department of Botany, University of Nigeria, Nsukka. The leaves were air-dried, pulverized and the powder was stored in an air-tight container pending extraction. Cold maceration technique was employed for extraction [5] using ethyl acetate as solvent. Exactly 2500ml (2.5 litres) of ethyl acetate was used to soak the *Moringa oleifera* powder in a sterile container and the container was sealed properly and allowed to stand for 48hours before filtration. The residue was rinsed with additional 2500ml of ethyl acetate to ensure exhaustive extraction, and a solution of the extract was obtained, and the solvent allowed to vapourize at room temperature.

Test Animals

Twenty (20) young female albino mice, *Mus musculus* (6 - 8 weeks old) purchased from the Animal House, Department of Pharmacology and Toxicology, University of Nigeria, Nsukka and kept under standard pathogen-free conditions in the animal facility of the department of pharmacology and toxicology. The animals were well fed with chick's grower mesh (vital feed) and water ad libitum throughout the study period. Ethical considerations with respect to handling of laboratory animals were duly followed in accordance withthe "NIH guidelines for laboratory animal care and use" [16] and the University of Nigeria regulations for laboratory animal use.

Typhoid Fever Vaccine Preparation

The vaccine used was prepared locally by heat denaturation method. Outlined below are the steps elaborating how the vaccine was prepared.

Collection and isolation of Salmonella typhi (ST)

3.6g of the agar was dissolved in 50ml of water. The mixture was poured into two petri dishes, each containing 20ml, and the remaining 10ml was poured in two test tubes, each containing 5ml, and allowed to set or gel. ST was obtained from the University of Nigeria Teaching Hospital, Ituku-Ozalla Enugu. Pure culture of the isolate, *Salmonella typhi*, was prepared by sub-culturing using a wireloop on the Salmonella-Shigella agar in the petri dishes by streak method and then were incubated for 24hours at 37°C. After incubation, distinct colonies were again sub-cultured into freshly prepared and sterilized nutrient broth and incubated for another 24hours.

Determination of bio-load

1ml of the broth culture of the microorganism was collected and transferred aseptically into a test tube containing 9ml of sterile water and this was labelled 10^{-1} (10-fold serial dilution); this was progressively done till 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-labelled 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, 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 resultant 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.

Determination of death time of microorganism

The cell culture was harvested and diluted in test tubes containing 10ml sterile water, and the suspensions were evenly distributed by shaking and were sterilized by mild heating in a pressure cooker. Suspensions were diluted using normal saline, centrifuged at 3000 rev/min for 5minutes, and the supernatant decanted, leaving behind the cells. The cells were washed twice with normal saline and resuspended in fresh normal saline. 2ml of the bacterial suspension was then transferred to a sterile test tube and placed in a water bath at a constant temperature of 56ºC. Loopfuls were transferred from the selected dilution test tube at time 0, into resuscitating test tubes containing nutrient broth at different time intervals (i.e. 10, 20, 30, 40, 50, 60, 70, 80, 90 minutes), i.e. subculturing into the respective resuscitating test tubes (labeled according to the time).The test tubes with their content were then heated at a temperature of about 55-60˚C. 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.

ST vaccine formulation

Vaccine containing 2.5×10^{8}cells/ml was prepared. The selected broth culture was centrifuged at 3000rpm for 5 minutes. The supernatant was aspirated and the cells washed twice with normal saline by centrifugation and aspiration of the supernatant in each case. The cells were then resuspended in a specified volume of normal saline (5ml) and heated (at the same temperature) for a period of time equivalent to the predetermined death time. The "formulated vaccine", was then aseptically transferred into bijou bottles and made up with normal saline, was labeled appropriately and stored in the refrigerator.

Vaccination of mice

The experimental animal were divided into five groups named A, B, C, D and E of four mice each and vaccinated intraperitoneally as follows; Group A received 0.4ml of formulated vaccine only (which contained 10^{8} cells), Group B received 10mg of the MO extract/kg body weight only, Group C received 10mg MO extract and 0.4ml of formulated vaccine, Group D received 0.05ml of ethyl acetate only(solvent for extraction) and then Group E received 0.4 ml of

normal saline only (solvent for vaccine constitution). The vaccination procedure was repeated once after two weeks. Blood samples were collected from each mouse by intraocular eye puncture using the method described by [17] at 1 week and 2 weeks post first vaccination and 1week post second vaccination.

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 weighing balance and the weight was recorded. This continued till the animals were sacrificed.

Challenge of animals with live *Salmonella typhi* (ST)

One-week after the second vaccination, the animals were challenged with 10^7 live *S. typhi* organisms contained in 0.04ml of the preparation, through the intraperitoneal route.

Blood culture

One-week post challenge with ST, blood samples were collected from each of the mice and 2-fold serial dilution of the blood samples was done by diluting 25µL of the blood sample with 25µL of normal saline. 10µL of each of the diluted blood sample was then aseptically placed on each of the 8 respective portions of the properly labeled agar plates containing freshly prepared nutrient agar. This diagnostic method was done for all the mice blood samples as described by [18]. The plates with its contents were incubated for 24 hours at 37°C and then examined for growth of microorganisms via colony count.

Statistical Analysis

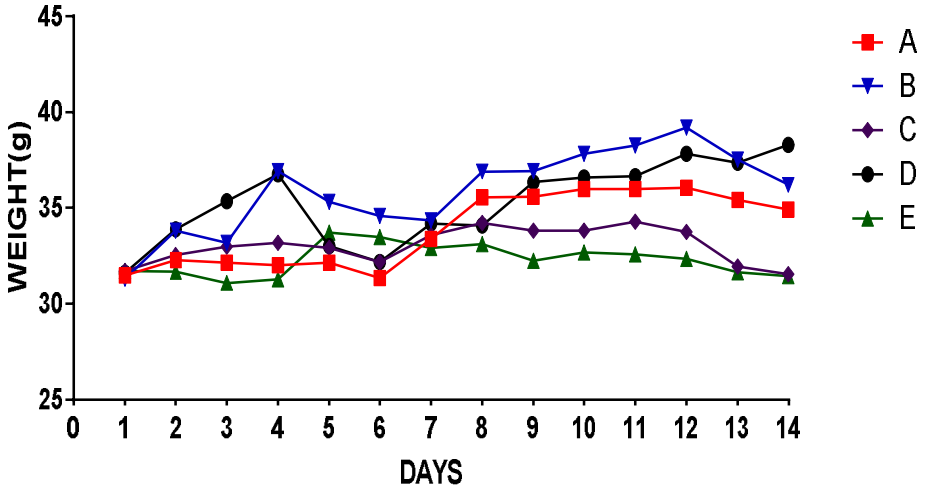
The data obtained was expressed as mean ± standard deviation (Mean ± SD). One-way analysis of variance (ANOVA) followed by Duncan post-hoc test were used to test for significance. Differences in mean were considered significant at $p < 0.05$. SPSS version 20 was used for the analysis.

RESULTS

Mice Body Weight

From the periodic mice body weight monitoring, mice body weight of treated groups showed a growth pattern that did not deviate significantly from those of the control group (Fig. 1). Fig. 1A demonstrates that the weight of mice group treated with our formulated ST vaccine only, increased progressively from day 6. Weight curve of mice group treated with Moringa leaf extract alone, showed an irregular but overall increase in mice weight throughout the period of study (fig.1B). Those treated with ST vaccine – MO combination demonstrated a slight increase in body weight from day 6, though there was a decline from day 12 through 14 (fig. 1C). There was a sharp decline in the body weight of mice group treated with ethyl acetate from day 4 to day 6; however, their body weight increased gradually from day 6 to day 14 (fig. 1D). Finally, body weight of mice in the control group was virtually uniform throughout the period of study (fig. 1E).

Figure 1. Mice body weight curve

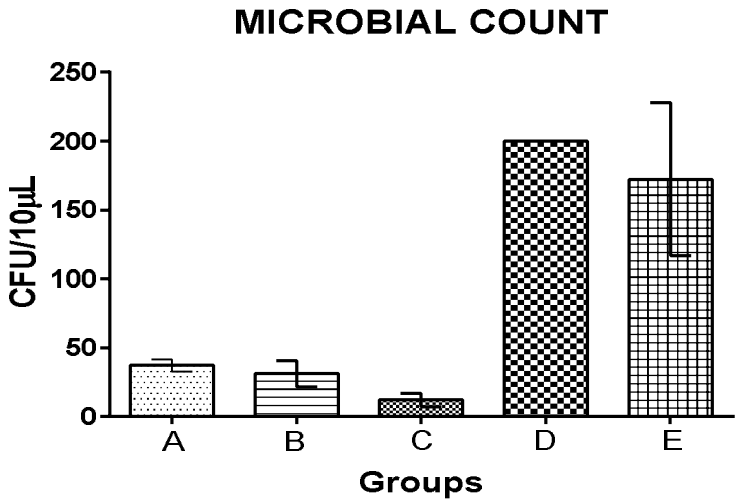


A= ST vaccine (0.4ml = 10^8 cells), B= MO extract (10mg), C= ST vaccine (0.4ml = 10^8 cells) + MO extract (10mg), D= Ethyl acetate (0.05ml), E= Normal saline (0.4ml)

Blood Culture

From the blood culture, distinct colony forming units (cfu) were observed, counted and recorded as shown in Fig. 2 and Table 1. Mice group treated with ST vaccine – MO combination demonstrateda significantly lower ($P < 0.05$) number of colony forming units than those of the control groups (group D and E). However, it was not significantly lower ($p > 0.05$) thanthe number of colony forming units in blood culture of mice groups treated with ST vaccine alone (group A) and those treated with MO leaf extract alone (group B). Interestingly, there was lower number of colony forming units in blood culture of mice groups treated with MO leaf extract alone (group B) when compared with those treated with our formulated ST vaccine alone (group A), though it was non-significant ($p > 0.05$).

Figure 2. Blood culture results



*A= ST vaccine (0.4ml = 10^8 cells) **, B= MO extract (10mg) **, C= ST vaccine (0.4ml = 10^8 cells) + MO extract (10mg) ***, D= Ethyl acetate (0.05ml) *, E= Normal saline (0.4ml) **

DISCUSSION

The immune system is able to spawn a massive variety of molecules and cells capable of specifically recognizing and eradicating virtually incessant variety of foreign pathogenic invaders [19]. Vaccination has proved to be the most efficient tool for empowering the immune system to preventa variety of infectious diseases including typhoid fever. The ultimate goal of vaccination, in addition to safety, is to generate a pathogen-specific immune response that would elicit robust humoral and cell-mediated immunity providing long-lasting protection against infection [20]. Upon recognition of the vaccine antigen, a cascade of reactions is triggered resulting in the release of inflammatory mediators which may include chemokines, activation of the complement pathway and also cellular recruitment; all these may lead to the development of signs and symptoms of local inflammation or allergy in the host [21]. These reactions would consequently result in elimination of infectious agent or pathogen from the living system. Low pH in the stomach poses a barrier to the oral route of administration of live attenuated S. typhi vaccine; hence we sought to use an alternative route of administration, the intraperitoneal route, during the study. One way of assessing the imminence of typhoid fever in a mammalian host is by determining the presence of *S. typhi* in blood samples of the host.

Table 1. Colony Forming Units

	N	Colony Forming Units
Group A	4	$37.25 \pm 4.50^{**}$
Group B	4	$31.25 \pm 9.43^{**}$
Group C	4	$12.25 \pm 4.85^{***}$
Group D	4	$200.00 \pm 0.00^{*}$
Group E	4	$172.25 \pm 55.50^{*}$

Results are expressed as mean ± S.D. (* > ** > ***)

Blood culture is said to be the most decisive method of typhoid fever diagnosis, generally after seven days of infection; more than 80% of patients are likely to test positive for ST during this period of infection [18]. Results from the blood culture test correlates relatively the potency of the treatments administered to the mice in the elimination of ST infection. Elevated levels of microbial load observed in mice

groups administered normal saline (172.25 ± 55.50 CFU) and in those administered 0.05ml ethyl acetate (200.0 ± 0.00 CFU) which are both control groups, implies that these treatment agents, expectedly, lacked antimicrobial or therapeutic potentials. This was consistent with the findings of (5), showing that there was elevated quantity of ST in blood culture of mice administered normal saline. MO-treated groups showed a significantly lower microbial load profile (31.25 ± 9.43 CFU) when compared to those of the negative control groups (D, E), indicating that MO has remarkable antimicrobial and immunomodulatory properties. This observation is consistent with the findings of [22], who reported a heightened antimicrobial activity against ST. This further supports the claim in folk medicine, about MO, as having antibacterial properties [14]. Again, there was a significant decline in the microbial load (37.25 ± 4.50 CFU) of mice treated with our formulated ST vaccine alone when compared to those of the control groups. This indicates that the ST vaccine is potent and has capacity to mediate animmunoprotective effect. This observation is consistent with the findings of (5), who reported a similarly formulated ST vaccine to have a prophylactic effect against ST infection. Furthermore, mice groups treated with our formulated ST vaccine in combination with MO extract demonstrated a much lower microbial load profile (12.25 ± 4.85 CFU), though non-significantly, when compared with those treated with the ST vaccine alone (37.25 ± 4.50 CFU). This implies that the MO leaves extract has a promising adjuvant property as its synergistic effect in reducing the microbial load of the mice was phenomenal. Moringa leaf extract was also shown to have upregulated the immunoprotective effect of a respiratory syncytial virus vaccine when administered in combination with it [23]. It also suggests that bioactive phytochemical(s) embedded in the MO extract has both immunomodulatory and adjuvant properties that could be of immense support to the development of better efficacious vaccines for myriad infectious diseases, when used in isolation or incorporated into vaccine formulations [15].

Weight curve results correlates the general physiological effect a prophylactic treatment exerts on a subject. Substantial loss in body weight of animals up to 10% of initial body weight, with the administration of extract is considered as toxic for its use [24]. Again, ST infection has been associated with weight loss [25]; hence, the need to monitor the body weight of the mice. Results showed that there was a slight progressive decline in the weight of control group administered normal saline (fig. 1E), which indicates that the S. typhi cells administered during the challenge were live and viable. This is in contrast with the observation of (5), who reported a sharp decline in the body weight of mice administered normal saline. This observation suggests that the mice model used in the experiment may have a very strong innate immune outfit which may be the reason why there was no sudden decline in the weight of mice administered normal saline. A progressive increase from day 6 was observed in the weight of control group administered ethyl acetate (fig. 1D). This observation suggests that ethyl acetate may have exerted a sterilizing effect (antimicrobial activity) which inhibited the proliferation of ST cellstemporarily and consequently, the mice having not been affected severely, gained weight progressively from day 6. However, a sharp decline was observed in the body weight of mice groups treated with the ST vaccine – MO combination from day 12 to day 14. It may be that the prophylactic effect of the ST vaccine – MO combinations led to a transient loss of appetite in the mice.Furthermore, weight curves of mice treated with the vaccine alone and those treated with MO showed a uniform weight gain pattern; there was irregular weight increase in the treated groups (fig 1A,1B).This observation also correlates the findings reported by [5, 23], which showed that a similarly formulated vaccine did not have an adverse effect on the mice as they gained weight in an irregular manner.This suggests that the prophylactic agents are relatively safe and could be used in treatment of typhoid fever.

Limitation of this study entails assessment of the humoral and cellular immune responses, especially looking out for the immunoglobulin titre levels and cytokines, to further illustrate the possible mode of action of our formulated ST vaccine in isolation and when combined with MO extract on the immune system.

CONCLUSION

The search for a more potent typhoid fever vaccine with a lasting immuno-protective effect is still on. Here, we evaluated the synergistic effect of MO leaves extract when co-administered with typhoid fever vaccine and our findings revealed that MO extract demonstrates a promising antimicrobial effect when combined with ST vaccine and may be evaluated for adjuvant properties for the vaccine in view of conferring a longer immunity. Hence, further studies on identification and isolation of the bioactive compound (s) responsible for antimicrobial and possible adjuvant properties is recommended.

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